17 Cell membrane receptors and cell signalling

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17.1 RECEPTORS FOR CELL SIGNALLING

17.1.1 Intercellular signal transduction

Cells in multicellular organisms need to be able to communicate with each other in order to respond to external stimuli and to coordinate their activities to achieve homeostasis. Such communication is termed **intercellular signalling** and is achieved by:

- The release by the 'signalling' cells of **signalling molecules**, referred to as **endogenous agonists** or **first messengers.**
- The specific recognition and binding of these agonists by **receptor molecules**, simply referred to as receptors, located either in the cell membrane or in the cytoplasm of the 'target' cell. Each cell membrane contains between 10^3 and 10^6 molecules of a given receptor. Binding of the agonist to a specific binding domain on the receptor changes the receptor from its inactive, **resting state**, to an active state. Exceptionally, a receptor may possess activity in the absence of agonist. Such receptors are said to possess **constitutive activity**.
- The initiation of a sequence of molecular events commonly involving interaction between the active receptor and other, so-called effector molecules, the whole process being referred to as **intracellular signal transduction** which terminates in the final cellular response.

Agonist signalling molecules range from the gas nitric oxide, amines, amino acids, nucleosides, nucleotides and lipids to hormones, growth factors, interleukins,

interferons and cytokines. These molecules are either lipophilic or hydrophilic. Lipophilic signalling molecules such as the steroid hormones (progesterone, oestrogen and testosterone) and non-steroid hormones (thyroxine and triiodothyronine) can readily cross the cell membrane, in which case they bind to receptors located in the cytoplasm. These receptors have two **binding domains**: an agonist-binding domain and a DNA-binding domain. Binding of the lipophilic agonist to its site results in a receptor–agonist complex that is able to pass through pores in the nuclear membrane into the nucleus where it interacts with a specific DNA sequence, termed a **hormone response element**, to regulate (activating or repressing) the transcription of downstream genes. For this reason, the receptors are referred to as **nuclear receptors**. In the majority of cases of intercellular signalling, however, the signalling agonist is hydrophilic and therefore incapable of diffusing across the cell membrane. The receptors for such agonists are therefore embedded in, and span, the cell membrane with the agonist-binding domain exposed on the extracellular side. This chapter will consider the molecular nature and mode of action of such cell surface receptors.

Cell membrane receptor proteins possess three distinct **domains**:

- *Extracellular domain*: This protrudes from the external surface of the membrane and contains all or part of the agonist-binding domain known as the **orthosteric agonist-binding site**.
- *Transmembrane domain*: This is inserted into the phospholipid bilayer of the membrane and may consist of several regions that loop repeatedly back and forth across the membrane. In some cases these loops form a channel for the 'gating' (hence the channel may be open or closed) of ions across the membrane, whilst in other receptors the loops create part of the orthosteric site.
- *Intracellular domain*: This region of the protein has to respond to the extracellular binding of the agonist to initiate the transduction process. In some cases it is the site of the activation of enzyme activity within the receptor protein, commonly kinase activity, or is the site that interacts with effector proteins.

The existence of three domains within receptor proteins reflects their **amphipathic** nature in that they contain regions of 19 to 24 amino acid residues possessing polar groups that are hydrophilic, and similar sized regions that are rich in non-polar groups that are hydrophobic and hence lipophilic. The hydrophobic regions, generally in the form of α-helixes, are the transmembrane regions that are inserted into the non-polar, long-chain fatty acid portion of the phospholipid bilayer of the membrane. Superfamilies of receptor proteins can be recognised from the precise number of transmembrane regions each possesses. In contrast, the hydrophilic regions of the receptor are exposed on the outside and inside of the membrane where they interact with the aqueous, hydrophilic environment.

17.1.2 Classification of cell membrane receptors

Studies of the structure of membrane receptors and of the mechanisms of their signal transduction have led to the identification of three main classes of cell membrane receptors:

- *Ligand-gated ion-channel receptors*: These are responsible for the selective movement of ions such as Na^+, K^+ and Cl^- across membranes. Binding of the agonist triggers the **gating** (opening) of a channel and the movement of ions across the membrane. This ion movement is a short-term, fast response that results in the propagation of a membrane potential wave. It may be excitatory and result in the depolarisation of the cell (e.g. the nicotinic acetylcholine and ionotropic glutamate receptors), or inhibitory (e.g. the γ-aminobutyric acid A ($GABA_A$) receptor). All receptors in this class consist of four or five homo- or heteromeric subunits. Responses produced by this class of receptors occur in fractions of a second.
- *G-protein-coupled receptors (GPCRs)*: Receptors in this class are linked to a G-protein that is trimeric (i.e. it has three subunits). Receptor activation by agonist binding triggers its interaction with a G-protein located within the cell membrane and protruding into the cytoplasm resulting in the exchange of GTP for GDP (hence the name G-protein) on one subunit that dissociates from the trimer causing the activation of an effector molecule such as adenylyl cyclase (also known as adenylate cyclase) that is part of an intricate network of intracellular transduction pathways. Responses produced by GPCRs occur in the timescale of minutes.
- *Protein kinase receptors*: These receptors all undergo agonist-stimulated autophosphorylation in their intracellular domain. This activates a kinase activity within this domain. The majority of activated receptor kinases catalyse the transfer of the γ-phosphate of ATP to the hydroxyl group of a tyrosine in the target effector protein, hence the term **receptor tyrosine kinases** (RTKs). This phosphorylation process controls the activity of many vital cell processes. Members of a minor subgroup of protein kinase receptors transfer the phosphate group of ATP to a serine or threonine group rather than tyrosine, hence the term **receptor serine** (or **threonine**) **kinases**. Responses produced by protein kinase receptors occur over a timescale of minutes to hours. Closely related to the protein kinase receptors is a group of receptors that lack intrinsic protein kinase activity but which recruit a non-receptor tyrosine kinase after agonist binding. The recruited kinase phosphorylates tyrosine residues in the receptors' intracellular domains then act as recognition sites for other effector proteins which, when activated, translocate to the cell nucleus where, in association with other regulatory proteins, they modify gene expression. Responses to these receptors occur within a timescale of minutes to hours.

Further details of the mechanism by which each of these three classes of receptor induce the transduction process are discussed in Section 17.5. There is evidence that many receptors exist in multiple **isoforms** that have subtly different physiological roles and that many receptors form homo- and/or heterodimers or oligomers that are sensitive to allosteric regulation and which function as partners to initiate interactions between the downstream signalling molecules triggered by each receptor. This **cross-talk** between receptors allows cells to integrate signalling information originating from various external sources and to respond to it with maximum regulatory efficiency.

Signalling co-receptors

In addition to the three groups of signalling receptors discussed above, there is a group of membrane receptors that bind agonists but which do not directly transduce a cellular signal but to do so they form a complex with a receptor from one of the above three classes. These receptors are termed **co-receptors** or **accessory receptors**. These co-receptors are:

- used by a large number of ligands including interleukin, epidermal growth factor and fibroblast growth factor;
- expressed ubiquitously within a given organism such that they are often the most abundant receptor for the agonists they bind;
- expressed with conserved structural features on the cell surface of a diverse range of organisms.

Eight families of co-receptors have been identified, each containing up to eight members. The agonists that they bind are promiscuous in that a given co-receptor may bind up to nine different agonists and a given agonist can bind to more than one co-receptor. Their central role in the regulation of cellular processes is evident from the observation that their mutation and/or altered expression is associated with such human diseases as certain cancers, inflammation and ischaemic heart disease. Mutations commonly cause a loss of co-receptor function leading to an autosomal dominant or recessive inherited disorder (see cytokine receptors, Section 17.4.4).

17.2 QUANTITATIVE ASPECTS OF RECEPTOR–LIGAND BINDING

17.2.1 Dose–response curves

The response of membrane receptors in their resting inactive state to exposure to an increasing concentration (dose) of agonist is a curve that has three distinct regions:

- an initial threshold below which little or no response is observed;
- a slope in which the response increases rapidly with increasing dose;
- a declining response with further increases in dose and a final maximum response.

Since such plots commonly span several hundred-fold variations in agonist concentration, they are best expressed in semi-logarithmic form (Fig. 17.1).

A dose–response curve for an inverse agonist (Section 17.2.2) acting on a receptor with constitutive activity would be a mirror image of that shown in Fig. 17.1 resulting in a progressive decrease in receptor activity.

Dose–response studies coupled with the observed transduction pathway have enabled molecules (ligands) that bind to a receptor to be placed into one or more of the following classes:

- *Full agonists*: These ligands increase the activity of the receptors and produce the same maximal response but they differ in the dose required to achieve it (Fig. 17.1).

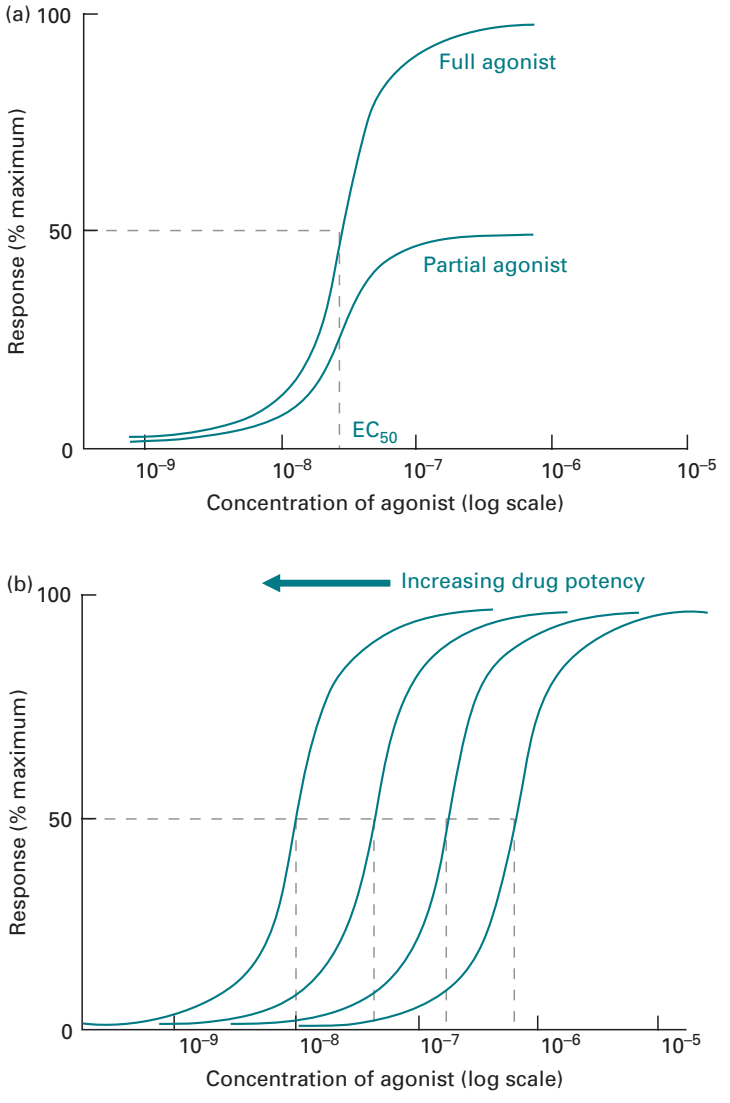

Fig. 17.1 Dose–response curves for receptor agonists. (a) The biological effect (% maximum response) and the concentration of a full agonist are plotted on a logarithmic scale. An equipotent partial agonist has a lower efficacy than a full agonist – it cannot achieve the maximum response even when all the receptors are occupied. EC_{50} is the concentration of agonist that produces 50% maximum effect. (b) Dose–response curves for four full agonist drugs of different potencies but equal efficacy. (Reproduced from Maxwell, S. R. J. and Webb, D. J. (2008). Receptor functions. *Medicine*, **36**, 344–349, by permission of Elsevier Science.)

- *Partial agonists*: These ligands also increase the activity of the receptors but do not produce the maximal response shown by full agonists even when present in large excess such that all the receptors are occupied (Fig. 17.1).
- *Inverse agonists*: These agonists decrease the activity of constitutively active receptors to their inactive state.

- *Partial inverse agonists*: These agonists decrease the activity of constitutively active receptors but not to their inactive state.
- *Protean agonists*: These agonists acting on receptors possessing constitutive activity display any response ranging from full agonism to full inverse agonism depending on the level of constitutive activity in the system and the relative efficacies of the constitutive activity and that induced by the agonist.
- *Biased agonists*: This form of agonist behaviour is found with receptors that can couple to two or more different G-proteins and as a consequence the agonist preferentially selects one of them thus favouring one specific transduction pathway.
- *Antagonists*: In the absence of agonists these ligands produce no change in the activity of the receptors. Three subclasses have been identified using the antagonist in the presence of an agonist and using receptors not possessing constitutive activity:
  (i) *Competitive reversible antagonists*: The antagonist competes with the agonist for the orthosteric sites so that the effect of the antagonist can be overcome by increasing the concentration of agonist (Fig. 17.2).
  (ii) *Non-competitive reversible antagonists*: The antagonist binds at a different site on the receptors to that of the orthosteric site so that the effect of the antagonist cannot be overcome by increasing the concentration of agonist.
  (iii) *Irreversible competitive antagonists*: The antagonist competes with the agonist for the orthosteric site but the antagonist forms a covalent bond with the site so that its effect cannot be overcome by increasing the concentration of agonist.
- *Allosteric modulators*: These bind to a site distinct from that of the orthosteric site and can only be detected using functional, as opposed to ligand-binding, assays. They are discussed more fully in Section 17.2.3.

As a result of this classification, ligand action on receptors can be characterised by a number of parameters:

- *Intrinsic activity*: This is a measure of the ability of an agonist to induce a response by the receptors. It is defined as the maximum response to the test agonist relative to the maximum response to a full agonist acting on the same receptors. All full agonists, by definition, have an intrinsic activity of 1 whereas partial agonists have an intrinsic activity of less than 1.
- *Efficacy (e)*: This is a measure of the inherent ability of an agonist to initiate a physiological response following binding to the orthosteric site. The initiation of a response is linked to the ability of the agonist to promote the formation of the active conformation of the receptors whereas for inverse agonists it is linked to their ability to promote the formation of the inactive conformation. While all full agonists must have a high efficacy their efficacy values will not necessarily be equal, in fact values of e have no theoretical maximum value. Partial agonists have a low efficacy, antagonists have zero efficacy and inverse agonists have negative efficacy.
- *Collateral efficacy*: This relates to the ability of the agonist to preferentially select one of the two or more possible transduction pathways displayed by the binding

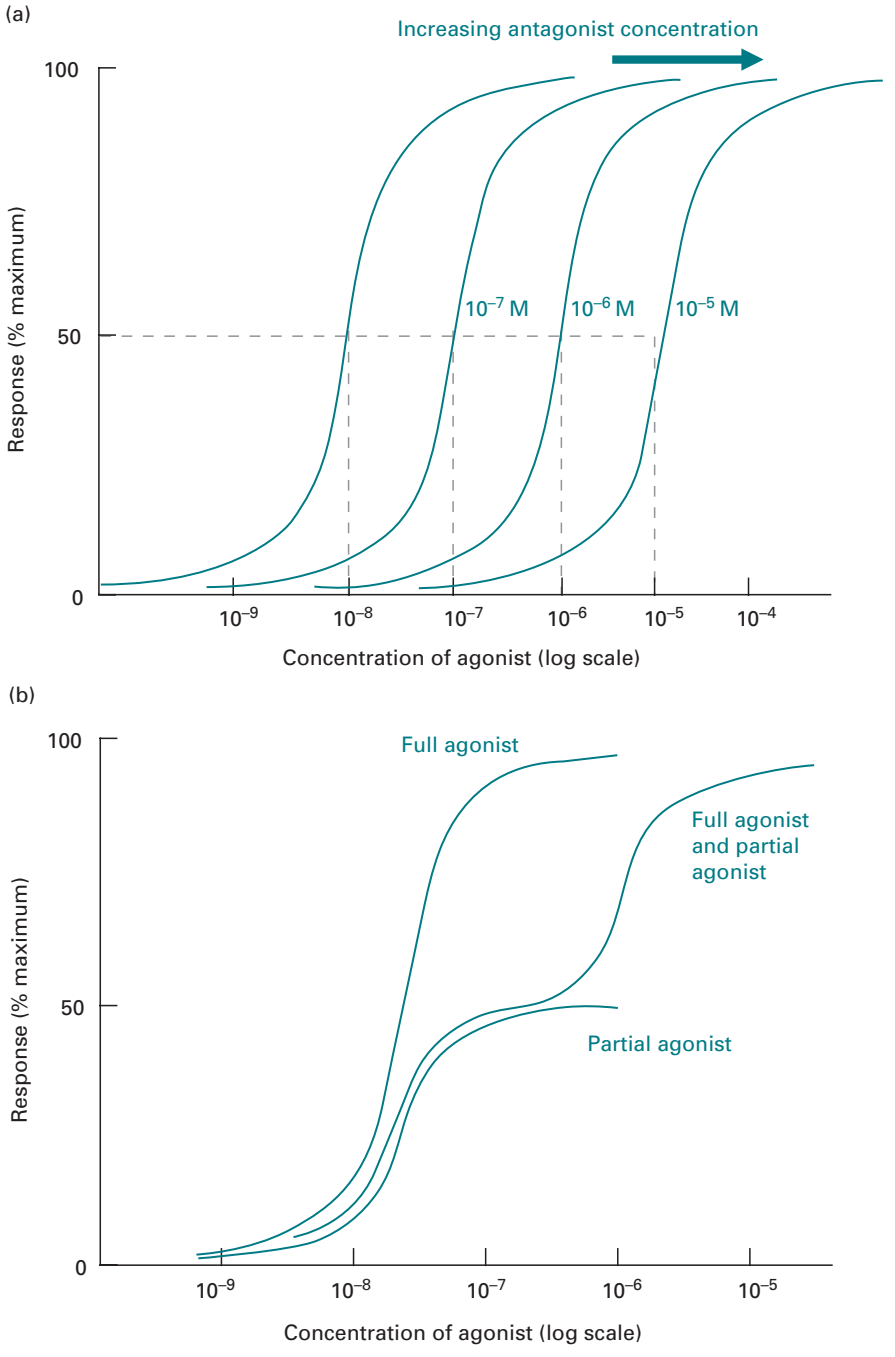

Fig. 17.2 Dose–response curves for receptor agonist. (a) In the presence of a competitive reversible antagonist. In the presence of a reversible antagonist, the dose–response curve for a full agonist is shifted to the right because the full agonist competes for receptor binding. The higher the concentration of antagonist the greater the shift, but the maximum response remains unchanged. below (b) In the presence of a partial agonist, the dose-response curve of the full agonist is shifted to the right. (Reproduced from Maxwell, S. R. J. and Webb, D. J. (2008). Receptor functions. *Medicine*, **36**, 344–349, by permission of Elsevier Science.)

receptors. It primarily relates to G-protein-coupled receptors that can bind to two or more G-proteins.

- *Potency*: This is a measure of the concentration of agonist required to produce the maximum effect; the more potent the agonist the smaller the concentration required. The potency of an agonist is related to the position of the sigmoidal curve on the log dose axis. It is expressed in a variety of forms including the **effective dose** or **concentration for 50% maximal response**, ED_{50} or EC_{50}. On a semi-logarithm plot, the value emerges as pED_{50} or pEC_{50} value (i.e. $-\log_{10} ED_{50}$). Thus an agonist with EC_{50} of 3×10^{-5} M would have a pEC_{50} of 4.8. The potency of a reversible antagonist is expressed by its pA_2 value, defined as negative logarithm of the concentration of antagonist that will produce a two-fold shift in the concentration–response curve for an agonist.
- *Affinity*: This is a measure of the concentration of agonist required to produce 50% binding. As will be shown in the following section, affinity is a reflection of both the rate of association of the ligand with the receptors and the rate of dissociation of the resulting complexes. The rate of association is a reflection of the three-dimensional interaction between the two and the rate of dissociation a reflection of the strength of binding within the complexes. Affinity of an agonist can be expressed by an **affinity** or **binding constant**, K_a, but is more commonly expressed as a **dissociation constant**, K_d, of the receptor–ligand complex where K_d is equal to the reciprocal of K_a. The affinity of receptors for an antagonist is expressed by the corresponding dissociation constant K_b.
- *Selectivity*: This is a measure of the ability of an agonist to discriminate between receptor subtypes. This is particularly important from a therapeutic perspective.
- *Functional selectivity*: This is a measure of the ability of the agonist to induce selective response from receptors capable of promoting more than one transduction activity.

17.2.2 Constitutive receptor activity, inverse agonists and receptor activation

The long-held view of receptor–agonist interaction was based on a **two-state model** that visualised that the binding of the agonist (A) by the receptor to form a receptor–agonist complex triggers a conformational change in the receptor that converts it from a **dormant** or **resting inactive state** (R) to an **active state** (R*):

$$\underset{\text{inactive}}{R + A} \longrightarrow \underset{\text{inactive}}{AR} \longrightarrow \underset{\text{active}}{AR^*} \longrightarrow \underset{\text{effector}}{\text{transduction via}}$$

The formation of the active state of the receptor initiates a transduction (linking) process in which the receptor activates an **effector protein**. The effector protein may be the receptor itself or a distinct protein that is either attached to the inside of the membrane or free in the cytoplasm. This activated effector either allows the passage of selected ions across the membrane thereby changing the membrane potential or it produces a **second messenger** which initiates a **cascade** of molecular events, involving molecules located on and/or at the internal surface of the cell

membrane or within the cell cytoplasm, that terminate in the final target cell response. Examples of second messengers are Ca^{2+}, cAMP, cGMP, 1,2-diacylglycerol and inositol-1,4,5-trisphosphate.

However, in 1989 it was discovered that opioid receptors in NG108 cells possessed activity in the absence of agonist. This receptor activity in the absence of agonist was termed **constitutive activity**, and synthetic ligands were identified that could bind to the receptor and decrease its constitutive activity in the absence of physiological agonist. Such ligands were termed **inverse agonists**. Subsequent *in vivo* and *in vitro* investigations with a wide range of receptor types, many of which were G-protein-coupled receptors, identified other examples of receptors with constitutive activity and showed that this activity may have a physiological role thereby confirming that constitutive activity is not solely a consequence of a mutation or overexpression of a receptor gene. Of particular interest was the observation that certain receptor mutations (known as **constitutively active mutants**, CAMs) were associated with such clinical disorders as retinitis pigmentosa, hyperthyroidism and some autoimmune diseases.

The **conformational selection model** of receptor action was formulated to rationalise the concomitant existence of active and inactive conformations. The model envisages that in the absence of agonist, receptors exist as an equilibrium mixture of inactive (R) and active (R*) forms and that the relative proportion of the two forms is determined by the associated equilibrium constant. An introduced ligand will preferentially bind to one conformation, thereby stabilising it, and causing a displacement of the equilibrium between the two forms. Agonists will preferentially bind to the R* state displacing the equilibrium to increase the proportion of the R* form. Partial agonists are deemed to have the ability to bind to both forms with a preference for the R* form again resulting in an increase in the R* form but by a smaller amount than that produced by full agonists. Inverse agonists preferentially bind to the R conformation, displacing the equilibrium and increasing the proportion of R. Partial inverse agonists can bind to both states with a preference for the R resulting in decrease in the proportion of R* form. Unlike the two-state model, the conformational selection model does not require the binding ligand to cause a conformational change in the receptor in order to alter the activity of the receptor.

Prior to the discovery of constitutive activity in some receptors, ligands were classified either as agonists or antagonists. This classification formed the basis of the understanding of the pharmacological action of many therapeutic agents and the development of new ones by the pharmaceutical industry. However, retrospective evaluation of ligands previously classified as antagonists but using receptors produced by cloning techniques and shown to possess constitutive activity revealed that many were actually inverse agonists and possessed negative efficacy whilst others were **neutral antagonists** in that they neither increased nor decreased the receptor activity. To date approximately 85% of all antagonists that have been re-evaluated have been shown to be inverse agonists. These observations can be rationalised in that:

- *agonism* is a behaviour characteristic of a particular ligand and can be demonstrated in the absence of any other ligand for the receptor,
- *antagonism* can only be demonstrated in the presence of an agonist,
- *inverse agonism* can only be demonstrated when the receptor possesses constitutive activity that can be reduced by the agent. In the absence of constitutive activity inverse agonists can only demonstrate simple competitive antagonism of a full or partial agonist.

Further understanding of the nature of constitutive activity and the mode of action of different agonists on a given receptor has come from studies on the histamine H_3 receptor (H_3R). This is a G-protein-coupled receptor, specifically coupling to G_i/G_o proteins (see Section 17.4.3). Constitutive activity has been found in both rat and human brain in which the activity inhibits histamine release from synaptosomes. Studies using the ligand proxyfan, previously classified as an antagonist, have assigned to it a spectrum of activities ranging from full agonist through partial agonist to partial inverse agonist and full inverse agonist. Such behaviour by a ligand has been classified as **protean agonism** and proxyfan as a **protean agonist.** The precise behaviour of proxyfan in a given study correlated with the level of constitutive activity of the system and the relative efficacy of the constitutively active state R* and that induced by the ligand, AR*. Thus in the absence of any R* or in the presence of AR* with a lower efficacy than that of R*, the ligand will display agonist activity. When both R* and AR* states are present with equal efficacy the ligand will display neutral antagonism and when the AR* state has a higher efficacy than R* the ligand will display inverse agonism. Such behaviour can only be explained by a **multistate model** in which multiple R* and AR* states of the receptor can be formed. Furthermore, studies using a range of agonists on the histamine H_3 receptor indicated that different ligands could promote the creation of distinct active states that can display differential signalling. A wider understanding of the mechanism by which receptors are activated by agonists is linked to the discovery, initially made with the α_{1B} adrenergic receptor, that certain mutations in the sequence of G-protein-coupled receptors caused a large increase in the constitutive activity of the receptor. This observation had the implication that there may be domains in the receptor that are crucial to the conservation of a receptor not displaying constitutive activity, and that the action of agonists was to release these constraints creating the active receptor. In mutant receptors possessing constitutive activity these constraints have been released as a result of the mutation. Studies have shown that the activation of inactive receptors by agonists proceeds by a series of conformational changes. The question as to whether agonists and inverse agonists switch the receptor in a linear 'on–off' scale or whether they operate by different mechanisms has been studied using the α_{2A} adrenergic receptor and a fluorescence resonance energy transfer (FRET) based approach. Differences in the kinetics and character of the conformational changes induced by these two classes of agonists provided clear evidence for distinct types of molecular switch. Moreover, full agonists and partial agonists also showed distinct differences indicating that receptors do not operate by a simple 'on–off' switch but rather that

they have several distinct conformational states and that these states can be switched with distinct kinetics by the various classes of agonist.

As previously pointed out, the pharmaceutical industry seeks to identify receptor agonists or antagonists that can be used for the treatment of specific clinical conditions. The potential role of inverse agonists in this respect remains to be fully evaluated. However, it is apparent that clinical conditions caused by a mutant receptor that has constitutive activity in contrast to the normal receptor that is only active in the presence of the physiological agonist could be treated with an inverse agonist that would eliminate the constitutive activity. Equally, use of an inverse agonist may be advantageous in conditions resulting from the overstimulation of the receptor due to the overproduction of the signalling agonist. At the present time many inverse agonists are used clinically although at the time of their development they were believed to be competitive antagonists.

17.2.3 Allosteric modulators

Studies using functional screening assays have demonstrated that many receptors possess **allosteric sites** distinct from the orthosteric agonist site. Such sites have been identified in monomeric receptors as well as those that form homo- and heterodimers. These allosteric sites are capable of binding **allosteric modulators** that exert one of three distinct effects (Fig. 17.3):

- alteration of the affinity of the agonist for its orthosteric site; or
- alteration of the efficacy (i.e. ability to produce the response of the receptor via its various effectors) of the agonist; or
- display an efficacy independent of the presence of an agonist. Such efficacy could be of the agonist or inverse agonist variety.

The binding to the allosteric site is characterised by its dissociation constant and by a cooperativity factor α, which is a thermodynamic measure of the strength of interaction between this site and the orthosteric site. Since these two sites are distinct, allosteric modulators induce unique conformational changes in the receptor and at least in principle these may alter the signalling, desensitisation and internalisation states induced by agonists binding at the orthosteric site. Recently, evidence has been obtained to indicate that allosteric interaction between distinct sites may operate by ligand-dependent changes in the dynamic properties of the receptor rather than simple conformational changes. This idea is based on the recognition that receptors, like enzymes, exist as assemblies of conformations the balance between which can be altered by ligand binding (Section 15.2.4). Ligands acting as allosteric modulators can be classified into one of four types on the basis of the effects they produce on the activity of the orthosteric site:

- *Allosteric agonist*: This is a ligand that binds to an allosteric site and mediates the activation of the receptor in the absence of the physiological agonist.
- *Allosteric enhancer*: This is a ligand that enhances the affinity or efficacy of an agonist acting on the orthosteric site without having any activity of its own.
- *Allosteric modulator*: This is a ligand that alters (increases or decreases) the activity of an agonist or antagonist acting on the orthosteric site without having any activity of

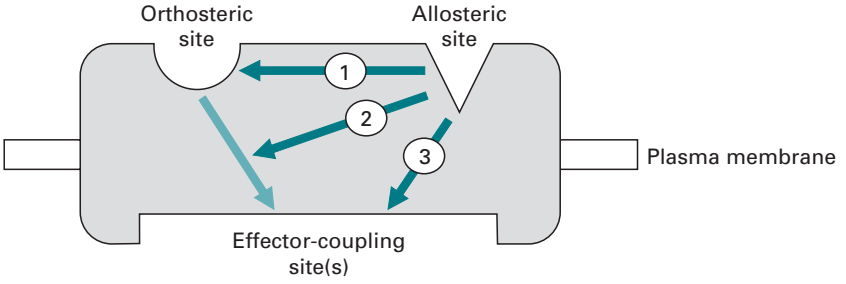

Fig. 17.3 Types of allosteric modulator. Allosteric ligands can affect receptor function in three general ways. (1) Allosteric modulation of orthosteric ligand-binding affinity; (2) allosteric modulation of orthosteric ligand efficacy; (3) direct allosteric agonism. (Reproduced from Langmead, C. J. and Christopoulos, A. (2006). Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox. *Trends in Pharmacological Sciences*, **27**, 475–481, by permission of Elsevier Science.)

its own in the absence of the agonist or antagonist. Allosteric enhancers are a subgroup of these allosteric modulators;

- *Ago-allosteric modulator*: This is a ligand that can act as both an allosteric agonist and as an allosteric modulator altering the efficacy and/or the potency of agonists acting on the orthosteric site.

The presence of allosteric sites is important in drug discovery since in principle drugs acting at this site may modify the physiological response transduced by the receptor (Section 18.2.2).

17.2.4 Quantitative characterisation of receptor–ligand binding

The previous discussion has shown that ligands capable of binding to a receptor may be of a variety of types. To fully characterise any ligand it is essential that its binding be expressed in quantitative terms. This is achieved by binding studies. If under the conditions of the binding studies the total concentration of ligand is very much greater than that of receptor (so-called **saturation conditions**), changes in ligand concentration due to receptor binding can be ignored but changes in the free (unbound) receptor concentration cannot. Hence if:

$[R_t]$ is the total concentration of receptor that determines the maximum binding capacity for the ligand
[L] is the free ligand concentration
[RL] is the concentration of receptor–ligand complex
then $[R_t - RL]$ is the concentration of free receptor.
At equilibrium, the forward and reverse reactions for ligand binding and dissociation will be equal:

$$k_{+1}([R_t] - [RL])[L] = k_{-1}[RL]$$

where k_{+1} is the association rate constant and k_{-1} is the dissociation rate constant.

Therefore:

$$\frac{k_{-1}}{k_{+1}} = K_d = \frac{1}{K_a} = \frac{([R_t] - [RL])[L]}{[RL]} \tag{17.1}$$

where K_d is the dissociation constant for RL and K_a is the association or affinity constant. Rearranging gives:

$$[RL] = \frac{[L][R_t]}{K_d + [L]} \tag{17.2}$$

Determination of K_d

Equation 17.2 is of the form of a rectangular hyperbola which predicts that ligand binding will reach a limiting value as the ligand concentration is increased and therefore that receptor binding is a saturable process. The equation is of precisely the same form as equations 15.1 and 15.2 that define the binding of the substrate to its enzyme in terms of K_m and V_{max}. For the experimental determination of K_d, equation 17.2 can be used directly by analysing the experimental data by non-linear regression curve-fitting programs (see Section 15.2.1). However, several linear transformations of equation 17.2 have been developed. One such is the **Scatchard** equation (17.3):

$$\frac{[RL]}{[L]} = \frac{[R_t]}{K_d} - \frac{[RL]}{K_d} \tag{17.3}$$

This equation predicts that a plot of [RL]/[L] against [RL] will be a straight-line slope $-1/K_d$ allowing K_d to be calculated. However, in many studies the relative molecular mass of the receptor protein is unknown so that the concentration term [RL] cannot be calculated in molar terms. In such cases it is acceptable to express the extent of ligand binding in any convenient unit (B), e.g. pmoles 10^{-6} cells, pmoles mg^{-1} protein or more simply as an observed change, for example in fluorescence ($\triangle F$), under the defined experimental conditions. Since maximum binding (B_{max}) will occur when all the receptor sites are occupied, i.e. when $[R_t] = B_{max}$, equation 17.3 can be written in the form:

$$\frac{B}{[L]} = \frac{B_{max}}{K_d} - \frac{B}{K_d} \tag{17.4}$$

Hence a plot of $B/[L]$ against B will be a straight line, slope $-1/K_d$ and intercept on the y-axis of B_{max}/K_d (Fig. 17.4). In cases where the relative molecular mass of the receptor protein is known the Scatchard equation can be expressed in the form:

$$\frac{B}{[L]B_{max}} = \frac{n}{K_d} - \frac{B}{B_{max}K_d} \tag{17.5}$$

where n is the number of independent ligand-binding sites on the receptor. The expression B/B_{max} is the number of moles of ligand bound to one mole of receptor. If this expression is defined as r, then:

$$\frac{r}{[L]} = \frac{n}{K_d} - \frac{r}{K_d} \tag{17.6}$$

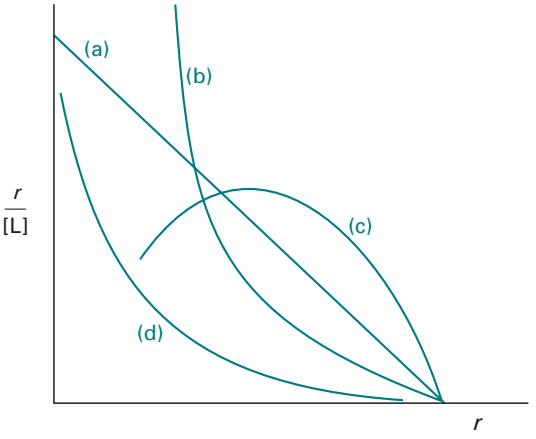

Fig. 17.4 Scatchard plot for (a) a single set of sites with no cooperativity, (b) two sets of sites with no cooperativity, (c) a single set of sites with positive cooperativity, and (d) a single set of sites with negative cooperativity.

In this case, a plot of $r/[L]$ against r will again be linear with a slope of $-1/K_d$ but in this case the intercept on the x-axis will be equal to the number of ligand-binding sites, n, on the receptor.

Alternative linear plots to the Scatchard plot are:

Lineweaver–Burk plot

$$\frac{1}{B} = \frac{1}{B_{max}} + \frac{K_d}{B_{max}[L]} \tag{17.7}$$

Hanes plot

$$\frac{[L]}{B} = \frac{K_d}{B_{max}} + \frac{[L]}{B_{max}} \tag{17.8}$$

In practice, Scatchard plots are most commonly carried out although statistically they are prone to error since the experimental variable B occurs in both the x and y terms so that linear regression of these plots overestimates both K_d and B_{max}. There is a view that linear transformations of the three types above are all inferior to the non-linear regression analysis of equation 17.2 since they all distort the experimental error. For example, linear regression assumes that the scatter of experimental points around the line obeys Gaussian distribution and that the standard deviation of the points is the same. In practice this is rarely true and as a consequence values of the slope and intercept are not the 'best' value. It can be seen from equation 17.2 that when the receptor sites are half saturated, i.e. $B = B_{max}/2$, then $[L] = K_d$. Hence K_d will have units of molarity.

The derivation of equation 17.2 is based on the assumption that there is a single set of homogeneous receptors and that there is no cooperativity between them in the binding of the ligand molecules. In practice, two other possibilities arise namely that there are two distinct populations of receptors each with different binding constants

and secondly that there is cooperativity in binding within a single population. In both cases the Scatchard plot will be curvilinear (Fig. 17.4). If cooperativity is suspected, it should be confirmed by a **Hill plot** which, in its non-kinetic form, is:

$$\log\left(\frac{Y}{1-Y}\right) = h\log[\mathrm{L}] - \log K_\mathrm{d} \qquad (17.9)$$

or

$$\log\left(\frac{B}{B_\mathrm{max}-B}\right) = h\log[\mathrm{L}] - \log K_\mathrm{d}$$

where Y is the fractional saturation of the binding sites (from 0–1) and h is the **Hill constant.** For a receptor with multiple binding sites that function independently $h = 1$, whereas for a receptor with multiple sites which are interdependent, h is either greater than 1 (**positive cooperativity**) or less than 1 (**negative cooperativity**). Scatchard plots that are biphasic due to ligand **multivalence** (i.e. multiple binding sites) rather than receptor cooperativity, are sometimes taken to indicate that the two extreme, and approximately linear, sections of the curvilinear plots represent high affinity (high bound/free ratio at low bound values) and low affinity (low bound/free ratio at high bound values) sites and that tangents drawn to these two sections of the curve can be used to calculate the associated K_d and B_max values. This is incorrect, and the correct values can only be obtained from the binding data by means of careful mathematical analysis generally undertaken by the use of special computer programs many of which are commercially available.

Determination of rate constants

To determine the dissociation rate constant, k_{-1} (units: time^{-1}) of any ligand for the receptor, some of the receptor–ligand complex (B_0) is allowed to form usually using the ligand labelled with a radioactive isotope. The availability of the remaining unoccupied receptors to the labelled ligand is then blocked by the addition of at least 100-fold excess of the unlabelled ligand or competitive antagonist and the rate of release (B_t) of the radiolabelled ligand from its binding site monitored as a function of time. This generally necessitates the separation of the bound and unbound fractions. The rate is given by the expression:

$$\frac{\mathrm{d}B_0}{\mathrm{d}t} = -k_{-1}B_0$$

and the equation governing the release by the expression:

$$B_t = B_0\mathrm{e}^{-k_{-1}t}$$

hence:

$$\log B_t = \log B_0 - 2.303k_{-1}t \qquad (17.10)$$

Thus a plot of $\log B_\mathrm{t}$ against time will give a straight line with a slope of $-2.303k_{-1}$ allowing k_{-1} to be estimated.

The association rate constant, k_{+1} (units: $M^{-1}time^{-1}$), is best estimated by the **approach to equilibrium method** by which the extent of agonist binding is monitored continuously until equilibrium is reached under conditions that are such that [L]>> [R_t] (this gives pseudo first-order conditions rather than second order; under these conditions [R_t] decreases with time but [L] remains constant.) Ligand binding increases asymptotically such that:

$$\log\left(\frac{B_{eq}}{B_{eq}-B_t}\right) = 2.303(k_{+1}[L]+k_{-1})t \qquad (17.11)$$

Thus a plot of log $B_{eq}/(B_{eq} - B_t)$ against time will be linear with a slope of $2.303(k_{+1}[L] + k_{-1})$ where B_{eq} and B_t are the ligand binding at equilibrium and time t respectively. From knowledge of k_{-1} (obtained by the method discussed above) and [L], the value of k_{+1} can be calculated from the slope.

The K_d values observed for a range of receptors binding to their physiological agonist are in the range 10^{-6}–10^{-11} M, which is indicative of a higher affinity than is typical of enzymes for their substrates. The corresponding k_{+1} rate constants are in the range 10^5–10^8 $M^{-1}min^{-1}$ and k_{-1} in the range 0.001–0.5 min^{-1}. Studies with G-protein-coupled receptors that form a tertiary complex (AR*G) have shown that the tertiary complex has a higher affinity for the agonist than has the binary complex (AR*). Receptor affinity for its agonist is also influenced by receptor interaction with various adaptor protein molecules present in the intracellular cell membrane. This is discussed more fully later.

It is relatively easy to calculate the number of receptors on cell membranes from binding data. The number is in the range 10^3–10^6 per cell. Although this may appear

Example 1 ANALYSIS OF LIGAND-BINDING DATA

Question The extent of the binding of an agonist to its membrane-bound receptor on intact cells was studied as a function of ligand concentration in the absence and presence of a large excess of unlabelled competitive antagonist. In all cases the extent of total ligand binding was such that there was no significant change in the total ligand concentration. What quantitative information about the binding of the ligand to the receptor can be deduced from this data?

	[Ligand] (nM)							
	40	60	80	120	200	500	1000	2000
Total ligand bound (pmoles 10^{-6} cells)	0.284	0.365	0.421	0.547	0.756	1.269	2.147	2.190
Ligand binding in presence of competitive antagonist (pmoles 10^{-6} cells)	0.054	0.068	0.084	0.142	0.243	0.621	1.447	1.460

Example 1 (*cont.*)

Answer To address this problem it is first necessary to calculate the specific binding of the ligand to the receptor (B_s). The use of a large excess of unlabelled competitive antagonist enables the non-specific binding to be measured. The difference between this and the total binding gives the specific binding. Once this is known, various graphical options are open to evaluate the data. The simplest is a plot of the specific ligand binding as a function of the total ligand binding. More accurate methods are those based on linear plots such as a Scatchard plot (equation 17.4) and a Lineweaver–Burk plot (equation 17.7). In addition, it is possible to carry out a Hill plot (equation 17.9) to obtain an estimate of the Hill constant, h. The derived data for each of these three plots are shown in the following table:

	[Ligand] (nM)							
	40	60	80	120	200	500	1000	2000
Total bound ligand (pmol 10^{-6} cells)	0.284	0.365	0.421	0.547	0.756	1.269	2.147	2.190
Non-specific binding (B_{ns}) (pmol 10^{-6} cells)	0.054	0.068	0.084	0.142	0.243	0.621	1.447	1.460
Specific binding (B_s) (pmol 10^{-6} cells)	0.230	0.297	0.337	0.405	0.513	0.648	0.700	0.730
$B_s/[L] \times 10^3$ (dm^3 10^{-6} cells)	5.75	4.95	4.21	3.37	2.56	1.30	0.70	0.43
$1/[B_s]$ (pmol 10^{-6} cells)$^{-1}$	4.35	3.37	2.97	2.47	1.95	1.54	1.43	1.37
$1/[L]$ $(nM)^{-1}$	0.0250	0.0170	0.0125	0.0083	0.0050	0.0020	0.0010	0.0005
$(B_{max} - B_s)$ (pmol 10^{-6} cells)	0.52	0.45	0.413	0.345	0.237	0.102	0.050	0.020
$B_s/(B_{max} - B_s)$	0.44	0.66	0.816	1.174	2.164	6.35	14.00	36.50
log $B_s/(B_{max} - B_s)$	−0.356	−0.180	−0.088	0.070	0.335	0.803	1.146	1.562
log [L]	1.60	1.78	1.90	2.08	2.30	2.70	3.00	3.30

The hyperbolic plot allows an estimate to be made of the maximum ligand binding, B_{max}. It is approximately 0.75 pmoles 10^{-6}cells. An estimate can then be made of K_d by reading the value of [L] that gives a ligand binding value of 0.5 B_{max} (0.375 pmoles 10^{-6} cells). It gives an approximate value for K_d of 100 nM.

A Scatchard plot obtained by regression analysis gives a correlation coefficient, r, of 0.996, B_{max} of 0.786 pmoles 10^{-6} cells and K_d of 97.3 nM. A Lineweaver–Burk plot gives a correlation coefficient, r, of 0.998, B_{max} of 0.746 pmoles 10^{-6} cells and K_d of 90.5 nM. Note that there is some variation between these three sets of calculated values and the ones given by the Lineweaver–Burk plot are more likely to be correct since, as previously pointed out, the Scatchard plot overestimates both values when the binding data are subjected to linear regression analysis.

The Hill plot based on a value of B_{max} of 0.75 pmoles 10^{-6} cells, gave a correlation coefficient of 0.998 and a value for the slope of 1.13. This is equal to the Hill constant, h.

Table 17.1 **G-protein coupling of 5-hydroxtryptamine (serotonin) receptors**

Family	G-protein coupling	Response
5-HT_1 (5 isoforms)	G_i/G_o coupled	Decreasing levels of cAMP
5-HT_2 (3 isoforms)	G_q/G_{11} coupled	Increasing cellular levels of IP_3 and DAG
5-HT_3 (not GPCR)	Ligand-gated Na^+ and K^+ cation channel	Depolarising plasma membrane
5-HT_4	G_s coupled	Increasing levels of cAMP
5-HT_{5A}	G_i/G_o coupled?	Inhibition of adenylyl cyclase
5-HT_6	G_s coupled	Stimulates adenylyl cyclase
5-HT_7	G_s coupled	Increasing levels of cAMP

large, it actually represents a small fraction of the total membrane protein. This partly explains why receptor proteins are sometimes difficult to purify. From knowledge of receptor numbers and the K_d values for the agonist, it is possible to calculate the occupancy of these receptors under normal physiological concentrations of the agonist. In turn it is possible to calculate how the occupancy and the associated cellular response will respond to changes in the circulating concentration of the agonist. The percentage response change will be greater the lower the normal occupancy of the receptors. This is seen from the shape of the dose–response curve within the physiological range of the agonist concentration. It is clear that if the normal occupancy is high, the response to change in agonist concentration is small. Under such conditions, the response is likely to be larger if the receptor–agonist binding is a positively cooperative process.

Receptor subclasses

Binding studies using both agonists and inverse agonists have identified receptor subclasses for a given endogenous agonist. As an example, over a dozen types of 5-hydroxytryptamine (5-HT, serotonin) receptor have been identified (Table 17.1). An interesting feature of some receptor subclasses is that not only do they have different binding characteristics, but they may also trigger opposing cellular responses. Thus there are three subclasses (β_1, β_2 and β_3) of β-adrenergic receptors with different amino acid composition, affinities for agonists and physiological responses but all of which are activated by adrenaline and noradrenaline. β_1-Adrenergic receptors mediate cardiac responses, β_2-adrenergic receptors are involved in skeletal and smooth muscle function, and β_3-adrenergic receptors are involved in metabolic responses. Selective synthetic agonists, such as salbutamol used in the treatment of asthma, readily discriminate between the three subclasses.

Quantitative characterisation of competitive antagonists

The ability of a competitive antagonist to reduce the response of a receptor to a given concentration of agonist can be quantified in two main ways:

- *IC_{50} value*: The antagonist concentration that reduces the response of the receptor, in the absence of the antagonist, to a given concentration of agonist by 50%.
- *K_b value*: The dissociation constant for the binding of the antagonist.

To determine an IC_{50} value, the standard procedure is to study the effect of increasing concentrations of antagonist on the response to a fixed concentration of agonist. In the absence of antagonist the response will be a maximum. As the antagonist concentration is increased the response will decrease in a manner that is a mirror image of a dose–response curve (Fig. 17.1). From the curve the antagonist concentration required to reduce the response by half (IC_{50}) can be determined. If this study is repeated for a series of increasing fixed agonist concentrations it will be evident that the IC_{50} value is critically dependent on the agonist concentration used, i.e. it is not an absolute value. In spite of this, it is commonly used, because of its simplicity, particularly in the screening of potential therapeutic agents. From knowledge of the IC_{50} value, in principle it is possible to calculate the K_b value using the *Cheng–Prusoff equation*:

$$K_b = \frac{IC_{50}}{(1 + [L]/K_d)} \tag{17.12}$$

where [L] is the concentration of agonist and K_d is its dissociation constant. It is evident from this equation that IC_{50} only approximates to K_b when [L] is very small and the denominator approaches 1. Although this equation is commonly used to calculate K_b values its application is subject to reservations primarily because inhibition curves do not confirm the nature of the antagonism but also because the application of the equation is subject to the concentration of agonist used relative to its EC_{50} value. Antagonist equilibrium constants, K_b, are best determined by application of the **Schildt equation**:

$$r = 1 + [B]/K_b \tag{17.13}$$

or in its logarithmic form:

$$\log(r - 1) = \log[B] - \log K_b \tag{17.14}$$

where [B] is the concentration of antagonist, and r is the *dose factor* that measures the amount by which the agonist concentration needs to be increased in the presence of the antagonist to produce the same response as that obtained in the absence of antagonist, the assumption being made that the same fraction of receptors needs to be activated in the presence and absence of the antagonist to produce a given response. Experimentally, the dose ratio is equal to the dose of agonist required to give 50% response in the presence of the given antagonist concentration divided by the EC_{50} value.

To carry out a Schildt plot, the receptor response to increasing concentrations of agonist for a series of fixed concentrations of antagonist is studied and the dose ratio for each concentration of antagonist calculated. The maximum response in all cases should be the same. Equation 17.14 then predicts that a plot of the log(r – 1) against log[B] should be a straight line of slope unity with an intercept on the abscissa equal to log K_b. If the slope is not unity then either the antagonist is not acting competitively or more complex interactions are occurring between the antagonist and the receptor, possibly allosteric in nature. It is important to note from the Schild equation that the value of K_b, unlike that of IC_{50}, is independent of the precise agonist used to generate the data and is purely a characteristic of the antagonist for the specific receptor. A limitation of a Schildt plot is that any error in measuring EC_{50} (i.e. the response in the absence of antagonist) will automatically influence the value of all the derived dose ratios. The intercept of a Schildt plot on the y-axis also gives the pA_2 value for the antagonist (p for negative logarithm; A for antagonist; and 2 for the dose ratio when the concentration of antagonist equals pA_2). The pA_2 will be equal to the value of $-\log K_b$ since at an antagonist concentration that gives a dose ratio of 2, the Schildt equation reduces to log[B] = log K_b. pA_2 is a measure of the **potency** of the antagonist. Software programs are commercially available for the analysis of ligand-binding data. Examples include: *Prism* (www.graphpad.com/curvefit); *Sigmaplot* (www.systat.com/products/sigmaplot); *Origin8* and *OriginPro 8* (www.originlab.com) and *Calcusyn* (www.biosoft.com).

Example 2 SCHILDT PLOT – CALCULATION OF A K_b AND pA_2 VALUE

Question Use the data in Fig. 17.2 to construct a Schildt plot. The left-hand plot is the receptor response to agonist binding in the absence of antagonist. The next three responses are in the presence of 10^{-7} M, 10^{-6} M and 10^{-5} M antagonist. Read off from the graph the concentration of agonist required to produce 50% maximum response in the absence and presence of the antagonist and calculate the dose ratio (r) at each of the three antagonist concentrations. Then plot a graph of log(r – 1) against log [antagonist] and hence calculate both pA_2 and K_b.

[Antagonist] (M)	10^{-7}	10^{-6}	10^{-5}
EC_{50} (M)	10^{-7}	10^{-6}	10^{-5}
r	10	100	1000
$r-1$	9	99	999
log (r – 1)	0.954	1.9956	2.999
log [B]	−7	−6	−5

Answer You will see that the Schildt plot is linear ($r = 0.9999$) and that it has a slope of 1.02 confirming the competitive nature of the antagonist. The extrapolation of the line to the y-axis gives a value of −8.12. This is equal to $-\log K_b$, hence $K_b = 7.58 \times 10^{-9}$ M and $pA_2 = 8.12$.

17.3 LIGAND-BINDING AND CELL-SIGNALLING STUDIES

17.3.1 Selection of ligand and receptor preparation

Receptor preparations

Preparations of receptors for ligand-binding studies may either leave the membrane intact or involve the disruption of the membrane and the release of the receptor with or without membrane fragments, some of which could form vesicles with variable receptor orientation and control mechanisms. Membrane receptor proteins show no or very little ligand-binding properties in the absence of phospholipid so that if a purified receptor protein is chosen, it must be introduced into a phospholipid vesicle for binding study purposes. The range of receptor preparations available for binding studies is shown in Table 17.2.

Kinetic studies aimed at the determination of individual rate constants are best carried out using isolated cells whilst studies of the number of receptors in intact tissue are best achieved by labelling the receptors with a radiolabel preferably using an irreversible competitive antagonist and applying the technique of quantitative autoradiography.

Ligands

A common technique for the study of ligand–receptor interaction is the use of a radiolabelled ligand with isotopes such as ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{135}I. Generally a high-specific-activity ligand is used as this minimises the problem of non-specific binding (see below). If a large number of ligands are being studied, such as in the screening of potential new therapeutic agents, the cost and time of producing the radiolabelled forms becomes virtually prohibitive and experimental techniques such as fluorescence spectroscopy and surface plasmon resonance spectroscopy have to be used. However, the use of radiolabelled ligands remains attractive as a means of distinguishing between orthosteric and allosteric ligands.

The technique of using radiolabelled ligand generally requires the separation of bound and unbound ligand once equilibrium has been achieved. This is most commonly achieved by techniques such as equilibrium dialysis and ultrafiltration exploiting the inability of receptor-bound ligand to cross a semi-permeable membrane, and by simple centrifugation exploiting the ability of the receptor-bound ligand to be pelleted by an applied small centrifugal field.

For the study of ligand–receptor interactions that occur on a sub-millisecond timescale, special approaches such as stopped-flow and quench-flow methods (Section 15.3.3) need to be adopted to deliver the ligand to the receptor. An alternative approach is the use of so-called **caged compounds**. These possess no inherent ligand properties but on **laser flash photolysis** with light of a specific wavelength, a protecting group masking a key functional group is instantaneously cleaved releasing the active ligand.

Table 17.2 **Receptor preparations for the study of receptor–ligand binding**

Receptor preparation	Comments
Tissue slices	5–50 μm thick, generally adhered to gelatine-coated glass slide. Good for study of receptor distribution.
Cell membrane preparation	Disrupt cells (from tissue or cultures) by sonication and isolate membrane fraction by centrifugation. Lack of cytoplasmic components may compromise receptor function. Used to study ligand binding and receptor distribution in lipid rafts and caveolae. Increasingly commonly used with cell lines transfected with human receptor genes.
Solubilised receptor preparation	Disrupt membrane with detergents and purify receptors by affinity chromatography using an immobilised competitive antagonist. Isolation from other membrane components may compromise studies.
Isolated cells	Release cells from tissue by mechanical or enzymatic (collagenase, trypsin) means. Cells may be in suspension or monolayers. May be complicated by presence of several cell types. Widely used for the study of a range of receptor functions. Allows ligand binding and cellular functional responses to be studied under the same experimental conditions.
Cultured cell lines	Very popular. Has advantage of cell homogeneity and ease of replication.
Recombinant receptors	Produced by cloning or mutagenesis techniques and inserted into specific cultured cell line including ones of human origin. Popular for the study of the effect of mutations on receptor function such as constitutive activity (CAMs) and cell signalling. Care needed to ensure that receptors have same functional characteristics (e.g. post-translational modification) as native cells.

17.3.2 Experimental procedures for ligand-binding studies

The general experimental approach for studying the kinetics of receptor–ligand binding and hence to determine the experimental values of the binding constants and the total number of binding sites is to incubate the receptor preparation with the ligand under defined conditions of temperature, pH and ionic concentration for a specific period of time that is sufficient to allow equilibrium to be attained. The importance of allowing the system to reach equilibrium cannot be overstated as equations 17.1 to 17.11 do not hold if equilibrium has not been attained. Using an appropriate analytical procedure, the bound and unbound forms of the ligand are then quantified or some associated change measured. This quantification may necessitate the separation of the bound and unbound fractions. The study is then repeated for a

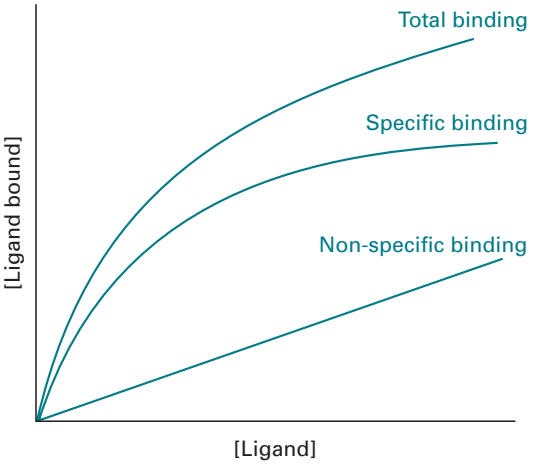

Fig. 17.5 Specific and non-specific binding of a ligand to a membrane receptor. Specific binding is normally hyperbolic and shows saturation. Non-specific binding is linear and is not readily saturated.

series of ligand concentrations to cover 10–90% of maximum binding at a fixed receptor concentration. The binding data are then analysed using equations 17.4 to 17.11 often in the form of a computer program many of which are available commercially. Most perform linear or non-linear least-squares regression analysis of the experimental data. Measurements may be made on a **steady-state** (single measurement) basis or a **time-resolved** (multiple measurements over a period of time) basis most commonly by stopped-flow or quench-flow procedures (Section 15.3.3).

Non-specific binding

A general problem in the study of receptor–ligand binding is the **non-specific binding** of the ligand to sites other than the orthosteric or allosteric binding sites. Such non-specific binding may involve the membrane lipids and other proteins either located in the membrane or released by the isolation procedure. The characteristic of non-specific binding is that it is non-saturable but is related approximately linearly to the total concentration of the ligand. Thus the observed ligand binding is the sum of the saturable (hyperbolic) specific binding to the receptor and the non-saturable (linear) binding to miscellaneous sites. The **specific binding** component is usually obtained indirectly either by carrying out the binding studies in the presence of an excess of non-labelled ligand (agonist or antagonist) if a labelled ligand is being used, or by using a large excess of an agonist or competitive antagonist in other studies. The presence of the excess unlabelled or competitive ligand will result in the specific binding sites not being available to the ligand under study and hence its binding would be confined to non-specific sites (Fig. 17.5). In practice, a concentration of the competitive ligand of at least 1000 times its K_d or K_b must be used and confirmation that under the conditions of the experiment, non-specific binding was being studied would be sought by repeating the study using a range of different and structurally dissimilar competitive ligands that should give consistent estimates of the non-specific binding.

Experimental techniques for the study of the binding of a ligand to a membrane receptor

Equations 17.1 to 17.11 allow the calculation of quantitative parameters that characterise the binding of a ligand to a receptor. Such parameters are fundamental to the understanding of the mechanism of the binding and its relationship to the subsequent cellular response. They also allow comparisons to be made of the comparative efficacy and affinity of a series of ligands for a common receptor, a process that is essential in the development of new drugs (Chapter 18). Numerous techniques are available for the study of ligand binding but those that are amenable to automation, do not require the bound and unbound fraction of ligand to be separated and do not require the use of radiolabelled ligand are generally the preferred methods. Examples are as follows.

Fluorescence spectroscopy

Fluorescence-based techniques are ideal for the study of ligand–receptor binding as they are ultra sensitive, being capable of studying binding involving a few or even individual ligand molecules and single receptors. The general principles of fluorescence spectroscopy are discussed in Chapter 12. The methods are based on either changes in the intrinsic fluorescence of the receptor protein tagged with a suitable fluorescent marker (fluor) or the induction of fluorescence in either the ligand or the receptor protein as a result of receptor–ligand binding. Commonly used fluors include fluorescein, rhodamine and the dye Fluo-3 (Table 4.3.) but a better alternative is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* or the red fluorescent protein of *Discosoma striata* either of which can be attached to receptor proteins by gene cloning without altering the normal function of the protein. The main advantage of using either of these two autofluorescent proteins is that no cofactors are required for fluorescence to occur hence the study protocols are relatively simple.

The most common forms of fluorescence spectroscopy applied to the study of receptor-ligand binding are:

- *Fluorescence resonance energy transfer* (FRET): This relies on the presence of two fluors in distinct locations within the receptor protein such that the emission spectrum of one and the excitation spectrum of the other overlap. In such circumstances, the emission light of one fluor may be absorbed by the second (hence energy transfer) and be emitted as part of its emission. The extent to which this may occur is proportional to $1/R^6$, where R is the distance between the two fluors and which is changed as a result of ligand binding.
- *Fluorescence anisotropy*: Anisotropy is the directional variation in optical properties, in this case fluorescence, along perpendicular and parallel axes. In this technique fluorescence is induced by plane-polarised (blue) light. Molecules of the fluor orientated parallel to this plane of polarisation will be excited preferentially. However, if some of these molecules rotate after the absorption of the light but before the fluorescence has time to occur some of the resulting fluorescence will be depolarised (i.e. no longer in one plane). The extent to which this occurs can be used to deduce information about the size, shape and flexibility of the protein carrying the fluor. It can also be used to monitor the binding of a ligand to the protein. The fluorescence

intensity is measured parallel (i.e. in the same plane) to the absorbed plane-polarised light and at right angles to it. From the two measurements it is possible to calculate the degree of fluorescence depolarisation and hence the fluorescence anisotropy, both of which are expressed in terms of the difference between the fluorescence parallel to the absorbed plane-polarised light and that perpendicular to it, the difference being expressed as a function of the sum of the fluorescence in the two planes.

- *Fluorescence cross-correlation spectroscopy* (FCCS): This technique differs from other forms of fluorescence spectroscopy in that it is not primarily concerned with fluorescence intensity, but rather with small spontaneous fluorescence fluctuations induced by molecules diffusing into and out of a small focal volume in aqueous solution. Such fluctuations are related to changes in the diffusion coefficient of each probe and hence can be correlated with receptor–ligand binding. Both the receptor and the ligand are labelled with spectrally distinct fluors and their interaction studied by confocal microscopy (Section 4.3.1). Two lasers are aligned to the same confocal point and used to excite the two fluors. Following binding, the receptor–ligand complex emits in both fluorescent wavelengths that are monitored to give a cross-correlation signal that is directly related to the concentration of the receptor–ligand complex.

Surface plasmon resonance (SPR) spectroscopy

The principles and experimental details of this technique are discussed in Section 13.3. The advantages of the technique are that it does not require the molecules to be fluorescent or radiolabelled; it can be used to study molecules as small as 100 Da and can be used with coloured or opaque solutions. Such studies with G-protein-coupled receptors have shown that agonists, inverse agonists and antagonists can readily be distinguished by the conformational changes they induce in the membrane in the region of the receptor. Specifically, agonists and inverse agonists increase membrane thickness (agonists more so than inverse agonists) by causing an elongation of the receptor whereas antagonists cause no change. **Plasmon-waveguide resonance** (PWR) **spectroscopy** is closely related to SPR spectroscopy. It is more sensitive than SPR spectroscopy and can be used to study receptor conformational changes in lipid bilayers. Thus a single lipid bilayer is deposited on the resonator surface and the receptor protein inserted from a detergent-solubilised solution. A solution containing the ligand is then passed over the layer allowing the binding process to be studied.

Isothermal titration calorimetry

This method is based on the measurement of the heat change, positive or negative, associated with the binding and of the relationship between the enthalpy change ($\triangle H$), Gibbs' free energy change ($\triangle G$), the entropy change ($\triangle S$), the number of molecules of ligand bound to each receptor protein (**stoichiometry**) (n) and the binding constant (K_a). The experimental details are discussed in Section 15.3.3. Its practical advantages are that it can be applied to the study of any receptor–ligand pair without the need for radiolabelling or the attachment of fluors, or the need to separate bound and unbound fractions.

Protein microarray technology

This approach to the study of receptor–ligand binding exploits the principle that assay systems that use a small amount of capture molecules (the ligand) and a small amount of target molecules (the receptor) can be more sensitive than systems that use a hundred times more material (Section 8.5.5). In this miniaturisation approach, the ligand is immobilised onto a small area of a solid phase, commonly a derivatised glass slide. The resulting 'microspot' contains a high density (concentration) of ligand but a very small amount of it. It is then incubated with the receptor, commonly fluorescently tagged, resulting in the binding of some of the receptor molecules. Since the microspot covers a small area there is effectively no change in the concentration of the unbound ligand in the sample even if its concentration was low and the binding affinity was high. This is true provided that $<0.1\ K_d$ of the ligand molecules are bound in the complex, where K_d is the dissociation constant for the complex. The ligand–receptor complex is then quantified by fluorimetric methods and the procedure repeated for a series of increasing microspot sizes (increasing ligand concentration) but such that the density of the capture molecules is constant. After each incubation excess receptor protein is washed away and the remaining complex analysed by surface-enhanced laser desorption/ionisation (SELDI) mass spectrometry, a variant of MALDI (Section 9.3.8). In practice, microspots are immobilised in rows on the solid support allowing the simultaneous analysis of hundreds or even thousands of samples.

Other methods used to study ligand–receptor binding

Other experimental methods that can be used to quantify receptor–ligand binding and hence characterise both the receptor and the ligand include:

- analytical ultracentrifugation by the sedimentation velocity (Section 3.5.1),
- scintillation proximity assay (Section 14.3.2) commonly using ^{3}H- or ^{125}I-labelled ligand,
- NMR (Section 13.5) observing either changes in the signal of the protein or of the ligand induced by receptor–ligand binding,
- X-ray crystallography (Section 13.6) either by co-crystallising the receptor and ligand or by soaking crystals of the receptor in solutions of the ligand.

17.4 MECHANISMS OF SIGNAL TRANSDUCTION

17.4.1 Cell signalling assays

In order to determine the details of a specific transduction pathway it is necessary to identify the proteins and other small effectors whose activity or concentration change in response to the activation of the receptor. This is possible using **functional screening assays** that are of two main types: **cell-free** (biochemical) and **cell-based** (cellular) (see Section 18.2.3 for further details). In practice, whilst cell-free

systems are rapid and relatively easy to interpret, cellular assays have several advantages:

- they do not require any pre-purification of the receptor protein which make them attractive for the screening of so-called orphan receptors whose cellular function and physiological ligand have yet to be determined;
- the conformation and hence the activity of the receptor protein is most likely to better reflect the physiological situation than is the case for biochemical cell-free assays;
- their use allows screening for potential therapeutic agents so as to quickly identify and reject compounds that possess any cytotoxic properties;
- they are readily adaptable to robotically controlled **high-throughput screening** or **high-content screening** protocols both of which are commonly based on fluorescence microscopy using cells in microtitre plates (Section 18.2.3).

Much of the work using these assays is aimed at the discovery of new drugs and so cells of human origin are the preferred targets. In practice such cells are expensive to culture and are not readily adapted to automated assays. For such reasons, micro-organisms are commonly used, with yeast being most commonly chosen because of the high degree of conservation of basic molecular and cellular mechanisms between yeast and human cells. Moreover, it is relatively easy to engineer yeast cells to incorporate human receptor proteins such as GPCRs, RTKs and ion-channels.

Numerous analytical techniques have been used to probe signalling pathways (Fig. 17.6). The majority are adaptations of standard techniques for studying protein structure, protein–ligand interaction and protein–protein interaction. As a result of their high sensitivity, fluorimetric techniques, particularly fluorescence resonance energy transfer and fluorescence correlation spectroscopy, feature prominently in these techniques. Techniques are now available for the site-specific post-translational labelling of proteins with small fluorescent tags. Commercial companies offer reagents, kits and services to facilitate the rapid identification of proteins associated with the activation of a particular receptor. The most common approaches include:

- The use of monoclonal antibodies for western blots, protein purification and immunocytochemistry. Western blots can identify changes in the expression of a specific protein in the pathway; immunocytochemistry can detect movement within the cell as a result of the activation of a pathway and the use of phosphospecific antibodies can detect the phosphorylation of a particular protein.
- The use of *knock-out* and *knock-in* strategies using mice or cell lines, commonly embryonic stem cells. In these techniques either the endogenous locus of the receptor gene is manipulated or a modified receptor gene with an appropriate promoter is expressed in the host. The effect of such action is then studied by techniques such as Southern and western blots.
- The use of microarray techniques – 'chips' are now commercially available on which all the proteins of the *Saccharomyces cerevisiae* proteome or a large proportion of those of the human proteome have been individually deposited (see Sections 8.5.4 and 8.5.5 for further details). The approach is particularly suitable for identifying

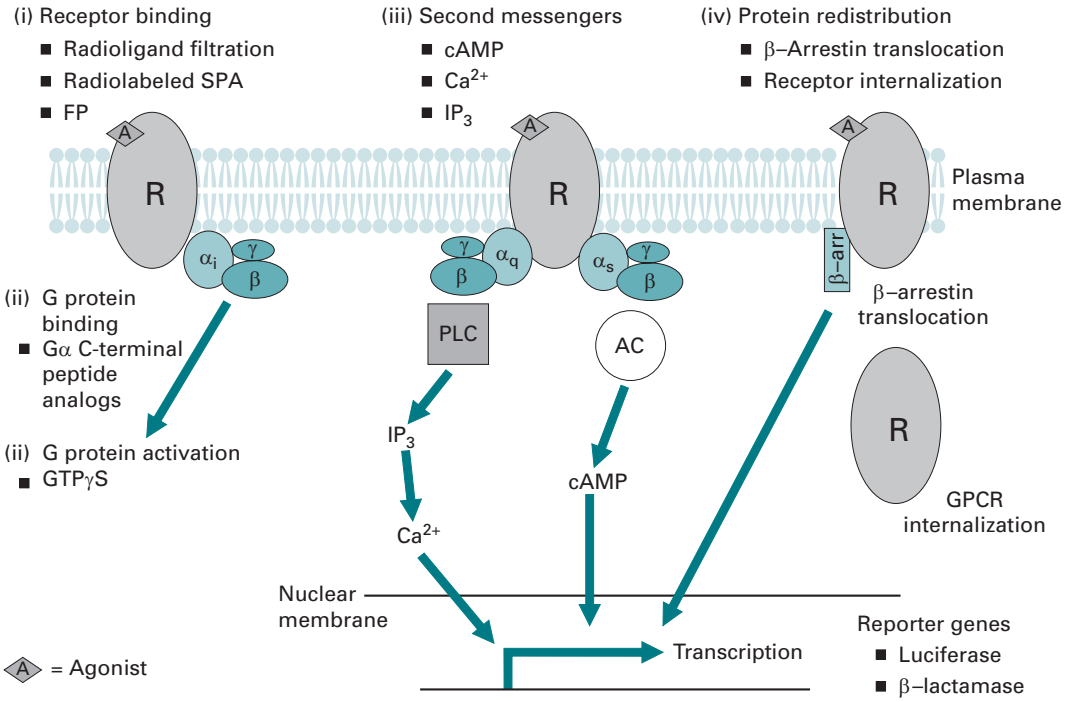

Fig. 17.6. Examples of GPCR screening approaches. The modulation of GPCR activity can be measured using a variety of assays, including (i) receptor binding; (ii) G-protein binding and activation; (iii) second-messenger signalling, including reporter systems that measure the response downstream; and (iv) protein redistribution. (i) Receptor binding can be assayed using filtration binding of a radiolabelled ligand using a radioactive ligand and a scintillation proximity assay (SPA), or using a fluorescently labelled ligand and fluorescence polarisation (FP) to monitor specific binding between the ligand and the receptor. Receptor activity can also be monitored using (ii) G-protein binding and activation with high-affinity peptides that mimic the Gα C-terminus or using GTPγS assays. Subsequently, Gα and Gβγ go on to activate their effector molecules, and (iii) second-messenger signalling pathways can be monitored. For example, the $G\alpha_q$ subunit can interact with phospholipase C (PLC), causing enzyme activation and the catalysis of phosphotidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP_3) which can then mobilise Ca^{2+}. Alternatively, the $G\alpha_s$ subunit can interact with and promote the activity of adenylyl cyclase (AC), resulting in the production of cAMP. When assaying for (iv) protein redistribution, either the translocation of β-arrestin (β-arr) or the internalisation of the GPCR can be monitored. (Reproduced from Gilchrist, A. (2007). Modulating G-protein-coupled receptors: from traditional pharmacology to allosterics. *Trends in Pharmacological Sciences*, **28**, 431–438, by permission of Elsevier Science.)

the likely substrates of the very large number of protein kinases involved in cell signalling and to study the role of ubiquitination in receptor recycling.

- The use of the **enzyme fragment complementation** (EFC) technique. This is commonly carried out using the enzyme β-galactosidase. The technique uses a small fragment of the enzyme and an inactive deletion mutant of the enzyme both of which are enzymically inactive. The two forms are attached either by chemical conjugation or by a recombination procedure to two proteins suspected of interacting in a particular signalling pathway. If the two proteins do interact they allow the two attached galactosidase forms to complement each other generating a heteromeric complex

capable of acting on an added substrate of the enzyme to release a fluorescent or chemiluminescent signal that can readily be detected and quantified. The technique has been particularly successful in the study of pathways that result in a nuclear signal since the resulting fluorescent signal will be located in the nucleus. In an alternative format, EFC can be used to monitor second messengers such as cAMP and hence be used to study GPCRs. In this case, cAMP is chemically conjugated to the galactosidase fragment and an antibody that binds to the conjugated cAMP added to the assay. The antibody sterically prevents the two galactosidase forms combining to form the active enzyme and hence produce a signal. However, as free cAMP is generated by the GPCR–adenylyl cyclase system it displaces the conjugated cAMP from the antibody allowing the two galactosidase fragments to complement and generate a signal thus allowing the cAMP to be quantified. Similar assays have been developed for tyrosine kinases and serine threonine kinases and the technique can be used to monitor protein expression changes in response to receptor activation. The assays are easily miniaturised and automated to give high throughput precision data.

17.4.2 Signal transduction through ligand-gated ion channels

Ligand-gated ion channels constitute one of the mechanisms for the control of the transmembrane movement of ions down their concentration gradient resulting in a change in membrane potential. This control of ion movement is exerted on the basis of ion type (anion or cation), ion charge and ion size. Binding of the ligand to the resting state of the receptor induces a conformational change in the receptor protein that results in the opening of the channel and the movement (gating) of ions. The channel remains open until either the ligand is removed or when, in the continued presence of the ligand, the receptor protein changes to its desensitised state in which the channel is closed. Since this mechanism of transduction is independent of any other membrane component or intracellular molecule, the cellular response to ligand binding is almost instantaneous. This class of membrane receptors includes numerous receptors that are involved in signal transmission between neurons, between glia and neurons and between neurons and muscles.

Four superfamilies of ligand-gated ion channels, classified on the basis of the number of transmembrane (TM) segments within the subunits (2TM, 3TM, 4TM and 6TM) have been identified. The 4TM family has been the most thoroughly investigated and all members shown to consist of five subunits (pentameric). Members of the family include:

- nicotinic acetylcholine receptors (nAChR) that are the primary excitatory receptors in skeletal muscle and the peripheral nervous system;
- serotonin (5-hydroxytryptamine) (5HT) receptors located in neurons;
- γ-aminobutyric acid receptors ($GABA_A$ and $GABA_B$) found in the cortex and which are inhibitory transmitters;
- glycine receptors found in the spinal cord and brainstem and which like the GABA receptors are inhibitory transmitters.

The five subunits of the nAChR receptor are of four types, α, β, γ and δ with a stoichiometry of $\alpha_2\beta\gamma\delta$. All five span the membrane four times mainly with α-helical

structure, but with some β-structure. Each α-helical region has been designated TM1–TM4 and the experimental evidence supports the view that the TM2 regions of each of the five subunits line the ion channel with the TM1 and TM3 regions forming a scaffold to support the channel. Binding studies have provided evidence for the allosteric binding of two molecules of agonist with a Hill constant of about 2. Affinity labelling studies have shown that the ligand-binding sites are in a cleft between two subunits. In both cases an α-subunit has the principal role in binding with the γ and δ subunits playing a minor role. The first ligand molecule binds to the α_1 subunit which is in contact with the δ subunit and the second ligand molecule to α_2 which is in contact with the γ subunit. Thus the two binding sites are not structurally identical. Genetically engineered variants of the five subunits have shown that the absence of the α subunit results in the lack of binding of acetylcholine thereby confirming the importance of this subunit.

Electron microscopic study of the nAChR on the *Torpedo* electric organ postsynaptic membrane has given an indication of the three-dimensional structure of the channel. It is funnel-shaped with a large proportion of the receptor outside the membrane protruding into the postsynaptic cleft. The channel is 25–30 Å wide at the entrance and only 6.4 Å wide at its narrowest point. Three rings of negatively charged amino acid residues, all on the four TM2 helices, line the narrow part of the channel and appear to determine its selectivity. The importance of these amino acid residues has been confirmed by mutagenicity studies. Detailed electrophysiological studies have revealed that channel opening involves the subtle rearrangement of three transmembrane α-helices, one in each of the TM1, TM2 and TM3 segments. The whole process is kinetically complex but the essential features may be represented as follows:

$$\begin{array}{lccccccccc} & A + R & \rightleftharpoons & AR + A & \rightleftharpoons & A_2R & \rightleftharpoons & A_2R^{*} & \rightleftharpoons & A_2R^{**} \\ \text{channel :} & \text{closed} & & \text{closed} & & \text{closed} & & \text{open} & & \text{desensitised} \end{array}$$

where A is acetylcholine and R the receptor containing two binding sites for acetylcholine, one on each α subunit.

Measurement of the numerous rate constants for these reversible processes has revealed that the rate constant for the opening of the channel is greater than the rate constants for the corresponding reverse process (i.e. the reversion to the closed conformation), for the dissociation of a ligand molecule from the closed conformation and for the transition from the open active state to the desensitised state. The consequence of this is that many opening and closing events of the channel occur before either the transition to the desensitised state or a molecule of ligand dissociates from the binding site. The mean channel open time for torpedo nAChR is 3.0 ms and the mean closed time 94 μs within the bursts of opening and closing activity. The desensitised receptor (R**) eventually reverts to the closed resting state (R).

Desensitisation may be linked to phosphorylation. All five subunits contain amino acid residues located between the TM3 and TM4 regions that are potential sites for phosphorylation, and phosphorylation of the receptor has been shown to occur at two serine residues on each of the γ and δ subunits. Each phosphate group introduces two negative oxygen atoms that could induce important conformational changes in the

receptor structure and desensitisation. Mutagenesis studies of these serine residues have shown that their replacement by non-polar amino acids minimises the susceptibility of the receptor to acetylcholine-induced desensitisation. In contrast, replacement of the serines by glutamates, which contain negatively charged carboxyl groups, permanently desensitises the receptor. This phosphorylation-induced modulation of receptor function is found in many other types of ion-transport proteins indicating a common mechanism, but it is not clear whether or not phosphorylation is an essential prerequisite for receptor desensitisation.

17.4.3 Signal transduction through G-protein-coupled receptors (GPCRs)

The fact that over 800 human G-protein-coupled receptors have been identified and verified emphasises their cellular importance. They are the largest group of signalling transduction receptors and are responsible for a wide range of physiological processes ranging from the transmission of light and odorant signals to hormonal action and neurotransmission. Dysregulation of GPCRs is associated with several clinical conditions such that many currently used drugs target these receptors (Section 18.2).

Although these receptors share a common 7TM structure (Fig. 17.7), their agonist-binding (orthosteric) domains vary considerably. For small agonists (e.g. adrenaline, histamine, dopamine, serotonin) the domain is partially embedded within a transmembrane helical structure but for large agonists, including the neuropeptides and chemokines, the domain may span the extracellular loops or be located near the N-terminal region.

Based on phylogenetic criteria, GPCRs have been classified into five families:

- *Rhodopsin family*: This is the largest and most widely studied family. It currently contains 672 verified members which are highly heterogeneous and have therefore been divided into four subgroups, α, β, γ and δ. The α subgroup contains many receptors that are involved in basic physiological functions and hence are targets for drug therapy. Examples include the H_1 and H_2 histamine receptors, the serotonin receptors 5-HT_{1A}, 5-HT_{1D} and 5-HT_{2A}, the adrenergic receptors 1A, 2A, B1 and B2 and muscarinic receptors.
- *Secretin family*: This family is characterised by a long N-terminal tail containing six cysteine residues linked by disulphide bridges and involved in ligand binding. The family has 15 members which include the glucagon, calcitonin and secretin receptors. They all activate adenylyl cyclase and couple through the same G_s protein. Their overexpression is commonly linked to human tumours.
- *Adhesion family*: This family contains 33 members all of which bind cell adhesion molecules such as integrins, cadherins and selectins, which are involved in the control of mitogenesis, differentiation and the immune system.
- *Glutamate family*: This family has 22 members all of which bind either glutamate or GABA. They are involved in neurotransmission in the brain by controlling the movement of ions such as Ca^{2+}, Na^+ and Cl^- through ion channels. Their action may be either excitatory or inhibitory. Members include the so-called metabotropic glutamate receptors (mGluR) of which eight have been identified and each shown

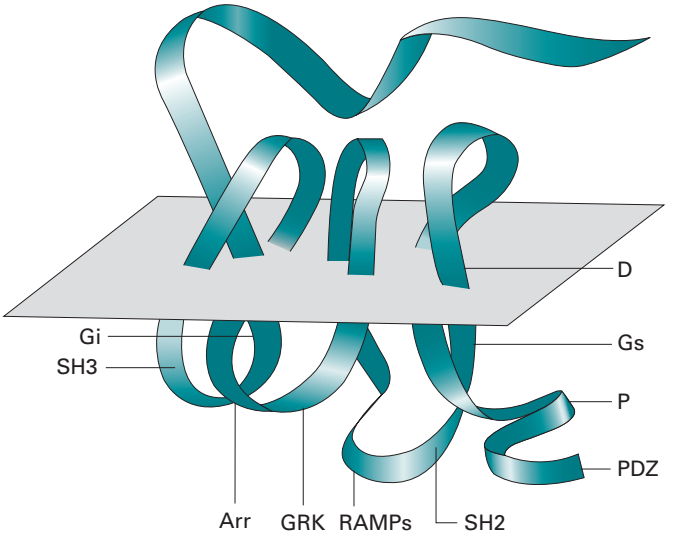

Fig. 17.7 Schematic diagram of a hypothetical G-protein-coupled receptor. Labels denote general regions of interaction of the receptor with other cellular proteins including different G-proteins (G_i and G_s), PDZ, SH2 and SH3-domain proteins, receptor-activity-modifying proteins (RAMPs), arrestin (Arr), G-protein-coupled receptor kinase (GRK), sites for dimerisation with other GPCRs (D), and phosphorylation sites that lead to uncoupling and internalisation (P). Any one of these active processes could be considered a form of expression of efficacy. The figure is a general description of various loci for protein interactions, but does not represent accurate locations, as, in most cases, these are not well characterised. (Reproduced from Kenakin, T. (2002). Efficacy at G-protein-coupled receptors. *Nature Reviews Drug Discovery*, **1**, 103–110, by permission of Nature Publishing Group.)

to be distributed in specific regions of the brain. In the resting state the channels are closed, but the binding of glutamate leads to the production of a second messenger that triggers the opening of the channel and the movement of ions. The channel then becomes desensitised and closes as the glutamate begins to dissociate returning the receptor to its resting state. The receptors are targets for a number of drugs such as the benzodiazepines. mGluRs contain an allosteric site that is a potential target for drugs involved in the treatment of Parkinson's disease (mGluR4), schizophrenia (mGluR5) and addiction (mGluR2).

- *Frizzled/Taste family*: These two subgroups of the same family have 11 and 25 members respectively. The Frizzled receptors are named after the *Drosophila* tissue polarity gene known as *frizzled*. The receptors bind Wnt glycoproteins involved in transduction pathways for the control of gene expression in embryonic development and regulation.

G-protein structure

G-proteins are heterotrimeric, consisting of one of each of three subunits, α (40–45 kDa), β (36–40 kDa) and γ (8 kDa), which are loosely attached to the inner surface of the cellular membrane through lipophilic tails on the α and γ subunits. The β and γ subunits are firmly attached to each other but the linkage to the α subunit is weaker. In addition to the binding site for the β subunit, the α subunit has a binding site

near the N-terminal end for the C-terminal region of the receptor and a guanine nucleotide-binding site that also possess GTPase activity. Although the α subunit contains the binding site for the receptor, binding only occurs when the α subunit is bound to the βγ dimer. Studies have revealed a very complex picture of the G-proteins. Sixteen human genes and splice variants encode α subunits giving rise to at least 28 distinct types; five genes encode β subunits and 14 genes the γ subunits. The potential number of different Gαβγ functional trimers is therefore very large.

G-protein subgroups

The most important are:

- the G_s subgroup which stimulate adenylyl cyclase. It includes G_{olf} coupled to olfactory receptors;
- the G_i subgroup which inhibit adenylyl cyclase and activate some Ca^{2+} and K^+ channels;
- the G_q subgroup which couple receptors to calcium mobilisation through phospholipase C_β that in turn generates the two second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG);
- the G_o subgroup which reduce the probability of opening of some voltage-gated Ca^{2+} channels involved in neurotransmitter release;
- the G_t subgroup which stimulate phosphodiesterase following light stimulation of the retina involving transducin; and
- the $G_{12/13}$ subgroup which is involved in the regulation of the cytoskeleton and processes related to movement. The subgroup activates inducible nitric oxide synthetase and the Na^+/H^+ exchanger. The action involves the low-molecular-weight protein Rho.

The G-protein α subunit family is divided into four subgroups based on sequence homology: $G_S\alpha$, $G_i/_o\alpha$, $G_q\alpha$ and $G_{12}/_{13}\alpha$. Each subgroup has been further divided into specific isotypes. The Gα subunit nomenclature is used to classify GPCRs, hence GPCRs are referred to as G_S-, G_i- or G_q- etc. reflecting their primary signalling pathway as discussed below. Since the number of human GPCRs is far greater than the number of human G-proteins it is obvious that each member of the Gα subgroups must be able to interact with many GPCRs.

The G-protein cycle

Agonist binding to the GPCR triggers a **G-protein cycle:**

- In the normal resting state, the trimeric G-protein has a molecule of GDP bound to the α subunit. At this stage the G-protein is not coupled to the receptor but it is firmly attached to the inner face of the cell membrane.
- An agonist binds to its binding site on the GPCR and induces a rapid allosteric conformational change that activates the G-protein binding site located in intracellular loops, resulting in a GPCR–G-protein complex. The complex interacts by diffusion translocation with a G-protein–adaptor complex that binds to the α subunit.

- Binding of the GPCR–G-protein complex to the G-protein–adaptor complex induces an allosteric conformational change in the guanine nucleotide-binding site on the α subunit of the G-protein causing the site to be more accessible to the cytosol where [GTP] > [GDP] resulting in the dissociation of the GDP and the formation of a transient 'empty state'.
- GTP binding to the nucleotide-binding site on the α subunit triggers a second, rapid conformational change in the α subunit causing the dissociation of GTPα subunit and leaving the Gβγ subunits as a dimer. Both the GTPα subunit and the Gβγ dimer remain attached to the cell membrane.
- GTPα subunit and/or the Gβγ dimer bind to an inactive effector molecule causing its activation or inhibition. Examples of such effector molecules include adenylyl cyclase, phospholipase C, cyclic nucleotide phosphodiesterases and a number of ion channels.
- Hydrolysis of GTP to GDP by the GTPase site of the α subunit, with the involvement of RGS proteins (see below for further details), terminates the activation or inhibition by reversing the conformational change originally induced by the receptor–agonist complex. This facilitates the dissociation of the α subunit from the effector and its reassociation with the Gβγ dimer, thus completing the cycle. Concomitantly, the binding of the receptor–agonist complex to the G-protein reduces the affinity of the receptor for its agonist encouraging its dissociation from its binding site resulting in the reformation of the inactive conformation of the receptor and hence terminating its binding to the G-protein.

Two important examples of the role of Gα-GTP as a transducer are the activation of the key enzymes adenylyl cyclase, that converts ATP to the second messenger cAMP, and phosphodiesterase (phospholipase C) that cleaves phosphatidylinositol-4, 5-bisphosphate (PIP_2), a component of the cytoplasmic side of the cell membrane, to two second messengers – inositol-1,4,5-trisphosphate and diacylglycerol. Most examples of the transducer role of the Gβγ dimer are linked to the activation of G_i and G_o. Examples include the activation of β-adrenergic receptor kinase (βARK), phospholipase A_2 and the K^+ channel GIRK (G-protein-activated inwardly rectifying potassium channel).

A given G-protein may be activated by a large number of different receptors (referred to as **G-protein promiscuity**), whilst a given receptor may interact with different G-proteins and/or produce more than one response (referred to as **receptor promiscuity**). A receptor capable of activating more than one type of G-protein and hence of initiating more than one response is referred to as a **pleiotropic receptor** (meaning it has multiple phenotypic expressions). An example is the human adenosine receptor that can couple to G_i, G_s and G_q. Some GPCRs are capable of binding several agonists each of which can induce a specific conformational change that preferentially selects a specific G-protein that in turn leads it to activate a specific transduction pathway. Examples are the 5-HT receptors, 13 of which have been identified to date and 12 shown to be GPCRs (Table 17.1). Such multiple roles for a given agonist have lead to the concepts of **functional selectivity** (Fig. 17.8) and **biased agonism** (Fig. 17.9).

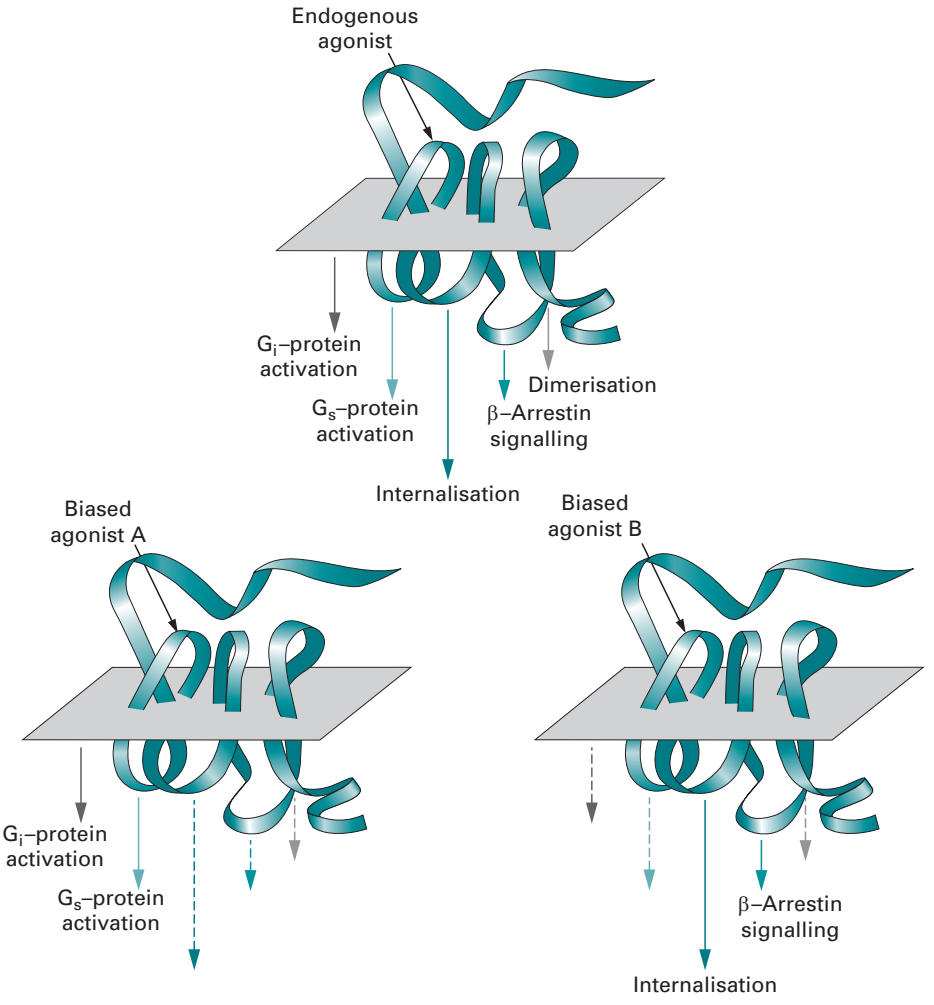

Fig. 17.8 Biased agonism. Whereas a natural agonist can activate the receptor to express all of its behaviour towards its cellular host, some agonists can stabilise active receptor conformations that trigger only some of these behaviours. Thus biased agonist A activates primary G-protein signalling pathways, whereas biased agonist B activates mainly G-protein-independent β-arrestin signalling and internalisation. (Reproduced from Kenakin, T. (2007). Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends in Pharmacological Sciences*, **28**, 407–412, by permission of Elsevier Science.)

GPCR dimerisation

Evidence has been obtained to indicate that GPCRs interact with other GPCRs to form either homo- or heterodimers. In the case of the receptors in families B and C, the evidence is that the functional activity (phenotype) of the receptors is linked to these forms but in the case of family A receptors this link is less clear. The functional unit of both the mGluR1 and $GABA_B$ receptors in family C, for example, is a homodimer and X-ray crystallographic studies on the glutamate receptor have indicated that the dimer exists as a dynamic equilibrium between two conformations, one ‘open’ the

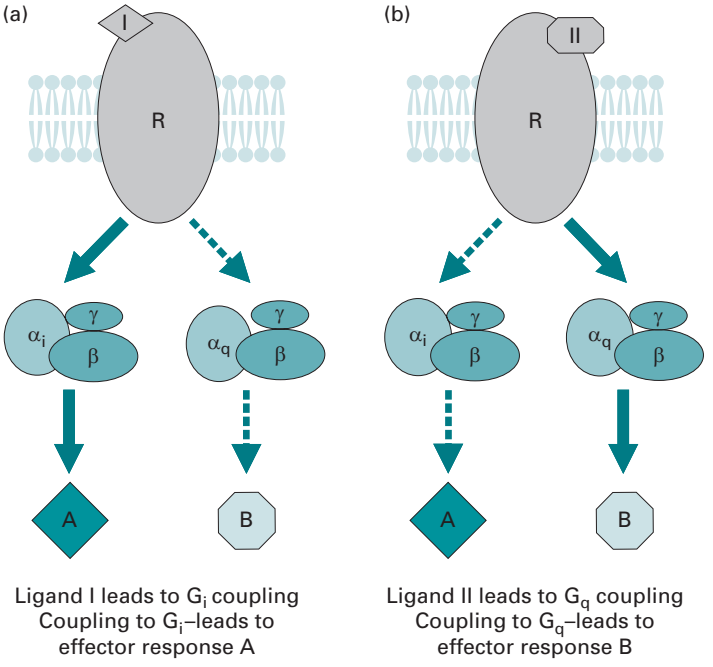

Fig. 17.9 Functional selectivity. GPCRs often couple to multiple G-proteins. Functional selectivity is seen when ligand binding influences which G-protein associates with the receptor by promoting distinct coupling efficiencies. For example, binding of a distinct ligand (I) leads to activation of G_i and effector responses initiated through this G protein (a), whereas binding of a different ligand (II) to the same GPCR leads to activation of G_q and to an alternative set of effector responses driven through this G-protein (b). (Reproduced from Gilchrist, A. (2007). Modulating G-protein-coupled receptors: from traditional pharmacology to allosterics. *Trends in Pharmacological Sciences*, **28**, 431–437, by permission of Elsevier Science.)

other 'closed', and that the role of glutamate is to stabilise the closed, active form. In contrast, the $GABA_B$ receptor is a heterodimer involving two receptors from family B. The explanation for the importance of this dimerisation of GPCRs is a topic of current research but a number of points have emerged so far:

- Firstly, it appears to occur early in the biosynthetic pathway of the receptors, specifically in the endoplasmic reticulum, and is essential for the trafficking of the receptors into the membrane.
- Secondly, molecular modelling studies with the rhodopsin receptor have demonstrated that it can only effectively interact with subunits of a G-protein if it is a homodimer emphasising the importance of molecular size and conformation in receptor activity.
- Thirdly, the formation of heterodimers facilitates the opportunity for crosstalk between the two protomers. Such crosstalk has been demonstrated for a number of receptors including histamine H_1 receptors and $GABA_B$ receptors and there is growing evidence of the general importance of such receptor crosstalk in maintaining specificity from signal to cellular response.

- Fourthly, the dimerisation affords the potential for further allosteric modification of ligand (agonist, G-protein, other proteins) binding on the individual protomers that may be linked to receptor crosstalk and hence cellular response (Section 17.2.3).

GPCR association with other proteins

In addition to forming dimers with other receptors, GPCRs also form associations with several groups of other proteins (Fig. 17.6) that are crucial to the signal activity and regulation of the receptor probably by allosteric and cooperative effects.

- *Receptor activity modifying proteins* (RAMPs): Three RAMPs (RAMPs1, 2 and 3) have been characterised and shown to be relatively small (RAMP1 is a 140 amino acid protein) with a single membrane-spanning domain, a large extracellular domain and a small intracellular domain. The RAMP–receptor heterodimer determines the specificity of the functional receptor. The dimers are formed in the endoplasmic reticulum and the RAMP remains associated with the receptor for the whole of the receptor's lifetime. RAMPs appear to be most important for the class B GPCRs.
- *G-protein-coupled receptor kinases (GRKs) and β-arrestins*: These two families of proteins are intimately involved in the control of GPCR activity. There are seven members of the GRK family (GRK 1–7) and four members of the β-arrestin family (β-arrestin 1–4). Their actions are coordinated in that the GRK phosphorylates the agonist-activated receptor at serine and threonine residues in the intracellular domain (Fig. 17.10) and this stimulates the binding of β-arrestin. This in turn uncouples the GPCR from its G-protein thereby desensitising the receptor in spite of the continuing presence of the agonist and simultaneously targets the receptor to clathrin-coated pits in the membrane and subsequent endocytosis (Section 17.5.2).
- *GPCR-interacting proteins* (GIPs): These proteins are involved in a number of key processes including (a) targeting GPCRs to specific cellular compartments, (b) the assembly of GPCRs into functional complexes called **receptosomes** and (c) the fine-tuning of the signalling of the GPCRs. Examples of these GIPs include the multi-PDZ proteins, the Shank family of proteins and the Homer proteins. As their name implies, multi-PDZ proteins possess a number of PDZ (PSD-95, Dig and ZO-1/2) domains each of which can bind to the C-terminal region of different receptor and effector proteins involved in the transduction of a given signal. The Shank proteins possess several protein–protein interaction motifs including the SH2 (Src-homology domain 2) motif which recognises and binds tyrosine-phosphorylated sequences (this includes some receptors with intrinsic protein kinase activity (Section 17.4.3) and the SH3 (Src-homology domain 3) motif which recognises and binds sequences that are rich in the amino acid proline). GPCRs therefore possess domains capable of recognising and binding to these various motifs (Fig. 17.6).

In addition to the three groups of proteins discussed above, a fourth group is also involved in the regulation of the GPCR-transduced signal, but rather than associating with the GPCR directly, proteins in this group interact with the associated G-protein and are therefore referred to as **regulation of G-protein signalling proteins**

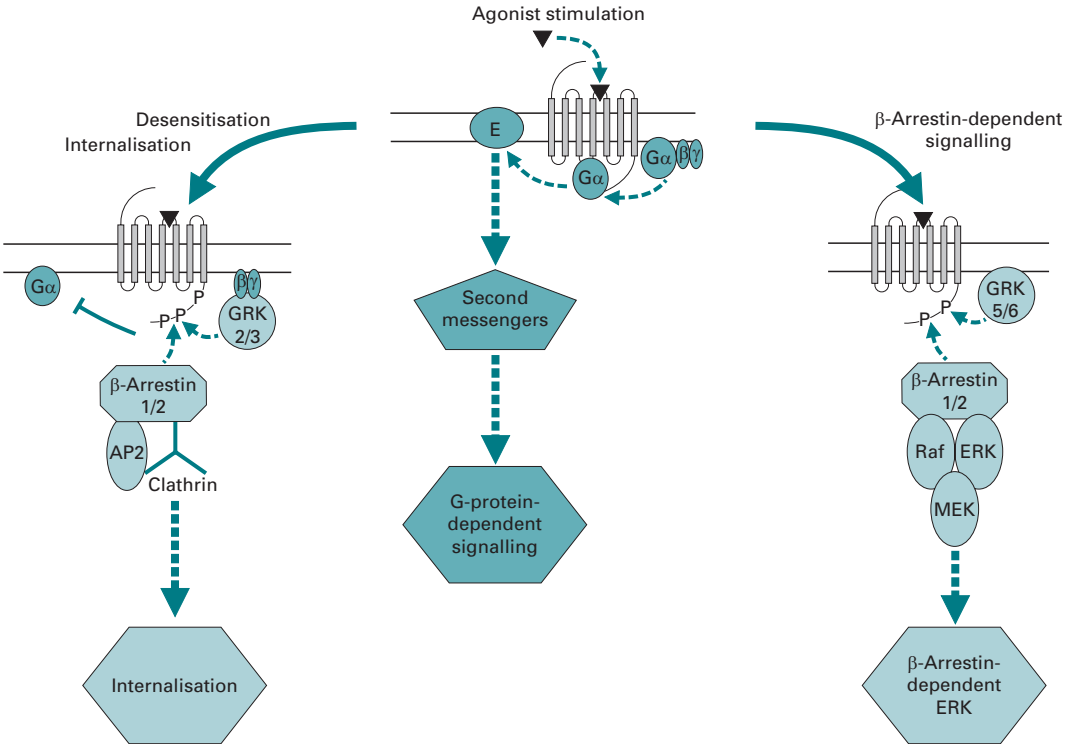

Fig. 17.10 Functional specialisation of the different GRKs. Upon agonist stimulation, GRK2 and GRK3 are recruited to the plasma membrane by interacting with Gβγ subunits. They have a predominant role in receptor phosphorylation, β-arrestin recruitment, desensitisation and internalisation. GRK5 and GRK6 are constitutively associated with the plasma membrane. They are both required for β-arrestin-dependent ERK activation, although the locus of their action might be at the receptor or downstream. In some systems, GRK5 and GRK6 can also mediate desensitisation and internalisation. E, second-messenger-generating enzyme. (Reproduced from Reiter, E. and Lefkowitz, R. J. (2006). GRKs and β-arrestins: roles in receptor silencing, trafficking and signalling. *Trends in Endocrinology and Metabolism*, **17**, 159–165, by permission of Elsevier Science.)

(RGS). This group of over 20 proteins can be divided into five subfamilies based on sequence homology. They all have two actions namely they reduce the binding of the Gα GTP to the effector and they act as GTPases accelerating the hydrolysis of GTP to GDP by a factor of over 2000-fold, hence they are also referred to as **GTP-accelerating proteins** (*GAPs*). As a consequence of this latter activity, RGSs effectively regulate the duration of the response resulting from the activation of a GPCR.

Kinetics of the activation of and signalling by GPCRs

Spectroscopic techniques, especially FRET, have been used to evaluate details of the kinetics and mechanisms of GPCR-mediated signals. Such studies have shown that agonist binding, the activation of the receptor and interaction with the G-protein all

occur within less than 50 ms, that the activation of the G-protein and its interaction with the effector protein occur within 500 ms, that the formation of second messengers such as cAMP can take up to 20 s, that the interaction of the GRK-phosphorylated receptor with β-arrestin can take up to 50 s and that a conformational change in β-arrestin may take up to 5 min. These results indicate that G-protein activation is the rate-limiting step in GPCR signalling. Interestingly, whereas full agonists activate the G-protein within 50 ms, partial agonists take up to 1 s and inverse agonists take about 1 s to reduce the intrinsic activity of a receptor. Such studies have also shown that some G_i-like G-proteins can initiate a signal without the need to dissociate whereas other G-proteins do appear to need to dissociate first.

17.4.4 Signal transduction through receptors with intrinsic protein kinase activity

It has been appreciated for over 20 years that phosphorylation coupled with dephosphorylation represents an important mechanism for the regulation of protein activity. A large number of intracellular kinases and phosphatases have been characterised and their regulatory action linked to conformational changes induced in the target protein as a result of the introduction or removal of a phosphate group. Control of protein activity by the kinase/phosphatase principle is found in a broad range of organisms, indicating its early evolution. It operates with the net consumption of ATP, but with the considerable gain in sensitivity, amplification and flexibility that more than compensate for the ATP consumed.

There are two main classes of receptor kinases based on the specificity of the induced protein kinase, namely receptor tyrosine kinases and serine/threonine receptor kinases.

Receptor tyrosine kinases

Twenty subclasses of receptor tyrosine kinases (RTKs) are known, based on their extra- and intracellular structures. The human genome contains approximately 90 RTK genes. Three of the best characterised are the epidermal growth factor receptor (EGFR) (also known as ErbB and HER), platelet derived growth factor (PDGF) receptor and the insulin receptor (IR). The vast majority of RTKs, including EGFR and PDGF, are single-chain, monomeric proteins in the absence of their agonist but they dimerise on agonist binding. However, a few, including the insulin receptor and the closely related insulin-like growth factor-1 (IGF-1) receptor, are permanently dimeric. Each monomer of the insulin receptor consists of an α and a β chain that are linked via a disulphide bridge making the functional dimer an $\alpha_2\beta_2$ tetramer (Fig. 17.11). The insulin receptor is involved in the regulation of lipid and protein metabolism in addition to its role in the maintenance of glucose homeostasis. EGFR regulates aspects of development including that of the nervous system, but mutant forms are linked to cancer.

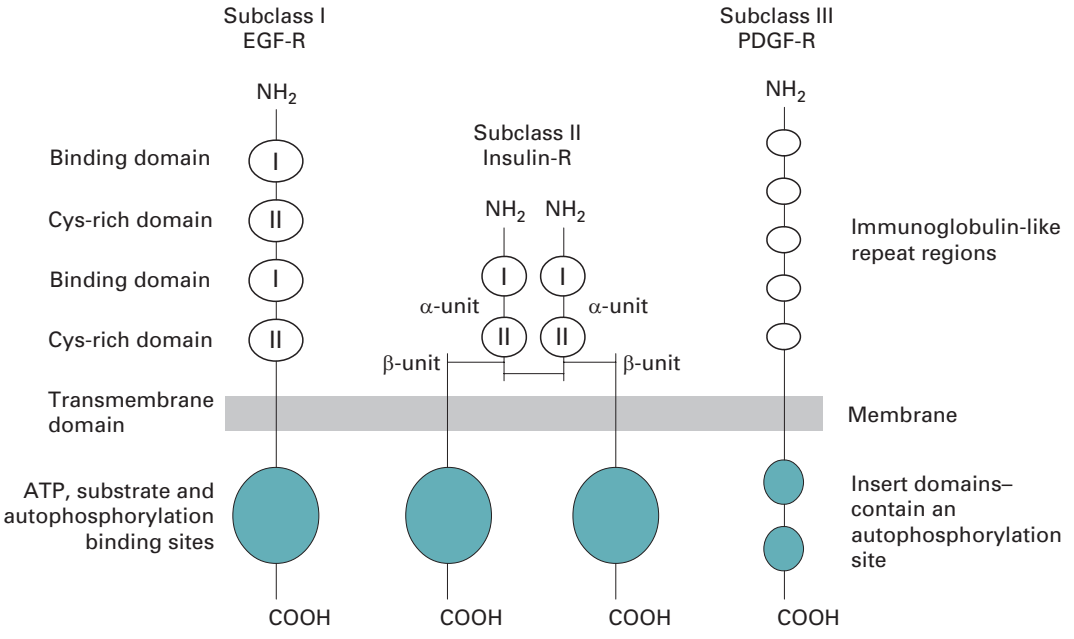

Fig. 17.11 Diagrammatic representation of three receptor tyrosine kinase subclasses. The EGF subclass contains two ligand-binding domains that are located in juxtaposition so that the ligands bind in a cleft between the two domains. The two cysteine-rich domains are both located near the membrane surface. On ligand binding, both the EGF and PDGF subclass receptors dimerise so that the intracellular tyrosine kinase domains possess elevated activity and enhanced binding affinity relative to the monomeric forms. The insulin subclass receptors are effectively dimeric, but, as with subclasses I and III, there is allosteric interaction between the two $\alpha\beta$ halves of each receptor on ligand binding. The tyrosine kinase domains of the three subclasses show the greatest degree of homology between the three subclasses.

Example 3 RECEPTORS AND CANCER THERAPY

Cancers are caused by a mutation that stimulates a cell to grow in an uncontrolled fashion. Inherited mutations cause a relatively small proportion of cancers; approximately 10% of breast cancers, for example, are the result of inherited mutations such as those caused by the *BRCA1* and *BRAC2* genes. A diversity of spontaneous mutations underlie most cancers and a given type of cancer may be the consequence of a range of mutations including single-letter changes to a codon, gene deletions, insertions and duplications and chromosomal rearrangements. The implication of this diversity of cause is that a given type of cancer such as breast cancer may be the result of changes in different molecular pathways and hence will need a range of drugs to be available to the clinician for the treatment of patients. The implication of this is that for the effective treatment of a given patient it is vital that the underlying cause is identified. Most current drug treatments target either a membrane receptor or an enzyme. Examples of receptor targets are given below:

Example 3 (*cont.*)

Drug	Target	Mechanism
Gefitinib (Iressa) – a small molecule	Binds to the ATP binding site of the tyrosine kinase domain of epidermal growth factor receptor (EGFR) in non-small-cell lung cancer	Inhibits the activation of the Ras signal transduction cascade thereby inhibiting tumour growth
Trastuzumab (Herceptin) – an antibody	HER2 receptor, a member of the EGFR family	Blocks HER2 receptor preventing it from forming dimers with similar receptors that cause a signal to the nucleus to stimulate the tumour to grow and divide
Tositumomab (Bexxar) – an antibody carrying ^{131}I	CD20 receptor on β-cells	Binds to receptor and the radioactive iodine isotope that it carries kills cancerous lymphoma cells
Rituximab (MabThera) – a chimeric monoclonal antibody	CD20 receptor on β-cells	Triggers the immune system to destroy healthy and lymphoma cells
Tamoxifen (Nolvadex) – a small molecule	Oestrogen receptor	Decreases DNA synthesis and inhibits oestrogen effects that cause the tumour to grow
Sorafenib (Nexavar) – a small prodrug molecule	Vascular endothelial growth factor receptor (VEGFR) and platlet-derived growth factor receptor (PDGFR) on cells lining blood vessels	Inhibits protein kinase activity of receptors. Also binds to c-kit receptors on cancer cell involved in cancer growth

Like the treatment for HIV/AIDS (Section 18.2.2) cancer therapy most commonly involves the use of a cocktail of drugs acting by different mechanisms.

Binding of insulin to its binding site (one on each of the α chains) causes a conformational change that activates the kinase activity. In the case of EGFRs, agonist binding causes either a conformational change that allows the occupied receptors to recognise each other resulting in their association to form homo- or heterodimers or stabilises a pre-existing equilibrium between monomers and dimers and induces conformational changes within the dimer. At this stage the kinase action of the receptor is inactive, but the dimerisation is rapidly followed by the mutual cross-autophosphorylation of one to three Tyr residues in the tyrosine kinase domains

on each monomer forming SH2 or phosphotyrosine binding (PTB) domains that serve as docking sites for effector proteins that possess SH2 sites causing the phosphorylation and activation of the effector. One such effector protein for the insulin receptor is insulin receptor substrate-1 (IRS). Effector proteins that bind either possess enzyme activity such as kinase, phosphatase, lipase and GTPase or act as adaptors that act to link the activated and phosphorylated receptor with other effectors. In addition, the effector may possess other binding domains to which further effectors can bind. This multiplicity of binding domains and bound effectors enable a web of signalling pathways and cascades to be established with the potential for transduction pathway branching to meet prevailing cellular demands.

Receptor serine and threonine kinases

Complementary to the tyrosine kinase group of receptors is a second group of protein kinase receptors characterised by their ability to autophosphorylate serine and threonine residues in the intracellular domain of the receptor. These protein serine/threonine kinase receptors are specific for members of the transforming growth factor (TGF)-β superfamily of receptors which regulate growth, differentiation, migration and cell adhesion. They are classified into a number of subgroups on the basis of their structure, particularly their serine/threonine kinase domain. They are all single transmembrane receptors that on ligand binding form hetero-oligomeric complexes between subgroup types. This stimulates autophosphorylation and activation of the serine/threonine kinase activity towards other cytosolic proteins that are components of the transduction pathway.

Like the G-protein-coupled receptors, receptor protein kinases stimulate numerous transduction pathways. The downstream members of these transduction pathways include the phospholipases and phosphoinositide kinases that are also involved in the G-protein transduction pathways. Receptor protein kinases in turn are subject to regulation by ubiquitin ligases, protein kinases and phosphatases and various adaptor proteins. Importantly, 30% of all RTKs are repeatedly found either mutated or overexpressed in a wide range of different human malignancies. EGFR, for example, is associated with breast cancers. One of a number of effectors unique to the receptor protein kinases is Ras, a membrane-bound guanosine binding protein with intrinsic GTPase activity, involved in cell growth and development in all eukaryotes. Activated Ras triggers the mitogen-activated protein kinase (MAP kinase cascade) which in turn phosphorylates multiple proteins in the nucleus including transcription factors that regulate gene expression in cell division, cell adhesion, apoptosis and cell migration. The Ras gene was the first oncogene to be discovered to be associated with a human cancer. It is the target for the treatment of various forms of cancer by the development of monoclonal antibodies.

Cytokine receptors

A group of receptors closely related to the protein kinase receptors are the **cytokine receptors**. Cytokines are a group of peptides and proteins intimately involved in cell signalling. They play key roles in the immune system and are involved in

immunological, inflammatory and infectious diseases. They have also been termed lymphokines, interleukins and chemokines. They act by binding to specific cytokine receptors that structurally resemble protein kinase receptors in that they exist as functional homo- or hetero-oligomers but they lack intrinsic kinase activity. Binding of the cytokine activates the receptor enabling it to recruit a non-receptor tyrosine kinase, such as a member of the src family or the Janus kinases family, to the cytoplasmic side of the receptor. Activation of this kinase by the receptor enables the receptor–kinase complex to recruit other effector proteins that trigger a signalling cascade linked to the up- or downregulation of genes and their transcription factors.

Protein tyrosine phosphatases

Crucial to the control of cell signalling involving receptor kinases is the existence of a group of protein tyrosine and serine/threonine phosphatases that can either deactivate or activate pathways by dephosphorylation of receptors or effectors. The human genome includes approximately 100 genes for tyrosine phosphatases. This is a similar number of genes as for RTKs suggesting that the two families are partners in the regulation of the signalling response with the kinases controlling the amplitude of the responses and the phosphatases their rate and duration. Some of the phosphatases are purely cytoplasmic whilst others are receptor-like and are referred to as **receptor tyrosine phosphatases** (RTPs). Most protein tyrosine phosphatases have two phosphatase domains for reasons that are not clear but their specificity may be linked to interaction between the two sites. The activity of the phosphatases appears to be linked to their own phosphorylation and a significant number have a SH2 domain similar to that of the receptor tyrosine kinases. There is currently great interest in protein tyrosine phosphatases as many have been shown to act a tumour suppressors in contrast to protein tyrosine kinases some of which act as oncoproteins.

17.4.5 Dynamics of signalling pathways

The classical view of receptor signal transduction is that the process is a **linear cascade**:

agonist⟶receptor⟶effector(s)⟶transduction⟶response

However, the current evidence is that such linear cascades are an oversimplification of the actual organisation. Most receptors appear to operate in complex and highly integrated **networks** that control several linked processes. GPCRs and PTKs that control many physiologically important pathways commonly merge to form integrated networks. Receptors in such networks share key components or **nodes** that mediate and modify the receptors' signals and which are the sites of crosstalk between the receptors that can result in signal divergence. Crosstalk between GPCRs and RTKs is common and may be bidirectional in that the GPCR may be 'upstream' or 'downstream' of the RTK signal transduction. This has given rise to the concept of **transactivation** by which a given receptor is activated by a ligand of a heterologous receptor that may belong to a different class of receptor with respect to the signal transduction mechanism. Thus an activated RTK may initiate GPCR signalling by causing

the dissociation of the Gα subunit from the Gβγ subunit allowing the Gα subunit to initiate signalling, for example by activating adenylyl cyclase, or the agonist-bound RTK may directly associate with a GPCR through scaffold molecules such as RGS, allowing the RTK to use components of GPCR signalling such as GRK/β-arrestin. A good example is the regulation of glucose metabolism involving the β-adrenergic receptor (a GPCR) and its regulation by the insulin receptor. The ability of the insulin receptor to counter the release of glucose by the action of catecholamines on the adrenergic receptor leads to the tight control of serum glucose levels. This control by the insulin receptor of the β-adrenergic receptor appears to operate by two (at least) mechanisms, one involving the internalisation of the β-adrenergic receptors via GRK/β-arrestin hence decreasing the sensitivity of the cell to circulating catecholamine, the other at the level of the activation of RGSs hence terminating the action of the activated β-adrenergic receptor. Crosstalk between the two GPCRs μ-opioid receptor (MOR) and α_{2A}-adrenergic receptor (α_{2A}AR) has been shown to proceed via a direct conformational change-induced inactivation of the noradrenaline-occupied α_{2A}AR by the binding of morphine to the MOR. The two receptors form a heterodimer and activate common transduction pathways mediated through the inhibition of G_i and G_o. The inhibition of the α_{2A}AR occurs within a subsecond of the morphine binding and terminates a downstream MAP kinase cascade induced by the α_{2A}AR.

Nodes characteristically consist of a group of related proteins that are essential for the receptor-mediated signal but such that two or more of these proteins have unique roles within the network and are therefore the source of divergence within the network (Fig. 17.12). Many networks contain cascades of cycles formed by two or more interconvertable forms of a signalling protein. This protein is modified by two opposing enzymes, commonly a kinase and a phosphatase for phosphoproteins or a guanosine nucleotide exchange factor (GEF) and a GTPase-activating protein (GAP) for G-proteins. Such cascades afford the property of **ultrasensitivity** to the input signal particularly under conditions in which the enzymes are operating near saturation.

17.5 RECEPTOR TRAFFICKING

17.5.1 Membrane structure

Detailed studies of cell membranes and of the area of the membrane occupied by signalling receptors have revealed that the membrane is very patchy with segregated regions or **microdomains** of different protein and lipid structure. One such type of these microdomains are **lipid rafts** that are dynamic structures rich in sphingolipids, glycosphingolipids, sphingomyelin and cholesterol and as a result are less fluid than the remainder of the membrane. Lipid rafts are involved in the organisation of receptors and their associated signal-transduction pathways. A subset of lipid rafts are **caveolae** characterised by their invaginated morphology produced as a result of cross-linking between a constituent protein called **caveolin**. There is evidence that a given receptor accumulates in either lipid rafts or caveolae but not both.

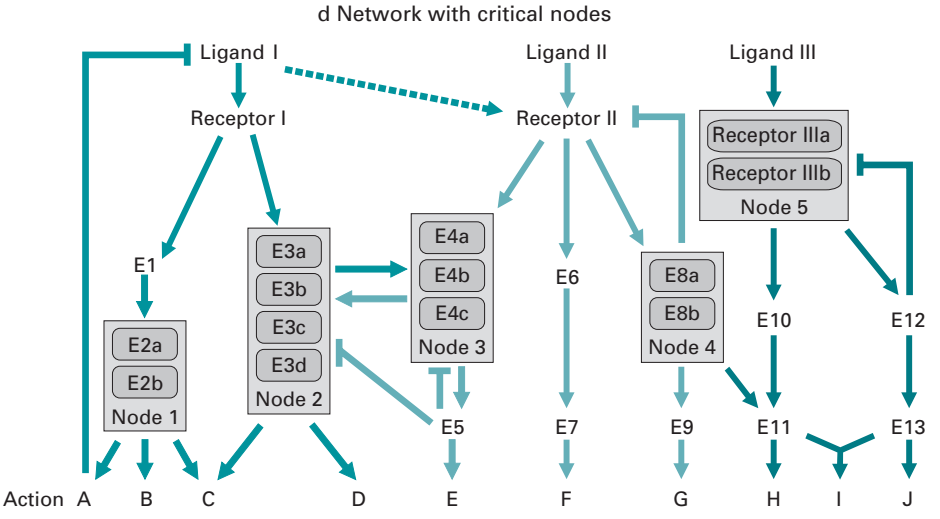

Fig. 17.12 Signalling pathways with critical nodes. Ligand binding to a receptor (I, II and III) activates the receptor and in turn activates (full arrows) the linked effectors (numbers beside an effector E indicate distinct proteins, the small letters indicate different isoforms of a given protein) leading to the transduction of one or more actions (A to J) involving different nodes. The actions may result in the inhibition of the action of the initiating ligand or of the receptor (blocked arrows). A ligand may also activate a second receptor but with less intensity (dotted arrows). Activated effectors may alternatively either network with other effectors (arrows) or inhibit other effectors (blocked arrows). (Adapted from Taniguchi, C. M., Emanuelli, B. and Kakn, C. R. (2006). Critical nodes on signalling pathways: insights into insulin action. *Nature Reviews Molecular Cell Biology*, **7**, 85–96, by permission of Nature Publishing Group.)

17.5.2 Receptor endocytosis

Endocytosis, also referred to as **endosomal trafficking**, is the process by which lipids and proteins, including receptors, are taken into the cell from the plasma membrane. It is a complex and dynamic process for which multiple pathways exist, each involving characteristic scaffold and accessory proteins. The most important pathways for membrane receptors are the clathrin-dependent and clathrin-independent pathways.

The internalisation and trafficking of receptors has been most thoroughly investigated using G-protein-coupled receptors, particularly the β_2-adrenergic receptor. Studies have revealed that the processes involve three stages:

- The recruitment of the receptors, normally with agonist bound to its orthosteric site (**agonist-dependent internalisation**) but in some cases in the absence of bound agonist (**agonist-independent internalisation**), to discrete endocytic sites such as rafts and caveolae in the membrane. In the case of the agonist-dependent route, the receptor is phosphorylated by a G-protein-coupled receptor kinase (GRK) and associated with β-arrestin prior to internalisation.
- The internalisation of the receptors to form an **early endosome,** also referred to as a **sorting endosome.**

- The sorting of the receptor proteins into specific domains within the endosome for either subsequent recycling to the membrane for reuse or for their degradation in lysosomes.

In the case of G-protein-coupled receptors, after binding the agonist and G-protein, the receptors undergo agonist-dependent phosphorylation by a GRK and this stimulates interaction with one of the β-arrestin family of protein adaptor complexes. These cytosolic adaptors facilitate the disruption of the interaction of the receptors with G-proteins and the recruitment of the receptors into **coated pits** in the membrane where they link the receptor to a protein called clathrin via a second adaptor protein. A large number of such second adaptors have been identified and shown to belong to one of two classes: multimeric adaptor proteins of which AP-2 is best known, and monomeric adaptor proteins (also known as CLASPs). This group is numerically the largest and includes Epsin1, AP180, Dab2 and CALM as well as β-arrestin. The adaptor protein interacts with phosphoinositides and promotes both the assembly of the coated pits and the recruitment of the activated receptors to them. There is evidence that scaffold proteins such as Eps15 are also involved in the assembly of the clathrin cage. Coated pits are regions in the membrane that are rich in clathrin that is located on the cytoplasmic side of the membrane. Clathrin consists of three heavy and three light chains which can polymerise to form a polymeric cage-like structure or lattice that links to the C-terminal end of the receptor. The polymeric clathrin network drives membrane deformation and the 'budding' of the coated pit to create an **endocytic vesicle**. This budding process involves the actin cytoskeleton that is a dynamic network of over 100 structural and regulatory proteins, and the GTPase dynamin. With the expenditure of GTP and in collaboration with a number of BAR proteins that are inserted into the membrane, the actin and dynamin promote the removal of the budding vesicle from the membrane. Once the free vesicle has formed, the clathrin coat is lost as a result of the action of one of the proteins of the heat shock protein 70 family. The vesicles then fuse to form an early endosome in which the bound agonist is removed by the prevailing mildly acidic (pH 6.2) conditions (Fig. 17.13).

Recent evidence indicates that RTKs also undergo endocytosis from special localised regions of the membrane termed **dorsal waves** that are large, circular protrusions in the cell membrane. This endocytic process does not involve clathrin but it does require specific adaptors such as Grb2, dynamin and components of the actin cytoskeleton that combine to release the early endosome by a mechanism similar to that for GPCRs.

There are two possible fates for the receptors within the early endosome:

- recycling to the membrane thereby restoring a functional receptor;
- proteolysis resulting in a net decrease in membrane receptor function, a process known as **downregulation**.

The pathways for endocytic sorting are determined by the operation of **sorting signals**. The main sorting signal appears to reside in the cytoplasmic region of the receptor itself. Thus for the β_2-adrenergic receptor, a PDZ-binding domain in the C-terminal region interacts with a protein called EBP50 with the result that the receptor undergoes recycling, the process also involving β-arrestin and a protein

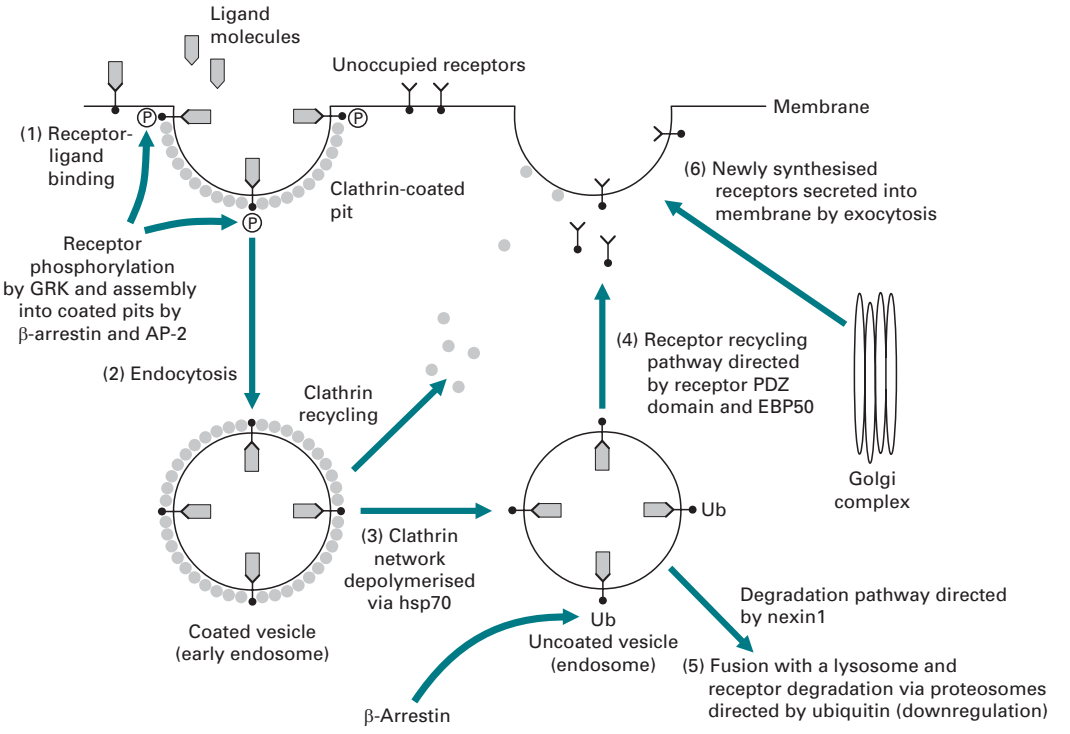

Fig. 17.13 Pathway of agonist-dependent G-protein-coupled receptor internalisation and endocytic sorting. (1) Occupied receptors are phosphorylated (P) by a G-protein-coupled receptor kinase (GRK) leading to the recruitment of β-arrestins. Arrestins serve as adaptor proteins by linking phosphorylated receptors to components of the transport machinery such as clathrin and adaptor protein AP-2 and their recruitment to clathrin-coated pits. (2) The coated pit 'buds' into the cytoplasm aided by the clathrin which forms a network leading to the formation of an endosome. Note that the cytoplasmic domain of the receptor remains exposed to the cytoplasm following endocytosis. (3) The clathrin network is depolymerised and the clathrin recycled to the inner membrane. (4) The receptors are dephosphorylated and as a result of the interaction of EBP50 with a PDZ domain on the receptor, traffic back to the cell surface resulting in functional resensitisation. Alternatively, (5) dephosphorylated receptors are tagged with ubiquitin (Ub) and enter the degradation pathway. Here they interact with nexin1 and ESCRTs which promote the fusion of the endosome with a lysosome and the degradation of the receptor by a number of proteasomes – a process known as downregulation. (6) The Golgi complex secretes newly synthesised receptor molecules to the outer membrane surface by exocytosis. The balance between receptor cycling, receptor degradation and receptor synthesis and exocytosis determines the number of functionally active receptors on the membrane surface at any time.

known as NSF. Chimeras of the receptor lacking the PDZ domain are directed to the degradative pathway. GPCRs sorted for recycling are first dephosphorylated by an endosome-associated phosphatase and recycled back to the membrane via the Golgi complex. In the case of receptors directed to degradation, the sorting signal directs the receptors to so-called **late endosomes** that have a multivesicular appearance referred to as **multivesicular bodies** (MVBs) that contain a number of protein complexes known as **endosomal sorting complex required for transport** (ESCRT-I-III) which promote the fusion of the endosome with a lysosome. The resulting decrease in pH within the vesicle to 5.3 facilitates the downregulation of the receptor by proteolysis.

There is evidence that this downregulation process is also dependent upon the ubiquitination of the receptor, a process that may include an active role for β-arrestin. Ubiquitin is known to 'tag' proteins for degradation, the process involving the action of a number of proteasomes (Section 15.5.4).

It has yet to be established just how universal the clathrin-linked and the non-clathrin-linked endocytotic pathways are to the large number of receptors of various cell types. What is very clear is that the expression, activation, regulation, desensitisation and endocytosis of receptors are all dependent on numerous protein–protein interactions, many of which occur at the plasma membrane interface, that each cause crucial conformational changes in the receptor and/or their regulators such as to couple receptor activity to current cellular and whole organism demands. It is equally evident that reversible multi-site phosphorylation plays a vital role in the regulation of the activity of receptors and their effectors.

The temporal variation in the number of cell surface receptors available for agonist binding is the net result of receptor trafficking and of new receptor synthesis, which takes place in the rough endoplasmic reticulum. A leader sequence in the protein results in its recognition and transport to the Golgi complex where it is glycosylated, packaged into coated vesicles and inserted into the membrane by **exocytosis**, in which clathrin plays a vital role. The balance between receptor synthesis, recycling and degradation is subject to various control mechanisms so that free receptor availability in the outer membrane meets current physiological needs. Temporal variation in cell membrane receptor numbers is also of significance in the clinical response to chronic drug administration that leads to the downregulation of receptor numbers, and in neurodegenerative conditions in which the release of the physiological agonist is deficient resulting in upregulation of receptor numbers.

17.6 SUGGESTIONS FOR FURTHER READING

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Useful websites

G-PROTEINS

www.gpcr.org/7tm

CELL SIGNALLING

www.cellsignal.com
www.signaling-gateway.org

RECEPTOR ENDOCYTOSIS

www.cytochemistry.net/cell-biology/recend.htm