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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. However, nothing resembling scientific principles was applied to therapeutics, which was known at that time as *materia medica*.[2] 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.[3] 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 that 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

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[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. Furthermore, some biological products (e.g. **epoietin**) show batch-to-batch variation in their chemical constitution that significantly affects their properties.

[2] The name persists today in some ancient universities, being attached to chairs of what we would call clinical pharmacology.
[3] Oliver Wendell Holmes, an eminent physician, wrote in 1860: '[I] 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).

plant 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).[4]

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.[5] 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, psychological treatments 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 implausible 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 that 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 chemical or physical interventions. They focus instead mainly on subjective malaise, which may be disease-associated or

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[4]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. 41). 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.

[5]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. 12); George Hitchings and Gertrude Elion, who described the antimetabolite principle and produced the first effective anticancer drugs (Ch. 56); 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.
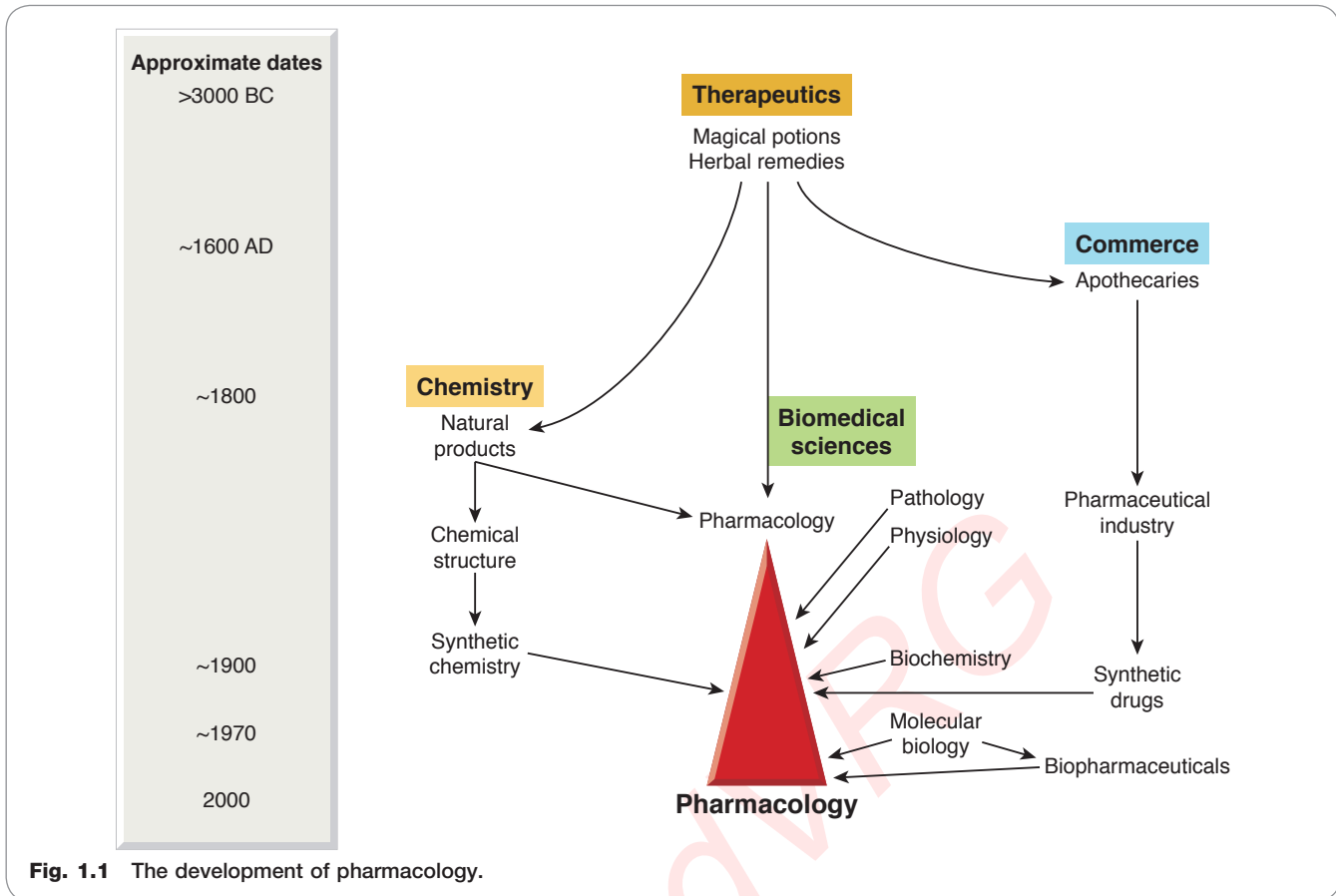
Fig. 1.1 The development of pharmacology.

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. Demand for 'alternative' therapies by the general public, alas, has little to do with demonstrable efficacy.[6]

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 intro-
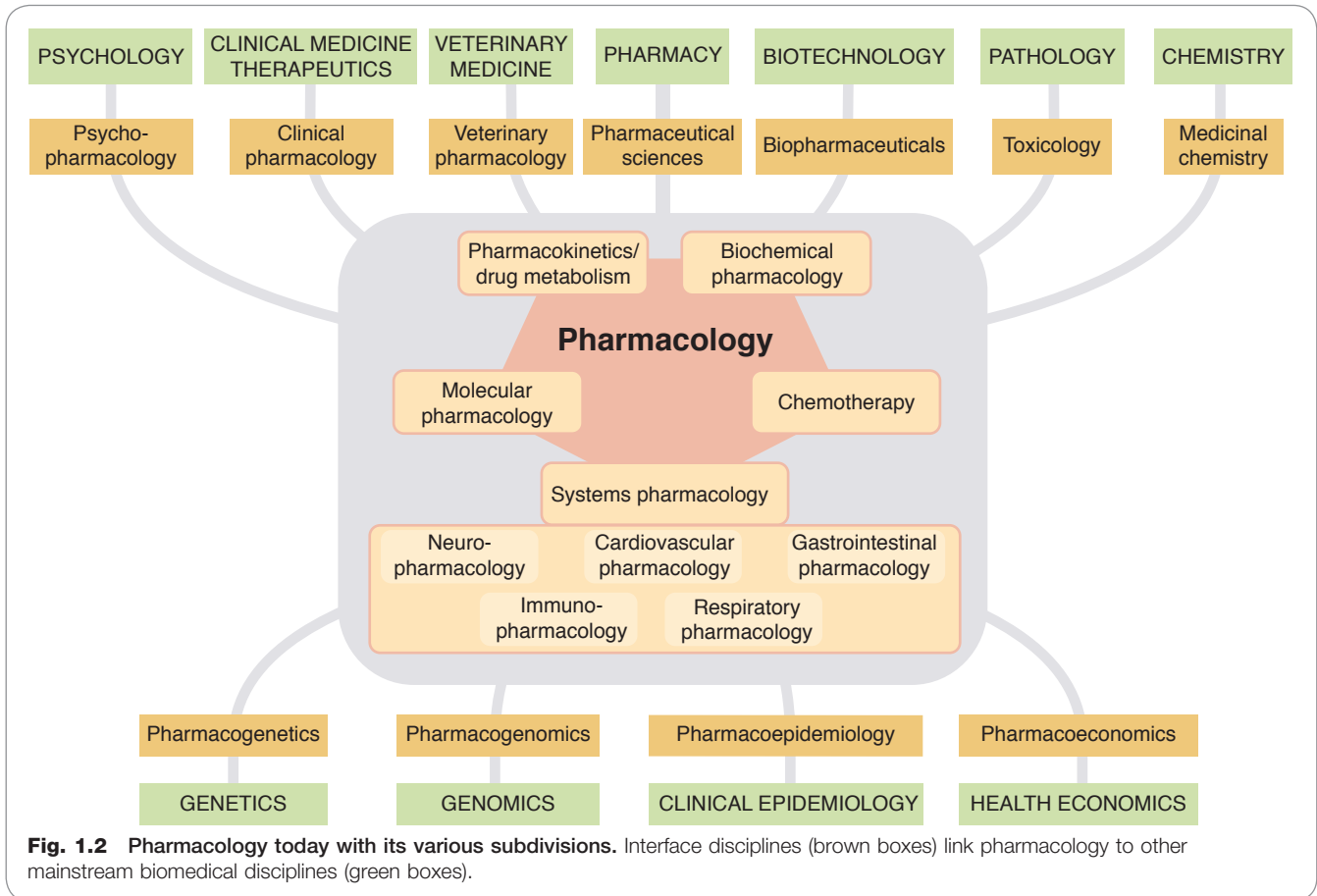
duced into the body, are very different from those of drug-based therapeutics and will require a different conceptual framework, which texts such as this will increasingly need to embrace if they are to stay abreast of modern medical treatment.

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

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[6]The UK Medicines and Healthcare Regulatory Agency (MHRA) requires detailed evidence of therapeutic efficacy based on controlled clinical trials before a new drug is registered, but no clinical trials data for homeopathic products or for the many herbal medicines that were on sale before the Medicines Act of 1968.

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

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, discussed in Chapter 11. 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. 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, potentially with far-reaching consequences for therapeutics (see Ch. 11).

Pharmacoepidemiology. This is the study of drug effects at the population level (see Strom, 2005). 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 detracts from the utility of a drug, even though its overall 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 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) and Rascati (2009).

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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 cell constituents 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. 41), 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 51 and 56), as well as mutagenic and carcinogenic agents (Ch. 57), interact directly with DNA rather than protein; bisphosphonates, used to treat osteoporosis (Ch. 36), bind to calcium salts in the bone matrix, rendering it toxic to osteoclasts, much like rat poison. There are also exceptions among the new generation of *biopharmaceutical drugs* that include nucleic acids, proteins and antibodies (see Ch. 59).

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.

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 – hormones, neurotransmitters, inflammatory mediators, etc.

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[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.

– 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 through which a response is transduced.

'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. 43), or the enzyme dihydrofolate reductase as the 'receptor' for **methotrexate** (Ch. 50). 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 be unable to function.

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 pharmacological receptors; we cannot usefully speak of 'agonists' for the other classes of drug target 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. 47) act by blocking monoamine transporters but are notorious for producing side effects (e.g. dry mouth) related to their ability to block various other 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. 42), cannabinoids (Ch. 19) and benzodiazepine tranquillisers (Ch. 44),

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 and protein crystallography 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. 30).[2] 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 Basic and Clinical Pharmacology (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 database of known receptor classes is available (see www.guidetopharmacology.org/), as well as a regularly updated summary (Alexander et al., 2013).

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 alter the receptor's behaviour towards the cell and elicit a tissue response. The molecular mechanisms associated with receptor activation are
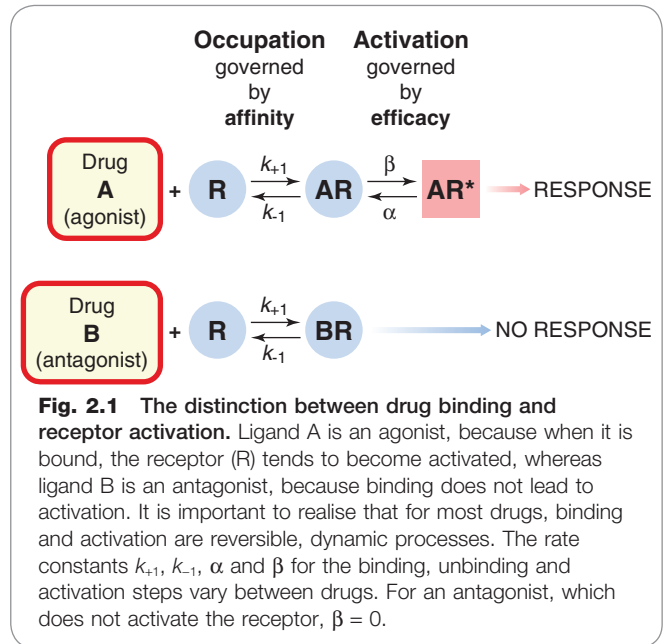

Fig. 2.1 The distinction between drug binding and receptor activation. Ligand A is an agonist, because when it is bound, the receptor (R) tends to become activated, whereas ligand B is an antagonist, because binding does not lead to activation. It is important to realise that for most drugs, binding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

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. 12 and 18). 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.

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. 18-20).

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 ^3H, ^{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 (i.e. when the rate of association [binding] and dissociation [unbinding] of the radioactive drug are equal). The bound radioactivity is measured after removal of the supernatant. In such experiments, the radiolabelled drug will exhibit both specific binding (i.e. binding to receptors, which is saturable as there are a

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[2]For this work, and the development of β-adrenoceptor antagonists using a similar experimental approach, Sir James Black was awarded the 1984 Nobel Prize in Physiology or Medicine.
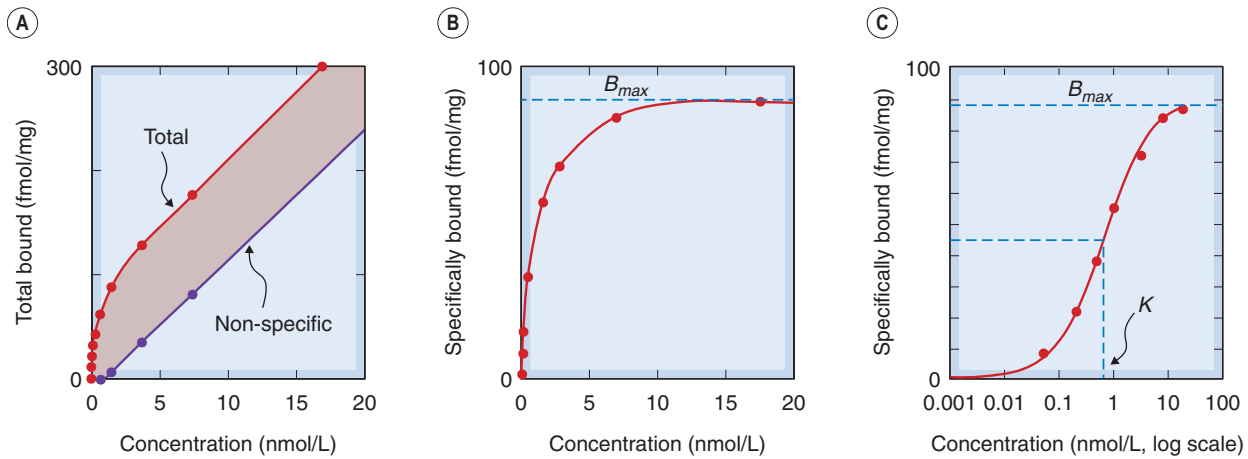
Fig. 2.2 **Measurement of receptor binding (β adrenoceptors in cardiac cell membranes).** The ligand was [^{3}H]-cyanopindolol, a derivative of pindolol (see Ch. 14). **[A]** Measurements of total and non-specific binding at equilibrium. Non-specific binding is measured in the presence of a saturating 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 as in **[B]** plotted against the concentration on a log scale. The sigmoid curve is a *logistic curve* representing the logarithmic scaling of the rectangular hyperbola plotted in panel **[B]** from which the binding parameters K and B_{max} can be determined.

finite number of receptors in the tissue) and a certain amount of 'non-specific binding' (i.e. drug taken up by structures other than receptors, which, at the concentrations used in such studies, is normally non-saturable), 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, C) 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. 2.13), 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 tissue. When combined with functional studies, binding measurements have proved very valuable. It has, for example, been confirmed that the *spare receptor hypothesis* (p. 10) 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. 17).

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. 46).

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 aficionados 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. This allows 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}). A logarithmic concentration or dose scale is often used. This transforms the curve from a rectangular hyperbola to a sigmoidal curve in which the mid portion is essentially linear (the importance of the slope of the linear portion will become apparent later in this chapter when we consider antagonism and partial agonists). The E_{max}, EC_{50} and slope parameters are useful for comparing different drugs that produce qualitatively similar effects (see Fig. 2.7 and 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 response produced is not, as a rule, directly proportional to receptor occupancy. This often arises because the maximum response of a tissue may be produced by agonists when they are bound to less than 100% of the receptors. Under these circumstances the tissue is said to possess spare receptors (see p. 10).

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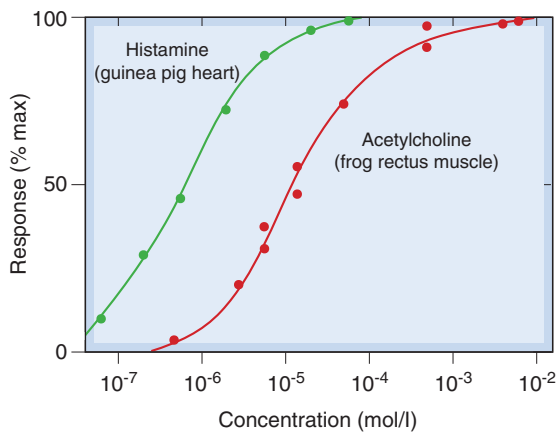
Fig. 2.3 Experimentally observed concentration–effect curves. Although the lines, drawn according to the binding equation 2.5, fit the points well, such curves do not give correct estimates of the affinity of drugs for receptors. This is because the relationship between receptor occupancy and response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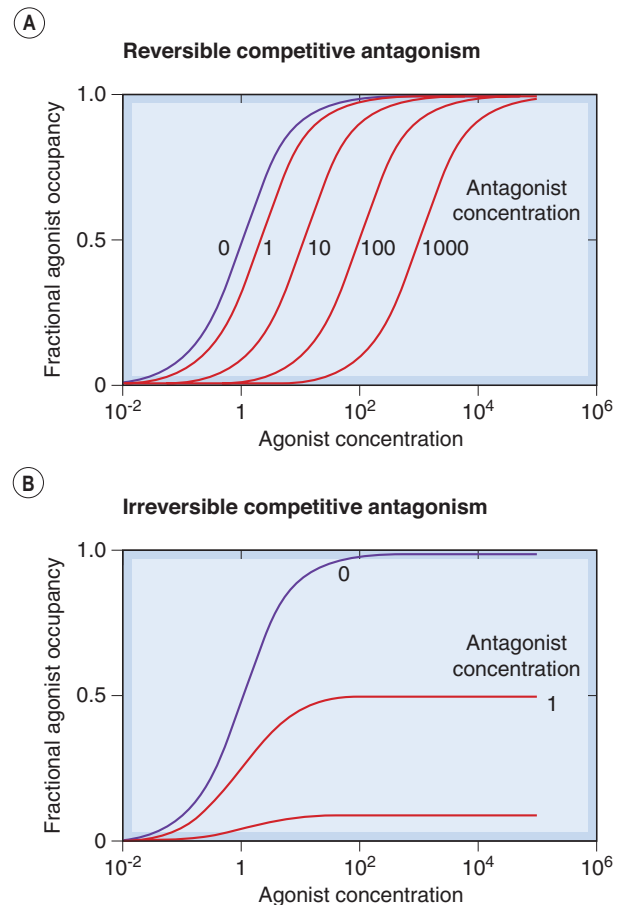

Fig. 2.4 Hypothetical agonist concentration–occupancy curves in the presence of reversible [A] and irreversible [B] competitive antagonists. The concentrations are normalised with respect to the equilibrium constants, K (i.e. 1.0 corresponds to a concentration equal to K and results in 50% occupancy).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

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). The problem is reduced but not entirely eradicated by the use of recombinant receptors expressed in cells in culture. 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.

SPARE RECEPTORS

▼ Stephenson (1956), studying the actions of acetylcholine analogues in isolated tissues, found that many full agonists were capable of 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. 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. But in fact it is highly efficient in 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.

COMPETITIVE ANTAGONISM

Though one drug can inhibit the response to another in several ways (see p. 15), 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 (i.e. proportion of receptors to which the agonist is bound) 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. 19) predicts that in the presence of a fixed concentration of the antagonist, the log concentration–effect curve for the agonist will be shifted to 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*,
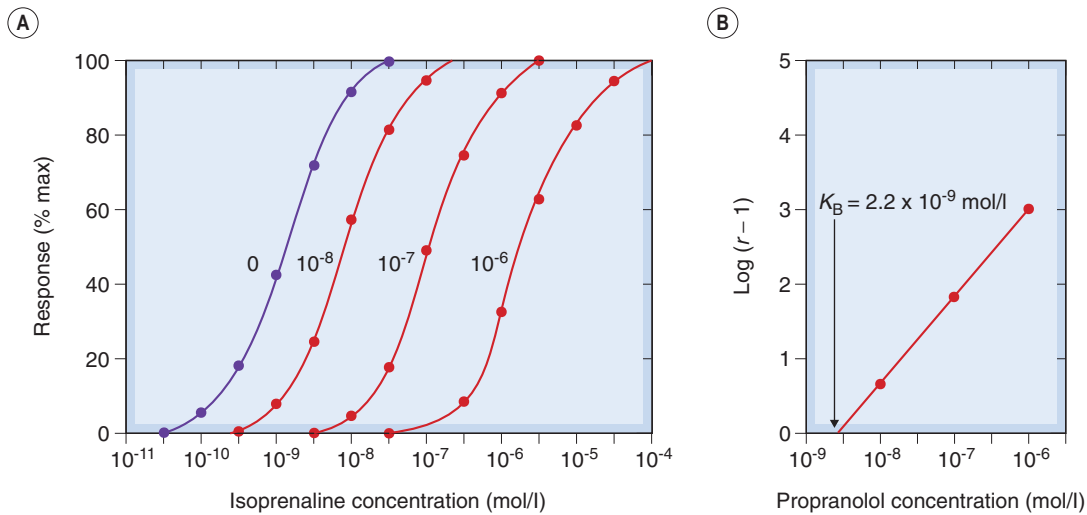
Fig. 2.5 **Competitive antagonism of isoprenaline by propranolol measured on isolated guinea pig atria.** [**A**] Concentration–effect curves at various propranolol concentrations (indicated on the curves). Note the progressive shift to the right without a change of slope or maximum. [**B**] Schild plot (equation 2.10). The equilibrium dissociation constant (K_B) for propranolol is given by the abscissal intercept, 2.2 × 10^{-9} mol/l. *(Results from Potter LT 1967 Uptake of propranolol by isolated guinea-pig atria. J Pharmacol Exp Ther 55, 91–100.)*

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. 19). These predictions are often borne out in practice (see Fig. 2.5A), providing a relatively simple method for determining the dissociation constant of the antagonist (K_B; Fig. 2.5B). 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 (as detailed 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 (i.e. antagonism can be overcome by increasing the concentration of the agonist)
- linear relationship between agonist dose ratio and antagonist concentration
- evidence of competition from binding studies.

Competitive antagonism is the most direct mechanism by which one drug can reduce the effect of another (or of an endogenous mediator).

▼ The characteristics of *reversible competitive antagonism* described above reflect the fact that agonist and competitive antagonist molecules do not stay bound to the receptor but bind and rebind continuously. 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 effectively 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.

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
- Antagonist affinity, measured in this way, is widely used as a basis for receptor classification.

IRREVERSIBLE COMPETITIVE ANTAGONISM

▼ *Irreversible competitive* (or *non-equilibrium*) *antagonism* occurs when the antagonist binds to the same site on the receptor as the agonist but 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.[3]

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

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 p. 17) is not always so clear. This is because of the phenomenon of spare receptors (see p. 10); 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).

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[3]This type of antagonism is sometimes called non-competitive, but that term is ambiguous and best avoided in this context.
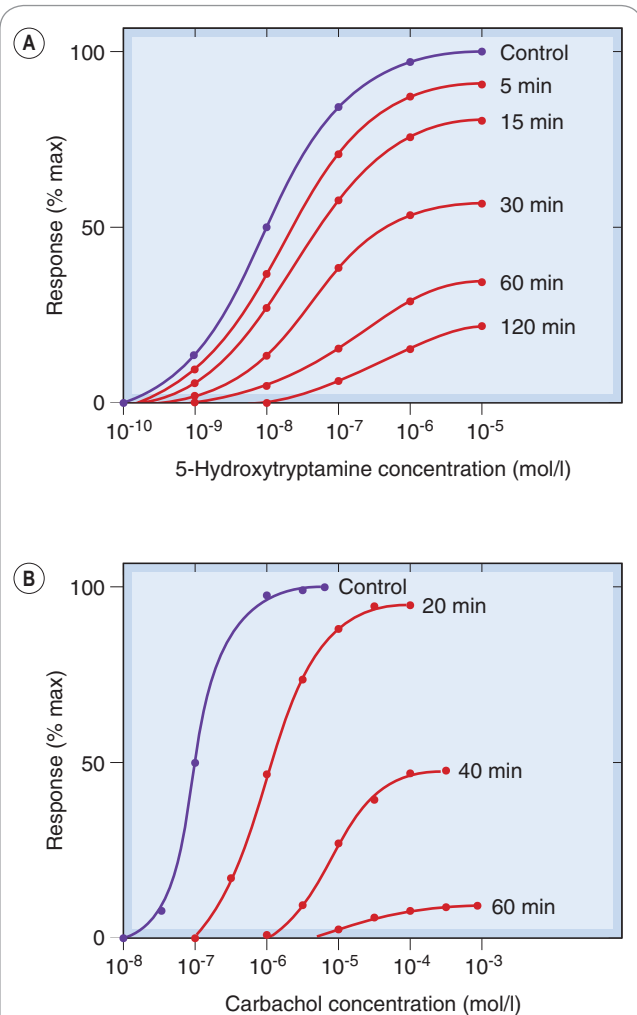
Fig. 2.6 Effects of irreversible competitive antagonists on agonist concentration–effect curves. [**A**] Rat stomach smooth muscle responding to 5-hydroxytryptamine at various times after addition of methysergide (10^{-9} mol/l). [**B**] Rabbit stomach responding to carbachol at various times after addition of dibenamine (10^{-5} mol/l). *(Panel [A] after Frankhuijsen AL, Bonta IL 1974 Eur J Pharmacol 26, 220; panel [B] after Furchgott RF 1965 Adv Drug Res 3, 21.)*

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. 30) and monoamine oxidase inhibitors (Ch. 47).

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 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 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 sub-maximal 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 response 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. 18) 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**, pro-duces a barely detectable response even at 100% occu-pancy, 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 full agonist is a drug whose efficacy[4] is sufficient that it pro-duces a maximal response when less than 100% of recep-tors are occupied. A partial agonist has lower efficacy, such that 100% occupancy elicits only a submaximal response.

▼ 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 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.

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[4]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.
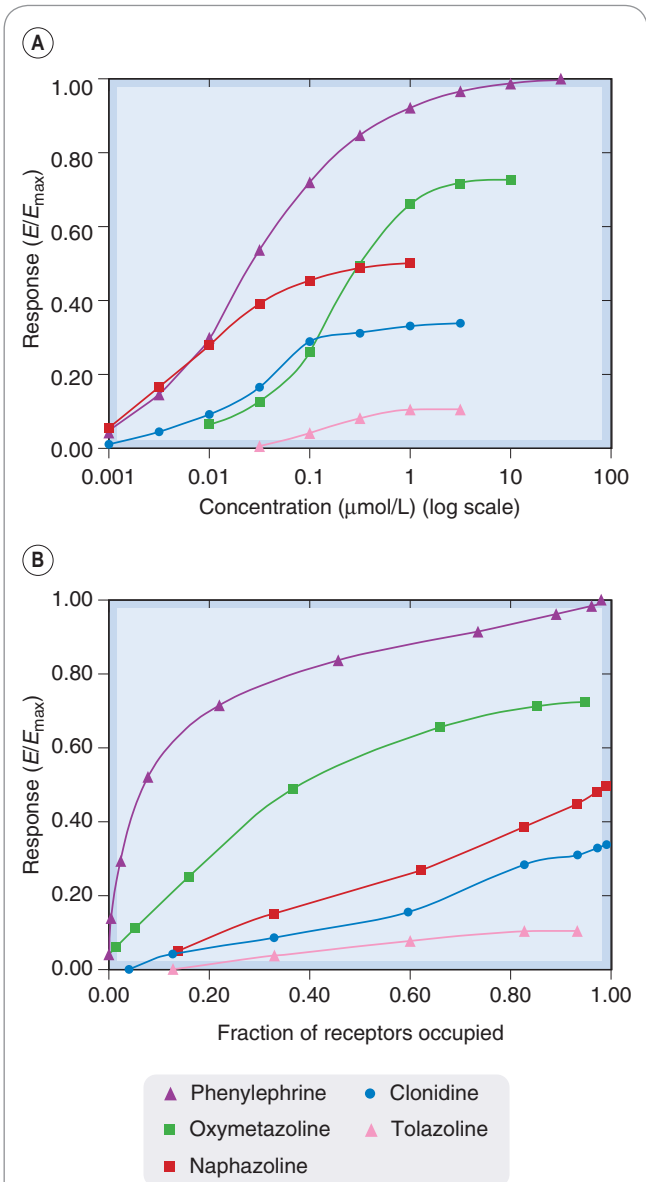
Fig. 2.7 Partial agonists. [**A**] Log concentration–effect curves for a series of α-adrenoceptor agonists causing contraction of an isolated strip of rabbit aorta. Phenylephrine is a full agonist. The others are partial agonists with different efficacies. The lower the efficacy of the drug the lower the maximum response and slope of the log concentration-response curve. [**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 RR Jr et al. 1979 J Pharmacol Exp Ther 209, 429–436.)*

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

(*constitutive activation*) may exist even when no ligand is present. These include receptors for benzodiazepines (see Ch. 44), cannabinoids (Ch. 19), 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 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), and which 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 10000 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). Neutral antagonists, by binding to the agonist binding site, will antagonise both agonists and inverse agonists. Inverse agonism was first observed at the benzodiazepine receptor (Ch. 44) but such drugs are proconvulsive and thus not therapeutically useful! 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, most 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
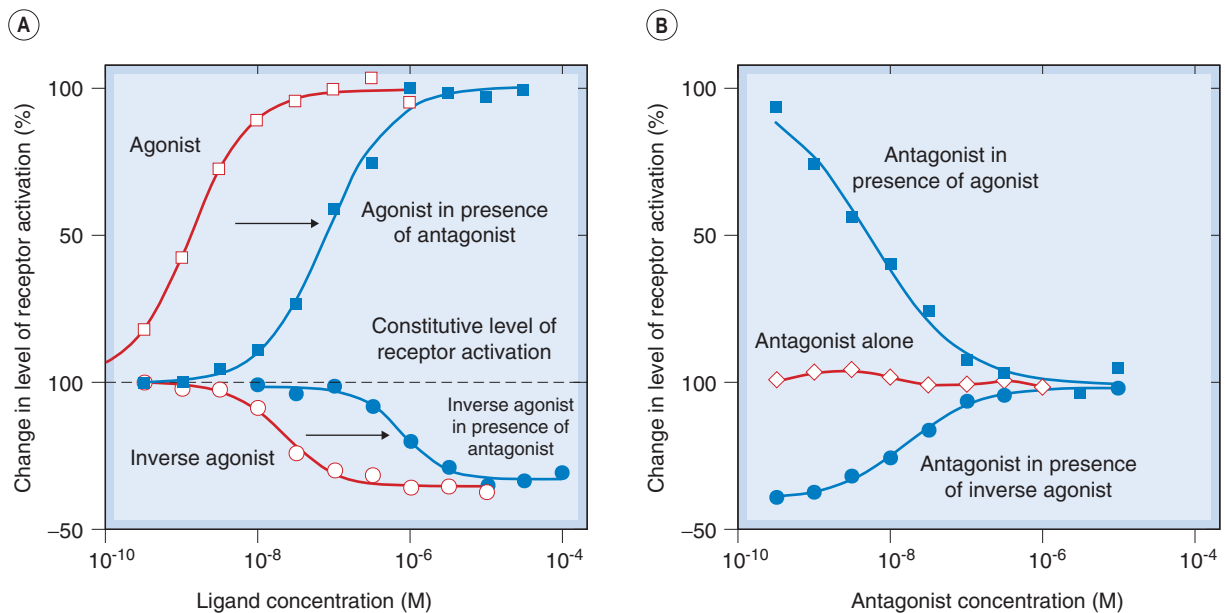
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Fig. 2.8   **Inverse agonism.** The interaction of a competitive antagonist with normal and inverse agonists in a system that shows receptor activation in the absence of any added ligands (constitutive activation). [**A**] The degree of receptor activation (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.)
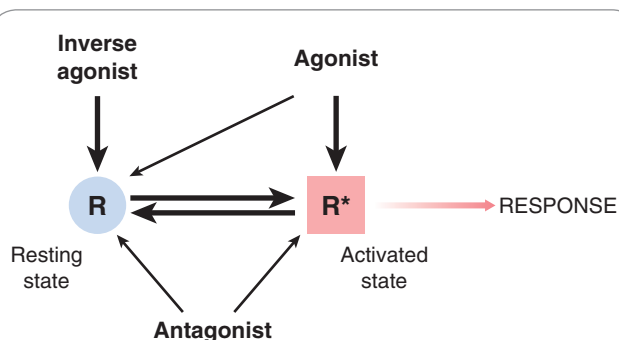

Fig. 2.9   **The two-state model.** The receptor is shown in two conformational states, 'resting' (R) and 'activated' (R*), which exist in equilibrium. Normally, when no ligand is present, the equilibrium lies far to the left, and few receptors are found in the R* state. For constitutively active receptors, 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.

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.

BIASED AGONISM

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).

Receptors that couple to second messenger systems (see Ch. 3) can couple to more than one intracellular effector pathway, giving rise to two or more simultaneous responses. One might expect that all agonists that activate the same receptor type would evoke the same array of responses (Fig. 2.10A). However it has become apparent that different agonists can exhibit *bias* for the generation of one response over another even although they are acting through the same receptor (Fig. 2.10B) probably because they stabilise different conformational states of the receptor. Agonist bias has become an important concept in pharmacology recently and might in future have important therapeutic implications (see Kelly, 2013).

Redefining and attempting to measure agonist efficacy for such a multistate model is problematic, however, and
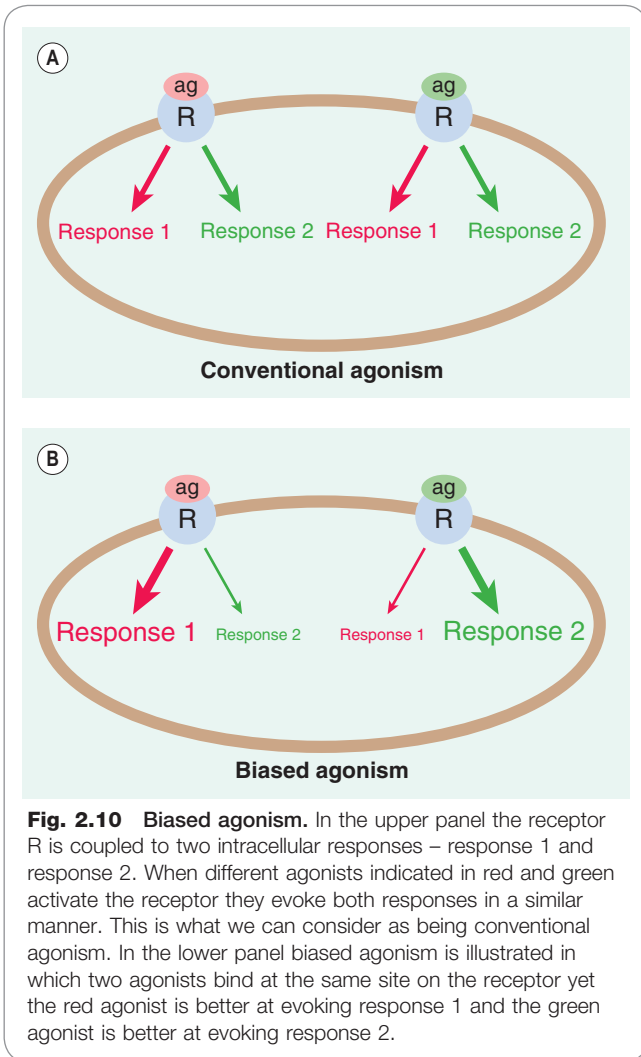
Fig. 2.10 Biased agonism. In the upper panel the receptor R is coupled to two intracellular responses – response 1 and response 2. When different agonists indicated in red and green activate the receptor they evoke both responses in a similar manner. This is what we can consider as being conventional agonism. In the lower panel biased agonism is illustrated in which two agonists bind at the same site on the receptor yet the red agonist is better at evoking response 1 and the green agonist is better at evoking response 2.

requires a more complicated state transition model than the two-state model described above. The errors, pitfalls and a possible way forward have recently been outlined by Kenakin and Christopoulos (2013).

ALLOSTERIC MODULATION

▼ In addition to the agonist binding site (now referred to as the *orthosteric* binding site), to which competitive antagonists also bind, receptor proteins possess many other (*allosteric*) binding sites (see Ch. 3) through which drugs can influence receptor function in various ways, by increasing or decreasing the affinity of agonists for the agonist binding site, by modifying efficacy or by producing a response themselves (Fig. 2.11). 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 (Fig. 2.11). 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 glycine at NMDA receptors (Ch. 38), benzodiazepines at GABA_A receptors (Ch. 38), **cinacalcet** at the Ca²⁺ receptor (Ch. 36) and **sulfonylurea** drugs at K_ATP channels (Ch. 31). One reason why allosteric modulation may be important to the pharmacologist and future drug development is that across families of receptors such as the muscarinic receptors (see Ch. 13) the orthosteric binding sites are very similar and it has

proven difficult to develop selective agonists and antagonists for individual subtypes. The hope is that there will be greater variation in the allosteric sites and that receptor-selective allosteric ligands can be developed. Furthermore, positive allosteric modulators will exert their effects only on receptors that are being activated by endogenous ligands and have no effect on those that are not activated. This might provide a degree of selectivity (e.g. in potentiating spinal inhibition mediated by endogenous opioids, see Ch. 42) and a reduction in side effect profile.

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*.
- *Allosteric modulators* bind to sites on the receptor other than the agonist binding site and can modify agonist activity.

OTHER FORMS OF DRUG ANTAGONISM

Other mechanisms can also account for inhibitory interactions between drugs.
The most important ones are:

- chemical antagonism
- pharmacokinetic antagonism
- block of receptor–response 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. 18).

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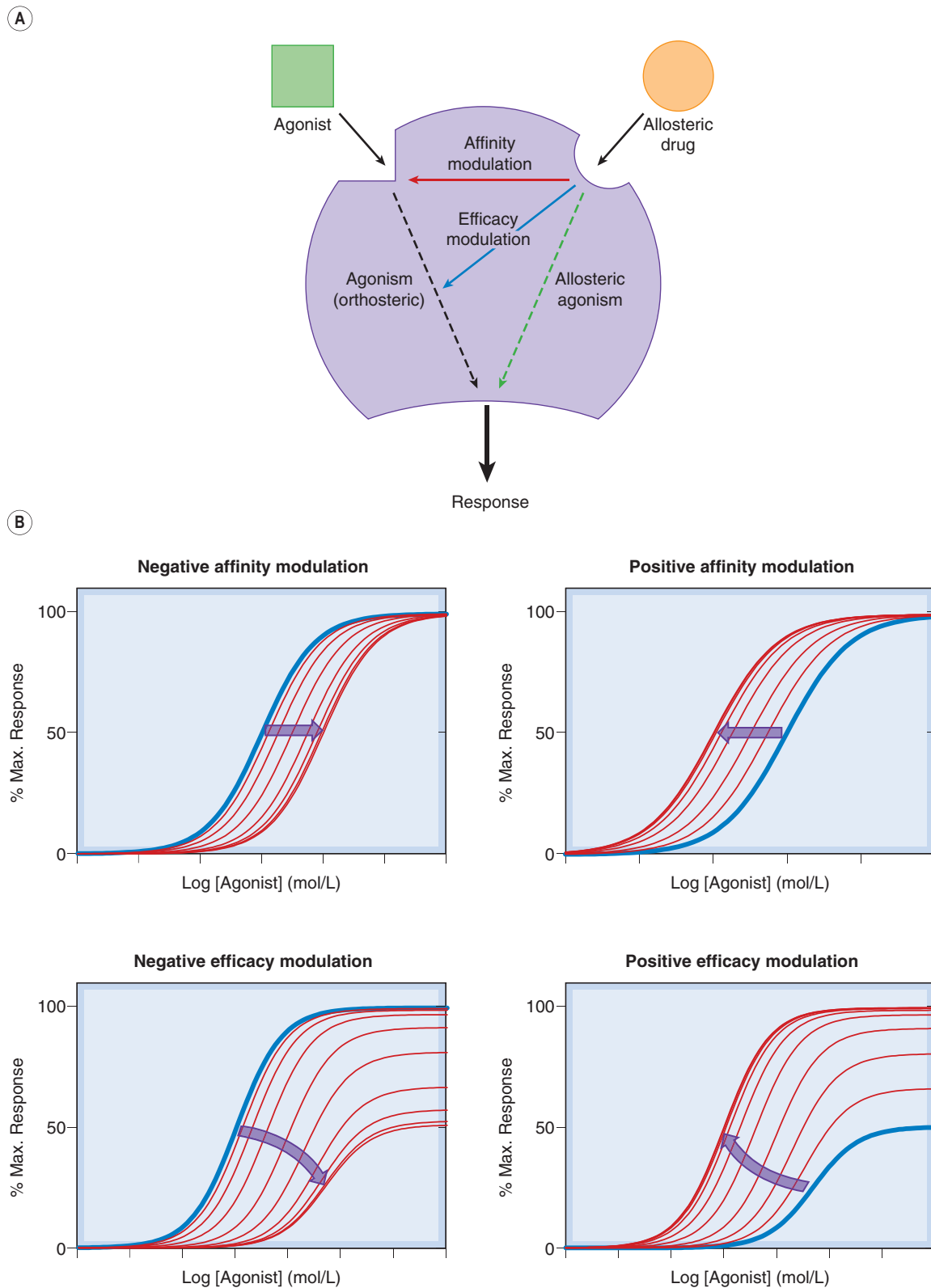
Fig. 2.11 Allosteric modulation. [**A**] Allosteric drugs bind at a separate site on the receptor to 'traditional' agonists (now often referred to as 'orthosteric' agonists). They can modify the activity of the receptor by (i) altering agonist affinity, (ii) altering agonist efficacy or (iii) directly evoking a response themselves. [**B**] Effects of affinity- and efficacy-modifying allosteric modulators on the concentration–effect curve of an agonist (blue line). In the presence of the allosteric modulator the agonist concentration–effect curve (now illustrated in red) is shifted in a manner determined by the type of allosteric modulator until a maximum effect of the modulator is reached. *(Panel [A] adapted with permission from Conn et al., 2009 Nature Rev Drug Discov 8, 41–54; panel [B] courtesy of A Christopoulos.)*

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 **phenytoin**, is given; see Chs 9 and 57). 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 57, are common and can be important in clinical practice.

BLOCK OF RECEPTOR–RESPONSE LINKAGE

Non-competitive antagonism describes the situation where the antagonist blocks at some point downstream from the agonist binding site on the receptor, and interrupts the chain of events that leads to the production of a response by the agonist. For example, **ketamine** enters the ion channel pore of the NMDA receptor (see Ch. 38) blocking it and thus preventing ion flux through the channels. Drugs such as **verapamil** and **nifedipine** prevent the influx of Ca^{2+} through the cell membrane (see Ch. 22) and thus block non-selectively the contraction of smooth muscle produced by drugs acting at any receptor that couples to these calcium channels. 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.

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.

> ### 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–response linkage
> - physiological antagonism (two agents producing opposing physiological effects).

DESENSITISATION AND TOLERANCE

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 hours, 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 50 and 56). Many different mechanisms can give rise to these phenomena. 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. 56).

CHANGE IN RECEPTORS

Among receptors directly coupled to ion channels (see Ch. 3), desensitisation is often rapid and pronounced. At the neuromuscular junction (Fig. 2.12A), the desensitised state is caused by a conformational change in the receptor, resulting in tight binding of the agonist molecule without 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. 2.12B). 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 considered further in Chapter 3. This type of desensitisation usually takes seconds to minutes to develop, and recovers 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 2.12B and is a slower process than the uncoupling described above. 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 normally depends on receptor phosphorylation and the subsequent binding of *arrestin* proteins to the phosphorylated receptor (see Ch. 3, Fig. 3.16). 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.

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

Fig. 2.12 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 adenylyl 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.
*(Panel [A] from Katz B, Thesleff S 1957 J Physiol 138, 63; panel
[B] from Perkins JP 1981 Trends Pharmacol Sci 2, 326.)*

terminals (see Chs 14 and 48), show marked tachyphylaxis
because the amine stores become depleted.

ALTERED DRUG METABOLISM

Tolerance to some drugs, for example **barbiturates** (Ch.
44) and **ethanol** (Ch. 49), occurs partly because repeated
administration of the same dose produces a progressively
lower plasma concentration, because of increased meta-
bolic 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 toler-
ance 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 nul-
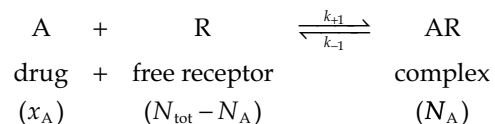lified by a homeostatic response. For example, the blood

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 experi-
ence that many side effects of drugs, such as nausea or
sleepiness, tend to subside even though drug administra-
tion is continued. We may assume that some kind of
physiological adaptation is occurring, presumably associ-
ated 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 (see
Colquhoun, 2006).

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 equilib-
rium, 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 reac-
tion can be represented by:

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

The Law of Mass Action (which states that the rate of a chemical
reaction is proportional to the product of the concentrations of reac-
tants) 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 *affinity constant* of binding is given by k_{+1}/k_{-1} and from equation
2.3 equals $N_A/x_A(N_{tot} - N_A)$. Unfortunately this has units of reciprocal
concentration (l/mol) which for some of us is a little hard to get our
heads around. Pharmacologists therefore tend to use the reciprocal
of the affinity constant, the *equilibrium dissociation constant* (K_A),
which has units of concentration (mol/l).

$$K_A = k_{-1}/k_{+1} = x_A (N_{tot} - N_A)/N_A \quad (2.4)$$

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

$$p_A = \frac{x_A}{x_A + k_{-1}/k_{+1}} = \frac{x_A}{x_A + K_A} \quad (2.5)$$
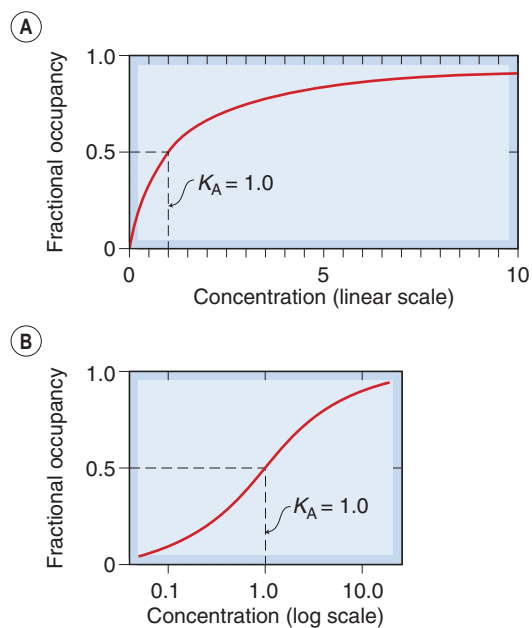
Fig. 2.13 Theoretical relationship between occupancy and ligand concentration. The relationship is plotted according to equation 2.5. [**A**] Plotted with a linear concentration scale, this curve is a rectangular hyperbola. [**B**] Plotted with a log concentration scale, it is a symmetrical sigmoid curve.

Thus if the equilibrium dissociation constant of a drug is known we can calculate the proportion of receptors it will occupy at any concentration.

Equation 2.5 can be written:

$$p_A = \frac{x_A / K_A}{x_A / K_A + 1} \tag{2.6}$$

This important result is known as the Hill–Langmuir equation.[5]

The *equilibrium dissociation constant*, 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 2.6 describes the relationship between occupancy and drug concentration, and it generates a characteristic curve known as a *rectangular hyperbola*, as shown in Figure 2.13A. It is common in pharmacological work to use a logarithmic scale of concentration; this converts the hyperbola to a symmetrical sigmoid curve (Fig. 2.13B).

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:

$$B = B_{max} x_A / (x_A + K_A) \tag{2.7}$$

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:

$$B / x_A = B_{max} / (K_A - B / K_A) \tag{2.8}$$

---

[5]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.

A plot of B / x_A against B (known as a *Scatchard plot*) 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 dissociation 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:

$$p_A = \frac{x_A / K_A}{x_A / K + x_B / K_B + 1} \tag{2.9}$$

Comparing this result with equation 2.5 shows that adding drug B, as expected, reduces the occupancy by drug A. Figure 2.4A (p. 10) 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 equation 2.9 shows that

$$r_A = (x_B / K_B) + 1 \tag{2.10}$$

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

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 (2.10) is known as the *Schild equation*, after the pharmacologist who first used it to analyse drug antagonism.

Equation 2.10 can be expressed logarithmically in the form:

$$\log(r_A - 1) = \log x_B - \log K_B \tag{2.11}$$

Thus a plot of log (r_A–1) against log x_B, usually called a Schild plot (as in Fig. 2.5 above), should give a straight line with unit slope (i.e. its gradient is equal to 1) 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 dissociation 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 dissociation constant of 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 an antagonist against all agonists that act on the same population of receptors.

These predictions have been verified for many examples of competitive antagonism (see 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 (as they shift the equilibrium between R and R*), 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. 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 the later review (Kenakin & Christopoulos, 2011) presents a detailed account of the value of quantification in the study of drug action.

Fig. 2.14 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.

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.

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.

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. 2.14). For example, activation of cardiac β adrenoceptors (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. Opioids (see Ch. 42) produce an immediate analgesic effect but, after a time, tolerance and dependence ensue, and in some
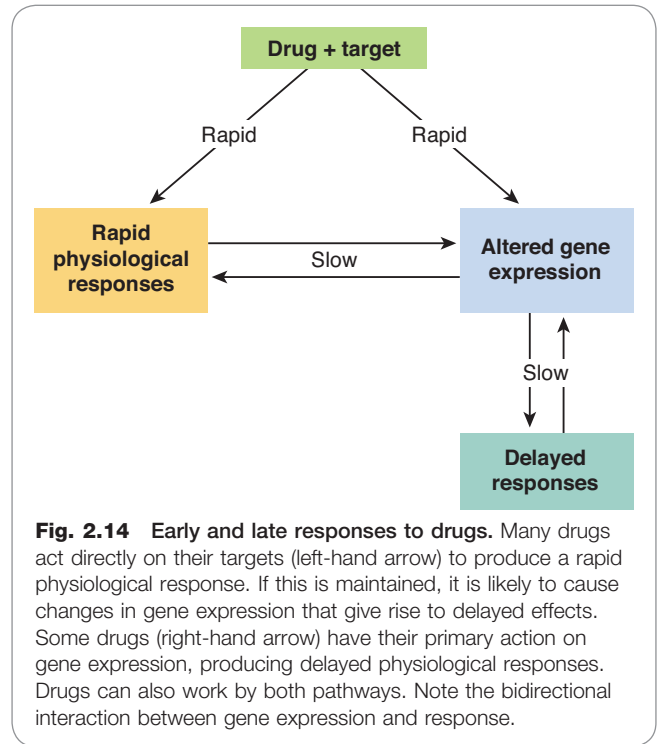
Drug effects

- 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. 28) or delayed responses (e.g. antidepressants; Ch. 47).

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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
- transporters (carrier molecules).

The great majority of important drugs act on one or other of these types of protein, but there are exceptions. For example, **colchicine** used to treat arthritic gout attacks (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 50–56), 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 of this book. Many therapeutically useful drugs act, either as agonists or antagonists, on receptors for known endogenous mediators. 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 and opioid receptors (see Chs 19 and 42), the endogenous mediators were identified later; in others, known as *orphan receptors* (see below) the mediator, if it exists, still remains unknown.
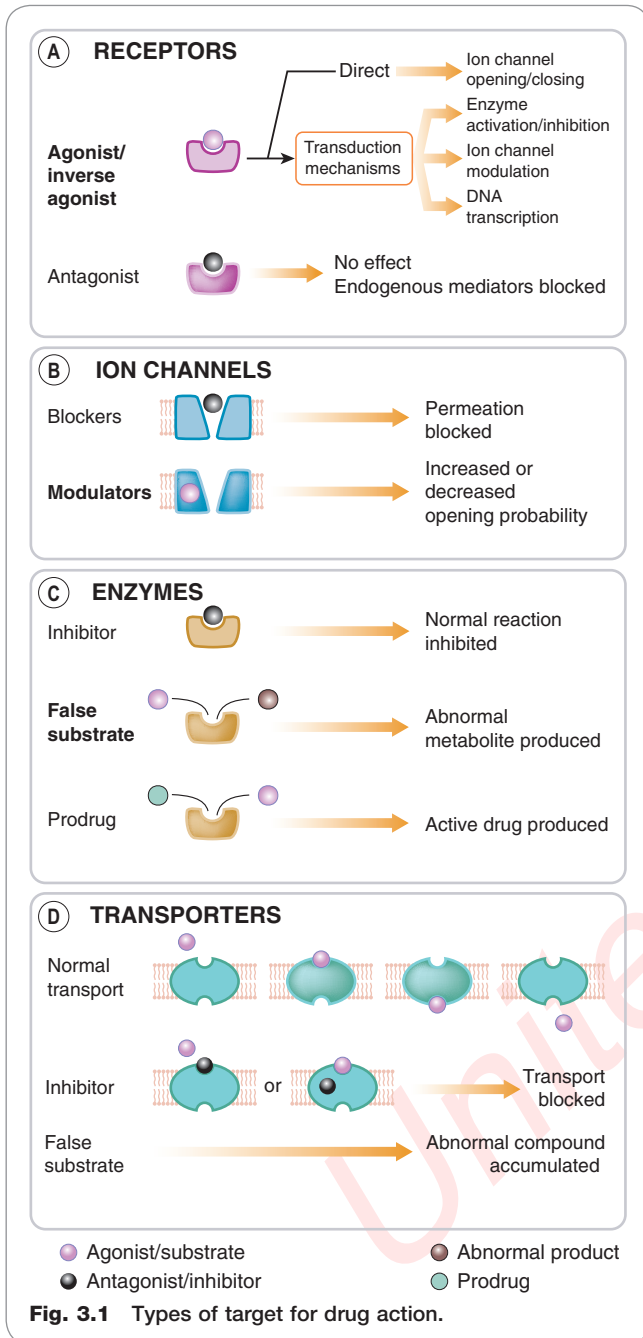
ION CHANNELS

Ion channels[1] are essentially gateways in cell membranes that selectively allow the passage of particular ions, and that 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 in several ways:

1. By binding to the channel protein itself, either to the ligand-binding (*orthosteric*) site of ligand-gated channels, or to other (*allosteric*) sites, or, in the simplest case, exemplified by the action of local anaesthetics on the voltage-gated sodium channel (see Ch. 43), the drug molecule plugs the channel physically (Fig. 3.1B), blocking ion permeation. Examples of drugs that bind to allosteric sites on the channel protein and thereby affect channel gating include:
   - **benzodiazepine** tranquillisers (see Ch. 44). These drugs bind to a region of the $GABA_A$ receptor–chloride channel complex (a ligand-gated channel) that is distinct from the GABA binding site and facilitate the opening of the channel by the inhibitory neurotransmitter GABA (see Ch. 38)

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[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 CM 2003 Voltage-gated K channels; http://www.stke.org).

Fig. 3.1 Types of target for drug action.

– vasodilator drugs of the **dihydropyridine** type (see Ch. 22), which inhibit the opening of L-type calcium channels (see Ch. 4)

– **sulfonylureas** (see Ch. 31) used in treating diabetes, which act on ATP-gated potassium channels of pancreatic β-cells and thereby enhance insulin secretion.

2. By an indirect interaction, involving a G protein and other intermediaries (see p. 30).

3. By altering the level of expression of ion channels on the cell surface. For example **gabapentin** reduces the insertion of neuronal calcium channels into the plasma membrane (Ch. 45).

A summary of the different ion channel families and their functions is given below.

ENZYMES

Many drugs are targeted on enzymes (Fig. 3.1C). 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). 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. 56).

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 (e.g. **enalapril** is converted by esterases to enalaprilat, which inhibits angiotensin converting enzyme). 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.

TRANSPORTERS

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 transporters 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, the uptake of neurotransmitter precursors (such as choline) or of neurotransmitters themselves (such as amines and amino acids) by nerve terminals, and the transport of drug molecules and their metabolites across cell membranes and epithelial barriers. We shall encounter transporters 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. 56). 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-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.

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

RECEPTOR PROTEINS

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.

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, with the sequencing of the entire genome of various species, including human, 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 p. 25) have been cloned. Endogenous ligands for many of these novel receptors identified by gene cloning are so far unknown, and they are described as 'orphan receptors'.[2] Identifying ligands for these presumed receptors is often difficult. However, there are examples (e.g. the cannabinoid receptor; see Ch. 19) where important endogenous ligands have been linked to hitherto orphan receptors, and others, such as PPARs (peroxisome proliferator-activated receptors) that have emerged as the targets of important therapeutic drugs (see Ch. 32) though the endogenous ligand remains unknown. 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, with many still to be fully characterised.

Receptors are proteins normally embedded in membrane lipid and thus have proven very difficult to crystallise. Obtaining crystals of a protein allows its structure to be analysed at very high resolution by X-ray diffraction techniques. Much of our knowledge of ligand-gated ion channel structure comes from work on the nicotinic acetylcholine receptor. Recent years have seen great strides made in crystallising other types of receptors. So far, much of the information obtained relates to how ligands bind to receptors (i.e. the extracellular domains), but we are now beginning to learn more about agonist-induced receptor conformational changes and how signalling is initiated (see Audet & Bouvier, 2012).

Now that the genes have been clearly identified, the emphasis has shifted to characterising the receptors pharmacologically and determining their molecular characteristics and physiological functions.
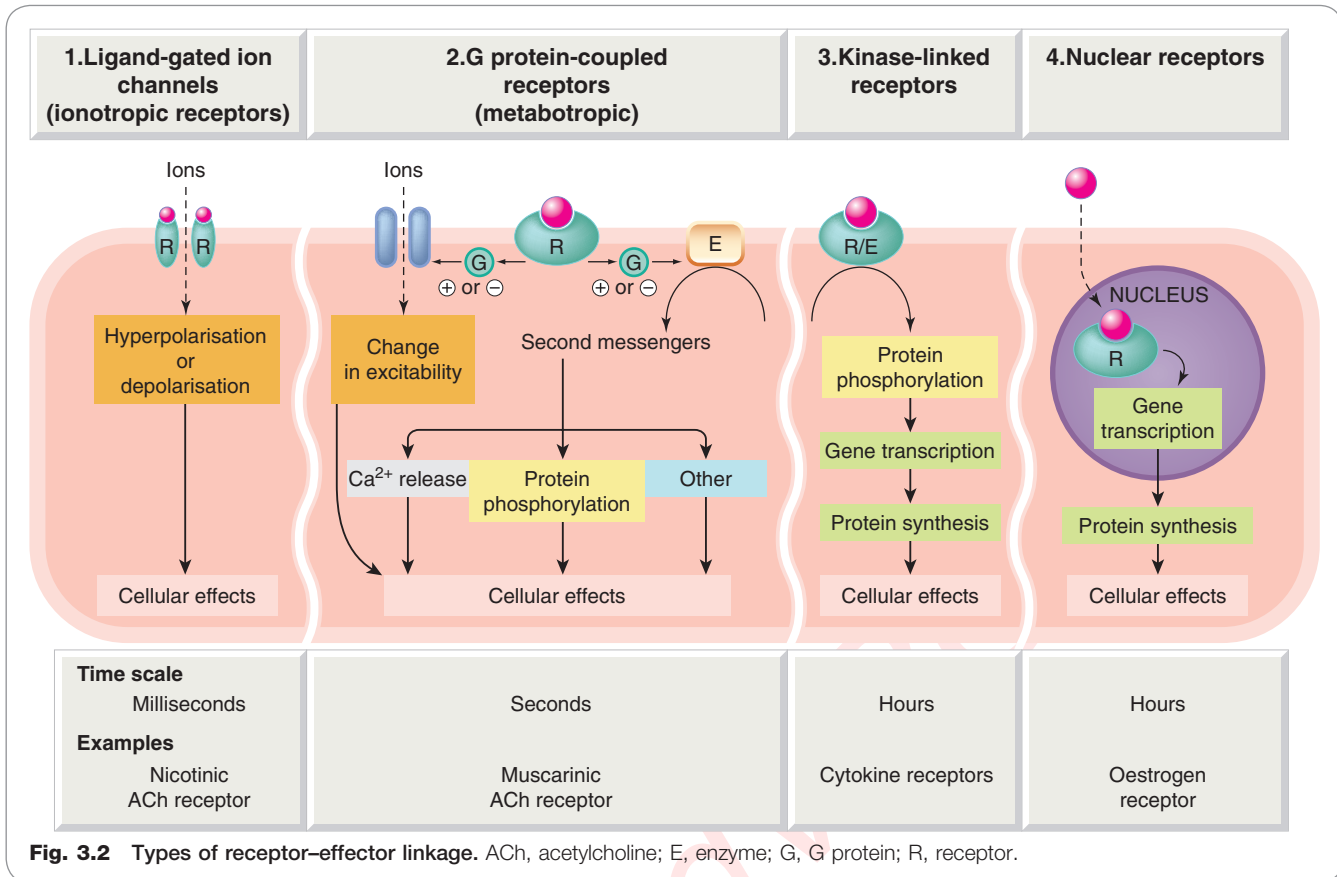
TYPES OF RECEPTOR

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 3.1).

- Type 1: **ligand-gated ion channels** (also known as **ionotropic** receptors).[3] 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 (Table 3.1).
- 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 primarily via a G protein (see p. 27). They constitute the largest family,[4] and include receptors for many hormones and slow transmitters (Table 3.1).
- 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

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[2]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. More information on orphan receptors can be found at www.guidetopharmacology.org/GRAC/FamilyDisplayForward ?familyId=115#16.

[3]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. 45); many are also drug targets, although not receptors in the strict sense.
[4]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. 3.2 **Types of receptor–effector linkage.** ACh, acetylcholine; E, enzyme; G, G protein; R, receptor.

single transmembrane helix. In many cases, the intracellular domain is enzymic in nature (with protein kinase or guanylyl cyclase activity).

- Type 4: **nuclear receptors.** These are receptors that regulate gene transcription.[5] 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 39.2), 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, which produces receptors with different binding characteristics and different signal transduction mechanisms, although its pharmacological relevance remains to be clarified. Another process 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

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[5]The term *nuclear receptor* is something of a misnomer, because some are actually located in the cytosol and migrate to the nuclear compartment when a ligand is present.

[6]Receptors for 5-hydroxytryptamine (see Ch. 15) are currently the champions with respect to diversity, with 14 cloned subtypes.
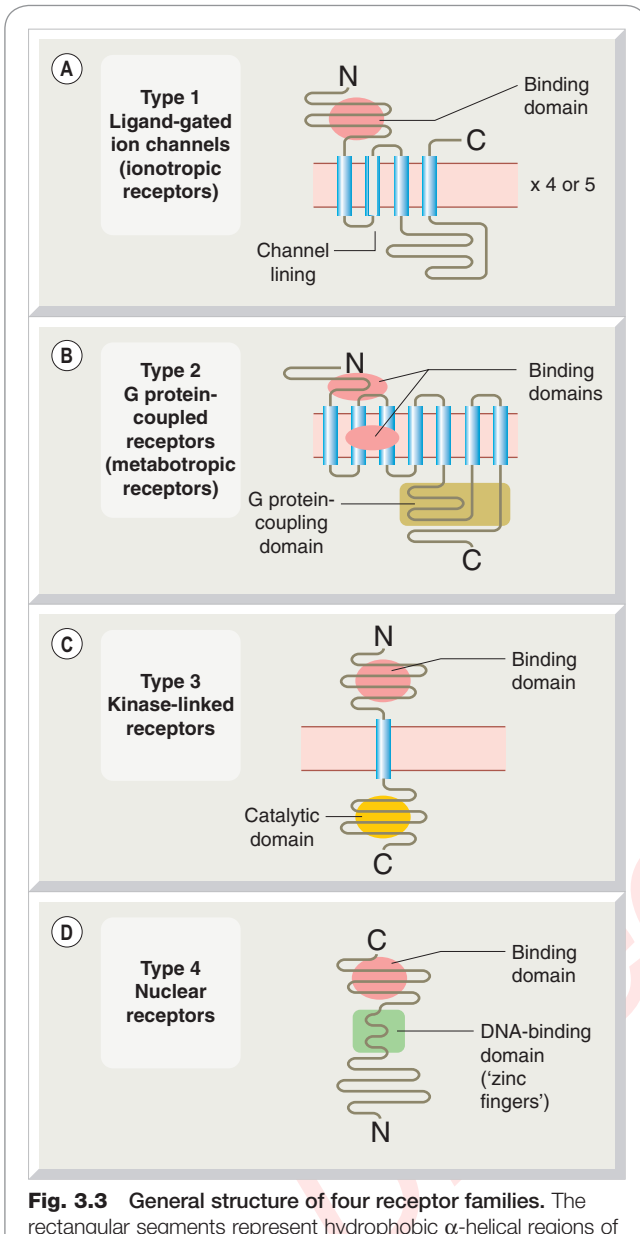
Fig. 3.3 General structure of four receptor families. The rectangular segments represent hydrophobic α-helical regions of the protein comprising approximately 20 amino acids, which form the membrane-spanning domains of the receptors. [**A**] Type 1: ligand-gated ion channels. The example illustrated here shows the subunit structure of the nicotinic acetylcholine receptor. The subunit structure of other ligand-gated ion channels is shown in Fig. 3.20. 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. [**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.

general. New receptor subtypes and isoforms continue to be discovered, and regular updates of the catalogue are available (www.guidetopharmacology.org/). The problems of classification, nomenclature and taxonomy resulting from this flood of data have been mentioned earlier.

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 promised. 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

In this general description of ligand-gated ion channel structure we will focus primarily on the nicotinic acetylcholine receptor, which we find at the skeletal neuromuscular junction (Ch. 13). It is the one we know most about and is similar in structure and function to other cys-loop receptors (so called because they have in their structure a large intracellular domain between transmembrane domains 3 and 4 containing multiple cystine residues [see Fig. 3.3A]), which also include the $GABA_A$ and glycine receptors (Ch. 38) as well as the 5-HT_3 (Chs 15 and 39) receptor. Other types of ligand-gated ion channel exist – namely ionotropic glutamate receptors (Ch. 38) and purinergic P2X receptors (Chs 16 and 39) – that differ in several respects from the nicotinic acetylcholine receptor.

MOLECULAR STRUCTURE

Ligand-gated ion channels have structural features in common with other ion channels, described on p. 45. The nicotinic acetylcholine receptor (Fig. 3.4), the first to be cloned, 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 (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 N-terminal region 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 (primarily Na^+ and K^+, although some types of nicotinic receptor are permeable to Ca^{2+} as well).

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

Table 3.1 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 or arrestin	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 receptor- and DNA-binding domains

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 and glycine). Other mutations affect properties such as gating and desensitisation of ligand-gated channels.

Other ligand-gated ion channels, such as glutamate receptors (see Ch. 38) and P2X receptors (see Ch. 39), whose structures are shown in Figure 3.5, have a different architecture. Ionotropic glutamate receptors are tetrameric and the pore is built from loops rather than transmembrane helices, in common with many other (non-ligand-gated) ion channels (see p. 29). P2X receptors are trimeric and each subunit has only two transmembrane domains (North, 2002). The nicotinic receptor and other cys-loop receptors are pentamers with two agonist binding sites on each receptor. Binding of one agonist molecule to one site increases the affinity of binding at the other site (positive co-operativity) and both sites need to be bound for the receptor to be activated and the channel to open. Some ionotropic glutamate receptors have as many as four agonist binding sites and P2X receptors have three but they appear to open when two agonist molecules are bound. Once again we realise that the simple model of receptor activation shown in Figure 2.1 is an oversimplification as it only considered one agonist molecule binding to produce a response. For two or more agonist molecules binding, more complex mathematical models are needed (see Colquhoun, 2006).
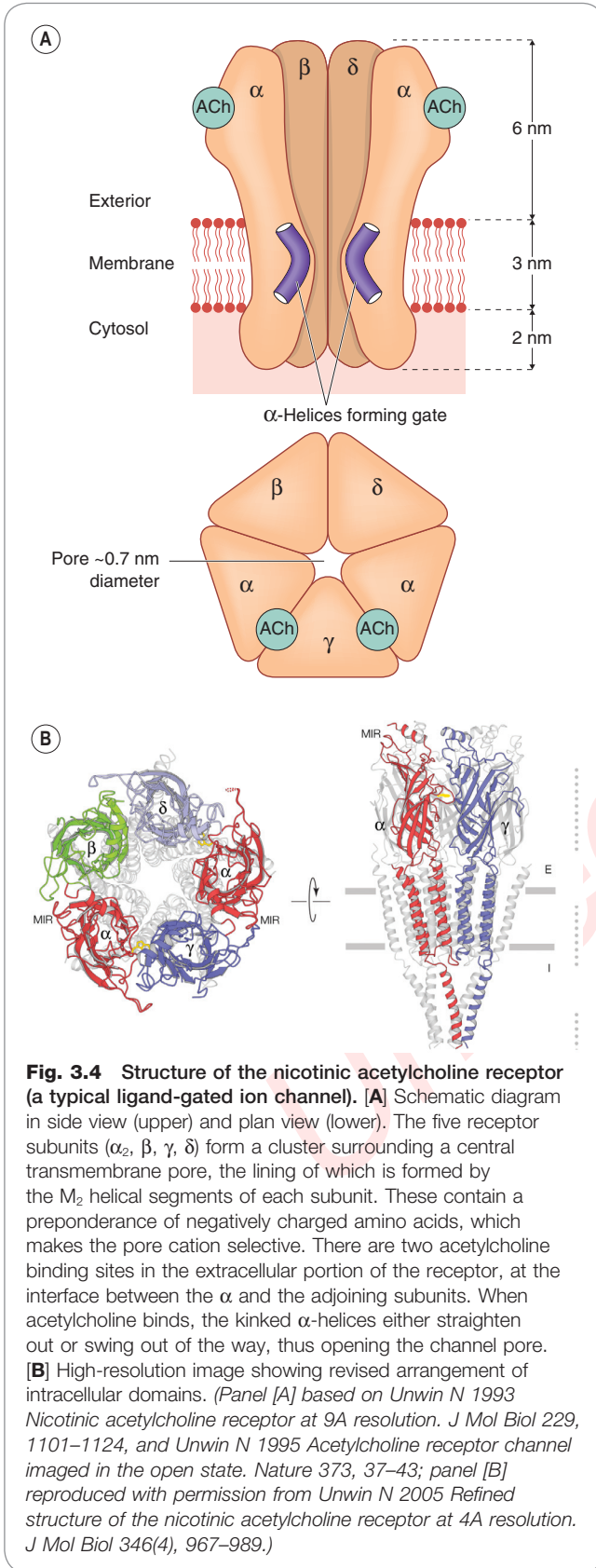
THE GATING MECHANISM

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. 13) or glutamate in the central nervous system (Ch. 38), cause an increase in Na$^+$ and K$^+$ permeability and in some instances Ca^{2+} permeability. At negative membrane potentials 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 ion 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.

▼ The patch clamp recording technique, devised by Neher and Sakmann, allows the very small current flowing through a single ion channel to be measured directly (Fig. 3.6), and the results have fully confirmed the previous interpretation of channel properties based on noise analysis made by Katz and Miledi in 1972. The patch clamp technique provides a view, rare 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. 30, Fig. 3.6). 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 (about 10^7 ions per second) to be compatible with a carrier mechanism. The channel conductance produced by different agonists is the same, whereas the mean channel lifetime varies. The ligand–receptor interaction scheme shown in Chapter 2 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 β/α ≫ 1, whereas for a drug of low efficacy β/α has a lower value.

At some ligand-gated ion channels the situation is more complicated because different agonists may cause individual channels to open to one or more of several distinct conductance levels (Fig. 3.6). This implies that there is more than one R* conformation. Furthermore, desensitisation of ligand-gated ion channels (see Ch. 2) also involves one or more additional agonist-induced conformational states. These findings necessitate some elaboration of the simple scheme 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.

TYPE 2: G PROTEIN-COUPLED RECEPTORS

The abundant GPCR family comprises many of the receptors that are familiar to pharmacologists, such as muscarinic AChRs, 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.

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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. [**B**] High-resolution image showing revised arrangement of intracellular domains. *(Panel [A] 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; panel [B] reproduced with permission from Unwin N 2005 Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J Mol Biol 346(4), 967–989.)*

> ### Ligand-gated ion channels
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> - 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), glutamate (NMDA) and ATP (P2X) receptors.

Many neurotransmitters, apart from peptides, can interact with both GPCRs and ligand-gated channels, allowing the same molecule to produce fast (through ligand-gated ion channels) and relatively slow (through GPCRs) 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 adreno-ceptor (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. Recently, the difficulties of crystallising GPCRs have been overcome (see Weis & Kobilka, 2008), allowing the use of the powerful technique of X-ray crystallography to study the molecular structure of these receptors in detail (Fig. 3.7). 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; Bockenhauer et al., 2011). This is starting to provide important information on agonist- and antagonist-bound receptor conformations as well as receptor–G protein interactions. From such studies we are gaining 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.

G protein-coupled receptors consist of a single polypeptide chain, usually of 350–400 residues, but can in some cases be up to 1100 residues. The general anatomy is shown in Figure 3.3B. Their characteristic structure

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[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.
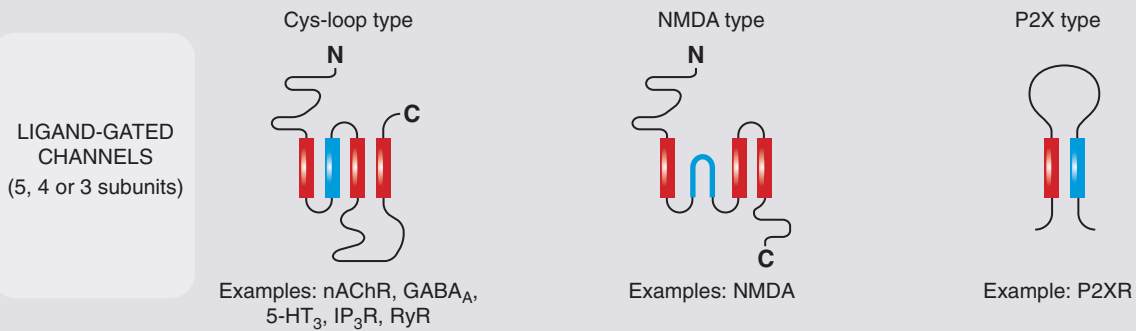
Fig. 3.5 **Molecular architecture of ligand-gated ion channels.** Red and blue rectangles represent membrane-spanning α-helices and blue hairpins represent the P loop pore-forming regions. Cys-loop receptors are pentameric, NMDA-type receptors are tetrameric and P2X receptors are trimeric. 5-HT$_3$, 5-hydroxytryptamine type 3 receptor; GABA$_A$, GABA type A receptor; IP$_3$R, inositol trisphosphate receptor; nAChR, nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartatic acid receptor; P2XR, purine P2X receptor; RyR, ryanodine receptor.

Table 3.2   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 www.guidetopharmacology.org.

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. There is considerable sequence homology between the members of one family, but little between different families. They share the same seven transmembrane 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 3.2). 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. Family C is the smallest, its main members being the metabotropic glutamate and GABA receptors (Ch. 38) and the Ca^{2+}-sensing receptors[8] (see Ch. 36).

▼ 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 p. 32, Fig. 3.9). 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.

Site-directed mutagenesis experiments show that the 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 type of G protein, and swapping parts of the cytoplasmic loop between different receptors alters their G protein selectivity. Phosphorylation of serine and threonine residues on the C-terminal tail and other intracellular domains by intracellular kinases can result in receptor desensitisation (see p. 36).

For small molecules, such as noradrenaline (norepinephrine) and acetylcholine, the ligand-binding domain of class A receptors is buried in the cleft between the α-helical segments within the membrane (Figs 3.3B and 3.7), similar to the slot occupied by retinal in the rhodopsin molecule.

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[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 mmol/l – 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. 36). This homeostatic mechanism is quite distinct from the mechanisms for regulating intracellular Ca^{2+}, discussed in Chapter 4.
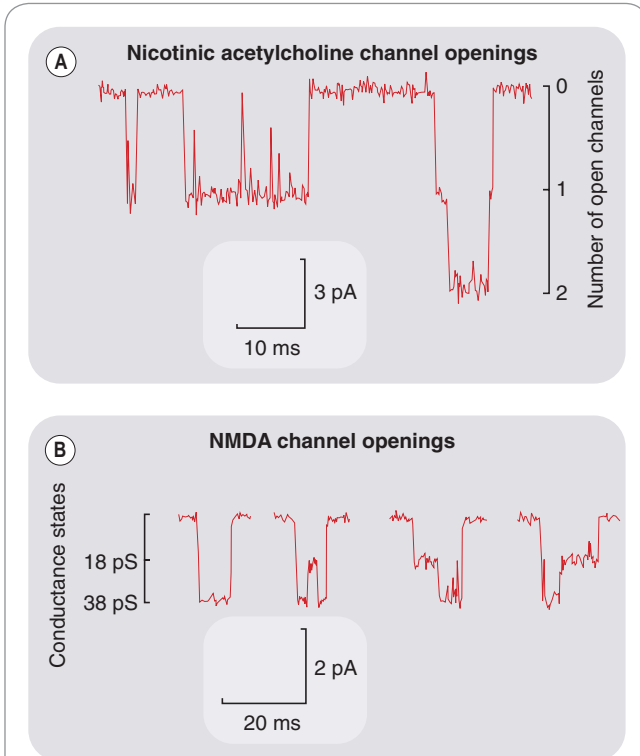
Fig. 3.6 Single channel openings recorded by the patch clamp technique. [**A**] Acetylcholine-operated ion channels at the frog motor endplate. The pipette, which was applied tightly to the surface of the membrane, contained 10 μmol/l ACh. The downward deflections show the currents flowing through single 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 with a discrete step from the first to the second. [**B**] Single-channel NMDA receptor currents recorded from cerebellar neurons in the outside-out patch conformation. NMDA was added to the outside of the patch to activate the channel. The channel opens to multiple conductance levels. In [**B**] the openings to the higher conductance level and the subsequent closings are smooth, indicating that one channel is opening (two channels would not be expected to open and close simultaneously) whereas in [**A**] there are discrete steps indicating two channels. *(Panel [A] courtesy of D Colquhoun and DC Ogden; panel [B] reproduced with permission from Cull-Candy SG & Usowicz MM 1987 Nature 325, 525–528.)*

Fig. 3.7 **Structure of the M_4 muscarinic receptor.** High resolution image showing the conformation of the M_4 muscarinic receptor bound with both an agonist (orthosteric) and a positive allosteric modulator. The brown cylinders represent the transmembrane domains. The full extent of the N- and C-terminal domains and the third intracellular loop are not shown. *(Courtesy of A Christopoulos.)*

Peptide ligands, such as substance P (Ch. 18), bind more superficially to the extracellular loops, as shown in Figure 3.3B. From crystal structures and 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]

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[9]In the past many lead compounds have come from screening huge chemical libraries (see Ch. 60). No inspiration was required, just robust assays, large computers and efficient robotics. Now with the generation of crystal structures we may be moving to a more sophisticated age in drug discovery.
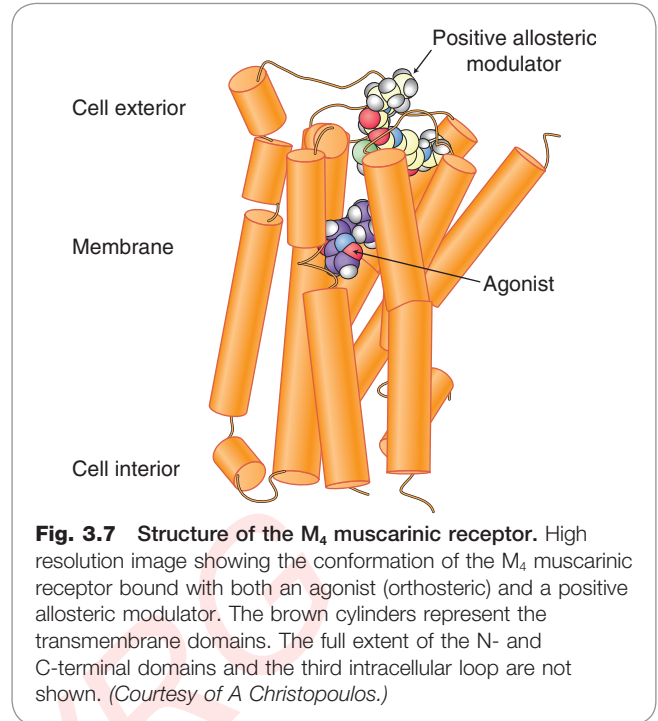
PROTEASE-ACTIVATED RECEPTORS

▼ 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. 3.8) 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. 42). 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 (Fig. 3.8), after which the receptor is internalised and degraded, to be replaced by newly synthesised protein.

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
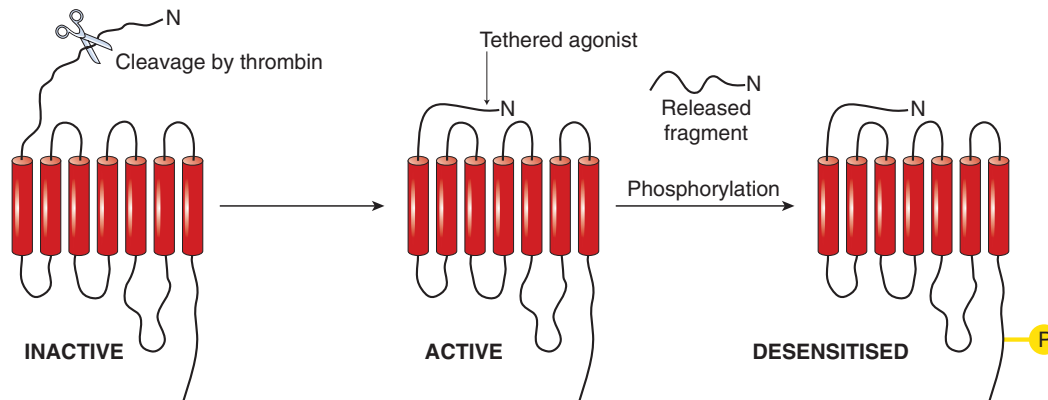
Fig. 3.8 **Activation of a protease-activated receptor by cleavage of the N-terminal extracellular domain.** Inactivation occurs by phosphorylation. Recovery requires resynthesis of the receptor.
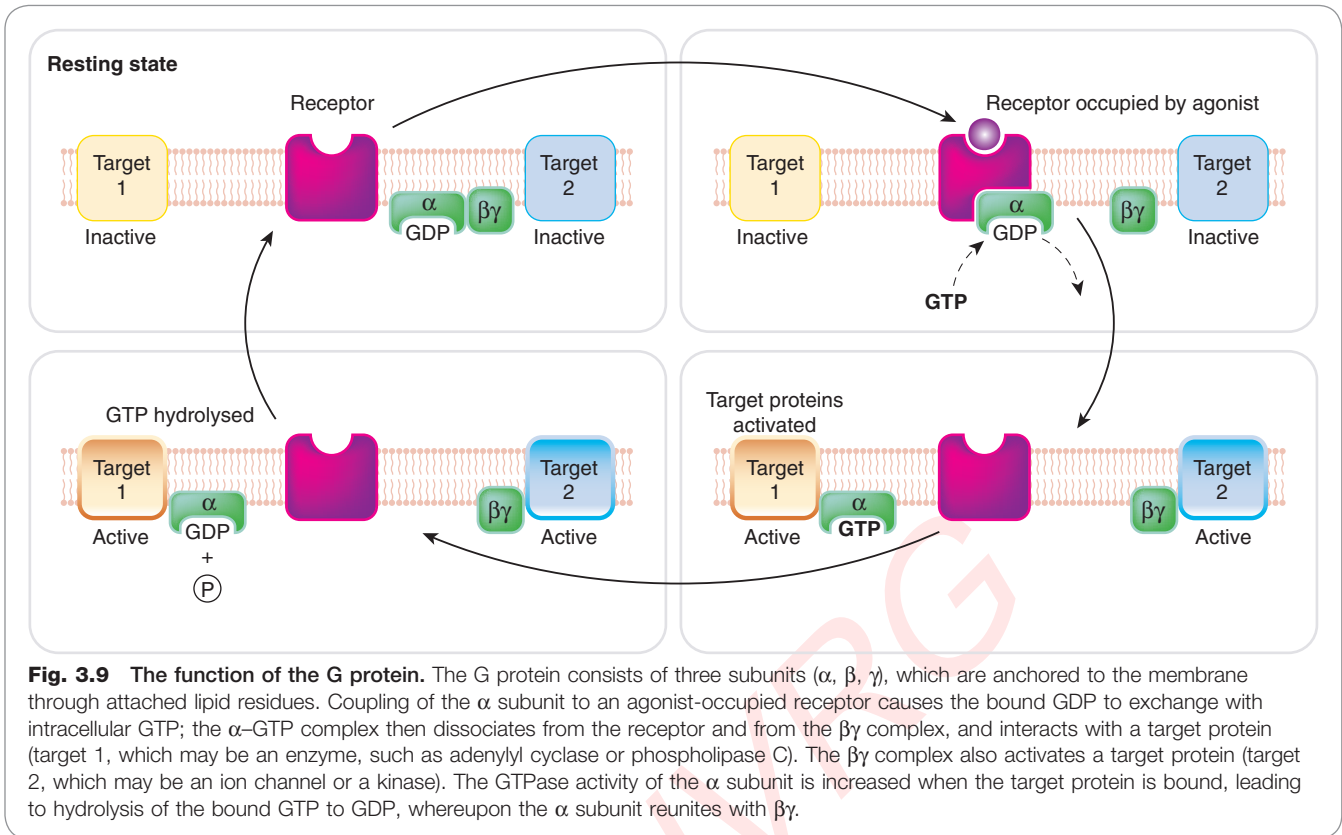

G protein-coupled receptors

- These are sometimes called metabotropic or seven-transmembrane-domain (7-TDM) receptors.
- Structures comprise seven membrane-spanning α-helices, often linked as dimeric structures.
- The third intracellular loop 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 binds GTP, dissociates and is then free to activate an effector (e.g. a membrane enzyme). 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.

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subunits: α, β and γ (Fig. 3.9). Guanine nucleotides bind to the α subunit, which has enzymic (GTPase) activity, catalysing the conversion of GTP to GDP. The β and γ subunits remain together as a βγ complex. The 'γ' subunit is 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. 3.9), the G protein exists as an αβγ trimer, which may or may not be precoupled to the receptor, 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), inducing a high affinity interaction of αβγ and the receptor. This agonist-induced interaction 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. 3.9). 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. 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 3.3). G protein activation 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. 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. In addition, there is a family of about 20 cellular proteins, regulators of G protein signalling (RGS) proteins (see review by Xie & Palmer, 2007), that possess a conserved sequence that binds specifically to α subunits to increase greatly their GTPase activity, so hastening the hydrolysis of GTP and inactivating the complex. RGS proteins thus exert an inhibitory effect on G protein signalling, a mechanism that is thought to have a regulatory function in many situations.

How is specificity of GPCR function 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

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Fig. 3.9 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 phospholipase C). The βγ complex also activates a target protein (target 2, which may be an ion channel or a kinase). 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 3.3 The main G protein subtypes and their functions[a]

Subtypes	Associated receptors	Main effectors	Notes
Gα subunits			
Gα$_s$	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
Gα$_i$	As for Gα$_s$, also opioid, cannabinoid receptors	Inhibits adenylyl cyclase, decreasing cAMP formation	Blocked by pertussis toxin, which prevents dissociation of αβγ complex
Gα$_o$	As for Gα$_s$, also opioid, cannabinoid receptors	? Limited effects of α subunit (effects mainly due to βγ subunits)	Blocked by pertussis toxin. Occurs mainly in nervous system
Gα$_q$	Amine, peptide and prostanoid receptors	Activates phospholipase C, increasing production of second messengers inositol trisphosphate and diacylglycerol (see p. 34 and 35)	
Gβγ subunits			
	All GPCRs	Activate potassium channels inhibit voltage-gated calcium channels activate GPCR kinases (GRKs, p. 36) activate mitogen-activated protein kinase cascade Interact with some forms of adenylyl cyclase and with phospholipase Cβ	Many βγ isoforms identified, but specific functions are not yet known

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. 3.10 Bidirectional control of a target enzyme, such as adenylyl cyclase by G_s and G_i. Heterogeneity of G proteins allows different receptors to exert opposite effects on a target enzyme.
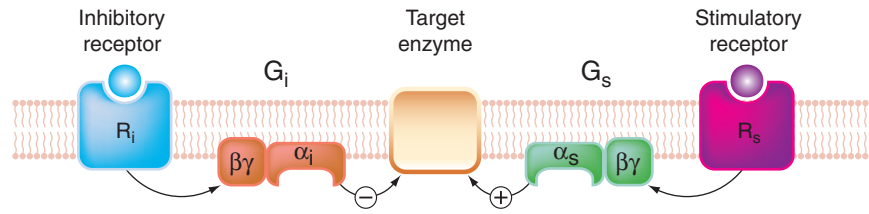
cells, produce opposite functional effects (Chs 13 and 14). The main reason is molecular variation within the α subunits, of which more than 20 subtypes have been identified[10] (Table 3.3). Four main classes of G protein (G_s, G_i, G_o and G_q) are of pharmacological importance. As summarised in Table 3.3, 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. 3.10).

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 3.3). 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 gastrointestinal epithelium, are due to the uncontrolled activation of adenylyl 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 Nahorski, 2006; Table 3.3), 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 regulates 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, see below). Many different drugs, hormones and neurotransmitters act on GPCRs and increase or decrease the catalytic activity of adenylyl cyclase, thus raising or lowering the concentration of cAMP within the cell. In mammalian cells there are 10 different molecular isoforms of the enzyme, some of which respond selectively to $G\alpha_s$ or $G\alpha_i$.

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 – primarily protein kinase A (PKA) in eukaryotic cells. Protein kinases regulate the function of many different cellular proteins by controlling protein phosphorylation. Figure 3.11 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. 42). Adenylyl cyclase can be activated directly by drugs such as **forskolin**, which is 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. Eleven PDE subtypes exist, of which some (e.g.

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[10]In humans there are 21 known subtypes of Gα, 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.
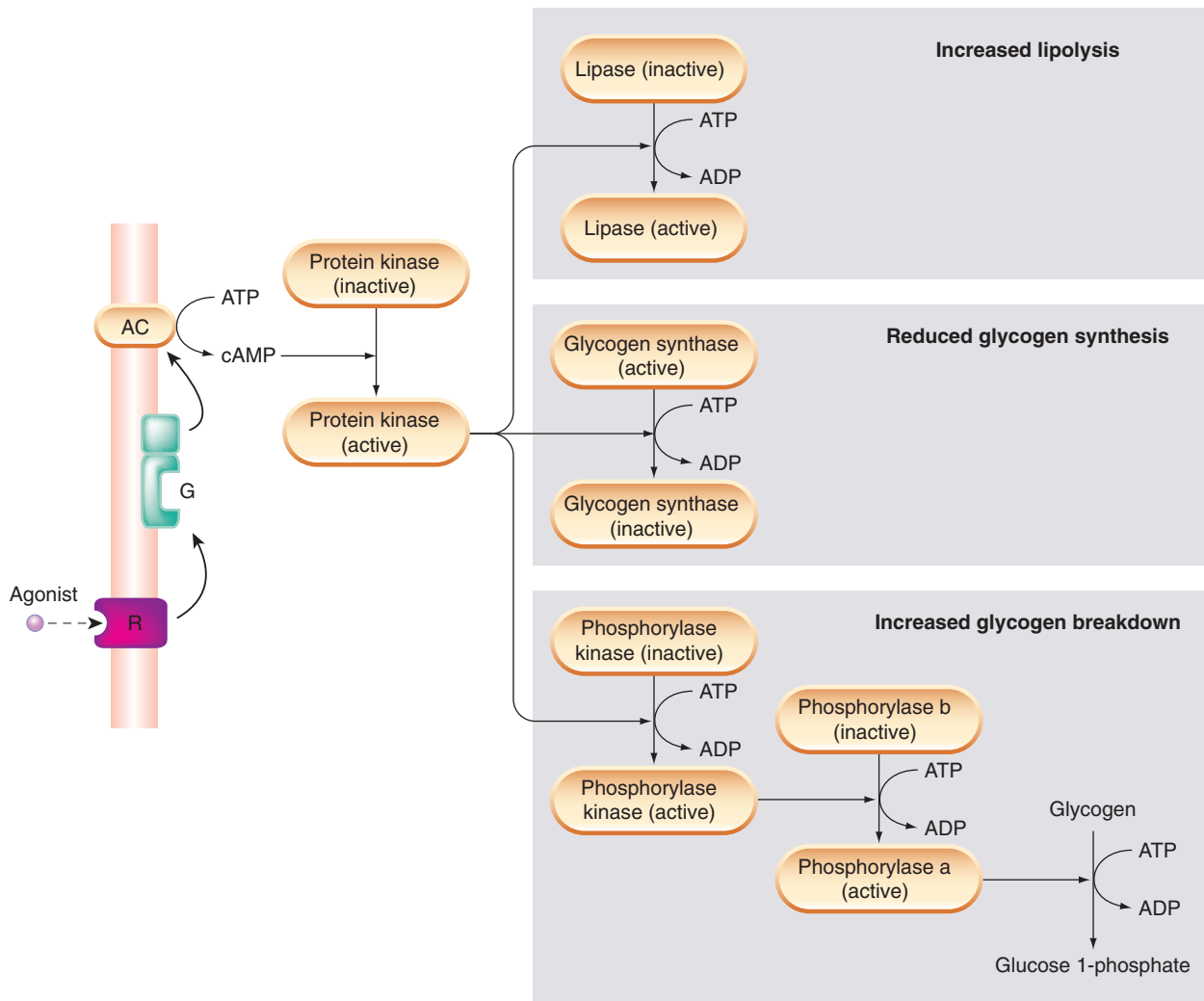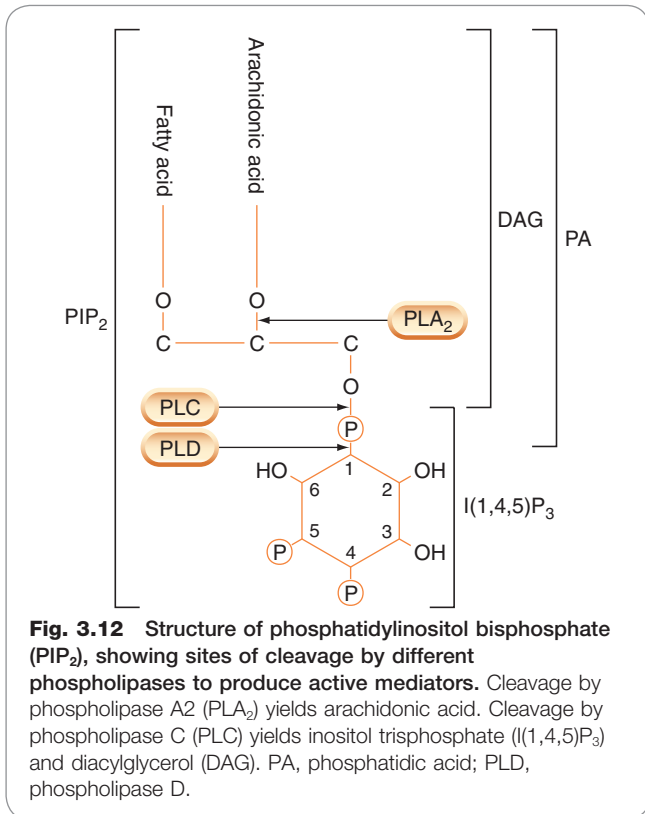
Fig. 3.11 Regulation of energy metabolism by cAMP. AC, adenylyl cyclase.

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 28 and 48). **Rolipram** (used to treat asthma; Ch. 28) 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. 35) 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. 3.12). Subsequently, Michell and Berridge found that many hormones that produce an increase in free 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. It was later 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. 3.13), both of which function as second messengers as discussed below (p. 35). The activation of PLCβ by various agonists is mediated through a G protein (G_q, see Table 3.3). After cleavage of PIP_2, the status quo is restored, as shown in Figure 3.13, DAG being phosphorylated to form phosphatidic acid (PA), while the IP_3 is dephosphorylated and then recoupled

Fig. 3.12 **Structure of phosphatidylinositol bisphosphate (PIP$_2$), showing sites of cleavage by different phospholipases to produce active mediators.** Cleavage by phospholipase A2 (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.

with PA to form PIP$_2$ once again.[11] Lithium, an agent used in psychiatry (see Ch. 47), blocks this recycling pathway (see Fig. 3.13).

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, 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$_3$ can be 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 some evidence suggests that it, and also higher inositol phosphates, may play 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 protein kinase, *protein kinase C* (PKC), which catalyses the phosphorylation of a variety of intracellular proteins. DAG, unlike the inositol phosphates, is highly lipophilic and remains within the membrane. It binds to a specific site on the PKC molecule, causing the enzyme to migrate from the cytosol to the cell membrane, thereby becoming activated. There are at least 10 different mammalian PKC subtypes, which have distinct cellular distributions and phosphorylate different proteins. Several 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. 18) 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. 39), act on many different functional proteins, such as ion channels, receptors, enzymes (including other kinases), transcription factors and cytoskeletal proteins. Protein phosphorylation by kinases plays a central role in signal transduction, and controls many different aspects of cell function. The DAG–PKC link provides a mechanism whereby GPCRs can mobilise this army of control freaks.

Ion channels as targets for G proteins

Another major function of G protein-coupled receptors is to control ion channel function directly by mechanisms that do not involve second messengers such as cAMP or inositol phosphates. Direct G protein–channel interaction, through the βγ subunits of G$_i$ and G$_o$ proteins, appears to be a general mechanism for controlling K$^+$ and Ca^{2+} channels. In cardiac muscle, for example, mAChRs enhance K$^+$ permeability in this way (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 certain K$^+$ channels – known as G protein-activated inwardly rectifying K$^+$ channels (GIRK) – or by inhibiting voltage-activated N and P/Q type Ca^{2+} channels and thus reducing neurotransmitter release (see Chs 4 and 42).

The main postulated roles of GPCRs in controlling enzymes and ion channels are summarised in Figure 3.14.

The Rho/Rho kinase system

▼ This 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. 22). Specific Rho kinase inhibitors (e.g. **fasudil**) are in development for a wide range of clinical indications – an area to watch.
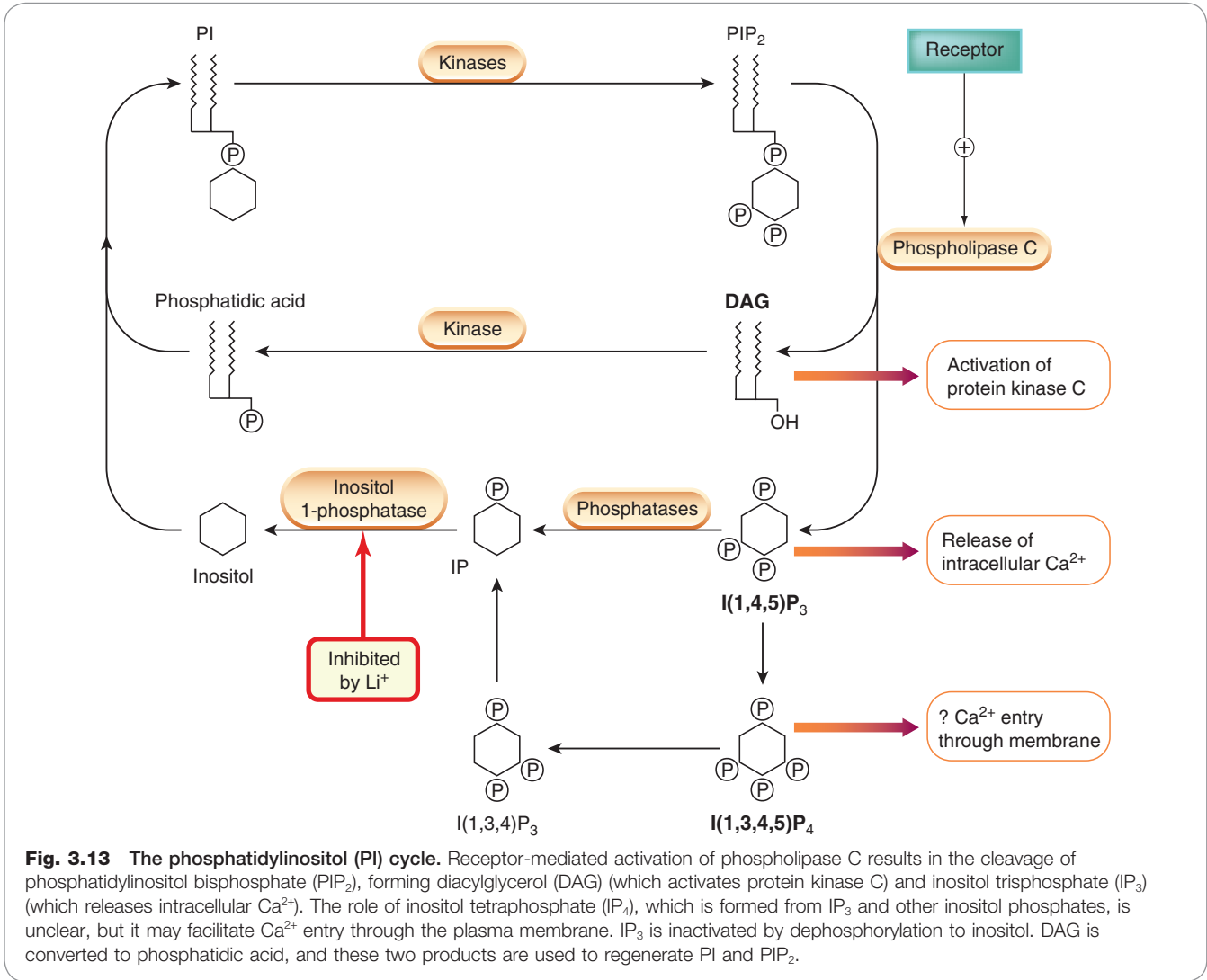
The MAP kinase system

▼ The MAP kinase system involves several signal transduction pathways (Fig. 3.15) that are activated not only by various cytokines and growth factors acting on kinase-linked receptors (see p. 40, Fig. 3.17), but also by ligands activating GPCRs. The coupling of GPCRs to different families of MAP kinases can involve G protein α and βγ subunits as well as Src and arrestins – proteins also involved in GPCR desensitisation (see p. 36) (Pierce & Lefkowitz, 2001). The MAP kinase system controls many processes involved in gene expression, cell division, apoptosis and tissue regeneration

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 further developments have necessitated a substantial overhaul of the basic model.

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[11]Alternative abbreviations for these mediators are PtdIns (PI), PtdIns (4,5)-P$_2$ (PIP$_2$), Ins (1,4,5)-P$_3$ (IP$_3$), and Ins (1,2,4,5)-P4 (IP$_4$).

Fig. 3.13 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.

GPCR desensitisation

▼ As described in Chapter 2, desensitisation is a feature of most GPCRs, and the mechanisms underlying it have been extensively studied. *Homologous desensitisation* is restricted to the receptors activated by the desensitising agonist, while *heterologous desensitisation* affects other GPCRs in addition. Two main processes are involved (see 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 specific membrane-bound GPCR kinases (GRKs) and by kinases such as PKA and PKC.

On receptor activation GRK2 and GRK3 are recruited to the plasma membrane by binding to free G protein βγ subunits. GRKs then phosphorylate the receptors in their activated (i.e. agonist-bound) state. The phosphorylated receptor serves as a binding site for arrestins, intracellular proteins that block the interaction between the receptor and the G proteins producing a selective *homologous desensitisation*. Arrestin binding also targets the receptor for endocytosis in clathrin-coated pits (Fig. 3.16). The internalised receptor can then either be dephosphorylated and reinserted into the plasma membrane (resensitisation) or trafficked to lysosomes for degradation (inactivation). This type of desensitisation seems to occur with most GPCRs but with subtle differences that fascinate the aficionados.

Phosphorylation by PKA and PKC at residues different from those targeted by GRKs generally leads to impaired coupling between the activated receptor and the G protein, so the agonist effect is reduced. This can give rise to either homologous or heterologous desensitisation, depending on whether or not receptors other than that for the desensitising agonist are simultaneously phosphorylated by the kinases, some of which are not very selective. Receptors phosphorylated by second messenger kinases are probably not internalised and are reactivated by dephosphorylation by phosphatases when the agonist is removed.

GPCR oligomerisation

▼ The earlier view that GPCRs exist and function as monomeric proteins (in contrast to ion channels, which generally comprise multimeric complexes; see p. 26) 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. 38). A similar situation arises with G protein-coupled glutamate receptors. Oddly, although such dimers have two potential agonist binding sites, one on each subunit, only one is functional and signalling is transmitted through the dimer to the other receptor in the dimer which couples to the G protein (see Fig 38.9).

Other GPCRs are functional as monomers but it now seems likely that most, if not all, GPCRs can exist as either homomeric or heteromeric oligomers (i.e. dimers or larger oligomers) (Prinster et al., 2005). Within the opioid receptor family (see Ch. 42), the μ receptor
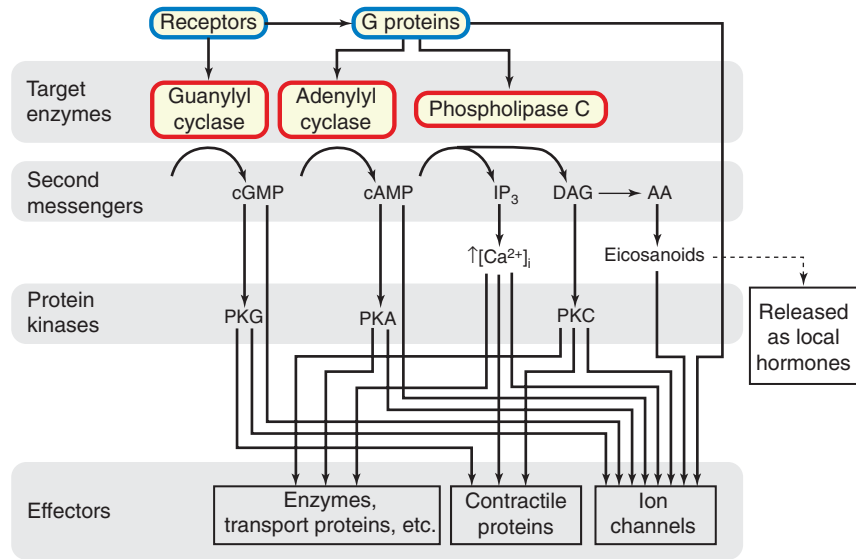
Fig. 3.14 G protein and second messenger control of cellular effector systems. AA, arachidonic acid; DAG, diacylglycerol; IP$_3$, inositol trisphosphate. Not shown in this diagram are signalling pathways where arrestins, rather than G proteins, link GPCRs to downstream events (see text).

Effectors controlled by G proteins

Two key second messenger pathways are controlled by receptors via G proteins:
- Adenylyl cyclase/cAMP:
  - can be activated or inhibited by pharmacological ligands, depending on the nature of the receptor and G protein
  - adenylyl 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:
- Ion channels:
  - opening potassium channels, resulting in membrane hyperpolarisation
  - inhibiting calcium channels, thus reducing neurotransmitter release.
- Phospholipase A$_2$ (and thus the formation of arachidonic acid and eicosanoids).

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was crystallised as a dimer and stable and functional heterodimers 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 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*. 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 (see Ch. 2), 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
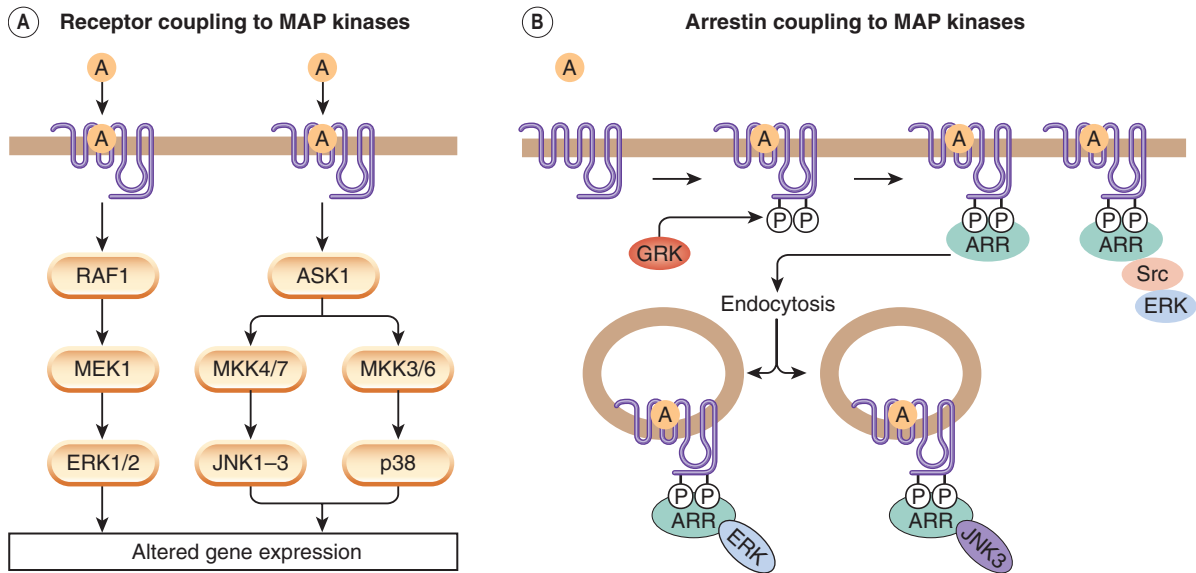
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Fig. 3.15 GPCR activation of MAP kinase cascade. [**A**] Sequential activation of the multiple components of the MAP kinase cascade. GPCR activation of MAP kinases can involve Gα and βγ subunits (not shown). [**B**] Activation of ERK and JNK3 through interaction with arrestins (βARR). Activation of ERK can occur either at the plasma membrane involving Src, or by direct activation after internalisation of the receptor/arrestin complex. ARR, arrestin; GRK, G protein-coupled receptor kinase.
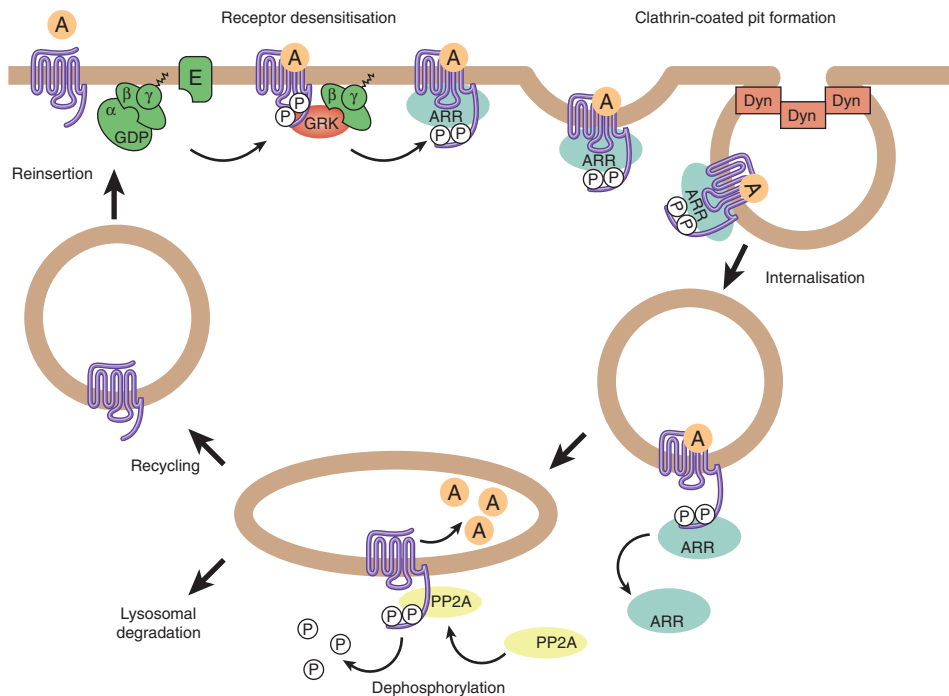

Fig. 3.16 Desensitisation and trafficking of G protein-coupled receptors (GPCRs). On prolonged agonist activation of the GPCR selective G protein-coupled receptor kinases (GRKs) are recruited to the plasma membrane and phosphorylate the receptor. Arrestin (ARR) then binds and trafficks the GPCR to clathrin-coated pits for subsequent internalisation into endosomes in a dynamin-dependent process. The GPCR is then dephosphorylated by a phosphatase (PP2A) and either recycled back to the plasma membrane or trafficked to lysosomes for degradation. ARR, arrestin; Dyn, dynamin; GRK, G protein-coupled receptor kinase; PP2A, phosphatase 2A.

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 angiotensin 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 *biased agonism*; see Ch. 2). Binding of arrestins to GPCRs initiates MAP kinase signalling, such that agonists that induce GRK/arrestin 'desensitisation'

will terminate some GPCR signalling but may also activate signalling through arrestins that may continue even after the receptor/arrestin complex has been internalised (see Fig. 3.15).

Biased agonism 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

▼ Receptor activity-modifying-proteins (RAMPs) are a family of membrane proteins that associate with some 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. 18) 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, **adrenomedulin**. 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 nearly all the examples involve peptide receptors, the calcium-sensing receptor being an exception. RAMPs are an example of how protein–protein interactions influence the pharmacological behaviour of the receptors in a highly selective way and may be novel targets for drug development (Sexton et al., 2012).

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. In this context, signalling mediated through arrestins bound to the receptor (see p. 38), rather than through G proteins, is important (see reviews by Pierce & Lefkowitz; 2001; Delcourt et al., 2007). Arrestins can act as an intermediary for GPCR activation of the MAP kinase cascade (see Fig. 3.15).

There are many examples where the various 'adapter proteins' that link receptors of the tyrosine kinase type to their effectors (see p. 40) can also interact with GPCRs (see Brzostowski & Kimmel, 2001), allowing the same effector systems to be regulated by receptors of either type.

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.

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 ligand-gated channels and GPCRs.

They are activated by a wide variety of protein mediators, including growth factors and cytokines (see Ch. 18), and hormones such as insulin (see Ch. 31) and leptin (Ch. 32), 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, linking a large extracellular ligand-binding domain to 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 review by 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 18.

The main types are as follows:

Receptor tyrosine kinases (RTKs). These receptors have the basic structure shown in Figure 3.17A, 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 lipopolysaccharides and play an important role in the body's reaction to infection (see Ch. 6). The insulin receptor (see Ch. 31) also belongs to the RTK class, although it has a more complex dimeric structure.

Receptor serine/threonine kinases. This smaller class is similar in structure to RTKs but they 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. 3.17B) lack intrinsic enzyme activity. When occupied, they activate various tyrosine kinases, such as Jak (the Janus kinase). Ligands for these receptors include cytokines such as **interferons** and **colony-stimulating factors** involved in immunological responses.

PROTEIN PHOSPHORYLATION AND KINASE CASCADE MECHANISMS

Protein phosphorylation (see Cohen, 2002) 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 proteins is known as the *SH2 domain proteins* (standing for Src homology, because they were
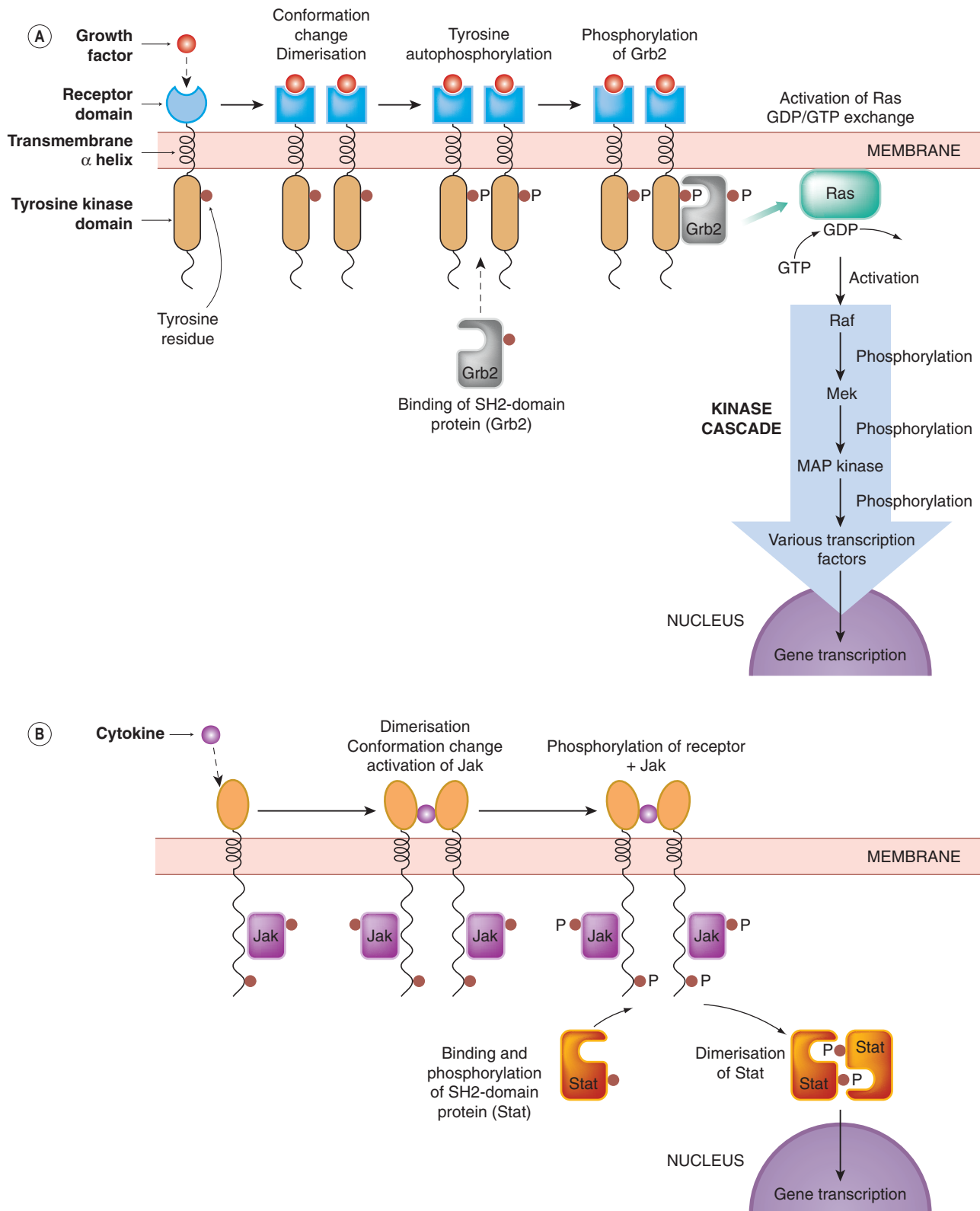
Fig. 3.17 **Transduction mechanisms of kinase-linked receptors.** The first step following agonist binding is dimerisation, which leads to autophosphorylation of the intracellular domain of each receptor. SH2-domain proteins then bind to the phosphorylated receptor and are themselves phosphorylated. Two well-characterised pathways are shown: [**A**] the growth factor (Ras/Raf/mitogen-activated protein [MAP] kinase) pathway (see also Ch. 5). Grb2 can also be phosphorylated but this negatively regulates its signalling. [**B**] Simplified scheme of the cytokine (Jak/Stat) pathway (see also Ch. 18). Some cytokine receptors may pre-exist as dimers rather than dimerise on cytokine binding. 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.

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 3.17.

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 p. 35). Other SH2-containin-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 Jin and Pawson (2012). *Nuclear factor kappa B* (NFκB) is a transcription factor that plays a key role in multiple disorders including inflammation and cancer (see Chs 17 and 56; 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 various proinflammatory genes.

▼ Two well-defined signal transduction pathways are summarised in Figure 3.17. The Ras/Raf pathway 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. 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 56). For more details, see review by Avruch (2007).

A second pathway, the Jak/Stat pathway (Fig. 3.17B) 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.

Other important mechanisms centre on *phosphatidylinositol-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 protein kinases, particularly protein kinase B (PKB, also known as Akt), have 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. A 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. 56).

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 18 and 29), resembles the receptor tyrosine kinase family and is activated in a similar way by dimerisation when the agonist is bound.

Figure 3.18 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
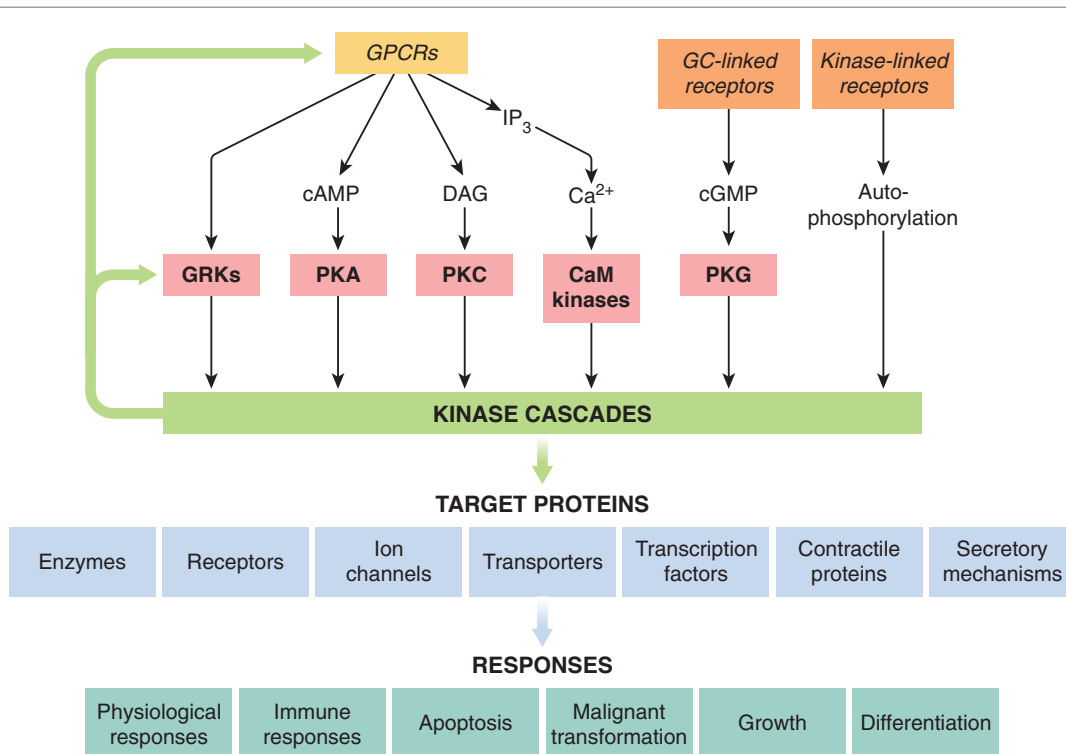
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Fig. 3.18 Central role of kinase cascades in signal transduction. Kinase cascades (e.g. those shown in Fig. 3.15) are activated by GPCRs, either directly or via different second messengers, by receptors that generate cGMP, or by kinase-linked receptors. 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.

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.

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 dephosphorylate proteins and thus reverse the effects of kinases.

TYPE 4: NUCLEAR RECEPTORS

By the 1970s, it was clear that receptors for steroid hormones such as oestrogen and the glucocorticoids (Ch. 33) 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. 34) and the fat-soluble vitamins D and A (retinoic acid), were found to act in a similar fashion. Comparisons of gene and protein sequence data led to the recognition that these receptors were members of a much larger family of related proteins. We now know these as the *nuclear receptor (NR) family*.

As well as NRs such as the glucocorticoid and retinoic acid receptor, whose ligands are 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 the *retinoid X receptor* (RXR), a receptor cloned on the basis of its similarity with the vitamin A receptor, and which was subsequently found to bind the vitamin A derivative 9-*cis*-retinoic acid. Over the intervening years, specific binding partners have been characterised for many NRs ('adopted orphans'; e.g. RXR) but for many others ('true orphans') these have yet to be identified – or perhaps do not exist as such, as one possible function of these receptors is their 'promiscuous' ability to bind to many related compounds (such as dietary factors) with low affinity.

Unlike the other receptors described in this chapter, the NRs can directly interact with DNA. For this reason we should really regard them as *ligand-activated transcription factors* that transduce signals by modifying gene transcription. Another unique property is that NRs are not

embedded in membranes like GPCRs or ion channels (although see Ch. 33), 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.

The NR superfamily probably evolved from a single distant evolutionary ancestral gene by duplication and other events. In man, there are at least 48 members but more 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 (Burris et al., 2013), being responsible for the biological effects of approximately 10–15% 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 predominantly 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. NRs also regulate expression of many drug metabolising enzymes and transporters. Many illnesses are associated with malfunctioning of the NR system, including inflammation, cancer, diabetes, cardiovascular disease, obesity and reproductive disorders (see Kersten et al., 2000; Murphy & Holder, 2000).

STRUCTURE OF NUCLEAR RECEPTORS

▼ All NRs are monomeric proteins that share a broadly similar structural design (see Fig. 3.19 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 regulatory capacity 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 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* (HREs) located in genes that are regulated by this family of receptors, but it also plays a part in regulating receptor dimerisation.

It is the highly flexible *hinge region* in the molecule that allows it to dimerise with other NRs. This can produce molecular complexes with diverse configurations, able to interact differently with DNA. Finally, the *C-terminal domain* contains the ligand-binding module

and is specific to each class of receptor. The AF2 region is important in ligand-dependent activation and is generally highly conserved although absent in *Rev-erbAα* and *Rev-erbAβ*, NRs that regulate metabolism as part of a circadian molecular clock mechanism. 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.

CONTROL OF GENE TRANSCRIPTION

▼HREs are 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 large complexes of other 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. The case of CAR is particularly interesting; like some types of G proteins described earlier in this chapter, CAR can form a constitutively active complex that is terminated when it binds its ligand. The mechanisms of negative gene regulation by NRs are particularly complex (see Santos et al., 2011 for a good account of this phenomenon).

CLASSIFICATION OF NUCLEAR RECEPTORS

NRs are usually classified into subfamilies according to their phylogenetic development. For our purposes, however, it is more useful to make another distinction between them based on their molecular action. Mechanistically then, the NR superfamily consists of two main classes (I and II), and two other minor groups of receptors (III, IV).

Class I consists largely of endocrine steroid receptors, including the glucocorticoid and mineralocorticoid receptors (GR and MR), as well as the oestrogen, progesterone and androgen receptors (ER, PR and AR, respectively). The hormones (e.g. glucocorticoids) recognised by these receptors generally act in a negative feedback fashion to control biological events (see Ch. 33 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) into the cell from the blood,
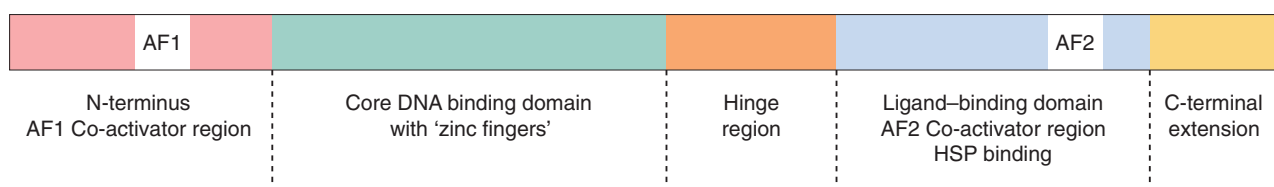

Fig. 3.19 Schematic diagram of a nuclear receptor. The heterogenous N-terminal domain harbours the AF1 (activation function 1) site. This binds cell-specific transcription factors that modify the properties of the receptor. The highly conserved core domain comprises two 'zinc fingers'; cysteine- (or cystine-/histidine-) rich loops in the amino acid chain that are held in a particular conformation by zinc ions and which are responsible for DNA recognition and binding. The flexible hinge region in the molecule allows the receptor to dimerise with other NRs, and the C-terminal domain, which contains the ligand-binding module, is specific to each class of receptor (see text for more details).

ligands bind their NR partner 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' HREs. Once bound, the NR recruits other proteins to form complexes that promote transcription of multiple genes. 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. 45). Indeed, PXR and CAR are akin to airport security guards who alert the bomb disposal squad when suspicious luggage is found. When they sense foreign molecules (xenobiotics), they induce drug-metabolising enzymes such as CYP3A (which is responsible for metabolising about 60% of all prescription drugs; see Ch. 9 and di Masi et al., 2009). They also bind some prostaglandins and nonsteroidal drugs, as well as the antidiabetic **thiazolidinediones** (see Ch. 31) and **fibrates** (see Ch. 23). Unlike the receptors in class I, these NRs almost always operate as heterodimers together with the retinoid X receptor (RXR).

Two types of heterodimer may then 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. Class II NRs are generally bound to co-repressor proteins. These dissociate when the ligand binds and allows recruitment of co-activator proteins and hence changes in gene transcription. They tend to mediate positive feedback effects (e.g. occupation of the receptor amplifies rather than inhibits a particular biological event).

Class III NRs are very similar to Class I in the sense that they form homodimers, but they can bind to HREs, which do not have an inverted repeat sequence. Class IV NRs may function as monomers or dimers but only bind to one HRE half site. Many of the remaining orphan receptors belong to these latter classes.

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).

Table 3.4 summarises the properties of some common NRs of importance to pharmacologists.

Table 3.4 Some common pharmacologically significant nuclear receptors

Receptor name	Abbreviation	Ligand	Drugs	Location	Ligand binding	Mechanism of action
Type I						
Androgen	AR	Tetosterone	All natural and synthetic glucocorticoids (Ch. 33), mineralocorticoids (Ch. 29) and sex steroids (Ch. 35) together with their antagonists (e.g. raloxifine, 4-hydroxy-tamoxifen and mifepristone)	Cytosolic	Homodimers	Translocation to nucleus. Binding to HREs with two half sites with an inverted sequence
Oestrogen	ERα,β	17β-oestradiol				
Glucocorticoid	GRα	Cortisol, corticosterone				
Progesterone	PR	Progesterone				
Mineralocorticoid	MR	Aldosterone				
Type II						
Retinoid X	RXR α,β,γ	9-*cis*-retinoic acid	Retinoid drugs (Ch. 27)	Nuclear	Heterodimers often with RXR	Complexed with co-repressors, which are displaced following ligand binding, allowing the binding of transactivators
Retinoic acid	RAR α,β,γ	Vitamin A				
Thyroid hormone	TR α,β	T3, T4	Thyroid hormone drugs			
Peroxisome proliferator	PPAR α,β,γ,δ	Fatty acids, prostaglandins	Rosiglitazone, pioglitazone			
Constitutive androstane	CAR	Androstane	Stimulation of CYP synthesis and alteration of drug metabolism			
Pregnane X	PXR	Xenobiotics				

Only examples from Classes I and II are included.

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Nuclear receptors

- A family of 48 soluble receptors that sense lipid and hormonal signals and modulate gene transcription.
- Their ligands are many and varied, including steroid drugs and hormone, thyroid hormones, vitamins A and D, various lipids and xenobiotics
- There are two main categories:
  - Class I NRs 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)
  - Class II NRs 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).
- 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 the target of approximately 10% of prescription drugs, and the enzymes that it regulates affect the pharmacokinetics of some 60% of all prescription drugs.

</div>

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, but drugs can act upon them to alter their ability to open and close.[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 Ch. 4). Electrophysiology, particularly the *voltage clamp technique*, 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 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 background 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

In the main these channels open when the cell membrane is depolarised.[13] 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 Fig. 3.5) are activated by binding of a chemical ligand to a site on the channel molecule. Fast neurotransmitters, such as glutamate, acetylcholine, GABA, 5-hydroxytryptamine and ATP (see Chs 13, 16 and 38) act in this way, binding to sites on the outside of the membrane. In addition there are also ligand-gated ion channels that do not respond to neurotransmitters but to changes in their local environment. For example, the TRPV1 channel on sensory nerves that mediates the pain-producing effect of the chilli pepper ingredient capsaicin responds to extracellular protons when tissue pH falls, as occurs in inflamed tissue, as well as to the physical stimulus, heat (see Ch. 42).

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[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.

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[13]There is always an exception to the rule! The members of the HCN family of potassium channels found in neurons and cardiac muscle cells are activated by hyperpolarisation.

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.
- Calcium-activated chloride channels, widely expressed in excitable and non-excitable cells where they are involved in diverse functions such as epithelial secretion of electrolytes and water, sensory transduction, regulation of neuronal and cardiac excitability and regulation of vascular tone.
- 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. 31), where they are part of the mechanism linking insulin secretion to blood glucose concentration.

Other examples of cell membrane 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

The main ones, IP$_3$ and **ryanodine** receptors (see Ch. 4), are a special class of ligand-gated calcium channels that are present on the endoplasmic or sarcoplasmic reticulum rather than the plasma membrane and control the release of Ca^{2+} from intracellular stores. Ca^{2+} can also be released from lysosomal stores by nicotinic acid adenine dinucleotide phosphate activating two pore domain calcium channels.

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 the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, to remain elevated even when the intracellular 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 voltage-gated sodium channel was cloned. The main structural subtypes are shown in Figure 3.20. All consist of several (often four) domains, which are similar or identical to each other,

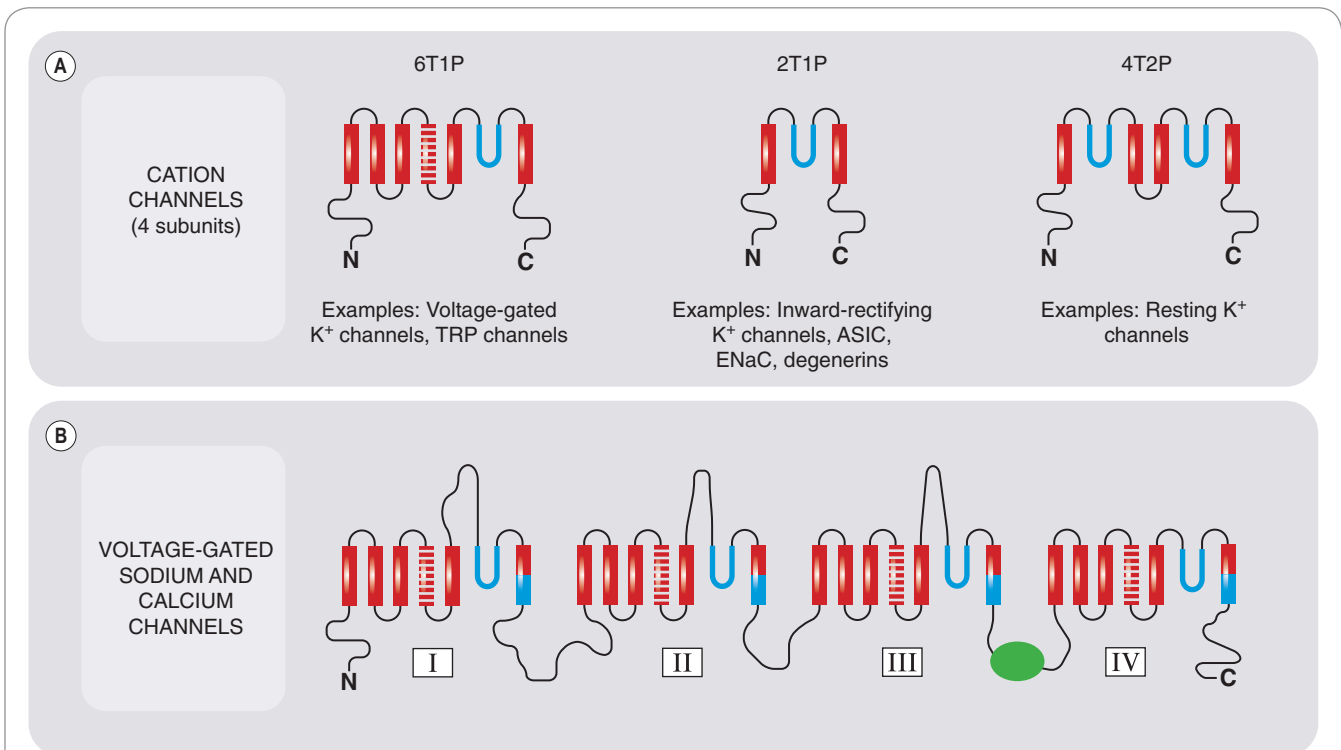**Fig. 3.20** **Molecular architecture of ion channels.** Red and blue rectangles represent membrane-spanning α-helices. Blue hairpins are pore loop (P) domains, present in many channels, blue rectangles being the pore-forming regions of the membrane-spanning α-helices. Cross-shaded rectangles represent 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. ASIC, acid-sensing ion channel; ENaC, epithelial sodium channel; TRP, transient receptor potential channel.

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.

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.[14] 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 3.20A, whereas others are classed as 'two-pore domain' channels, because each subunit contains two P loops.

The various architectural motifs shown in Figure 3.20 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. 31) regulate the channel. 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.

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. 3.21). Ion channel pharmacology is likely to be a fertile source of future new drugs.

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 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
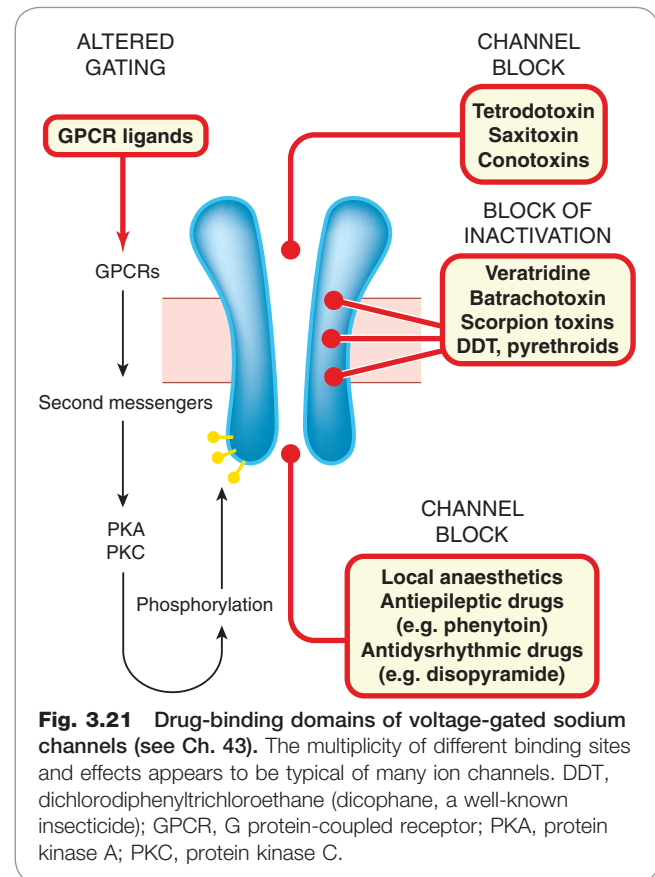

Fig. 3.21 **Drug-binding domains of voltage-gated sodium channels (see Ch. 43).** The multiplicity of different binding sites and effects appears to be typical of many ion channels. DDT, dichlorodiphenyltrichloroethane (dicophane, a well-known insecticide); GPCR, G protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C.

these intracellular mediators. Increased $[Ca^{2+}]_i$ opens certain types of potassium and chloride 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. 31) act selectively on ATP-gated potassium channels.

Figure 3.21 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 induces adaptive responses, which, particularly with drugs that act on the central nervous system, can limit their effectiveness as in opioid tolerance (see Ch. 42) or

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[14]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.

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can be the basis for therapeutic efficacy. In the latter instance this may take the form of a very slow onset of the therapeutic effect (e.g. with antidepressant drugs; see Ch. 47). 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. 56).

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, ion channels 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 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 29 and 33) 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. 28) and a poor prognosis in patients with cardiac failure (Ch. 21). 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).

Mutations in ligand-gated ion channels ($GABA_A$ and nicotinic) and other ion channels (Na^+ and K^+) that alter their function give rise to some forms of idiopathic epilepsy (see Ch. 45 and Guerrini et al., 2003).

Research on genetic polymorphisms affecting receptors, signalling molecules, ion channels and effector enzymes is continuing apace, and it is expected that a clearer understanding of the variability between individuals in their disease susceptibility and response to therapeutic drugs (see Ch. 57) will result in the foreseeable future.

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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 Nestler et al. (2008), Berridge (2012) and Kandel et al. (2013).

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 100 nmol/l. The Ca^{2+} concentration in extracellular fluid, $[Ca^{2+}]_o$, is about 2.4 mmol/l, 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 Clapham, 2007; Berridge, 2009).
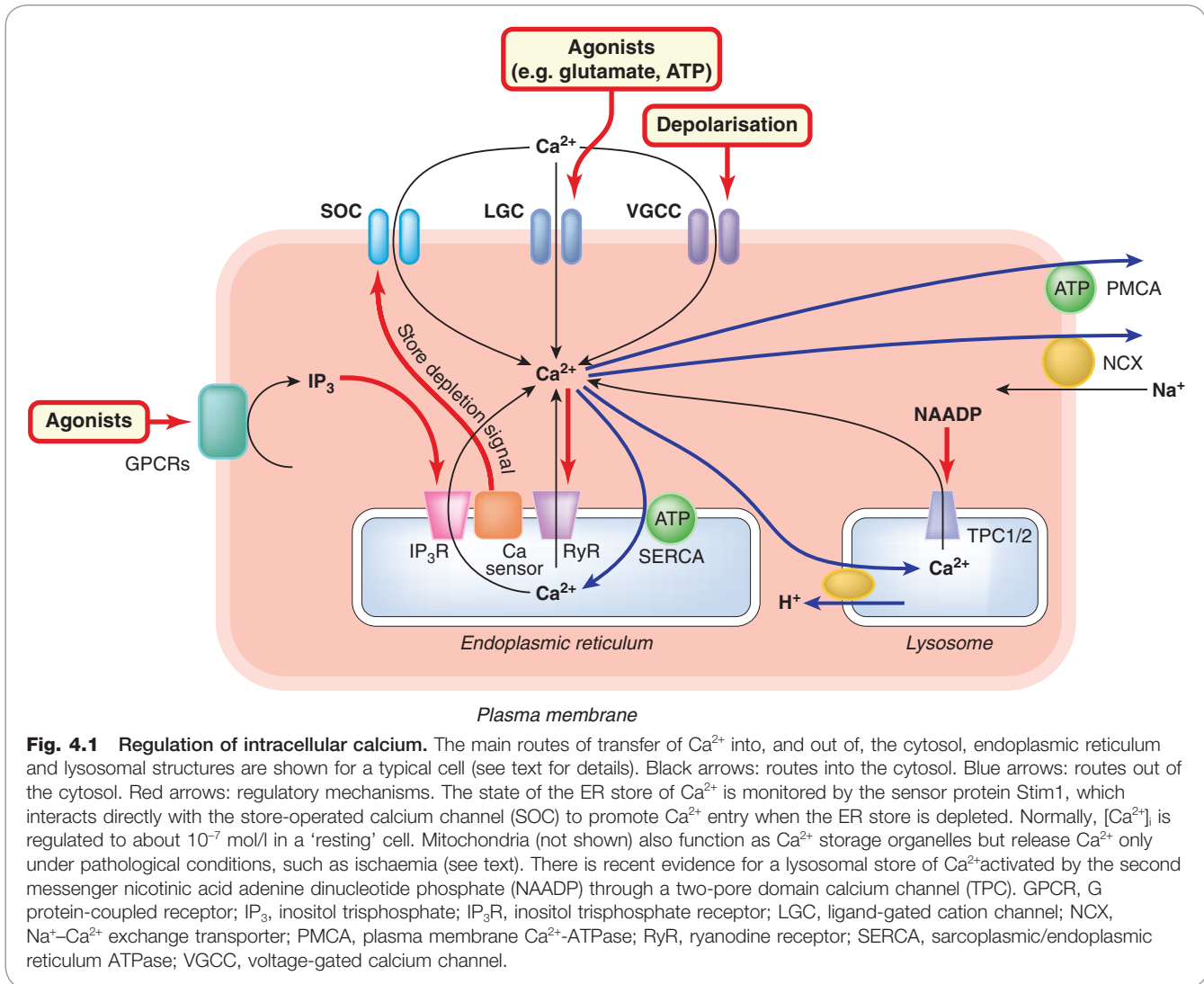
CALCIUM ENTRY MECHANISMS

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*, p. 53).

VOLTAGE-GATED CALCIUM CHANNELS

The pioneering work of Hodgkin and Huxley on the ionic basis of the nerve action potential (see p. 55) 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 not conduct Na^+ or K^+; they are ubiquitous in excitable cells

Fig. 4.1 Regulation of intracellular calcium. The main routes of transfer of Ca^{2+} into, and out of, the cytosol, endoplasmic reticulum and lysosomal structures 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 recent evidence for a lysosomal store of Ca^{2+}activated by the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) through a two-pore domain calcium channel (TPC). 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.

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 and two subunits from the same gene, α2δ, linked by a disulfide bond) 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 p. 60 and 61), and N channels (and also P/Q) are involved in neurotransmitter and hormone release, while T channels mediate Ca^{2+} entry into neurons around the resting membrane potential and can control the rate of repolarisation of neurons and cardiac cells as well as various Ca^{2+}-dependent functions such as regulation of other channels, enzymes, etc. Clinically used drugs that act directly on some forms of calcium channel 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 42 and 45). 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

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[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*. Although P stands for *Purkinje* – this type of channel was first observed in cerebellar Purkinje cells – it continued the alphabetical sequence (missing out O of course) and so the next discovered were termed Q and R.

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; and 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. 42)
	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-4A (component of funnel-web spider venom)
	R	Low threshold Fast inactivation	Neurons and dendrites Control of firing patterns	Blocked by low concentrations of SNX-482 (a toxin from a member of the tarantula family)
Inositol-trisphosphate	IP$_3$ receptor	Activated by Ca^{2+} and ATP in the presence of IP$_3$	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 skeletal muscle via dihydropyridine receptor of T-tubules. Activated by Ca^{2+} in cardiac muscle	Located in endoplasmic/sarcoplasmic reticulum. Pathway for Ca^{2+} release in striated muscle	Activated by caffeine and ATP in the presence of Ca^{2+} Ryanodine both activates (low concentrations) and closes (high concentrations) the channel. Also closed by Mg^{2+}, K$^+$ channel blockers and dantrolene Mutations may lead to drug-induced malignant hypothermia, sudden cardiac death and central core disease
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

receptor of the NMDA type (Ch. 38), 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 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. 40).

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 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$_3$R) 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$_3$R 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 $[Ca^{2+}]_i$.
* *Ryanodine receptors* (RyR) are so called because they were first identified through the specific blocking action of the plant alkaloid **ryanodine**. There are three isoforms – RyR1–3 (Van Petegem, 2012) that are expressed in many different cell types. RyR1 is highly expressed in skeletal muscle, RyR2 in the heart, and RyR3 in brain neurons. In skeletal muscle RyRs on the SR are physically coupled to *dihydropyridine receptors* on the T-tubules (see Fig. 4.9); this coupling results in Ca^{2+} release following the action potential in the muscle fibre. In other muscle types RyRs respond to Ca^{2+} that enters the cell through membrane calcium channels by a mechanism known as *calcium-induced calcium release* (CICR).

The functions of IP$_3$Rs 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. 48) 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. 41), which is associated with inherited abnormalities in the RyR protein.

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 adenine 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 two-pore domain calcium channels.

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 Morgan et al., 2011).

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 40) 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 either by binding directly to these proteins or through a Ca^{2+}-binding protein that 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

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[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.
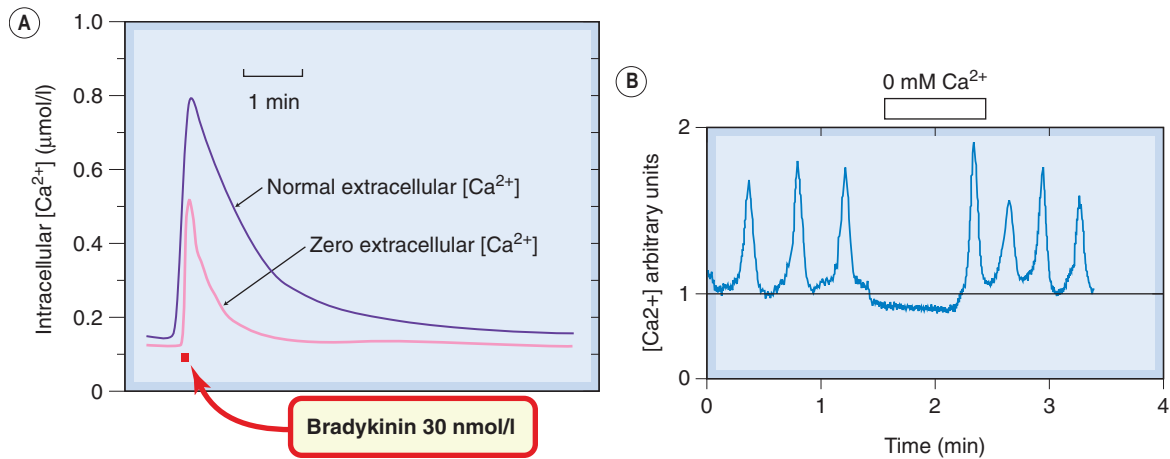
Fig. 4.2 **[A] Increase in intracellular free calcium concentration in response to receptor activation.** The records were obtained from a single rat sensory neuron grown in tissue culture. The cells were loaded with the fluorescent Ca^{2+} indicator Fura-2, and the signal from a single cell monitored with a fluorescence microscope. A brief exposure to the peptide bradykinin, which causes excitation of sensory neurons (see Ch. 42), 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 GM Burgess and A Forbes.)* **[B] Spontaneous intracellular 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 N, Hollywood M, Sargeant G 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), lysosomes 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.

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.

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 skeletal, 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 skeletal 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 control-ling the state of its interior, and it requires a continuous supply of energy to do so. In relation to the topics dis-cussed in this chapter, the following characteristics are especially important:

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

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 gener-ally 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 p. 57–60) 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^+ perme-ability causes an inward (depolarising) current of Na^+ ions, whereas an increase in K^+ permeability causes an outward (repolarising) 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 poten-tial 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, repo-larisation is assisted by the opening of voltage-dependent
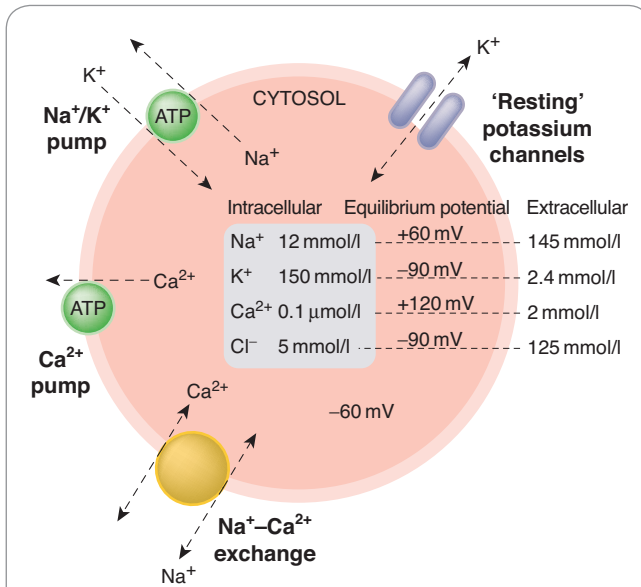
Fig. 4.3 **Simplified diagram showing the ionic balance of a typical 'resting' cell.** The main transport mechanisms that maintain the ionic gradients across the plasma membrane are the ATP-driven Na^+–K^+ and Ca^{2+} pumps and the Na^+–Ca^{2+} exchange transporter. The membrane is relatively permeable to K^+, because some types of potassium channel 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.
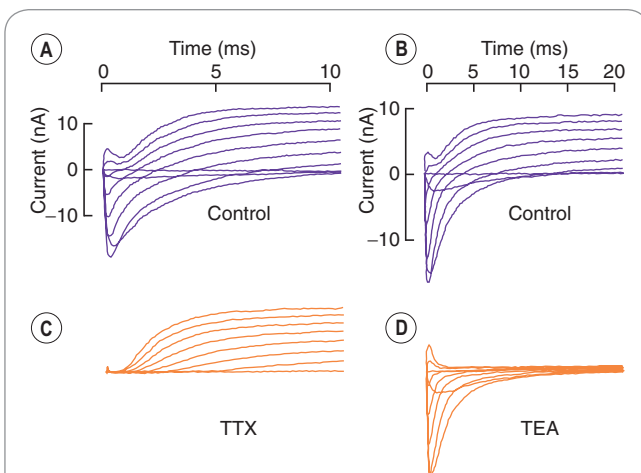
Fig. 4.4 **Separation of sodium and potassium currents in the nerve membrane.** Voltage clamp records from the node of Ranvier of a single frog nerve fibre. At time 0, the membrane potential was stepped to a depolarised level, ranging from −60 mV (lower trace in each series) to +60 mV (upper trace in 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.)*
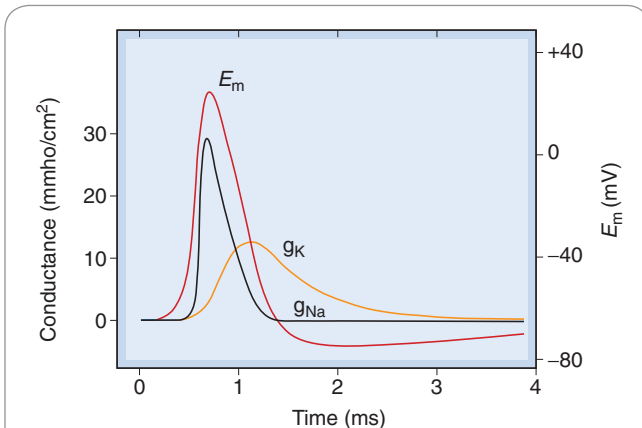
Fig. 4.5 **Behaviour of sodium and potassium channels during a conducted action potential.** Rapid opening of sodium channels occurs during the action potential upstroke. Delayed opening of potassium channels, and inactivation of sodium channels, causes repolarisation. E_m, membrane potential; g_{Na}, g_K, membrane conductance to Na^+, K^+.

K^+ 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. Subsequently (see Hille, 2001), voltage-gated calcium channels (see Fig. 4.1) were discovered. These function in basically the same way as sodium channels if on a slightly slower timescale; 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 on p. 50-54.

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. Inherited mutations of channel proteins are responsible for a wide variety of (mostly rare) neurological and other genetic disorders (see Ashcroft, 2000, 2006).
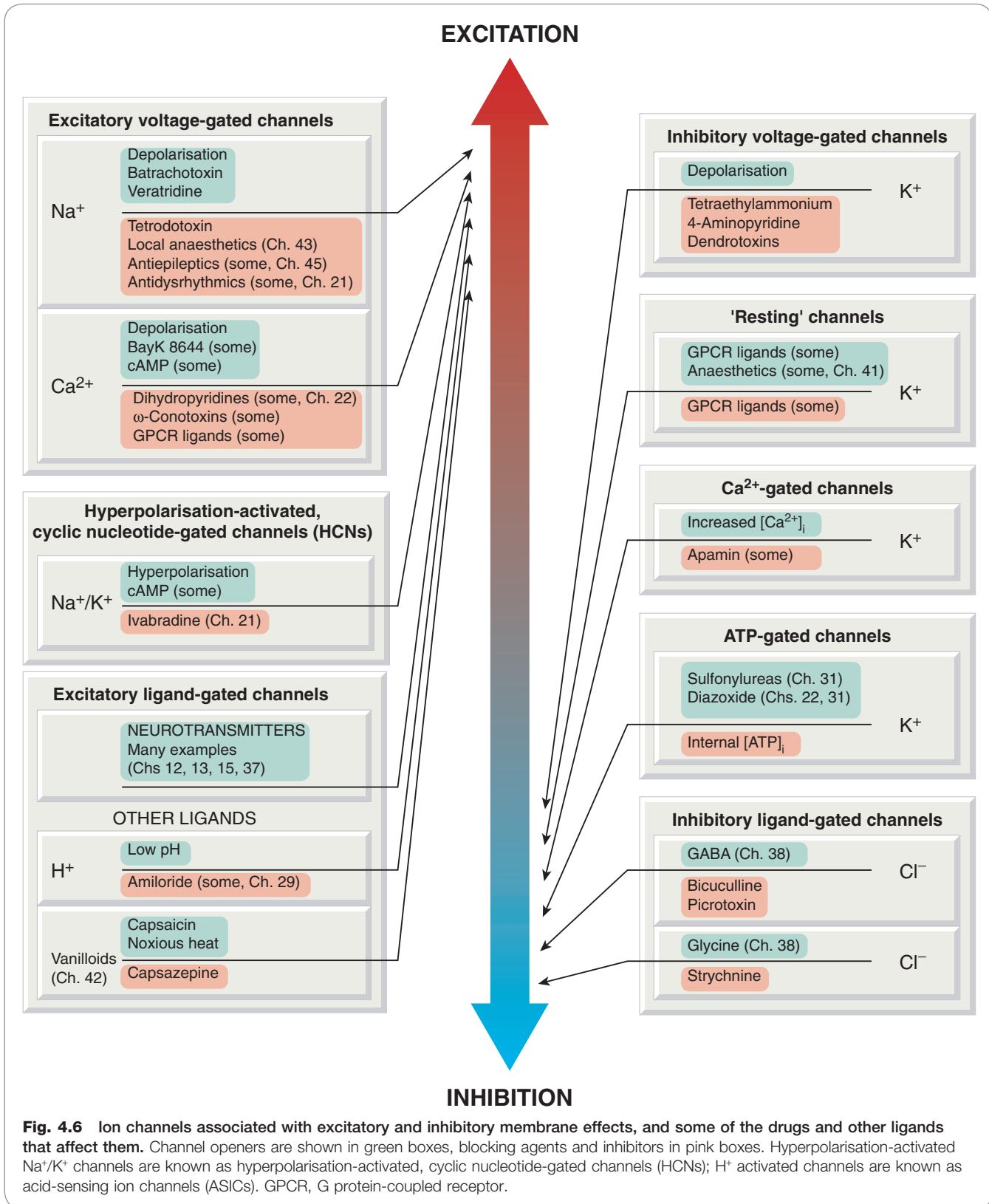
USE DEPENDENCE AND VOLTAGE DEPENDENCE

▼ 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. 43), antidysrhythmic drugs (Ch. 21) and antiepileptic drugs (Ch. 45), 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. 45), 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. 43) 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

EXCITATION

Excitatory voltage-gated channels

Na$^+$
- Depolarisation
Batrachotoxin
Veratridine
- Tetrodotoxin
Local anaesthetics (Ch. 43)
Antiepileptics (some, Ch. 45)
Antidysrhythmics (some, Ch. 21)

Ca^{2+}
- Depolarisation
BayK 8644 (some)
cAMP (some)
- Dihydropyridines (some, Ch. 22)
ω-Conotoxins (some)
GPCR ligands (some)

**Hyperpolarisation-activated,
cyclic nucleotide-gated channels (HCNs)**

Na$^+$/K$^+$
- Hyperpolarisation
cAMP (some)
- Ivabradine (Ch. 21)

Excitatory ligand-gated channels

- NEUROTRANSMITTERS
Many examples
(Chs 12, 13, 15, 37)

OTHER LIGANDS

H$^+$
- Low pH
- Amiloride (some, Ch. 29)

Vanilloids
(Ch. 42)
- Capsaicin
Noxious heat
- Capsazepine

Inhibitory voltage-gated channels

- Depolarisation
- Tetraethylammonium
4-Aminopyridine
Dendrotoxins
K$^+$

'Resting' channels

- GPCR ligands (some)
Anaesthetics (some, Ch. 41)
- GPCR ligands (some)
K$^+$

Ca^{2+}-gated channels

- Increased [Ca^{2+}]$_i$
- Apamin (some)
K$^+$

ATP-gated channels

- Sulfonylureas (Ch. 31)
Diazoxide (Chs. 22, 31)
- Internal [ATP]$_i$
K$^+$

Inhibitory ligand-gated channels

- GABA (Ch. 38)
- Bicuculline
Picrotoxin
Cl$^-$

- Glycine (Ch. 38)
- Strychnine
Cl$^-$

INHIBITION

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. Hyperpolarisation-activated Na$^+$/K$^+$ channels are known as hyperpolarisation-activated, cyclic nucleotide-gated channels (HCNs); H$^+$ activated channels are known as acid-sensing ion channels (ASICs). GPCR, G protein-coupled receptor.

voltage-gated sodium channels. The early voltage clamp studies by Hodgkin and Huxley on the squid giant axon, described on p. 55, 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. 43) to label and purify the channel proteins, and subsequently to clone them. Sodium channels consist of a central, pore forming α subunit (shown in Fig. 3.20) and two auxiliary β subunits. Nine α-subunits (Na$_V$1.1 through Na$_V$1.9) and four β subunits have been identified in mammals. The α subunits contain four similar domains each comprising six
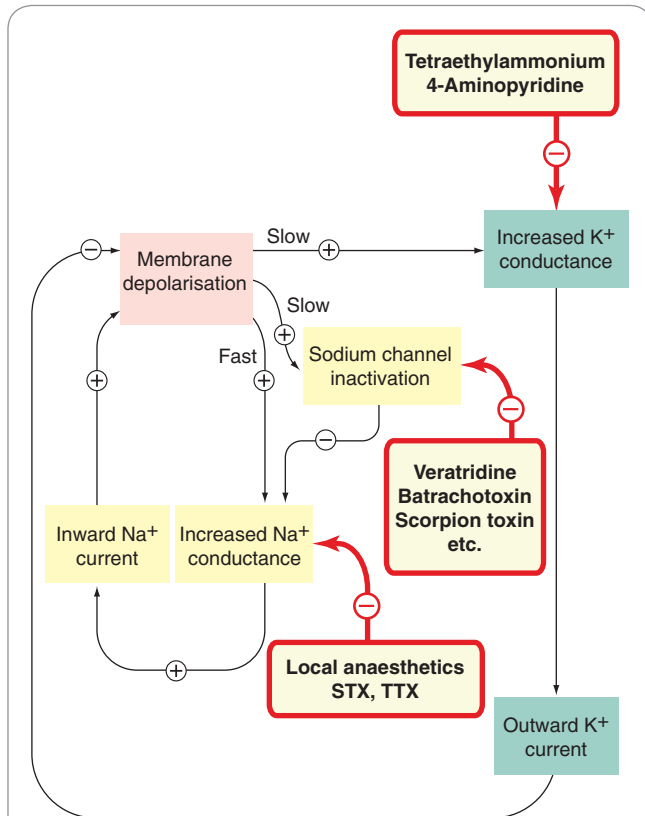
Fig. 4.7 Sites of action of drugs and toxins that affect channels involved in action potential generation. Many other mediators affect these channels indirectly via membrane receptors, through phosphorylation or altered expression. STX, saxitoxin; TTX, tetrodotoxin.
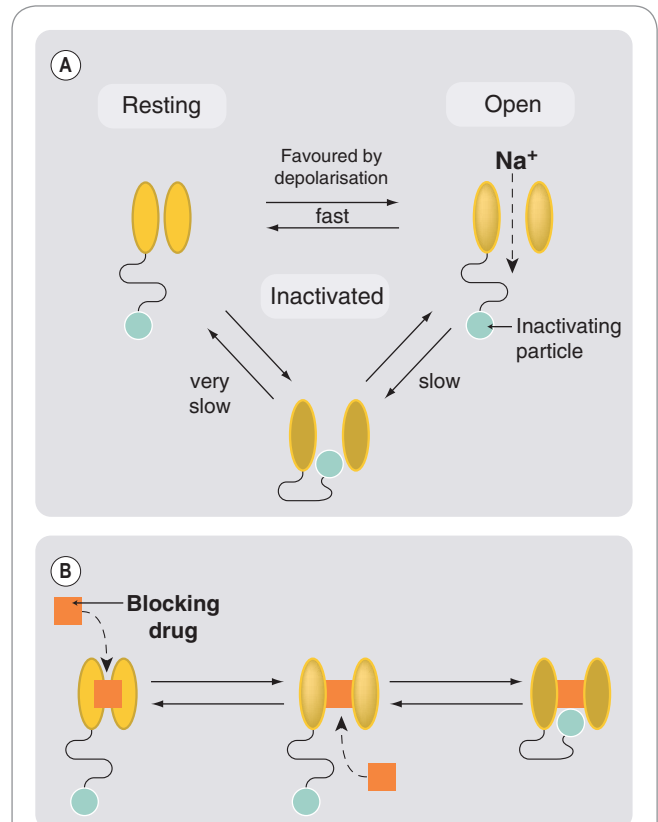

Fig. 4.8 Resting, activated and inactivated states of voltage-gated channels, exemplified by the sodium channel. [**A**] Membrane depolarisation causes a rapid transition from the resting (closed) state to the open state. The inactivating particle (part of the intracellular domain of the channel protein) is then able to block the channel. With prolonged depolarization, below the threshold for opening, channels can go directly from resting to inactivated without opening. [**B**] Some blocking drugs (such as tetrodotoxin) block the channel from the outside, like a plug, whereas others (such as local anaesthetics and antiepileptic drugs) enter from the inside of the cell and often show preference for the open or inactivated states, and thus affect the kinetic behaviour of the channels, with implications for their clinical application.

membrane-spanning helices (reviewed by 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. This is explained by the relative insensitivity of some α subunits ($Na_v1.5$, $Na_v1.8$ and $Na_v1.9$) to tetrodotoxin. Changes in the level of expression of some sodium channel subunits is thought to underlie the hyperexcitability of sensory neurons in different types of neuropathic pain (see Ch. 42).

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.

POTASSIUM CHANNELS

In a typical resting cell (see p. 55, Fig. 4.3), 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 some 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

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 Some are blocked by tertiapin (from honey bee venom)	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 sulfonylurea receptor (SUR) results in modulation by sulfonylureas (e.g. glibenclamide) which close channel, and by K⁺ channel openers (e.g. diazoxide, minoxidil) 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. isoflurane) No selective blocking agents Modulation by GPCR agonists and antagonists	The nomenclature is misleading, especially when they are incorrectly referred to as two-pore channels

GPCR, G protein-coupled receptor.

[a]K⁺ channel structures (see Fig 3.20) 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.

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), Jenkinson (2006), Alexander et al. (2013).

▼ Potassium channels fall into three main classes (Table 4.2),[3] of which the structures are shown in Figure 3.20.

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[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.

- *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 electro-physiologists, and others such as Ca^{2+}-activated potassium channels and two subtypes that are important in the heart, HERG and LQT channels. 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. 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. 31) and smooth muscle relaxant drugs, such as **minoxidil** 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 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 **isoflurane** (Ch. 41).

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. Drug-induced prolongation of the *QT* interval is an unwanted side effect. Nowadays new drugs are screened for this property at an early stage in the development process (see Ch. 60). Certain familial types of deafness and epilepsy are associated with mutations in voltage-gated potassium channels (Ashcroft, 2000, 2006).

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 inactivation 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.

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 and skeletal muscle contractility are also the targets of important drug effects.

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 endplate (see Ch. 13), to the rest of the fibre. The T-tubule membrane contains voltage-gated calcium channels termed dihydropyridine receptors (DHPRs),[4] that respond to membrane depolarisation conducted passively along the T-tubule when the plasma membrane is invaded by an action potential. DHPRs 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. Direct coupling between the DHPRs of the T-tubule and the RyRs of the SR (as shown in Fig. 4.9) causes the opening of the RyRs on membrane depolarisation. 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.

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. 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. T-tubules in cardiac muscle contain L-type calcium channels, which open during this plateau and allow Ca^{2+} to enter. This Ca^{2+} entry acts on RyRs (a different molecular type from those of skeletal muscle) to release Ca^{2+} from the SR (Fig. 4.9).

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[4]Although these are, to all intents and purposes, just a form of L-type calcium channel the term dihydropyridine receptor (DHPR) is used to reflect that they are not identical to the L-type channels in neurons and cardiac muscle.
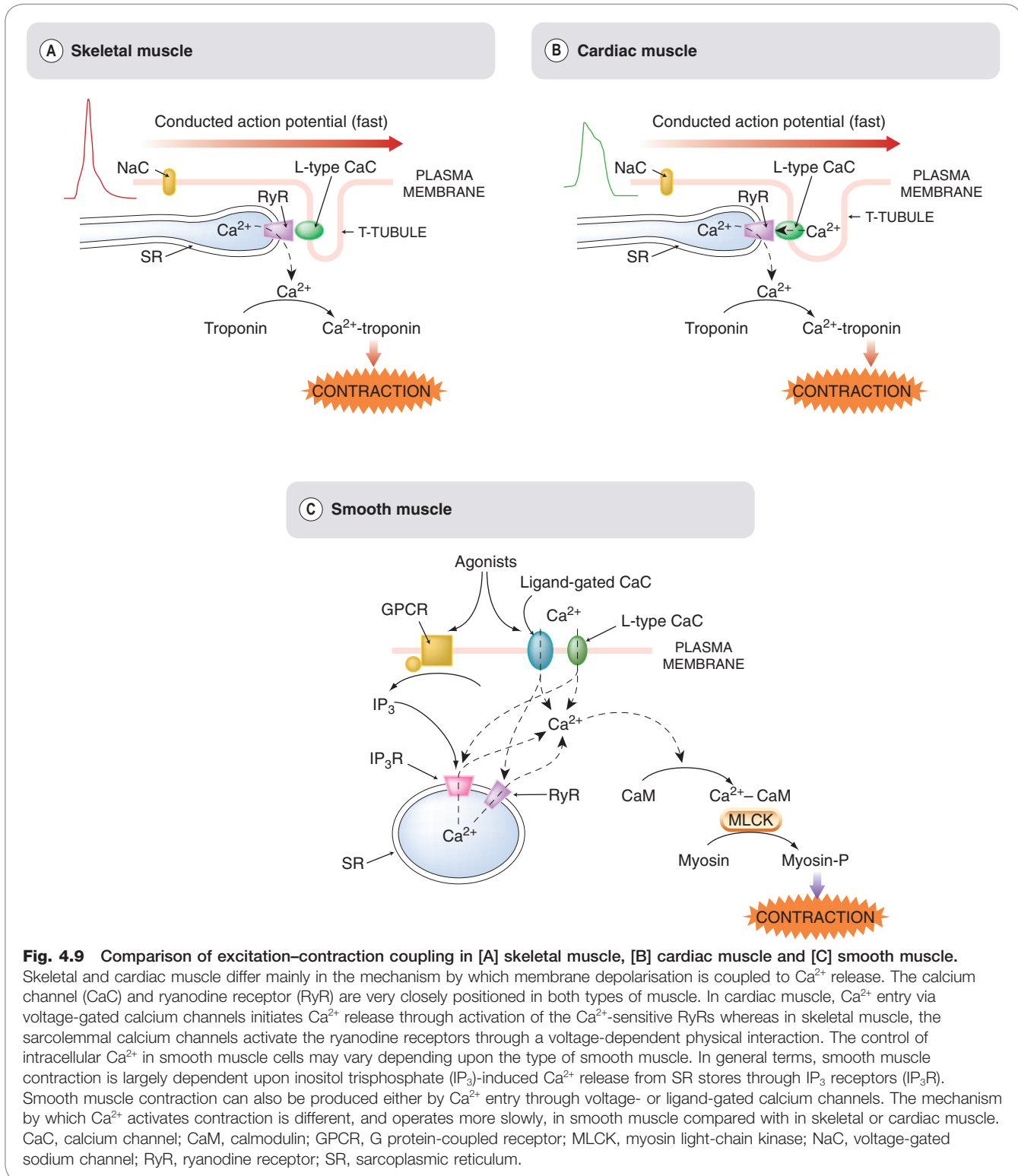
Fig. 4.9 Comparison of excitation–contraction coupling in [A] skeletal muscle, [B] cardiac muscle and [C] smooth muscle. Skeletal and cardiac muscle differ mainly in the mechanism by which membrane depolarisation is coupled to Ca^{2+} release. The calcium channel (CaC) and ryanodine receptor (RyR) are very closely positioned in both types of muscle. In cardiac muscle, Ca^{2+} entry via voltage-gated calcium channels initiates Ca^{2+} release through activation of the Ca^{2+}-sensitive RyRs whereas in skeletal muscle, the sarcolemmal calcium channels activate the ryanodine receptors through a voltage-dependent physical interaction. The control of intracellular Ca^{2+} in smooth muscle cells may vary depending upon the type of smooth muscle. In general terms, smooth muscle contraction is largely dependent upon inositol trisphosphate (IP_3)-induced Ca^{2+} release from SR stores through IP_3 receptors (IP_3R). Smooth muscle contraction can also be produced either by Ca^{2+} entry through voltage- or ligand-gated calcium channels. The mechanism by which Ca^{2+} activates contraction is different, and operates more slowly, in smooth muscle compared with in skeletal or cardiac muscle. CaC, calcium channel; CaM, calmodulin; GPCR, G protein-coupled receptor; MLCK, myosin light-chain kinase; NaC, voltage-gated sodium channel; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

With minor differences, the subsequent mechanism by which Ca^{2+} activates the contractile machinery is the same as in skeletal muscle. Ca^{2+}-induced Ca^{2+} release via RyRs may play a role in some forms of cardiac arrhythmia. It has been suggested that the antidysrhythmic effects of **flecainide** and β blockers may be due, in part, to their ability to reduce this release. Mutations of RyRs are implicated in various disorders of skeletal and cardiac muscle function (see Priori & Napolitano, 2005).

SMOOTH MUSCLE

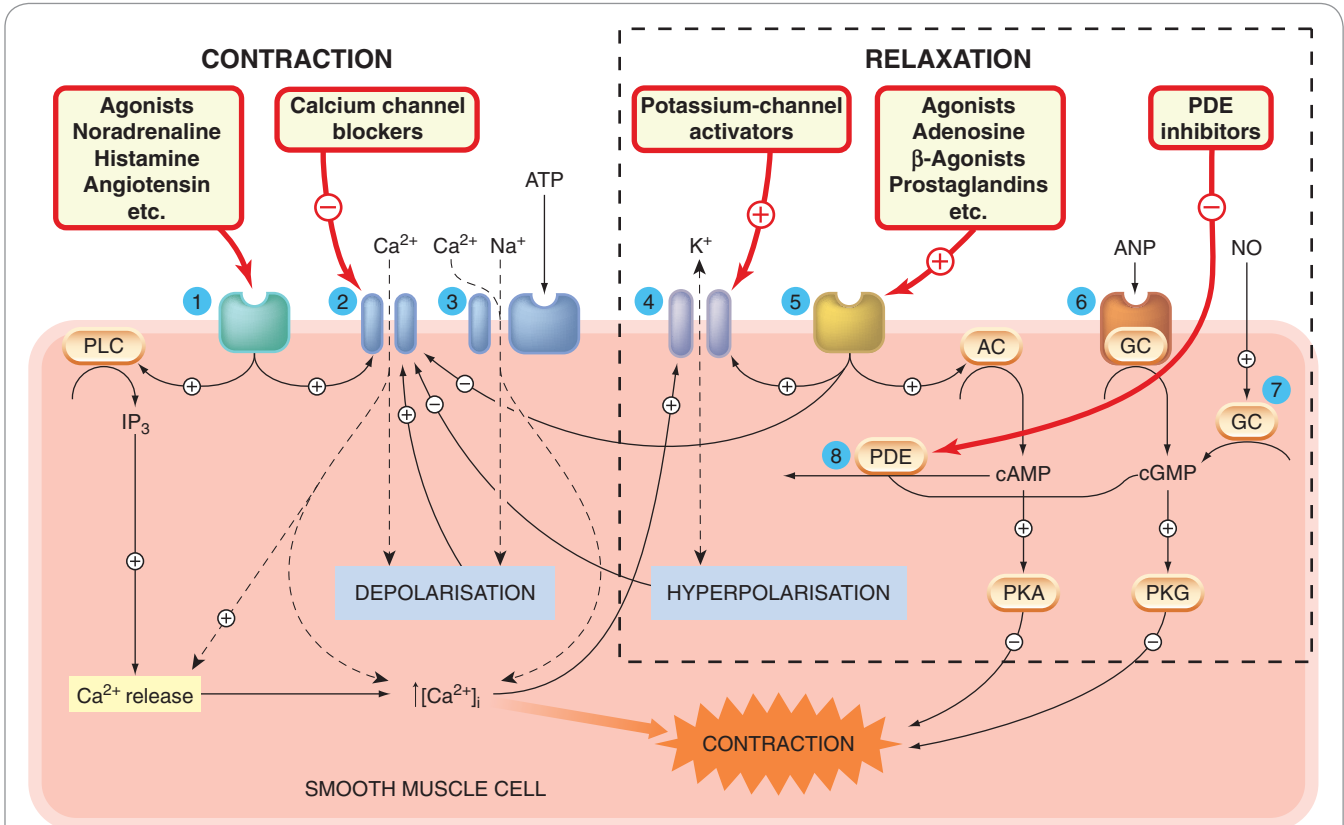The properties of smooth muscle vary considerably in different organs, and the mechanisms linking membrane

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, adenylyl cyclase; PKA, protein kinase A; PKG, protein kinase G; PLC, phospholipase C.

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 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. RyRs are also present in many smooth muscle cells and calcium-induced Ca^{2+} release via these channels may play a role in generating muscle contraction (Fig. 4.9) or couple to plasma membrane calcium-activated K^+ channels to

regulate membrane potential and thereby Ca^{2+} entry through voltage-gated calcium channels (Fig. 4.10).

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. 61, Fig. 4.9). 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.

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.

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 comprises all the conventional neurotransmitters and neuromodulators (see Chs 12 and 36), and many hormones. It also includes secreted proteins such as cytokines and various growth factors (Ch. 18).
- Mediators that are produced on demand and are released by diffusion or by membrane carriers.[5] This group includes nitric oxide (Ch. 20) and many lipid mediators (e.g. prostanoids, Ch. 17) and endocannabinoids (Ch. 19), which are released from the postsynaptic cell to act retrogradely on nerve terminals.

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. 18) and *angiotensin* (Ch. 22), which are peptides produced by protease-mediated cleavage of circulating proteins.

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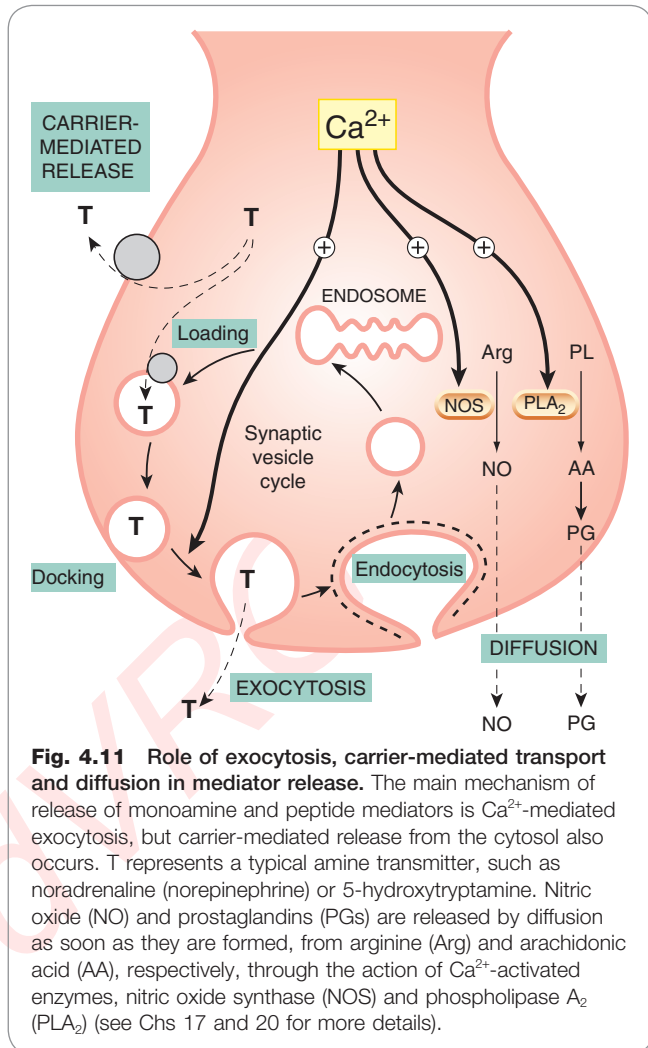

Fig. 4.11   Role of exocytosis, carrier-mediated transport and diffusion in mediator release. The main mechanism of release of monoamine and peptide mediators is Ca^{2+}-mediated exocytosis, but carrier-mediated release from the cytosol also occurs. T represents a typical amine transmitter, such as noradrenaline (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).

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 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 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,

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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/Q type (see p. 52, Table 4.1), 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. Elsewhere, where speed is less critical, Ca^{2+} may come from intracellular stores, 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, 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.
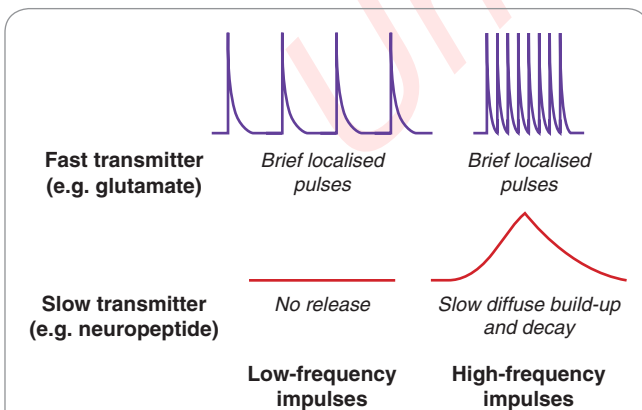

Fig. 4.12 **Time course and frequency dependence of the release of 'fast' and 'slow' transmitters.** Fast transmitters (e.g. glutamate) are stored in synaptic vesicles that are 'docked' close to voltage-gated calcium channels in the membrane of the nerve terminal, and are released in a short burst when 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.

Having undergone exocytosis, the empty vesicle[6] 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.

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 (see Fig. 4.11). Drugs such as **amphetamines**, which release amines from central and peripheral nerve terminals (see Chs 14 and 39), 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 from the cytosol 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 Fig 12.5 and 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 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.

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[6]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*).

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. 29). 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. 29), 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
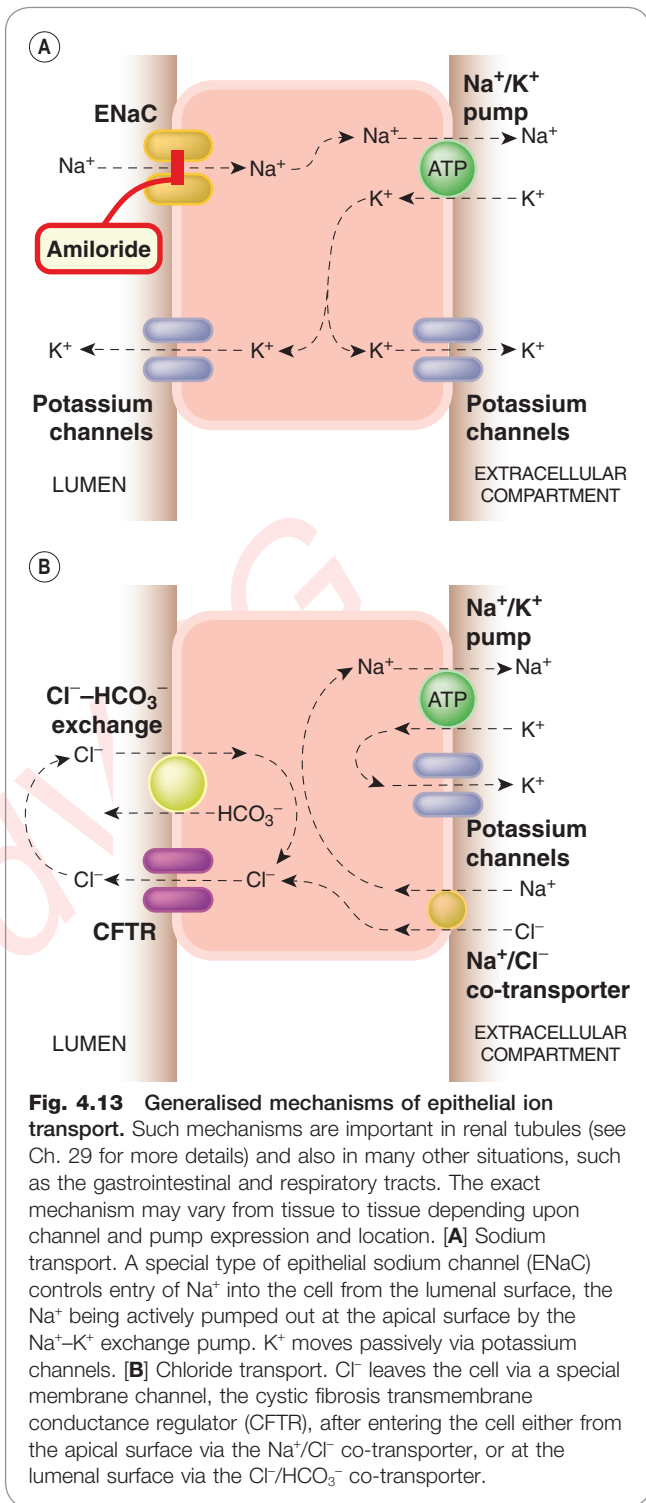
Fig. 4.13 Generalised mechanisms of epithelial ion transport. Such mechanisms are important in renal tubules (see Ch. 29 for more details) and also in many other situations, such as the gastrointestinal and respiratory tracts. The exact mechanism may vary from tissue to tissue depending upon channel and pump expression and location. [**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.

significant therapeutic advance. So far, no therapeutic 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

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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 [CFTR]), 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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Cell proliferation, apoptosis, repair and regeneration

5

OVERVIEW

About 10 billion new cells are created daily through cell division and this must be counterbalanced by the elimination of a similar number from the body in an ordered manner. This chapter explains how this is managed. We deal with the life and death of the cell – the processes of replication, proliferation, apoptosis, repair and regeneration and how these relate to the actions of drugs. We begin with cell replication. We explain how stimulation by growth factors causes cells to divide and then consider the interaction of these cells with the extracellular matrix which regulates further cell proliferation. We describe the crucial 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 culminate in its demise. We explain how these processes relate to the repair of damaged tissue, to the possibility of its regeneration and whether there is scope for modulating this with novel drugs.

CELL PROLIFERATION

Cell proliferation is, of course, a fundamental biological event. It is integral to many physiological and pathological processes including growth, healing, repair, hypertrophy, hyperplasia and the development of tumours. Because cells need oxygen to survive, *angiogenesis* (the development of new blood vessels) necessarily accompanies many of these processes.

Proliferating cells go through what is termed *the cell cycle*, during which they replicate all their components and then divide into two identical daughter cells. The process is tightly regulated by signalling pathways including 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

In the adult, few cells divide repeatedly and most remain in a quiescent phase outside the cycle in the phase termed G_0 (Fig. 5.1). Some cells such as neurons and skeletal muscle cells spend all their lifetime in G_0 whereas others, including bone marrow cells and the epithelium of the gastrointestinal tract, divide daily.

The cell cycle is an ordered sequential series of phases (Fig. 5.1). These are known as:

- G_1: preparation for DNA synthesis
- S: DNA synthesis and chromosome duplication
- G_2: preparation for division
- M: mitosis, 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 the critical S phase and M phases. Entry into each of these phases is tightly regulated at *check points* (restriction points) at the start of the S and M phases. DNA damage stops the cycle at one or other of these check points and the integrity of this process is critical for the maintenance of genetic stability. Failure of the check points to stop the cycle when it is appropriate to do so is a hallmark of cancer.

Quiescent cells enter G_1 after exposure to chemical mediators, some of which are associated with damage. For example, a wound can stimulate a quiescent skin cell to divide, thus repairing the lesion. The impetus for a cell to enter the cycle (i.e. to move from G_0 into G_1) may be *growth factors* acting on *growth factor receptors*, though the action of other types of ligands on G protein-coupled receptors (see Ch. 3) can also initiate the process.

Growth factors stimulate the synthesis of both positive regulators of the cell cycle that control the changes necessary for cell division and negative regulators that counterbalance the positive regulators. The maintenance of normal cell numbers in tissues and organs requires a balance between the positive and the negative regulatory signals. *Apoptosis*[1] also controls cell numbers.
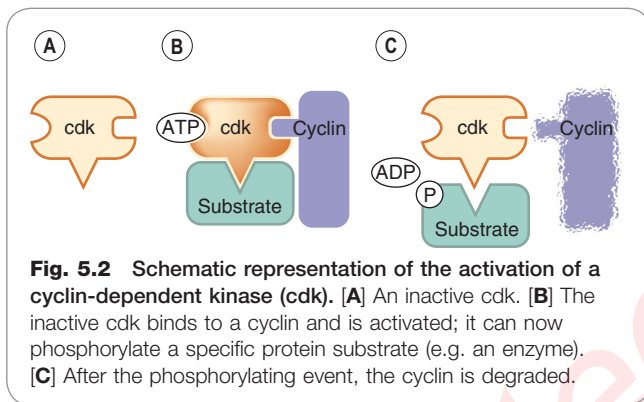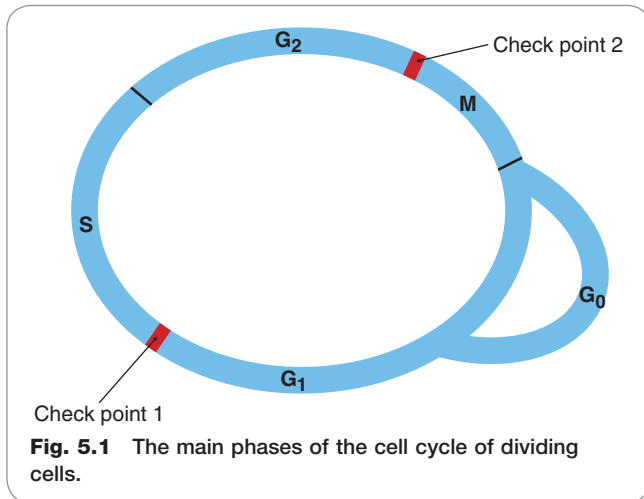
POSITIVE REGULATORS OF THE CELL CYCLE

The cycle begins when a growth factor acts on a quiescent cell, provoking it to divide. Growth factors stimulate production of two families of proteins, namely *cyclins* and serine/threonine protein kinases called *cyclin-dependent kinases* (cdks), coded for by the *delayed response* genes. The cdks sequentially phosphorylate various enzymes – activating some and inhibiting others – to coordinate the progression of the cell through the cycle.

Each cdk is inactive and must bind to a cyclin, before it can phosphorylate its target protein(s). After the phosphorylation event the cyclin is degraded (Fig. 5.2) by the *ubiquitin/protease system*. Here, several enzymes sequentially add small molecules of ubiquitin to the cyclin. The resulting ubiquitin polymer acts as an 'address label' that directs the cyclin to the *proteasome* where it is degraded.

There are eight main groups of cyclins. According to the 'classical model' of the cell cycle (see Satayanarayana & Kaldis, 2009), those of principal importance in the control of the 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

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[1] The term is originally a Greek word that describes the falling of leaves or petals from plants.

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 binds to a cyclin and is activated; it can now phosphorylate a specific protein substrate (e.g. an enzyme). [**C**] After the phosphorylating event, the cyclin is degraded.

and 6; and cyclin E, cdk 2. Precise timing of each step is essential and many cycle proteins are degraded after they have carried out their functions. The actions of the cyclin/cdk complexes throughout the cell cycle are depicted in Figure 5.3.

The activity of these cyclin/cdk complexes is negatively modulated 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*[2] – is hypophosphorylated. This restrains the cell cycle at check point 1 by inhibiting the expression of several proteins critical for further cycle progression. The Rb protein accomplishes this by binding to transcription factors controlling the expression of the genes that code for proteins essential for DNA replication during S phase, such as cyclins E and A, DNA polymerase, thymidine kinase and dihydrofolate reductase.

Growth factor action on a cell in G_0 propels it into G_1, which prepares the cell for S phase. The concentration of cyclin D increases and the cyclin D/cdk complex phosphorylates and activates the proteins required for DNA replication.

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, past check point 1, into S phase.

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

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

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 are at this point a tangled mass in 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 of the cell (see Fig. 5.3).
- *Anaphase*. A specialised cytoskeletal 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, nuclear 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, which restrains the cell cycle while it is hypophosphorylated.

Inhibitors of the cdks also serve as negative regulators, their main action being at check point 1. There are two known families of inhibitors: the *CIP family* (cdk inhibitory proteins, also termed KIP or kinase inhibitory proteins) – proteins p21, p27 and p57; and the *Ink family* (inhibitors of kinases) – proteins p16, p19 and p15.

Protein p21 is a good example of the role of a cyclin/cdk inhibitor. It 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 the p53 protein, a transcription factor found in only low concentrations in normal healthy cells. However, following 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

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[2]So named because mutations of the *Rb* gene are associated with retinoblastoma tumours.
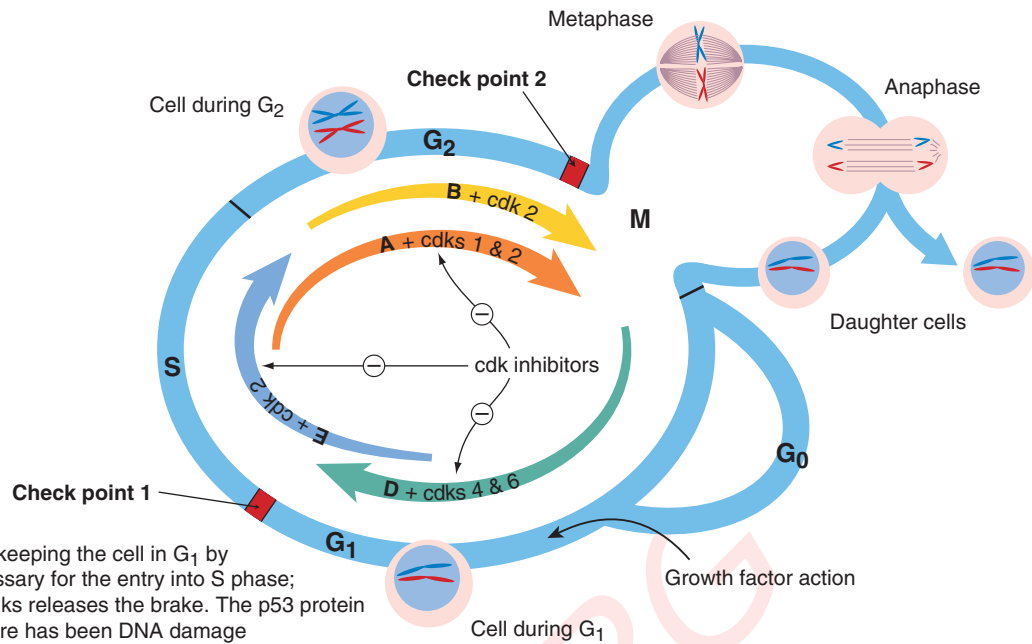
Rb acts as a brake here, keeping the cell in G_1 by inhibiting the genes necessary for the entry into S phase; phosphorylation by the cdks releases the brake. The p53 protein stops the cycle here if there has been DNA damage

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 the cdk action 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* arrest 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.

is unsuccessful, the *p53* gene triggers apoptosis – cell suicide.

Inhibition of the cycle at check point 2

DNA damage can arrest the cycle 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 and Swanton (2004).

INTERACTIONS BETWEEN CELLS, GROWTH FACTORS AND THE EXTRACELLULAR MATRIX

Cell proliferation is regulated by the integrated interplay between growth factors, cells, the extracellular matrix (ECM) and the matrix metalloproteinases (MMPs). The ECM is secreted by the cells and provides a supportive framework. It also profoundly influences cell behaviour by signalling through the cell's integrins. Matrix expression by cells is regulated by growth factors and cytokines (see Verrecchia & Mauviel, 2007; Järveläinen et al., 2009). The activity of some growth factors is, in turn, determined by the matrix, because they are sequestered by matrix components and released by proteases (e.g. MMPs) secreted by the cells.

The action of growth factors acting through receptor tyrosine kinases or receptor-coupled kinases (see Ch. 3) is a fundamental part of these processes. Important examples include *fibroblast growth factor* (FGF), *epidermal growth*

The cell cycle

- The term *cell cycle* refers to the sequence of events that take place within a cell as it prepares for division. The quiescent or resting state is called G_0.
- Growth factor action stimulates a cell in G_0 to enter the cycle.
- The phases of the cell cycle are:
  - G_1: preparation for DNA synthesis
  - S: DNA synthesis
  - G_2: preparation for division
  - M, mitosis: division into two daughter cells.
- In G_0 phase, a hypophosphorylated protein, coded for by the *Rb* gene, arrests the cycle 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 specific proteins termed cyclins.
- Four main cyclins D, E, A and B, together with their cdk complexes drive the cycle; cyclin D/cdk also releases the Rb protein-mediated inhibition.

There are protein inhibitors of cdks in the cell. Protein p21 is particularly important; it is expressed when DNA damage triggers transcription of gene *p53* and arrests the cycle at check point 1.

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, e.g. *collagen species* (the main proteins of the matrix) and *elastin*.
- Non-fibre-forming elements, e.g. proteoglycans, glucoproteins and adhesive proteins such as *fibronectin*. Proteoglycans have a growth-regulating role, in part by functioning as a reservoir of sequestered growth factors. Others are associated with the cell surface, where they 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 cell surface integrins.

Other proteins in the ECM are *thrombospondin* and *osteopontin*, 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 TGF-β.

▼ The ECM is a target for drug action. Both beneficial and adverse effects have been reported. Thus glucocorticoids decrease collagen synthesis in chronic inflammation and cyclo-oxygenase (COX)-2 inhibitors can modify fibrotic processes through a proposed action on TGF-β. Statins can decrease fibrosis by inhibiting angiotensin-induced connective tissue growth factor production (Rupérez et al., 2007) and reduce MMP expression. This may contribute to their effects in cardiovascular diseases (Tousoulis et al., 2010). 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 that regulate tissue repair.

THE ROLE OF INTEGRINS

▼ Integrins are transmembrane kinase-linked receptors (see Ch. 3) comprising α and β subunits. Interaction with the ECM elements (e.g. fibronectin) triggers 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). Following integrin stimulation an adapter protein and an enzyme (*focal adhesion kinase*), activate the kinase cascade that comprises the growth factor signalling pathway. There is extensive cross-talk between the integrin and growth factor pathways (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 activation inhibits apoptosis (see below), further facilitating growth factor action (see reviews by Gahmberg et al., 2009 and Barczyk et al., 2010).

Several monoclonal antibodies are targeted at integrins, including **natalizumab**, used to treat multiple sclerosis and **abciximab**, an antithrombotic (Ch. 24).

THE ROLE OF MATRIX METALLOPROTEINASES

▼ Degradation of the ECM by metalloproteinases is necessary for tissue growth, repair and remodelling. When growth factors stimulate a cell to enter the cell cycle, they also stimulate the secretion of metalloproteinases (as inactive precursors), which then sculpt the matrix, producing the local changes necessary to accommodate the increased cell numbers. Metalloproteinases in turn release growth factors from the ECM and, in some cases (e.g. interleukin [IL]-1β), process 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.
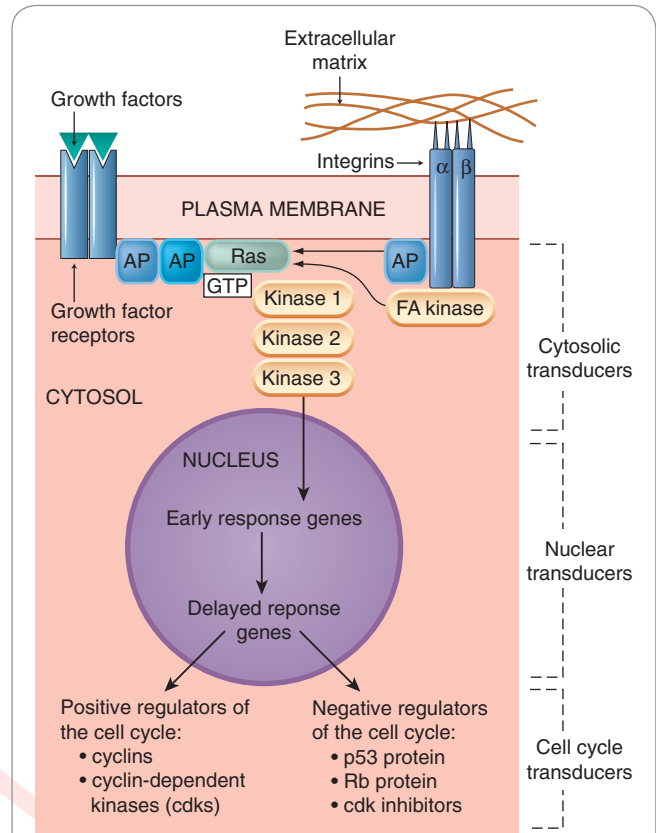
Fig. 5.4 Simplified diagram of the effect of growth factors on a cell in G_0. The overall effect of growth factor action is the generation of the cell cycle transducers. A cell such as the one depicted will then embark on G_1 phase of the cell cycle. Most growth factor receptors have integral tyrosine kinase (see Fig. 3.17). These receptors dimerise, then cross-phosphorylate their 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 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 protein.

In addition to their physiological function, metalloproteinases are involved in the tissue destruction that accompanies 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 (Skiles et al., 2004; Clark et al., 2008; Marastoni et al., 2008). Because of this, much effort has gone into developing synthetic MMP inhibitors for treating cancers and inflammatory disorders, although 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 this, new tissues (including tumours) cannot grow. Angiogenic stimuli include cytokines and various growth factors, in particular *vascular*

Interactions between cells, growth factors and the matrix

- Cells secrete the components of the extracellular matrix (ECM) and become embedded in this tissue.
- The ECM influences the growth and behaviour of the cells. It also acts as a reservoir of growth factors.
- Integrins are transmembrane cellular receptors that can interact with elements of the ECM. They modulate growth factor signalling pathways and also mediate cytoskeletal adjustments within the cell.
- Growth factors cause cells to release metalloproteinases that degrade the local matrix so that it can accommodate the increase in cell numbers.
- Metalloproteinases release growth factors from the ECM and can activate some that are present in precursor form.

endothelial growth factor (VEGF). The sequence of events in angiogenesis is as follows:

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

A monoclonal antibody, **bevacizumab**, which neutralises VEGF, is used as adjunct treatment for various cancers (see Ch. 56), and following 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. It is regulated by a built-in genetically programmed self-destruct mechanism consisting of specific sequence of biochemical events. It is thus unlike *necrosis*, which is disorganised disintegration of damaged cells that releases substances that trigger the inflammatory response.[3]

Apoptosis plays an essential role in embryogenesis, shaping organs during development by eliminating cells that have become redundant. It is the mechanism that each day unobtrusively removes some 10 billion cells from the human body. It is involved in numerous physiological events, including 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 that could become malignant.

Impaired apoptosis is also implicated in the pathophysiology of many conditions, including:

- chronic neurodegenerative diseases such as Alzheimer's, multiple sclerosis and Parkinson's disease (Ch. 40)
- conditions with acute tissue damage or cell loss, such as myocardial infarction (Ch. 21), stroke and spinal cord injury (Ch. 40)
- depletion of T cells in HIV infection (Ch. 52)
- osteoarthritis (Ch. 36)
- haematological disease, such as aplastic anaemia (Ch. 25)
- evasion of the immune response by cancer cells and resistance to cancer chemotherapy (Ch. 56)
- autoimmune/inflammatory diseases such as myasthenia gravis (Ch. 13), rheumatoid arthritis (Ch. 26), and bronchial asthma (Ch. 28)
- viral infections with ineffective eradication of virus-infected cells (Ch. 52).

▼ 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 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.) are required for cell survival and viability. The self-destruct mechanism is automatically triggered unless it is actively and continuously inhibited by these antiapoptotic factors. Different cell types require differing sets of survival factors, which function only locally. If a cell strays or is dislodged from the area protected by its paracrine survival signals, it will die.

Withdrawal of these 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* and by DNA damage. But it is generally accepted that cell proliferation processes and apoptosis are tightly integrated.

MORPHOLOGICAL CHANGES IN APOPTOSIS

As the cell dies it 'rounds up', the chromatin condenses into dense masses, the cytoplasm shrinks and there is blebbing of the plasma membrane. Finally, mediated by a family of proteolytic enzymes known as caspases, the cell is transformed into a cluster of membrane-bound entities. This cellular 'corpse' displays 'eat me' signals, such as phosphatidylserine on its surface, which are recognized by macrophages, which then phagocytose the remains. It is important that these cellular fragments are enclosed by a membrane because otherwise the release of cell constituents could trigger an inflammatory reaction. An additional safeguard against this is that phagocytosing macrophages release anti-inflammatory mediators such as TGF-β, Annexin-1 and IL-10.

THE MAJOR PLAYERS IN APOPTOSIS

The repertoire of reactions in apoptosis is extremely complex and varies between species and cell types. Yet it

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[3]There are other forms of programmed cell death (PCD) including *autophagy* and (confusingly) *programmed necrosis*. Here we will focus on apoptosis, also known as 'Type I PCD'.
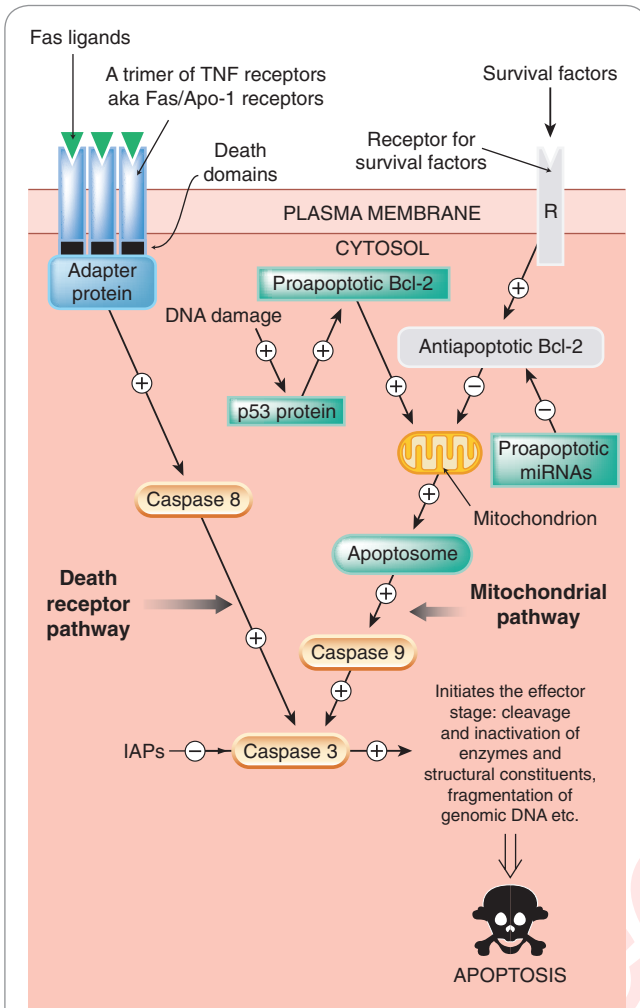
Fig. 5.5 **A simplified diagram of the two main signalling pathways in apoptosis.** The 'death receptor' pathway is activated when death receptors such as members of the tumour necrosis factor (TNF) family are stimulated by specific death ligands. This recruits adapter proteins that activate initiator 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 releases 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 restrains apoptosis by inhibiting the mitochondrial 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 (shown in grey), this antiapoptotic drive is withdrawn. IAP, inhibitor of apoptosis.

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 desirable targets for drugs used to treat many proliferative diseases.

Only a simple outline of apoptosis can be given here. Portt et al. (2011) have reviewed the whole area in detail. 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 all of which contain a characteristic motif recognised by the caspases), inactivating some and activating others. A cascade of about nine different caspases are required, some functioning as initiators that transmit the initial apoptotic signals, and others 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 that cuts genomic DNA between the nucleosomes, generating DNA fragments of approximately 180 base pairs.

However, 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).

Besides the caspases, another pathway can be triggered by *apoptotic initiating factor* (AIF), a protein released from the mitochondria that enters the nucleus and triggers cell suicide.

PATHWAYS TO APOPTOSIS

There are two main routes to cell death: stimulation of death receptors by external ligands (the *extrinsic pathway*) and an internal *mitochondrial pathway*. Both routes activate initiator caspases and converge on a final common effector caspase pathway.

THE EXTRINSIC 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 include 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 a ligand such as tumour necrosis factor (TNF) itself or TRAIL[4] causes them to trimerise and recruit an adapter protein that binds to their death domains. The resulting complex activates caspase 8 (and probably caspase 10), which in turn activate the effector caspases (Fig. 5.5).

The mitochondrial pathway

This pathway can be triggered by DNA damage or by withdrawal of cell survival factors or other factors. In

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[4]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.

some way, the cell can 'audit' such damage and decide whether to initiate the apoptotic pathway. It is possible that *promyelocytic leukaemia bodies*, large complexes of proteins in the nucleus, participate in this task (Wyllie, 2010), although how they do so is not clear.

Regulating the apoptotic event are the members of the Bcl-2 protein family, a group of proteins with homologous domains allowing interactions between individual members. If the cell selects the apoptotic route, the p53 protein activates p21 and proapoptotic members of the Bcl-2 family – Bid, Bax and Bak. In addition to these proapoptotic individuals, this family has antiapoptotic members (e.g. Bcl-2 itself).[5] These factors compete with each other on the surface of the mitochondria and the outcome depends upon the relative concentrations of these molecular players. In the case of a proapoptotic signal, oligomers of Bax and or Bak form pores in the mitochondrial membrane through which proteins such as cytochrome c can leak.

When released, cytochrome c complexes with a protein termed Apaf-1 (apoptotic protease-activating factor-1) and the two then combine with procaspase 9 and activates it. This latter enzyme orchestrates the effector caspase pathway. The triumvirate 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. The withdrawal of survival factors can cause death in several different ways depending on the cell type. A common mechanism is tipping the balance between Bcl-2 family members leading to loss of the antiapoptotic protein action, with the resultant unopposed action of the proapoptotic Bcl-2 proteins (see Fig. 5.5).

The two main cell death pathways are connected to each other, in that caspase 8 in the death receptor pathway can activate the proapoptotic Bcl-2 proteins 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. They are 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 some 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), as well as carcinogenesis, metastasis and resistance to cancer therapies (Wurdinger & Costa, 2007; Garzon et al., 2009). 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 heralded as targets for new drug development for a variety of disease states (Liu et al., 2008; Stenvang et al., 2008; Tsai & Yu, 2010).

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

Apoptosis

- Apoptosis is programmed cell death. It is an essential biological process and critical for (e.g.) embryogenesis and tissue homeostasis.
- Apoptosis depends upon a cascade of proteases called caspases. Two sets of initiator caspases converge on a set of effector caspases, which bring about the apoptotic event.
- Two main pathways activate the effector caspases: the death receptor pathway and the mitochondrial pathway.
  - Stimulation of the tumour necrosis factor receptor family initiates the death receptor pathway. The main initiator 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 sub-pathway that releases cytochrome c from the mitochondrion. 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 factors causes cell death through the mitochondrial pathway.
- The effector caspases (e.g. caspase 3) initiate a cascade of proteases that cleave cell constituents, DNA, cytoskeletal components, enzymes, etc. This reduces the cell to a cluster of membrane-bound entities that are eventually phagocytosed by macrophages.

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. 56)
- regeneration of tissues.

The role of cell proliferation and apoptosis in the first two processes listed is self-evident and needs no further comment. Their involvement in immune tolerance is discussed briefly above but the other processes require further discussion.

REPAIR AND HEALING

Repair occurs when tissues are damaged or lost. 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.

There is considerable overlap between the mechanisms activated in inflammation and repair. Both entail an ordered series of events including 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 appropriate for the particular tissue involved. TGF-β is a key regulator of several of these processes.

> ### Repair, healing and regeneration
>
> - Repair and healing occurs when tissues are damaged. It is a common sequel to inflammation. Connective tissue cells, white blood cells and blood vessels are commonly involved.
> - Regeneration is the replacement of the tissue or organ that has been damaged or lost. It depends upon the presence of a pool of primitive stem cells that have the potential to develop into any cell in the body. Complete regeneration of a tissue or organ is rare in mammals. The more rapid repair processes – often accompanied by scarring – usually make good the damage. This may be an evolutionary trade-off in mammals for the lost power of regeneration.
> - However, it might be possible to activate regenerative pathways in mammals – at least to some extent and in some organs.

HYPERPLASIA

Hyperplasia (cell proliferation and matrix expansion) is a hallmark of chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (Chs 6 and 26), psoriasis, chronic ulcers and chronic obstructive lung disease. It also underlies the bronchial hyper-reactivity of chronic asthma (Ch. 28) and glomerular nephritis.

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

Growth factor signalling systems, antiapoptotic pathways and cell cycle controllers are of increasing interest as targets for novel approaches to the treatment of cancer. See Chapter 56.

STEM CELLS AND REGENERATION

Regeneration of tissue replaces that lost following damage or disease and allows restoration of function. Many animals (e.g. amphibians) have impressive regenerative powers and can even regrow an entire organ such as a limb or a tail. The essential process is the activation of *stem cells* – a pool of undifferentiated cells that have the potential to develop into any of the more specialised cells in the body ('totipotent' or 'pluripotent' cells). Not only do amphibians have a plentiful supply of these primitive cells but many of their more specialised cells can de-differentiate, becoming stem cells again. These can then multiply and retrace the fetal developmental pathways that generated the organ, proliferating again and again and eventually differentiating into the various cell types needed to replace the missing structure.

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

Almost alone among mammalian organs, the liver has significant ability to replace itself. It can regenerate to its original size in a remarkably short time, provided that at least 25% has been left intact.[6] The mature parenchymal liver cells participate in this process as well as all the other cellular components of the liver.

It is necessary to distinguish *embryonic stem cells* (ES cells) from *adult stem cells* and *progenitor cells*. ES cells are the true pluripotent cells of the embryo which are able to differentiate into any other cell type. Adult stem cells (AS cells) have a more restricted capability whereas progenitor cells are able to differentiate only into a single cell type. ES cells are absent in the adult mammal, but AS cells are present, although they are few in number. 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 (with a few exceptions) an unalterable situation, but recent work has suggested that it might be possible to activate the regenerative pathways in mammals – at least to some extent and in some organs. Replacement of entire limbs is manifestly not possible in humans, but regeneration of limited amounts of tissue or of a small part of an organ may well be feasible. For this to happen, it is 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 de-differentiate. This can occur in some mammals under special circumstances. However, it may be that repair is the Janus face of regeneration, 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:

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[6]There is an account of liver regeneration in Greek mythology. 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. During the night, however, 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 seems unrealistically fast – rat liver takes 2 weeks or more to get back to the original size after 66% hepatectomy.

- 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-resident 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 to proliferate. Other essential processes include those already discussed such as angiogenesis, activation of MMPs and interaction between the matrix and fibronectin to link all the new elements together. The 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 restore regenerative ability could be of immense therapeutic value. Stem cell therapy has become an attractive prospect for treating all manner of diseases, ranging from erectile dysfunction and urinary incontinence to heart disease and neurodegeneration. Animal studies have confirmed that this is a potentially rewarding area although routine stem cell therapy in humans is still a distant prospect. The literature is daunting but the following examples provide an insight into the obstacles and aspirations of the field: repair of damaged heart muscle (Ch. 21; see Lovell & Mathur, 2011), repair of retinal degeneration (Ong & da Cruz, 2012), stroke (Banerjee et al., 2011) and replacement of insulin-secreting cells to treat type I diabetes mellitus (Ch. 31; Voltarelli et al., 2007).

THERAPEUTIC PROSPECTS

Theoretically, all the processes described in this chapter could constitute useful targets for new drug development. Below, we list 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 being tested for chronic lymphocytic leukaemia.
- **Obatoclax**, a small molecule inhibitor of Bcl-2 action, is being tested for treating haematological malignancies. For details see MacFarlane (2009).
- MicroRNA technology could also be used to promote apoptosis (see Fig. 5.5).
- Monoclonal agonist antibodies to the death receptor ligand TRAIL (e.g. **lexatumumab**) are undergoing clinical trials for treatment of 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. 3).
- One of the most cancer-specific genes codes for an endogenous caspase inhibitor, *survivin*. This occurs in high concentrations in certain tumours and a small molecule suppressor of survivin is in clinical trial (Giaccone & Rajan, 2009), the objective being to induce cancer cell suicide.

Inhibiting apoptosis might prevent or treat a wide range of common degenerative disorders. Unfortunately, success in developing such inhibitors for clinical use has so far proved elusive and a number have been found to lack efficacy in clinical trials. Current areas of interest include the following:

- Blocking the PD-1 death receptor with a targeted antibody 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 treating myocardial infarction, stroke, liver disease, organ transplantation and sepsis. **Emricasan** is one such candidate undergoing trials in patients requiring liver transplants.

ANGIOGENESIS AND METALLOPROTEINASES

The search for clinically useful anti-angiogenic drugs and 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: bevacizumab, a monoclonal antibody that neutralises VEGF, which is also used to treat age-related macular degeneration, a disease also 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 anti-angiogenic properties and can induce differentiation (Dickson & Schwartz, 2009).

Some compounds affect upstream pathways for cdk activation and may find a use in cancer treatment. Examples are **perifosine** (although its future is uncertain at the moment) 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. 56).

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. 56).

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