1 What is pharmacology?

OVERVIEW

In this introductory chapter, we explain how pharmacology came into being and evolved as a scientific discipline, and describe the present day structure of the subject and its links to other biomedical sciences. The structure that has emerged forms the basis of the organisation of the rest of the book. Readers in a hurry to get to the here-and-now of pharmacology can safely skip this chapter.

WHAT IS A DRUG?

For the purposes of this book, a drug can be defined as *a chemical substance of known structure, other than a nutrient or an essential dietary ingredient,*[1] *which, when administered to a living organism, produces a biological effect.*

A few points are worth noting. Drugs may be synthetic chemicals, chemicals obtained from plants or animals, or products of genetic engineering. A *medicine* is a chemical preparation, which usually but not necessarily contains one or more drugs, administered with the intention of producing a therapeutic effect. Medicines usually contain other substances (excipients, stabilisers, solvents, etc.) besides the active drug, to make them more convenient to use. To count as a drug, the substance must be administered as such, rather than released by physiological mechanisms. Many substances, such as insulin or thyroxine, are endogenous hormones but are also drugs when they are administered intentionally. Many drugs are not used in medicines but are nevertheless useful research tools. In everyday parlance, the word *drug* is often associated with addictive, narcotic or mind-altering substances—an unfortunate negative connotation that tends to bias uninformed opinion against any form of chemical therapy. In this book, we focus mainly on drugs used for therapeutic purposes but also describe important examples of drugs used as experimental tools. Although poisons fall strictly within the definition of drugs, they are not covered in this book.

ORIGINS AND ANTECEDENTS

Pharmacology can be defined as the study of the effects of drugs on the function of living systems. As a science, it was born in the mid-19th century, one of a host of new biomedical sciences based on principles of experimentation rather than dogma that came into being in that remarkable period. Long before that—indeed from the dawn of civilisation—herbal remedies were widely used, pharmacopoeias were written, and the apothecaries' trade flourished, but nothing resembling scientific principles was applied to therapeutics. Even Robert Boyle, who laid the scientific foundations of chemistry in the middle of the 17th century, was content, when dealing with therapeutics (*A Collection of Choice Remedies*, 1692), to recommend concoctions of worms, dung, urine and the moss from a dead man's skull. The impetus for pharmacology came from the need to improve the outcome of therapeutic intervention by doctors, who were at that time skilled at clinical observation and diagnosis but broadly ineffectual when it came to treatment.[2] Until the late 19th century, knowledge of the normal and abnormal functioning of the body was too rudimentary to provide even a rough basis for understanding drug effects; at the same time, disease and death were regarded as semisacred subjects, appropriately dealt with by authoritarian, rather than scientific, doctrines. Clinical practice often displayed an obedience to authority and ignored what appear to be easily ascertainable facts. For example, cinchona bark was recognised as a specific and effective treatment for malaria, and a sound protocol for its use was laid down by Lind in 1765. In 1804, however, Johnson declared it to be unsafe until the fever had subsided, and he recommended instead the use of large doses of calomel (mercurous chloride) in the early stages—a murderous piece of advice which was slavishly followed for the next 40 years.

The motivation for understanding what drugs can and cannot do came from clinical practice, but the science could be built only on the basis of secure foundations in physiology, pathology and chemistry. It was not until 1858 that Virchow proposed the cell theory. The first use of a structural formula to describe a chemical compound was in 1868. Bacteria as a cause of disease were discovered by Pasteur in 1878. Previously, pharmacology hardly had the legs to stand on, and we may wonder at the bold vision of Rudolf Buchheim, who created the first pharmacology institute (in his own house) in Estonia in 1847.

In its beginnings, before the advent of synthetic organic chemistry, pharmacology concerned itself exclusively with understanding the effects of natural substances, mainly plant extracts—and a few (mainly toxic) chemicals such as mercury and arsenic. An early development in chemistry was the purification of active compounds from plants. Friedrich Sertürner, a young German apothecary, purified morphine from opium in 1805. Other substances quickly followed, and, even though their structures were unknown, these compounds showed that chemicals, not magic or vital forces, were responsible for the effects that plant

[1] Like most definitions, this one has its limits. For example, there are a number of essential dietary constituents, such as iron and various vitamins, that are used as medicines.

[2] Oliver Wendell Holmes, an eminent physician, wrote in 1860: '… firmly believe that if the whole materia medica, as now used, could be sunk to the bottom of the sea, it would be all the better for mankind and the worse for the fishes.' (See Porter, 1997.)

extracts produced on living organisms. Early pharmacologists focused most of their attention on such plant-derived drugs as quinine, digitalis, atropine, ephedrine, strychnine and others (many of which are still used today and will have become old friends by the time you have finished reading this book).[3]

PHARMACOLOGY IN THE 20TH AND 21ST CENTURIES

Beginning in the 20th century, the fresh wind of synthetic chemistry began to revolutionise the pharmaceutical industry, and with it the science of pharmacology. New synthetic drugs, such as barbiturates and local anaesthetics, began to appear, and the era of antimicrobial chemotherapy began with the discovery by Paul Ehrlich in 1909 of arsenical compounds for treating syphilis. Further breakthroughs came when the sulfonamides, the first antibacterial drugs, were discovered by Gerhard Domagk in 1935, and with the development of penicillin by Chain and Florey during the Second World War, based on the earlier work of Fleming.

These few well-known examples show how the growth of synthetic chemistry, and the resurgence of natural product chemistry, caused a dramatic revitalisation of therapeutics in the first half of the 20th century. Each new drug class that emerged gave pharmacologists a new challenge, and it was then that pharmacology really established its identity and its status among the biomedical sciences.

In parallel with the exuberant proliferation of therapeutic molecules—driven mainly by chemistry—which gave pharmacologists so much to think about, physiology was also making rapid progress, particularly in relation to chemical mediators, which are discussed in depth elsewhere in this book. Many hormones, neurotransmitters and inflammatory mediators were discovered in this period, and the realisation that chemical communication plays a central role in almost every regulatory mechanism that our bodies possess immediately established a large area of common ground between physiology and pharmacology, for interactions between chemical substances and living systems were exactly what pharmacologists had been preoccupied with from the outset. The concept of 'receptors' for chemical mediators, first proposed by Langley in 1905, was quickly taken up by pharmacologists such as Clark, Gaddum, Schild and others and is a constant theme in present day pharmacology (as you will soon discover as you plough through the next two chapters). The receptor concept, and the technologies developed from it, have had a massive impact on drug discovery and therapeutics. Biochemistry also emerged as a distinct science early in the 20th century, and the discovery of enzymes and the delineation of biochemical pathways provided yet another framework for understanding drug effects. The picture of pharmacology that emerges from this brief glance at history (Fig. 1.1) is of a subject evolved from ancient prescientific therapeutics, involved in commerce from the 17th century onwards, and which gained respectability by donning the trappings of science as soon as this became possible in the mid-19th century. Signs of its carpetbagger past still cling to pharmacology, for the pharmaceutical industry has become very big business and much pharmacological research nowadays takes place in a commercial environment, a rougher and more pragmatic place than the glades of academia.[4] No other biomedical 'ology' is so close to Mammon.

ALTERNATIVE THERAPEUTIC PRINCIPLES

Modern medicine relies heavily on drugs as the main tool of therapeutics. Other therapeutic procedures such as surgery, diet, exercise, etc. are also important, of course, as is deliberate non-intervention, but none is so widely applied as drug-based therapeutics.

Before the advent of science-based approaches, repeated attempts were made to construct systems of therapeutics, many of which produced even worse results than pure empiricism. One of these was *allopathy*, espoused by James Gregory (1735–1821). The favoured remedies included blood letting, emetics and purgatives, which were used until the dominant symptoms of the disease were suppressed. Many patients died from such treatment, and it was in reaction against it that Hahnemann introduced the practice of *homeopathy* in the early 19th century. The guiding principles of homeopathy are:

- like cures like
- activity can be enhanced by dilution.

The system rapidly drifted into absurdity: for example, Hahnemann recommended the use of drugs at dilutions of $1:10^{60}$, equivalent to one molecule in a sphere the size of the orbit of Neptune.

Many other systems of therapeutics have come and gone, and the variety of dogmatic principles that they embodied have tended to hinder rather than advance scientific progress. Currently, therapeutic systems that have a basis which lies outside the domain of science are actually gaining ground under the general banner of 'alternative' or 'complementary' medicine. Mostly, they reject the 'medical model', which attributes disease to an underlying derangement of normal function that can be defined in biochemical or structural terms, detected by objective means, and influenced beneficially by appropriate chemi-

[3]A handful of synthetic substances achieved pharmacological prominence long before the era of synthetic chemistry began. Diethyl ether, first prepared as 'sweet oil of vitriol' in the 16th century, and nitrous oxide, prepared by Humphrey Davy in 1799, were used to liven up parties before being introduced as anaesthetic agents in the mid-19th century (see Ch. 40). Amyl nitrite (see Ch. 21) was made in 1859 and can claim to be the first 'rational' therapeutic drug; its therapeutic effect in angina was predicted on the basis of its physiological effects—a true 'pharmacologist's drug' and the smelly forerunner of the nitrovasodilators that are widely used today. Aspirin (Ch. 26), the most widely used therapeutic drug in history, was first synthesised in 1853, with no therapeutic application in mind. It was rediscovered in 1897 in the laboratories of the German company Bayer, who were seeking a less toxic derivative of salicylic acid. Bayer commercialised aspirin in 1899 and made a fortune.

[4]Some of our most distinguished pharmacological pioneers made their careers in industry: for example, Henry Dale, who laid the foundations of our knowledge of chemical transmission and the autonomic nervous system (Ch. 11); George Hitchings and Gertrude Elion, who described the antimetabolite principle and produced the first effective anticancer drugs (Ch. 54); and James Black, who introduced the first β-adrenoceptor and histamine H_2-receptor antagonists (Chs 13 and 17). It is no accident that in this book, where we focus on the scientific principles of pharmacology, most of our examples are products of industry, not of nature.

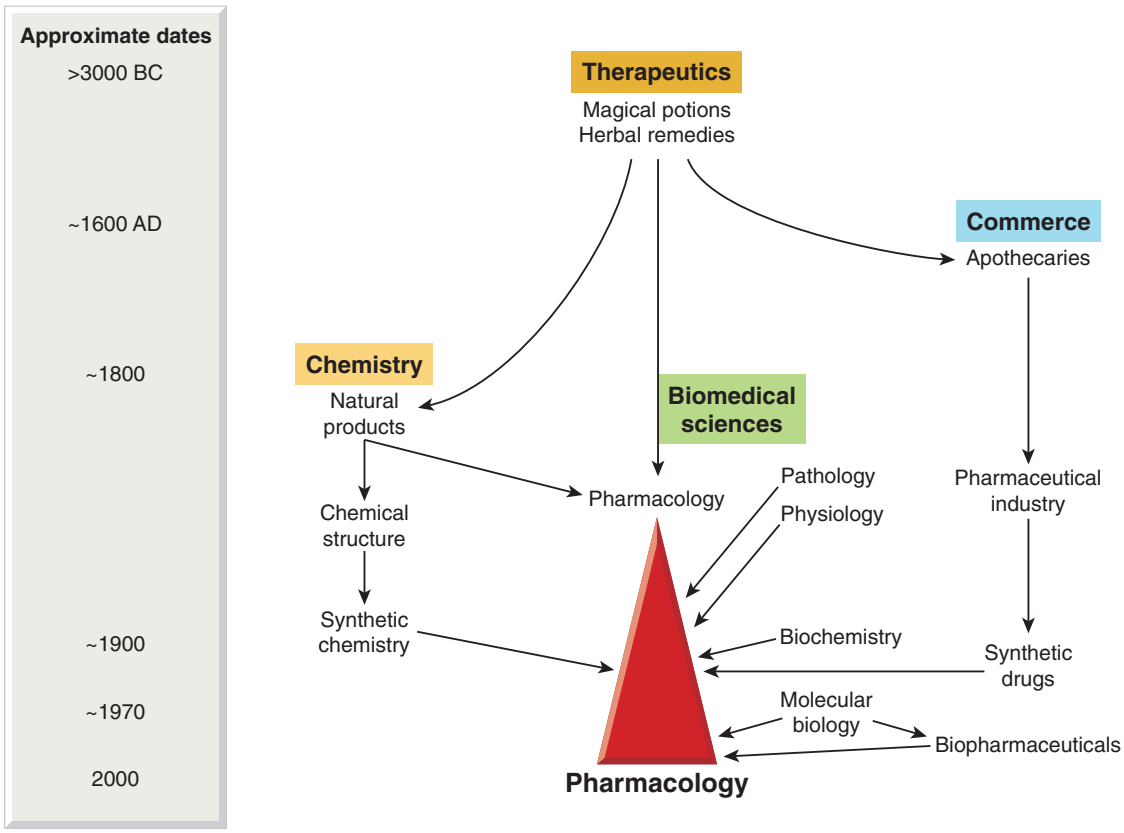

Fig. 1.1 The development of pharmacology.

cal or physical interventions. They focus instead mainly on subjective malaise, which may be disease-associated or not. Abandoning objectivity in defining and measuring disease goes along with a similar departure from scientific principles in assessing therapeutic efficacy and risk, with the result that principles and practices can gain acceptance without satisfying any of the criteria of validity that would convince a critical scientist, and that are required by law to be satisfied before a new drug can be introduced into therapy. Public acceptance, alas, has little to do with demonstrable efficacy.[5]

THE EMERGENCE OF BIOTECHNOLOGY

Since the 1980s, biotechnology has emerged as a major source of new therapeutic agents in the form of antibodies, enzymes and various regulatory proteins, including hormones, growth factors and cytokines (see Buckel, 1996; Walsh, 2003). Although such products (known as *biopharmaceuticals*) are generally produced by genetic engineering rather than by synthetic chemistry, the pharmacological principles are essentially the same as for conventional drugs. Looking further ahead, gene- and cell-based therapies (Ch. 59), although still in their infancy, will take therapeutics into a new domain. The principles governing the design, delivery and control of functioning artificial genes introduced into cells, or of engineered cells introduced into the body, are very different from those of drug-based therapeutics and will require a different conceptual framework, which texts such as this will increasingly need to embrace if they are to stay abreast of modern medical treatment.

PHARMACOLOGY TODAY

As with other biomedical disciplines, the boundaries of pharmacology are not sharply defined, nor are they constant. Its exponents are, as befits pragmatists, ever ready to poach on the territory and techniques of other disciplines. If it ever had a conceptual and technical core that it could really call its own, this has now dwindled almost to the point of extinction, and the subject is defined by its purpose—to understand what drugs do to living organisms, and more particularly how their effects can be applied to therapeutics—rather than by its scientific coherence.

Figure 1.2 shows the structure of pharmacology as it appears today. Within the main subject fall a number of compartments (neuropharmacology, immunopharmacology, pharmacokinetics, etc.), which are convenient, if not watertight, subdivisions. These topics form the main subject matter of this book. Around the edges are several interface disciplines, not covered in this book, which form important two-way bridges between pharmacology and other fields of biomedicine. Pharmacology tends to have

[5]Antiscientific populism and commercial pressures recently caused the UK Medicines and Healthcare Regulatory Agency (MHRA) to approve a homeopathic product, despite the lack of evidence that it worked.

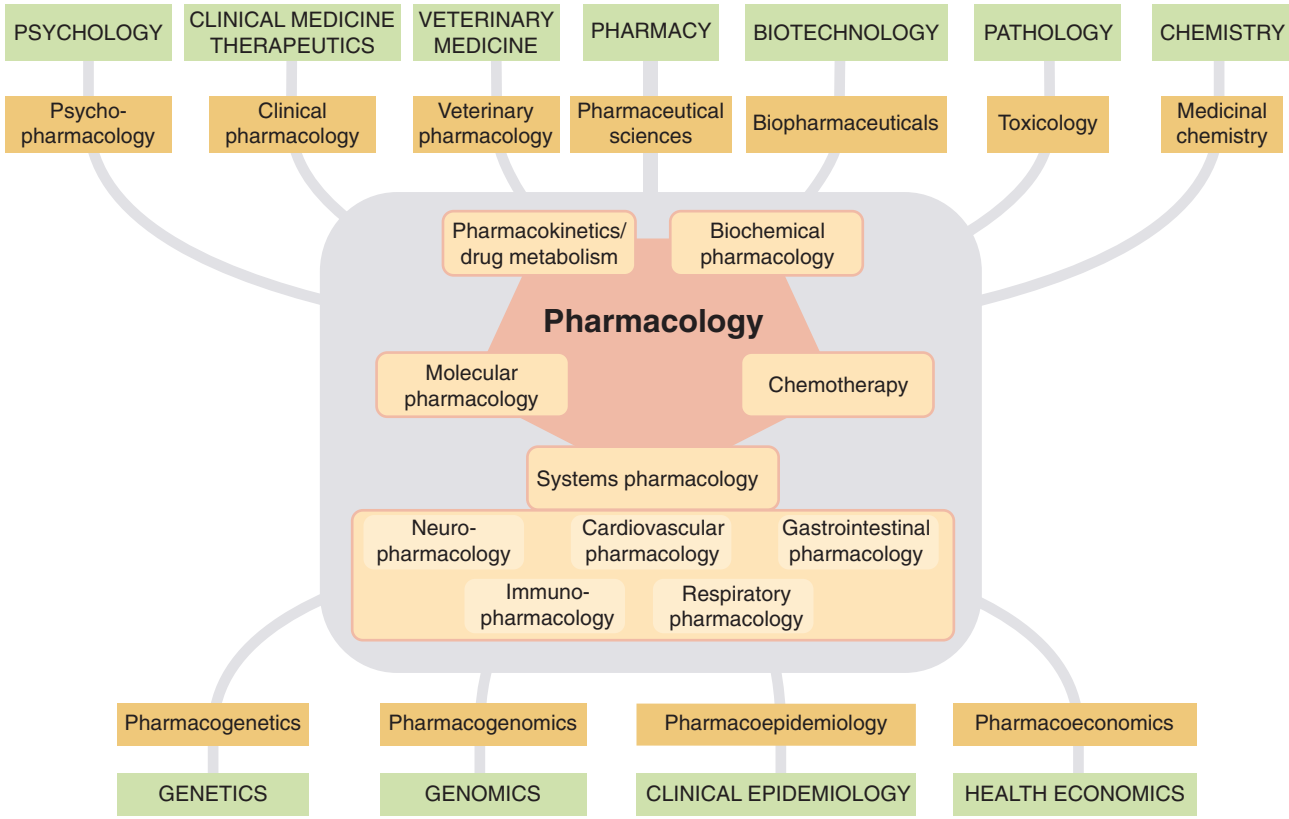

Fig. 1.2 Pharmacology today with its various subdivisions. Interface disciplines (brown boxes) link pharmacology to other mainstream biomedical disciplines (green boxes).

more of these than other disciplines. Recent arrivals on the fringe are subjects such as pharmacogenomics, pharmacoepidemiology and pharmacoeconomics.

Biotechnology. Originally, this was the production of drugs or other useful products by biological means (e.g. antibiotic production from microorganisms or production of monoclonal antibodies). Currently in the biomedical sphere, biotechnology refers mainly to the use of recombinant DNA technology for a wide variety of purposes, including the manufacture of therapeutic proteins, diagnostics, genotyping, production of transgenic animals, etc. The many non-medical applications include agriculture, forensics, environmental sciences, etc.

Pharmacogenetics. This is the study of genetic influences on responses to drugs. Originally, pharmacogenetics focused on familial idiosyncratic drug reactions, where affected individuals show an abnormal—usually adverse—response to a class of drug (see Nebert & Weber, 1990). It now covers broader variations in drug response, where the genetic basis is more complex.

Pharmacogenomics. This recent term overlaps with pharmacogenetics, describing the use of genetic information to guide the choice of drug therapy on an individual basis. The underlying principle is that differences between individuals in their response to therapeutic drugs can be predicted from their genetic make-up. Examples that confirm this are steadily accumulating (see Ch. 11). So far, they mainly involve genetic polymorphism of drug-metabolising enzymes or receptors (see Weinshilboum & Wang, 2004; Swen et al., 2007). Ultimately, linking specific gene variations with variations in therapeutic or unwanted effects of a particular drug should enable the tailoring of therapeutic choices on the basis of an individual's genotype. Steady improvements in the cost and feasibility of individual genotyping will increase its applicability, with far-reaching consequences for therapeutics.[6]

Pharmacoepidemiology. This is the study of drug effects at the population level (see Strom, 2000). It is concerned with the variability of drug effects between individuals in a population, and between populations. It is an increasingly important topic in the eyes of the regulatory authorities who decide whether or not new drugs can be licensed for therapeutic use. Variability between individuals or populations has an adverse effect on the utility of a drug, even though its mean effect level may be satisfactory. Pharmacoepidemiological studies also take into account patient compliance and other factors that apply when the drug is used under real-life conditions.

Pharmacoeconomics. This branch of health economics aims to quantify in economic terms the cost and benefit of drugs used therapeutically. It arose from the concern of many governments to provide for healthcare from tax revenues, raising questions of what therapeutic procedures

[6]An interesting recent example concerns a newly introduced anticancer drug, **gefitinib**, which is highly effective in treating lung cancer but works in only about 10% of cases. Responders have mutations in the receptor tyrosine kinase (see Ch. 3) that is the target of this drug, and can be identified in advance by genotyping (see Lynch et al., 2004).

represent the best value for money. This, of course, raises fierce controversy, because it ultimately comes down to putting monetary value on health and longevity. As with pharmacoepidemiology, regulatory authorities are increasingly requiring economic analysis, as well as evidence of individual benefit, when making decisions on licensing. For more information on this complex subject, see Drummond et al. (1997), Rascati (2009).

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2 How drugs act: general principles

OVERVIEW

The emergence of pharmacology as a science came when the emphasis shifted from describing what drugs do to explaining how they work. In this chapter, we set out some general principles underlying the interaction of drugs with living systems (Ch. 3 goes into the molecular aspects in more detail). The interaction between drugs and cells is described, followed by a more detailed examination of different types of drug–receptor interaction. We are still far from the holy grail of being able to predict the pharmacological effects of a novel chemical substance, or to design *ab initio* a chemical to produce a specified therapeutic effect; nevertheless, we can identify some important general principles, which is our purpose in this chapter.

INTRODUCTION

To begin with, we should gratefully acknowledge Paul Ehrlich for insisting that drug action must be explicable in terms of conventional chemical interactions between drugs and tissues, and for dispelling the idea that the remarkable potency and specificity of action of some drugs put them somehow out of reach of chemistry and physics and required the intervention of magical 'vital forces'. Although many drugs produce effects in extraordinarily low doses and concentrations, low concentrations still involve very large numbers of molecules. One drop of a solution of a drug at only 10^{-10} mol/l still contains about 3×10^9 drug molecules, so there is no mystery in the fact that it may produce an obvious pharmacological response. Some bacterial toxins (e.g. diphtheria toxin) act with such precision that a single molecule taken up by a target cell is sufficient to kill it.

One of the basic tenets of pharmacology is that drug molecules must exert some chemical influence on one or more constituents of cells in order to produce a pharmacological response. In other words, drug molecules must get so close to these constituent cellular molecules that the two interact chemically in such a way that the function of the latter is altered. Of course, the molecules in the organism vastly outnumber the drug molecules, and if the drug molecules were merely distributed at random, the chance of interaction with any particular class of cellular molecule would be negligible. Pharmacological effects, therefore, require, in general, the non-uniform distribution of the drug molecule within the body or tissue, which is the same as saying that drug molecules must be 'bound' to particular constituents of cells and tissues in order to produce an effect. Ehrlich summed it up thus: '*Corpora non agunt nisi fixata*' (in this context, 'A drug will not work unless it is bound').[1]

These critical binding sites are often referred to as 'drug targets' (an obvious allusion to Ehrlich's famous phrase 'magic bullets', describing the potential of antimicrobial drugs). The mechanisms by which the association of a drug molecule with its target leads to a physiological response constitute the major thrust of pharmacological research. Most drug targets are protein molecules. Even general anaesthetics (see Ch. 40), which were long thought to produce their effects by an interaction with membrane lipid, now appear to interact mainly with membrane proteins (see Franks, 2008). All rules need exceptions, and many antimicrobial and antitumour drugs (Chs 50 and 55), as well as mutagenic and carcinogenic agents (Ch. 57), interact directly with DNA rather than protein; bisphosphonates, used to treat osteoporosis (Ch. 35), bind to calcium salts in the bone matrix, rendering it toxic to osteoclasts, much like rat poison.

PROTEIN TARGETS FOR DRUG BINDING

Four main kinds of regulatory protein are commonly involved as primary drug targets, namely:

- receptors
- enzymes
- carrier molecules (transporters)
- ion channels.

There are some exceptions, particularly among the new generation of *biopharmaceutical drugs* (see Ch. 59). Furthermore, many drugs bind (in addition to their primary targets) to plasma proteins (see Ch. 8) and other tissue proteins, without producing any obvious physiological effect. Nevertheless, the generalisation that most drugs act on one or other of the four types of protein listed above serves as a good starting point.

Further discussion of the mechanisms by which such binding leads to cellular responses is given in Chapters 3–4.

DRUG RECEPTORS

WHAT DO WE MEAN BY RECEPTORS?

▼ As emphasised in Chapter 1, the concept of receptors is central to pharmacology, and the term is most often used to describe the target molecules through which soluble physiological mediators—

[1]There are, if one looks hard enough, exceptions to Ehrlich's dictum—drugs that act without being bound to any tissue constituent (e.g. osmotic diuretics, osmotic purgatives, antacids and heavy metal chelating agents). Nonetheless, the principle remains true for the great majority.

Targets for drug action

- A drug is a chemical applied to a physiological system that affects its function in a specific way.
- With few exceptions, drugs act on target proteins, namely:
  - receptors
  - enzymes
  - carriers
  - ion channels.
- The term *receptor* is used in different ways. In pharmacology, it describes protein molecules whose function is to recognise and respond to endogenous chemical signals. Other macromolecules with which drugs interact to produce their effects are known as *drug targets*.
- Specificity is reciprocal: individual classes of drug bind only to certain targets, and individual targets recognise only certain classes of drug.
- No drugs are completely specific in their actions. In many cases, increasing the dose of a drug will cause it to affect targets other than the principal one, and this can lead to side effects.

hormones, neurotransmitters, inflammatory mediators, etc.—produce their effects. Examples such as acetylcholine receptors, cytokine receptors, steroid receptors, and growth hormone receptors abound in this book, and generally the term *receptor* indicates a recognition molecule for a chemical mediator.

'Receptor' is sometimes used to denote *any* target molecule with which a drug molecule (i.e. a foreign compound rather than an endogenous mediator) has to combine in order to elicit its specific effect. For example, the voltage-sensitive sodium channel is sometimes referred to as the 'receptor' for **local anaesthetics** (see Ch. 42), or the enzyme dihydrofolate reductase as the 'receptor' for **methotrexate** (Ch. 49). The term *drug target*, of which receptors are one type, is preferable in this context.

In the more general context of cell biology, the term receptor is used to describe various cell surface molecules (such as T-cell receptors, integrins, Toll receptors, etc; see Ch. 6) involved in the cell-to-cell interactions that are important in immunology, cell growth, migration and differentiation, some of which are also emerging as drug targets. These receptors differ from conventional pharmacological receptors in that they respond to proteins attached to cell surfaces or extracellular structures, rather than to soluble mediators.

Various carrier proteins are often referred to as receptors, such as the *low-density lipoprotein receptor* that plays a key role in lipid metabolism (Ch. 23) and the transferrin receptor involved in iron absorption (Ch. 25). These entities have little in common with pharmacological receptors. Though quite distinct from pharmacological receptors, these proteins play an important role in the action of drugs such as *statins* (Ch. 23).

RECEPTORS IN PHYSIOLOGICAL SYSTEMS

Receptors form a key part of the system of chemical communication that all multicellular organisms use to coordinate the activities of their cells and organs. Without them, we would resemble a bucketful of amoebae.

Some fundamental properties of receptors are illustrated by the action of **adrenaline** (epinephrine) on the heart. Adrenaline first binds to a receptor protein (the *β-adrenoceptor*, see Ch. 14) that serves as a recognition site for adrenaline and other catecholamines. When it binds to the receptor, a train of reactions is initiated (see Ch. 3) leading to an increase in force and rate of the heartbeat. In the absence of adrenaline, the receptor is functionally silent. This is true of most receptors for endogenous mediators (hormones, neurotransmitters, cytokines, etc.), although there are examples (see Ch. 3) of receptors that are 'constitutively active'—that is, they exert a controlling influence even when no chemical mediator is present.

There is an important distinction between *agonists*, which 'activate' the receptors, and *antagonists*, which combine at the same site without causing activation, and block the effect of agonists on that receptor. The distinction between agonists and antagonists only exists for receptors with this type of physiological regulatory role; we cannot usefully speak of 'agonists' for the more general class of drug targets described above.

The characteristics and accepted nomenclature of pharmacological receptors are described by Neubig et al. (2003). The origins of the receptor concept and its pharmacological significance are discussed by Rang (2006).

DRUG SPECIFICITY

For a drug to be useful as either a therapeutic or a scientific tool, it must act selectively on particular cells and tissues. In other words, it must show a high degree of binding site specificity. Conversely, proteins that function as drug targets generally show a high degree of ligand specificity; they bind only molecules of a certain precise type.

These principles of binding site and ligand specificity can be clearly recognised in the actions of a mediator such as **angiotensin** (Ch. 22). This peptide acts strongly on vascular smooth muscle, and on the kidney tubule, but has very little effect on other kinds of smooth muscle or on the intestinal epithelium. Other mediators affect a quite different spectrum of cells and tissues, the pattern in each case reflecting the specific pattern of expression of the protein receptors for the various mediators. A small chemical change, such as conversion of one of the amino acids in angiotensin from L to D form, or removal of one amino acid from the chain, can inactivate the molecule altogether, because the receptor fails to bind the altered form. The complementary specificity of ligands and binding sites, which gives rise to the very exact molecular recognition properties of proteins, is central to explaining many of the phenomena of pharmacology. It is no exaggeration to say that the ability of proteins to interact in a highly selective way with other molecules—including other proteins—is the basis of living machines. Its relevance to the understanding of drug action will be a recurring theme in this book.

Finally, it must be emphasised that no drug acts with complete specificity. Thus tricyclic antidepressant drugs (Ch. 46) act by blocking monoamine transporters but are notorious for producing side effects (e.g. dry mouth) related to their ability to block various receptors. In general, the lower the potency of a drug and the higher the dose needed, the more likely it is that sites of action other than the primary one will assume significance. In clinical terms, this is often associated with the appearance of unwanted side effects, of which no drug is free.

Since the 1970s, pharmacological research has succeeded in identifying the protein targets of many different types of drug. Drugs such as opioid analgesics (Ch. 41), cannabinoids (Ch. 18) and benzodiazepine tranquillisers (Ch.43),

whose actions had been described in exhaustive detail for many years, are now known to target well-defined receptors, which have been fully characterised by gene-cloning techniques (see Ch. 3).

RECEPTOR CLASSIFICATION

▼ Where the action of a drug can be associated with a particular receptor, this provides a valuable means for classification and refinement in drug design. For example, pharmacological analysis of the actions of histamine (see Ch. 17) showed that some of its effects (the H_1 effects, such as smooth muscle contraction) were strongly antagonised by the competitive histamine antagonists then known. Black and his colleagues suggested in 1970 that the remaining actions of histamine, which included its stimulant effect on gastric secretion, might represent a second class of histamine receptor (H_2). Testing a number of histamine analogues, they found that some were selective in producing H_2 effects, with little H_1 activity. By analysing which parts of the histamine molecule conferred this type of specificity, they were able to develop selective H_2 antagonists, which proved to be potent in blocking gastric acid secretion, a development of major therapeutic significance (Ch. 29). Two further types of histamine receptor (H_3 and H_4) were recognised later.

Receptor classification based on pharmacological responses continues to be a valuable and widely used approach. Newer experimental approaches have produced other criteria on which to base receptor classification. The direct measurement of ligand binding to receptors (see below) has allowed many new receptor subtypes to be defined that could not easily be distinguished by studies of drug effects. Molecular cloning (see Ch. 3) provided a completely new basis for classification at a much finer level of detail than can be reached through pharmacological analysis. Finally, analysis of the biochemical pathways that are linked to receptor activation (see Ch. 3) provides yet another basis for classification.

The result of this data explosion was that receptor classification suddenly became much more detailed, with a proliferation of receptor subtypes for all the main types of ligand. As alternative molecular and biochemical classifications began to spring up that were incompatible with the accepted pharmacologically defined receptor classes, the International Union of Pharmacological Sciences (IUPHAR) convened expert working groups to produce agreed receptor classifications for the major types, taking into account the pharmacological, molecular and biochemical information available. These wise people have a hard task; their conclusions will be neither perfect nor final but are essential to ensure a consistent terminology. To the student, this may seem an arcane exercise in taxonomy, generating much detail but little illumination. There is a danger that the tedious lists of drug names, actions and side effects that used to burden the subject will be replaced by exhaustive tables of receptors, ligands and transduction pathways. In this book, we have tried to avoid detail for its own sake and include only such information on receptor classification as seems interesting in its own right or is helpful in explaining the actions of important drugs. A comprehensive IUPHAR database of known receptor classes is available (see http://www.iuphar-db.org), as well as a regularly updated summary (Alexander et al., 2009).

DRUG–RECEPTOR INTERACTIONS

Occupation of a receptor by a drug molecule may or may not result in *activation* of the receptor. By activation, we mean that the receptor is affected by the bound molecule in such a way as to elicit a tissue response. The molecular mechanisms associated with receptor activation are discussed in Chapter 3. Binding and activation represent two distinct steps in the generation of the receptor-mediated response by an agonist (Fig. 2.1). If a drug binds to the receptor without causing activation and thereby prevents the agonist from binding, it is termed a *receptor antagonist*. The tendency of a drug to bind to the receptors is governed by its *affinity*, whereas the tendency for it, once bound, to activate the receptor is denoted by its *efficacy*. These terms are defined more precisely below (p. 13). Drugs of high potency generally have a high affinity for the receptors and thus occupy a significant proportion of the receptors even at low concentrations. Agonists also possess significant efficacy, whereas antagonists, in the simplest case, have zero efficacy. Drugs with intermediate levels of efficacy, such that even when 100% of the receptors are occupied the tissue response is submaximal, are known as *partial agonists*, to distinguish them from *full agonists*, the efficacy of which is sufficient that they can elicit a maximal tissue response. These concepts, though clearly an oversimplified description of events at the molecular level (see Ch. 3), provide a useful basis for characterising drug effects.

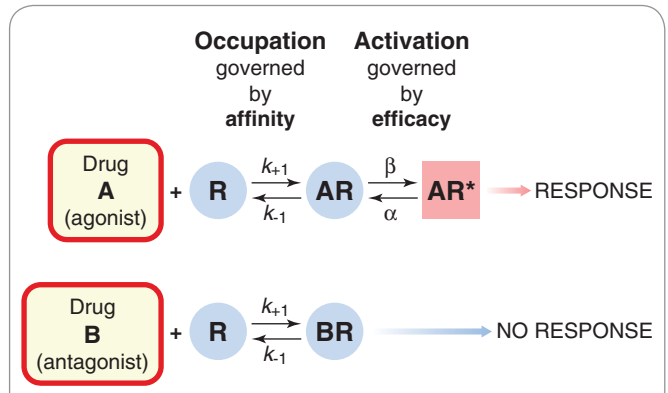

Fig. 2.1 The distinction between drug binding and receptor activation. Ligand A is an agonist, because when it is bound, the receptor (R) tends to become activated, whereas ligand B is an antagonist, because binding does not lead to activation. The rate constants k_{+1}, k_{-1}, α and β for the binding and activation steps vary between drugs. For an antagonist, which does not activate the receptor, β = 0.

We now discuss certain aspects in more detail, namely drug binding, agonist concentration–effect curves, competitive antagonism, partial agonists and the nature of efficacy. Understanding these concepts at a qualitative level is sufficient for many purposes, but for more detailed analysis a quantitative formulation is needed (see p. 16).

THE BINDING OF DRUGS TO RECEPTORS

▼ The binding of drugs to receptors can often be measured directly by the use of drug molecules (agonists or antagonists) labelled with one or more radioactive atoms (usually ^{3}H, ^{14}C or ^{125}I). The usual procedure is to incubate samples of the tissue (or membrane fragments) with various concentrations of radioactive drug until equilibrium is reached. The bound radioactivity is measured after removal of the supernatant.

In such experiments, there is invariably a certain amount of 'non-specific binding' (i.e. drug taken up by structures other than receptors), which obscures the specific component and needs to be kept to a minimum. The amount of non-specific binding is estimated by measuring the radioactivity taken up in the presence of a saturating concentration of a (non-radioactive) ligand that inhibits completely the binding of the radioactive drug to the receptors, leaving behind the non-specific component. This is then subtracted from the total binding to give an estimate of specific binding (Fig. 2.2). The *binding curve* (Fig. 2.2B) defines the relationship between concentration and the amount of drug bound (B), and in most cases it fits well to the relationship predicted theoretically (see Fig. 2.11, below), allowing the affinity of the drug for the receptors to be estimated, as well as the *binding capacity* (B_{max}), representing the density of receptors in the

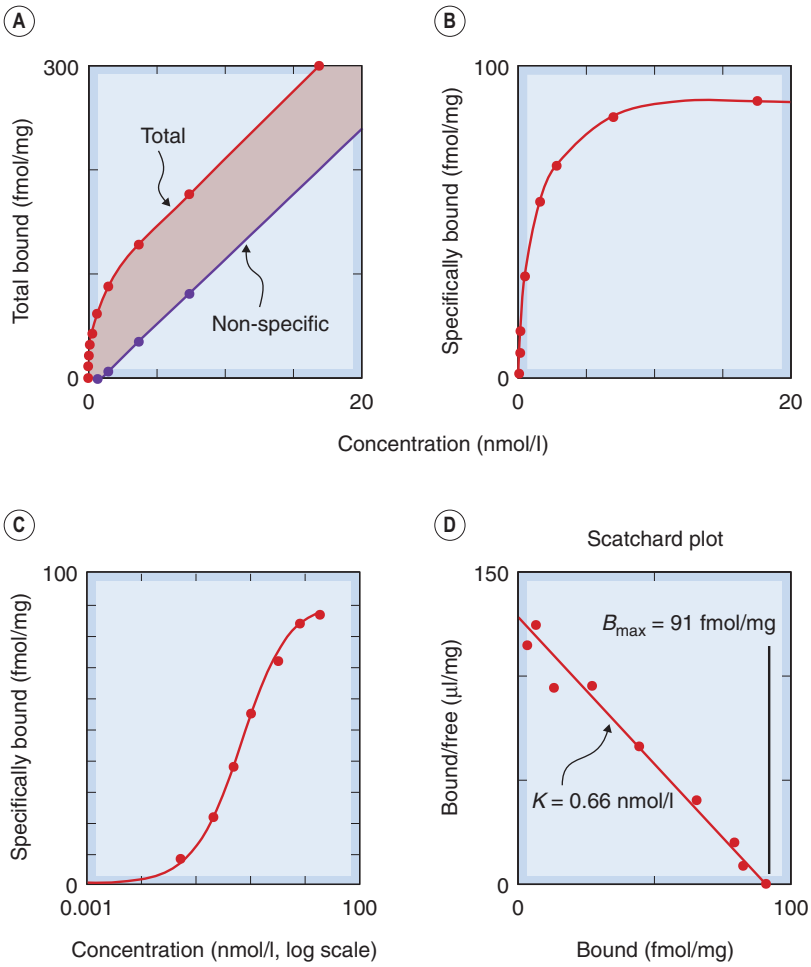

Fig. 2.2 Measurement of receptor binding (β adrenoceptors in cardiac cell membranes). The ligand was [^{3}H]-cyanopindolol, a derivative of pindolol (see Ch. 14). [**A**] Measurements of total and non-specific binding at equilibrium. Non-specific binding is measured in the presence of a saturating concentration of a non-radioactive β-adrenoceptor agonist, which prevents the radioactive ligand from binding to β adrenoceptors. The difference between the two lines represents specific binding. [**B**] Specific binding plotted against concentration. The curve is a rectangular hyperbola (equation 2.5). [**C**] Specific binding plotted against concentration (log scale). The sigmoid curve is a *logistic curve* representing the logarithmic scaling of the rectangular hyperbola plotted in panel B. [**D**] Scatchard plot (equation 2.7). This gives a straight line from which the binding parameters K and B_{max} can be calculated.

tissue. When combined with functional studies, binding measurements have proved very valuable. It has, for example, been confirmed that the *spare receptor hypothesis* (p. 13) for muscarinic receptors in smooth muscle is correct; agonists are found to bind, in general, with rather low affinity, and a maximal biological effect occurs at low receptor occupancy. It has also been shown, in skeletal muscle and other tissues, that denervation leads to an increase in the number of receptors in the target cell, a finding that accounts, at least in part, for the phenomenon of *denervation supersensitivity*. More generally, it appears that receptors tend to increase in number, usually over the course of a few days, if the relevant hormone or transmitter is absent or scarce, and to decrease in number if it is in excess, a process of adaptation to drugs or hormones resulting from continued administration (see p. 15).

Non-invasive imaging techniques, such as *positron emission tomography* (PET), can also be used to investigate the distribution of receptors in structures such as the living human brain. This technique has been used, for example, to measure the degree of dopamine receptor blockade produced by antipsychotic drugs in the brains of schizophrenic patients (see Ch. 45).

Binding curves with agonists often reveal an apparent heterogeneity among receptors. For example, agonist binding to muscarinic receptors (Ch. 13) and also to β-adrenoceptors (Ch. 14) suggests at least two populations of binding sites with different affinities. This may be because the receptors can exist either unattached or coupled within the membrane to another macromolecule, the G-protein (see Ch. 3), which constitutes part of the transduction system through which the receptor exerts its regulatory effect. Antagonist binding does not show this complexity, probably because antagonists, by their nature, do not lead to the secondary event of G-protein coupling. Because agonist binding results in activation, agonist affinity has proved to be a surprisingly elusive concept, about which afficionados love to argue.

THE RELATION BETWEEN DRUG CONCENTRATION AND EFFECT

Although binding can be measured directly, it is usually a biological response, such as a rise in blood pressure, contraction or relaxation of a strip of smooth muscle in an organ bath, the activation of an enzyme, or a behavioural response, that we are interested in, and this is often plotted as a *concentration–effect curve* (in vitro) or *dose–response curve* (in vivo), as in Figure 2.3. Such curves allow us to estimate *the maximal response* that the drug can produce (E_{max}), and the concentration or dose needed to produce a 50% maximal response (EC_{50} or ED_{50}), parameters that are useful for comparing the potencies of different drugs that produce qualitatively similar effects (see Ch. 7). Although they look similar to the binding curve in Figure 2.2C, concentration–effect curves cannot be used to measure the affinity of agonist drugs for their receptors, because the physiological response produced is not, as a rule, directly proportional to receptor occupancy. For an integrated physiological response, such as a rise in arterial blood pressure produced by adrenaline (epinephrine), many factors interact. Adrenaline (see Ch. 14) increases cardiac output and constricts some blood vessels while dilating others, and the change in arterial pressure itself evokes a superimposed reflex

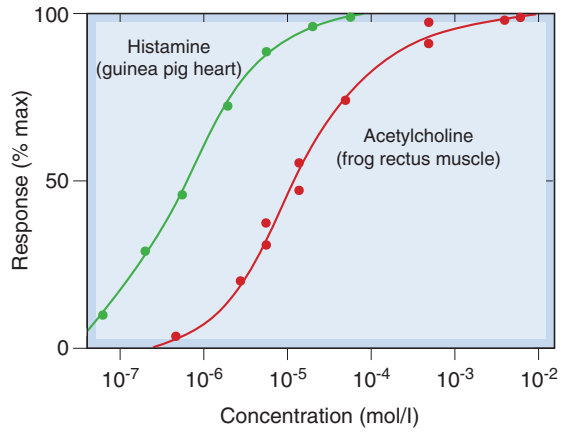

Fig. 2.3 Experimentally observed concentration–effect curves. Although the lines, drawn according to the binding equation 2.5, fit the points well, such curves do not give correct estimates of the affinity of drugs for receptors. This is because the relationship between receptor occupancy and response is usually non-linear.

response. The final effect is clearly not a direct measure of receptor occupancy in this instance, and the same is true of most drug-induced effects.

In interpreting concentration–effect curves, it must be remembered that the concentration of the drug at the receptors may differ from the known concentration in the bathing solution. Agonists may be subject to rapid enzymic degradation or uptake by cells as they diffuse from the surface towards their site of action, and a steady state can be reached in which the agonist concentration at the receptors is very much less than the concentration in the bath. In the case of acetylcholine, for example, which is hydrolysed by cholinesterase present in most tissues (see Ch. 13), the concentration reaching the receptors can be less than 1% of that in the bath, and an even bigger difference has been found with noradrenaline (norepinephrine), which is avidly taken up by sympathetic nerve terminals in many tissues (Ch. 14). Thus, even if the concentration–effect curve, as in Figure 2.3, looks just like a facsimile of the binding curve (Fig. 2.2C), it cannot be used directly to determine the affinity of the agonist for the receptors.

COMPETITIVE ANTAGONISM

Though one drug can inhibit the response to another in several ways (see below), competition at the receptor level is particularly important, both in the laboratory and in the clinic, because of the high potency and specificity that can be achieved.

In the presence of a competitive antagonist, the agonist occupancy at a given agonist concentration is reduced, because the receptor can accommodate only one molecule at a time. However, because the two are in competition, raising the agonist concentration can restore the agonist occupancy (and hence the tissue response). The antagonism is therefore said to be *surmountable*, in contrast to other types of antagonism (see below) where increasing the agonist concentration fails to overcome the blocking effect. A simple theoretical analysis (see p. 17) predicts that in the presence of a fixed concentration of the antagonist, the log concentration–effect curve for the agonist will be shifted to

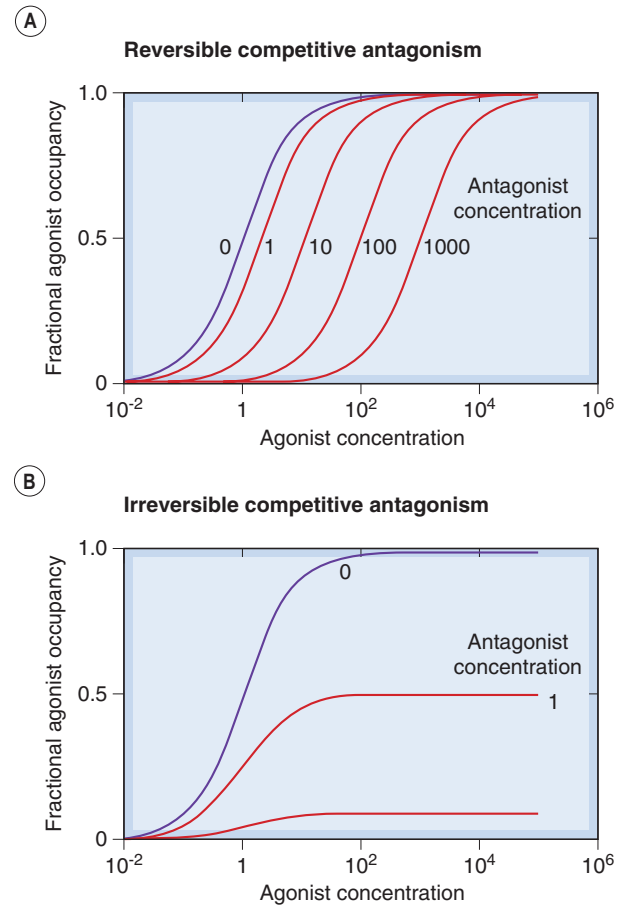

Fig. 2.4 Hypothetical agonist concentration–occupancy curves in the presence of reversible [A] and irreversible [B] competitive antagonists. The concentrations are normalised with respect to the equilibrium constants, *K* (i.e. 1.0 corresponds to a concentration equal to *K* and results in 50% occupancy). Note that increasing the agonist concentration overcomes the effect of a reversible antagonist (i.e. the block is surmountable), so that the maximal response is unchanged, whereas the effect of an irreversible antagonist is unsurmountable and full agonist occupancy cannot be achieved.

the right, without any change in slope or maximum—the hallmark of competitive antagonism (Fig. 2.4A). The shift is expressed as a *dose ratio*, *r*, (the ratio by which the agonist concentration has to be increased in the presence of the antagonist in order to restore a given level of response). Theory predicts that the dose ratio increases linearly with the concentration of the antagonist (see p. 17). These predictions are often borne out in practice (see Fig. 2.5), and examples of competitive antagonism are very common in pharmacology. The surmountability of the block by the antagonist may be important in practice, because it allows the functional effect of the agonist to be restored by an increase in concentration. With other types of antagonism (see below), the block is usually insurmountable.

The salient features of competitive antagonism are:

- shift of the agonist log concentration–effect curve to the right, without change of slope or maximum
- linear relationship between agonist dose ratio and antagonist concentration
- evidence of competition from binding studies.

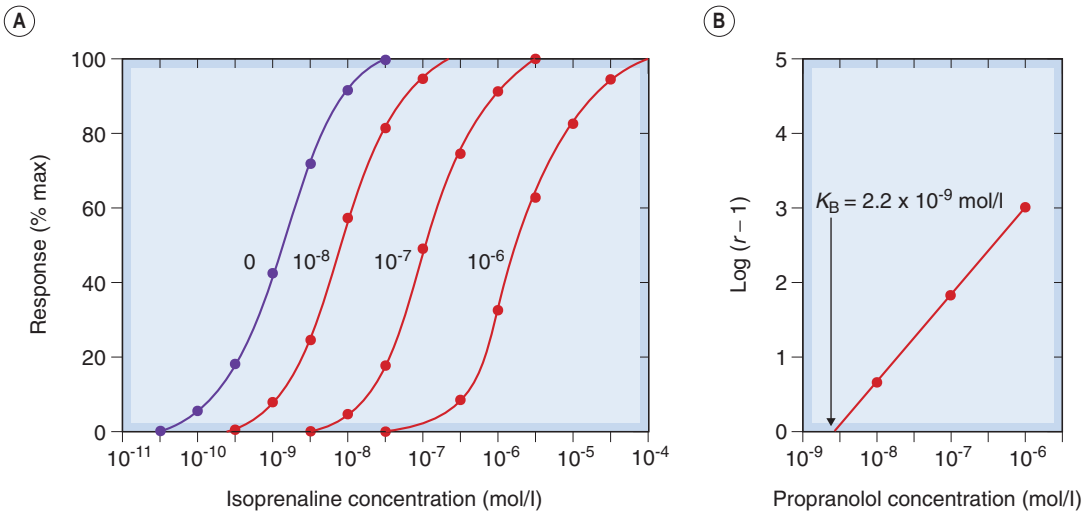

Fig. 2.5 Competitive antagonism of isoprenaline by propranolol measured on isolated guinea pig atria. [A] Concentration–effect curves at various propranolol concentrations (indicated on the curves). Note the progressive shift to the right without a change of slope or maximum. [B] Schild plot (equation 2.10). The equilibrium constant (K) for propranolol is given by the abscissal intercept, 2.2×10^{-9} mol/l. (Results from Potter L T 1967 Uptake of propranolol by isolated guinea-pig atria. J Pharmacol Exp Ther 55: 91–100.)

Competitive antagonism is the most direct mechanism by which one drug can reduce the effect of another (or of an endogenous mediator), and several examples are listed in Table 3.1.

▼ The characteristics of reversible competitive antagonism described above reflect the fact that the rate of dissociation of the antagonist molecules is sufficiently high that a new equilibrium is rapidly established on addition of the agonist. In effect, the agonist is able to displace the antagonist molecules from the receptors, although it cannot, of course, evict a bound antagonist molecule. Displacement occurs because, by occupying a proportion of the vacant receptors, the agonist reduces the rate of association of the antagonist molecules; consequently, the rate of dissociation temporarily exceeds that of association, and the overall antagonist occupancy falls.

Irreversible, or *non-equilibrium*, competitive antagonism occurs when the antagonist dissociates very slowly, or not at all, from the receptors, with the result that no change in the antagonist occupancy takes place when the agonist is applied.[2]

The predicted effects of reversible and irreversible antagonists are compared in Figure 2.4.

Competitive antagonism

- Reversible competitive antagonism is the commonest and most important type of antagonism; it has two main characteristics:
  - in the presence of the antagonist, the agonist log concentration–effect curve is shifted to the right without change in slope or maximum, the extent of the shift being a measure of the *dose ratio*
  - the dose ratio increases linearly with antagonist concentration; the slope of this line is a measure of the affinity of the antagonist for the receptor.
- Antagonist affinity, measured in this way, is widely used as a basis for receptor classification.

[2]This type of antagonism is sometimes called non-competitive, but that term is ambiguous and best avoided in this context.

In some cases (Fig. 2.6A), the theoretical effect is accurately reproduced, but the distinction between reversible and irreversible competitive antagonism (or even non-competitive antagonism; see below) is not always so clear. This is because of the phenomenon of spare receptors (see p. 13); if the agonist occupancy required to produce a maximal biological response is very small (say 1% of the total receptor pool), then it is possible to block irreversibly nearly 99% of the receptors without reducing the maximal response. The effect of a lesser degree of antagonist occupancy will be to produce a parallel shift of the log concentration–effect curve that is indistinguishable from reversible competitive antagonism (Fig. 2.6B).

Irreversible competitive antagonism occurs with drugs that possess reactive groups that form covalent bonds with the receptor. These are mainly used as experimental tools for investigating receptor function, and few are used clinically. Irreversible enzyme inhibitors that act similarly are clinically used, however, and include drugs such as aspirin (Ch. 26), omeprazole (Ch. 29) and monoamine oxidase inhibitors (Ch. 46).

ALLOSTERIC EFFECTS

▼ In addition to the agonist binding site, to which competitive antagonists bind, receptor proteins possess many other (allosteric) binding sites (see Ch. 3) through which drugs can influence receptor function in various ways, increasing or decreasing the affinity of agonists for the agonist binding site, or by modifying efficacy. Depending on the direction of the effect, the ligands may be allosteric antagonists or allosteric facilitators of the agonist effect, and the effect may be to alter the slope and maximum of the agonist log concentration–effect curve. This type of allosteric modulation of receptor function has attracted much attention recently (see review by May et al., 2007), and may prove to be more widespread than previously envisaged. Well-known examples of allosteric facilitation include the action of glycine (allosteric ligand) on glutamate receptors and of benzodiazepines on $GABA_A$ receptors (Ch. 37).

PARTIAL AGONISTS AND THE CONCEPT OF EFFICACY

So far, we have considered drugs either as agonists, which in some way activate the receptor when they occupy it, or

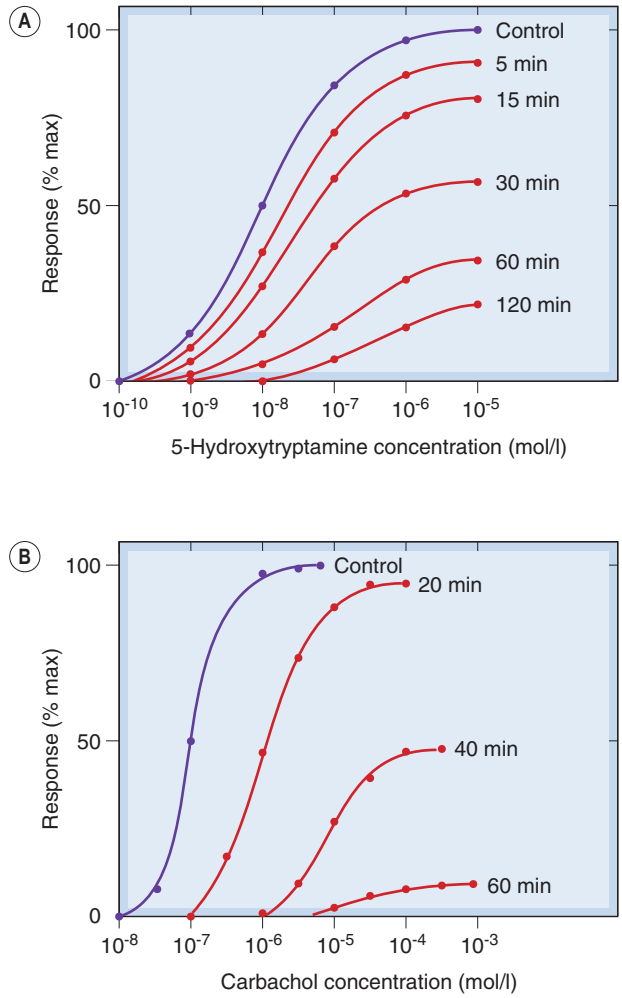

Fig. 2.6 Effects of irreversible competitive antagonists on agonist concentration–effect curves. [**A**] Rat stomach smooth muscle responding to 5-hydroxytryptamine at various times after addition of methysergide (10^{-9} mol/l). [**B**] Rabbit stomach responding to carbachol at various times after addition of dibenamine (10^{-5} mol/l). ([**A**] After Frankhuijsen A L, Bonta I L 1974 Eur J Pharmacol 26: 220; [**B**] After Furchgott R F 1965 Adv Drug Res 3: 21.)

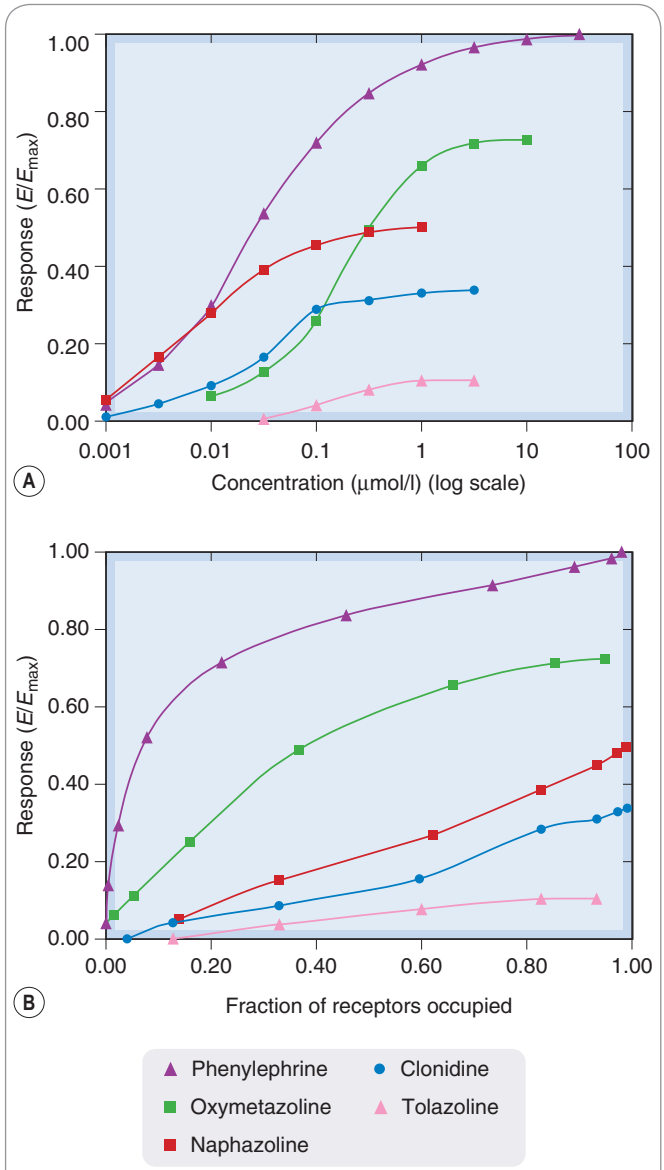

Fig. 2.7 Partial agonists. [**A**] Log concentration–effect curves for a series of α-adrenoceptor agonists causing contraction of an isolated strip of rabbit aorta. **Phenylephrine** is a full agonist. The others are partial agonists with different efficacies. [**B**] The relationship between response and receptor occupancy for the series. Note that the full agonist, phenylephrine, produces a near-maximal response when only about half the receptors are occupied, whereas partial agonists produce submaximal responses even when occupying all of the receptors. The efficacy of **tolazoline** is so low that it is classified as an α-adrenoceptor antagonist (see Ch. 14). In these experiments, receptor occupancy was not measured directly, but was calculated from pharmacological estimates of the equilibrium constants of the drugs. (Data from Ruffolo et al. 1979 J Pharmacol Exp Ther 209: 429–436.)

as antagonists, which cause no activation. However, the ability of a drug molecule to activate the receptor is actually a graded, rather than an all-or-nothing, property. If a series of chemically related agonist drugs acting on the same receptors is tested on a given biological system, it is often found that the largest response that can be produced by the drug in high concentration differs from one drug to another. Some compounds (known as *full agonists*) can produce a maximal response (the largest response that the tissue is capable of giving), whereas others (*partial agonists*) can produce only a submaximal response. Figure 2.7A shows concentration effect curves for several α-adrenoceptor agonists (see Ch. 14) which cause contraction of isolated strips of rabbit aorta. The full agonist **phenylephrine** produced the maximal effect of which the tissue was capable; the other compounds could only produce submaximal responses and are partial agonists. The difference between full and partial agonists lies in the relationship between receptor occupancy and response. In the experiment shown in Figure 2.7 it was possible to estimate the affinity of the various drugs for the receptor, and hence (based on the theoretical model described later; p. 17) to calculate the fraction of receptors occupied (known as *occupancy*) as a function of drug concentration. Plots of response as a function of occupancy for the different compounds are shown in Figure 2.7B, showing that for partial agonists the

response at a given level of occupancy is less than for full agonists. The weakest partial agonist, **tolazoline**, produces a barely detectable response even at 100% occupancy, and is usually classified as a *competitive antagonist* (see p. 10 and Ch. 14).

These differences can be expressed quantitatively in terms of *efficacy* (e), a parameter originally defined by Stephenson (1956) that describes the 'strength' of the agonist–receptor complex in evoking a response of the tissue. In the simple scheme shown in Figure 2.1, efficacy describes the tendency of the drug–receptor complex to adopt the active (AR*), rather than the resting (AR) state. A drug with zero efficacy (e = 0) has no tendency to cause receptor activation, and causes no tissue response. A drug with efficacy[3] is a full agonist, while partial agonists lie in between.

▼ Subsequently, it was appreciated that characteristics of the tissue (e.g. the number of receptors that it possesses and the nature of the coupling between the receptor and the response; see Ch. 3), as well as of the drug itself, were important, and the concept of *intrinsic efficacy* was developed (see Jenkinson, 1996; Kenakin, 1997), which can account for a number of anomalous findings. For example, depending on tissue characteristics, a given drug may appear as a full agonist in one tissue but a partial agonist in another, and drugs may differ in their relative agonist potencies in different tissues, though the receptor is the same.

It would be nice to be able to explain what efficacy means in physical terms, and to understand why one drug may be an agonist while another, chemically very similar, is an antagonist. We are beginning to understand the molecular events underlying receptor activation (described in Ch. 3) but can still give no clear answer to the question of why some ligands are agonists and some are antagonists, although the simple theoretical two-state model described below provides a useful starting point.

Despite its uncertain mechanistic basis, efficacy is a concept of great practical importance. **Adrenaline** (epinephrine) and **propranolol** (see Ch. 14) have comparable affinities for the β-adrenoceptor but differ in efficacy. Woebetide the doctor—and the student, for that matter—who confuses them. Efficacy matters!

CONSTITUTIVE RECEPTOR ACTIVATION AND INVERSE AGONISTS

▼ Although we are accustomed to thinking that receptors are activated only when an agonist molecule is bound, there are examples (see De Ligt et al., 2000) where an appreciable level of activation may exist even when no ligand is present. These include receptors for benzodiazepines (see Ch. 43), cannabinoids (Ch. 18), serotonin (Ch. 15) and several other mediators. Furthermore, receptor mutations occur—either spontaneously, in some disease states (see Bond & Ijzerman, 2006) or experimentally created (see Ch. 4)—that result in appreciable activation in the absence of any ligand (*constitutive activation*). Resting activity may be too low to have any effect under normal conditions but become evident if receptors are overexpressed, a phenomenon clearly demonstrated for β-adrenoceptors (see Bond et al., 1995), a result that may prove to have major pathophysiological implications. Thus if, say, 1% of receptors are active in the absence of any agonist, in a normal cell expressing perhaps 10 000 receptors, only 100 will be active. Increasing the expression level 10-fold will result in 1000 active receptors, producing a significant effect. Under these conditions, it may be possible for a ligand to reduce the level of constitutive activation; such drugs are known as *inverse agonists* (Fig. 2.8; see De Ligt et al., 2000) to distinguish them from *neutral antagonists*, which do not by themselves affect the level of activation. Inverse agonists can be regarded as drugs with negative efficacy, to distinguish them from agonists (positive efficacy) and neutral antagonists (zero efficacy). New examples of constitutively active receptors and inverse agonists are emerging with increasing frequency (mainly among G-protein-coupled receptors; Seifert & Wenzel-Seifert, 2002). In theory, an inverse agonist, by silencing constitutively active receptors, should be more effective than a neutral antagonist in disease states associated with receptor mutations or with receptor-directed autoantibodies that result in enhanced constitutive activation. These include certain types of hyperthyroidism, precocious puberty and parathyroid diseases (see Bond & Ijzerman, 2006). This remains to be verified, but it turns out that most of the receptor antagonists in clinical use are actually inverse agonists when tested in systems showing constitutive receptor activation. However, most receptors—like cats—show a preference for the inactive state, and for these there is no practical difference between a competitive antagonist and an inverse agonist. It remains to be seen whether the inverse agonist principle will prove to be generally important in therapeutics, but interest is running high. So far, nearly all the examples come from the family of G-protein-coupled receptors (see Ch. 3 and the review by Costa & Cotecchia, 2005), and it is not clear whether similar phenomena occur with other receptor families.

The following section describes a simple model that explains full, partial and inverse agonism in terms of the relative affinity of different ligands for the resting and activated states of the receptor.

The two-state receptor model

▼ As illustrated in Figure 2.1, agonists and antagonists both bind to receptors, but only agonists activate them. How can we express this difference, and account for constitutive activity, in theoretical terms? The two-state model (Fig. 2.9) provides a simple but useful approach.

As shown in Figure 2.1, we envisage that the occupied receptor can switch from its 'resting' (R) state to an activated (R*) state, R* being favoured by binding of an agonist but not an antagonist molecule.

As described above, receptors may show constitutive activation (i.e. the R* conformation can exist without any ligand being bound), so the added drug encounters an equilibrium mixture of R and R* (Fig. 2.9). If it has a higher affinity for R* than for R, the drug will cause a shift of the equilibrium towards R* (i.e. it will promote activation and be classed as an agonist). If its preference for R* is very large, nearly all the occupied receptors will adopt the R* conformation and the drug will be a full agonist (positive efficacy); if it shows only a modest degree of selectivity for R* (say 5–10-fold), a smaller proportion of occupied receptors will adopt the R* conformation and it will be a partial agonist; if it shows no preference, the prevailing R:R* equilibrium will not be disturbed and the drug will be a neutral antagonist (zero efficacy), whereas if it shows selectivity for R it will shift the equilibrium towards R and be an inverse agonist (negative efficacy). We can therefore think of efficacy as a property determined by the relative affinity of a ligand for R and R*, a formulation known as the *two-state model*, which is useful in that it puts a physical interpretation on the otherwise mysterious meaning of efficacy, as well as accounting for the existence of inverse agonists.

A major problem with the two-state model is that, as we now know, receptors are not actually restricted to two distinct states but have much greater conformational flexibility, so that there is more than one inactive and active conformation. The different conformations that they can adopt may be preferentially stabilised by different ligands, and may produce different functional effects by activating different signal transduction pathways (see Ch. 3). Redefining efficacy for such a multistate model is difficult, however, and requires a more complicated state transition model than that described here.

SPARE RECEPTORS

▼ Stephenson (1956), studying the actions of acetylcholine analogues in isolated tissues, found that many full agonists were capable of

[3]In Stephenson's formulation, efficacy is the reciprocal of the occupancy needed to produce a 50% maximal response, thus e = 25 implies that a 50% maximal response occurs at 4% occupancy. There is no theoretical upper limit to efficacy.

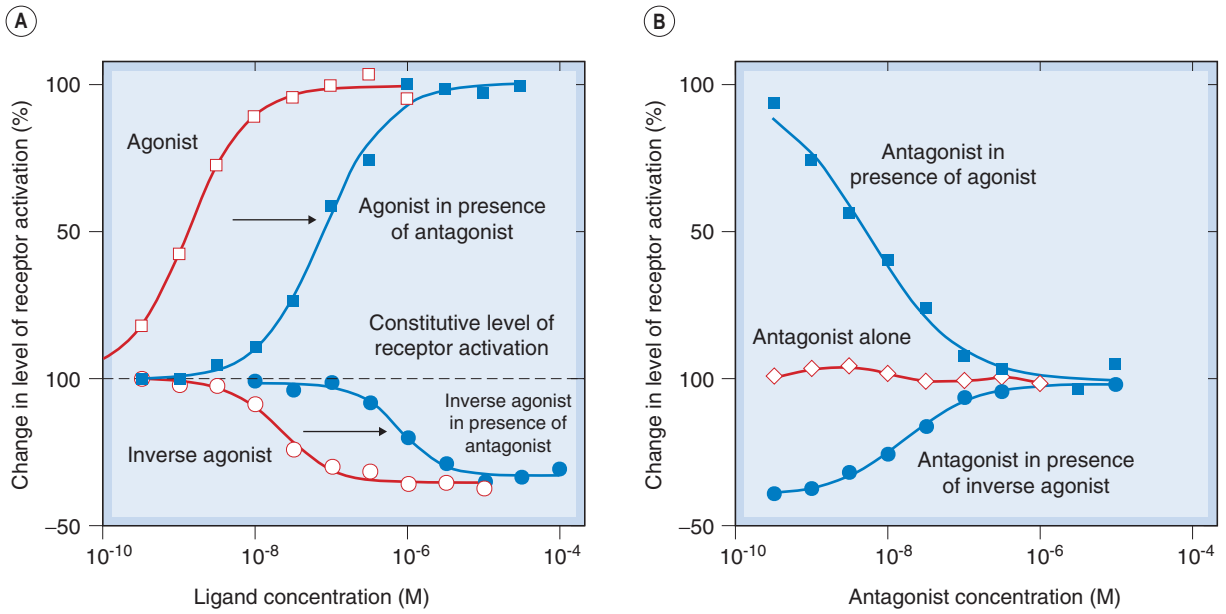

Fig. 2.8 Inverse agonism. The interaction of a competitive antagonist with normal and inverse agonists in a system that shows receptor activation in the absence of any added ligands (constitutive activation). [**A**] The degree of receptor activation (vertical scale) increases in the presence of an agonist (open squares) and decreases in the presence of an inverse agonist (open circles). Addition of a competitive antagonist shifts both curves to the right (closed symbols). [**B**] The antagonist on its own does not alter the level of constitutive activity (open symbols), because it has equal affinity for the active and inactive states of the receptor. In the presence of an agonist (closed squares) or an inverse agonist (closed circles), the antagonist restores the system towards the constitutive level of activity. These data (reproduced with permission from Newman-Tancredi A et al. 1997 Br J Pharmacol 120: 737–739) were obtained with cloned human 5-hydroxytryptamine (5-HT) receptors expressed in a cell line. (Agonist, 5-carboxamidotryptamine; inverse agonist, spiperone; antagonist, WAY 100635; ligand concentration [M = mol/l]; see Ch. 15 for information on 5-HT receptor pharmacology.)

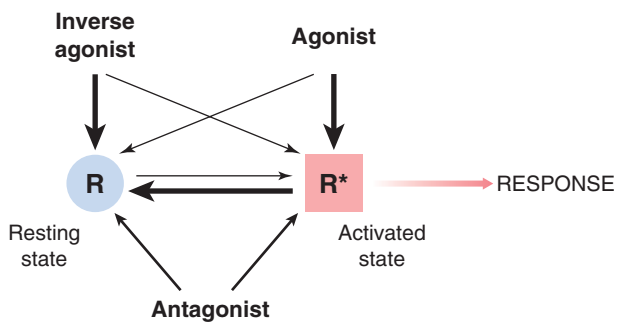

Fig. 2.9 The two-state model. The receptor is shown in two conformational states, 'resting' (R) and 'activated' (R*), which exist in equilibrium. Normally, when no ligand is present, the equilibrium lies far to the left, and few receptors are found in the R* state. For constitutively active receptors, an appreciable proportion of receptors adopt the R* conformation in the absence of any ligand. Agonists have higher affinity for R* than for R, so shift the equilibrium towards R*. The greater the relative affinity for R* with respect to R, the greater the efficacy of the agonist. An inverse agonist has higher affinity for R than for R* and so shifts the equilibrium to the left. A 'neutral' antagonist has equal affinity for R and R* so does not by itself affect the conformational equilibrium but reduces by competition the binding of other ligands.

Agonists, antagonists and efficacy

- Drugs acting on receptors may be *agonists* or *antagonists*.
- Agonists initiate changes in cell function, producing effects of various types; antagonists bind to receptors without initiating such changes.
- Agonist potency depends on two parameters: *affinity* (i.e. tendency to bind to receptors) and *efficacy* (i.e. ability, once bound, to initiate changes that lead to effects).
- For antagonists, efficacy is zero.
- *Full agonists* (which can produce maximal effects) have high efficacy; *partial agonists* (which can produce only submaximal effects) have intermediate efficacy.
- According to the two-state model, efficacy reflects the relative affinity of the compound for the resting and activated states of the receptor. Agonists show selectivity for the activated state; antagonists show no selectivity. This model, although helpful, fails to account for the complexity of agonist action.
- *Inverse agonists* show selectivity for the resting state of the receptor, this being of significance only in situations where the receptors show *constitutive activity*.

eliciting maximal responses at very low occupancies, often less than 1%. This means that the mechanism linking the response to receptor occupancy has a substantial reserve capacity. Such systems may be said to possess *spare receptors*, or a receptor reserve. This is common with drugs that elicit smooth muscle contraction but less so for other types of receptor-mediated response, such as secretion, smooth muscle relaxation or cardiac stimulation, where the effect is more nearly proportional to receptor occupancy. The existence of spare receptors does not imply any functional subdivision of the receptor pool, but merely that the pool is larger than the number needed to evoke a full response. This surplus of receptors over the number actually needed might seem a wasteful biological arrangement. It means, however, that a given number of agonist–receptor complexes, corresponding to a given level of biological response, can be reached with a lower concentration of hormone or neurotransmitter than would be the case if fewer receptors were provided. Economy of hormone or transmitter secretion is thus achieved at the expense of providing more receptors.

DRUG ANTAGONISM AND SYNERGISM

Frequently, the effect of one drug is reduced or enhanced in the presence of another. Competitive antagonism, described earlier, is a common and important mechanism, which will be encountered frequently in this book. However, a variety of other mechanisms can account for inhibitory or facilitatory interactions between drugs. The following list includes the most important ones:

- chemical antagonism
- pharmacokinetic antagonism
- block of receptor–effector linkage
- physiological antagonism.

CHEMICAL ANTAGONISM

Chemical antagonism refers to the uncommon situation where the two substances combine in solution; as a result, the effect of the active drug is lost. Examples include the use of chelating agents (e.g. **dimercaprol**) that bind to heavy metals and thus reduce their toxicity, and the use of the neutralising antibody **infliximab** which has an anti-inflammatory action due to its ability to sequester the inflammatory cytokine, tumour necrosis factor (TNF; see Ch. 17).

PHARMACOKINETIC ANTAGONISM

Pharmacokinetic antagonism describes the situation in which the 'antagonist' effectively reduces the concentration of the active drug at its site of action. This can happen in various ways. The rate of metabolic degradation of the active drug may be increased (e.g. the reduction of the anticoagulant effect of **warfarin** when an agent that accelerates its hepatic metabolism, such as **phenobarbital**, is given; see Chs 9 and 56). Alternatively, the rate of absorption of the active drug from the gastrointestinal tract may be reduced, or the rate of renal excretion may be increased. Interactions of this sort, discussed in more detail in Chapter 56, are common and can be important in clinical practice.

BLOCK OF RECEPTOR–EFFECTOR LINKAGE

Non-competitive antagonism describes the situation where the antagonist blocks at some point, downstream from the receptor, the chain of events that leads to the production of a response by the agonist. For example, drugs such as **verapamil** and **nifedipine** prevent the influx of Ca^{2+} through the cell membrane (see Ch. 22) and thus block non-specifically the contraction of smooth muscle produced by other drugs. As a rule, the effect will be to reduce the slope and maximum of the agonist log concentration–response curve although it is quite possible for some degree of rightward shift to occur as well.

Types of drug antagonism

Drug antagonism occurs by various mechanisms:
- chemical antagonism (interaction in solution)
- pharmacokinetic antagonism (one drug affecting the absorption, metabolism or excretion of the other)
- competitive antagonism (both drugs binding to the same receptors); the antagonism may be reversible or irreversible
- interruption of receptor–effector linkage
- physiological antagonism (two agents producing opposing physiological effects).

PHYSIOLOGICAL ANTAGONISM

Physiological antagonism is a term used loosely to describe the interaction of two drugs whose opposing actions in the body tend to cancel each other. For example, **histamine** acts on receptors of the parietal cells of the gastric mucosa to stimulate acid secretion, while **omeprazole** blocks this effect by inhibiting the proton pump; the two drugs can be said to act as physiological antagonists.

DESENSITISATION AND TACHYPHYLAXIS

Often, the effect of a drug gradually diminishes when it is given continuously or repeatedly. *Desensitisation* and *tachyphylaxis* are synonymous terms used to describe this phenomenon, which often develops in the course of a few minutes. The term *tolerance* is conventionally used to describe a more gradual decrease in responsiveness to a drug, taking days or weeks to develop, but the distinction is not a sharp one. The term *refractoriness* is also sometimes used, mainly in relation to a loss of therapeutic efficacy. *Drug resistance* is a term used to describe the loss of effectiveness of antimicrobial or antitumour drugs (see Chs 49 and 55). Many different mechanisms can give rise to this type of phenomenon. They include:

- change in receptors
- translocation of receptors
- exhaustion of mediators
- increased metabolic degradation of the drug
- physiological adaptation
- active extrusion of drug from cells (mainly relevant in cancer chemotherapy; see Ch. 55).

CHANGE IN RECEPTORS

Among receptors directly coupled to ion channels (see Ch. 3), desensitisation is often rapid and pronounced. At the neuromuscular junction (Fig. 2.10A), the desensitised state is caused by a conformational change in the receptor, resulting in tight binding of the agonist molecule without

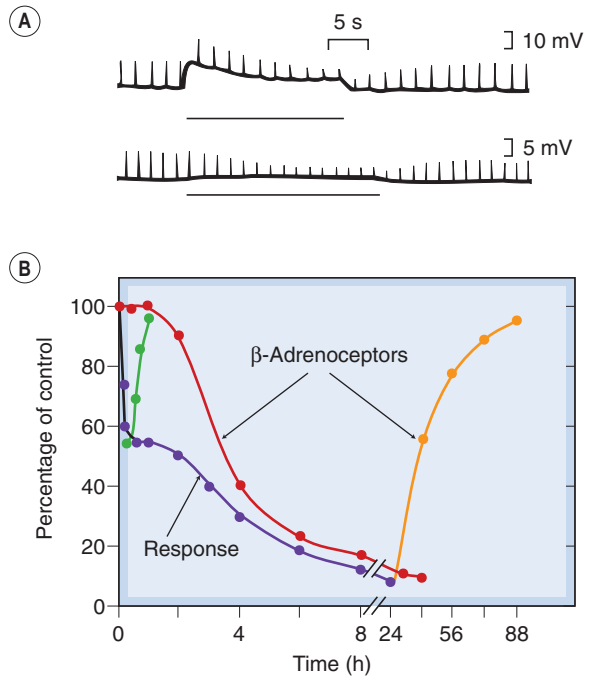

Fig. 2.10 Two kinds of receptor desensitisation. [**A**] Acetylcholine (ACh) at the frog motor endplate. Brief depolarisations (upward deflections) are produced by short pulses of ACh delivered from a micropipette. A long pulse (horizontal line) causes the response to decline with a time course of about 20 s, owing to desensitisation, and it recovers with a similar time course. [**B**] β-Adrenoceptors of rat glioma cells in tissue culture. Isoproterenol (1 μmol/l) was added at time zero, and the adenylate cyclase response and β-adrenoceptor density measured at intervals. During the early uncoupling phase, the response (blue line) declines with no change in receptor density (red line). Later, the response declines further concomitantly with disappearance of receptors from the membrane by internalisation. The green and orange lines show the recovery of the response and receptor density after the isoproterenol is washed out during the early or late phase. (From: [**A**] Katz B, Thesleff S 1957 J Physiol 138: 63; [**B**] Perkins J P 1981 Trends Pharmacol Sci 2: 326.)

the opening of the ionic channel. Phosphorylation of intracellular regions of the receptor protein is a second, slower mechanism by which ion channels become desensitised.

Most G-protein-coupled receptors (see Ch. 3) also show desensitisation (see Fig. 2.10B). Phosphorylation of the receptor interferes with its ability to activate second messenger cascades, although it can still bind the agonist molecule. The molecular mechanisms of this 'uncoupling' are described by Lefkowitz et al. (1998) and considered further in Chapter 3. This type of desensitisation usually takes a few minutes to develop, and recovers at a similar rate when the agonist is removed.

It will be realised that the two-state model in its simple form, discussed earlier, needs to be further elaborated to incorporate additional 'desensitised' states of the receptor.

TRANSLOCATION OF RECEPTORS

Prolonged exposure to agonists often results in a gradual decrease in the number of receptors expressed on the cell surface, as a result of *internalisation* of the receptors. This is shown for β-adrenoceptors in Figure 2.10B and is a slower process than the uncoupling described above. In studies on cell cultures, the number of β-adrenoceptors can fall to about 10% of normal in 8 h in the presence of a low concentration of **isoprenaline**, and recovery takes several days. Similar changes have been described for other types of receptor, including those for various peptides. The internalised receptors are taken into the cell by endocytosis of patches of the membrane, a process that also depends on receptor phosphorylation. This type of adaptation is common for hormone receptors and has obvious relevance to the effects produced when drugs are given for extended periods. It is generally an unwanted complication when drugs are used clinically, but it can be exploited. For example, **gonadotrophin-releasing hormone** (see Ch. 34) is used to treat endometriosis or prostatic cancer; given continuously, this hormone paradoxically inhibits gonadotrophin release (in contrast to the normal stimulatory effect of the physiological secretion, which is pulsatile).

EXHAUSTION OF MEDIATORS

In some cases, desensitisation is associated with depletion of an essential intermediate substance. Drugs such as **amphetamine**, which acts by releasing amines from nerve terminals (see Chs 14 and 47), show marked tachyphylaxis because the amine stores become depleted.

ALTERED DRUG METABOLISM

Tolerance to some drugs, for example **barbiturates** (Ch. 43) and **ethanol** (Ch. 48), occurs partly because repeated administration of the same dose produces a progressively lower plasma concentration, because of increased metabolic degradation. The degree of tolerance that results is generally modest, and in both of these examples other mechanisms contribute to the substantial tolerance that actually occurs. On the other hand, the pronounced tolerance to **nitrovasodilators** (see Chs 20 and 22) results mainly from decreased metabolism, which reduces the release of the active mediator, nitric oxide.

PHYSIOLOGICAL ADAPTATION

Diminution of a drug's effect may occur because it is nullified by a homeostatic response. For example, the blood pressure-lowering effect of **thiazide diuretics** is limited because of a gradual activation of the renin–angiotensin system (see Ch. 22). Such homeostatic mechanisms are very common, and if they occur slowly the result will be a gradually developing tolerance. It is a common experience that many side effects of drugs, such as nausea or sleepiness, tend to subside even though drug administration is continued. We may assume that some kind of physiological adaptation is occurring, presumably associated with altered gene expression resulting in changes in the levels of various regulatory molecules, but little is known about the mechanisms involved.

QUANTITATIVE ASPECTS OF DRUG–RECEPTOR INTERACTIONS

▼ Here we present some aspects of so-called *receptor theory*, which is based on applying the Law of Mass Action to the drug–receptor interaction and which has served well as a framework for interpreting a large body of quantitative experimental data.

The binding reaction

▼ The first step in drug action on specific receptors is the formation of a reversible drug–receptor complex, the reactions being governed by the Law of Mass Action. Suppose that a piece of tissue, such as heart muscle or smooth muscle, contains a total number of receptors, N_{tot}, for an agonist such as adrenaline. When the tissue is exposed to adrenaline at concentration x_A and allowed to come to equilibrium, a certain number, N_A, of the receptors will become occupied, and the number of vacant receptors will be reduced to $N_{tot} - N_A$. Normally, the number of adrenaline molecules applied to the tissue in solution greatly exceeds N_{tot}, so that the binding reaction does not appreciably reduce x_A. The magnitude of the response produced by the adrenaline will be related (even if we do not know exactly how) to the number of receptors occupied, so it is useful to consider what quantitative relationship is predicted between N_A and x_A. The reaction can be represented by:

$$\begin{array}{ccccc} A & + & R & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & AR \\ \text{drug} & + & \text{free receptor} & & \text{complex} \\ (x_A) & & (N_{tot} - N_A) & & (N_A) \end{array}$$

The Law of Mass Action (which states that the rate of a chemical reaction is proportional to the product of the concentrations of reactants) can be applied to this reaction.

$$\text{Rate of forward reaction} = k_{+1}x_A(N_{tot} - N_A) \quad (2.1)$$

$$\text{Rate of backward reaction} = k_{-1}N_A \quad (2.2)$$

At equilibrium, the two rates are equal:

$$k_{+1}x_A(N_{tot} - N_A) = k_{-1}N_A \quad (2.3)$$

The proportion of receptors occupied, or occupancy (p_A), is N_A/N_{tot}, which is independent of N_{tot}.

$$p_A = \frac{x_A}{x_A + k_{-1}/k_{+1}} \quad (2.4)$$

Defining the equilibrium constant for the binding reaction, $K_A = k_{-1}/k_{+1}$, equation 2.4 can be written:

$$p_A = \frac{x_A/K_A}{x_A/K_A + 1} \quad (2.5)$$

This important result is known as the Hill–Langmuir equation.[4]

The *equilibrium* constant,[5] K_A, is a characteristic of the drug and of the receptor; it has the dimensions of concentration and is numerically equal to the concentration of drug required to occupy 50% of the sites at equilibrium. (Verify from equation 2.5 that when $x_A = K_A$, $p_A = 0.5$.) The higher the affinity of the drug for the receptors, the lower will be the value of K_A. Equation 2.5 describes the relationship between occupancy and drug concentration, and it generates a characteristic curve known as a *rectangular hyperbola*, as shown in Figure 2.11A. It is common in pharmacological work to use a logarithmic scale of concentration; this converts the hyperbola to a symmetrical sigmoid curve (Fig. 2.11B).

The same approach is used to analyse data from experiments in which drug binding is measured directly (see p. 9, Fig. 2.2). In this case, the relationship between the amount bound (B) and ligand concentration (x_A) should be:

$$B = B_{max}x_A/(x_A + K_A) \quad (2.6)$$

where B_{max} is the total number of binding sites in the preparation (often expressed as pmol/mg of protein). To display the results in linear form, equation 2.6 may be rearranged to:

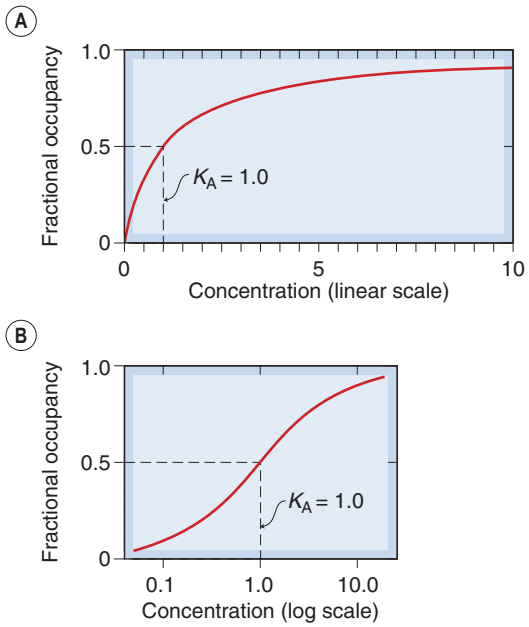

Fig. 2.11 Theoretical relationship between occupancy and ligand concentration. The relationship is plotted according to equation 2.5. [**A**] Plotted with a linear concentration scale, this curve is a rectangular hyperbola. [**B**] Plotted with a log concentration scale, it is a symmetrical sigmoid curve.

$$B/x_A = B_{max}/(K_A - B/K_A) \quad (2.7)$$

A plot of B/x_A against B (known as a *Scatchard plot*; Fig. 2.2C) gives a straight line from which both B_{max} and K_A can be estimated. Statistically, this procedure is not without problems, and it is now usual to estimate these parameters from the untransformed binding values by an iterative non-linear curve-fitting procedure.

To this point, our analysis has considered the binding of one ligand to a homogeneous population of receptors. To get closer to real-life pharmacology, we must consider (a) what happens when more than one ligand is present, and (b) how the tissue response is related to receptor occupancy.

Binding when more than one drug is present

▼ Suppose that two drugs, A and B, which bind to the same receptor with equilibrium constants K_A and K_B, respectively, are present at concentrations x_A and x_B. If the two drugs compete (i.e. the receptor can accommodate only one at a time), then, by application of the same reasoning as for the one-drug situation described above, the occupancy by drug A is given by:

$$p_A = \frac{x_A/K_A}{x_A/K + x_B/K_B + 1} \quad (2.8)$$

Comparing this result with equation 2.5 shows that adding drug B, as expected, reduces the occupancy by drug A. Figure 2.4A shows the predicted binding curves for A in the presence of increasing concentrations of B, demonstrating the shift without any change of slope or maximum that characterises the pharmacological effect of a competitive antagonist (see Fig. 2.5). The extent of the rightward shift, on a logarithmic scale, represents the ratio (r_A, given by x_A'/x_A where x_A' is the increased concentration of A) by which the concentration of A must be increased to overcome the competition by B. Rearranging 2.8 shows that

$$r_A = (x_B/K_B) + 1 \quad (2.9)$$

Thus r_A depends only on the concentration and equilibrium constant of the competing drug B, not on the concentration or equilibrium constant of A.

[4]A V Hill first published it in 1909, when he was still a medical student. Langmuir, a physical chemist working on gas adsorption, derived it independently in 1916. Both subsequently won Nobel prizes. Until recently, it was known to pharmacologists as the Langmuir equation, even though Hill deserves the credit.

[5]The equilibrium constant is sometimes called the dissociation constant. Some authors prefer to use the reciprocal of K_A, referred to as an affinity constant, in these expressions, which can cause confusion to the unwary.

If A is an agonist, and B is a competitive antagonist, and we assume that the response of the tissue will be an unknown function of p_A, then the value of r_A determined from the shift of the agonist concentration–effect curve at different antagonist concentrations can be used to estimate the equilibrium constant K_B for the antagonist. Such pharmacological estimates of r_A are commonly termed *agonist dose ratios* (more properly concentration ratios, although most pharmacologists use the older term). This simple and very useful equation (2.9) is known as the *Schild equation*, after the pharmacologist who first used it to analyse drug antagonism.

Equation 2.9 can be expressed logarithmically in the form:

$$\log(rA-1) = \log x_B - \log K_B \qquad (2.10)$$

Thus a plot of log $(r_A - 1)$ against log x_B, usually called a Schild plot (as in Fig. 2.5), should give a straight line with unit slope and an abscissal intercept equal to log K_B. Following the pH and pK notation, antagonist potency can be expressed as a pA_2 value; under conditions of competitive antagonism, $pA_2 = -\log K_B$. Numerically, pA_2 is defined as the negative logarithm of the molar concentration of antagonist required to produce an agonist dose ratio equal to 2. As with pH notation, its principal advantage is that it produces simple numbers, a pA_2 of 6.5 being equivalent to a K_B of 3.2×10^{-7} mol/l.

For competitive antagonism, r shows the following characteristics:

- It depends only on the concentration and equilibrium constant of the antagonist, and not on the size of response that is chosen as a reference point for the measurements (so long as it is submaximal).
- It does not depend on the equilibrium constant for the agonist.
- It increases linearly with x_B, and the slope of a plot of $(r_A - 1)$ against x_B is equal to $1/K_B$; this relationship, being independent of the characteristics of the agonist, should be the same for all agonists that act on the same population of receptors.

These predictions have been verified for many examples of competitive antagonism (Fig. 2.5).

In this section, we have avoided going into great detail and have oversimplified the theory considerably. As we learn more about the actual molecular details of how receptors work to produce their biological effects (see Ch. 3), the shortcomings of this theoretical treatment become more obvious. The two-state model can be incorporated without difficulty, but complications arise when we include the involvement of G-proteins (see Ch. 3) in the reaction scheme, and when we allow for the fact that receptor 'activation' is not a simple on–off switch, as the two-state model assumes, but may take different forms. It is as though the same receptor can turn on a tap or a light bulb, depending on which agonist does the talking. Despite strenuous efforts by theoreticians to allow for such possibilities, the molecules always seem to remain one step ahead. Nevertheless, this type of basic theory applied to the two-state model remains a useful basis for developing quantitative models of drug action. The book by Kenakin (1997) is recommended as an introduction, and his later review (Kenakin, 2002) presents a more elaborate theoretical approach.

Binding of drugs to receptors

- Binding of drugs to receptors necessarily obeys the *Law of Mass Action*.
- At equilibrium, receptor occupancy is related to drug concentration by the *Hill–Langmuir equation* (2.5).
- The higher the affinity of the drug for the receptor, the lower the concentration at which it produces a given level of occupancy.
- The same principles apply when two or more drugs compete for the same receptors; each has the effect of reducing the apparent affinity for the other.

THE NATURE OF DRUG EFFECTS

In discussing how drugs act in this chapter, we have focused mainly on the consequences of receptor activation. Details of the receptors and their linkage to effects at the cellular level are described in Chapter 3. We now have a fairly good understanding at this level. It is important, however, particularly when considering drugs in a therapeutic context, that their direct effects on cellular function generally lead to secondary, delayed effects, which are often highly relevant in a clinical situation in relation to both therapeutic efficacy and harmful effects (see Fig. 2.12). For example, activation of a β-adrenoceptor in the heart (see Chs 3 and 21) causes rapid changes in the functioning of the heart muscle, but also slower (minutes to hours) changes in the functional state of the receptors (e.g. desensitisation), and even slower (hours to days) changes in gene expression that produce long-term changes (e.g. hypertrophy) in cardiac structure and function. Similarly, **antidepressant drugs**, which have immediate effects on transmitter metabolism in the brain (see Ch. 46) take weeks to produce therapeutic benefit. **Opioids** (see Ch. 41) produce an immediate analgesic effect but, after a time, tolerance and dependence ensue, and in some cases long-term addiction. In these and many other examples, the nature of the intervening mechanism is unclear, although as a general rule any long-term phenotypic change necessarily involves alterations of gene expression. Drugs are often used to treat chronic conditions, and understanding long-term as well as acute drug effects is becoming increasingly important. Pharmacologists have traditionally tended to focus on short-term physiological responses, which are much easier to study, rather than on delayed effects. The focus is now clearly shifting.

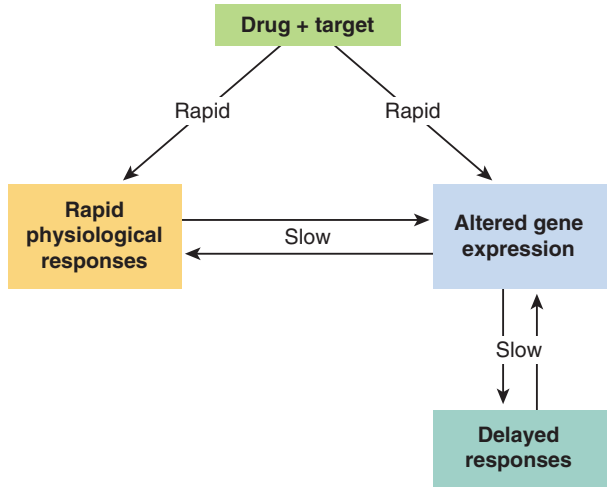

Fig. 2.12 Early and late responses to drugs. Many drugs act directly on their targets (left-hand arrow) to produce a rapid physiological response. If this is maintained, it is likely to cause changes in gene expression that give rise to delayed effects. Some drugs (right-hand arrow) have their primary action on gene expression, producing delayed physiological responses. Drugs can also work by both pathways. Note the bidirectional interaction between gene expression and response.

Drug effects

- Drugs act mainly on cellular targets, producing effects at different functional levels (e.g. biochemical, cellular, physiological and structural).
- The direct effect of the drug on its target produces acute responses at the biochemical, cellular or physiological levels.
- Acute responses generally lead to *delayed long-term effects*, such as desensitisation or down-regulation of receptors, hypertrophy, atrophy or remodelling of tissues, tolerance, dependence and addiction.
- Long-term delayed responses result from changes in gene expression, although the mechanisms by which the acute effects bring this about are often uncertain.
- Therapeutic effects may be based on acute responses (e.g. the use of bronchodilator drugs to treat asthma; Ch. 27) or delayed responses (e.g. antidepressants; Ch. 46).

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3 How drugs act: molecular aspects

OVERVIEW

In this chapter, we move from the general principles of drug action outlined in Chapter 2 to the molecules that are involved in recognising chemical signals and translating them into cellular responses. Molecular pharmacology is advancing rapidly, and the new knowledge is changing our understanding of drug action and also opening up many new therapeutic possibilities, further discussed in other chapters.

First, we consider the types of target proteins on which drugs act. Next, we describe the main families of receptors and ion channels that have been revealed by cloning and structural studies. Finally, we discuss the various forms of receptor–effector linkage (signal transduction mechanisms) through which receptors are coupled to the regulation of cell function. The relationship between the molecular structure of a receptor and its functional linkage to a particular type of effector system is a principal theme. In the next two chapters, we see how these molecular events alter important aspects of cell function—a useful basis for understanding the effects of drugs on intact living organisms. We go into more detail than is necessary for understanding today's pharmacology at a basic level, intending that students can, if they wish, skip or skim these chapters without losing the thread; however, we are confident that tomorrow's pharmacology will rest solidly on the advances in cellular and molecular biology that are discussed here.

TARGETS FOR DRUG ACTION

The protein targets for drug action on mammalian cells (Fig. 3.1) that are described in this chapter can be broadly divided into:

- receptors
- ion channels
- enzymes
- carrier molecules (transporters).

The great majority of important drugs act on one or other of these types of protein, but there are exceptions. For example, **colchicine** (Ch. 26) interacts with the structural protein tubulin, while several immunosuppressive drugs (e.g. **ciclosporin**, Ch. 26) bind to cytosolic proteins known as immunophilins. Therapeutic antibodies that act by sequestering cytokines (protein mediators involved in inflammation; see Ch. 26) are also used. Targets for chemotherapeutic drugs (Chs 49–55), where the aim is to suppress invading microorganisms or cancer cells, include DNA and cell wall constituents as well as other proteins.

RECEPTORS

Receptors (Fig. 3.1A) are the sensing elements in the system of chemical communications that coordinates the function of all the different cells in the body, the chemical messengers being the various hormones, transmitters and other mediators discussed in Section 2. Many therapeutically useful drugs act, either as agonists or antagonists, on receptors for known endogenous mediators. Some examples are given in Table 3.1. In most cases, the endogenous mediator was discovered before—often many years before—the receptor was characterised pharmacologically and biochemically, but in recent years, many receptors have been identified initially on the basis of their pharmacological or molecular characteristics. In some cases, such as the cannabinoid receptors (see Ch. 18), the endogenous mediator was identified later; in many others, known as *orphan receptors* (see below) the mediator—if it exists—remains unknown.

ION CHANNELS

Ion channels[1] are essentially gateways in cell membranes, which selectively allow the passage of particular ions, and which are induced to open or close by a variety of mechanisms. Two important types are *ligand-gated channels* and *voltage-gated channels*. The former open only when one or more agonist molecules are bound, and are properly classified as receptors, since agonist binding is needed to activate them. Voltage-gated channels are gated by changes in the transmembrane potential rather than by agonist binding.

In general, drugs can affect ion channel function either by binding to the channel protein itself (to the ligand-binding site of ligand-gated channels, or to other parts of the channel molecule), or they may affect channel function by an indirect interaction, involving a G-protein and other intermediaries (see below). In the simplest case, exemplified by the action of local anaesthetics on the voltage-gated sodium channel (see Ch. 42), the drug molecule plugs the channel physically (Fig. 3.1B), blocking ion permeation.

Examples of drugs that bind to accessory (*allosteric*) sites on the channel protein and thereby affect channel gating include:

- vasodilator drugs of the **dihydropyridine** type (see Ch. 22), which inhibit the opening of L-type calcium channels (see Ch. 4)
- **benzodiazepine** tranquillisers (see Ch. 43). These drugs bind to a region of the $GABA_A$ receptor–chloride channel complex (a ligand-gated channel; see above) that is distinct from the GABA binding site. Most

[1] 'Ion channels and the electrical properties they confer on cells are involved in every human characteristic that distinguishes us from the stones in a field.' (Armstrong C M 2003 Voltage-gated K channels; http://www.stke.org.)

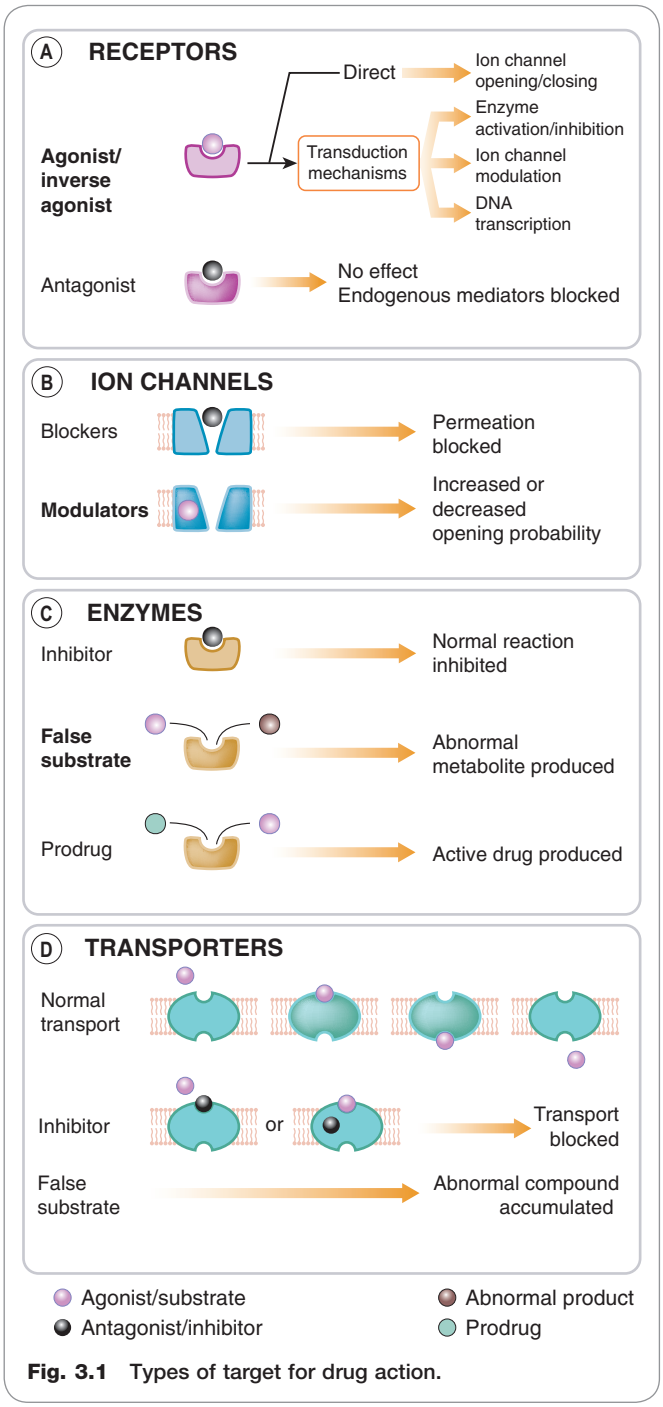

Fig. 3.1 Types of target for drug action.

benzodiazepines facilitate the opening of the channel by the inhibitory neurotransmitter GABA (see Ch. 37), but some inverse agonists are known that have the opposite effect, causing anxiety rather than tranquillity

- **sulfonylureas** (see Ch. 30) used in treating diabetes, which act on ATP-gated potassium channels of pancreatic β-cells and thereby enhance insulin secretion.

A summary of the different ion channel families and their functions is given below (p. 43).

ENZYMES

Many drugs are targeted on enzymes (Fig. 3.1C), examples being given in Table 3.1. Often, the drug molecule is a substrate analogue that acts as a competitive inhibitor of the enzyme (e.g. **captopril**, acting on angiotensin-converting enzyme; Ch. 22); in other cases, the binding is irreversible and non-competitive (e.g. **aspirin**, acting on cyclo-oxygenase; Ch. 26). The immunophilin to which **ciclosporin** binds (see above) has enzymic activity as an isomerase that catalyses the *cis–trans* isomerisation of proline residues in proteins, a reaction that is important in allowing expressed proteins to fold correctly. Inhibition of this enzymic activity is one of the mechanisms by which ciclosporin causes immunosuppression. Drugs may also act as false substrates, where the drug molecule undergoes chemical transformation to form an abnormal product that subverts the normal metabolic pathway. An example is the anticancer drug **fluorouracil**, which replaces uracil as an intermediate in purine biosynthesis but cannot be converted into thymidylate, thus blocking DNA synthesis and preventing cell division (Ch. 55).

It should also be mentioned that drugs may require enzymic degradation to convert them from an inactive form, the prodrug (see Ch. 9), to an active form. Examples are given in Table 9.3. Furthermore, as discussed in Chapter 57, drug toxicity often results from the enzymic conversion of the drug molecule to a reactive metabolite. Paracetamol (see Ch. 26) causes liver damage in this way. As far as the primary action of the drug is concerned, this is an unwanted side reaction, but it is of major practical importance.

TRANSPORT PROTEINS

The movement of ions and small organic molecules across cell membranes generally occurs either through channels (see above), or through the agency of a transport protein, because the permeating molecules are often too polar (i.e. insufficiently lipid soluble) to penetrate lipid membranes on their own (Fig. 3.1D). Many such carriers are known; examples of particular pharmacological importance include those responsible for the transport of ions and many organic molecules across the renal tubule, the intestinal epithelium and the blood–brain barrier, the transport of Na^+ and Ca^{2+} out of cells, and the uptake of neurotransmitter precursors (such as choline) or of neurotransmitters themselves (such as noradrenaline, 5-hydroxytryptamine [5-HT], glutamate and peptides) by nerve terminals, and the transport of drug molecules and their metabolites across cell membranes and epithelial barriers. We shall encounter them frequently in later chapters.

In many cases, hydrolysis of ATP provides the energy for transport of substances against their electrochemical gradient. Such transport proteins include a distinct ATP binding site, and are termed ABC (ATP-binding cassette) transporters. Important examples include the sodium pump (Na^+-K^+-ATPase; see Ch. 4) and '*multi-drug-resistance*' (MDR) transporters that eject cytotoxic drugs from cancer and microbial cells, conferring resistance to these therapeutic agents (see Ch. 55). In other cases, including the neurotransmitter transporters, the transport of organic molecules is coupled to the transport of ions (usually Na^+), either in the same direction (*symport*) or in the opposite direction (*antiport*), and therefore relies on the electrochemical gradient for Na^+ generated by the ATP-

Table 3.1 Some examples of targets for drug action

Type of target	Effectors		See Chapter
Receptors	**Agonists**	**Antagonists**	
Nicotinic ACh receptor	Acetylcholine Nicotine Varenicline	Tubocurarine α-Bungarotoxin	13 48
β-Adrenoceptor	Noradrenaline Isoprenaline	Propranolol	14
Histamine (H_1 receptor)	Histamine	Mepyramine	26
Opiate (μ-receptor)	Morphine	Naloxone	41
Dopamine (D_2 receptor)	Dopamine Bromocriptine	Chlorpromazine	38, 46
Oestrogen receptor	Ethinylestradiol	Tamoxifen	34
Epidermal growth factor receptor		Trastuzumab	59
Ion channels	**Blockers**	**Modulators**	
Voltage-gated Na^+ channels	Local anaesthetics Tetrodotoxin	Veratridine	42
Renal tubule Na^+ channels	Amiloride	Aldosterone	28
Voltage-gated Ca^{2+} channels	Divalent cations (e.g. Cd^{2+})	Dihydropyridines	21, 22 41
ATP-sensitive K^+ channels	ATP	Sulphonylureas	30
GABA-gated Cl^- channels	Picrotoxin	Benzodiazepines	43
Enzymes	**Inhibitors**		
Acetylcholinesterase	Neostigmine		13
Cyclo-oxygenase	Aspirin		26
Angiotensin-converting enzyme	Captopril		22
HMG-CoA reductase	Simvastatin		23
Monoamine oxidase-A	Iproniazid		46
Phosphodiesterase type V	Sildenafil		34
Dihydrofolate reductase	Trimethoprim Methotrexate		53 55
Thymidine kinase	Aciclovir		51
HIV protease	Saquinavir		51
Transport proteins	**Inhibitors**	**False substrates**	
Noradrenaline transporter (membrane)	Tricyclic antidepressants Cocaine	 Amphetamine Methyldopa	46 47, 48 14, 45 22
Weak acid carrier (renal tubule)	Probenecid		28
$Na^+/K^+/2Cl^-$ co-transporter (loop of Henle)	Loop diuretics		28
Proton pump (gastric mucosa)	Omeprazole		29
MDR transporter	Verapamil		55
Others			
Immunophilins	Ciclosporin Tacrolimus		26
Tubulin	Colchicine Taxol		26 55

Note: These are representative examples, and by no means a complete list. Other biochemical targets for drugs used in chemotherapy are discussed in Chapters 49–55.

driven sodium pump. The carrier proteins embody a recognition site that makes them specific for a particular permeating species, and these recognition sites can also be targets for drugs whose effect is to block the transport system. Some examples are given in Table 3.1.

The importance of transport proteins as a source of individual variation in the pharmacokinetic characteristics of various drugs is becoming increasingly recognised (see Ch. 10).

RECEPTOR PROTEINS

ISOLATION AND CLONING OF RECEPTORS

In the 1970s, pharmacology entered a new phase when receptors, which had until then been theoretical entities, began to emerge as biochemical realities following the development of receptor-labelling techniques (see Ch. 2), which made it possible to extract and purify the receptor material. This approach was first used successfully on the nicotinic acetylcholine receptor (see Ch. 13), where advantage was taken of two natural curiosities. The first was that the electric organs of many fishes, such as rays (*Torpedo* sp.) and electric eels (*Electrophorus* sp.) consist of modified muscle tissue in which the acetylcholine-sensitive membrane is extremely abundant, and these organs contain much larger amounts of acetylcholine receptor than any other tissue. The second was that the venom of snakes of the cobra family contains polypeptides that bind with very high specificity to nicotinic acetylcholine receptors. These substances, known as α-toxins, can be labelled and used to assay the receptor content of tissues and tissue extracts. The best known is **α-bungarotoxin**, the main component of the venom of the Malayan banded krait (*Bungarus multicinctus*).[2] Treatment of muscle or electric tissue with non-ionic detergents renders the membrane-bound receptor protein soluble, and it can then be purified by the technique of affinity chromatography. Similar approaches have now been used to purify a great many hormone and neurotransmitter receptors, as well as ion channels, carrier proteins and other kinds of target molecules.

▼ Once receptor proteins were isolated and purified, it was possible to analyse the amino acid sequence of a short stretch, allowing the corresponding base sequence of the mRNA to be deduced and full-length DNA to be isolated, by conventional cloning methods, starting from a cDNA library obtained from a tissue source rich in the receptor of interest. The first receptor clones were obtained in this way, but subsequently expression cloning and cloning strategies based on sequence homologies, which do not require prior isolation and purification of the receptor protein, were widely used, and now several hundred receptors of all four structural families (see below) have been cloned. Endogenous ligands for many of these 'receptor-like' molecules identified by gene cloning are so far unknown, and they are described as 'orphan receptors'.[3] Identifying ligands for these presumed receptors is often difficult. However, there are examples (e.g. the cannabinoid receptor; see Ch. 18) where important endogenous ligands have been linked to hitherto orphan receptors, and others, such as PPARs (peroxisome proliferator-activated receptors), which have emerged as the targets of important therapeutic drugs (see Ch. 30) though the endogenous ligand remains unknown. Several endogenous peptide ligands for orphan receptors have been identified (see Davenport, 2003), whose physiological and possible therapeutic significance is under investigation. There is optimism that novel therapeutic agents will emerge by targeting this pool of unclaimed receptors.

Much information has been gained by introducing the cloned DNA encoding individual receptors into cell lines, producing cells that express the foreign receptors in a functional form. Such engineered cells allow much more precise control of the expressed receptors than is possible with natural cells or intact tissues, and the technique is widely used to study the binding and pharmacological characteristics of cloned receptors. Expressed human receptors, which often differ in their sequence and pharmacological properties from their animal counterparts, can be studied in this way.

The cloning of receptors revealed many molecular variants (subtypes) of known receptors, which had not been evident from pharmacological studies. This produced some taxonomic confusion, but in the long term molecular characterisation of receptors is essential. Barnard, one of the high priests of receptor cloning, was undaunted by the proliferation of molecular subtypes among receptors that pharmacologists had thought that they understood. He quoted Thomas Aquinas: 'Types and shadows have their ending, for the newer rite is here'. The newer rite, Barnard confidently asserted, was molecular biology. Analysis of the human and other mammalian genomes suggests that many hundreds of receptor-like genes are present, of which only a minority so far have a pharmacological identity. Now that the genes have been clearly identified, and the full molecular inventory established, the emphasis has shifted to characterising the receptors pharmacologically and determining their physiological functions.

TYPES OF RECEPTOR

Receptors elicit many different types of cellular effect. Some of them are very rapid, such as those involved in synaptic transmission, operating within milliseconds, whereas other receptor-mediated effects, such as those produced by thyroid hormone or various steroid hormones, occur over hours or days. There are also many examples of intermediate timescales—catecholamines, for example, usually act in a matter of seconds, whereas many peptides take rather longer to produce their effects. Not surprisingly, very different types of linkage between the receptor occupation and the ensuing response are involved. Based on molecular structure and the nature of this linkage (the transduction mechanism), we can distinguish four receptor types, or superfamilies (see Figs 3.2 and 3.3; Table 3.2).

- Type 1: **Ligand-gated ion channels** (also known as **ionotropic** receptors).[4] The chain of discoveries culminating in the molecular characterisation of these receptors is described by Halliwell (2007). Typically, these are the receptors on which fast neurotransmitters act. Examples include the nicotinic acetylcholine receptor (nAChR; see Ch. 13); $GABA_A$ receptor (see Ch. 37); and glutamate receptors of the NMDA, AMPA and kainate types (see Ch. 37).

[2]Nature has had the good sense to keep these heavily armed fishes and snakes well apart. Ironically enough, *B. multicinctus* is now officially an endangered species, threatened by scientists' demand for its venom. Evolution for survival can go one step too far.

[3]An oddly Dickensian term that seems inappropriately condescending, because we can assume that these receptors play defined roles in physiological signalling—their 'orphanhood' reflects our ignorance, not their status.

[4]Here, focusing on receptors, we include ligand-gated ion channels as an example of a receptor family. Other types of ion channels are described later (p. 43); many are also drug targets, although not receptors in the strict sense.

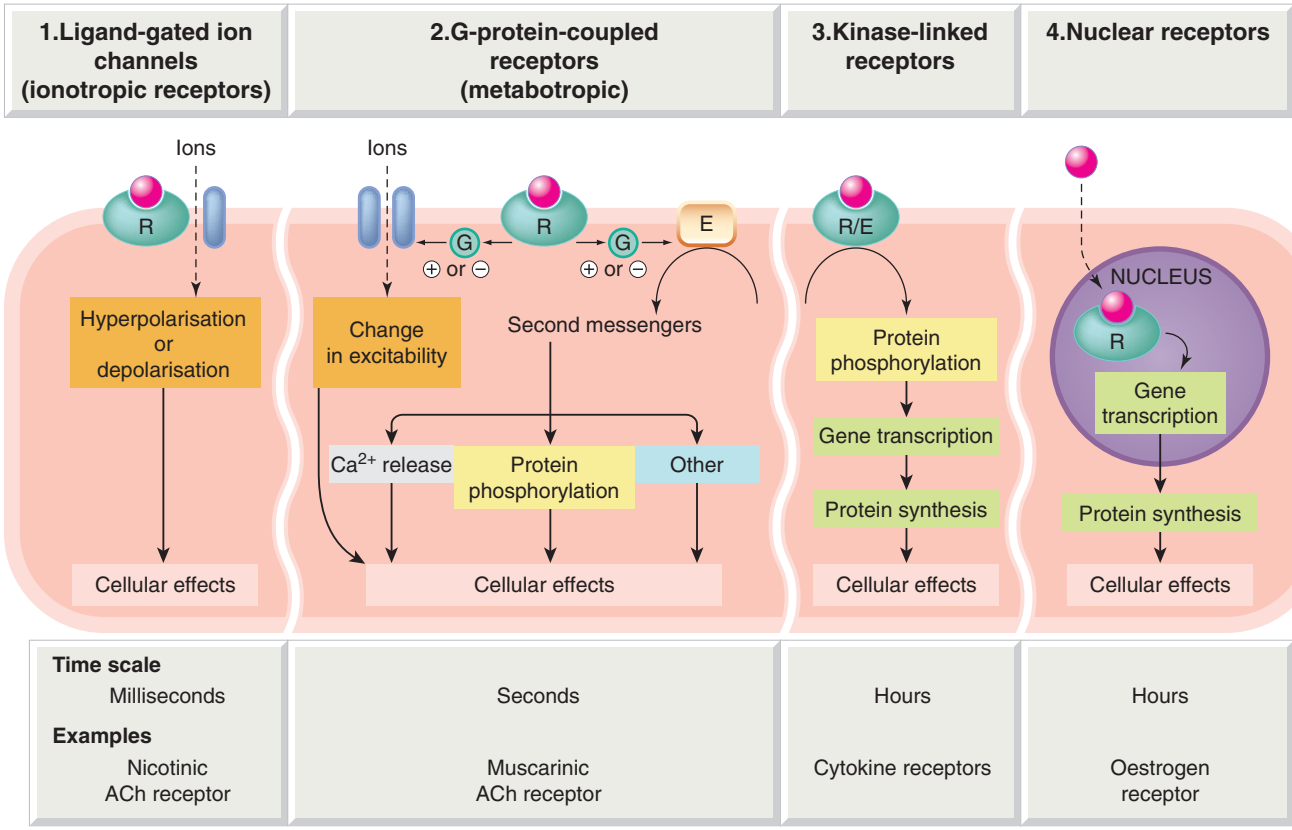

Fig. 3.2 **Types of receptor–effector linkage.** ACh, acetylcholine; E, enzyme; G, G-protein; R, receptor.

Table 3.2 **The four main types of receptor**

	Type 1: ligand-gated ion channels	Type 2: G-protein-coupled receptors	Type 3: receptor kinases	Type 4: nuclear receptors
Location	Membrane	Membrane	Membrane	Intracellular
Effector	Ion channel	Channel or enzyme	Protein kinases	Gene transcription
Coupling	Direct	G-protein	Direct	Via DNA
Examples	Nicotinic acetylcholine receptor, $GABA_A$ receptor	Muscarinic acetylcholine receptor, adrenoceptors	Insulin, growth factors, cytokine receptors	Steroid receptors
Structure	Oligomeric assembly of subunits surrounding central pore	Monomeric or oligomeric assembly of subunits comprising seven transmembrane helices with intracellular G-protein-coupling domain	Single transmembrane helix linking extracellular receptor domain to intracellular kinase domain	Monomeric structure with separate receptor- and DNA-binding domains

- Type 2: **G-protein-coupled receptors** (GPCRs). These are also known as **metabotropic receptors** or **7-transmembrane (7-TM or heptahelical) receptors**. They are membrane receptors that are coupled to intracellular effector systems via a G-protein (see below). They constitute the largest family,[5] and include receptors for many hormones and slow transmitters, for example the muscarinic acetylcholine receptor (mAChR; see Ch. 13), adrenoceptors (see Ch. 14) and chemokine receptors (see Ch. 17).
- Type 3: **kinase-linked and related receptors**. This is a large and heterogeneous group of membrane receptors responding mainly to protein mediators. They comprise an extracellular ligand-binding domain linked to an intracellular domain by a single transmembrane helix. In many cases, the intracellular domain is enzymic in nature (with protein kinase or guanylyl cyclase activity). Type 3 receptors include

[5]There are 865 human GPCRs comprising 1.6% of the genome (Fredriksson & Schiöth, 2005). Nearly 500 of these are believed to be odorant receptors involved in smell and taste sensations, the remainder being receptors for known or unknown endogenous mediators—enough to keep pharmacologists busy for some time yet.

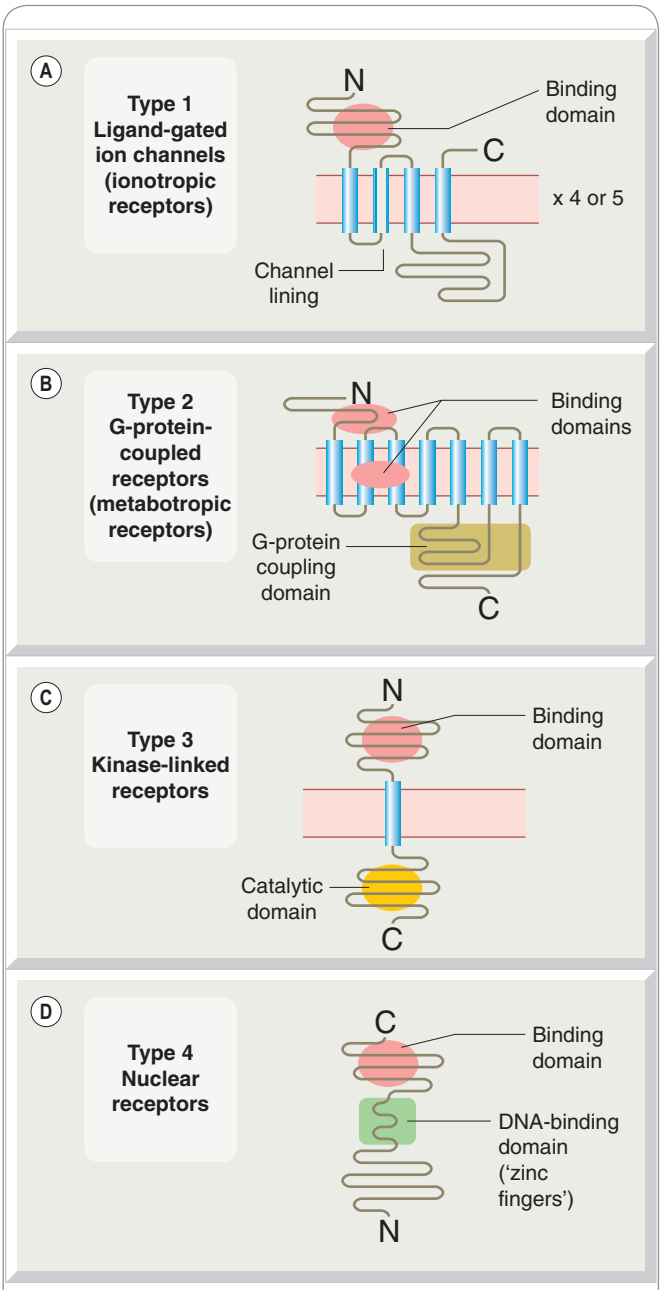

Fig. 3.3 General structure of four receptor families. The rectangular segments represent hydrophobic α-helical regions of the protein comprising approximately 20 amino acids, which form the membrane-spanning domains of the receptors. [**A**] Type 1: ligand-gated ion channels. Many ligand-gated ion channels comprise four or five subunits of the type shown, the whole complex containing 16–20 membrane-spanning segments surrounding a central ion channel. Other structural types are shown in Fig. 3.18. [**B**] Type 2: G-protein-coupled receptors. [**C**] Type 3: kinase-linked receptors. Most growth factor receptors incorporate the ligand-binding and enzymatic (kinase) domains in the same molecule, as shown, whereas cytokine receptors lack an intracellular kinase domain but link to cytosolic kinase molecules. Other structural variants also exist. [**D**] Type 4: nuclear receptors that control gene transcription.

those for insulin and for various cytokines and growth factors (see Chs 17 and 32); the receptor for atrial natriuretic factor (ANF; Chs 21 and 22) is the main example of the guanylyl cyclase type. The two kinds are very similar structurally, even though their transduction mechanisms differ.

- Type 4: **nuclear receptors**. These are receptors that regulate gene transcription. The term *nuclear receptors* is something of a misnomer, because some are actually located in the cytosol and migrate to the nuclear compartment when a ligand is present. They include receptors for steroid hormones (see Ch. 32), thyroid hormone (Ch. 33) and other agents such as retinoic acid and vitamin D. Receptors of this type also recognise many foreign molecules, inducing the expression of enzymes that metabolise them.

MOLECULAR STRUCTURE OF RECEPTORS

The molecular organisation of typical members of each of these four receptor superfamilies is shown in Figure 3.3. Although individual receptors show considerable sequence variation in particular regions, and the lengths of the main intracellular and extracellular domains also vary from one to another within the same family, the overall structural patterns and associated signal transduction pathways are very consistent. The realisation that just four receptor superfamilies provide a solid framework for interpreting the complex welter of information about the effects of a large proportion of the drugs that have been studied has been one of the most refreshing developments in modern pharmacology.

RECEPTOR HETEROGENEITY AND SUBTYPES

Receptors within a given family generally occur in several molecular varieties, or subtypes, with similar architecture but significant differences in their sequences, and often in their pharmacological properties.[6] Nicotinic acetylcholine receptors are typical in this respect; distinct subtypes occur in different brain regions (see Table 38.2), and these differ from the muscle receptor. Some of the known pharmacological differences (e.g. sensitivity to blocking agents) between muscle and brain acetylcholine receptors correlate with specific sequence differences; however, as far as we know, all nicotinic acetylcholine receptors respond to the same physiological mediator and produce the same kind of synaptic response, so why many variants should have evolved is still a puzzle.

▼ Much of the sequence variation that accounts for receptor diversity arises at the genomic level, i.e. different genes give rise to distinct receptor subtypes. Additional variation arises from alternative mRNA splicing, which means that a single gene can give rise to more than one receptor isoform. After translation from genomic DNA, the mRNA normally contains non-coding regions (introns) that are excised by mRNA splicing before the message is translated into protein. Depending on the location of the splice sites, splicing can result in inclusion or deletion of one or more of the mRNA coding regions, giving rise to long or short forms of the protein. This is an important source of variation, particularly for GPCRs (see Kilpatrick et al., 1999), which produces receptors with different binding characteristics and different signal transduction mechanisms, although its pharmacological relevance remains to be clarified. Another process

[6]Receptors for 5-HT (see Ch. 15) are currently the champions with respect to diversity, with 14 cloned subtypes.

that can produce different receptors from the same gene is mRNA editing, which involves the mischievous substitution of one base in the mRNA for another, and hence a small variation in the amino acid sequence of the receptor.

Molecular heterogeneity of this kind is a feature of all kinds of receptors—indeed of functional proteins in general. New receptor subtypes and isoforms continue to be discovered, and regular updates of the catalogue are available (Alexander et al., 2009; IUPHAR Receptor Database and Channel Compendium). The problems of classification, nomenclature and taxonomy resulting from this flood of data have been mentioned earlier (p. 8). From the pharmacological viewpoint, where our concern is to understand individual drugs and what they do to living organisms, and to devise better ones, it is important that we keep molecular pharmacology in perspective. The 'newer rite' has proved revelatory in many ways, but the sheer complexity of the ways in which molecules behave means that we have a long way to go before reaching the reductionist Utopia that molecular biology promises. When we do, this book will get much shorter. In the meantime, we try to pick out the general principles without getting too bogged down in detail.

We will now describe the characteristics of each of the four receptor superfamilies.

TYPE 1: LIGAND-GATED ION CHANNELS

MOLECULAR STRUCTURE

These molecules have structural features in common with other ion channels, described on p. 45 (Ashcroft, 2000). The nicotinic acetylcholine receptor (Fig. 3.4), the first to be cloned, has been studied in great detail (see Karlin, 1993). It consists of a pentameric assembly of different subunits, of which there are four types, termed α, β, γ and δ, each of molecular weight (M_r) 40–58 kDa. The subunits show marked sequence homology, and each contains four membrane-spanning α-helices, inserted into the membrane as shown in Figure 3.4B. The pentameric structure (α_2, β, γ, δ) possesses two acetylcholine binding sites, each lying at the interface between one of the two α subunits and its neighbour. Both must bind acetylcholine molecules in order for the receptor to be activated. This receptor is sufficiently large to be seen in electron micrographs, and Figure 3.4B shows its structure, based mainly on a high-resolution electron diffraction study (Unwin, 1993, 1995; Miyazawa et al., 2003). Each subunit spans the membrane four times, so the channel comprises no fewer than 20 membrane-spanning helices surrounding a central pore.

▼ The two acetylcholine-binding sites lie on the extracellular parts of the two α subunits. One of the transmembrane helices (M_2) from each of the five subunits forms the lining of the ion channel (Fig. 3.4). The five M_2 helices that form the pore are sharply kinked inwards halfway through the membrane, forming a constriction. When acetylcholine molecules bind, a conformation change occurs in the extracellular part of the receptor (see review by Gay & Yakel, 2007), which twists the α subunits, causing the kinked M_2 segments to swivel out of the way, thus opening the channel (Miyazawa et al., 2003). The channel lining contains a series of anionic residues, making the channel selectively permeable to cations.

The use of site-directed mutagenesis, which enables short regions, or single residues, of the amino acid sequence to be altered, has shown that a mutation of a critical residue in the M_2 helix changes the channel from being cation selective (hence excitatory in the context of synaptic function) to being anion selective (typical of receptors for inhibitory transmitters such as GABA). Other mutations affect properties such as gating and desensitisation of ligand-gated channels.

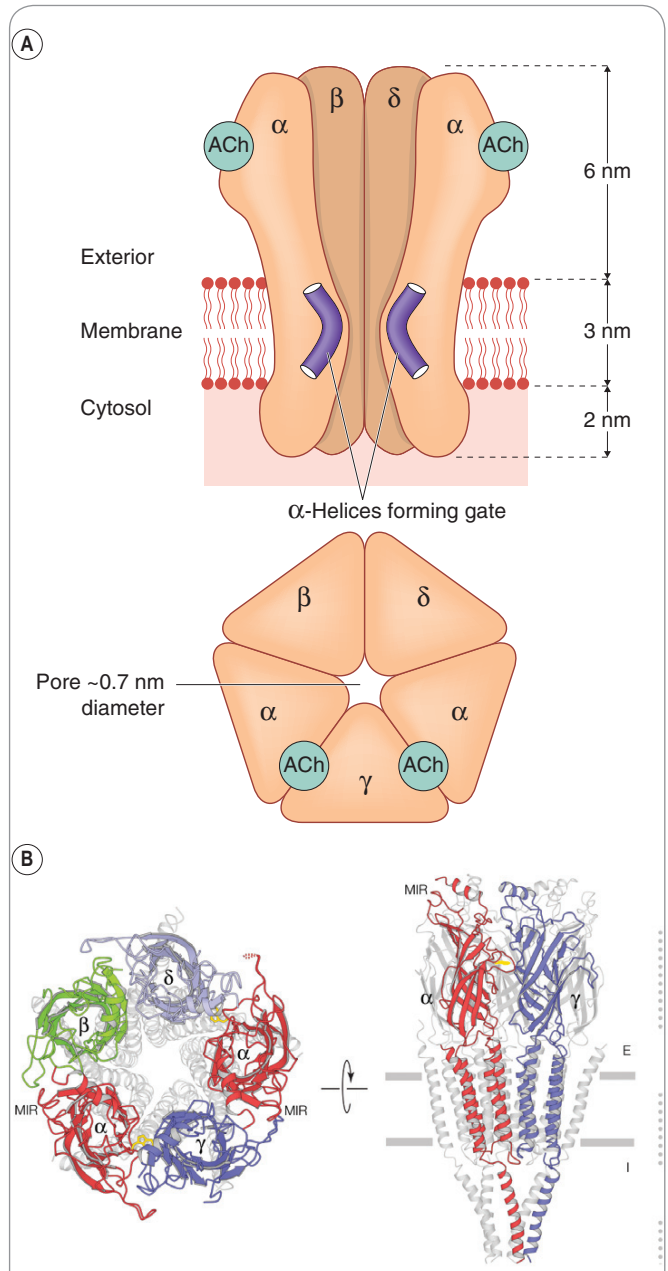

Fig. 3.4 Structure of the nicotinic acetylcholine receptor (a typical ligand-gated ion channel). [**A**] Schematic diagram in side view (upper) and plan view (lower). The five receptor subunits (α_2, β, γ, δ) form a cluster surrounding a central transmembrane pore, the lining of which is formed by the M_2 helical segments of each subunit. These contain a preponderance of negatively charged amino acids, which makes the pore cation selective. There are two acetylcholine binding sites in the extracellular portion of the receptor, at the interface between the α and the adjoining subunits. When acetylcholine binds, the kinked α-helices either straighten out or swing out of the way, thus opening the channel pore. (Based on Unwin N 1993 Nicotinic acetylcholine receptor at 9A resolution. J Mol Biol 229: 1101–1124, and Unwin N 1995 Acetylcholine receptor channel imaged in the open state. Nature 373: 37–43.) [**B**] High-resolution image showing revised arrangement of intracellular domains. (Reproduced with permission from Unwin N 2005 Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J Mol Biol Mar 4;346(4):967–989.)

Receptors for other fast transmitters, such as $GABA_A$ receptors (Ch. 37), 5-HT (Ch. 15) and glycine receptors (Ch. 37), are built on the same five-subunit pattern, and form the group of *cys-loop* receptors. Other ligand-gated ion channels, such as glutamate receptors (see Ch. 37) and the 'capsaicin receptor' (TRPV1; see Ch. 41), whose structures are shown in Figure 3.18, have a different (*P-loop*) architecture, in which the pore is built from loops rather than transmembrane helices (see p. 45), in common with many other (non-ligand-gated) ion channels.

THE GATING MECHANISM

Receptors of this type control the fastest synaptic events in the nervous system, in which a neurotransmitter acts on the postsynaptic membrane of a nerve or muscle cell and transiently increases its permeability to particular ions. Most excitatory neurotransmitters, such as acetylcholine at the neuromuscular junction (Ch. 12) or glutamate in the central nervous system (Ch. 37), cause an increase in Na^+ and K^+ permeability. This results in a net inward current carried mainly by Na^+, which depolarises the cell and increases the probability that it will generate an action potential. The action of the transmitter reaches a peak in a fraction of a millisecond, and usually decays within a few milliseconds. The sheer speed of this response implies that the coupling between the receptor and the ionic channel is a direct one, and the molecular structure of the receptor–channel complex (see above) agrees with this. In contrast to other receptor families (see below), no intermediate biochemical steps are involved in the transduction process.

▼ A breakthrough by Katz and Miledi in 1972 made it possible for the first time to study the properties of individual ligand-gated channels by the use of noise analysis. Studying the action of acetylcholine at the motor endplate, they observed that small random fluctuations of membrane potential were superimposed on the steady depolarisation produced by acetylcholine (Fig. 3.5). These fluctuations arise because, in the presence of an agonist, there is a dynamic equilibrium between open and closed ion channels. In the steady state, the rate of opening balances the rate of closing, but from moment to moment the number of open channels will show random fluctuations about the mean. By measuring the amplitude of these fluctuations, the conductance of a single ion channel can be calculated, and by measuring their frequency (usually in the form of a spectrum in which the noise power of the signal is plotted as a function of frequency), the average duration for which a single channel stays open (mean open time) can be calculated. In the case of acetylcholine acting at the endplate, the channel conductance is about 20 picosiemens (pS), which is equivalent to an influx of about 10^7 ions per second through a single channel under normal physiological conditions, and the mean open time is 1–2 ms. The magnitude of the single channel conductance confirms that permeation occurs through a physical pore through the membrane, because the ion flow is too large to be compatible with a carrier mechanism. The channel conductance produced by different acetylcholine-like agonists is the same, whereas the mean channel lifetime varies.

The simple scheme shown in Fig. 2.1 is a useful model for ion channel gating. The conformation R*, representing the open state of the ion channel, is thought to be the same for all agonists, accounting for the finding that the channel conductance does not vary. Kinetically, the mean open time is determined mainly by the closing rate constant, α, and this varies from one drug to another. As explained in Chapter 2, an agonist of high efficacy that activates a large proportion of the receptors that it occupies will be characterised by $\beta/\alpha >> 1$, whereas for a drug of low efficacy β/α has a lower value.

The patch clamp recording technique, devised by Neher and Sakmann, allows the very small current flowing through a single ionic channel to be measured directly (Fig. 3.6), and the results have fully confirmed the interpretation of channel properties based on noise analysis. This technique provides a view, unique in biology, of the physiological behaviour of individual protein molecules in real time, and has given many new insights into the gating reactions and permeability characteristics of both ligand-gated channels and voltage-gated channels (see p. 43). Single-channel recording has shown that many agonists cause individual channels to open to one or more of several distinct conductance levels. In the case of glutamate-activated channels, it appears that different agonists produce different receptor conformations associated with different channel conductances (Jin et al., 2003). Desensitisation of ligand-gated ion channels also involves one or more additional agonist-induced conformational states. These findings necessitate some elaboration of the simple scheme of Figure 2.1, in which only a single open state, R*, is represented, and are an example of the way in which the actual behaviour of receptors makes our theoretical models look a little threadbare.

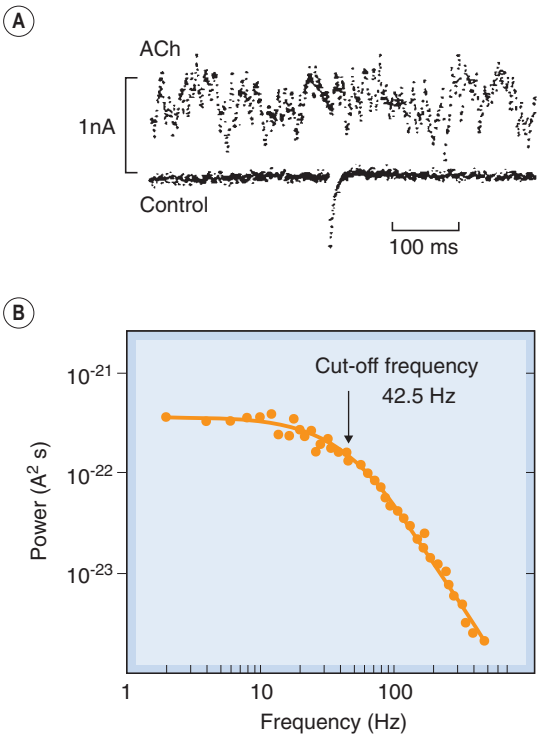

Fig. 3.5 Acetylcholine-induced noise at the frog motor endplate. [**A**] Records of membrane current recorded at high gain under voltage clamp. The upper noise record was recorded during the application of acetylcholine (ACh) from a micropipette. The lower record was obtained in the absence of ACh, the blip in the middle being caused by the spontaneous release of a packet of ACh from the motor nerve. The steady (DC) component of the ACh signal has been removed by electronic filtering, leaving the high-frequency noise signal. [**B**] Power spectrum of ACh-induced noise recorded in a similar experiment to that shown above. The spectrum is calculated by Fourier analysis and fitted with a theoretical (Lorentzian) curve that corresponds to the expected behaviour of a single population of channels whose lifetime varies randomly. The cut-off frequency (at which the power is half of its limiting low-frequency value) enables the mean channel lifetime to be calculated. (From [**A**] Anderson C R, Stevens C F 1973 J Physiol 235: 655; [**B**] Ogden D C et al. 1981 Nature 289: 596.)

Ligand-gated ion channels

- These are sometimes called ionotropic receptors.
- They are involved mainly in fast synaptic transmission.
- There are several structural families, the commonest being heteromeric assemblies of four or five subunits, with transmembrane helices arranged around a central aqueous channel.
- Ligand binding and channel opening occur on a millisecond timescale.
- Examples include the nicotinic acetylcholine, GABA type A ($GABA_A$) and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors.

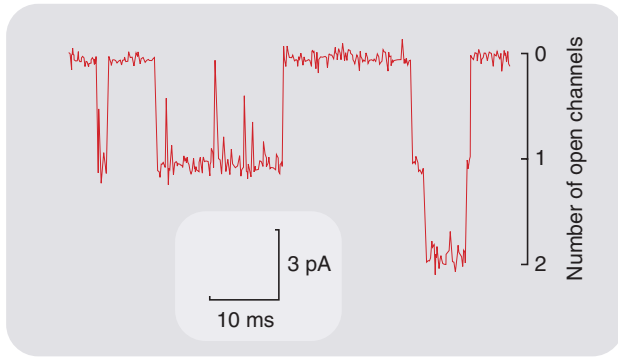

Fig. 3.6 Single acetylcholine-operated ion channels at the frog motor endplate recorded by the patch clamp technique. The pipette, which was applied tightly to the surface of the membrane, contained 10 μmol/l ACh. The downward deflections show the currents flowing through single ion channels in the small patch of membrane under the pipette tip. Towards the end of the record, two channels can be seen to open simultaneously. The conductance and mean lifetime of these channels agrees well with indirect estimates from noise analysis (see Fig. 3.5). (Courtesy of D Colquhoun and D C Ogden.)

TYPE 2: G-PROTEIN-COUPLED RECEPTORS

The abundant GPCR family comprises many of the receptors that are familiar to pharmacologists, such as mAChRs, adrenoceptors, dopamine receptors, 5-HT receptors, opioid receptors, receptors for many peptides, purine receptors and many others, including the chemoreceptors involved in olfaction and pheromone detection, and also many 'orphans' (see Fredriksson & Schiöth, 2005). For most of these, pharmacological and molecular studies have revealed a variety of subtypes. All have the characteristic heptahelical structure.

Many neurotransmitters, apart from peptides, can interact with both GPCRs and ligand-gated channels, allowing the same molecule to produce a wide variety of effects. Individual peptide hormones, on the other hand, generally act either on GPCRs or on kinase-linked receptors (see below), but rarely on both, and a similar choosiness applies to the many ligands that act on nuclear receptors.[7]

The human genome includes genes encoding about 400 GPCRs (excluding odorant receptors), which constitute the commonest single class of targets for therapeutic drugs, and it is thought that many promising therapeutic drug targets of this type remain to be identified. For a short review, see Hill (2006).

MOLECULAR STRUCTURE

The first GPCR to be fully characterised was the β-adrenoceptor (Ch. 14), which was cloned in 1986. Molecular biology caught up very rapidly with pharmacology, and all of the receptors that had been identified by their pharmacological properties have now been cloned. What seemed revolutionary in 1986 is now commonplace, and nowadays any aspiring receptor has to be cloned before it is taken seriously.

G-protein-coupled receptors consist of a single polypeptide chain of up to 1100 residues whose general anatomy is shown in Figure 3.3B. Their characteristic structure comprises seven transmembrane α-helices, similar to those of the ion channels discussed above, with an extracellular N-terminal domain of varying length, and an intracellular C-terminal domain.

GPCRs are divided into three distinct families (see Schwartz, 1996). There is considerable sequence homology between the members of one family, but none between different families. They share the same seven-helix (heptahelical) structure, but differ in other respects, principally in the length of the extracellular N terminus and the location of the agonist binding domain (Table 3.3). Family A is by far the largest, comprising most monoamine, neuropeptide and chemokine receptors. Family B includes receptors for some other peptides, such as calcitonin and glucagon (see Ch. 19). Family C is the smallest, its main members being the metabotropic glutamate and GABA receptors (Ch. 37) and the Ca^{2+}-sensing receptors[8] (see Ch. 35).

▼ The understanding of the function of receptors of this type owes much to studies of a closely related protein, *rhodopsin*, which is responsible for transduction in retinal rods. This protein is abundant in the retina, and much easier to study than receptor proteins (which are anything but abundant); it is built on an identical plan to that shown in Figure 3.3 and also produces a response in the rod (hyperpolarisation, associated with inhibition of Na^+ conductance) through a mechanism involving a G-protein (see below). The most obvious difference is that a photon, rather than an agonist molecule, produces the response. In effect, rhodopsin can be regarded as incorporating its own inbuilt agonist molecule, namely *retinal*, which isomerises from the *trans* (inactive) to the *cis* (active) form when it absorbs a photon.

[7]Examples of promiscuity are increasing, however. Steroid hormones, normally faithful to nuclear receptors, make the occasional pass at ion channels and other targets (see Falkenstein et al., 2000), and some eicosanoids act on nuclear receptors as well as GPCRs. Nature is quite open minded, although such examples are liable to make pharmacologists frown and students despair.

[8]The Ca^{2+}-sensing receptor (see Conigrave et al., 2000) is an unusual GPCR that is activated, not by conventional mediators, but by extracellular Ca^{2+} in the range of 1–10 mM—an extremely low affinity in comparison with other GPCR agonists. It is expressed by cells of the parathyroid gland, and serves to regulate the extracellular Ca^{2+} concentration by controlling parathyroid hormone secretion (Ch. 35). This homeostatic mechanism is quite distinct from the mechanisms for regulating intracellular Ca^{2+} discussed in Chapter 4.

Site-directed mutagenesis experiments show that the long third cytoplasmic loop is the region of the molecule that couples to the G-protein, because deletion or modification of this section results in receptors that still bind ligands but cannot associate with G-proteins or produce responses. Usually, a particular receptor subtype couples selectively with a particular G-protein, and swapping parts of the cytoplasmic loop between different receptors alters their G-protein selectivity.

For small molecules, such as noradrenaline (norepinephrine), the ligand-binding domain of class A receptors is buried in the cleft between the α-helical segments within the membrane (Fig. 3.3B), similar to the slot occupied by retinal in the rhodopsin molecule. Peptide ligands, such as substance P (Ch. 19) bind more superficially to the extracellular loops, as shown in Figure 3.3B. By single-site mutagenesis experiments, it is possible to map the ligand-binding domain of these receptors, and the hope is that it may soon be possible to design synthetic ligands based on knowledge of the receptor site structure—an important milestone for the pharmaceutical industry, which has relied up to now mainly on the structure of endogenous mediators (such as histamine) or plant alkaloids (such as morphine) for its chemical inspiration.[9] Recently, the difficulties of crystallising type A GPCRs have been overcome, allowing the use of the powerful technique of X-ray crystallography to study the molecular structure of these receptors in detail (see Weis & Kobilka, 2008). Also, fluorescence methods have been developed to study the kinetics of ligand binding and subsequent conformational changes associated with activation (see Lohse et al., 2008). From such studies we should gain a clearer picture of the mechanism of activation of GPCRs and the factors determining agonist efficacy, as well as having a better basis for designing new GPCR ligands.

Table 3.3 G-protein-coupled receptor families[a]

Family	Receptors[b]	Structural features
A: rhodopsin family	The largest group. Receptors for most amine neurotransmitters, many neuropeptides, purines, prostanoids, cannabinoids, etc.	Short extracellular (N terminal) tail. Ligand binds to transmembrane helices (amines) or to extracellular loops (peptides)
B: secretin/ glucagon receptor family	Receptors for peptide hormones, including secretin, glucagon, calcitonin	Intermediate extracellular tail incorporating ligand-binding domain
C: metabotropic glutamate receptor/ calcium sensor family	Small group Metabotropic glutamate receptors, $GABA_B$ receptors, Ca^{2+}-sensing receptors	Long extracellular tail incorporating ligand-binding domain

[a] A fourth distinct family includes many receptors for pheromones but no pharmacological receptors.
[b] For full lists, see http://www.iuphar-db.org.

Protease-activated receptors

▼ Although activation of GPCRs is normally the consequence of a diffusible agonist, it can be the result of protease activation. Four types of protease-activated receptors (PARs), have been identified (see review by Ramachandran & Hollenberg, 2008). Many proteases, such as thrombin (a protease involved in the blood-clotting cascade; see Ch. 24), activate PARs by snipping off the end of the extracellular N-terminal tail of the receptor (Fig. 3.7) to expose five or six N-terminal residues that bind to receptor domains in the extracellular loops, functioning as a 'tethered agonist'. Receptors of this type occur in many tissues (see Ramachandran & Hollenberg, 2008), and they appear to play a role in inflammation and other responses to tissue damage where tissue proteases are released. One of the family of PARs, PAR-2, is activated by a protease released from mast cells, and is expressed on sensory neurons. It is thought to play a role in inflammatory pain (see Ch. 41). A PAR molecule can be activated only once, because the cleavage cannot be reversed, so continuous resynthesis of receptor protein is necessary. Inactivation occurs by a further proteolytic cleavage that frees the tethered ligand, or by desensitisation, involving phosphorylation (see below), after which the receptor is internalised and degraded, to be replaced by newly synthesised protein.

[9] Many lead compounds in recent years have come from screening huge chemical libraries (see Ch. 56). No inspiration is required, just robust assays, large computers and efficient robotics.

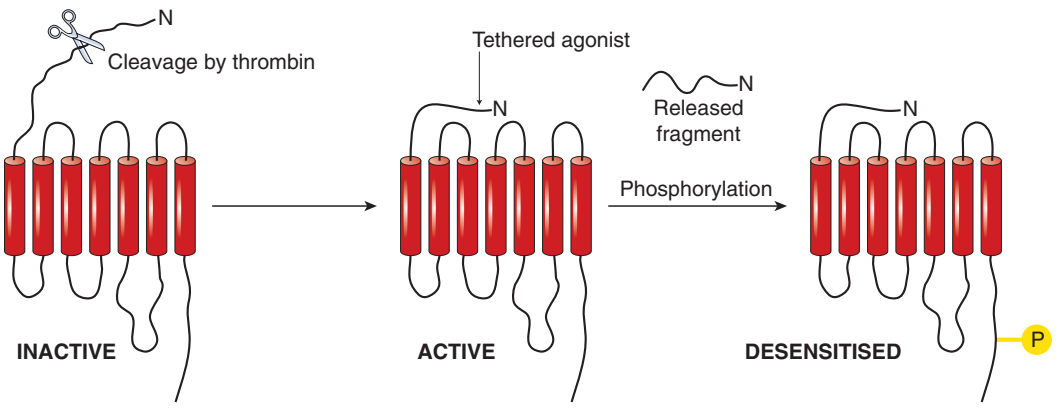

Fig. 3.7 Activation of a protease-activated receptor by cleavage of the N-terminal extracellular domain. Inactivation occurs by phosphorylation. Recovery requires resynthesis of the receptor.

G-protein-coupled receptors

- These are sometimes called metabotropic receptors.
- Structures comprise seven membrane-spanning α-helices, often linked as dimeric structures.
- One of the intracellular loops is larger than the others and interacts with the G-protein.
- The G-protein is a membrane protein comprising three subunits (α, β, γ), the α subunit possessing GTPase activity.
- When the trimer binds to an agonist-occupied receptor, the α subunit dissociates and is then free to activate an effector (a membrane enzyme or ion channel). In some cases, the βγ subunit is the activator species.
- Activation of the effector is terminated when the bound GTP molecule is hydrolysed, which allows the α subunit to recombine with βγ.
- There are several types of G-protein, which interact with different receptors and control different effectors.
- Examples include muscarinic acetylcholine receptors, adrenoceptors, neuropeptide and chemokine receptors, and protease-activated receptors.

G-PROTEINS AND THEIR ROLE

G-proteins comprise a family of membrane-resident proteins whose function is to recognise activated GPCRs and pass on the message to the effector systems that generate a cellular response. They represent the level of middle management in the organisational hierarchy, intervening between the receptors—choosy mandarins alert to the faintest whiff of their preferred chemical—and the effector enzymes or ion channels—the blue-collar brigade that gets the job done without needing to know which hormone authorised the process. They are the go-between proteins, but were actually called G-proteins because of their interaction with the guanine nucleotides, GTP and GDP. For more detailed information on the structure and functions of G-proteins, see reviews by Milligan & Kostenis (2006) and Oldham & Hamm (2008). G-proteins consist of three subunits: α, β and γ (Fig. 3.8). Guanine nucleotides bind to the α subunit, which has enzymic activity, catalysing the conversion of GTP to GDP. The β and γ subunits remain together as a βγ complex. All three subunits are anchored to the membrane through a fatty acid chain, coupled to the G-protein through a reaction known as *prenylation*. G-proteins appear to be freely diffusible in the plane of the membrane, so a single pool of G-protein in a cell can interact with several different receptors and effectors in an essentially promiscuous fashion. In the 'resting' state (Fig. 3.8), the G-protein exists as an unattached αβγ trimer, with GDP occupying the site on the α subunit. When a GPCR is activated by an agonist molecule, a conformational change occurs, involving the cytoplasmic domain of the receptor (Fig. 3.3B), causing it to acquire high affinity for αβγ. Association of αβγ with the receptor occurs within about 50 ms, causing the bound GDP to dissociate and to be replaced with GTP (GDP–GTP exchange), which in turn causes dissociation of the G-protein trimer, releasing α-GTP and βγ subunits; these are the 'active' forms of the G-protein, which diffuse in the membrane and can associate with various enzymes and ion channels, causing activation of the target (Fig. 3.8). It was originally thought that only the α subunit had a signalling function, the βγ complex serving merely as a chaperone to keep the flighty α subunits out of range of the various effector proteins that they might otherwise excite. However, the βγ complexes actually make assignations of their own, and control effectors in much the same way as the α subunits (see Clapham & Neer, 1997). Association of α or βγ subunits with target enzymes or channels can cause either activation or inhibition, depending on which G-protein is involved (see Table 3.4).

Signalling is terminated when the hydrolysis of GTP to GDP occurs through the GTPase activity of the α subunit. The resulting α–GDP then dissociates from the effector, and reunites with βγ, completing the cycle. Attachment of the α subunit to an effector molecule actually increases its GTPase activity, the magnitude of this increase being different for different types of effector. Because GTP hydrolysis is the step that terminates the ability of the α subunit to produce its effect, regulation of its GTPase activity by the effector protein means that the activation of the effector tends to be self-limiting. The mechanism results in amplification because a single agonist–receptor complex can activate several G-protein molecules in turn, and each of these can remain associated with the effector enzyme for long enough to produce many molecules of product. The product (see below) is often a 'second messenger', and further amplification occurs before the final cellular response is produced.

How is specificity achieved so that each kind of receptor produces a distinct pattern of cellular responses? With a common pool of promiscuous G-proteins linking the various receptors and effector systems in a cell, it might seem that all specificity would be lost, but this is clearly not the case. For example, mAChRs and β-adrenoceptors, both of which occur in cardiac muscle cells, produce opposite functional effects (Chs 13 and 14). The main reason is molecular variation within the α subunits, of which more than 20 subtypes have been identified[10] (see Wess, 1998; Table 3.4). Four main classes of G-protein (G_s, G_i, G_o and G_q) are of pharmacological importance. As summarised in Table 3.4, they show selectivity with respect to both the receptors and the effectors with which they couple, having specific recognition domains in their structure complementary to specific G-protein-binding domains in the receptor and effector molecules. G_s and G_i produce, respectively, stimulation and inhibition of the enzyme *adenylyl cyclase* (Fig. 3.9).

The α subunits of these G-proteins differ in structure. One functional difference that has been useful as an experimental tool to distinguish which type of G-protein is involved in different situations concerns the action of two bacterial toxins, *cholera toxin* and *pertussis toxin* (see Table 3.4). These toxins, which are enzymes, catalyse a conjugation reaction (ADP ribosylation) on the α subunit of G-proteins. Cholera toxin acts only on G_s, and it causes persistent activation. Many of the symptoms of cholera, such as the excessive secretion of fluid from the

[10]In humans there are 21 known subtypes of Gα, there are 6 of Gβ and 12 of Gγ, providing, in theory, about 1500 variants of the trimer. We know little about the role of different α, β and γ subtypes, but it would be rash to assume that the variations are functionally irrelevant. By now, you will be unsurprised (even if somewhat bemused) by such a display of molecular heterogeneity, for it is the way of evolution.

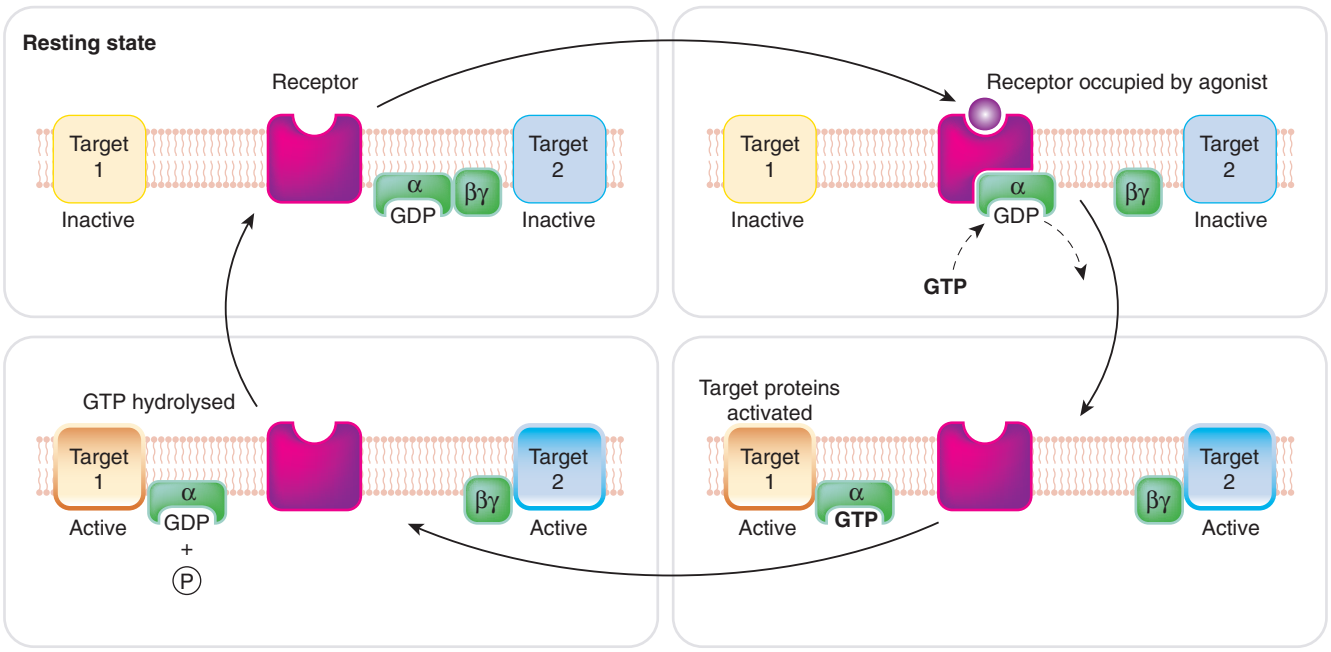

Fig. 3.8 The function of the G-protein. The G-protein consists of three subunits (α, β, γ), which are anchored to the membrane through attached lipid residues. Coupling of the α subunit to an agonist-occupied receptor causes the bound GDP to exchange with intracellular GTP; the α–GTP complex then dissociates from the receptor and from the βγ complex, and interacts with a target protein (target 1, which may be an enzyme, such as adenylyl cyclase, or an ion channel). The βγ complex may also activate a target protein (target 2). The GTPase activity of the α subunit is increased when the target protein is bound, leading to hydrolysis of the bound GTP to GDP, whereupon the α subunit reunites with βγ.

Table 3.4 The main G-protein subtypes and their functions[a]

Subtypes	Associated receptors	Main effectors	Notes
Gα subunits			
$G\alpha_s$	Many amine and other receptors (e.g. catecholamines, histamine, serotonin)	Stimulates adenylyl cyclase, causing increased cAMP formation	Activated by cholera toxin, which blocks GTPase activity, thus preventing inactivation
$G\alpha_i$	As for $G\alpha_s$, also opioid, cannabinoid receptors	Inhibits adenylyl cyclase, decreasing cAMP formation	Blocked by pertussis toxin, which prevents dissociation of αβγ complex
$G\alpha_o$	As for $G\alpha_s$, also opioid, cannabinoid receptors	?Limited effects of α subunit (effects mainly due to βγ subunits)	Blocked by pertussis toxin. Occurs mainly in nervous system
$G\alpha_q$	Amine, peptide and prostanoid receptors	Activates phospholipase C, increasing production of second messengers inositol trisphosphate and diacylglycerol (see p. 33)	—
Gβγ subunits	All GPCRs	As for Gα subunits (see above). Also: • activate potassium channels • inhibit voltage-gated calcium channels • activate GPCR kinases (GRKs, p. 36) • activate mitogen-activated protein kinase cascade	Many βγ isoforms identified, but specific functions are not yet known Gβγ-mediated effects probably require higher levels of GPCR activation than Gα-mediated effects

GPCR, G-protein-coupled receptor.
[a]This table lists only those isoforms of major pharmacological significance. Many more have been identified, some of which play roles in olfaction, taste, visual transduction and other physiological functions (see Offermanns, 2003).

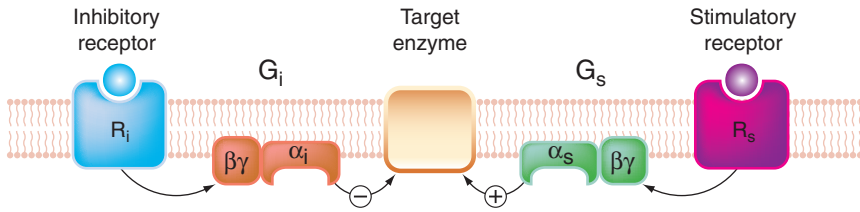

Fig. 3.9 Bidirectional control of a target enzyme, such as adenylate cyclase by G_s and G_i. Heterogeneity of G-proteins allows different receptors to exert opposite effects on a target enzyme.

gastrointestinal epithelium, are due to the uncontrolled activation of adenylate cyclase that occurs. Pertussis toxin specifically blocks G_i and G_o by preventing dissociation of the G-protein trimer.

TARGETS FOR G-PROTEINS

The main targets for G-proteins, through which GPCRs control different aspects of cell function (see Milligan, 1995; Nahorski, 2006; Table 3.4), are:

- *adenylyl cyclase*, the enzyme responsible for cAMP formation
- *phospholipase C*, the enzyme responsible for inositol phosphate and diacylglycerol (DAG) formation
- *ion channels*, particularly calcium and potassium channels
- *Rho A/Rho kinase*, a system that controls the activity of many signalling pathways controlling cell growth and proliferation, smooth muscle contraction, etc.
- *Mitogen-activated protein kinase* (MAP kinase), a system that controls many cell functions, including cell division.

The adenylyl cyclase/cAMP system

The discovery by Sutherland and his colleagues of the role of cAMP (cyclic 3′,5′-adenosine monophosphate) as an intracellular mediator demolished at a stroke the barriers that existed between biochemistry and pharmacology, and introduced the concept of second messengers in signal transduction. cAMP is a nucleotide synthesised within the cell from ATP by the action of a membrane-bound enzyme, adenylyl cyclase. It is produced continuously and inactivated by hydrolysis to 5′-AMP by the action of a family of enzymes known as phosphodiesterases (PDEs). Many different drugs, hormones and neurotransmitters act on GPCRs and produce their effects by increasing or decreasing the catalytic activity of adenylyl cyclase, thus raising or lowering the concentration of cAMP within the cell. There are nine different molecular isoforms of the enzyme, some of which respond selectively to $G\alpha_s$ or $G\alpha_i$ (see Simonds, 1999).

Cyclic AMP regulates many aspects of cellular function including, for example, enzymes involved in energy metabolism, cell division and cell differentiation, ion transport, ion channels, and the contractile proteins in smooth muscle. These varied effects are, however, all brought about by a common mechanism, namely the activation of *protein kinases* by cAMP. Protein kinases regulate the function of many different cellular proteins by controlling protein phosphorylation (see p. 39) Figure 3.10 shows how increased cAMP production in response to β-adrenoceptor activation affects enzymes involved in glycogen and fat metabolism in liver, fat and muscle cells. The result is a coordinated response in which stored energy in the form of glycogen and fat is made available as glucose to fuel muscle contraction.

Other examples of regulation by cAMP-dependent protein kinases include the increased activity of voltage-gated calcium channels in heart muscle cells (see Ch. 21). Phosphorylation of these channels increases the amount of Ca^{2+} entering the cell during the action potential, and thus increases the force of contraction of the heart.

In smooth muscle, cAMP-dependent protein kinase phosphorylates (thereby inactivating) another enzyme, *myosin-light-chain kinase*, which is required for contraction. This accounts for the smooth muscle relaxation produced by many drugs that increase cAMP production in smooth muscle (see Ch. 4).

As mentioned above, receptors linked to G_i rather than G_s inhibit adenylyl cyclase, and thus reduce cAMP formation. Examples include certain types of mAChR (e.g. the M_2 receptor of cardiac muscle; see Ch. 13), α_2 adrenoceptors in smooth muscle (Ch. 14) and opioid receptors (see Ch. 41). Adenylyl cyclase can be activated directly by certain agents, including **forskolin** and fluoride ions, agents that are used experimentally to study the role of the cAMP system.

Cyclic AMP is hydrolysed within cells by *phosphodiesterases* (PDEs), an important and ubiquitous family of enzymes (see Beavo, 1995, for review). Eleven PDE subtypes exist, of which some (e.g. PDE_3 and PDE_4) are cAMP selective, while others (e.g. PDE_5) are cGMP selective. Most are weakly inhibited by drugs such as methylxanthines (e.g. **theophylline** and **caffeine**; see Chs 27 and 47). **Rolipram** (used to treat asthma; Ch. 27) is selective for PDE_4, expressed in inflammatory cells; **milrinone** (used to treat heart failure; Ch. 21) is selective for PDE_3, which is expressed in heart muscle; **sildenafil** (better known as Viagra; Ch. 34) is selective for PDE_5, and consequently enhances the vasodilator effects of nitrous oxide (NO) and drugs that release NO, whose effects are mediated by cGMP (see Ch. 20). The similarity of some of the actions of these drugs to those of sympathomimetic amines (Ch. 14) probably reflects their common property of increasing the intracellular concentration of cAMP. Selective inhibitors of the various PDEs are being developed, mainly to treat cardiovascular and respiratory diseases.

The phospholipase C/inositol phosphate system

The *phosphoinositide* system, an important intracellular second messenger system, was first discovered in the 1950s by Hokin and Hokin, whose recondite interests centred on the mechanism of salt secretion by the nasal glands of seabirds. They found that secretion was accompanied by increased turnover of a minor class of membrane phospholipids known as phosphoinositides (collectively known as PIs; Fig. 3.11). Subsequently, Michell and Berridge found that many hormones that produce an increase in free

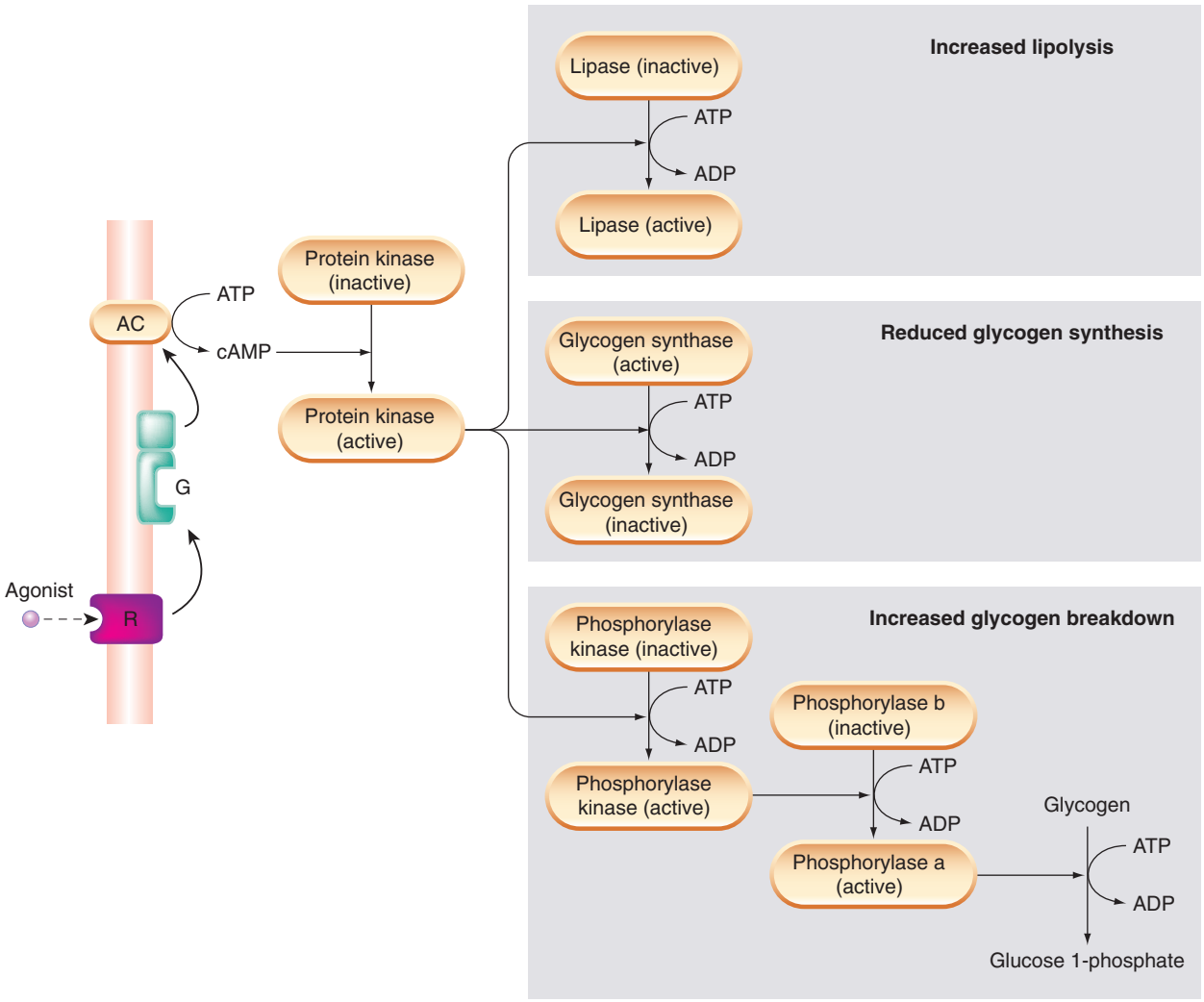

Fig. 3.10 **Regulation of energy metabolism by cAMP.** AC, adenylyl cyclase.

intracellular Ca^{2+} concentration (which include, for example, muscarinic agonists and α-adrenoceptor agonists acting on smooth muscle and salivary glands, and **vasopressin** acting on liver cells) also increase PI turnover. Subsequently, it was found that one particular member of the PI family, namely phosphatidylinositol (4,5) bisphosphate (PIP_2), which has additional phosphate groups attached to the inositol ring, plays a key role. PIP_2 is the substrate for a membrane-bound enzyme, phospholipase Cβ (PLCβ), which splits it into *diacylglycerol* (DAG) and *inositol (1,4,5) trisphosphate* (IP_3; Fig. 3.12), both of which function as second messengers as discussed below. The activation of PLCβ by various agonists is mediated through a G-protein (G_q, see Table 3.4). After cleavage of PIP_2, the status quo is restored as shown in Figure 3.12, DAG being phosphorylated to form phosphatidic acid (PA), while the IP_3 is dephosphorylated and then recoupled with PA to form PIP_2 once again.[11] **Lithium**, an agent used in psychiatry (see Ch. 46), blocks this recycling pathway (see Fig. 3.12).

[11]Alternative abbreviations for these mediators are PtdIns (PI), PtdIns (4,5)-P_2 (PIP_2), Ins (1,4,5)-P_3 (IP3), and Ins (1,2,4,5)-P4 (IP_4).

Inositol phosphates and intracellular calcium

Inositol (1,4,5) trisphosphate (IP_3) is a water-soluble mediator that is released into the cytosol and acts on a specific receptor—the IP_3 receptor—which is a ligand-gated calcium channel present on the membrane of the endoplasmic reticulum. The main role of IP_3, described in more detail in Chapter 4, is to control the release of Ca^{2+} from intracellular stores. Because many drug and hormone effects involve intracellular Ca^{2+}, this pathway is particularly important. IP_3 is converted inside the cell to the (1,3,4,5) tetraphosphate, IP_4, by a specific kinase. The exact role of IP_4 remains unclear, but recent evidence suggests that it, and also higher inositol phosphates, plays a role in controlling gene expression.

Diacylglycerol and protein kinase C

Diacylglycerol is produced as well as IP_3 whenever receptor-induced PI hydrolysis occurs. The main effect of DAG is to activate a membrane-bound protein kinase, *protein kinase C* (PKC), which catalyses the phosphorylation of a variety of intracellular proteins (see Nishizuka, 1988; Walaas & Greengard, 1991). DAG, unlike the inositol phosphates, is highly lipophilic and remains within the

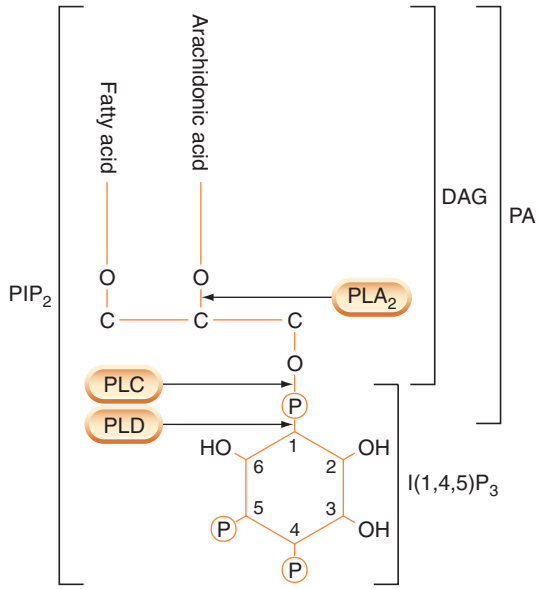

Fig. 3.11 Structure of phosphatidylinositol bisphosphate (PIP_2), showing sites of cleavage by different phospholipases to produce active mediators. Cleavage by phospholipase A_2 (PLA_2) yields arachidonic acid. Cleavage by phospholipase C (PLC) yields inositol trisphosphate ($I(1,4,5)P_3$) and diacylglycerol (DAG). PA, phosphatidic acid; PLD, phospholipase D.

membrane. It binds to a specific site on the PKC molecule, which migrates from the cytosol to the cell membrane in the presence of DAG, thereby becoming activated. There are 10 different mammalian PKC subtypes, which have distinct cellular distributions and phosphorylate different proteins. Most are activated by DAG and raised intracellular Ca^{2+}, both of which are produced by activation of GPCRs. PKCs are also activated by phorbol esters (highly irritant, tumour-promoting compounds produced by certain plants), which have been extremely useful in studying the functions of PKC. One of the subtypes is activated by the lipid mediator arachidonic acid (see Ch. 17) generated by the action of phospholipase A_2 on membrane phospholipids, so PKC activation can also occur with agonists that activate this enzyme. The various PKC isoforms, like the tyrosine kinases discussed below (p. 37), act on many different functional proteins, such as ion channels, receptors, enzymes (including other kinases), transcription factors and cytoskeletal proteins. Kinases in general play a central role in signal transduction, and control many different aspects of cell function. The DAG–PKC link provides a channel whereby GPCRs can mobilise this army of control freaks.

Ion channels as targets for G-proteins

G-protein-coupled receptors can control ion channel function directly by mechanisms that do not involve second messengers such as cAMP or inositol phosphates. Direct G-protein–channel interaction was first shown for cardiac muscle, but appears to be a general mechanism for controlling K^+ and Ca^{2+} channels (see Wickham & Clapham, 1995). In cardiac muscle, for example, mAChRs are known to enhance K^+ permeability (thus hyperpolarising the cells and inhibiting electrical activity; see Ch. 21). Similar mechanisms operate in neurons, where many inhibitory drugs such as opioid analgesics reduce excitability by opening K^+ channels or inhibiting Ca^{2+} channels (see Ch. 41). These actions are produced by direct interaction between the βγ subunit of G_0 and the channel, without the involvement of second messengers.

Effectors controlled by G-proteins

Two key pathways are controlled by receptors via G-proteins. Both can be activated or inhibited by pharmacological ligands, depending on the nature of the receptor and G-protein.

- Adenylate cyclase/cAMP:
  - adenylate cyclase catalyses formation of the intracellular messenger cAMP
  - cAMP activates various protein kinases that control cell function in many different ways by causing phosphorylation of various enzymes, carriers and other proteins.
- Phospholipase C/inositol trisphosphate (IP_3)/diacylglycerol (DAG):
  - catalyses the formation of two intracellular messengers, IP_3 and DAG, from membrane phospholipid
  - IP_3 acts to increase free cytosolic Ca^{2+} by releasing Ca^{2+} from intracellular compartments
  - increased free Ca^{2+} initiates many events, including contraction, secretion, enzyme activation and membrane hyperpolarisation
  - DAG activates protein kinase C, which controls many cellular functions by phosphorylating a variety of proteins.

Receptor-linked G-proteins also control:

- phospholipase A_2 (and thus the formation of arachidonic acid and eicosanoids)
- ion channels (e.g. potassium and calcium channels, thus affecting membrane excitability, transmitter release, contractility, etc.).

The Rho/Rho kinase system

▼ This recently discovered signal transduction pathway (see Bishop & Hall, 2000) is activated by certain GPCRs (and also by non-GPCR mechanisms), which couple to G-proteins of the $G_{12/13}$ type. The free G-protein α subunit interacts with a *guanosine nucleotide exchange factor*, which facilitates GDP–GTP exchange at another GTPase, Rho. Rho–GDP, the resting form, is inactive, but when GDP–GTP exchange occurs, Rho is activated, and in turn activates Rho kinase. Rho kinase phosphorylates many substrate proteins and controls a wide variety of cellular functions, including smooth muscle contraction and proliferation, angiogenesis and synaptic remodelling. By enhancing hypoxia-induced pulmonary artery vasoconstriction, activation of Rho kinase is thought to be important in the pathogenesis of pulmonary hypertension (see Ch. 21). Specific Rho kinase inhibitors (e.g. **fasudil**) are in development for a wide range of clinical indications—an area to watch.

The MAP kinase system

▼ This signal transduction pathway (see below and Fig. 3.15) is activated not only by various cytokines and growth factors acting on kinase-linked receptors (see p. 37), but also by GPCR ligands. It controls many processes involved in cell division, apoptosis and tissue regeneration.

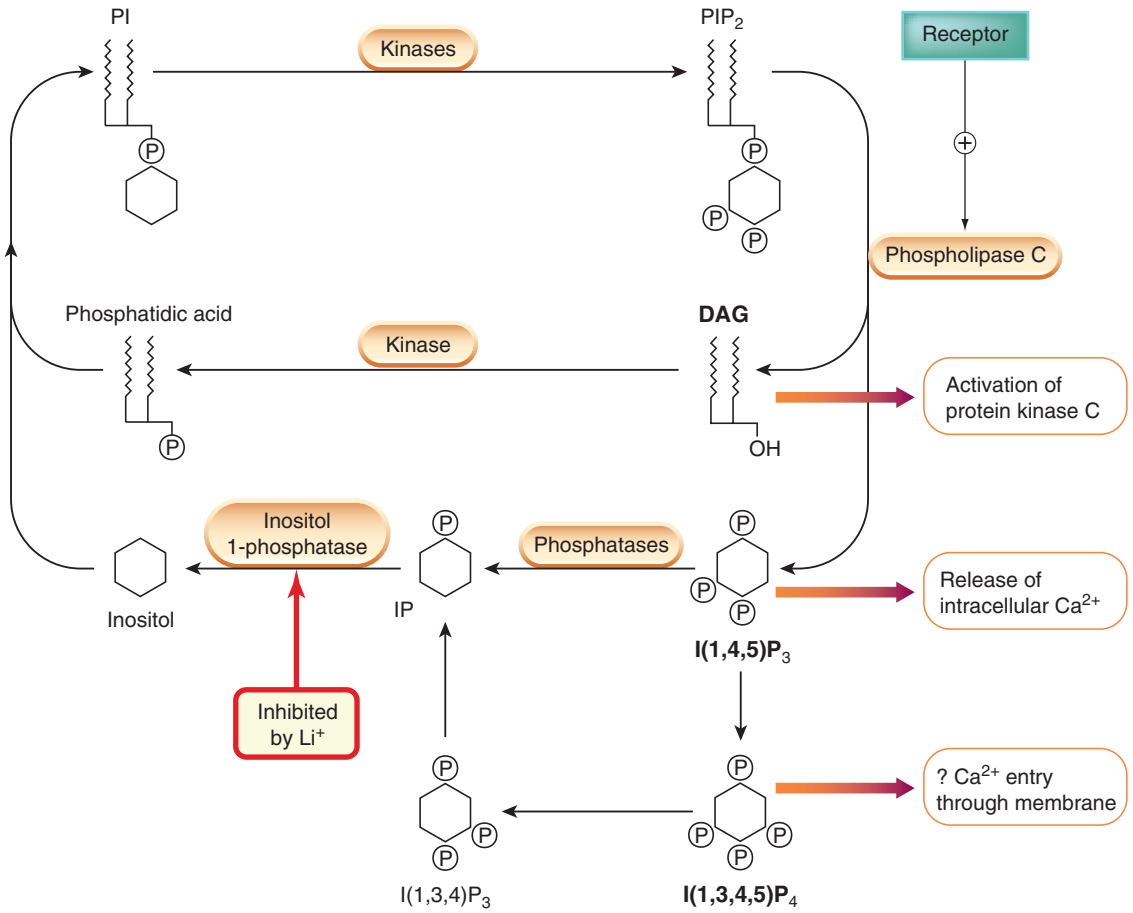

Fig. 3.12 The phosphatidylinositol (PI) cycle. Receptor-mediated activation of phospholipase C results in the cleavage of phosphatidylinositol bisphosphate (PIP_2), forming diacylglycerol (DAG) (which activates protein kinase C) and inositol trisphosphate (IP_3) (which releases intracellular Ca^{2+}). The role of inositol tetraphosphate (IP_4), which is formed from IP_3 and other inositol phosphates, is unclear, but it may facilitate Ca^{2+} entry through the plasma membrane. IP_3 is inactivated by dephosphorylation to inositol. DAG is converted to phosphatidic acid, and these two products are used to regenerate PI and PIP_2.

The main postulated roles of GPCRs in controlling enzymes and ion channels are summarised in Figure 3.13.

DESENSITISATION

▼ As described in Chapter 2, desensitisation is a feature of all GPCRs, and the mechanisms underlying it have been extensively studied. Two main processes are involved (see Koenig & Edwardson, 1997; Ferguson, 2001; Kelly et al., 2008):

- receptor phosphorylation
- receptor internalisation (endocytosis).

The sequence of GPCRs includes certain residues (serine and threonine), mainly in the C-terminal cytoplasmic tail, which can be phosphorylated by kinases such as protein kinase A (PKA), PKC and specific membrane-bound GPCR kinases (GRKs).

Phosphorylation by PKA and PKC, which are activated by many GPCRs, generally leads to impaired coupling between the activated receptor and the G-protein, so the agonist effect is reduced. These kinases are not very selective, so receptors other than that for the desensitising agonist will also be affected. This effect, whereby one agonist can desensitise other receptors, is known as *heterologous desensitisation*, and is generally weak and short lasting (see Fig. 3.14).

Phosphorylation by GRKs (Fig. 3.14) is receptor-specific to a greater or lesser degree, and affects mainly receptors in their activated (i.e. agonist-bound) state, resulting in *homologous desensitisation*. The residues that GRKs phosphorylate are different from those targeted by other kinases, and the phosphorylated receptor serves as a binding site for β-arrestins, intracellular proteins that block the interaction with G-proteins and also target the receptor for endocytosis, producing a more profound and long-lasting desensitisation. The first GRK to be identified was the β-adrenoceptor kinase, BARK, but several others have since been discovered, and this type of desensitisation seems to occur with most GPCRs. Besides initiating desensitisation, GRKs and arrestins are also involved as intermediates in various other GPCR-mediated signalling pathways that are distinct from those involving G-proteins (see Reiter & Lefkowitz, 2006). For example, binding of β-arrestin to GPCRs can recruit Src proteins, which in turn activate the *MAP kinase cascade*, which plays an important role in controlling cell division (see p. 39).

FURTHER DEVELOPMENTS IN GPCR BIOLOGY

▼ By the early 1990s, we thought we had more or less got the measure of GPCR function, as described above. Since then, the plot has thickened, and recent developments (see review by Pierce et al., 2002) have necessitated a substantial overhaul of the basic model, whose implications for pharmacology in the future are not yet clear. Those wishing to stick to the basic story of GPCR function can safely skip this section.

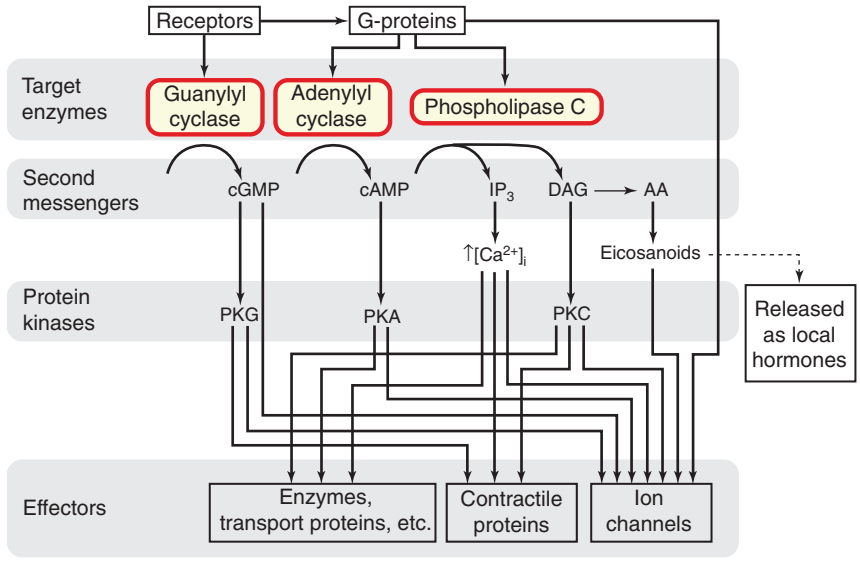

Fig. 3.13 G-protein and second messenger control of cellular effector systems. AA, arachidonic acid; DAG, diacylglycerol; IP_3, inositol trisphosphate. Not shown in this diagram are signalling pathways where arrestins, rather than G-proteins, link GPCRs to downstream events (see text).

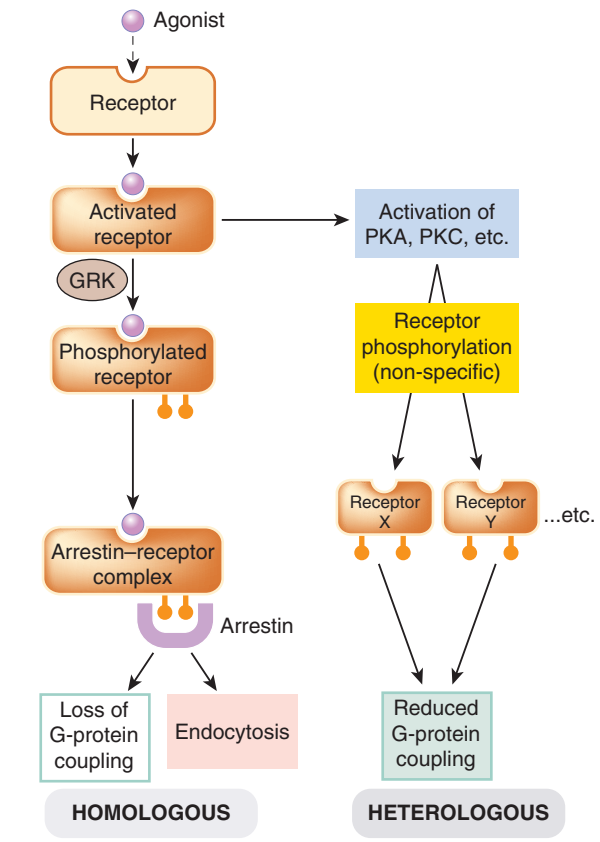

Fig. 3.14 Desensitisation of G-protein-coupled receptors (GPCRs). Homologous (agonist-specific) desensitisation involves phosphorylation of the activated receptor by a specific kinase (GPCR kinase, GRK). The phosphorylated receptor (P-R) then binds to arrestin, causing it to lose its ability to associate with a G-protein, and to undergo endocytosis, which removes the receptor from the membrane. Heterologous (cross-) desensitisation occurs as a result of phosphorylation of one type of receptor as a result of activation of kinases by another. PKA and PKC, protein kinase A and C, respectively.

GPCR dimerisation

▼ The conventional view that GPCRs exist and function as monomeric proteins (in contrast to ion channels, which generally form multimeric complexes; see p. 44) was first overturned by work on the $GABA_B$ receptor. Two subtypes of this GPCR exist, encoded by different genes, and the functional receptor consists of a heterodimer of the two (see Ch. 37). It now seems likely that most, if not all, GPCRs exist as oligomers (Prinster et al., 2005). Within the opioid receptor family (see Ch. 41), stable and functional dimers of κ and δ receptors, whose pharmacological properties differ from those of either parent, have been created in cell lines. More diverse GPCR combinations have also been found, such as that between dopamine (D_2) and somatostatin receptors, on which both ligands act with increased potency. Roaming even further afield in search of functional assignations, the dopamine receptor D_5 can couple directly with a ligand-gated ion channel, the $GABA_A$ receptor, inhibiting the function of the latter without the intervention of any G-protein (Liu et al., 2000). These interactions have so far been studied mainly in engineered cell lines, but they also occur in native cells. Functional dimeric complexes between angiotensin (AT_1) and bradykinin (B_2) receptors occur in human platelets and show greater sensitivity to angiotensin than 'pure' AT_1 receptors (AbdAlla et al., 2001). In pregnant women suffering from hypertension (pre-eclamptic toxaemia), the number of these dimers increases due to increased expression of B_2 receptors, resulting—paradoxically—in increased sensitivity to the vasoconstrictor action of angiotensin. This is the first instance of the role of dimerisation in human disease.

It is too early to say what impact this newly discovered versatility of GPCRs in linking up with other receptors to form functional combinations will have on conventional pharmacology and therapeutics, but it could be considerable.

Constitutively active receptors

▼ G-protein-coupled receptors may also be constitutively (i.e. spontaneously) active in the absence of any agonist (see Ch. 2 and review by Costa & Cotecchia, 2005). This was first shown for the β-adrenoceptor (see Ch. 14), where mutations in the third intracellular loop, or simply overexpression of the receptor, result in constitutive receptor activation. There are now many examples of native GPCRs that show constitutive activity when expressed in vitro (see Teitler et al., 2002). The histamine H_3 receptor also shows constitutive activity in vivo, and this may prove to be a quite general phenomenon. It means that inverse agonists, which suppress this basal activity, may exert effects distinct from those of neutral antagonists, which block agonist effects without affecting basal activity.

Agonist specificity

▼ It was thought that the linkage of a particular GPCR to a particular signal transduction pathway depends mainly on the structure of the receptor, particularly in the region of the third intracellular loop, which confers specificity for a particular G-protein, from which the rest of the signal transduction pathway follows. This would imply, in line with the two-state model discussed in Chapter 2, that all agonists acting on a particular receptor stabilise the same activated (R*) state and should activate the same signal transduction pathway, and produce the same type of cellular response. It is now clear that this is an oversimplification. In many cases, for example with agonists acting on opioid receptors, or with inverse agonists on β-adrenoceptors, the cellular effects are qualitatively different with different ligands, implying the existence of more than one—probably many—R* states (sometimes referred to as *agonist trafficking* or *protean agonism*, see Kenakin, 2002). This has profound implications—indeed heretical to many pharmacologists, who are accustomed to think of agonists in terms of their affinity and efficacy, and nothing else; it will add a new dimension to the way in which we think about drug efficacy and specificity (see Kelly et al., 2008).

RAMPs and RGS proteins

▼ Receptor activity-modifying proteins (RAMPs) are a family of membrane proteins that associate with GPCRs and alter their functional characteristics. They were discovered in 1998 when it was found that the functionally active receptor for the neuropeptide **calcitonin gene-related peptide** (CGRP) (see Ch. 19) consisted of a complex of a GPCR—called calcitonin receptor-like receptor (CRLR)—that by itself lacked activity, with another membrane protein (RAMP1). More surprisingly, CRLR when coupled with another RAMP (RAMP2) showed a quite different pharmacology, being activated by an unrelated peptide, **adrenomedullin**. In other words, the agonist specificity is conferred by the associated RAMP as well as by the GPCR itself. More RAMPs have emerged, and so far (see Parameswaran & Spielman, 2006) nearly all the examples involve peptide receptors.

Regulators of G-protein signalling (RGS) proteins (see review by Xie & Palmer 2007) are a family of about 20 cellular proteins that possess a conserved sequence that binds specifically to Gα subunits. They increase greatly the GTPase activity of the active GTP–Gα complex, so hastening the hydrolysis of GTP and inactivating the complex. They thus exert an inhibitory effect on G-protein signalling, a mechanism that is thought to have a regulatory function in many situations. RAMPs and RGS proteins are two examples where protein–protein interactions influence the pharmacological behaviour of the receptors in a highly selective way.

G-protein-independent signalling

▼ In using the term G-protein-coupled receptor to describe the class of receptors characterised by their heptahelical structure, we are following conventional textbook dogma but neglecting the fact that G-proteins are not the only link between GPCRs and the various effector systems that they regulate. The example of direct linkage between GPCRs and ion channels was mentioned above. There are also many examples where the various 'adapter proteins' that link receptors of the tyrosine kinase type to their effectors (see below) can also interact with GPCRs (see Brzostowski & Kimmel, 2001), allowing the same effector systems to be regulated by receptors of either type. In this context, the coupling of β-arrestins (see above), rather than G-proteins, to the activated GPCR, or phosphorylation of the C-terminal region of the GPCR by GRKs, produces a recognition site for molecules of the signal transduction pathway, analogous to the functioning of the kinase-linked receptors (see below; reviews by Bockaert & Pin, 1999; Delcourt et al., 2007).

In summary, the simple dogma that underpins much of our current understanding of GPCRs, namely,

> one GPCR gene—one GPCR protein—one functional GPCR—one G-protein—one response

is showing distinct signs of wear. In particular:

- one gene, through alternative splicing, RNA editing, etc., can give rise to more than one receptor protein
- one GPCR protein can associate with others, or with other proteins such as RAMPs, to produce more than one type of functional receptor
- different agonists may affect the receptor in different ways and elicit qualitatively different responses
- the signal transduction pathway does not invariably require G-proteins, and shows cross-talk with tyrosine kinase-linked receptors (see below).

G-protein-coupled receptors are evidently versatile and adventurous molecules around which much modern pharmacology revolves, and nobody imagines that we have reached the end of the story.

TYPE 3: KINASE-LINKED AND RELATED RECEPTORS

These membrane receptors are quite different in structure and function from either the ligand-gated channels or the GPCRs. They mediate the actions of a wide variety of protein mediators, including growth factors and cytokines (see Chs 17, 19), and hormones such as insulin (see Ch. 30) and leptin (Ch. 31), whose effects are exerted mainly at the level of gene transcription. Most of these receptors are large proteins consisting of a single chain of up to 1000 residues, with a single membrane-spanning helical region, associated with a large extracellular ligand-binding domain, and an intracellular domain of variable size and function. The basic structure is shown in Figure 3.3C, but many variants exist (see below). Over 100 such receptors have been cloned, and many structural variations exist. For more detail, see reviews by Schenk & Snaar-Jakelska (1999) and Hubbard & Miller (2007). They play a major role in controlling cell division, growth, differentiation, inflammation, tissue repair, apoptosis and immune responses, discussed further in Chapters 5 and 17.

The main types are as follow.

- *Receptor tyrosine kinases (RTKs).* These receptors have the basic structure shown in Figure 3.15A, incorporating a tyrosine kinase moiety in the intracellular region. They include receptors for many growth factors, such as **epidermal growth factor** and **nerve growth factor**, and also the group of *Toll-like receptors* that recognise bacterial lipopolysaccarides and play an important role in the body's reaction to infection (see Ch. 17). The insulin receptor (see Ch. 30) also belongs to the RTK class, although it has a more complex dimeric structure.
- *Serine/threonine kinases*. This smaller class is similar in structure to RTKs but phosphorylate serine and/or threonine residues rather than tyrosine. The main example is the receptor for **transforming growth factor** (TGF).
- *Cytokine receptors*. These receptors (Fig. 3.15B) lack intrinsic enzyme activity. When occupied, they associate with, and activate, a cytosolic tyrosine kinase, such as Jak (the Janus kinase) or other kinases. Ligands for these receptors include cytokines such as **interferons** and **colony-stimulating factors** involved in immunological responses.

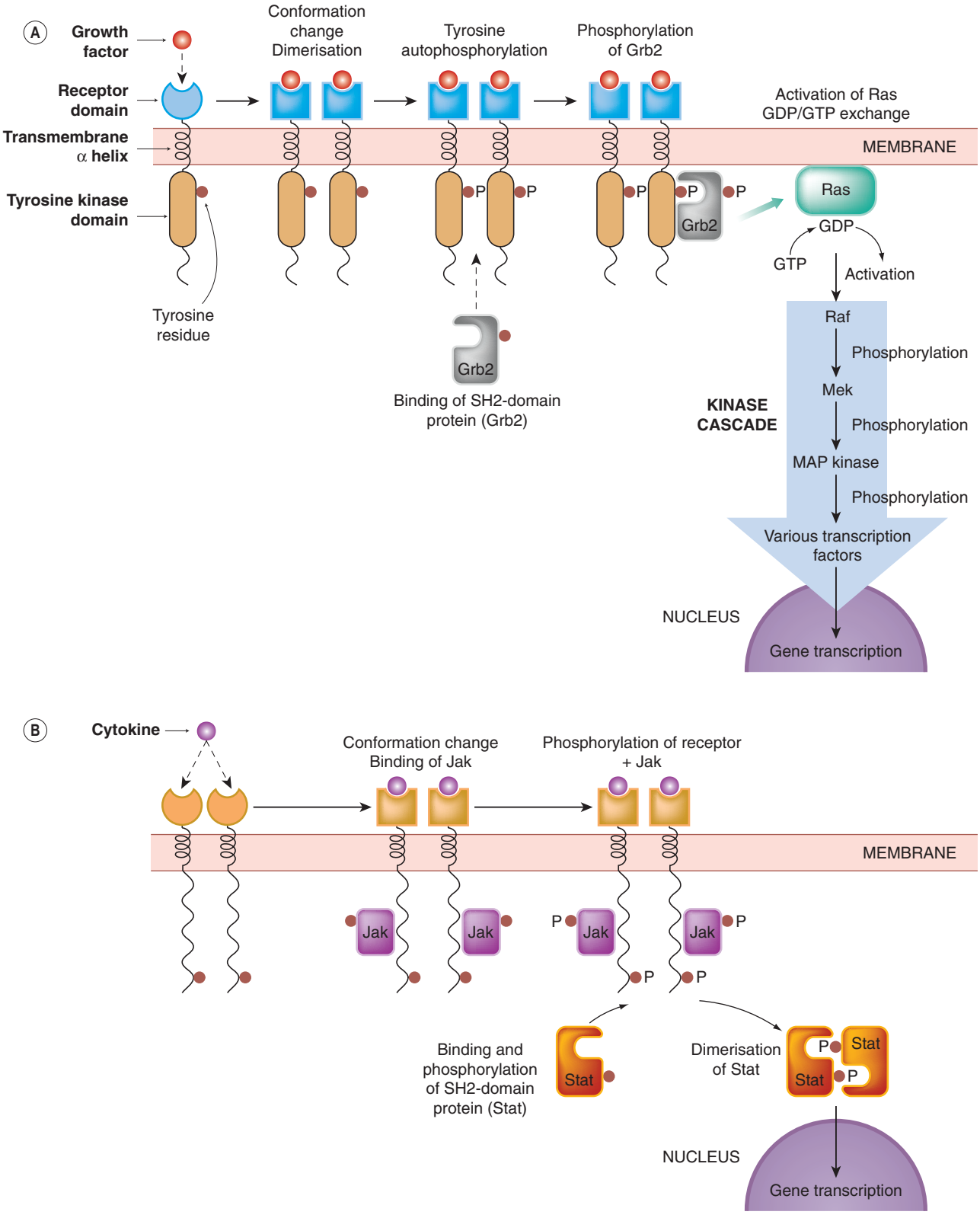

Fig. 3.15 Transduction mechanisms of kinase-linked receptors. The first step following agonist binding is dimerisation, which leads to autophosphorylation of the intracellular domain of each receptor. SH2-domain proteins then bind to the phosphorylated receptor and are themselves phosphorylated. Two well-characterised pathways are shown: [**A**] the growth factor (Ras/Raf/mitogen-activated protein [MAP] kinase) pathway (see also Ch. 5); [**B**] the cytokine (Jak/Stat) pathway (see also Ch. 17). Several other pathways exist, and these phosphorylation cascades interact with components of G-protein systems.

Kinase-linked receptors

- Receptors for various growth factors incorporate tyrosine kinase in their intracellular domain.
- Cytokine receptors have an intracellular domain that binds and activates cytosolic kinases when the receptor is occupied.
- The receptors all share a common architecture, with a large extracellular ligand-binding domain connected via a single membrane-spanning helix to the intracellular domain.
- Signal transduction generally involves dimerisation of receptors, followed by autophosphorylation of tyrosine residues. The phosphotyrosine residues act as acceptors for the SH2 domains of a variety of intracellular proteins, thereby allowing control of many cell functions.
- They are involved mainly in events controlling cell growth and differentiation, and act indirectly by regulating gene transcription.
- Two important pathways are:
  - the Ras/Raf/mitogen-activated protein (MAP) kinase pathway, which is important in cell division, growth and differentiation
  - the Jak/Stat pathway activated by many cytokines, which controls the synthesis and release of many inflammatory mediators.
- A few hormone receptors (e.g. atrial natriuretic factor) have a similar architecture and are linked to guanylyl cyclase.

PROTEIN PHOSPHORYLATION AND KINASE CASCADE MECHANISMS

One of the major principles to emerge over the last 10–20 years (see Cohen, 2002) is that protein phosphorylation is a key mechanism for controlling the function of proteins (e.g. enzymes, ion channels, receptors, transport proteins) involved in regulating cellular processes. Phosphorylation and dephosphorylation are accomplished by *kinases* and *phosphatases*, respectively—enzymes of which several hundred subtypes are represented in the human genome—which are themselves subject to regulation dependent on their phosphorylation status. Much effort is currently being invested in mapping the complex interactions between signalling molecules that are involved in drug effects and pathophysiological processes such as oncogenesis, neurodegeneration, inflammation and much else. Here we can present only a few pharmacologically relevant aspects of what has become an enormous subject.

In many cases, ligand binding to the receptor leads to dimerisation. The association of the two intracellular kinase domains allows a mutual autophosphorylation of intracellular tyrosine residues to occur. The phosphorylated tyrosine residues then serve as high-affinity docking sites for other intracellular proteins that form the next stage in the signal transduction cascade. One important group of such 'adapter' proteins is known as the *SH2 domain proteins* (standing for Src homology, because it was first identified in the Src oncogene product). These possess a highly conserved sequence of about 100 amino acids, forming a recognition site for the phosphotyrosine residues of the receptor. Individual SH2 domain proteins, of which many are now known, bind selectively to particular receptors, so the pattern of events triggered by particular growth factors is highly specific. The mechanism is summarised in Figure 3.15.

What happens when the SH2 domain protein binds to the phosphorylated receptor varies greatly according to the receptor that is involved; many SH2 domain proteins are enzymes, such as protein kinases or phospholipases. Some growth factors activate a specific subtype of phospholipase C (PLCγ), thereby causing phospholipid breakdown, IP_3 formation and Ca^{2+} release (see above). Other SH2-containing proteins couple phosphotyrosine-containing proteins with a variety of other functional proteins, including many that are involved in the control of cell division and differentiation. The end result is to activate or inhibit, by phosphorylation, a variety of transcription factors that migrate to the nucleus and suppress or induce the expression of particular genes. For more detail, see Pawson (2002). Nuclear factor kappa B (NFκB) is a transcription factor that plays a key role in inflammatory responses (see Ch. 17; Karin et al., 2004). It is normally present in the cytosol complexed with an inhibitor (IκB). Phosphorylation of IκB occurs when a specific kinase (IKK) is activated in response to various inflammatory cytokines and GPCR agonists. This results in dissociation of IκB from NFκB and migration of NFκB to the nucleus, where it switches on a wide variety of proinflammatory genes.

▼ Two well-defined signal transduction pathways are summarised in Figure 3.15. The Ras/Raf pathway (Fig. 3.15A) mediates the effect of many growth factors and mitogens. Ras, which is a proto-oncogene product, functions like a G-protein, and conveys the signal (by GDP/GTP exchange) from the SH2-domain protein, Grb, which is phosphorylated by the RTK. Activation of Ras in turn activates Raf, which is the first of a sequence of three serine/threonine kinases, each of which phosphorylates, and activates, the next in line. The last of these, mitogen-activated protein (MAP) kinase, (which is also activated by GPCRs, see above), phosphorylates one or more transcription factors that initiate gene expression, resulting in a variety of cellular responses, including cell division. This three-tiered MAP kinase cascade forms part of many intracellular signalling pathways involved in a wide variety of disease processes, including malignancy, inflammation, neurodegeneration, atherosclerosis and much else. The kinases form a large family, with different subtypes serving specific roles. They are thought to represent an important target for future therapeutic drugs. Many cancers are associated with mutations in the genes coding for proteins involved in this cascade, leading to activation of the cascade in the absence of the growth factor signal (see Chs 5 and 55). For more details, see reviews by Marshall (1996), Schenk & Snaar-Jakelska (1999), Avruch (2007).

A second pathway, the Jak/Stat pathway (Fig. 3.15B) is involved in responses to many cytokines. Dimerisation of these receptors occurs when the cytokine binds, and this attracts a cytosolic tyrosine kinase unit (Jak) to associate with, and phosphorylate, the receptor dimer. Jaks belong to a family of proteins, different members having specificity for different cytokine receptors. Among the targets for phosphorylation by Jak are a family of transcription factors (Stats). These are SH2-domain proteins that bind to the phosphotyrosine groups on the receptor–Jak complex, and are themselves phosphorylated. Thus activated, Stat migrates to the nucleus and activates gene expression (see Ihle, 1995).

Other important mechanisms centre on *phosphaditylinositol-3-kinase* (PI_3 kinases, see Vanhaesebroeck et al., 1997), a ubiquitous enzyme family that is activated both by GPCRs and RTKs and attaches a phosphate group to position 3 of PIP_2 to form PIP_3. Other kinases, particularly protein kinase B (PKB, also known as Akt), have

Protein phosphorylation in signal transduction

- Many receptor-mediated events involve protein phosphorylation, which controls the functional and binding properties of intracellular proteins.
- Receptor-linked tyrosine kinases, cyclic nucleotide-activated tyrosine kinases and intracellular serine/threonine kinases comprise a 'kinase cascade' mechanism that leads to amplification of receptor-mediated events.
- There are many kinases, with differing substrate specificities, allowing specificity in the pathways activated by different hormones.
- Desensitisation of G-protein-coupled receptors occurs as a result of phosphorylation by specific receptor kinases, causing the receptor to become non-functional and to be internalised.
- There is a large family of phosphatases that act to reverse the effects of kinases.

recognition sites for PIP_3 and are thus activated, controlling a wide variety of cellular functions, including apoptosis, differentiation, proliferation and trafficking. Akt also causes nitric oxide synthase activation in the vascular endothelium (see Ch. 20).

Recent work on signal transduction pathways has produced a bewildering profusion of molecular detail, often couched in a jargon that is apt to deter the faint-hearted. Perseverance will be rewarded, however, for there is no doubt that important new drugs, particularly in the areas of inflammation, immunology and cancer, will come from the targeting of these proteins (see Cohen, 2002). A recent breakthrough in the treatment of chronic myeloid leukaemia was achieved with the introduction of the first specific kinase inhibitor, **imatinib**, a drug that inhibits a specific tyrosine kinase involved in the pathogenesis of the disease (see Ch. 55).

The membrane-bound form of *guanylyl cyclase*, the enzyme responsible for generating the second messenger cGMP in response to the binding of natriuretic peptides (see Chs 19 and 21), resembles the tyrosine kinase family and is activated in a similar way by dimerisation when the agonist is bound (see Lucas et al., 2000).

Figure 3.16 illustrates the central role of protein kinases in signal transduction pathways in a highly simplified and schematic way. Many, if not all, of the proteins involved, including the receptors and the kinases themselves, are substrates for kinases, so there are many mechanisms for feedback and cross-talk between the various signalling pathways. Given that there are over 500 protein kinases, and similarly large numbers of receptors and other signalling molecules, the network of interactions can look bewilderingly complex. Dissecting out the details has become a major theme in cell biology. For pharmacologists, the idea of a simple connection between receptor and response, which guided thinking throughout the 20th century, is undoubtedly crumbling, although it will take some time before the complexities of signalling pathways are assimilated into a new way of thinking about drug action.

TYPE 4: NUCLEAR RECEPTORS

The fourth type of receptors we will consider belong to the *nuclear receptor (NR) family*. By the 1970s, it was clear that receptors for steroid hormones such as oestrogen and the glucocorticoids were present in the cytoplasm of cells and translocated into the nucleus after binding with their steroid partner. Other hormones, such as the thyroid hormone T_3 (Ch. 33) and the fat-soluble vitamins D and A (retinoic acid), were found to act in a similar fashion. Comparisons of genome and protein sequence data led to the recognition that they were members of a much larger family of related proteins. As well as NRs such as the glucocorticoid and retinoic acid receptor, whose ligands were well characterised, this family includes a great many (40%) *orphan receptors*—receptors with no known well-defined ligands. The first of these to be described, in the 1990s, was *RXR*, a receptor cloned on the basis of its similarity with the vitamin A receptor and that was subsequently found to bind the vitamin A derivative 9-*cis*-retinoic acid. Over the intervening years, binding partners have been identified for many NRs ('adopted orphans'; e.g. RXR) but the ligands of many others ('true orphans') have yet to be identified, or perhaps do not exist as such.

Today, it is convenient to regard the entire NR family as *ligand-activated transcription factors* that transduce signals by modifying gene transcription. Unlike the receptors described in the preceding sections of this chapter, the nuclear receptors are not embedded in membranes (although see below), but are present in the soluble phase of the cell. Some, such as the steroid receptors, become mobile in the presence of their ligand and can translocate from the cytoplasm to the nucleus, while others such as the RXR probably dwell mainly within the nuclear compartment. Some NRs, while unliganded, act to constitutively repress some genes (e.g. RXR).

In man, there are at least 48 NR genes (although only about half code for liganded receptors) but more NR proteins may arise through alternative splicing events. While this represents a rather small proportion of all receptors (less than 10% of the total number of GPCRs), the NRs are very important drug targets, being responsible for the biological effects of approximately 10% of all prescription drugs. They can recognise an extraordinarily diverse group of substances (mostly small hydrophobic molecules), which may exhibit full or partial agonist, antagonist or inverse agonist activity. Some NRs are involved predominately in endocrine signalling but many act as lipid sensors and are thus crucial links between our dietary and metabolic status and the expression of genes that regulate the metabolism and disposition of lipids. They also regulate expression of many drug metabolic enzymes and transporters. Many illnesses are associated with malfunctioning of the NR system, including inflammation, cancer, diabetes, cardiovascular disease, obesity and reproductive disorders (see Murphy & Holder, 2000, Kersten et al., 2000).

STRUCTURE OF NUCLEAR RECEPTORS

▼ All NRs are monomeric proteins that share a broadly similar structural design (see Fig. 3.17 and Bourguet et al., 2000, for further details). The *N-terminal domain* displays the most heterogeneity. It harbours the *AF1* (activation function 1) site that binds to other cell-specific transcription factors in a ligand-independent way and modifies the binding or activity of the receptor itself. Alternative splicing of genes may yield several receptor isoforms each with slightly different N-terminal regions. The *core domain* of the receptor is highly conserved and consists of the structure responsible for DNA recognition and binding. At the molecular level, this comprises two *zinc*

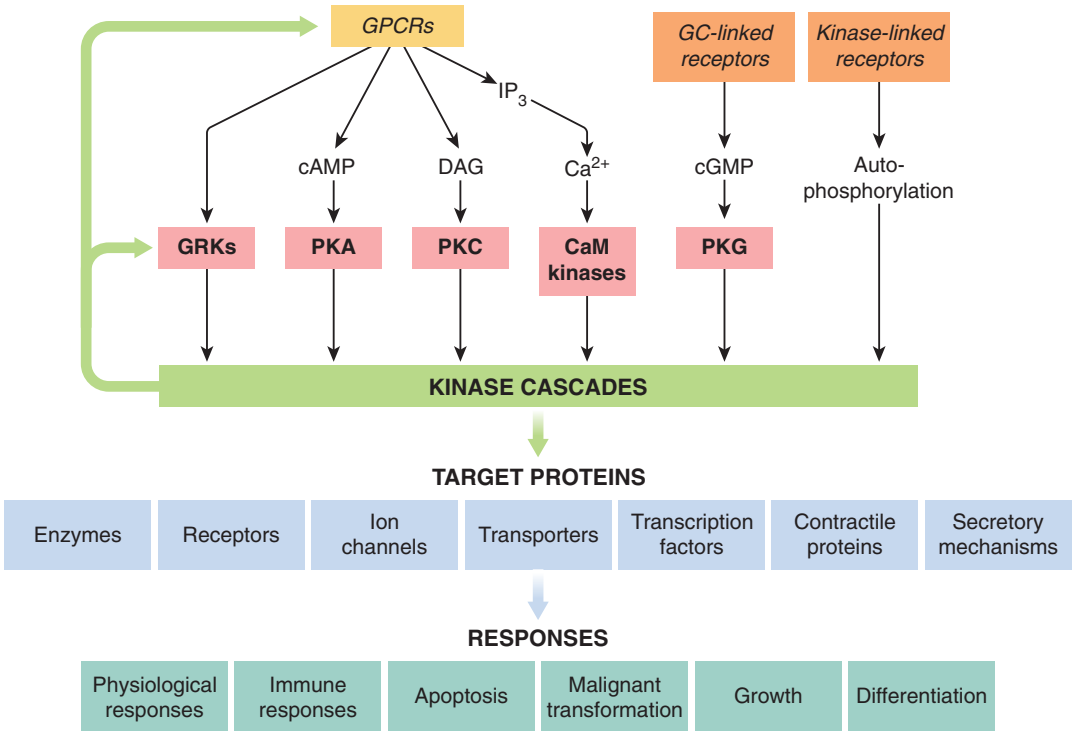

Fig. 3.16 Central role of kinase cascades in signal transduction. Kinase cascades (e.g. those shown in Fig. 3.15) are activated by GPCRs, either directly or via different second messengers, by receptors that generate cGMP, or by kinase-linked receptors. The kinase cascades regulate various target proteins, which in turn produce a wide variety of short- and long-term effects. CaM kinase, Ca^{2+}/calmodulin-dependent kinase; DAG, diacylglycerol; GC, guanylyl cyclase; GRK, GPCR kinase; IP_3, inositol trisphosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase.

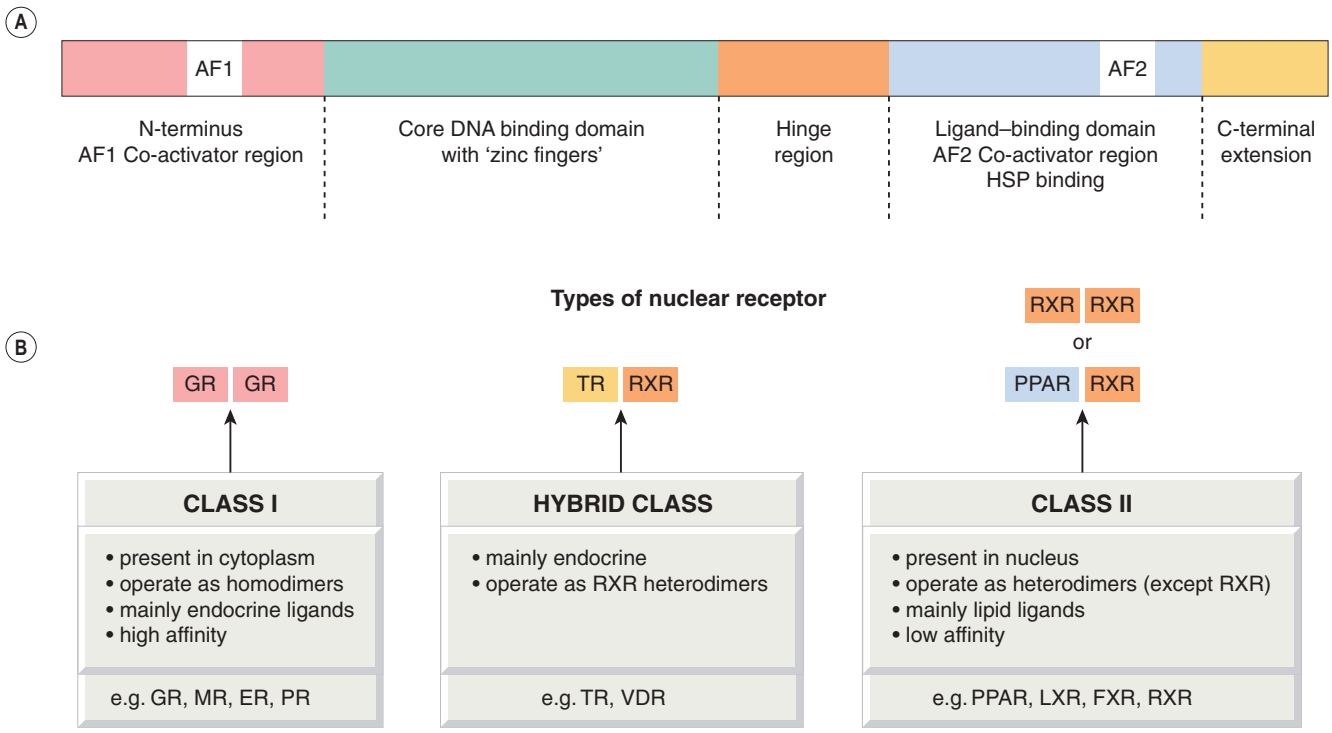

Fig. 3.17 Nuclear receptors. [**A**] Structure of a nuclear receptor, showing the different domains. [**B**] The two main classes of nuclear receptors. ER, oestrogen receptor; FXR, farnesoid receptor; GR, glucocorticoid receptor; HSP, heat shock protein; LXR, liver oxysterol receptor; MR, mineralocorticoid receptor; PPAR, peroxisome proliferator receptor; PR, prolactin receptor; RXR, retinoid receptor; TR, thyroid receptor; VDR, vitamin D receptor.

fingers—cysteine- (or cystine-/histidine-) rich loops in the amino acid chain that are held in a particular conformation by zinc ions. The main function of this portion of the molecule is to recognise and bind to the *hormone response elements* located in genes that are regulated by this family of receptors, but it also plays a part in regulating receptor dimerisation as well.

It is the highly flexible *hinge region* in the molecule that allows it to dimerise with other NRs and also to exhibit DNA binding in a variety of configurations. Finally, the *C-terminal domain* contains the ligand-binding module and is specific to each class of receptor. A highly conserved AF2 region is important in ligand-dependent activation. Also located near the C-terminal are motifs that contain nuclear localisation signals and others that may, in the case of some receptors, bind *accessory heat shock* and other proteins.

CLASSIFICATION OF NUCLEAR RECEPTORS

The NR superfamily consists of two *main* classes (I and II), together with a third that shares some of the characteristics of both (see Fig. 3.17 and Germain et al., 2006, for further details). Class I consists largely of receptors for the steroid hormones, including the glucocorticoid and mineralocorticoid receptors (GR and MR), as well as the oestrogen, progesterone and androgen receptors (ER, PR and AR, respectively). These receptors generally recognise hormones (e.g. glucocorticoids) that act in a negative feedback fashion to control biological events (see Ch. 32 for more details). In the absence of their ligand, these NRs are predominantly located in the cytoplasm, complexed with heat shock and other proteins and possibly reversibly attached to the cytoskeleton or other structures. Following diffusion (or possibly transportation) from the blood into the cell, their ligand partner binds to their NR with high affinity. These liganded receptors generally form homodimers and translocate to the nucleus, where they can *transactivate* or *transrepress* genes by binding to 'positive' or 'negative' hormone response elements (see below). Large numbers of genes can be regulated in this way by a single ligand. For example, it is estimated that the activated GR itself can regulate transcription of ~1% of the genome either directly or indirectly.

Class II NRs function in a slightly different way. Their ligands are generally lipids already present to some extent within the cell. This group includes the *peroxisome proliferator-activated receptor* (PPAR) that recognises fatty acids; the *liver oxysterol receptor* (LXR) that recognises and acts as a cholesterol sensor, the *farnesoid (bile acid) receptor* (FXR), a *xenobiotic receptor* (SXR; in rodents the PXR) that recognises a great many foreign substances, including therapeutic drugs, and the constitutive *androstane receptor* (CAR), which not only recognises the steroid androstane but also some drugs such as **phenobarbital** (see Ch. 43). These latter NRs are akin to airport security guards who alert the bomb disposal squad when suspicious luggage is found. They induce drug-metabolising enzymes such as CYP3A (which is responsible for metabolising about 60% of all prescription drugs; see Ch. 9 and Synold et al., 2001), and also bind some prostaglandins and non-steroidal drugs, as well as the antidiabetic **thiazolidinediones** (see Ch. 30) and **fibrates** (see Ch. 23). Unlike the receptors in class I, these NRs almost always operate as heterodimers together with the retinoid receptor (RXR). They tend to mediate positive feedback effects (e.g. occupation of the receptor amplifies rather than inhibits a particular biological event). When class II monomeric receptors bind to RXR, two types of heterodimer may be formed: a *non-permissive heterodimer*, which can be activated only by the RXR ligand itself, and the *permissive heterodimer*, which can be activated either by retinoic acid itself or by its partner's ligand.

A third group of NRs is really a subgroup of class II in the sense that they form obligate heterodimers with RXR, but rather than sensing lipids, they play a part in endocrine signalling. The group includes the *thyroid hormone receptor* (TR), the *vitamin D receptor* (VDR) and the *retinoic acid receptor* (RAR).

Nuclear receptors

- A family of 48 soluble receptors that sense lipid and hormonal signals and modulate gene transcription.
- Two main categories:
  - those that are present in the cytoplasm, form homodimers in the presence of their partner, and migrate to the nucleus. Their ligands are mainly endocrine in nature (e.g. steroid hormones)
  - those that are generally constitutively present in the nucleus and form heterodimers with the retinoid X receptor. Their ligands are usually lipids (e.g. the fatty acids).
- A third subgroup transduce mainly endocrine signals but function as heterodimers with retinoid X receptor (e.g. the thyroid hormone).
- The liganded receptor complexes initiate changes in gene transcription by binding to hormone response elements in gene promoters and recruiting co-activator or co-repressor factors.
- The receptor family is responsible for the pharmacology of approximately 10%, and the enzymes that it regulates affect the pharmacokinetics of some 60% of all prescription drugs.

CONTROL OF GENE TRANSCRIPTION

▼ Hormone response elements are the short (four or five base pairs) sequences of DNA to which the NRs bind to modify gene transcription. They are usually present symmetrically in pairs or half sites, although these may be arranged together in different ways (e.g. simple repeats or inverted repeats). Each NR exhibits a preference for a particular *consensus sequence* but because of the family homology, there is a close similarity between these sequences.

Once in the nucleus, the ligand-bound receptor recruits further proteins including *co-activators* or *co-repressors* to modify gene expression through its AF1 and AF2 domains. Some of these co-activators are enzymes involved in chromatin remodelling such as histone acetylase/deacetylase which, together with other enzymes, regulate the unravelling of the DNA to facilitate access by polymerase enzymes and hence gene transcription. Co-repressor complexes are recruited by some receptors and comprise histone deacetylase and other factors that cause the chromatin to become tightly packed, preventing further transcriptional activation. Some unliganded class II receptors such as TR and VDR are constitutively bound to these repressor complexes in the nucleus, thus 'silencing' the gene. The complex dissociates on ligand binding, permitting an activator complex to bind. The case of CAR is particularly interesting; like some types of G-proteins described earlier in this chapter, CAR also forms a constitutively active complex that is terminated when it binds its ligand.

The discussion here must be taken only as a broad guide to the action of NRs, as many other types of interaction have also been discovered. For example, some receptors

may bring about non-genomic actions by directly interacting with factors in the cytosol, or they may be covalently modified by phosphorylation or by protein–protein interactions with other transcription factors such that their function is altered (see Falkenstein et al., 2000). In addition, there is good evidence for separate membrane and other types of receptor that can bind some steroid hormones such as oestrogen (see Walters & Nemere, 2004). This intricate network of receptors and their nuclear and cytosolic interactions serves as a subtle regulator of blood lipids as well as transducing the effects of hormones that have arrived from distant tissues. Much remains to be discovered about this interesting and complex family of receptor proteins.

ION CHANNELS AS DRUG TARGETS

We have discussed ligand-gated ion channels as one of the four main types of drug receptor. There are many other types of ion channel that represent important drug targets, even though they are not generally classified as 'receptors' because they are not the immediate targets of fast neurotransmitters.[12]

Here we discuss the structure and function of ion channels at the molecular level; their role as regulators of cell function is described in Chapter 4.

Ions are unable to penetrate the lipid bilayer of the cell membrane, and can get across only with the help of membrane-spanning proteins in the form of channels or transporters. The concept of ion channels was developed in the 1950s on the basis of electrophysiological studies on the mechanism of membrane excitation (see below). Electrophysiology, particularly the *voltage clamp technique* (see Ch. 4) remains an essential tool for studying the physiological and pharmacological properties of ion channels. Since the mid-1980s, when the first ion channels were cloned by Numa in Japan, much has been learned about the structure and function of these complex molecules. The use of tight-seal ('patch clamp') recording, which allows the behaviour of individual channels to be studied in real time, has been particularly valuable in distinguishing channels on the basis of their conductance and gating characteristics. Accounts by Hille (2001), Ashcroft (2000) and Catterall (2000) give more information.

Ion channels consist of protein molecules designed to form water-filled pores that span the membrane, and can switch between open and closed states. The rate and direction of ion movement through the pore is governed by the electrochemical gradient for the ion in question, which is a function of its concentration on either side of the membrane, and of the membrane potential. Ion channels are characterised by:

- their selectivity for particular ion species, determined by the size of the pore and the nature of its lining
- their gating properties (i.e. the nature of the stimulus that controls the transition between open and closed states of the channel)
- their molecular architecture.

ION SELECTIVITY

Channels are generally either cation selective or anion selective. The main cation-selective channels are selective for Na^+, Ca^{2+} or K^+, or non-selective and permeable to all three. Anion channels are mainly permeable to Cl^-, although other types also occur. The effect of modulation of ion channels on cell function is discussed in Chapter 4.

GATING

VOLTAGE-GATED CHANNELS

These channels open when the cell membrane is depolarised. They form a very important group because they underlie the mechanism of membrane excitability (see Ch. 4). The most important channels in this group are selective sodium, potassium or calcium channels.

Commonly, the channel opening (activation) induced by membrane depolarisation is short lasting, even if the depolarisation is maintained. This is because, with some channels, the initial activation of the channels is followed by a slower process of inactivation.

The role of voltage-gated channels in the generation of action potentials and in controlling other cell functions is described in Chapter 4.

LIGAND-GATED CHANNELS

These (see above) are activated by binding of a chemical ligand to a site on the channel molecule. Fast neurotransmitters, such as glutamate, acetylcholine, GABA and ATP (see Chs 13, 16 and 37) act in this way, binding to sites on the outside of the membrane. The *vanilloid receptor* TRPV1 mediates the pain-producing effect of **capsaicin** on sensory nerves (as well as responding to low pH and heat; see Ch. 41).

Some ligand-gated channels in the plasma membrane respond to intracellular rather than extracellular signals, the most important being the following:

- Calcium-activated potassium channels, which occur in most cells and open, thus hyperpolarising the cell, when $[Ca^{2+}]_i$ increases.
- ATP-sensitive potassium channels, which open when the intracellular ATP concentration falls because the cell is short of nutrients. These channels, which are quite distinct from those mediating the excitatory effects of extracellular ATP, occur in many nerve and muscle cells, and also in insulin-secreting cells (see Ch. 30), where they are part of the mechanism linking insulin secretion to blood glucose concentration.

Other examples of channels that respond to intracellular ligands include arachidonic acid-sensitive potassium channels and DAG-sensitive calcium channels, whose functions are not well understood.

CALCIUM RELEASE CHANNELS

These are present on the endoplasmic or sarcoplasmic reticulum rather than the plasma membrane. The main ones, IP_3 and **ryanodine** receptors (see Ch. 4) are a special

[12]In truth, the distinction between ligand-gated channels and other ion channels is an arbitrary one. In grouping ligand-gated channels with other types of receptor in this book, we are respecting the historical tradition established by Langley and others, who first defined receptors in the context of the action of acetylcholine at the neuromuscular junction. The advance of molecular biology may force us to reconsider this semantic issue in the future, but for now we make no apology for upholding the pharmacological tradition.

class of ligand-gated calcium channels that control the release of Ca^{2+} from intracellular stores.

STORE-OPERATED CALCIUM CHANNELS

When the intracellular Ca^{2+} stores are depleted, 'store-operated' channels (SOCs) in the plasma membrane open to allow Ca^{2+} entry. The mechanism by which this linkage occurs involves interaction of a Ca^{2+}-sensor protein in the endoplasmic reticulum membrane with a dedicated Ca^{2+} channel in the plasma membrane (see Potier & Trebak, 2008). In response to GPCRs that elicit Ca^{2+} release, the opening of these channels allows $[Ca^{2+}]_i$ to remain elevated even when the stores are running low, and also provides a route through which the stores can be replenished (see Ch. 4).

MOLECULAR ARCHITECTURE OF ION CHANNELS

▼ Ion channels are large and elaborate molecules. Their characteristic structural motifs have been revealed as knowledge of their sequence and structure has accumulated since the mid-1980s, when the first ligand-gated channel (the nicotinic acetylcholine receptor) and the first voltage-gated sodium channel were cloned. The main structural subtypes are shown in Figure 3.18. All consist of several (often four) domains, which are similar or identical to each other, organised either as an oligomeric array of separate subunits, or as one large protein. Each subunit or domain contains a bundle of two to six membrane-spanning helices. Most ligand-gated channels have the basic structure shown in Figure 3.18A, comprising a pentameric array of non-identical subunits, each consisting of four transmembrane helices, of which one—the M_2 segment—from each subunit lines the pore. The large extracellular N-terminal region contains the ligand-binding region. Several exceptions to this basic design for ligand-gated channels have emerged recently. They include (see Fig. 3.18) the glutamate NMDA receptor (Ch. 37), and the vanilloid receptor (a channel that responds not only to chemicals of the vanilloid class, but also to heat and protons; see Ch. 41). In these, as in many other types of channel, the pore-forming part of the molecule consists of a hairpin loop—the pore (P) loop—between two of the helices.

Voltage-gated channels generally include one transmembrane helix that contains an abundance of basic (i.e. positively charged) amino acids. When the membrane is depolarised, so that the interior of the cell becomes less negative, this region—the voltage sensor—moves slightly towards the outer surface of the membrane, which has the effect of opening the channel (see Bezanilla, 2008). Many voltage-activated channels also show *inactivation*, which happens when an intracellular appendage of the channel protein moves to plug the channel from the inside. Voltage-gated sodium and calcium channels are remarkable in that the whole structure with four six-helix domains consists of a single huge protein molecule, the domains being linked together by intracellular loops of varying length. Potassium channels comprise the most numerous and heterogeneous class.[13] Voltage-gated potassium channels resemble sodium channels, except that they are made up of four subunits rather than a single long chain. The class of potassium channels known as 'inward rectifier channels' because of their biophysical properties has the two-helix structure shown in Figure 3.18C, whereas others are classed as 'two-pore domain' channels, because each subunit contains two P loops.

The various architectural motifs shown in Figure 3.18 only scrape the surface of the molecular diversity of ion channels. In all cases, the individual subunits come in several molecular varieties, and these can unite in different combinations to form functional channels as *hetero-oligomers* (as distinct from *homo-oligomers* built from identical subunits). Furthermore, the channel-forming structures described are usually associated with other membrane proteins, which significantly affect their functional properties. For example, the ATP-gated potassium channel exists in association with the *sulfonylurea receptor* (SUR), and it is through this linkage that various drugs (including antidiabetic drugs of the sulfonylurea class; see Ch. 30) regulate the channel (see Ashcroft & Gribble, 2000). Good progress is being made in understanding the relation between molecular structure and ion channel function, but we still have only a fragmentary understanding of the physiological role of many of these channels. Many important drugs exert their effects by influencing channel function, either directly or indirectly.

[13]The human genome encodes more than 70 distinct potassium channel subtypes—either a nightmare or a golden opportunity for the pharmacologist, depending on one's perspective.

PHARMACOLOGY OF ION CHANNELS

▼ Many drugs and physiological mediators described in this book exert their effects by altering the behaviour of ion channels. Here we outline the general mechanisms as exemplified by the pharmacology of voltage-gated sodium channels (Fig. 3.19). Ion channel pharmacology is likely to be a fertile source of future new drugs (see Clare et al., 2000).

The gating and permeation of both voltage-gated and ligand-gated ion channels is modulated by many factors, including the following.

- *Ligands that bind directly to various sites on the channel protein*. These include many neurotransmitters, and also a variety of drugs and toxins that act in different ways, for example by blocking the channel or by affecting the gating process, thereby either facilitating or inhibiting the opening of the channel.
- *Mediators and drugs that act indirectly, mainly by activation of GPCRs*. The latter produce their effects mainly by affecting the state of phosphorylation of individual amino acids located on the intracellular region of the channel protein. As described above, this modulation involves the production of second messengers that activate protein kinases. The opening of the channel may be facilitated or inhibited, depending on which residues are phosphorylated. Drugs such as β-adrenoceptor agonists (Ch. 14) affect calcium and potassium channel function in this way, producing a wide variety of cellular effects.
- *Intracellular signals, particularly Ca^{2+} and nucleotides such as ATP and GTP* (see Ch. 4). Many ion channels possess binding sites for these intracellular mediators. Increased $[Ca^{2+}]_i$ opens certain types of potassium channels, and inactivates voltage-gated calcium channels. As described in Chapter 4, $[Ca^{2+}]_i$ is itself affected by the function of ion channels and GPCRs. Drugs of the sulfonylurea class (see Ch. 30) act selectively on ATP-gated potassium channels.

Figure 3.19 summarises the main sites and mechanisms by which drugs affect voltage-gated sodium channels, a typical example of this type of drug target.

CONTROL OF RECEPTOR EXPRESSION

Receptor proteins are synthesised by the cells that express them, and the level of expression is itself controlled, via the pathways discussed above, by receptor-mediated events. We can no longer think of the receptors as the fixed elements in cellular control systems, responding to changes in the concentration of ligands, and initiating effects through the signal transduction pathway—they are themselves subject to regulation. Short-term regulation of receptor function generally occurs through *desensitisation*, as discussed above. Long-term regulation occurs through *an increase or decrease of receptor expression*. Examples of this type of control include the proliferation of various postsynaptic receptors after denervation (see Ch. 12), the upregulation of various G-protein-coupled and cytokine receptors in response to inflammation (see Ch. 17), and the induction of growth factor receptors by certain tumour viruses (see Ch. 5). Long-term drug treatment invariably

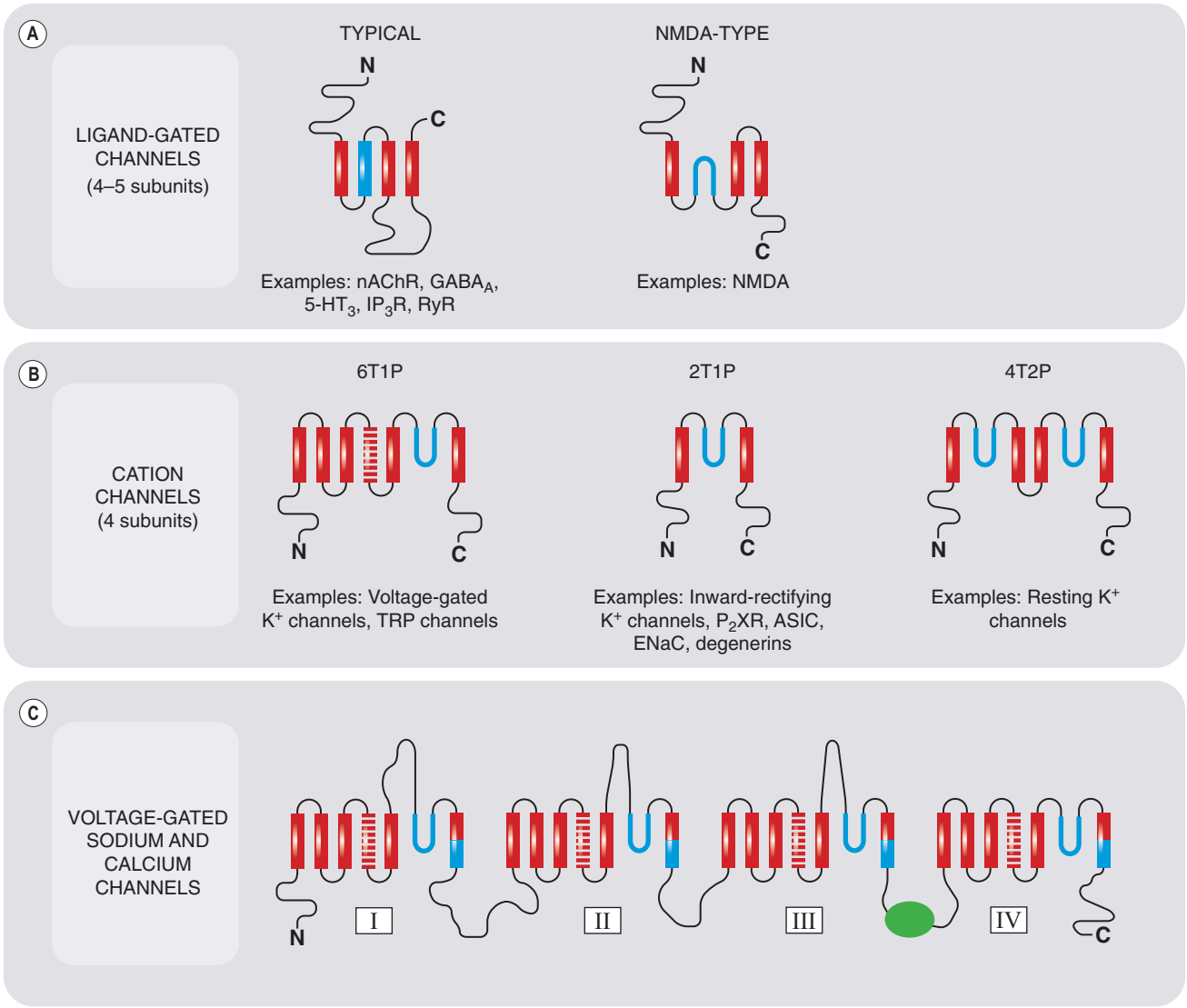

Fig. 3.18 Molecular architecture of ion channels. Red and blue rectangles represent membrane-spanning α-helices. Blue hairpins are pore loop (P) domains, present in many channels, blue rectangles being the pore-forming regions of the membrane-spanning α-helices. Cross-shaded rectangles represent the voltage-sensing regions of voltage-gated channels. The green symbol represents the inactivating particle of voltage-gated sodium channels. Potassium channel nomenclature is based on the number of transmembrane helices (T) and pore-forming loops (P) in each subunit. Further information on ion channels is given in Chapter 4. 5-HT$_3$, 5-hydroxytryptamine type 3 receptor; ASIC, acid-sensing ion channel; ENaC, epithelial sodium channel; GABA$_A$, GABA type A receptor; IP$_3$R, inositol trisphosphate receptor; nAChR, nicotinic acetylcholine receptor; P$_{2X}$R, purine P$_{2X}$ receptor; RyR, ryanodine receptor.

induces adaptive responses, which, particularly with drugs that act on the central nervous system, are often the basis for therapeutic efficacy. They may take the form of a very slow onset of the therapeutic effect (e.g. with antidepressant drugs; see Ch. 46), or the development of drug dependence (Ch. 48). It is likely that changes in receptor expression, secondary to the immediate action of the drug, are involved in delayed effects of this sort—a kind of 'secondary pharmacology' whose importance is only now becoming clearer. The same principles apply to drug targets other than receptors (ion channels, enzymes, transporters, etc.) where adaptive changes in expression and function follow long-term drug administration, resulting, for example, in resistance to certain anticancer drugs (Ch. 55).

RECEPTORS AND DISEASE

Increasing understanding of receptor function in molecular terms has revealed a number of disease states directly linked to receptor malfunction. The principal mechanisms involved are:

- autoantibodies directed against receptor proteins
- mutations in genes encoding receptors and proteins involved in signal transduction.

An example of the former is *myasthenia gravis* (see Ch. 13), a disease of the neuromuscular junction due to autoantibodies that inactivate nicotinic acetylcholine receptors. Autoantibodies can also mimic the effects of agonists, as in

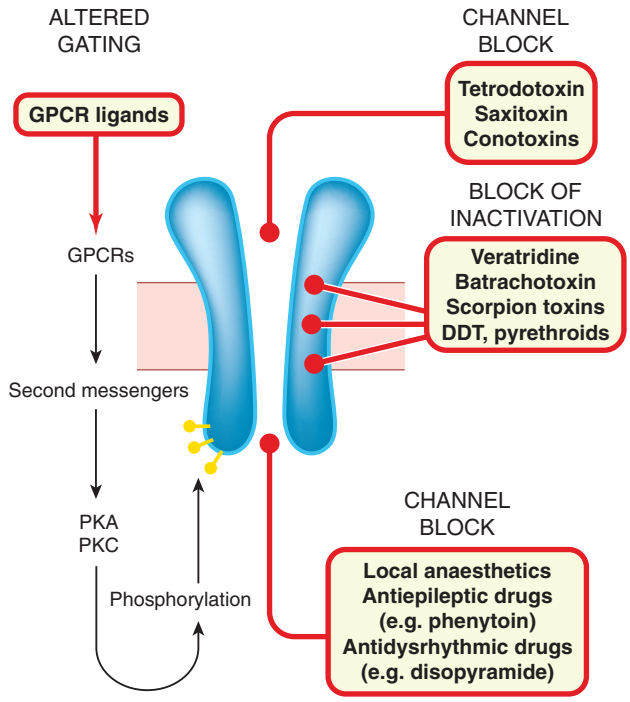

Fig. 3.19 Drug-binding domains of voltage-gated sodium channels (see Ch. 42). The multiplicity of different binding sites and effects appears to be typical of many ion channels. DDT, dichlorodiphenyltrichloroethane (dicophane, a well-known insecticide); GPCR, G-protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C.

many cases of thyroid hypersecretion, caused by activation of **thyrotropin** receptors. Activating antibodies have also been discovered in patients with severe hypertension (α-adrenoceptors), cardiomyopathy (β-adrenoceptors), and certain forms of epilepsy and neurodegenerative disorders (glutamate receptors).

Inherited mutations of genes encoding GPCRs account for various disease states (see Spiegel & Weinstein, 2004; Thompson et al., 2005). Mutated **vasopressin** and **adrenocorticotrophic hormone** receptors (see Chs 28 and 32) can result in resistance to these hormones. Receptor mutations can result in activation of effector mechanisms in the absence of agonists. One of these involves the receptor for thyrotropin, producing continuous oversecretion of thyroid hormone; another involves the receptor for luteinising hormone and results in precocious puberty. Adrenoceptor polymorphisms are common in humans, and recent studies suggest that certain mutations of the β_2-adrenoceptor, although they do not directly cause disease, are associated with a reduced efficacy of β-adrenoceptor agonists in treating asthma (Ch. 27) and a poor prognosis in patients with cardiac failure (Ch. 22). Mutations in G-proteins can also cause disease (see Spiegel & Weinstein, 2004). For example, mutations of a particular Gα subunit cause one form of *hypoparathyroidism*, while mutations of a Gβ subunit result in hypertension. Many cancers are associated with mutations of the genes encoding growth factor receptors, kinases and other proteins involved in signal transduction (see Ch. 5).

Apart from these examples, the high expectations that the numerous polymorphisms described for GPCRs and other receptors would provide a clear understanding of the variability between individuals in their disease susceptibility and response to therapeutic drugs (see Chs 56 and 57) have been, so far, largely unfulfilled, but research activity in this area continues apace.

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4 How drugs act: cellular aspects—excitation, contraction and secretion

OVERVIEW

The link between a drug interacting with a molecular target and its effect at the pathophysiological level, such as a change in blood glucose concentration or the shrinkage of a tumour, involves events at the cellular level. Whatever their specialised physiological function, cells generally share much the same repertoire of signalling mechanisms. In the next three chapters, we describe the parts of this repertoire that are of particular significance in understanding drug action at the cellular level. In this chapter, we describe mechanisms that operate mainly over a short timescale (milliseconds to hours), particularly excitation, contraction and secretion, which account for many physiological responses; Chapter 5 deals with the slower processes (generally days to months), including cell division, growth, differentiation and cell death, that determine the body's structure and constitution; Chapter 6 describes host defence mechanisms.

The short-term regulation of cell function depends mainly on the following components and mechanisms, which regulate, or are regulated by, the free concentration of Ca^{2+} in the cytosol, $[Ca^{2+}]_i$:

- **ion channels and transporters in the plasma membrane**
- **the storage and release of Ca^{2+} by intracellular organelles**
- **Ca^{2+}-dependent regulation of a variety of functional proteins, including enzymes, contractile proteins and vesicle proteins.**

More detailed coverage of the topics presented in this chapter can be found in Nicholls et al. (2001), Levitan & Kaczmarek (2002) and Nestler et al. (2008).

Because $[Ca^{2+}]_i$ plays such a key role in cell function, a wide variety of drug effects results from interference with one or more of these mechanisms. If love makes the human world go round, $[Ca^{2+}]_i$ does the same for cells. Knowledge of the molecular and cellular details is extensive, and here we focus on the aspects that help to explain drug effects.

REGULATION OF INTRACELLULAR CALCIUM

Ever since the famous accident by Sidney Ringer's technician, which showed that using tap water rather than distilled water to make up the bathing solution for isolated frog hearts would allow them to carry on contracting, the role of Ca^{2+} as a major regulator of cell function has never been in question. Many drugs and physiological mechanisms operate, directly or indirectly, by influencing $[Ca^{2+}]_i$. Here we consider the main ways in which it is regulated, and later we describe some of the ways in which $[Ca^{2+}]_i$ controls cell function. Details of the molecular components and drug targets are presented in Chapter 3, and descriptions of drug effects on integrated physiological function are given in later chapters.

The study of Ca^{2+} regulation took a big step forward in the 1970s with the development of optical techniques based on the Ca^{2+}-sensitive photoprotein *aequorin*, and fluorescent dyes such as *Fura*-2, which, for the first time, allowed free $[Ca^{2+}]_i$ to be continuously monitored in living cells with a high level of temporal and spatial resolution.

Most of the Ca^{2+} in a resting cell is sequestered in organelles, particularly the *endoplasmic* or *sarcoplasmic reticulum* (ER or SR) and the mitochondria, and the free $[Ca^{2+}]_i$ is kept to a low level, about 10^{-7} M. The Ca^{2+} concentration in tissue fluid, $[Ca^{2+}]_o$, is about 2.4 mM, so there is a large concentration gradient favouring Ca^{2+} entry. $[Ca^{2+}]_i$ is kept low (a) by the operation of active transport mechanisms that eject cytosolic Ca^{2+} through the plasma membrane and pump it into the ER, and (b) by the normally low Ca^{2+} permeability of the plasma and ER membranes. Regulation of $[Ca^{2+}]_i$ involves three main mechanisms:

- control of Ca^{2+} entry
- control of Ca^{2+} extrusion
- exchange of Ca^{2+} between the cytosol and the intracellular stores.

These mechanisms are described in more detail below and are summarised in Figure 4.1 (see reviews by Clapham, 2007; Berridge, 2009).

CALCIUM ENTRY MECHANISMS

There are four main routes by which Ca^{2+} enters cells across the plasma membrane:

- voltage-gated calcium channels
- ligand-gated calcium channels
- store-operated calcium channels (SOCs)
- Na^+–Ca^{2+} exchange (can operate in either direction; see *Calcium extrusion mechanisms*, below).

VOLTAGE-GATED CALCIUM CHANNELS

The pioneering work of Hodgkin and Huxley on the ionic basis of the nerve action potential (see below) identified voltage-dependent Na^+ and K^+ conductances as the main participants. It was later found that some invertebrate nerve and muscle cells could produce action potentials that depended on Ca^{2+} rather than Na^+, and it was then found that vertebrate cells also possess voltage-activated calcium channels capable of allowing substantial amounts of Ca^{2+} to enter the cell when the membrane is depolarised. These voltage-gated channels are highly selective for Ca^{2+} (although they also conduct Ba^{2+} ions, which are often used as a substitute in electrophysiological experiments), and do

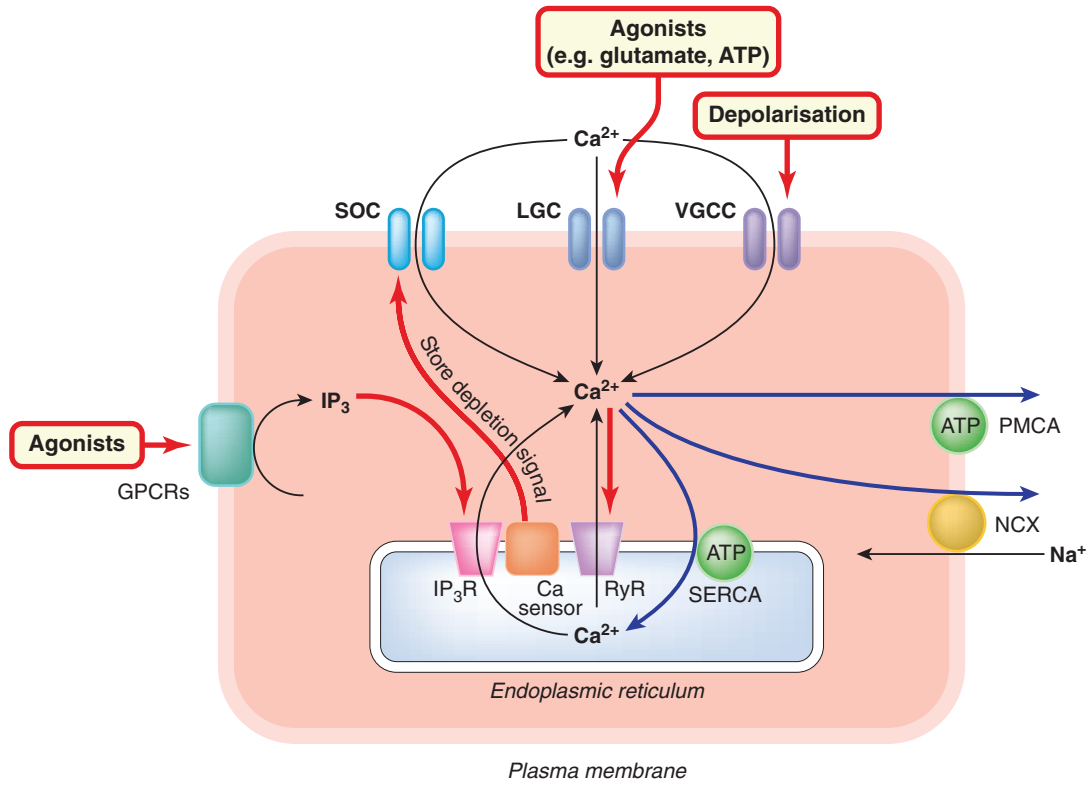

Fig. 4.1 Regulation of intracellular calcium. The main routes of transfer of Ca^{2+} into, and out of, the cytosol and endoplasmic reticulum are shown for a typical cell (see text for details). Black arrows: routes into the cytosol. Blue arrows: routes out of the cytosol. Red arrows: regulatory mechanisms. The state of the ER store of Ca^{2+} is monitored by the sensor protein Stim1, which interacts directly with the store-operated calcium channel (SOC) to promote Ca^{2+} entry when the ER store is depleted. Normally, $[Ca^{2+}]_i$ is regulated to about 10^{-7} mol/l in a 'resting' cell. Mitochondria (not shown) also function as Ca^{2+} storage organelles but release Ca^{2+} only under pathological conditions, such as ischaemia (see text). There is also evidence for an intracellular store (not shown) activated by the second messenger nicotinic acid dinucleotide phosphate. GPCR, G-protein-coupled receptor; IP_3, inositol trisphosphate; IP_3R, inositol trisphosphate receptor; LGC, ligand-gated cation channel; NCX, Na^+–Ca^{2+} exchange transporter; PMCA, plasma membrane Ca^{2+}-ATPase; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum ATPase; VGCC, voltage-gated calcium channel.

not conduct Na^+ or K^+; they are ubiquitous in excitable cells and cause Ca^{2+} to enter the cell whenever the membrane is depolarised, for example by a conducted action potential.

A combination of electrophysiological and pharmacological criteria have revealed five distinct subtypes of voltage-gated calcium channels: L, T, N, P/Q and R.[1] The subtypes vary with respect to their activation and inactivation kinetics, their voltage threshold for activation, their conductance, and their sensitivity to blocking agents, as summarised in Table 4.1. The molecular basis for this heterogeneity has been worked out in some detail. The main pore-forming subunits (termed α1, see Fig. 3.4) occur in at least 10 molecular subtypes, and they are associated with other subunits (β, γ, δ) that also exist in different forms. Different combinations of these subunits give rise to the different physiological subtypes. In general, L channels are particularly important in regulating contraction of cardiac and smooth muscle (see below), and N channels (and also P/Q) are involved in neurotransmitter and hormone release, while T channels mediate Ca^{2+} entry into neurons and thereby control various Ca^{2+}-dependent functions such as regulation of other channels, enzymes, etc. Clinically used drugs that act directly on these channels include the group of 'Ca^{2+} antagonists' consisting of *dihydropyridines* (e.g. **nifedipine**), **verapamil** and **diltiazem** (used for their cardiovascular effects; see Chs 21 and 22), and also **gabapentin** and **pregabalin** (used to treat pain and epilepsy; see Chs 41 and 44). Many drugs affect calcium channels indirectly by acting on G-protein-coupled receptors (see Ch. 3). A number of toxins act selectively on one or other type of calcium channel (Table 4.1), and these are used as experimental tools.

LIGAND-GATED CHANNELS

Most ligand-gated cation channels (see Ch. 3) that are activated by excitatory neurotransmitters are relatively non-selective, and conduct Ca^{2+} ions as well as other cations. Most important in this respect is the glutamate receptor of the NMDA type (Ch. 37), which has a particularly high permeability to Ca^{2+} and is a major contributor to Ca^{2+} uptake by postsynaptic neurons (and also glial cells) in the central nervous system. Activation of this receptor can readily cause so much Ca^{2+} entry that the cell dies, mainly

[1]P and Q are so similar that they usually get lumped together. The terminology is less than poetic: L stands for *long-lasting*; T stands for *transient*; N stands for *neither long-lasting nor transient*; and P, Q and R carry on alphabetically from N, with O (of course) omitted.

Table 4.1 Types and functions of Ca^{2+} channels

Gated by	Main types	Characteristics	Location and function	Drug effects
Voltage	L	High activation threshold Slow inactivation	Plasma membrane of many cells Main Ca^{2+} source for contraction in smooth and cardiac muscle	Blocked by **dihydropyridines**, **verapamil**, **diltiazem** **Calciseptine** (peptide from snake venom) Activated by **BayK 8644**
	N	Low activation threshold Slow inactivation	Main Ca^{2+} source for transmitter release by nerve terminals	Blocked by **ω-conotoxin** (component of *Conus* snail venom) and **ziconotide** (marketed preparation of ω-conotoxin used to control pain) (Ch. 41)
	T	Low activation threshold Fast inactivation	Widely distributed Important in cardiac pacemaker and atria (role in dysrhythmias), also neuronal firing patterns	Blocked by **mibefradil**
	P/Q	Low activation threshold Slow inactivation	Nerve terminals Transmitter release	Blocked by **ω-agatoxin** (component of funnel web spider venom)
	R	Low threshold Fast inactivation	Neurons and dendrites Control of firing patterns	
Inositol-trisphosphate	IP_3 receptor		Located in endoplasmic/sarcoplasmic reticulum Mediates Ca^{2+} release produced by GPCR activation	Not directly targeted by drugs Some experimental blocking agents known Responds to GPCR agonists and antagonists in many cells
Ca^{2+}	Ryanodine receptor	Directly activated in striated muscle via dihydropyridine receptor of T-tubules	Located in endoplasmic/sarcoplasmic reticulum. Mediates Ca^{2+}-evoked Ca^{2+} release in muscle. Also activated by the second messenger cyclic ADP ribose	Activated by **caffeine** (high concentrations Blocked by **ryanodine** Mutations may lead to drug-induced malignant hypothermia
Store depletion	Store-operated channels	Activated by sensor protein that monitors level of ER Ca^{2+} stores	Located in plasma membrane	Activated indirectly by agents that deplete intracellular stores (e.g. GPCR agonists, **thapsigargin**) Not directly targeted by drugs

through activation of Ca^{2+}-dependent proteases but also by triggering *apoptosis* (see Ch. 5). This mechanism, termed *excitotoxicity*, probably plays a part in various neurodegenerative disorders (see Ch. 39).

For many years, there was dispute about the existence of 'receptor-operated channels' in smooth muscle, responding directly to mediators such as adrenaline (epinephrine), acetylcholine and histamine. Now it seems (see Berridge, 2009) that the P2x receptor (see Ch. 3), activated by ATP, is the only example of a true ligand-gated channel in smooth muscle, and this constitutes an important route of entry for Ca^{2+}. As mentioned above, many mediators acting on G-protein-coupled receptors, affect Ca^{2+} entry indirectly, mainly by regulating voltage-gated calcium channels or potassium channels.

STORE-OPERATED CALCIUM CHANNELS (SOCs)

SOCs are very low-conductance channels that occur in the plasma membrane and open to allow entry when the ER stores are depleted, but are not sensitive to cytosolic $[Ca^{2+}]_i$. The linkage between the ER and the plasma membrane—for long a puzzle—was recently found to involve a Ca^{2+}-sensor protein (*Stim1*) in the ER membrane, which connects directly to the channel protein (*Orai1*) in the plasma membrane (see Clapham, 2007).

Like the ER and SR channels, these channels can serve to amplify the rise in $[Ca^{2+}]_i$ resulting from Ca^{2+} release from the stores. So far, only experimental compounds are known to block these channels, but efforts are being made to develop specific blocking agents for therapeutic use as relaxants of smooth muscle.

CALCIUM EXTRUSION MECHANISMS

Active transport of Ca^{2+} outwards across the plasma membrane, and inwards across the membranes of the ER or SR, depends on the activity of distinct Ca^{2+}-dependent ATPases,[2] similar to the Na^+/K^+-dependent ATPase that

[2]Clapham (2007) likens these pumps to Sisyphus, condemned endlessly to push a stone up a hill (also consuming ATP, no doubt), only for it to roll down again.

pumps Na^+ out of the cell in exchange for K^+. **Thapsigargin** (derived from a Mediterranean plant, *Thapsia garganica*) specifically blocks the ER pump, causing loss of Ca^{2+} from the ER. It is a useful experimental tool but has no therapeutic significance.

Calcium is also extruded from cells in exchange for Na^+, by Na^+–Ca^{2+} exchange. The transporter that does this has been fully characterised and cloned, and (as you would expect) comes in several molecular subtypes whose functions remain to be worked out. The exchanger transfers three Na^+ ions for one Ca^{2+}, and therefore produces a net depolarising current when it is extruding Ca^{2+}. The energy for Ca^{2+} extrusion comes from the electrochemical gradient for Na^+, not directly from ATP hydrolysis. This means that a reduction in the Na^+ concentration gradient resulting from Na^+ entry will reduce Ca^{2+} extrusion by the exchanger, causing a secondary rise in $[Ca^{2+}]_i$, a mechanism that is particularly important in cardiac muscle (see Ch. 21). **Digoxin,** which inhibits Na^+ extrusion, acts on cardiac muscle in this way (Ch. 21), causing $[Ca^{2+}]_i$ to increase.

CALCIUM RELEASE MECHANISMS

There are two main types of calcium channel in the ER and SR membrane, which play an important part in controlling the release of Ca^{2+} from these stores.

- The *inositol trisphosphate receptor* (IP_3R) is activated by inositol trisphosphate (IP_3), a second messenger produced by the action of many ligands on G-protein-coupled receptors (see Ch. 3). IP_3R is a ligand-gated ion channel, although its molecular structure differs from that of ligand-gated channels in the plasma membrane (see Mikoshiba, 2007). This is the main mechanism by which activation of G-protein-coupled receptors causes an increase in $[Ca^{2+}]_i$.
- The *ryanodine receptor* (RyR) is so called because it was first identified through the specific blocking action of the plant alkaloid **ryanodine**. It is particularly important in skeletal muscle, where there is direct coupling between the RyRs of the SR and the *dihydropyridine receptors* of the T-tubules (see below); this coupling results in Ca^{2+} release following the action potential in the muscle fibre. RyRs are also present in other types of cell that lack T-tubules; they are activated by a small rise in $[Ca^{2+}]_i$, producing the effect known as *calcium-induced calcium release* (CICR), which serves to amplify the Ca^{2+} signal produced by other mechanisms such as opening of calcium channels in the plasma membrane. CICR means that release tends to be regenerative, because an initial puff of Ca^{2+} releases more, resulting in localised 'sparks' or 'waves' of Ca^{2+} release (see Berridge, 1997).

The functions of IP_3Rs and RyRs are modulated by a variety of other intracellular signals (see Berridge et al., 2003), which affect the magnitude and spatiotemporal patterning of Ca^{2+} signals. Fluorescence imaging techniques have revealed a remarkable level of complexity of Ca^{2+} signals, and much remains to be discovered about the importance of this patterning in relation to physiological and pharmacological mechanisms. The Ca^{2+} sensitivity of RyRs is increased by **caffeine**, causing Ca^{2+} release from the SR even at resting levels of $[Ca^{2+}]_i$. This is used experimentally but rarely happens in humans, because the other pharmacological effects of caffeine (see Ch. 47) occur at much lower doses. The blocking effect of **dantrolene**, a compound related to ryanodine, is used therapeutically to relieve muscle spasm in the rare condition of *malignant hyperthermia* (see Ch. 40), which is associated with inherited abnormalities in the RyR protein. There are as yet few other examples of drugs that directly affect these Ca^{2+} release mechanisms.

A typical $[Ca^{2+}]_i$ signal resulting from activation of a G-protein-coupled receptor is shown in Figure 4.2. The response produced in the absence of extracellular Ca^{2+} represents release of intracellular Ca^{2+}. The larger and more prolonged response when extracellular Ca^{2+} is present shows the contribution of SOC-mediated Ca^{2+} entry. The various positive and negative feedback mechanisms that regulate $[Ca^{2+}]_i$ give rise to a variety of temporal and spatial oscillatory patterns (Fig. 4.2B) that are responsible for spontaneous rhythmic activity in smooth muscle and nerve cells (see Berridge, 2009).

OTHER SECOND MESSENGERS

▼ Two intracellular metabolites, cyclic ADP-ribose (cADPR) and nicotinic acid dinucleotide phosphate (NAADP; see Fliegert et al., 2007), formed from the ubiquitous coenzymes nicotinamide adenine dinucleotide (NAD) and NAD phosphate, also affect Ca^{2+} signalling. cADPR acts by increasing the sensitivity of RyRs to Ca^{2+}, thus increasing the 'gain' of the CICR effect. NAADP releases Ca^{2+} from lysosomes by activating channels not yet identified but evidently distinct from the IP_3R and RyR.

The levels of these messengers in mammalian cells may be regulated mainly in response to changes in the metabolic status of the cell, although the details are not yet clear. Abnormal Ca^{2+} signalling is involved in many pathophysiological conditions, such as ischaemic cell death, endocrine disorders and cardiac dysrhythmias, where the roles of cADPR and NAADP, and their interaction with other mechanisms that regulate $[Ca^{2+}]_i$, are the subject of much current work (see Berridge et al., 2003).

THE ROLE OF MITOCHONDRIA

▼ Under normal conditions, mitochondria accumulate Ca^{2+} passively as a result of the intramitochondrial potential, which is strongly negative with respect to the cytosol. This negativity is maintained by active extrusion of protons, and is lost—thus releasing Ca^{2+} into the cytosol—if the cell runs short of ATP, for example under conditions of hypoxia. This only happens in extremis, and the resulting Ca^{2+} release contributes to the cytotoxicity associated with severe metabolic disturbance. Cell death resulting from brain ischaemia or coronary ischaemia (see Chs 21 and 39) involves this mechanism, along with others that contribute to an excessive rise in $[Ca^{2+}]_i$.

CALMODULIN

Calcium exerts its control over cell functions by virtue of its ability to regulate the activity of many different proteins, including enzymes (particularly kinases and phosphatases), channels, transporters, transcription factors, synaptic vesicle proteins and many others. In most cases, a Ca^{2+}-binding protein serves as an intermediate between Ca^{2+} and the regulated functional protein, the best known such binding protein being the ubiquitous *calmodulin* (see Clapham, 2007). This regulates at least 40 different functional proteins—indeed a powerful fixer. Calmodulin is a dimer, with four Ca^{2+} binding sites. When all are occupied, it undergoes a conformational change, exposing a 'sticky' hydrophobic domain that lures many proteins into association, thereby affecting their functional properties.

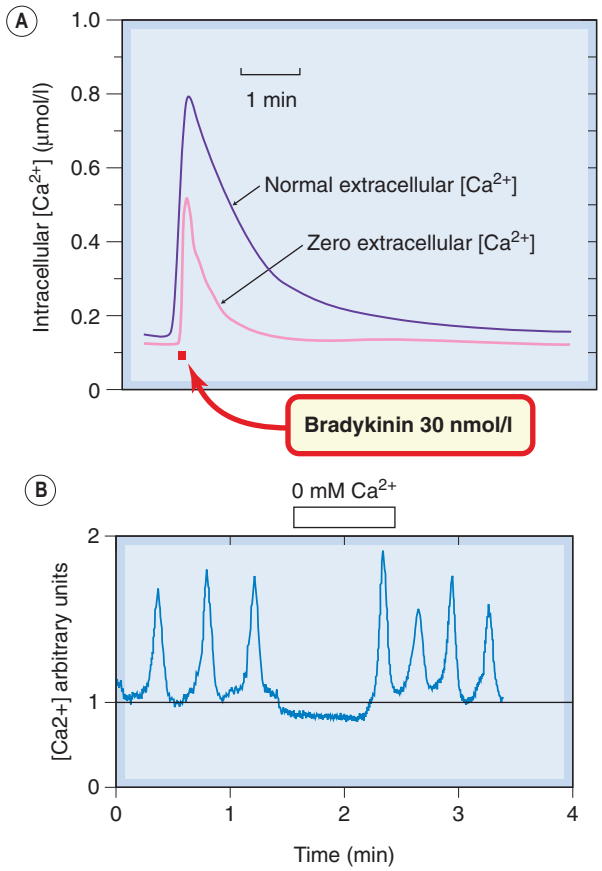

Fig. 4.2 **[A] Increase in intracellular calcium concentration in response to receptor activation.** The records were obtained from a single rat sensory neuron grown in tissue culture. The cells were loaded with the fluorescent Ca^{2+} indicator Fura-2, and the signal from a single cell monitored with a fluorescence microscope. A brief exposure to the peptide bradykinin, which causes excitation of sensory neurons (see Ch. 41), causes a transient increase in $[Ca^{2+}]_i$ from the resting value of about 150 nmol/l. When Ca^{2+} is removed from the extracellular solution, the bradykinin-induced increase in $[Ca^{2+}]_i$ is still present but is smaller and briefer. The response in the absence of extracellular Ca^{2+} represents the release of stored intracellular Ca^{2+} resulting from the intracellular production of inositol trisphosphate. The difference between this and the larger response when Ca^{2+} is present extracellularly is believed to represent Ca^{2+} entry through store-operated ion channels in the cell membrane. (Figure kindly provided by G M Burgess and A Forbes, Novartis Institute for Medical Research.) **[B] Spontaneous calcium oscillations in pacemaker cells from the rabbit urethra that regulate the rhythmic contractions of the smooth muscle.** The signals cease when external Ca^{2+} is removed, showing that activation of membrane Ca^{2+} channels is involved in the mechanism. (From McHale et al. 2006 J Physiol 570:23–28.)

Calcium regulation

- Intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, is critically important as a regulator of cell function.
- Intracellular Ca^{2+} is determined by (a) Ca^{2+} entry; (b) Ca^{2+} extrusion; and (c) Ca^{2+} exchange between the cytosol, endoplasmic or sarcoplasmic reticulum (ER, SR) and mitochondria.
- Calcium entry occurs by various routes, including voltage- and ligand-gated calcium channels and Na^+–Ca^{2+} exchange.
- Calcium extrusion depends mainly on an ATP-driven Ca^{2+} pump.
- Calcium ions are actively taken up and stored by the ER/SR, from which they are released in response to various stimuli.
- Calcium ions are released from ER/SR stores by (a) the second messenger IP_3 acting on IP_3 receptors; or (b) increased $[Ca^{2+}]_i$ itself acting on ryanodine receptors, a mechanism known as Ca^{2+}-induced Ca^{2+} release.
- Other second messengers, cyclic ADP ribose and nicotinic acid dinucleotide phosphate, also promote the release of Ca^{2+} from Ca^{2+} stores.
- Depletion of ER/SR Ca^{2+} stores promotes Ca^{2+} entry through the plasma membrane, via store-operated channels.
- Calcium ions affect many aspects of cell function by binding to proteins such as calmodulin, which in turn bind other proteins and regulate their function.

EXCITATION

Excitability describes the ability of a cell to show a regenerative all-or-nothing electrical response to depolarisation of its membrane, this membrane response being known as an action potential. It is a characteristic of most neurons and muscle cells (including striated, cardiac and smooth muscle) and of many endocrine gland cells. In neurons and muscle cells, the ability of the action potential, once initiated, to propagate to all parts of the cell membrane, and often to spread to neighbouring cells, explains the importance of membrane excitation in intra- and intercellular signalling. In the nervous system, and in striated muscle, action potential propagation is the mechanism responsible for communication over long distances at high speed, indispensable for large, fast-moving creatures. In cardiac and smooth muscle, as well as in some central neurons, spontaneous rhythmic activity occurs. In gland cells, the action potential, where it occurs, serves to amplify the signal that causes the cell to secrete. In each type of tissue, the properties of the excitation process reflect the special characteristics of the ion channels that underlie the process. The molecular nature of ion channels, and their importance as drug targets, is considered in Chapter 3; here we discuss the cellular processes that depend primarily on ion channel function. For more detail, see Hille (2001).

THE 'RESTING' CELL

The resting cell is not resting at all but very busy controlling the state of its interior, and it requires a continuous supply of energy to do so. In relation to the topics discussed in this chapter, the following characteristics are especially important:

- membrane potential
- permeability of the plasma membrane to different ions
- intracellular ion concentrations, especially $[Ca^{2+}]_i$.

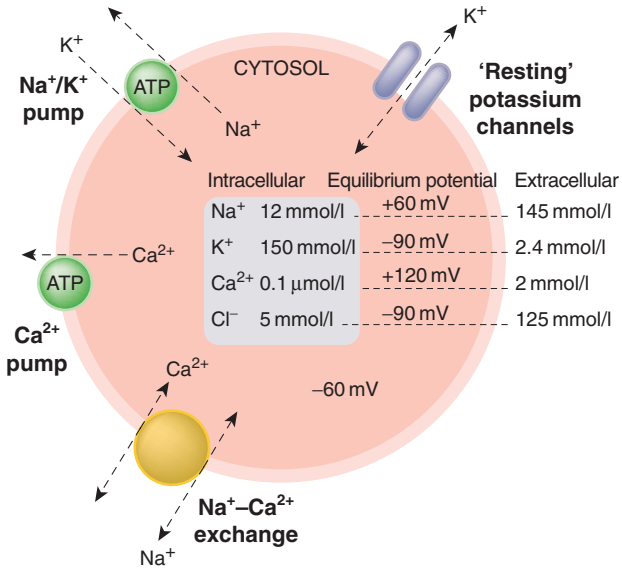

Fig. 4.3 Simplified diagram showing the ionic balance of a typical 'resting' cell. The main transport mechanisms that maintain the ionic gradients across the plasma membrane are the ATP-driven Na^+–K^+ and Ca^{2+} pumps and the Na^+–Ca^{2+} exchange transporter. The membrane is relatively permeable to K^+, because potassium channels are open at rest, but impermeable to other cations. The unequal ion concentrations on either side of the membrane give rise to the 'equilibrium potentials' shown. The resting membrane potential, typically about –60 mV but differing between different cell types, is determined by the equilibrium potentials and the permeabilities of the various ions involved, and by the 'electrogenic' effect of the transporters. For simplicity, anions and other ions, such as protons, are not shown, although these play an important role in many cell types.

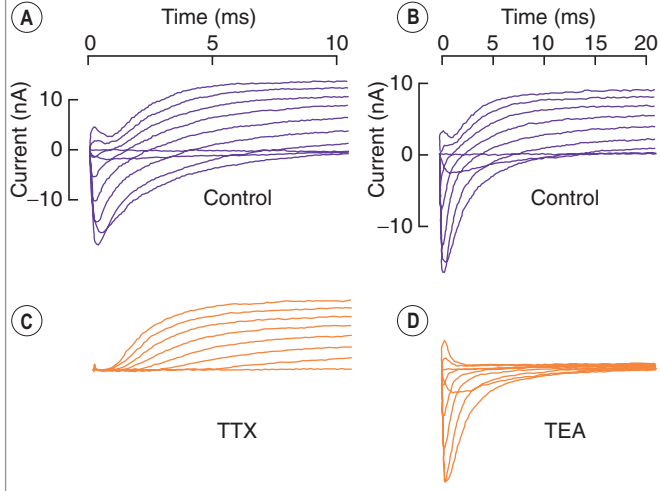

Fig. 4.4 Separation of sodium and potassium currents in the nerve membrane. Voltage clamp records from the node of Ranvier of a single frog nerve fibre. At time 0, the membrane potential was stepped to a depolarised level, ranging from –60 mV (lower trace in each series) to +60 mV (upper trace in each series) in 15-mV steps. [**A**] [**B**] Control records from two fibres. [**C**] Effect of tetrodotoxin (TTX), which abolishes Na^+ currents. [**D**] Effect of tetraethylammonium (TEA), which abolishes K^+ currents. (From Hille B 1970. Ionic channels in nerve membranes. Prog Biophys Mol Biol 21: 1–32.)

Under resting conditions, all cells maintain a negative internal potential between about –30 mV and –80 mV, depending on the cell type. This arises because (a) the membrane is relatively impermeable to Na^+, and (b) Na^+ ions are actively extruded from the cell in exchange for K^+ ions by an energy-dependent transporter, the Na^+ pump (or Na^+–K^+-ATPase). The result is that the intracellular K^+ concentration, $[K^+]_i$, is higher, and $[Na^+]_i$ is lower, than the respective extracellular concentrations. In many cells, other ions, particularly Cl^-, are also actively transported and unequally distributed across the membrane. In many cases (e.g. in neurons), the membrane permeability to K^+ is relatively high, and the membrane potential settles at a value of –60 to –80 mV, close to the equilibrium potential for K^+ (Fig. 4.3). In other cells (e.g. smooth muscle), anions play a larger part, and the membrane potential is generally lower (–30 to –50 mV) and less dependent on K^+.

ELECTRICAL AND IONIC EVENTS UNDERLYING THE ACTION POTENTIAL

Our present understanding of electrical excitability rests firmly on the work of Hodgkin, Huxley and Katz on squid axons, published in 1949–1952. Their experiments (see Katz, 1966) revealed the existence of voltage-gated ion channels (see above) and showed that the action potential is generated by the interplay of two processes:

1. a rapid, transient increase in Na^+ permeability that occurs when the membrane is depolarised beyond about –50 mV
2. a slower, sustained increase in K^+ permeability.

Because of the inequality of Na^+ and K^+ concentrations on the two sides of the membrane, an increase in Na^+ permeability causes an inward (depolarising) current of Na^+ ions, whereas an increase in K^+ permeability causes an outward current. The separability of these two currents can be most clearly demonstrated by the use of drugs blocking sodium and potassium channels, as shown in Figure 4.4. During the physiological initiation or propagation of a nerve impulse, the first event is a small depolarisation of the membrane, produced either by transmitter action or by the approach of an action potential passing along the axon. This opens sodium channels, allowing an inward current of Na^+ ions to flow, which depolarises the membrane still further. The process is thus a regenerative one, and the increase in Na^+ permeability is enough to bring the membrane potential close to E_{Na}. The increased Na^+ conductance is transient, because the channels inactivate rapidly and the membrane returns to its resting state.

In many types of cell, including most nerve cells, repolarisation is assisted by the opening of voltage-dependent potassium channels. These function in much the same way as sodium channels, but their activation kinetics are about 10 times slower and they do not inactivate appreciably. This means that the potassium channels open later than the sodium channels, and contribute to the rapid termination of the action potential. The behaviour of the sodium and potassium channels during an action potential is shown in Figure 4.5.

The foregoing account, based on Hodgkin and Huxley's work 60 years ago, involves only Na^+ and K^+ channels.

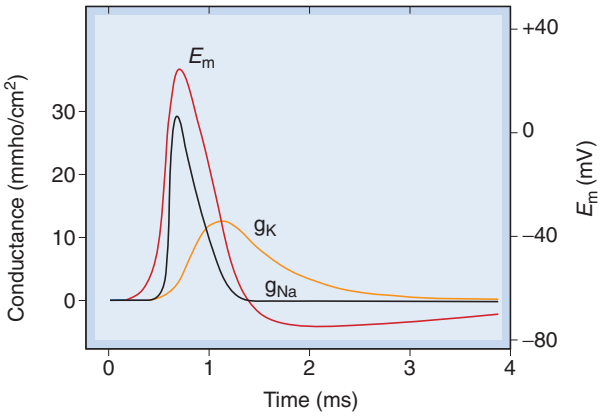

Fig. 4.5 Behaviour of sodium and potassium channels during a conducted action potential. Rapid opening of sodium channels occurs during the action potential upstroke. Delayed opening of potassium channels, and inactivation of sodium channels, causes repolarisation. E_m, membrane potential; g_{Na}, g_K, membrane conductance to Na^+, K^+.

Subsequently (see Hille, 2001), voltage-gated calcium channels (see Fig. 4.1) were discovered. These function in basically the same way as sodium channels; they contribute to action potential generation in many cells, particularly cardiac and smooth muscle cells, but also in neurons and secretory cells. Ca^{2+} entry through voltage-gated calcium channels plays a key role in intracellular signalling, as described above.

CHANNEL FUNCTION

The discharge patterns of excitable cells vary greatly. Skeletal muscle fibres are quiescent unless stimulated by the arrival of a nerve impulse at the neuromuscular junction. Cardiac muscle fibres discharge spontaneously at a regular rate (see Ch. 21). Neurons may be normally silent, or they may discharge spontaneously, either regularly or in bursts; smooth muscle cells show a similar variety of firing patterns. The frequency at which different cells normally discharge action potentials also varies greatly, from 100 Hz or more for fast-conducting neurons, down to about 1 Hz for cardiac muscle cells. These very pronounced functional variations reflect the different characteristics of the ion channels expressed in different cell types. Rhythmic fluctuations of $[Ca^{2+}]_i$ underlie the distinct firing patterns that occur in different types of cell (see Berridge, 2009).

Drugs that alter channel characteristics, either by interacting directly with the channel itself or indirectly through second messengers, affect the function of many organ systems, including the nervous, cardiovascular, endocrine, respiratory and reproductive systems, and are a frequent theme in this book. Here we describe some of the key mechanisms involved in the regulation of excitable cells.

In general, action potentials are initiated by membrane currents that cause depolarisation of the cell. These currents may be produced by synaptic activity, by an action potential approaching from another part of the cell, by a sensory stimulus or by spontaneous *pacemaker* activity. The tendency of such currents to initiate an action potential is governed by the *excitability* of the cell, which depends mainly on the state of (a) the voltage-gated sodium and/or calcium channels, and (b) the potassium channels of the resting membrane. Anything that increases the number of available sodium or calcium channels, or reduces their activation threshold, will tend to increase excitability, whereas increasing the resting K^+ conductance reduces it. Agents that do the reverse, by blocking channels or interfering with their opening, will have the opposite effect. Some examples are shown in Figures 4.6 and 4.7 and in Table 4.1. Inherited mutations of channel proteins are responsible for a wide variety of (mostly rare) neurological and other genetic disorders (see Ashcroft, 2000, 2006).

USE DEPENDENCE AND VOLTAGE DEPENDENCE

▼ Voltage-gated channels can exist in three functional states (Fig. 4.8): *resting* (the closed state that prevails at the normal resting potential), *activated* (the open state favoured by brief depolarisation) and *inactivated* (the blocked state resulting from a trap door-like occlusion of the open channel by a floppy intracellular appendage of the channel protein). After the action potential has passed, many sodium channels are in the inactivated state; after the membrane potential returns to its resting value, the inactivated channels take time to revert to the resting state and thus become available for activation once more. In the meantime, the membrane is temporarily *refractory*. Each action potential causes the channels to cycle through these states. The duration of the refractory period determines the maximum frequency at which action potentials can occur. Drugs that block sodium channels, such as local anaesthetics (Ch. 42), antidysrhythmic drugs (Ch. 21) and antiepileptic drugs (Ch. 44), commonly show a selective affinity for one or other of these functional states of the channel, and in their presence the proportion of channels in the high-affinity state is increased. Of particular importance are drugs that bind most strongly to the inactivated state of the channel and thus favour the adoption of this state, thus prolonging the refractory period and reducing the maximum frequency at which action potentials can be generated. This type of block is called *use dependent*, because the binding of such drugs increases as a function of the rate of action potential discharge, which governs the rate at which inactivated—and therefore drug-sensitive—channels are generated. This is important for some antidysrhythmic drugs (see Ch. 21) and for antiepileptic drugs (Ch. 44), because high-frequency discharges can be inhibited without affecting excitability at normal frequencies. Drugs that readily block sodium channels in their resting state (e.g. local anaesthetics, Ch. 42) prevent excitation at low as well as high frequencies.

Most sodium channel-blocking drugs are cationic at physiological pH and are therefore affected by the voltage gradient across the cell membrane. They block the channel from the inside, so that their blocking action is favoured by depolarisation. This phenomenon, known as *voltage dependence*, is also of relevance to the action of antidysrhythmic and antiepileptic drugs, because the cells that are the seat of dysrhythmias or seizure activity are generally somewhat depolarised and therefore more strongly blocked than 'healthy' cells. Similar considerations apply also to drugs that block potassium or calcium channels, but we know less about the importance of use and voltage dependence for these than we do for sodium channels.

SODIUM CHANNELS

In most excitable cells, the regenerative inward current that initiates the action potential results from activation of voltage-gated sodium channels. The early voltage clamp studies by Hodgkin and Huxley on the squid giant axon, described above, revealed the essential functional properties of these channels. Later, advantage was taken of the potent and highly selective blocking action of **tetrodotoxin** (TTX, see Ch. 42) to label and purify the channel protein, and subsequently to clone it, revealing the complex structure shown in Figure 3.18, with four similar domains each comprising six membrane-spanning helices (reviewed by

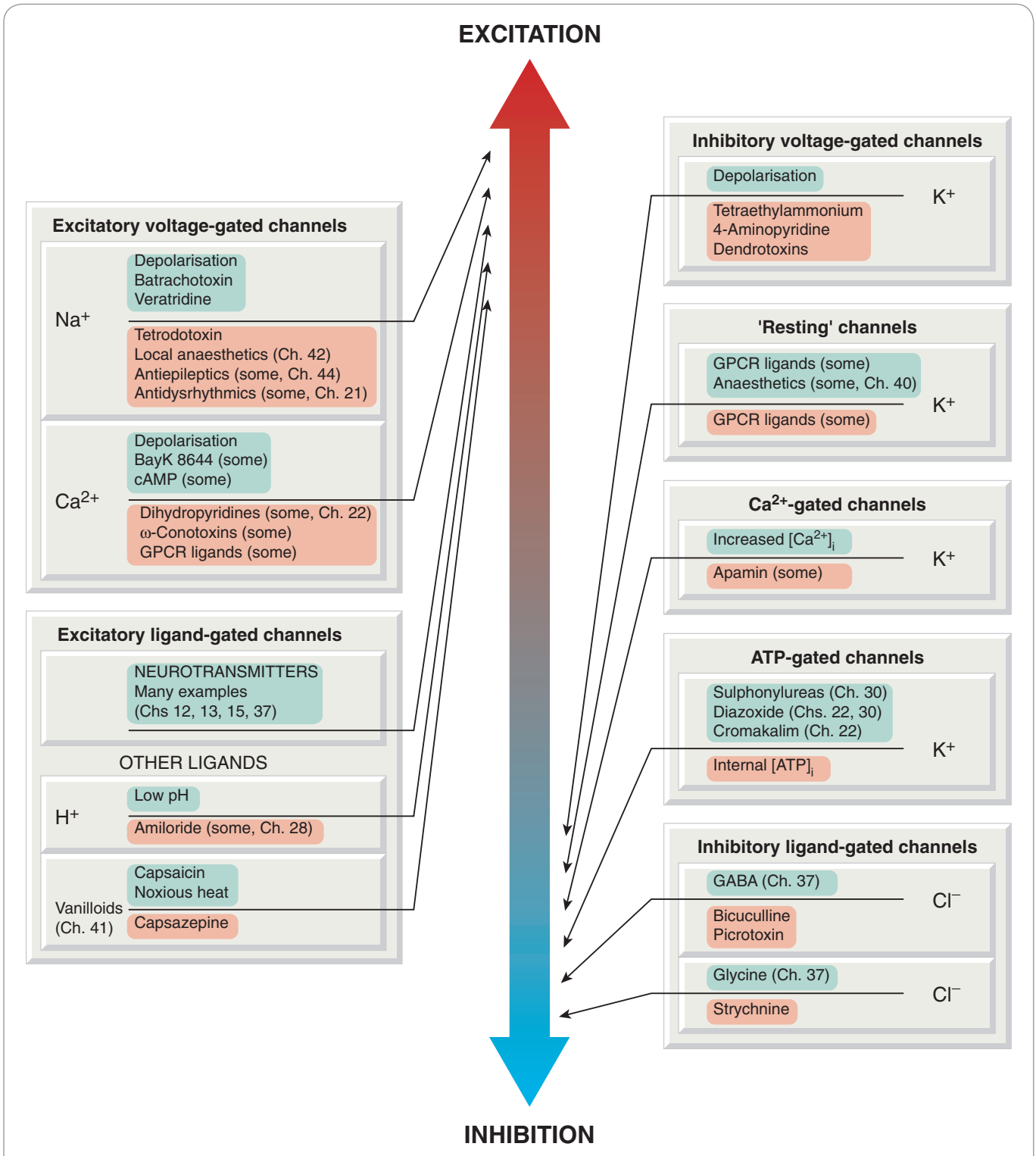

Fig. 4.6 Ion channels associated with excitatory and inhibitory membrane effects, and some of the drugs and other ligands that affect them. Channel openers are shown in green boxes, blocking agents and inhibitors in pink boxes. GPCR, G-protein-coupled receptor.

Catterall, 2000). One of these helices, S4, contains several basic amino acids and forms the voltage sensor, and moves outwards, thus opening the channel, when the membrane is depolarised. One of the intracellular loops is designed to swing across and block the channel when S4 is displaced, thus inactivating the channel.

It was known from physiological studies that the sodium channels of heart and skeletal muscle differ in various ways from those of neurons. In particular, cardiac sodium channels (and also those of some sensory neurons) are relatively insensitive to TTX, and slower in their kinetics, compared with most neuronal sodium channels. Nine distinct

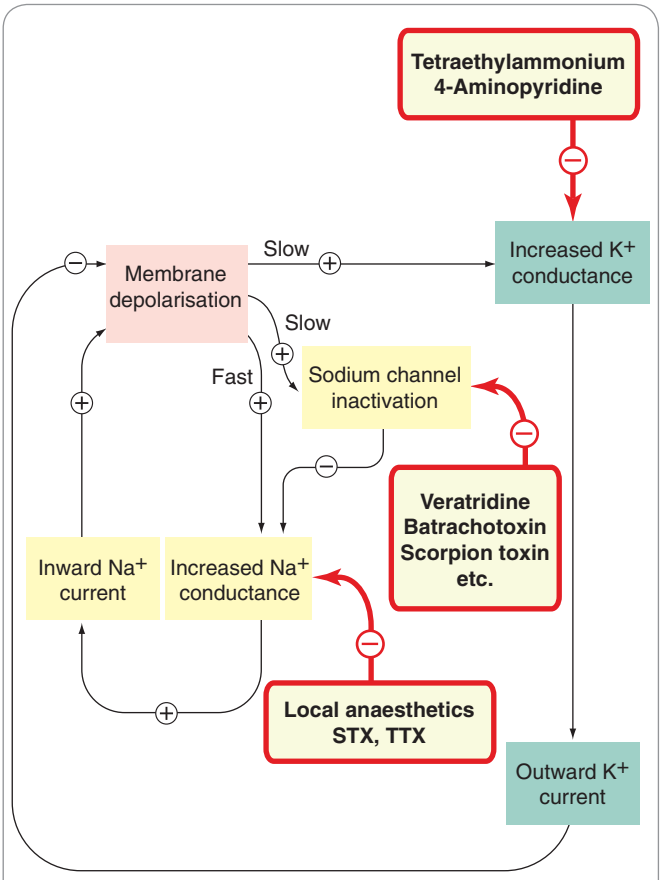

Fig. 4.7 Sites of action of drugs and toxins that affect channels involved in action potential generation. Many other mediators affect these channels indirectly via membrane receptors, through phosphorylation or altered expression. STX, saxitoxin; TTX, tetrodotoxin.

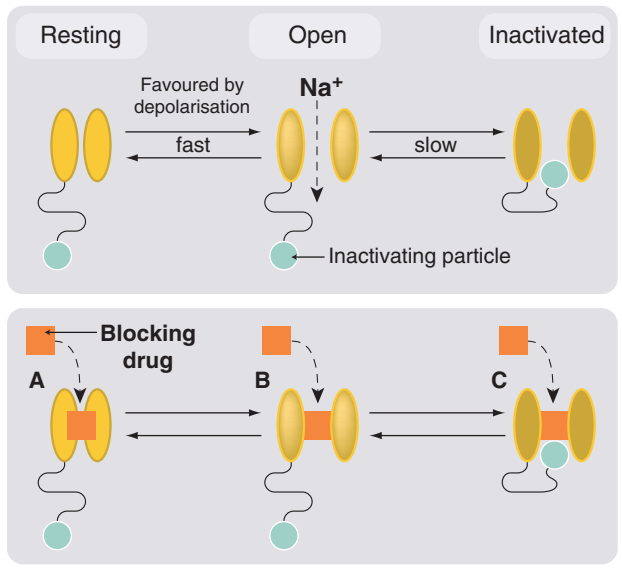

Fig. 4.8 Resting, activated and inactivated states of voltage-gated channels, exemplified by the sodium channel. Membrane depolarisation causes a rapid transition from the resting (closed) state to the open state. The inactivating particle (part of the intracellular domain of the channel protein) is then able to block the channel. Blocking drugs (e.g. local anaesthetics and antiepileptic drugs) often show preference for one of the three channel states, and thus affect the kinetic behaviour of the channels, with implications for their clinical application.

molecular subtypes have so far been identified, more than enough to explain the functional diversity.

In addition to channel blocking compounds such as tetrodotoxin, other compounds affect sodium channel gating. For example, the plant alkaloid **veratridine** and the frog skin poison **batrachotoxin** cause persistent activation, while various scorpion toxins prevent inactivation, mechanisms resulting in enhanced neuronal excitability.

Therapeutic agents that act by blocking sodium channels include local anaesthetic drugs (Ch. 42), antiepileptic drugs (Ch. 44) and antidysrhythmic drugs (Ch. 21). The sodium channel-blocking actions of these drugs were in most cases discovered long after their clinical applications were recognised; many of them lack specificity and produce a variety of unwanted side effects. The use of induced mutations in cloned sodium channels expressed in cell lines is now revealing which regions of the very large channel molecule are involved in the binding of particular agents, knowledge that should allow more specific drugs to be designed in the future.

POTASSIUM CHANNELS

In a typical resting cell (see above), the membrane is selectively permeable to K^+, and the membrane potential (about –60 mV) is somewhat positive to the K^+ equilibrium (about –90 mV). This resting permeability comes about because potassium channels are open. If more potassium channels open, the membrane hyperpolarises and the cell is inhibited, whereas the opposite happens if potassium channels close. As well as affecting excitability in this way, potassium channels also play an important role in regulating the duration of the action potential and the temporal patterning of action potential discharges; altogether, these channels play a central role in regulating cell function. As mentioned in Chapter 3, the number and variety of potassium channel subtypes is extraordinary, implying that evolution has been driven by the scope for biological advantage to be gained from subtle variations in the functional properties of these channels. A recent résumé lists over 60 different pore-forming subunits, plus another 20 or so auxiliary subunits. An impressive evolutionary display, maybe, but hard going for most of us. Here we outline the main types that are known to be important pharmacologically. For more details, and information on potassium channels and the various drugs and toxins that affect them, see Shieh et al. (2000) and Jenkinson (2006).

▼ Potassium channels fall into three main classes (Table 4.2),[3] of which the structures are shown in Figure 3.18.

[3]Potassium channel terminology is confusing, to put it mildly. Electrophysiologists have named K^+ currents prosaically on the basis of their functional properties (I_{KV}, I_{KCa}, I_{KATP}, I_{KIR}, etc.); geneticists have named genes somewhat fancifully according to the phenotypes associated with mutations (shaker, ether-a-go-go, etc.), while molecular biologists have introduced a rational but unmemorable nomenclature on the basis of sequence data (KCNK, KCNQ, etc., with numerical suffixes). The rest of us have to make what we can of the unlovely jargon of labels such as HERG (which—don't blink—stands for Human Ether-a-go-go Related Gene), TWIK, TREK and TASK.

Table 4.2 Types and functions of K^+ channels

Structural class[a]	Functional subtypes[b]	Functions	Drug effects	Notes
Voltage-gated (6T, 1P)	Voltage-gated K^+ channels	Action potential repolarisation Limits maximum firing frequency	Blocked by **tetraethylammonium, 4-aminopyridine** Certain subtypes blocked by **dendrotoxins** (from mamba snake venom)	Subtypes in the heart include HERG and LQT channels, which are involved in congenital and drug-induced dysrhythmias Other subtypes may be involved in inherited forms of epilepsy
	Ca^{2+}-activated K^+ channels	Inhibition following stimuli which increase $[Ca^{2+}]_i$	Certain subtypes blocked by **apamin** (from bee venom), and **charybdotoxin** (from scorpion venom)	Important in many excitable tissues to limit repetitive discharges, also in secretory cells
Inward rectifying (2T, 1P)	G-protein-activated	Mediate effects of many GPCRs which cause inhibition by increasing K^+ conductance	GPCR agonists and antagonists No important direct interactions	Other inward rectifying K^+ channels important in kidney
	ATP-sensitive	Found in many cells Channels open when [ATP] is low, causing inhibition Important in control of insulin secretion	Association of one subtype with the sulphonylorea receptor (SUR) results in modulation by sulphonylureas (e.g. **glibenclamide**) which close channel, and by K^+ channel openers (e.g. **diazoxide, pinacidil**) which relax smooth muscle	
Two-pore domain (4T, 2P)	Several subtypes identified (TWIK, TRAAK, TREK, TASK, etc.)	Most are voltage insensitive; some are normally open and contribute to the 'resting' K^+ conductance Modulated by GPCRs	Certain subtypes are activated by volatile anaesthetics (e.g. **halothane**) No selective blocking agents Modulation by GPCR agonists and antagonists	Recently discovered, so knowledge is fragmentary as yet

GPCR, G-protein-coupled receptor.
[a] K^+ channel structures (see Fig 3.17) are defined according to the number of transmembrane helices (T) and the number of pore-forming loops (P) in each α subunit. Functional channels contain several subunits (often four) which may be identical or different, and they are often associated with accessory (β) subunits.
[b] Within each functional subtype, several molecular variants have been identified, often restricted to particular cells and tissues. The physiological and pharmacological significance of this heterogeneity is not yet understood.

- Voltage-gated potassium channels, which possess six membrane-spanning helices, one of which serves as the voltage sensor, causing the channel to open when the membrane is depolarised. Included in this group are channels of the shaker family, accounting for most of the voltage-gated K^+ currents familiar to electrophysiologists, and others such as Ca^{2+}-activated potassium channels and two subtypes that are important in the heart, HERG and LQT channels. Disturbance of these channels, either by genetic mutations or by unwanted drug effects, is a major factor in causing cardiac dysrhythmias, which can cause sudden death (see Ch. 21). Many of these channels are blocked by drugs such as **tetraethylammonium** and **4-aminopyridine**.
- Inwardly rectifying potassium channels, so called because they allow K^+ to pass inwards much more readily than outwards (see review by Reimann & Ashcroft, 1999). These have two membrane-spanning helices and a single pore-forming loop (P loop). These channels are regulated by interaction with G-proteins (see Ch. 3) and mediate the inhibitory effects of many agonists acting on G-protein-coupled receptors. Certain types are important in the heart, particularly in regulating the duration of the cardiac action potential (Ch . 21); others are the target for the action of **sulfonylureas** (antidiabetic drugs that stimulate insulin secretion by blocking them; see Ch. 30) and smooth muscle relaxant drugs, such as **cromakalim** and **diazoxide**, which open them (see Ch. 22).
- Two-pore domain potassium channels, with four helices and two P loops (see review by Goldstein et al., 2001). These show outward rectification and therefore exert a strong repolarising influence, opposing any

Ion channels and electrical excitability

- Excitable cells generate an all-or-nothing action potential in response to membrane depolarisation. This occurs in most neurons and muscle cells, and also in some gland cells. The ionic basis and time course of the response varies between tissues.
- The regenerative response results from the depolarising current associated with opening of voltage-gated cation channels (mainly Na^+ and Ca^{2+}). It is terminated by spontaneous closure of these channels accompanied by opening of K^+ channels.
- These voltage-gated channels exist in many molecular varieties, with specific functions in different types of cell.
- The membrane of the 'resting' cell is relatively permeable to K^+ but impermeable to Na^+ and Ca^{2+}. Drugs or mediators that open K^+ channels reduce membrane excitability, as do inhibitors of Na^+ or Ca^{2+} channel function. Blocking K^+ channels or activating Na^+ or Ca^{2+} channels increases excitability.
- Cardiac muscle cells, some neurons and some smooth muscle cells generate spontaneous action potentials whose amplitude, rate and rhythm is affected by drugs that affect ion channel function.

tendency to excitation. They may contribute to the resting K^+ conductance in many cells, and are susceptible to regulation via G-proteins; certain subtypes have been implicated in the action of volatile anaesthetics such as **halothane** (Ch. 40).

Inherited abnormalities of potassium channels (channelopathies) contribute to a rapidly growing number of cardiac, neurological and other diseases. These include the *long QT syndrome* associated with mutations in cardiac voltage-gated potassium channels, causing episodes of ventricular arrest that can result in sudden death. Certain familial types of deafness and epilepsy are associated with mutations in voltage-gated potassium channels. (Ashcroft, 2000, 2006).

MUSCLE CONTRACTION

Effects of drugs on the contractile machinery of smooth muscle are the basis of many therapeutic applications, for smooth muscle is an important component of most physiological systems, including blood vessels and the gastrointestinal, respiratory and urinary tracts. For many decades, smooth muscle pharmacology with its trademark technology—the isolated organ bath—held the centre of the pharmacological stage, and neither the subject nor the technology shows any sign of flagging, even though the stage has become much more crowded. Cardiac muscle contractility is also the target of important drug effects, whereas striated muscle contractility is only rarely affected by drugs.

Although in each case the basic molecular basis of contraction is similar, namely an interaction between actin and myosin, fuelled by ATP and initiated by an increase in $[Ca^{2+}]_i$, there are differences between these three kinds of muscle that account for their different responsiveness to drugs and chemical mediators.

These differences (Fig. 4.9) involve (a) the linkage between membrane events and increase in $[Ca^{2+}]_i$, and (b) the mechanism by which $[Ca^{2+}]_i$ regulates contraction.

SKELETAL MUSCLE

Skeletal muscle possesses an array of transverse T-tubules extending into the cell from the plasma membrane. The action potential of the plasma membrane depends on voltage-gated sodium channels, as in most nerve cells, and propagates rapidly from its site of origin, the motor end-plate (see Ch. 13), to the rest of the fibre. The T-tubule membrane contains L-type calcium channels, which respond to membrane depolarisation conducted passively along the T-tubule when the plasma membrane is invaded by an action potential. These calcium channels are located extremely close to *ryanodine receptors* (RyRs; see Ch. 3) in the adjacent SR membrane, and activation of these RyRs causes release of Ca^{2+} from the SR. There is evidence of direct coupling between the calcium channels of the T-tubule and the RyRs of the SR (as shown in Fig. 4.9); however, Ca^{2+} entry through the T-tubule channels into the restricted zone between these channels and associated RyRs may also contribute. Through this link, depolarisation rapidly activates the RyRs, releasing a short puff of Ca^{2+} from the SR into the sarcoplasm. The Ca^{2+} binds to troponin, a protein that normally blocks the interaction between actin and myosin. When Ca^{2+} binds, troponin moves out of the way and allows the contractile machinery to operate. Ca^{2+} release is rapid and brief, and the muscle responds with a short-lasting 'twitch' response. This is a relatively fast and direct mechanism compared with the arrangement in cardiac and smooth muscle (see below), and consequently less susceptible to pharmacological modulation. The few examples of drugs that directly affect skeletal muscle contraction are shown in Table 4.1.

CARDIAC MUSCLE

Cardiac muscle (see review by Bers, 2002) differs from skeletal muscle in several important respects. The nature of the cardiac action potential, the ionic mechanisms underlying its inherent rhythmicity, and the effects of drugs on the rate and rhythm of the heart are described in Chapter 21. Cardiac muscle cells lack T-tubules, and there is no direct coupling between the plasma membrane and the SR. The cardiac action potential varies in its configuration in different parts of the heart, but commonly shows a 'plateau' lasting several hundred milliseconds following the initial rapid depolarisation. The plasma membrane contains many L-type calcium channels, which open during this plateau and allow Ca^{2+} to enter the cell, although not in sufficient quantities to activate the contractile machinery directly. Instead, this initial Ca^{2+} entry acts on RyRs (a different molecular type from those of skeletal muscle) to release Ca^{2+} from the SR, producing a secondary and much larger wave of Ca^{2+}. Because the RyRs of cardiac muscle are themselves activated by Ca^{2+}, the $[Ca^{2+}]_i$ wave is a regenerative, all-or-nothing event. The initial Ca^{2+} entry that triggers this event is highly dependent on the action potential duration, and on the functioning of the membrane L-type channels. Some of the drugs that affect it are shown in Table 4.1. With minor differences, the mechanism

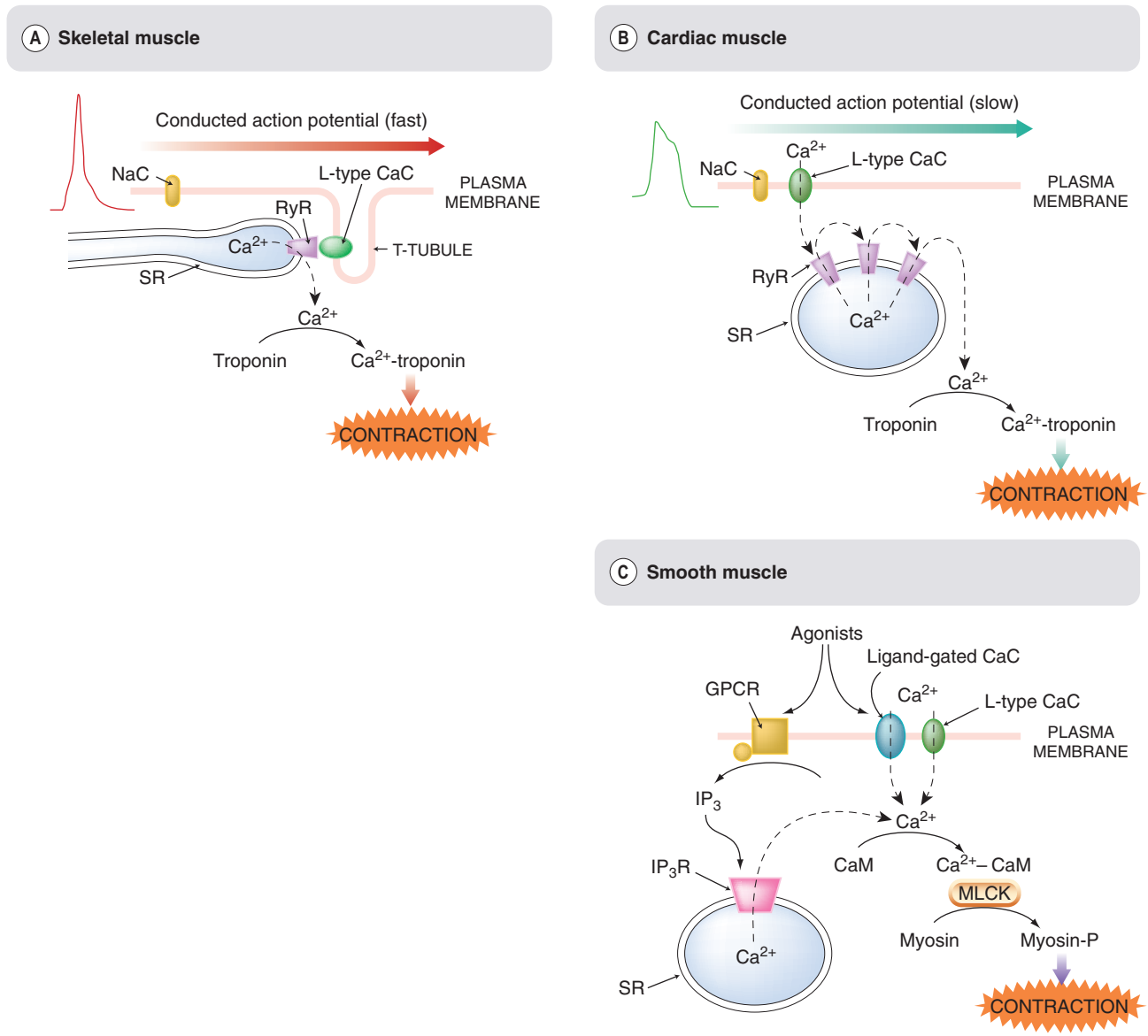

Fig. 4.9 Comparison of excitation–contraction coupling in [A] striated muscle, [B] cardiac muscle and [C] smooth muscle. Striated and cardiac muscle differ mainly in the mechanism by which membrane depolarisation is coupled to Ca^{2+} release. In striated muscle, the T-tubule membrane is coupled closely to the sarcoplasmic reticulum (SR) via the L-type CaC and the ryanodine receptor (RyR). In cardiac muscle, Ca^{2+} entry via voltage-gated calcium channels initiates a regenerative release through activation of the Ca^{2+}-sensitive RyRs. In smooth muscle, contraction can be produced either by Ca^{2+} entry through voltage- or ligand-gated calcium channels, or by inositol trisphosphate (IP_3)-mediated Ca^{2+} release from the SR. The mechanism by which Ca^{2+} activates contraction is different, and operates more slowly, in smooth muscle compared with in striated or cardiac muscle. CaC, calcium channel; CaM, calmodulin; SR, sarcoplasmic reticulum; GPCR, G-protein-coupled receptor; MLCK, myosin light-chain kinase; NaC, voltage-gated sodium channel; RyR, ryanodine receptor.

by which Ca^{2+} activates the contractile machinery is the same as in skeletal muscle. Mutations of ryanodine receptors are implicated in various disorders of skeletal and cardiac muscle function (see Priori & Napolitano, 2005), but so far, no therapeutically useful drugs have emerged from this line of inquiry.

SMOOTH MUSCLE

The properties of smooth muscle vary considerably in different organs, and the mechanisms linking membrane events and contraction are correspondingly variable and more complex than in other kinds of muscle. Spontaneous rhythmic activity occurs in many organs, by mechanisms producing oscillations of $[Ca^{2+}]_i$ (see Berridge, 2009). The action potential of smooth muscle is generally a rather lazy and vague affair compared with the more military behaviour of skeletal and cardiac muscle, and it propagates through the tissue much more slowly and uncertainly. The action potential is, in most cases, generated by L-type calcium channels rather than by voltage-gated sodium channels, and this is one important route of Ca^{2+} entry. In addition, many smooth muscle cells possess P2x receptors, ligand-gated cation channels, which allow Ca^{2+} entry when

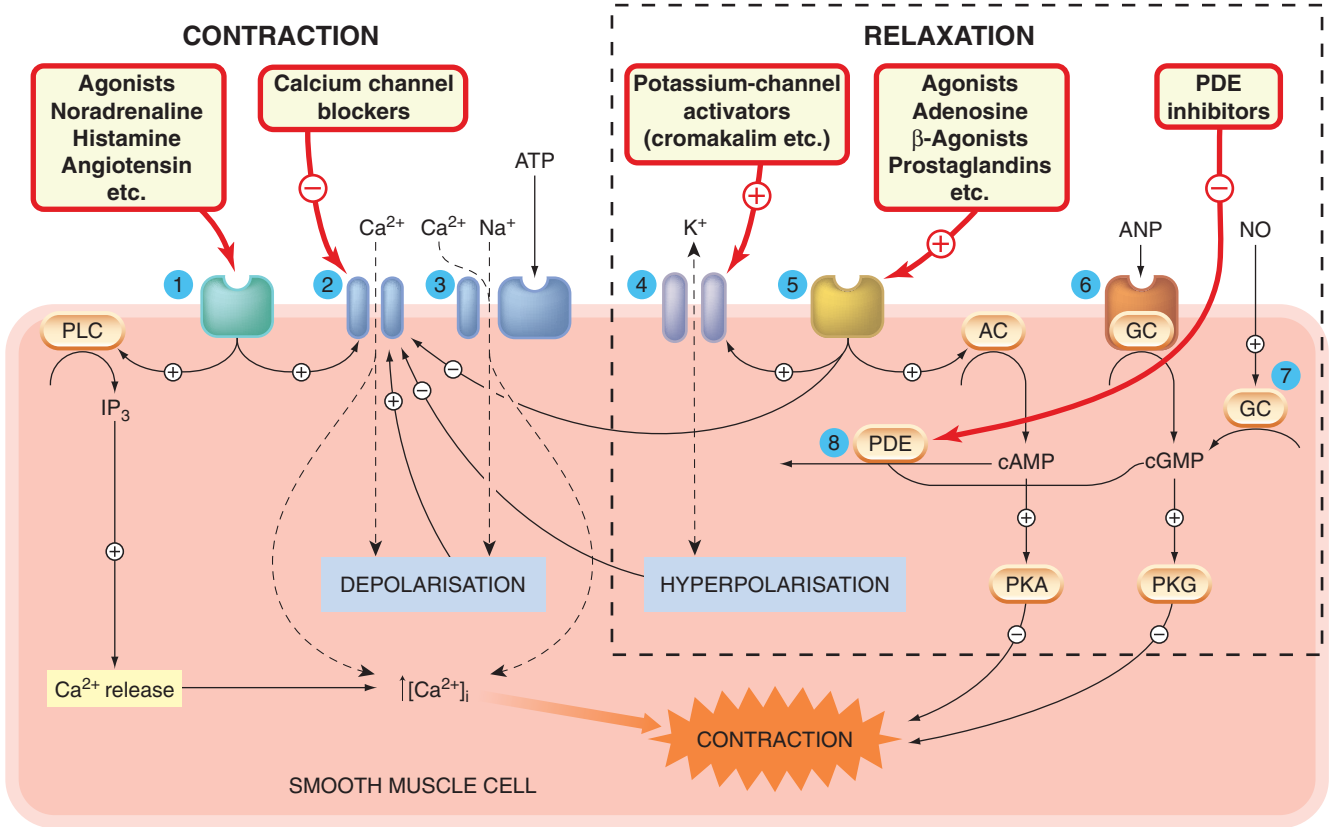

Fig. 4.10 Mechanisms controlling smooth muscle contraction and relaxation. 1. G-protein-coupled receptors for excitatory agonists, mainly regulating inositol trisphosphate formation and calcium channel function. 2. Voltage-gated calcium channels. 3. P2x receptor for ATP (ligand-gated cation channel). 4. Potassium channels. 5. G-protein-coupled receptors for inhibitory agonists, mainly regulating cAMP formation and potassium and calcium channel function. 6. Receptor for atrial natriuretic peptide (ANP), coupled directly to guanylyl cyclase (GC). 7. Soluble guanylyl cyclase, activated by nitric oxide (NO). 8. Phosphodiesterase (PDE), the main route of inactivation of cAMP and cGMP. AC, adenylate cyclase; PKA, protein kinase A; PKG, protein kinase G; PLC, phospholipase C.

activated by ATP released from autonomic nerves (see Ch. 12). Smooth muscle cells also store Ca^{2+} in the ER, from which it can be released when the IP_3R is activated (see Ch. 3). IP_3 is generated by activation of many types of G-protein-coupled receptor. Thus, in contrast to skeletal and cardiac muscle, Ca^{2+} release and contraction can occur in smooth muscle when such receptors are activated without necessarily involving depolarisation and Ca^{2+} entry through the plasma membrane.

The contractile machinery of smooth muscle is activated when the *myosin light chain* undergoes phosphorylation, causing it to become detached from the actin filaments. This phosphorylation is catalysed by a kinase, *myosin light-chain kinase* (MLCK), which is activated when it binds to Ca^{2+}–calmodulin (see p. 52). A second enzyme, *myosin phosphatase*, reverses the phosphorylation and causes relaxation. The activity of MLCK and myosin phosphatase thus exerts a balanced effect, promoting contraction and relaxation, respectively. Both enzymes are regulated by cyclic nucleotides (cAMP and cGMP; see Ch. 3), and many drugs that cause smooth muscle contraction or relaxation mediated through G-protein-coupled receptors or through guanylyl cyclase-linked receptors act in this way. Figure 4.10 summarises the main mechanisms by which drugs control smooth muscle contraction. The complexity of these control mechanisms and interactions explains why pharmacologists have been entranced for so long by smooth muscle. Many therapeutic drugs work by contracting or relaxing smooth muscle, particularly those affecting the cardiovascular, respiratory and gastrointestinal systems, as discussed in later chapters, where details of specific drugs and their physiological effects are given.

RELEASE OF CHEMICAL MEDIATORS

Much of pharmacology is based on interference with the body's own chemical mediators, particularly neurotransmitters, hormones and inflammatory mediators. Here we discuss some of the common mechanisms involved in the release of such mediators, and it will come as no surprise that Ca^{2+} plays a central role. Drugs and other agents that affect the various control mechanisms that regulate $[Ca^{2+}]_i$ will therefore also affect mediator release, and this accounts for many of the physiological effects that they produce.

Chemical mediators that are released from cells fall into two main groups (Fig. 4.11):

- Mediators that are preformed and packaged in storage vesicles—sometimes called storage granules—from which they are released by *exocytosis*. This large group

Muscle contraction

- Muscle contraction occurs in response to a rise in $[Ca^{2+}]_i$.
- In skeletal muscle, depolarisation causes rapid Ca^{2+} release from the sarcoplasmic reticulum (SR); in cardiac muscle, Ca^{2+} enters through voltage-gated channels, and this initial entry triggers further release from the SR; in smooth muscle, the Ca^{2+} signal is due partly to Ca^{2+} entry and partly to IP_3-mediated release from the SR.
- In smooth muscle, contraction can occur without action potentials, for example when agonists at G-protein-coupled receptors lead to IP_3 formation.
- Activation of the contractile machinery in smooth muscle involves phosphorylation of the myosin light chain, a mechanism that is regulated by a variety of second messenger systems.

comprises all the conventional neurotransmitters and neuromodulators (see Chs 12 and 36), and many hormones. It also includes secreted proteins such as cytokines (Ch. 17) and various growth factors (Ch. 19).

- Mediators that are produced on demand and are released by diffusion or by membrane carriers. This group includes nitric oxide (Ch. 20) and many lipid mediators (e.g. prostanoids, Ch. 17, and endocannabinoids, Ch. 18).[4]

Calcium ions play a key role in both cases, because a rise in $[Ca^{2+}]_i$ initiates exocytosis and is also the main activator of the enzymes responsible for the synthesis of diffusible mediators.

In addition to mediators that are released from cells, some are formed from precursors in the plasma, two important examples being *kinins* (Ch. 17) and *angiotensin* (Ch. 22), which are peptides produced by protease-mediated cleavage of circulating proteins.

EXOCYTOSIS

Exocytosis, occurring in response to an increase of $[Ca^{2+}]_i$, is the principal mechanism of transmitter release (see Fig. 4.11) in the peripheral and central nervous systems, as well as in endocrine cells and mast cells. The secretion of enzymes and other proteins by gastrointestinal and exocrine glands and by vascular endothelial cells is also basically similar. Exocytosis (see Burgoyne & Morgan, 2002) involves fusion between the membrane of synaptic vesicles and the inner surface of the plasma membrane. The vesicles are preloaded with stored transmitter, and release occurs in discrete packets, or quanta, each representing the contents of a single vesicle. The first evidence for this (see Nicholls et al., 2000) came from the work of Katz and his colleagues in the 1950s, who recorded spontaneous 'miniature endplate potentials' at the frog neuromuscular junction, and showed that each resulted from the spontaneous release of a packet of the transmitter, acetylcholine. They

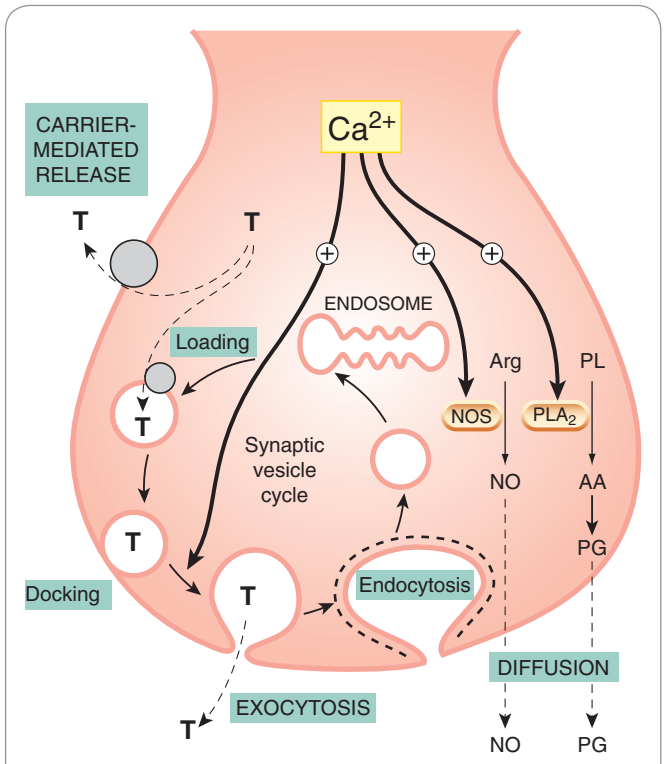

Fig. 4.11 Role of exocytosis, carrier-mediated transport and diffusion in mediator release. The main mechanism of release of monoamine and peptide mediators is Ca^{2+}-mediated exocytosis, but carrier-mediated release from the cytosol also occurs. T represents a typical amine transmitter, such as noradrenaline (norepinephrine) or 5-hydroxytryptamine. Nitric oxide (NO) and prostaglandins (PGs) are released by diffusion as soon as they are formed, from arginine (Arg) and arachidonic acid (AA), respectively, through the action of Ca^{2+}-activated enzymes, nitric oxide synthase (NOS) and phospholipase A_2 (PLA_2) (see Chs 17 and 20 for more details).

also showed that release evoked by nerve stimulation occurred by the synchronous release of several hundred such quanta, and was highly dependent on the presence of Ca^{2+} in the bathing solution. Unequivocal evidence that the quanta represented vesicles releasing their contents by exocytosis came from electron microscopic studies, in which the tissue was rapidly frozen in mid-release, revealing vesicles in the process of extrusion, and from elegant electrophysiological measurements showing that membrane capacitance (reflecting the area of the presynaptic membrane) increased in a stepwise way as each vesicle fused, and then gradually returned as the vesicle membrane was recovered from the surface. There is also biochemical evidence showing that, in addition to the transmitter, other constituents of the vesicles are released at the same time.

▼ In nerve terminals specialised for fast synaptic transmission, Ca^{2+} enters through voltage-gated calcium channels, mainly of the N and P type (see above), and the synaptic vesicles are 'docked' at active zones—specialised regions of the presynaptic membrane from which exocytosis occurs, situated close to the relevant calcium channels and opposite receptor-rich zones of the postsynaptic membrane (see Stanley, 1997). Elsewhere, where speed is less critical, Ca^{2+} may come from intracellular stores as described above, and the spatial organisation of active zones is less clear. It is common for secretory cells, including neurons, to release more than one mediator (for example,

[4]Carrier-mediated release can also occur with neurotransmitters that are stored in vesicles but is quantitatively less significant than exocytosis (see Ch. 13).

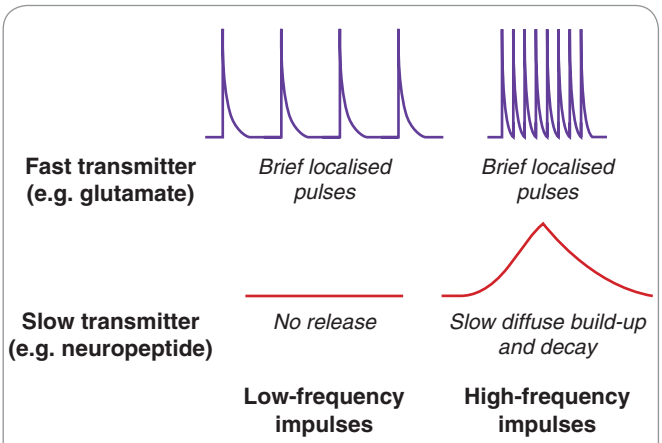

Fig. 4.12 Time course and frequency dependence of the release of 'fast' and 'slow' transmitters. Fast transmitters (e.g. glutamate) are stored in synaptic vesicles that are 'docked' close to voltage-gated calcium channels in the membrane of the nerve terminal, and are released in a short burst when the membrane is depolarised (e.g. by an action potential). Slow transmitters (e.g. neuropeptides) are stored in separate vesicles further from the membrane. Release is slower, because they must first migrate to the membrane, and occurs only when $[Ca^{2+}]_i$ builds up sufficiently.

a 'fast' transmitter such as glutamate and a 'slow' transmitter such as a neuropeptide) from different vesicle pools (see Ch. 12). The fast transmitter vesicles are located close to active zones, while the slow transmitter vesicles are further away. Release of the fast transmitter, because of the tight spatial organisation, occurs as soon as the neighbouring calcium channels open, before the Ca^{2+} has a chance to diffuse throughout the terminal, whereas release of the slow transmitter requires the Ca^{2+} to diffuse more widely. As a result, release of fast transmitters occurs impulse by impulse, even at low stimulation frequencies, whereas release of slow transmitters builds up only at higher stimulation frequencies. The release rates of the two therefore depend critically on the frequency and patterning of firing of the presynaptic neuron (Fig. 4.12). In non-excitable cells (e.g. most exocrine and endocrine glands), the slow mechanism predominates and is activated mainly by Ca^{2+} release from intracellular stores.

Calcium causes exocytosis by binding to the vesicle-bound protein *synaptotagmin*, and this favours association between a second vesicle-bound protein, *synaptobrevin*, and a related protein, *synaptotaxin*, on the inner surface of the plasma membrane. This association brings the vesicle membrane into close apposition with the plasma membrane, causing membrane fusion. This group of proteins, known collectively as SNAREs, plays a key role in exocytosis.

Having undergone exocytosis, the empty vesicle[5] is recaptured by endocytosis and returns to the interior of the terminal, where it fuses with the larger endosomal membrane. The endosome buds off new vesicles, which take up transmitter from the cytosol by means of specific transport proteins and are again docked on the presynaptic membrane. This sequence, which typically takes several minutes, is controlled by various trafficking proteins associated with the plasma membrane and the vesicles, as well as cytosolic proteins. Further details about exocytosis and vesicle recycling are given by Nestler et al. (2008) and Südhof (2004). So far, there are few examples of drugs that affect transmitter release by interacting with synaptic proteins, although the botulinum neurotoxins (see Ch. 13) produce their effects by proteolytic cleavage of SNARE proteins.

[5]The vesicle contents may not always discharge completely. Instead, vesicles may fuse transiently with the cell membrane and release only part of their contents (see Burgoyne & Morgan, 2002) before becoming disconnected (termed *kiss-and-run exocytosis*).

Mediator release

- Most chemical mediators are packaged into storage vesicles and released by exocytosis. Some are synthesised on demand and released by diffusion or the operation of membrane carriers.
- Exocytosis occurs in response to increased $[Ca^{2+}]_i$ as a result of a Ca^{2+}-mediated interaction between proteins of the synaptic vesicle and the plasma membrane, causing the membranes to fuse.
- After releasing their contents, vesicles are recycled and reloaded with transmitter.
- Many secretory cells contain more than one type of vesicle, loaded with different mediators and secreted independently.
- Stored mediators (e.g. neurotransmitters) may be released directly from the cytosol independently of Ca^{2+} and exocytosis by drugs that interact with membrane transport mechanisms.
- Non-stored mediators, such as prostanoids and nitric oxide, are released by increased $[Ca^{2+}]_i$, which activates the enzymes responsible for their synthesis.

NON-VESICULAR RELEASE MECHANISMS

If this neat and tidy picture of transmitter packets ready and waiting to pop obediently out of the cell in response to a puff of Ca^{2+} seems a little too good to be true, rest assured that the picture is not quite so simple. Acetylcholine, noradrenaline (norepinephrine) and other mediators can leak out of nerve endings from the cytosolic compartment, independently of vesicle fusion, by utilising carriers in the plasma membrane (Fig. 4.11). Drugs such as amphetamines, which release amines from central and peripheral nerve terminals (see Chs 14 and 38), do so by displacing the endogenous amine from storage vesicles into the cytosol, whence it escapes via the monoamine transporter in the plasma membrane, a mechanism that does not depend on Ca^{2+}.

Nitric oxide (see Ch. 20) and arachidonic acid metabolites (e.g. prostaglandins; Ch. 17) are two important examples of mediators that are released by diffusion across the membrane or by carrier-mediated extrusion, rather than by exocytosis. The mediators are not stored but escape from the cell as soon as they are synthesised. In both cases, the synthetic enzyme is activated by Ca^{2+}, and the moment-to-moment control of the rate of synthesis depends on $[Ca^{2+}]_i$. This kind of release is necessarily slower than the classic exocytotic mechanism, but in the case of nitric oxide is fast enough for it to function as a true transmitter (see Ch. 20).

EPITHELIAL ION TRANSPORT

Fluid-secreting epithelia include the renal tubule, salivary glands, gastrointestinal tract and airways epithelia. In each case, epithelial cells are arranged in sheets separating the interior (blood-perfused) compartment from the exterior lumen compartment, into which, or from which, secretion takes place. Fluid secretion involves two main mechanisms, which often coexist in the same cell and indeed interact with each other. Greger (2000) and Ashcroft (2000) give more

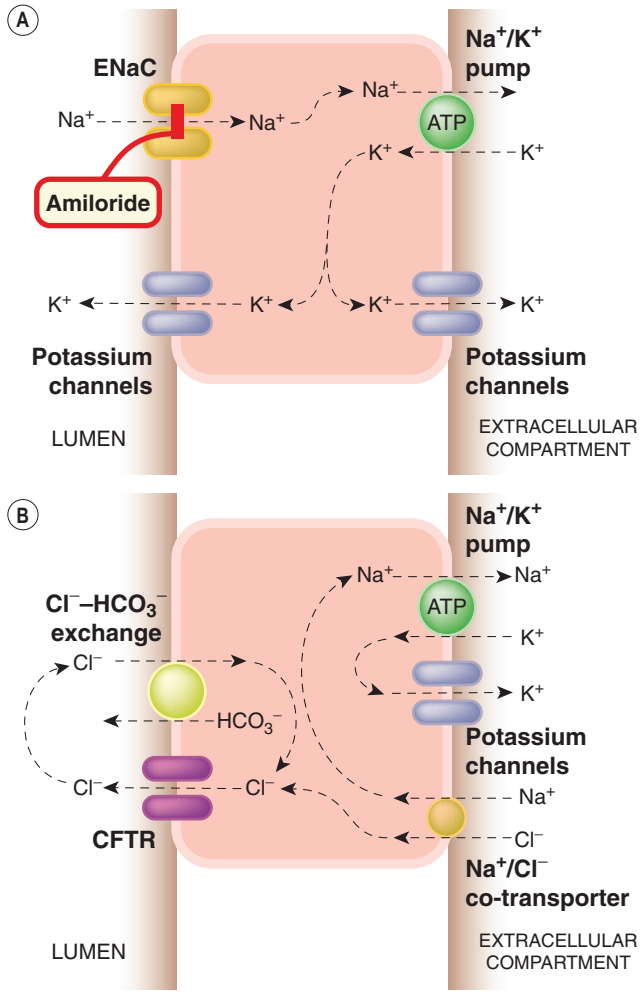

Fig. 4.13 Mechanisms of epithelial ion transport. Such mechanisms are important in renal tubules (see Ch. 28 for more details) and also in many other situations, such as the gastrointestinal and respiratory tracts. [**A**] Sodium transport. A special type of epithelial sodium channel (ENaC) controls entry of Na^+ into the cell from the lumenal surface, the Na^+ being actively pumped out at the apical surface by the Na^+–K^+ exchange pump. K^+ moves passively via potassium channels. [**B**] Chloride transport. Cl^- leaves the cell via a special membrane channel, the cystic fibrosis transmembrane conductance regulator (CFTR), after entering the cell either from the apical surface via the Na^+/Cl^- co-transporter, or at the lumenal surface via the Cl^-/HCO_3^- co-transporter.

detailed accounts. The two mechanisms (Fig. 4.13) are concerned, respectively, with Na^+ transport and Cl^- transport.

In the case of Na^+ transport, secretion occurs because Na^+ enters the cell passively at one end and is pumped out actively at the other, with water following passively. Critical to this mechanism is a class of highly regulated epithelial sodium channels (ENaCs) that allow Na^+ entry.

Epithelial sodium channels (see De la Rosa et al., 2000) are widely expressed, not only in epithelial cells but also in neurons and other excitable cells, where their function is largely unknown. They are regulated mainly by **aldosterone**, a hormone produced by the adrenal cortex that enhances Na^+ reabsorption by the kidney (Ch. 28). Aldosterone, like other steroid hormones, exerts its effects by regulating gene expression (see Ch. 3), and causes an increase in ENaC expression, thereby increasing the rate of Na^+ and fluid transport. ENaCs are selectively blocked by certain diuretic drugs, notably **amiloride** (see Ch. 28), a compound that is widely used to study the functioning of ENaCs in other situations.

Chloride transport is particularly important in the airways and gastrointestinal tract. In the airways, it is essential for fluid secretion, whereas in the colon it mediates fluid reabsorption, the difference being due to the different arrangement of various transporters and channels with respect to the polarity of the cells. The simplified diagram in Figure 4.13B represents the situation in the pancreas, where secretion depends on Cl^- transport. The key molecule in Cl^- transport is the *cystic fibrosis transmembrane conductance regulator* (CFTR; see Hwang & Sheppard, 1999), so named because early studies on the inherited disorder cystic fibrosis showed it to be associated with impaired Cl^- conductance in the membrane of secretory epithelial cells, and the CFTR gene, identified through painstaking genetic linkage studies and isolated in 1989, was found to encode a Cl^--conducting ion channel. Severe physiological consequences follow from the impairment of secretion, particularly in the airways but also in many other systems, such as sweat glands and pancreas. Studies on the disease-associated mutations of the CFTR gene have revealed much about the molecular mechanisms involved in Cl^- transport, but as yet no significant therapeutic advance. So far, no drugs are known that interact specifically with CFTRs.

Both Na^+ and Cl^- transport are regulated by intracellular messengers, notably by Ca^{2+} and cAMP, the latter exerting its effects by activating protein kinases and thereby causing phosphorylation of channels and transporters. CFTR itself is activated by cAMP. In the gastrointestinal tract, increased cAMP formation causes a large increase in the rate of fluid secretion, an effect that leads to the copious diarrhoea produced by cholera infection (see Ch. 3) and also by inflammatory conditions in which prostaglandin formation is increased (see Ch. 17). Activation of G-protein-coupled receptors, which cause release of Ca^{2+}, also stimulates secretion, possibly also by activating CFTR. Many examples of therapeutic drugs that affect epithelial secretion by activating or blocking G-protein-coupled receptors appear in later chapters.

Epithelial ion transport

- Many epithelia (e.g. renal tubules, exocrine glands and airways) are specialised to transport specific ions.
- This type of transport depends on a special class of epithelial sodium channels (ENaCs) which allow Na^+ entry into the cell at one surface, coupled to active extrusion of Na^+, or exchange for another ion, from the opposite surface.
- Anion transport depends on a specific chloride channel (the cystic fibrosis transmembrane conductance regulator), mutations of which result in cystic fibrosis.
- The activity of channels, pumps and exchange transporters is regulated by various second messengers and nuclear receptors, which control the transport of ions in specific ways.

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5 Cell proliferation, apoptosis, repair and regeneration

OVERVIEW

This chapter deals with cell proliferation, apoptosis, repair and regeneration and how these relate to the actions of drugs dealt with in this book. About 10 billion new cells are manufactured in the body daily through cell division—an output that must be counterbalanced by the elimination of a similar number of cells. We deal first with the changes that occur within an individual cell when, after stimulation by growth factors, it gears up to divide into two daughter cells. We then consider the interaction of cells, growth factors and the extracellular matrix in cell proliferation. We describe the phenomenon of apoptosis (the programmed series of events that lead to cell death), outlining the changes that occur in a cell that is preparing to die, and the intracellular pathways that lead to its demise. We consider how these processes relate to the repair of damaged tissue and the possibility of its regeneration. Lastly, we consider the pathophysiological significance of these events, and implications for the potential development of clinically useful drugs.

CELL PROLIFERATION

Cell proliferation is involved in many physiological and pathological processes including growth, healing, repair, hypertrophy, hyperplasia and the development of tumours. *Angiogenesis* (the development of new blood vessels) necessarily occurs during many of these processes.

Proliferating cells go through what is termed the cell cycle, during which the cell replicates all its components and then bisects itself into two identical daughter cells. Important components of the signalling pathways in proliferating cells are receptor tyrosine kinases or receptor-linked kinases, and the mitogen-activated protein kinase (MAP kinase) cascade (see Ch. 3). In all cases, the pathways eventually lead to transcription of the genes that control the cell cycle.

THE CELL CYCLE

The cell cycle is an ordered series of events consisting of several sequential phases (Fig. 5.1). These are:

- G_1: preparation for DNA synthesis
- S: DNA synthesis and chromosome duplication
- G_2: preparation for division
- mitosis (M): division into two daughter cells.

In cells that are dividing continuously, G_1, S and G_2 comprise *interphase*—the phase between one mitosis and the next.

Cell division requires the controlled timing of two critical events of the cell cycle: S phase (DNA replication) and M phase (mitosis). Entry into each of these phases is closely regulated, and there are two 'check points' (restriction points) in the cycle at the start of S and M, respectively. DNA damage results in the cycle being stopped at one or other of these. The integrity of the check points is critical for the maintenance of genetic stability and failure of the check points to stop the cycle when it is appropriate to do so is a hallmark of cancer.

In the adult, most cells do not constantly divide; most spend a varying amount of time in a quiescent phase outside the cycle in the phase termed G_0 (Fig. 5.1). Neurons and skeletal muscle cells spend all their lifetime in G_0; bone marrow cells and the lining cells of the gastrointestinal tract divide daily.

Quiescent cells can be activated into G_1 by chemical stimuli associated with damage; for example, a quiescent skin cell can be stimulated by a wound into dividing and repairing the lesion. The impetus for a cell to start off on the cell cycle (i.e. to move from G_0 into G_1) can be provided by several stimuli, the most important being *growth factors* acting on growth factor receptors, though the action of ligands on G-protein-coupled receptors (see Ch. 3) can also stimulate the cell to embark on the cell cycle.

Growth factors stimulate the production of signals of two types:

1. Positive regulators of the cell cycle that control the changes necessary for cell division.
2. Negative regulators that control the positive regulators.

The maintenance of normal cell numbers in tissues and organs requires that there be a balance between the positive regulatory forces and the negative regulatory forces. Apoptosis also has a role in the control of cell numbers (see below).

POSITIVE REGULATORS OF THE CELL CYCLE

The cycle starts when a growth factor acts on a quiescent cell, provoking it to divide. Growth factors stimulate production of the cell cycle regulators, which are coded for by the delayed response genes.

Two families of proteins, *cyclins* and *cyclin-dependent kinases* (cdks), control progress through the cycle. The cdks, functioning sequentially, phosphorylate various proteins (e.g. enzymes)—activating some and inhibiting others—to coordinate their activities.

Each cdk is inactive until it binds to a cyclin, the binding enabling the cdk to phosphorylate the protein(s) necessary for a particular step in the cycle. It is the cyclin that determines which protein(s) is phosphorylated. After the phosphorylation event has taken place, the cyclin is degraded (Fig. 5.2) by the ubiquitin/protease system. This involves several enzymes acting sequentially to add small molecules of ubiquitin to the cyclin, with the resulting ubiquitin polymer acting as an 'address label' that directs the cyclin to the proteasome where it is degraded.

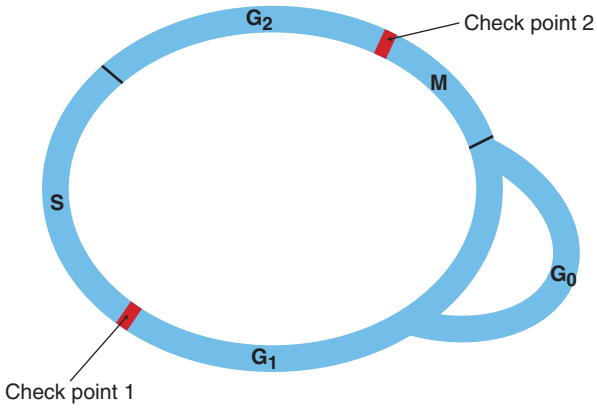

Fig. 5.1 The main phases of the cell cycle of dividing cells.

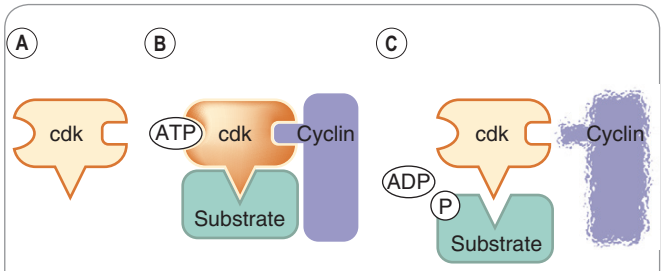

Fig. 5.2 Schematic representation of the activation of a cyclin-dependent kinase (cdk). [**A**] An inactive cdk. [**B**] The inactive cdk is activated by being bound to a cyclin; it can now phosphorylate a protein substrate (e.g. an enzyme). [**C**] After the phosphorylating event, the cyclin is degraded.

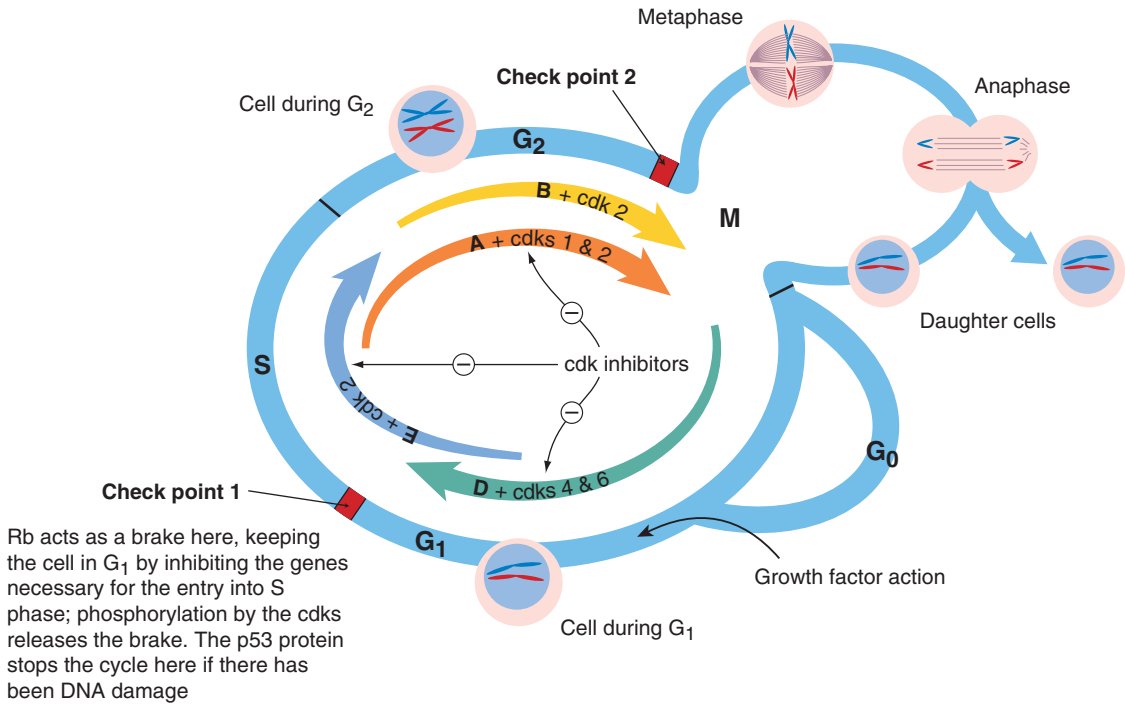

Fig. 5.3 Schematic diagram of the cell cycle, showing the role of the cyclin/cyclin-dependent kinase (cdk) complexes. The processes outlined in the cycle occur inside a cell such as the one shown in Figure 5.4. A quiescent cell (in G_0 phase), when stimulated to divide by growth factors, is propelled into G_1 phase and prepares for DNA synthesis. Progress through the cycle is determined by sequential action of the cyclin/cdk complexes—depicted here by coloured arrows, the arrows being given the names of the relevant cyclins: D, E, A and B. The cdks are given next to the relevant cyclins. The thickness of each arrow represents the intensity of action of the cdk at that point in the cycle. The activity of the cdks is regulated by cdk inhibitors. If there is DNA damage, the products of the tumour suppressor gene *p53* stop the cycle at check point 1, allowing for repair. If repair fails, apoptosis (see Fig. 5.5) is initiated. The state of the chromosomes is shown schematically in each G phase—as a single pair in G_1, and each duplicated and forming two daughter chromatids in G_2. Some changes that occur during mitosis (metaphase, anaphase) are shown in a subsidiary circle. After the mitotic division, the daughter cells may enter G_1 or G_0 phase. Rb, retinoblastoma gene.

There are eight main groups of cyclins. Those important in the control of the cell cycle are cyclins A, B, D and E. Each cyclin is associated with and activates a particular cdk. Cyclin A activates cdks 1 and 2; cyclin B, cdk 1; cyclin D, cdks 4 and 6; and cyclin E, cdk 2. Precise timing of each activity is essential, and many cycle proteins are degraded after they have carried out their functions. The actions of the cyclin/cdk complexes in the cell cycle are depicted in Figure 5.3.

The activity of these cyclin/cdk complexes is modulated by various negative regulatory forces (considered below), most of which act at one or other of the two check points.

In quiescent G_0 cells, cyclin D is present in low concentration, and an important regulatory protein—the *Rb*

protein[1]—is hypophosphorylated. Hypophosphorylated Rb holds the cell cycle in check at check point 1 by inhibiting the expression of several proteins critical for cell cycle progression. The Rb protein accomplishes this by binding to transcription factors, which control the expression of the genes that code for cyclins E and A, for DNA polymerase, for thymidine kinase, for dihydrofolate reductase, etc.—all essential for DNA replication during S phase.

Growth factor action on a cell in G_0 propels it into G_1, the phase in which the cell is preparing for S phase by synthesising the messenger RNAs and proteins needed for DNA replication.

During G_1, the concentration of cyclin D increases and the cyclin D/cdk complex phosphorylates and activates the necessary proteins.

In mid-G_1, the cyclin D/cdk complex phosphorylates the Rb protein, releasing a transcription factor that activates the genes for the components essential for the next phase—DNA synthesis.The action of the cyclin E/cdk complex is necessary for transition from G_1 to S phase, i.e. past check point 1.

Once past check point 1, into the S-phase, the processes that have been set in motion cannot be reversed, and the cell is committed to continue with DNA replication and mitosis. Cyclin E/cdk and cyclin A/cdk regulate progress through S phase, phosphorylating and thus activating proteins/enzymes involved in DNA synthesis.

In G_2 phase, the cell, which now has double the number of chromosomes, must duplicate all other cellular components for allocation to the two daughter cells. Synthesis of the necessary messenger RNAs and proteins occurs.

Cyclin A/cdk and cyclin B/cdk complexes are active during G_2 phase and are necessary for entry into M phase, i.e. for passing check point 2. The presence of cyclin B/cdk complexes in the nucleus is required for mitosis to commence.

Mitosis occurs in four stages:

- *Prophase*. The duplicated chromosomes (which have up to this point formed a tangled mass filling the nucleus) condense, each now consisting of two daughter chromatids (the original chromosome and a copy). These are released into the cytoplasm as the nuclear membrane disintegrates.
- *Metaphase*. The chromosomes are aligned at the equator (see Fig. 5.3).
- *Anaphase*. A specialised device, the mitotic apparatus, captures the chromosomes and draws them to opposite poles of the dividing cell (see Fig. 5.3).
- *Telophase*. A nuclear membrane forms round each set of chromosomes. Finally, the cytoplasm divides between the two forming daughter cells. Each daughter cell will be in G_0 phase and will remain there unless stimulated into G_1 phase as described above.

During metaphase, the cyclin A and B complexes phosphorylate cytoskeletal proteins, histones and possibly components of the spindle (the microtubules along which the chromatids are pulled during metaphase).

NEGATIVE REGULATORS OF THE CELL CYCLE

One of the main negative regulators is the Rb protein (see above) that—while it is hypophosphorylated—holds the cycle in check. Inhibitors of the cdks also serve as negative regulators, their main action being at check point 1.

There are two families of inhibitors:

1. The *CIP family* (cdk inhibitory proteins, also termed KIP or kinase inhibitory proteins)—proteins p21, p27 and p57.
2. The *Ink family* (inhibitors of kinases)—proteins p16, p19 and p15.

The action of p21 serves as an example of the role of a cyclin/cdk inhibitor. Protein p21 is under the control of the p53 gene—a particularly important negative regulator which is relevant in carcinogenesis—that operates at check point 1.

Inhibition of the cycle at check point 1

The p53 gene has been called the 'guardian of the genome'. It codes for a transcription factor—the p53 protein. In normal healthy cells, the steady-state concentration of the p53 protein is low. But when there is DNA damage, the protein accumulates and activates the transcription of several genes, one of which codes for p21. Protein p21 inactivates cyclin/cdk complexes, thus preventing Rb phosphorylation, which means that the cycle is arrested at check point 1. This allows for DNA repair. If the repair is successful, the cycle proceeds past check point 1 into S phase. If the repair is unsuccessful, the p53 gene triggers apoptosis—cell suicide (see below).

Inhibition of the cycle at check point 2

DNA damage can result in the cycle being stopped at check point 2, but the mechanisms involved are poorly understood. Inhibition of the accumulation of cyclin B/cdk complex in the nucleus seems to be a factor.

For more detail on the control of the cell cycle, see under *MicroRNAs* (below) and Swanton (2004).

The cell cycle

- The term *cell cycle* refers to the sequence of events that take place within a cell as it tools up for division.
- The phases of the cell cycle are:
  - G_1: preparation for DNA synthesis
  - S: DNA synthesis
  - G_2: preparation for division
  - mitosis: division into two daughter cells.
- Growth factor action stimulates a quiescent cell—said to be in G_0—to divide, i.e. to start on G_1 phase.
- In G_0 phase, a hypophosphorylated protein, coded for by the Rb gene, holds the cycle in check by inhibiting expression of critical factors necessary for DNA replication.
- Progress through the cycle is controlled by specific kinases (cyclin-dependent kinases; cdks) that are activated by binding to proteins termed cyclins.
- Four main cyclin/cdk complexes involving cyclins D, E, A and B drive the cycle; the first complex, cyclin D/cdk, releases the Rb protein-mediated inhibition.
- Various families of proteins act as cdk inhibitors. Important is protein p21, which is expressed when DNA damage causes transcription of gene p53. The p21 protein stops the cycle at check point 1.

[1]So named because mutations of the Rb gene are associated with retinoblastoma tumours.

INTERACTIONS BETWEEN CELLS, GROWTH FACTORS AND THE EXTRACELLULAR MATRIX

During cell proliferation, there is integrated interplay between growth factors, cells, the *extracellular matrix* (ECM), and the *matrix metalloproteinases* (MMPs, see below). The ECM supplies the supporting framework for the cells and is secreted by the cells themselves. It also profoundly influences cell behaviour through the cell's *integrins* (see below). Matrix expression is regulated by the action on the cell of growth factors and cytokines (see Verrecchia & Mauviel, 2007; Järveläinen et al., 2009). The activation status of some growth factors is, in turn, determined by the matrix, because they are sequestered by interaction with matrix components and released by enzymes (e.g. MMPs) secreted by the cells.

The action of growth factors—which act through receptor tyrosine kinases or receptor-coupled kinases (see Ch. 3) initiating the cell cycle—is a fundamental part of these processes. There are numerous growth factors, important examples being *fibroblast growth factor* (FGF), *epidermal growth factor* (EGF), *platelet-dependent growth factor* (PDGF), *vascular endothelial growth factor* (VEGF) and *transforming growth factor* (TGF)-β.

The main components of the extracellular matrix are:

- Fibre-forming elements, eg. *collagen species* (the main proteins of the matrix), and *elastin*.
- Non-fibre-forming, e.g. proteoglycans, glucoproteins and adhesive proteins (e.g. *fibronectin*). Proteoglycans have a growth-regulating role, in part by functioning as a reservoir of sequestrated growth factors (as specified above). Some proteoglycans are associated with the cell surface, where they help to bind cells to the matrix. Adhesive proteins link the various elements of the matrix together, and also form links between the cells and the matrix through integrins on the cells (see below).

Other proteins in the ECM are *thrombospondin* (Ch. 24) and *osteopontin* (Ch. 35) which are not structural elements but modulate cell–matrix interactions and repair processes. The production of the ECM components is regulated by growth factors, particularly transforming growth factor-β (TGF-β).

▼ Until recently, the importance of the ECM in drug action has been overlooked. Both beneficial and adverse effects of some drugs are due to effects on the ECM. Thus glucocorticoids decrease collagen synthesis in chronic inflammation, cyclo-oxygenase (COX)-2 inhibitors can modify fibrotic processes through a proposed action on TGF-β and statins can decrease fibrosis by inhibiting angiotensin-induced connective tissue growth factor production (Rupérez et al., 2007). The action of statins (see Ch. 23) in reducing circulating MMPs and decreasing MMP expression may contribute to their effects in cardiovascular diseases (Tousoulis et al., 2009). The adverse actions of some drugs attributable to an effect on the ECM include the osteoporosis and skin thinning caused by glucocortoicoids (discussed in Järveläinen et al., 2009). The ECM is also an important target in the search for new drugs.

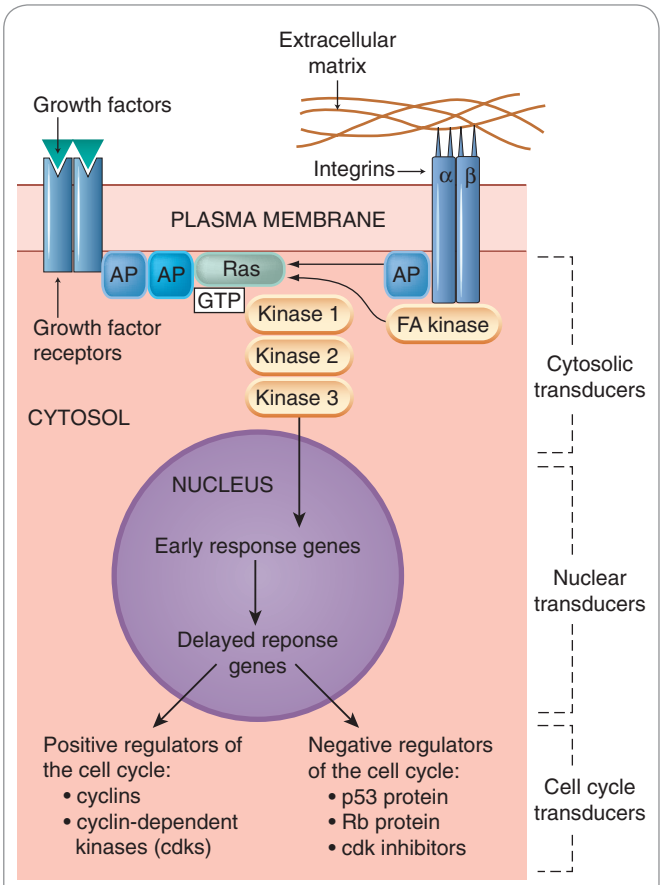

Fig. 5.4 Simplified diagram of the effect of growth factors on a cell in G_0. The overall effect of growth factor action is the generation of the cell cycle transducers. A cell such as the one depicted will then embark on G_1 phase of the cell cycle. Most growth factor receptors have integral tyrosine kinase (see Fig. 3.15). These receptors dimerise (form pairs), then phosphorylate each other's tyrosine residues. The early cytosolic transducers include proteins that bind to the phosphorylated tyrosine residues. Optimum effect requires cooperation with integrin action. Integrins (which have α and β subunits) connect the extracellular matrix with intracellular signalling pathways and also with the cell's cytoskeleton (not shown here). G-protein-coupled receptors can also stimulate cell proliferation, because their intracellular pathways can connect with the Ras/kinase cascade (not shown). AP, adapter protein; FA kinase, focal adhesion kinase; Rb, retinoblastoma.

THE ROLE OF INTEGRINS

▼ Integrins are transmembrane kinase-linked receptors (see Ch. 3), with α and β subunits that on interaction with the ECM elements outside the cell (e.g. fibronectin) mediate various cell responses, such as cytoskeletal rearrangement (not considered here) and co-regulation of growth factor function. Intracellular signalling by both growth factor receptors and integrins is important for optimal cell proliferation (Fig. 5.4). Integrin stimulation activates an intracellular transduction pathway, which, through an adapter protein and an enzyme (*focal adhesion kinase*), can activate the kinase cascade that forms part of the growth factor signalling pathway. Cross-talk between the integrin and growth factor pathways occurs by several other means as well (Streuli & Akhtar, 2009). Autophosphorylation of growth factor receptors (Ch. 3) is enhanced by integrin activation, and integrin-mediated adhesion to the extracellular matrix (Fig. 5.4) not only suppresses the concentrations of cdk inhibitors, but is required for the expression of cyclins A and D, and therefore for the progression of the cell cycle. Furthermore, integrin action stimulates apoptosis-inhibiting signals (see below), further facilitating growth factor action. See reviews by Gamberg et al., 2009 and Barczyk et al., 2010.

Several recently-introduced monoclonal antibodies are targeted at integrins, including **natalizumab**, used for multiple sclerosis (Baker & Hagg, 2007) and **abciximab**, an antithrombotic (Ch. 24).

Interactions between cells, growth factors and the matrix

- Cells are embedded in the extracellular matrix (ECM), which is secreted by the cells themselves.
- The ECM profoundly influences the cells through the cells' integrins; it also forms a store of growth factors by sequestering them.
- Integrins are transmembrane receptors that, on interaction with elements of the ECM, cooperate with growth factor signalling pathways (this is necessary for optimum cell division) and also mediate cytoskeletal adjustments within the cell.
- On stimulation with growth factors, cells release metalloproteinases that degrade the local matrix in preparation for the increase in cell numbers.
- Metalloproteinases release growth factors from the ECM and can activate some that are present in precursor form.

THE ROLE OF MATRIX METALLOPROTEINASES

▼ Degradation of the extracellular matrix by metalloproteinases is necessary during the growth, repair and remodelling of tissues. These enzymes are secreted as inactive precursors by local cells. When growth factors stimulate a cell to enter the cell cycle, they also stimulate the secretion of metalloproteinases, which then sculpt the matrix—producing the local changes necessary for the resulting increase in cell numbers. Metalloproteinases in turn play a part in releasing growth factors from the matrix as described above and, in some cases (e.g. interleukin [IL]-1β), in processing them from precursor to active form.

The action of these enzymes is regulated by TIMPS (tissue inhibitors of metalloproteinases), which are also secreted by local cells.

In addition to the physiological function outlined above, metalloproteinases are involved in the tissue destruction that occurs in various diseases, such as rheumatoid arthritis, osteoarthritis, periodontitis, macular degeneration and myocardial restenosis. They also have a critical role in the growth, invasion and metastasis of tumours, etc. See reviews by Clark et al. (2008), Skiles et al. (2004) and Marastoni et al. (2008). Much effort has gone into developing synthetic MMP inhibitors for treating cancers and inflammatory disorders, but clinical trials so far have shown limited efficacy and significant adverse effects (see Fingleton, 2008). **Doxycycline**, an antibiotic, also inhibits MMPs, and is used experimentally for this purpose.

ANGIOGENESIS

Angiogenesis, which normally accompanies cell proliferation, is the formation of new capillaries from existing small blood vessels, without which new tissues, including tumours, cannot grow. Angiogenic stimuli, in the context of cell proliferation, include the action of various growth factors and cytokines, in particular *vascular endothelial growth factor* (VEGF). The sequence of events is as follows:

1. The basement membrane is degraded locally by proteases.
2. Endothelial cells migrate out, forming a sprout.
3. Endothelial cells following the leading cells proliferate under the influence of VEGF.
4. Matrix is laid down around the new capillary.

A monoclonal antibody, **bevacizumab**, directed against VEGF, is used as adjunct treatment for various cancers (see Ch. 55), and also, by injection into the eye, to treat age-related macular degeneration, a condition in which retinal blood vessels proliferate, causing blindness.

APOPTOSIS AND CELL REMOVAL

Apoptosis is cell suicide by a built-in self-destruct mechanism consisting of a genetically programmed sequence of biochemical events. It is thus unlike necrosis, which is disorganised disintegration of damaged cells resulting in products that trigger the inflammatory response. For a detailed review see Aslan & Thomas (2009).

Apoptosis plays an essential role in embryogenesis, helping to shape organs during development by eliminating cells that have become redundant. It is the mechanism that each day unobtrusively removes 10 billion cells from the human body. It is involved in numerous physiological events: the shedding of the intestinal lining, the death of time-expired neutrophils and the turnover of tissues as the newborn infant grows to maturity. It is the basis for the development of self-tolerance in the immune system (Ch. 6) and acts as a first-line defence against carcinogenic mutations by purging cells with abnormal DNA that could become malignant.

Disturbed apoptosis is also implicated in the pathophysiology of many conditions. Conditions associated with excessive apoptosis include:

- chronic neurodegenerative diseases such as Alzheimer's, multiple sclerosis and Parkinson's disease (Ch. 39)
- conditions with acute tissue damage or cell loss such as myocardial infarction (Ch. 21), stroke and spinal cord injury (Ch. 39)
- depletion of T cells in HIV infection (Ch. 51)
- osteoarthritis (Ch. 35)
- haematological disease such as aplastic anaemia (Ch. 24).

Examples of defective apoptosis include:

- evasion of the immune response by cancer cells and resistance to cancer chemotherapy (Ch. 55)
- autoimmune/inflammatory diseases such as myasthenia gravis (Ch. 13), rheumatoid arthritis (Ch. 26), and bronchial asthma (Ch. 27)
- viral infections with ineffective eradication of virus-infected cells (Ch. 51).

▼ Apoptosis is particularly important in the regulation of the immune response and in the many conditions in which it is an underlying component. There is recent evidence that T cells have a negative regulatory pathway controlled by surface *programmed cell death receptors* (e.g. the PD-1 receptor), and that there is normally a balance between the stimulatory pathways triggered by antigens and this negative regulatory apoptosis-inducing pathway. The balance is important in the maintenance of peripheral tolerance. A disturbance of this balance is seen in autoimmune disease, in the 'exhaustion' of T cells in chronic viral diseases such as HIV, and possibly in tumour escape from immune destruction (Zha et al., 2004).

Apoptosis is *a default response*, i.e. continuous active signalling by tissue-specific trophic factors, cytokines and hormones, and cell-to-cell contact factors (adhesion molecules, integrins, etc.) may be required for cell survival and viability, and the self-destruct mechanism is automatically triggered unless it is actively and continuously inhibited by

these anti-apoptotic factors. Different cell types require differing sets of survival factors, which function only locally. If a cell strays or is dislodged from the area where its paracrine survival signals operate, it will die.

Withdrawal of these cell survival factors—which has been termed 'death by neglect'—is not the only pathway to apoptosis (see Fig. 5.5). The death machinery can be activated by ligands that stimulate *death receptors* ('death by design') and by DNA damage. But it is generally accepted that cell proliferation processes and apoptosis are tightly connected (see below).

MORPHOLOGICAL CHANGES IN APOPTOSIS

As the cell dies it rounds up, the chromatin condenses into dense masses, the cytoplasm shrinks, there is blebbing of the plasma membrane, and finally, by the action of a family of proteolytic enzymes known as *caspases* (see below), there is transformation of the cell into a cluster of membrane-bound entities, the corpse of the cell, which display 'eat me' signals—surface exposure of phosphatidylserine, etc. Macrophages recognise these signals and phagocytose the remains. The fact that the remains are membrane bound is important because release of the internal cell constituents could trigger an unwanted inflammatory reaction. An additional safeguard against this is that macrophages engaged in the clearance of the cell corpses release anti-inflammatory mediators such as TGF-β and IL-10.

THE MAJOR PLAYERS IN APOPTOSIS

The repertoire of reactions in apoptosis is extremely complex and can vary not only between species but between cell types. Yet it could be that the pivotal reaction(s) that lead to either cell survival or cell death are controlled by a single gene or combination of genes. If so, these genes could be attainable targets in the development of drugs for many proliferative diseases. The use of gene silencing by RNA interference (RNAi) technology permits very efficient and precise block of gene expression (see Ch. 59) and is being used to identify antiapoptotic genes.

Only a simple outline of the complex apoptotic repertoire of reactions can be given here. The major players are the *caspases*—a family of cysteine proteases present in the cell in inactive form. These undertake delicate protein surgery, selectively cleaving a specific set of target proteins (enzymes, structural components), inactivating some and activating others. A cascade of about nine different caspases takes part in bringing about apoptosis, some functioning as initiators that transmit the initial apoptotic signals, and some being responsible for the final phase of cell death (Fig. 5.5).

The executioner caspases (e.g. caspase 3) cleave and inactivate cell constituents such as the DNA repair enzymes, protein kinase C, and cytoskeletal components. A DNAase is activated and cuts genomic DNA between the nucleosomes, generating DNA fragments of approximately 180 base pairs.

Besides the caspases, another pathway involves a protein termed *apoptotic initiating factor* (AIF) that is released from the mitochondria, enters the nucleus and triggers cell suicide.

Not all caspases are death-mediating enzymes; some have a role in the processing and activating of cytokines (e.g. caspase 8 is active in processing the inflammatory cytokines IL-1 and IL-18).

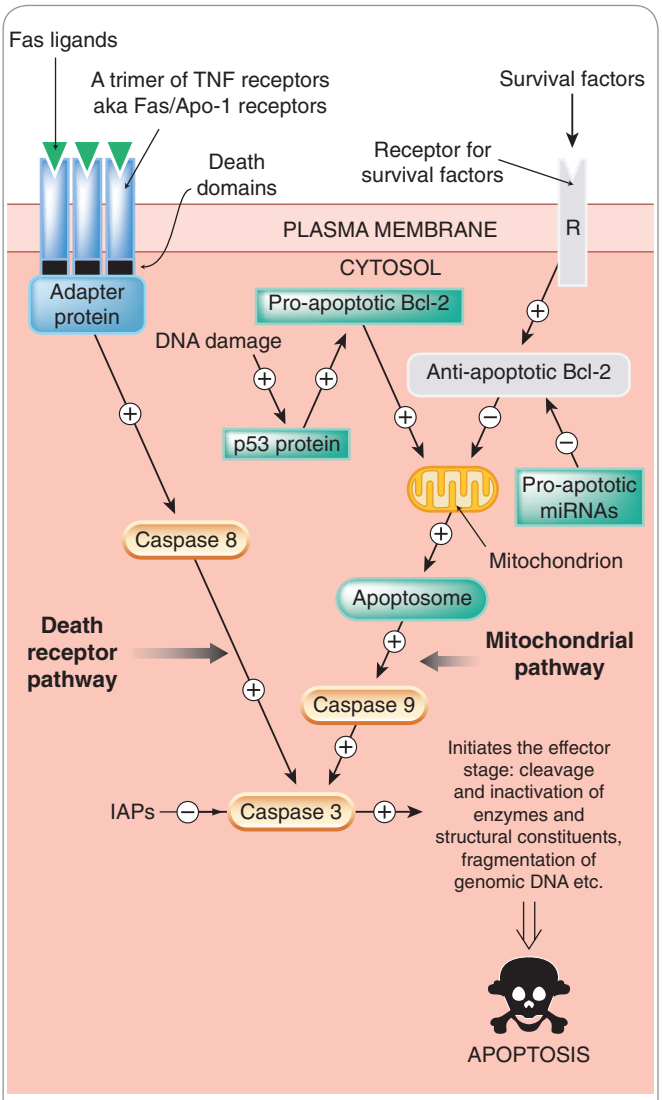

Fig. 5.5 Simplified diagram of the two main signalling pathways in apoptosis. The death receptor pathway is activated when death receptors such as members of the tumour necrosis factor (TNF) family are stimulated by specific death ligands. This recruits adapter proteins that activate initiator caspases (e.g. caspase 8), which in turn activate effector caspases such as caspase 3. The mitochondrial pathway is activated by diverse signals, one being DNA damage. In the presence of DNA damage that cannot be repaired, the p53 protein (see text and Figs 5.3 and 5.4) activates a subpathway that results in release of cytochrome c from the mitochondrion, with subsequent involvement of the apoptosome and activation of an initiator caspase, caspase 9. The apoptosome is a complex of procaspase 9, cytochrome c and apoptotic-activating protease factor-1 (Apaf-1). Both these pathways converge on the effector caspase (e.g. caspase 3), which brings about the demise of the cell. The survival factor subpathway normally holds apoptosis at bay by inhibiting the mitochondrion pathway through activation of the antiapoptotic factor Bcl-2. The receptor labelled 'R' represents the respective receptors for trophic factors, growth factors, cell-to-cell contact factors (adhesion molecules, integrins), etc. Continuous stimulation of these receptors is necessary for cell survival/proliferation. If this pathway is non-functional (as depicted here by being shown in grey), this antiapoptotic drive is withdrawn. IAP, inhibitor of apoptosis.

PATHWAYS TO APOPTOSIS

There are two main routes to cell death, one involving stimulation of death receptors by external ligands, and one arising within the cell and involving the mitochondria. Both these routes activate initiator caspases and both converge on a final common effector caspase pathway.

THE DEATH RECEPTOR PATHWAY

Lurking in the plasma membrane of most cell types are members of the tumour necrosis factor receptor (TNFR) superfamily (also known as Fas receptors), which function as death receptors (Fig. 5.5). Important family members are TNFR-1 and CD95 (also known as Fas ligands or Apo-1), but there are many others (e.g . PD-1, a death receptor that can be induced on activated T cells, as discussed above).

Each receptor has a 'death domain' in its cytoplasmic tail. Stimulation of the receptors by an external ligand such as tumour necrosis factor (TNF) itself or TRAIL[2] causes them to get together in threes (trimerise), and recruit an adapter protein that complexes with the trimer by associating with the death domains. The resulting complex activates caspase 8, an initiator caspase that in turn activates the effector caspases (Fig. 5.5).

THE MITOCHONDRIAL PATHWAY

This pathway can be called into action in two principal ways: by DNA damage and by withdrawal of the action of cell survival factors.

In the presence of DNA damage that cannot be repaired, the p53 protein activates a subpathway involving the p21 protein (see above) and proapoptotic members of the Bcl-2 protein family—Bid, Bax and Bak. In addition to these proapoptotic individuals, this family has antiapoptotic members (e.g. Bcl-2 itself, the first of these regulators to be discovered).[3] They meet at the surface of mitochondria and compete with each other. The proapoptotic branch of the family (e.g. Bax) promotes release of cytochrome c from the mitochondria; the antiapoptotic branch inhibits this. The released cytochrome c complexes with a protein termed Apaf-1 (apoptotic protease-activating factor-1), and the two then combine with procaspase 9 and activate it. This latter enzyme orchestrates the effector caspase pathway. The three-party composite of cytochrome c, Apaf-1 and procaspase 9 is termed the *apoptosome* (Fig. 5.5). See Riedl & Salvesen (2007).

Nitric oxide (see Ch. 20) is another mediator that can have proapoptotic and antiapoptotic actions.

In normal cells, survival factors (specified above) continuously activate antiapoptotic mechanisms, and the withdrawal of survival factors can cause death in several different ways depending on the cell type. But a common mechanism is a tipping of the balance between Bcl-2 family members leading to loss of the stimulation of antiapoptotic protein action, with resultant unopposed action of the proapoptotic Bcl-2 proteins (see Fig. 5.5).

The two main pathways to cell death are connected to each other, in that caspase 8 in the death receptor pathway can activate the proapoptotic Bcl-2 and thus activate the mitochondrial pathway.

MicroRNAs, the cell cycle and apoptosis

MicroRNAs (miRNAs), discovered only in the past decade, are a family of small non-coding RNAs present in the genomes of plants and animals and now known to inhibit the expression of genes coding for cell cycle regulation, apoptosis (Fig 5.5), cell differentiation and development (Carleton et al., 2007; Lynam-Lennon et al., 2009). About 3% of human genes encode for miRNA and it is proposed that up to 30% of human genes coding for proteins are regulated by miRNAs. Altered miRNA expression is now believed to be linked to a variety of diseases including diabetes, obesity, Alzheimer's, cardiovascular system diseases, inflammatory conditions, neurodegenerative diseases (Barbato et al., 2009) and various cancers (Wurdinger & Costa, 2007). Dysregulation of miRNA is believed to be involved in carcinogenesis, metastasis and resitance to cancer therapies (Garzon et al., 2009). There is in fact evidence that miRNAs are also believed to function as oncogenes and/or tumour suppressor genes and to regulate T cells (Zhou et al., 2009). Not surprisingly, miRNAs are being regarded as targets for new drug development for a variety of disease states (Liu et al., 2008; Stenvang et al., 2008; Tsai & Yu, 2010).

Apoptosis

- Apoptosis is programmed cell death, essential in embryogenesis and tissue homeostasis; it is brought about principally by a cascade of proteases—the caspases. Two sets of initiator caspases converge on a set of effector caspases.
- There are two main pathways to activation of the effector caspases: the death receptor pathway and the mitochondrial pathway.
  - The death receptor pathway involves stimulation of members of the tumour necrosis factor receptor family; and the main initiator caspase is caspase 8.
  - The mitochondrial pathway is activated by internal factors such as DNA damage, which results in transcription of gene *p53*. The p53 protein activates a subpathway that results in release from the mitochondrion of cytochrome c. This in turn complexes with protein Apaf-1, and together they activate initiator caspase 9.
- In undamaged cells, survival factors (cytokines, hormones, cell-to-cell contact factors) continuously activate antiapoptotic mechanisms. Withdrawal of survival factor stimulation causes cell death through the mitochondrial pathway.
- The effector caspases (e.g. caspase 3) start a pathway that results in cleavage of cell constituents, DNA, cytoskeletal components, enzymes, etc. This reduces the cell to a cluster of membrane-bound entities that are eventually phagocytosed by macrophages.

[2]TRAIL is **t**umour necrosis factor-**α**–**r**elated **a**poptosis-**i**nducing **l**igand, of course; what else? See Janssen et al. (2005) for discussion of a role of TRAIL. PD-L1, a ligand for the PD-1 receptor, is found on all haemopoietic cells and many other tissues.

[3]Another brake on the cell death mechanisms is a family of caspase-inhibiting proteins called IAPs (inhibitors of apoptosis proteins).

PATHOPHYSIOLOGICAL IMPLICATIONS

As mentioned above, cell proliferation and apoptosis are involved in many physiological and pathological processes. These are:

- the growth of tissues and organs in the embryo and later during childhood
- the replenishment of lost or time-expired cells such as leukocytes, gut epithelium and uterine endometrium
- immunological responses, including development of immunological tolerance to host proteins
- repair and healing after injury or inflammation
- the hyperplasia (increase in cell number and in connective tissue) associated with chronic inflammatory, hypersensitivity and autoimmune diseases (Ch. 6)
- the growth, invasion and metastasis of tumours (Ch. 55)
- regeneration of tissues.

The role of cell proliferation and apoptosis in the first two processes listed is self evident and needs no further comment, and their involvement in immune tolerance is discussed briefly above. But the other processes need further comment.

REPAIR AND HEALING

Repair occurs when there has been damage or loss of tissue; it is also implicated in the resolution of the local inflammatory reaction to a pathogen or chemical irritant. In some instances, damage or tissue loss can lead to regeneration, which is quite different to repair and is considered separately below.

In repair and healing, there is an ordered series of events involving cell migration, angiogenesis, proliferation of connective tissue cells, synthesis of extracellular matrix and finally remodelling—all coordinated by the growth factors and cytokines that are relevant for the particular tissue involved. TGF-β is a key cytokine in several of these processes.

There is considerable overlap between the inflammatory reaction and repair in terms of the cells and mechanisms activated.

HYPERPLASIA

Hyperplasia (cell proliferation and matrix expansion) are hallmarks of chronic inflammatory, hypersensitivity and autoimmune diseases such as rheumatoid arthritis (Chs. 6, 17 & 26), psoriasis, chronic ulcers, chronic obstructive lung disease, the processes underlying the bronchial hyperreactivity of chronic asthma (Ch. 27) and glomerular nephritis. The cells that take part and the events themselves are described in more detail in Chapter 6.

Cell proliferation and apoptotic events are also implicated in atherosclerosis (Ch. 23), restenosis and myocardial repair after infarction (Ch. 21).

THE GROWTH, INVASION AND METASTASIS OF TUMOURS

Perturbations in the growth factor signalling pathways, the antiapoptotic pathways and the function of the cell cycle controllers have an important role in the pathogenesis of malignancy. New understanding of this is leading to novel approaches to the treatment of cancer. See below and Chapter 55.

Repair, healing and regeneration

- Repair and healing occur when there has been damage or loss of tissue and are also implicated in the resolution of the local inflammatory reaction to a pathogen or chemical irritant. It involves the activation and proliferation of connective tissue cells, white blood cells and blood vessels.
- Regeneration is the replacement of the tissue or organ that has been damaged or lost. It involves the activation of primitive stem cells that have the potential to develop into any cell in the body. Regeneration of a tissue or organ is rare in mammals. If a mammal is injured or has its tissue removed, repair processes—often with subsequent scarring—usually make good the damage.
- It may be that repair (with rapid closure of the defect after tissue loss) is an evolutionary trade-off in mammals for the lost power of regeneration. But recent work has suggested that it might be possible to activate in mammals the original regenerative pathways—at least to some extent and in some organs.

STEM CELLS AND REGENERATION

Regeneration after damage or tissue loss implies restitution or replacement of the area so that it is identical to what was there before.

Many animals (e.g. amphibians and other lower orders) have an impressive power to regenerate their tissues, even to regrow an organ such as a limb. The essential process is the activation of *stem cells*—undifferentiated cells that have the potential to develop into any or most of the specialised cells in the body. Amphibians have a plentiful supply of these primitive cells in their organs and, furthermore, many of their specialised cells can dedifferentiate to become stem cells. These stem cells then multiply and retrace the pathways that generated the organ (e.g. a limb) during fetal life, proliferating again and again and eventually differentiating into the various cell types needed to replace the missing part.

However, during evolution, mammals have lost this ability and now have regenerative capacity in only a few tissues. Blood cells, intestinal epithelium and the outer layers of the skin are replaced continuously throughout life. Of the more discrete organs, there is a low degree of turnover and replacement of cells in such organs as liver, kidney and bone. This is in essence physiological renewal and is effected by local tissue-specific stem cells.

Almost alone, the liver has significant ability to replace itself if much of it is removed. It can regenerate to its original size in a remarkably short time, provided that at least 25% has been left intact.[4] And the mature parenchymal

[4]There is an account of liver regeneration in Greek myths. Prometheus stole the secret of fire from Zeus and gave it to mankind. To punish him, Zeus had him shackled to a crag in the Caucasus, and every day an eagle tore at his flesh and devoured much of his liver. But during the night, it regenerated and in the morning was whole again. The legend doesn't say whether the requisite 25% was left after the eagle had had its fill, and the regeneration described is unphysiologically speedy—rat liver takes 2 weeks or more to get back to the original size after 66% hepatectomy.

liver cells participate in this process as well as all the other cellular components of the liver.

Although stem cells are known to exist in most tissues in adult mammals, they are very few in number, the vast majority of cells in most tissues being irreversibly differentiated. If a mammal is injured or its tissue is removed, repair processes—often with subsequent scarring—usually make good the damage. It seems that rapid closure of the defect after tissue loss (which is much more speedily accomplished by repair mechanisms) takes priority over regeneration.

Until recently, it was assumed that this was an unalterable situation, except for a few examples, some mentioned above. But recent work has suggested that it might be possible to activate in mammals the original regenerative pathways—at least to some extent and in some organs. Regeneration of a lost limb as happens in amphibians is manifestly not possible in humans, but regeneration of limited areas of a tissue or of a small part of an organ may well be feasible. For this to happen, it would be necessary to encourage some stem cells to proliferate, develop and differentiate at the relevant sites. Or—and this is a rather more remote prospect in humans—to persuade some local specialised cells to dedifferentiate. This can occur in some mammals under special circumstances (see below). However, it may be that repair is the Janus face of regeneration, repair being an evolutionary trade-off in mammals for the lost power of regeneration.

▼ Where are the relevant stem cells that could be coaxed into regenerative service? Various possibilities are being vigorously investigated and in some cases tested clinically. These include:

- embryonic stem cells (limited availability and serious ethical issues)
- bone marrow-derived mesenchymal stem cells (Huang et al., 2009; Stapenbeck & Miyoshi, 2009)
- muscle-derived stem cells (Sinanan et al., 2006)
- human-induced pluripotent stem cells (Nishikawa et al., 2008)
- tissue-residing progenitor cells.

For a tissue such as the liver to regenerate, local tissue-specific stem cells must be stimulated by growth factors to enter the cell cycle and continue to proliferate. Other essential processes are:

- angiogenesis to supply the necessary blood vessels
- activation of MMPs and growth factors to replace the matrix in which the new cells are embedded
- interaction between matrix and integrins and fibronectin to link the new elements together.

Concomitant replacement of components of the lost connective tissue (fibroblasts, macrophages, etc.) would also be necessary.

Because most tissues do not regenerate spontaneously, mechanisms that could awaken the lost regenerative ability could be of immense value in numerous diseases. Two areas where recent progress has been reported include the regeneration of heart muscle after an infarction (Ch. 21) and replacement of insulin-secreting cells for the treatment of type I diabetes mellitus (Ch. 30).

HEART MUSCLE

Until recently it was assumed that cardiac muscle had no power to regenerate. But in a particular strain of mouse, when part of the heart is damaged by freezing, repair processes do not start up; instead, the area is replaced by regeneration within a few months. Regeneration of heart tissue also occurs in dogs after acute heart failure. Mitosis of myocytes is seen in the normal human heart, and proliferation of myocytes immediately after infarction has been reported. Indeed, the sequence of events described above has been shown to occur during the process of remodelling after myocardial infarction in rodents (Nian et al., 2004).

More recently, stem cell therapy has been shown to improve ventricular function in the failing heart (Gaetani et al., 2009) and to reduce infarct size and end systolic function in patients with myocardial infarction (Piepoli & Capucci, 2009).

INSULIN-SECRETING CELLS

The results of ongoing clinical trials in patients with type I diabetes suggest that haemopoietic stem cell transplantation can remove the need for daily insulin injections (Voltarelli et al., 2007).

THERAPEUTIC PROSPECTS

Considerable effort is being expended on finding compounds that will inhibit or modify the processes described in this chapter. So far there are few in clinical use, the main examples being those mentioned earlier, but it is likely that such agents will figure strongly in the pharmacology of the next decade, much work being aimed at developing new drugs for cancer therapy. Theoretically, all the processes could constitute targets for new drug development. Here we concentrate on those approaches that are proving or are likely to prove fruitful.

APOPTOTIC MECHANISMS

Compounds that could modify apoptosis are being intensively investigated (Melnikova & Golden, 2004; MacFarlane, 2009). Here we can only outline some of the more important approaches.

Drugs that promote apoptosis by various mechanisms were heralded as a potential new approach to cancer treatment, and are actively being studied, though none has yet been approved for clinical use. Potential proapoptotic therapeutic approaches need to be targeted precisely to the diseased tissue to avoid the obvious risks of damaging other tissues. Examples include the following:

- An antisense compound against Bcl-2 (**oblimersen**) is in phase III trial for chronic lymphocytic leukaemia.
- **Obatoclax**, a small molecule inhibitor of Bcl-2 action, is in Phase I/II trial for haematological malignancies. For details see MacFarlane (2009).
- MicroRNA technology could also be used to promote apoptosis (see Fig. 5.5).
- Two monoclonal agonist antibodies to the death receptor ligand TRAIL (**mapatumumab** and **lexatumumab**) are in Phase I/II trial against solid tumours and lymphomas (MacFarlane, 2009).
- A new drug, **bortezomib**, which inhibits the proteasome, is available for the treatment of selected cancers. It causes the build-up of Bax, an apoptotic promoter protein of the Bcl-2 family that acts by inhibiting antiapoptotic Bcl-2. Bortezomib acts partly by inhibiting NFκB action (see Ch. 17).
- An endogenous caspase inhibitor, *survivin*, occurs in high concentration in certain tumours, its gene being

one of the most cancer-specific genes in the genome. A small molecule suppressor of survivin is in clinical trial (Giaccone & Rajan, 2009), the object being to free caspases to induce cancer cell suicide.

Despite the appeal of inhibiting apoptosis as a means of preventing or treating a wide range of common degenerative disorders, success in developing inhibitors for clinical use has so far proved elusive, and a number of such compounds have been found to lack efficacy in clinical trials:

- The use of a blocking antibody to the PD-1 death receptor is a potentially fruitful new avenue to explore for the treatment of HIV, hepatitis B and hepatitis C infections, as well as other chronic infections and some cancers that express the ligand for PD-1 (Williams & Bevan, 2006).
- Several caspase inhibitors are under investigation for use in the treatment of myocardial infarction, stroke, liver disease, organ transplantation and sepsis. **Emricasan** (IDN-6556) is undergoing trials in patients needing liver transplants.

ANGIOGENESIS AND METALLOPROTEINASES

Metalloproteinases and angiogenesis have critical roles in physiological (e.g. growth, repair) and pathological processes (e.g. tumour growth, chronic inflammatory conditions). The search for clinically useful MMP inhibitors is continuing, but has not so far been successful. At present, only one new drug has been approved for use in cancer treatment: the antiangiogenesis compound **bevacizumab**, a monoclonal antibody that acts against VEGF (see above) which is also used to treat age-related macular degeneration, a disease of the retina associated with excessive proliferation of retinal blood vessels.

CELL CYCLE REGULATION

The main endogenous positive regulators of the cell cycle are the cdks. Several small molecules that inhibit cdks by targeting the ATP-binding sites of these kinases have been developed; an example is **flavopiridol**, currently in clinical trials, which inhibits all the cdks, causing arrest of the cell cycle; it also promotes apoptosis, has antiangiogenic ability and can induce differentiation (Dickson & Schwartz, 2009).

Some compounds affect upstream pathways for cdk activation and may find uses in cancer treatment. Examples are **perifosine** (currently in development for cancer treatment) and **lovastatin** (a cholesterol-lowering drug, see Ch. 23, which may also have anticancer properties).

Bortezomib, a boronate compound, covalently binds the proteasome, inhibiting the degradation of proapoptotic proteins. It is used in treating multiple myeloma (see Ch. 55).

Of the various components of the growth factor signalling pathway, receptor tyrosine kinases, the Ras protein and cytoplasmic kinases have been the subjects of most interest. Kinase inhibitors recently introduced for cancer treatment include **imatinib**, **gefitinib** and **erlotinib** (see Ch. 55).

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