REVIEW ARTICLE

Regulatory mechanisms that modulate signalling by G-protein-coupled receptors

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The large and functionally diverse group of G-protein-coupled receptors includes receptors for many different signalling molecules, including peptide and non-peptide hormones and neurotransmitters, chemokines, prostanooids and proteinases. Their principal function is to transmit information about the extracellular environment to the interior of the cell by interacting with the heterotrimeric G-proteins, and they thereby participate in many aspects of regulation. Cellular responses to agonists of these receptors are usually rapidly attenuated. Mechanisms of signal attenuation include removal of agonists from the extracellular fluid, receptor desensitization, endocytosis and down-regulation. Agonists are removed by dilution, uptake by transporters and enzymic degradation. Receptor desensitization is mediated by receptor phosphorylation by G-protein receptor kinases and second-messenger kinases, interaction of phosphorylated receptors with arrestins and receptor uncoupling from

G-proteins. Agonist-induced receptor endocytosis also contributes to desensitization by depleting the cell surface of high-affinity receptors, and recycling of internalized receptors contributes to resensitization of cellular responses. Receptor down-regulation is a form of desensitization that occurs during continuous, long-term exposure of cells to receptor agonists. Down-regulation, which may occur during the development of drug tolerance, is characterized by depletion of the cellular receptor content, and is probably mediated by alterations in the rates of receptor degradation and synthesis. These regulatory mechanisms are important, as they govern the ability of cells to respond to agonists. A greater understanding of the mechanisms that modulate signalling may lead to the development of new therapies and may help to explain the mechanism of drug tolerance.

INTRODUCTION

The superfamily of G-protein-coupled receptors (GPCRs) includes receptors for hormones and neurotransmitters, paracrine substances and inflammatory mediators, certain proteinases, a large number of taste and odorant molecules, and even photons and calcium ions [1]. These receptors have seven sequences of 20–25 hydrophobic residues that form an α -helix and span the plasma membrane, an extracellular N-terminus, three extracellular loops, three intracellular loops and an intracellular C-terminal tail. Their principal function is to transmit information about the extracellular environment to the interior of the cell, and they do this by interacting with the heterotrimeric G-proteins. Therefore these receptors and their agonists play an essential role in intercellular signalling and participate in numerous aspects of regulation.

Cellular responses to agonists of the GPCRs are usually rapidly attenuated. It is of great importance to understand the mechanisms of attenuation, because they regulate intercellular signalling, defects result in uncontrolled stimulation and disease, and several of the regulatory steps are attractive therapeutic targets. In common with many important biological processes, there are multiple parallel mechanisms and considerable apparent redundancy. Thus signals are attenuated by mechanisms that operate at the level of the agonist, the receptor, the G-proteins and at numerous downstream steps of the signalling pathway. This review focuses on regulatory mechanisms that operate at the level of the agonist and its receptor (Figure 1). Agonist removal from the extracellular fluid by uptake or degradation is the earliest mechanism of attenuation. Receptor desensitization,

which occurs during short-term (seconds to minutes) exposure of cells to agonists, is mediated by uncoupling of activated receptors from G-proteins, and effectively terminates the signalling. Receptor endocytosis depletes the plasma membrane of high-affinity receptors and contributes to both desensitization and resensitization of signalling. Receptor down-regulation is a loss of receptors from a cell that results from long-term (hours to days) continuous exposure of cells to agonists. Rather than discuss these mechanisms for all GPCRs, the review concentrates on regulation of signalling by hormones and neurotransmitters, although references will be made to other systems where the knowledge is more complete.

AGONIST REMOVAL FROM THE EXTRACELLULAR FLUID

Several processes contribute to the removal of hormones and neurotransmitters from the extracellular fluid, but their relative importance depends on the nature of the agonist. For all agonists, dilution in the extracellular fluid and subsequent excretion reduces concentrations to levels that are too low to produce detectable responses in target cells. Uptake by high-affinity transporters is one of the most widespread mechanisms for removal of agonists of GPCRs and ligand-gated ion channels from the synapse. This serves to effectively prevent further stimulation, and allows for the efficient re-use of the agonist if it is taken up into the nerve of origin. Extracellular degradation is the major mechanism for removing acetylcholine and peptides from the extracellular fluid. Acetylcholine released from cholinergic nerve terminals is degraded by acetylcholinesterase in the

Abbreviations used: GPCR, G-protein-coupled receptor; GRK, G-protein receptor kinase; β_2 -AR, β_2 -adrenergic receptor; β ARK, β -adrenergic receptor kinase; NK1-R, neurokinin 1 receptor; SP, substance P; NEP, neutral endopeptidase; PKA, protein kinase A; PKC, protein kinase C; TMD, transmembrane domain; CRE, cAMP response element; CREB, CRE binding protein; RACKs, receptors for activated C-kinases.

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Figure 1 Mechanisms that attenuate signalling by GPCRs

(a) Removal of agonists by dilution, uptake by transporter proteins and enzymic degradation is the first step in terminating the signal. (b) Phosphorylation of activated receptors by GRKs and subsequent interaction of the receptor with arrestins interdicts interactions with G-proteins and effectively terminates the signal, causing rapid desensitization. (c) Agonist-induced endocytosis depletes the plasma membrane of high-affinity receptors, and may represent a second phase of desensitization. (d) Down-regulation occurs during prolonged exposure of cells to agonists, and may be mediated by increased degradation and diminished synthesis of receptors.

synaptic cleft to give the inactive products acetate and choline; choline is taken up into the neuron by transporters to be re-used for the synthesis of acetylcholine. Peptides are degraded more slowly than acetylcholine by a family of cell-surface proteinases. The slower degradation of peptides may account for their long-term and widespread effects.

Uptake mechanisms

Since there are several comprehensive reviews of the neurotransmitter transporters, we will only summarize their salient features [2–5].

Transporter families

The transporters that are responsible for removing neurotransmitters from the extracellular fluid can be divided into families on the basis of their primary structure and ion-dependence.

The transporters for the monoamines [dopamine, noradrenaline, 5-hydroxytryptamine (serotonin)] and amino-acid-like neurotransmitters (γ -aminobutyric acid, glycine) rely on Na^+ and Cl^- transport for their activity, and the transmembrane Na^+ gradient provides the energy for the uptake of neurotransmitters. Comparisons of the protein sequences of the cloned transporters reveal remarkable structural similarity, with twelve stretches of 20–24 hydrophobic residues that probably represent membrane-spanning domains, and six extracellular and five intracellular loops [5,6]. There are no signal sequences for promoting insertion of the proteins into the plasma membrane, and the N- and C-termini are believed to be intracellular. The transporters in this group have about 20% of their amino acids in common, and almost 40% if conservative substitutions are considered. Thus there is quite remarkable structural similarity given the different molecules that they transport. Some of these transporters exist as subtypes; for example, different γ -aminobutyric acid transporters are products of distinct genes and differ in their pharmacological properties and distribution.

The glutamate transporter is distinct from the aforementioned transporters in that it depends upon Na^+ and K^+ transport for glutamate uptake [7]. Three glutamate transporters have been cloned and found to have six to eight transmembrane domains (TMDs). The amino acid sequences of these proteins are quite similar, but they are not related to the Na/Cl -dependent transporters.

Biological functions

High-affinity transporters remove neurotransmitters with binding constants in the micromolar range. They are often located on neurons and glial cells, and are thus ideally placed to remove neurotransmitters at sites of release and action (Figure 2a). For example, GAT-1, a high-affinity γ -aminobutyric acid transporter, is expressed at the plasma membrane of numerous axonal terminals of neurons in the central nervous system that also express the γ -aminobutyric acid-synthesizing enzyme glutamate decarboxylase [8].

The importance of transporters in terminating neurotransmission has been well documented by numerous studies using inhibitors. Certain powerful psychoactive drugs exert their effects by inhibiting transporters and thereby prolonging the effects of the neurotransmitters. Thus cocaine blocks the uptake of noradrenaline and dopamine, and the tricyclic antidepressants block the uptake of 5-hydroxytryptamine. The function of transporters has recently been probed by using homologous recombination to

Figure 2 Mechanisms that remove neurotransmitters from the synapse

(a) Monoamines and modified amino acids, exemplified by dopamine (DA), are removed by high-affinity transporters that are expressed by neurons and glial cells. These remove neurotransmitters from the synapse and retrieve them for re-use by the nerve of origin. **(b)** Acetylcholine (ACh) is rapidly degraded by acetylcholinesterase to inactive acetate and choline. Choline is taken up by a choline transporter to be re-used for synthesis of acetylcholine. **(c)** Neuropeptides, exemplified by SP, are degraded by ectoenzymes, such as NEP, to fragments that are usually devoid of biological activity.

delete transporter genes [9]. Homozygote mice lacking the dopamine transporter exhibit highly elevated locomotor behaviour. In wild-type mice, cocaine stimulates locomotor activity, but it has no effect in mice lacking the dopamine transporter, confirming that the transporter is the target of this drug. Together these results indicate that the dopamine transporter plays a major role in the control of locomotion by regulating dopaminergic tone in the basal ganglia and nucleus accumbens. In the brains of the knockout mice, dopamine D1 and D2 receptors are down-regulated. This adaptive response is probably due to the elevated levels of dopamine in the extracellular fluid, and underlines the importance of dampening dopaminergic neurotransmission. In diseases where effective levels of dopamine are reduced, such as Parkinson's syndrome, blockade of the dopamine transporter may be therapeutically beneficial.

Extracellular degradation of ligands

Degradation of acetylcholine

Acetylcholine released from nerve terminals is not taken up, but instead is degraded by acetylcholinesterase (Figure 2b). The structure, location and function of acetylcholinesterase will be briefly summarized (for reviews, see [10,11]).

The first convincing evidence for chemical neurotransmission was obtained by Loewi in 1921, with the demonstration that electrical stimulation of the vagal nerve inhibited contraction of the heart by release of an inhibitory substance, which was subsequently identified as acetylcholine [12]. Soon afterwards Loewi and Navratil [13] showed that the drug eserine owes its powerful pharmacological effect (of magnifying and prolonging the otherwise transient synaptic effects of acetylcholine) to inhibition of the rapid degradation of acetylcholine by acetylcholinesterase. Similarly, eserine converts the twitch response to electrical stimulation of nerves supplying skeletal muscle into a tetanic contraction [14]. Thus it is well established that the effects of acetylcholine are terminated by enzymic degradation.

Acetylcholinesterase is expressed in multiple forms, including monomers (G_1), dimers (G_2) and tetramers (G_4) of the catalytic subunits, as well as asymmetrical forms of one (A_4), two (A_8) or three (A_{12}) tetramers attached to a three-stranded collagen-like tail (Figure 3a). The catalytic subunits are derived from a single

gene by alternative RNA splicing. Although various forms of acetylcholinesterase are widely distributed throughout the body, they display specific subcellular distributions. Many of the asymmetrical forms are found at the cell surface in association with the extracellular matrix; G_1 forms are found in the endoplasmic reticulum; and G_2 dimers and G_4 tetramers are anchored to the plasma membrane. This specialized subcellular distribution relates to the regulation of cholinergic neurotransmission by acetylcholinesterase. Thus in the neuromuscular junction A_{12} , which is attached to the basal lamina, controls the amount of acetylcholine that reaches the cholinergic receptors of the muscle cells, whereas the plasmalemma-associated G_4 may remove acetylcholine from the synapse and thus terminate the signal.

As previously noted, inhibition of acetylcholinesterase has profound effects, emphasizing its importance in regulating cholinergic neurotransmission. Inhibition prolongs synaptic transmission, which ultimately desensitizes cholinergic receptors and results in paralysis and death due to respiratory failure. Indeed, nerve gases such as Sarin are acetylcholinesterase inhibitors. Acetylcholinesterase activity may also be affected in disease states. Myasthenia gravis (severe muscle weakness) is an autoimmune disorder that is caused by the production of antibodies to the nicotinic receptor. However, some forms of the disease are associated with the absence of acetylcholinesterase from postsynaptic membranes [15].

Degradation of peptide hormones and neurotransmitters

Although many peptides are taken up by their target cells during agonist-induced endocytosis of their receptors (see below), this occurs after stimulation of the receptor and desensitization, and does not regulate concentrations of peptides in the extracellular fluid. Instead, peptides are removed from the extracellular fluid by enzymic degradation, principally by cell-surface peptidases (Figure 2c) (see [16,17] for reviews). The active site of these peptidases projects into the extracellular fluid, and they are ideally placed to degrade neuropeptides at the cell surface in the vicinity of their receptors and thereby restrict the number of intact peptide molecules that are available to interact with receptors and initiate signalling.

Figure 3 Degradation of acetylcholine and neuropeptides by cell-surface enzymes

(a) Structure of acetylcholinesterase in the neuromuscular junction. The tetramers of the catalytic subunits are attached by disulphide bonds to the collagen tail, which is thought to bind to heparin sulphate proteoglycan in the basal lamina. Modified from [236] with permission of the authors and publishers. (b) Primary structure of NEP, indicating the membrane-spanning domain and the active-site domain. Modified from [18] with permission of the authors and publishers. The table shows cleavage sites (↓) and affinities for the hydrolysis of several favourable substrates by NEP. K_m values are from [24,237].

There are several well characterized neuropeptide-degrading enzymes, but here we will briefly review recent studies of neutral endopeptidase (NEP; EC 3.4.24.11), also known as endopeptidase-24.11, enkephalinase, common acute lymphoblastic leukaemia antigen, CD10 and neprilysin (Figure 3b). This enzyme is selected since it has been extensively studied and can be regarded as the prototypical neuropeptide-degrading enzyme (see [18] for a review).

NEP is one of several peptidases that were originally identified in the brush border of the kidney, and was subsequently identified in numerous tissues that are regulated by neuropeptides, including the brain and the gastrointestinal tract [19–21]. NEP has a single transmembrane domain of 22 residues, an intracellular N-terminal tail of 27 residues and a large extracellular C-terminus that contains the active site [22,23]. In contrast with the high specificity of acetylcholinesterase, NEP degrades several peptides, albeit with graded affinity. Preferred residues in the P1' site are hydrophobic, such as Phe, Leu, Val, Tyr and Trp, and favourable substrates include substance P (SP), enkephalins, gastrin-releasing peptide, somatostatin, cholecystokinin and bradykinin [24–27]. However, the affinity of NEP for its substrate peptides is far lower than those of receptors for neuropeptides. For example, the K_m of NEP for SP (32 μ M) is almost 10 000-fold less than the affinity of SP for the neurokinin 1 receptor (NK1-R) (K_d 3.5 nM). Despite this difference in affinity, NEP degrades SP and

thereby attenuates SP binding and signalling if it is co-expressed in the same cells as the NK1-R [28]. In contrast, NEP has smaller effects on SP binding and signalling when it is expressed in different cells from the NK1-R. Thus NEP must be strategically placed and present in a high concentration if it is to degrade SP at the cell surface, decrease the concentration of peptide in the vicinity of the NK1-R and thereby attenuate SP signalling.

Comparisons of the distribution of NEP with that of potential substrates and neuropeptide receptors provide important information about the functions of this enzyme [29–31]. NEP is present in the caudate-putamen, globus pallidus, olfactory tubercle and substantia nigra, and high levels are found in the choroid plexus. It is principally located at the plasma membranes of axons and dendrites, whereas glial cells are rarely stained [32]. NEP is co-localized with opioids and their receptors and with SP in certain brain regions. However, in other regions where NEP is expressed these peptides are absent, indicating that here the enzyme degrades other peptides. The localization of NEP to the plasma membrane of SP-containing boutons in the pig substantia nigra supports a role for NEP in inactivation of the tachykinins [33]. In the periphery, NEP is located in many non-neuronal cells including smooth-muscle cells, endothelial cells and various epithelial cells [34]. Detailed ultrastructural studies simultaneously comparing the localization of NEP, neuropeptides and receptors are lacking, although the recent availability of antibodies to tachykinin and opioid receptors should permit such analyses. Such studies would provide important information about the probable range over which neuropeptides may exert their effects. The ability of a peptide to regulate distant cells is likely to depend on the susceptibility of the peptide to degradation as well as the affinities of receptors on target cells, since dilution will inevitably occur during diffusion. Some neuropeptides are capable of diffusing considerable distances in neural tissues. This has been directly studied in the spinal cord by use of antibody-coated microprobes to detect neuropeptides in the extracellular fluid with high spatial resolution. Of particular interest is the comparison of levels of SP and neurokinin A in the spinal cord of cats [35,36]. Both tachykinins are released from primary afferents (where they are co-localized) in the superficial dorsal horn by noxious stimulation of the paw. However, SP is only detected at sites of release and is transient, whereas neurokinin A can be detected in the whole depth of the dorsal horn and persists beyond the duration of the stimulus. This difference is related to variability in the susceptibility of these peptides to degradation, since microinjection of peptidase inhibitors results in SP being detected over the whole of the dorsal horn. Presumably the slower degradation of neurokinin A enables this peptide to influence distant cells. Therefore, in contrast with the non-peptide neurotransmitters, which act briefly and locally, signalling by peptides can be prolonged and widespread.

The involvement of NEP in terminating the biological actions of neuropeptides has been extensively studied by using selective inhibitors. In a manner reminiscent of the effects of eserine on cholinergic neurotransmission, NEP inhibitors potentiate the biological actions of neuropeptides in many systems [18,27,37]. The recent availability of NEP knockout mice has permitted investigation of the role of NEP without the use of peptidase inhibitors, which may have non-specific effects and which are difficult to administer for prolonged periods [38]. These animals have been used to assess the role of NEP in terminating the pro-inflammatory effects of SP and bradykinin, which mediate neurogenic inflammation [39]. SP and bradykinin stimulate plasma extravasation in wild-type mice by interacting with the NK1-R and the bradykinin B2 receptor respectively. Remarkably, there is a high level of plasma extravasation in NEP

knockout mice under basal conditions, which is attenuated by administration of recombinant NEP and by antagonists of the NK1-R and B2 receptors. These findings suggest that genetic deletion of NEP results in diminished degradation of SP and bradykinin, which cause plasma extravasation by interacting with specific receptors on endothelial cells. The results imply that there is continuous release of tachykinins and kinins and that NEP maintains low basal levels of these peptides. These findings are of possible clinical interest, since NEP is down-regulated in inflamed tissues, which may contribute to uncontrolled inflammation [40,41].

RECEPTOR DESENSITIZATION

The early events of signalling by GPCRs, exemplified by the generation of second messengers such as cAMP and inositol 1,4,5-trisphosphate, are usually rapidly attenuated by regulatory processes collectively known as receptor desensitization (Figure 1b; see [42,43] for reviews). Two types of desensitization can be distinguished on the basis of the underlying mechanism. Homologous desensitization is mediated by agonist-dependent activation of the same receptor, whereas heterologous desensitization is caused by activation of a different receptor. An important component of desensitization, which occurs within seconds to minutes of receptor activation, is uncoupling of the activated receptor from its G-proteins by receptor phosphorylation. Two classes of protein kinases mediate this phosphorylation. A unique class of serine/threonine protein kinases, namely G-protein receptor kinases (GRKs), mediate agonist-dependent phosphorylation of GPCRs and initiate homologous desensitization, which depends on their functional co-factors, the arrestins. Second-messenger-dependent kinases [protein kinase C (PKC) and protein kinase A (PKA)] mediate

agonist-independent phosphorylation of receptors and initiate heterologous desensitization. The GRKs have the unusual feature of phosphorylating only agonist-occupied receptors, which distinguishes them from second-messenger kinases (see [44–46] for reviews).

Desensitization by GRKs

GRK families

The GRK family includes GRK1 or rhodopsin kinase, GRK2 or β -adrenergic receptor kinase-1 (β ARK1), GRK3 or β ARK2, GRK4 or IT11-A, GRK5 and GRK6 [47–51] (Figure 4). The family is expanded by the existence of splice variants; for example, there are four splice variants of GRK4 [52]. Comparisons of the primary sequences indicate marked sequence similarity, and there are several functional domains (Figure 4a). There is a centrally located catalytic domain of approx. 240 residues with considerable sequence identity between GRKs, which includes the conserved catalytic Asp-Leu-Gly sequence and the Gly-Xaa-Gly-Xaa-Xaa-Gly-Xaa_{10–16}-Lys motif involved in ATP binding and found in all protein kinases [51]. The N-terminus may serve to identify substrate specificity, while the C-terminus is involved in targeting of the cytoplasmic GRKs to the plasma membrane [53].

GRK receptor specificity

Studies using reconstituted receptors and kinases have shown that GRK1–GRK6 are capable of phosphorylating rhodopsin or the β_2 -adrenergic receptor (β_2 -AR) [48,50–52,54]. β ARK1/2 are of particular interest, since they phosphorylate many other GPCRs, including the muscarinic m1 [55], m2 [56] and m3 [57] receptors, the adrenergic α_2A , α_2B [58] and β_1 [59] receptors, the

Figure 4 Structure and function of the GRKs

(a) Structure of the GRKs based on rhodopsin kinase. The N-terminal domain is in black and the catalytic domain in dark pink. (b) Characteristics of the GRKs. Rho, rhodopsin; AR, adrenergic receptor; m₂AChR, muscarinic acetylcholine receptor; Th-R, thrombin receptor; D-R, dopamine receptor; LH-R, luteinizing-hormone receptor. Modified from [44] with the permission of the publishers. See the text for references.

dopaminergic D1A receptor [60], the NK1-R [61] and the thrombin receptor [62]. Thus β ARK1/2 phosphorylate receptors that couple to distinct G-proteins: G_s for the β_2 -AR, G_i for the α_2 -adrenergic receptor and $G_{q/11}$ for the m1, m3 and m5 muscarinic receptors and the NK1-R. Problems with the use of reconstituted systems for the analysis of GRK specificity include possible loss of cofactors or the use of a less favourable test substrate, such as the readily available rhodopsin. For example, early studies with GRK4 failed to show activity on rhodopsin [63]. However, GRK4 was recently found to phosphorylate the β_2 -AR and to augment desensitization of the luteinizing-hormone/chorionic-gonadotropin receptor in the presence of the lipid cofactor phosphatidylinositol 4,5-bisphosphate [52]. Lipid cofactors may be required for other GRKs.

GRK phosphorylation sites

Although GPCRs are known to be phosphorylated after agonist binding, with few exceptions the phosphorylation sites have not been identified. There are several explanations for our lack of precise information in this area. Firstly, although deductions about the sites of phosphorylation can be made from receptor mutation studies, unequivocal identification requires receptor purification and biochemical analysis, which is particularly challenging because the GPCRs are substantially hydrophobic and naturally expressed at low levels. Secondly, in contrast with the well defined consensus sites for second-messenger kinases, it has not been possible to identify consensus sites for phosphorylation by the GRKs. Thirdly, although studies using receptor fragments or reconstituted systems have permitted the mapping of potential sites for phosphorylation, recent observations *in vivo* using physiological stimuli indicate that receptors are phosphorylated differently depending on the nature of the stimulus and the cell type.

Initial studies of substrate determinants for rhodopsin kinase and β ARK1/2 used synthetic peptides resembling sequences from the C-terminal tail and the third intracellular loop of various receptors, and identified an Glu/Asp-Xaa-Ser phosphorylation consensus site [64–66]. However, intact receptors are better substrates than receptor fragments, and GRKs that are bound to an activated receptor are capable of phosphorylating synthetic peptides which are poor substrates for free kinases [66,67]. Thus GRK activity is greatly enhanced by binding to an activated receptor, and the substrate specificity of GRKs relies on the structure of the activated receptor as well as on the actual amino acid sequence surrounding the phosphorylation site.

Most information about substrate specificity and phosphorylation sites of intact receptors is derived from studies of rhodopsin and rhodopsin kinase. The identity of rhodopsin phosphorylation sites has been determined by analysis of a C-terminal peptide derived from isolated light-activated rod outer segments [68–70]. The initial sites of phosphorylation were identified at Ser³³⁸ or Ser³⁴³. β -ARK1/2 and GRK5 phosphorylated the same residues [46]. The lack of phosphorylation of the theoretically favourable Ser³³⁴ by these GRKs might be explained by its predicted location near the membrane surface, while Ser³³⁸ and Ser³⁴³ are predicted to face the aqueous medium, where they may be more accessible to water-soluble kinases [71]. This emphasizes that the specificity of GRKs depends on the three-dimensional structure of the receptor as well as the primary sequence.

Studies on intact animals using light as a physiological stimulus indicate that rhodopsin is phosphorylated at only a single residue, Ser³³⁸, Ser³⁴³ or Ser³³⁴, agreeing with earlier findings that a single

phosphorylation reaction is sufficient for the quenching of photo-transduction [72]. Interestingly, Ser³³⁸ is primarily phosphorylated after light flashes, and Ser³³⁴ after continuous illumination [72]. Factors contributing to the limited phosphorylation of rhodopsin may include binding of arrestin to phosphorylated rhodopsin and reduction of the photolysed chromophore [73]. In addition, the calcium-dependent interaction of recoverin, a member of a family of neuronal specific calcium-binding proteins, with rhodopsin kinase may further inhibit phosphorylation [74]. Similar calcium-binding proteins may negatively regulate other members of the GRK family and restrict their ability to multi-phosphorylate receptors in intact systems.

Studies of other GPCRs are complicated by the fact that second-messenger-dependent kinases as well as GRKs contribute to phosphorylation. Using reconstituted systems and site-directed mutagenesis, the β_2 -AR has been shown to be phosphorylated within the C-terminal tail by β ARK1/2 and in the C-terminal region of the third intracellular loop by PKA [75], although accurate mapping of individual phosphorylation sites has not been accomplished. Both PKC and GRKs participate in phosphorylation of the cholecystokinin A receptor, but the characteristics of receptor phosphorylation depend on the nature of the stimulus and whether the receptor is expressed in transfected cells or is naturally expressed [76,77]. These studies emphasize that receptors may be differently regulated in different cell lines, which may relate to variability in the cellular complement of kinases and phosphatases acting on the receptor.

Role of GRKs in receptor desensitization

For a GRK to mediate homologous desensitization of a receptor, it must be capable of phosphorylating the target receptor, appropriately localized and targeted to the plasma membrane in the vicinity of activated receptors, and mediate desensitization to physiological concentrations of agonists.

The functional importance of GRKs in receptor desensitization has been determined by modulation of the activity by various strategies. Overexpression of β ARK1 enhances desensitization of the β_2 -AR to high concentrations of agonist [78] and increases agonist-induced phosphorylation and desensitization of the δ -opioid receptor [79]. In contrast, overexpression of a dominant-negative β ARK1 mutant attenuates desensitization of the β_2 -AR, the δ -opioid receptor and the κ -opioid receptor [79–81]. Treatment of permeabilized cells with heparin, which inhibits β ARK1, blocks phosphorylation and desensitization of the β_2 -AR [82], and treatment of permeabilized olfactory cells with antibodies to β ARK1 reduces phosphorylation and desensitization of olfactory receptors [83,84].

GRKs must be appropriately localized at the plasma membrane if they are to mediate desensitization of cell-surface receptors. The mechanism by which cytosolic GRKs are translocated to the plasma membrane after receptor activation varies for the different kinases. Rhodopsin kinase contains a C-terminal CAAX motif that specifies farnesylation of the Cys residue, which increases its lipophilicity and is required for light-dependent translocation of the kinase and full enzymic activity [85,86]. β ARK1/2 are rapidly translocated from the cytosol to the plasma membrane after agonist stimulation [87,88]. They contain a 125-residue extension at the C-terminus that interacts with prenylated $\beta\gamma$ subunits of trimeric G-proteins [53,88,89]. Free $\beta\gamma$ subunits are only found in the plasma membrane at sites of receptor activation, so that this is an extremely precise mechanism for targeting these kinases to activated receptors. However, most GRK5 is normally present at the cell surface, and

Figure 5 Molecular architecture of arrestins

The functional domains are indicated by shading. R1 is an N-terminal positively charged domain of 25–29 residues that interacts with the negative R2 domain at the C-terminus. A (N-terminal) is a domain of approx. 130 residues that is involved in recognition of the activation state of the receptor. P is a positively charged stretch of approx. 20 residues that serves as a major phosphorylation recognition site. A (C-terminal) represents 10–30 residues of retinal arrestin also involved in recognition of the activation state. S is a domain of 120–150 residues forming a secondary binding site involved in receptor interaction in response to the occupancy of the primary phosphorylation- and activation-recognition binding sites. R2 comprises 60–85 residues and functions as a regulatory domain that interacts with R1. Modified from [108] with the permission of the publishers.

there appears to be direct interaction of a C-terminal domain with phospholipid head groups of the membrane [46]. Translocation of GRK6 depends on palmitoylation of cysteine residues in the C-terminus [46]. Palmitoylation of GRK4 also occurs and may be responsible for its predominant localization in the plasma membrane [52].

In addition to cytosolic and plasma-membrane pools, the GRKs may exist in other cellular compartments. β ARK1 has been detected in the endoplasmic reticulum of hepatocytes by cell fractionation and ultrastructural analysis [90]. This may serve as an intracellular store, or β ARK1 may phosphorylate proteins in the endoplasmic reticulum. There is a high-affinity binding site for β ARK1 ($K_d = 20$ nM) that may target the kinase to the endoplasmic reticulum [91]. This unidentified anchor protein may function similarly to the targeting proteins for second-messenger kinases [92].

The localization of GRKs provides insight into their possible functions. The presence of rhodopsin kinase in rod and cone outer segments suggests that its major function is in the phosphorylation of light-activated rhodopsin [93]. In contrast, β ARK1/2 are expressed in many tissues in a similar fashion (Figure 4b). High expression is found in brain, heart and spleen, followed by lung and kidney [48]. β ARK1 is usually more highly expressed and, in addition, very high levels of this kinase are found in monocytes and granulocytes [94]. β ARK1/2 are also found in neuronal cell bodies in the brain and in pre-synaptic and post-synaptic locations, with the latter predominating [95]. This suggests that β ARK1/2 are likely to function in the desensitization of many receptors, especially those activated at synapses. Since β ARK1/2 mediate homologous desensitization of the β_2 -AR to high concentrations of agonists, they are most likely to participate in the homologous desensitization of receptors that are exposed to high concentrations of agonists, a hypothesis that is supported by the synaptic localization of these proteins [95]. Demonstration of the co-localization of the GRKs and GPCRs is necessary in order to determine if these kinases are capable of regulating receptors under physiological circumstances. Such analyses are lacking, but are now possible given the increasing availability of receptor antisera.

Arrestins

The arrestins are the functional cofactors of the GRKs (for reviews, see [96,97]).

Arrestin families

There are several members of the arrestin family. Arrestin or S antigen from bovine retina binds to phosphorylated rhodopsin and thereby quenches signalling [98]. The observation that the ability of preparations of β ARK1 to desensitize the β_2 -AR diminished during purification suggested the existence of a

cofactor that was lost during purification [99]. β -Arrestin-1 and β -arrestin-2 (also called arrestin3) were subsequently identified and found to prevent activation of the β_2 -AR preferentially over rhodopsin [100,101]. Cone arrestin is similar in location and substrate specificity to arrestin [102,103]. There are several splice variants of the arrestins which sometimes show tissue-specific expression. For example, in spleen, lung, pituitary gland and kidney the major form of β -arrestin-1 lacks eight residues which are present in β -arrestin-1 in the cortex, cerebellum, striatum, pineal gland, retina and heart [104,105]. A splice variant of β -arrestin-2 contains an 11-amino-acid insert [104]. Comparison of the primary structures of arrestins from several species indicates that there is marked sequence similarity [106]. There are several conserved domains that may be important for receptor recognition, binding and intramolecular reactions (Figure 5). These domains have been characterized by mutagenesis and generation of chimaeric molecules [107–109].

Role of arrestins in receptor desensitization

The arrestins bind to phosphorylated receptors and are presumed to disrupt the interaction between the receptor and G-proteins, and to thereby mediate homologous desensitization. The functions of arrestins and GRKs have been extensively examined using purified proteins in reconstituted systems. Although retinal arrestin somewhat enhances β ARK1-mediated desensitization of the β_2 -AR [99], β -arrestin inhibits signalling of the β ARK1-phosphorylated β_2 -AR by > 75 % [110]. Together, β -arrestin and β ARK1 strongly inhibit β_2 -AR-induced GTPase activity and adenylate cyclase [111,112]. However, β -arrestins can also bind the agonist-activated non-phosphorylated β_2 -AR, albeit with much lower affinity. Thus β -arrestin binds to the phosphorylated β_2 -AR at a 1:1 stoichiometric ratio and with high affinity (K_d 1.8 nM), approx. 30-fold greater than that with which it binds to dephosphorylated receptors [112]. The potency with which β -arrestin inhibits the function of the β_2 -AR is increased 10-fold by β ARK1-induced receptor phosphorylation, but is unaffected by PKA-induced phosphorylation, which suggests that β -arrestin contributes to homologous, but not heterologous, desensitization [111].

A variety of strategies have been used to probe the function of arrestins in receptor desensitization in more intact systems. Desensitization of the β_2 -AR to high concentrations of isoprenaline is amplified by overexpression of β -arrestin-1 and β ARK1 [78]. Similar methods have been used to show that β -arrestins contribute to the desensitization of other receptors, such as the β_1 -adrenergic receptor [59]. In contrast, incubation of olfactory cilia with neutralizing antibodies to β -arrestin-2 and β ARK2 attenuated desensitization of odorant receptors [84]. Direct evidence for the functional significance of arrestins in intact animals comes from studies on *Drosophila*. Mutations of

the genes for *Drosophila* arrestins cause a defect in the ability of the animal to inactivate meta-rhodopsin, and after 5 days of light/dark exposure result in retinal degeneration [113]. The importance of arrestins in humans is indicated by the finding that a homozygous 1 bp deletion in the arrestin gene is a frequent cause of Oguchi disease, a congenital form of night blindness [114].

Analysis of the cellular and subcellular distributions of the arrestins provides further information about their possible functions. Arrestin and cone arrestin are principally located in the retina and the pineal gland, but both β -arrestin-1 and β -arrestin-2 are widely distributed and may participate in the regulation of many receptors. High levels are found in rat neuronal tissues, rat spleen, heart, liver, lung and mononuclear cells [94,101,115]. This distribution is not dissimilar to that of β ARK1/2. Ultrastructural studies revealed β -arrestin-2 immunoreactivity in multivesicular bodies of neurons, suggesting that β -arrestins interact with endocytosed receptors [101]. Detailed analyses of agonist-mediated alterations in the distribution of arrestins and receptors are lacking, although such studies are likely to provide new information about the function of the arrestins.

Desensitization by second-messenger kinases

Second-messenger kinases also contribute to the desensitization of receptors for hormones and neurotransmitters. PKA induces phosphorylation and heterologous desensitization of the β_2 -AR [116–118]. Desensitization of the responses of S49 lymphoma cells to isoprenaline are markedly reduced in kin^- cells, which lack PKA, indicating that PKA also mediates homologous desensitization of the β_2 -AR [119]. Like the GRKs, PKA is also translocated from the cytosol to a particulate fraction after agonist exposure [92]. PKC has been implicated in the desensitization of several receptors, including the m1 muscarinic receptor, the vasopressin receptor and the angiotensin II receptor [55,120,121]. Upon stimulation, PKC undergoes translocation from the cytosol to different sites, including the plasma membrane [92]. This translocation requires receptors for activated C-kinases (RACKs), one of which is a homologue of the β subunit of G-proteins. These receptors target PKC isoenzymes to target GPCRs, causing desensitization.

In addition to these second-messenger kinases, a 40 kDa kinase from porcine brain may phosphorylate phospholipase C-linked receptors [122]. This kinase, termed MAK, phosphorylates the muscarinic receptor at a serine residue. It is not inhibited by inhibitors of PKA or PKC. It is inhibited by heparin, which inhibits β ARK1, but not by zinc chloride, which also inhibits β ARK1. It is unclear if this represents a member of a novel family of receptor kinases.

Second-messenger kinases may participate in desensitization by regulating other kinases. For example, in human peripheral T cells, activation of PKC up-regulates GRKs [123]. On activation by phytohaemagglutinin, GRK activity increases 2–4-fold and the mRNA for β ARK1/2 increase similarly, although GRK5 and GRK6 are unaffected. Thus second-messenger kinases can alter levels of selected GRKs and, subsequently, the responsiveness of a cell to multiple agonists long after the second-messenger kinases were initially activated.

Desensitization of the β_2 -AR may be mediated by different kinases in different cell lines [124]. Thus in muscle cells and transfected Chinese hamster ovary cells, β ARK1 is of principal importance, while in rat osteosarcoma cells PKA but not β ARK1 is involved, and in human epidermoid carcinoma cells both PKA and β ARK1 are involved. The complex interactions of these different enzymes may be altered by the concentration of ligand,

thus an *in vivo* analysis is required to show what occurs under physiological conditions.

Desensitization by receptor palmitoylation

Most GPCRs have one or two cysteines located 12–15 residues C-terminal to TMD VII. Palmitoylation of these cysteines has been demonstrated for several GPCRs [125–131]. Post-translational acylation anchors the N-terminal portion of the cytoplasmic tail to the plasma membrane, creating a fourth intracellular loop. The acyl group is attached by a labile thioester bond to the cysteine residues, which allows the process to be reversed and gives cells the potential to control this modification [132]. Thus the palmitoylation state of the β_2 -AR and the D1 dopaminergic receptor is influenced by agonist exposure [128,133]. There is evidence that the palmitoylation of a GPCR may affect its desensitization. For example, a non-palmitoylated mutant β_2 -AR (C341G) was largely uncoupled from the G-protein, and had an elevated basal level of phosphorylation and a decreased rate of agonist-promoted desensitization [134,135]. This was due to enhanced phosphorylation of a PKA site close to Cys-341 in the unpalmitoylated state. Dynamic depalmitoylation in response to agonist could, therefore, enhance receptor phosphorylation and desensitization [135]. Similar results have been obtained for an analogous mutant of the dopamine 1 receptor [128]. However, palmitoylcysteines do not have a universal role in GPCR desensitization, since deletion of the cysteine in the α_{2A} -adrenergic receptor does not affect agonist-stimulated desensitization [136].

Differences in the importance of the palmitoylation state in desensitization may be explained by the different G α subunits or GRKs coupled to particular receptors. Although all G α subunits contain palmitate, they differ in their requirement for prior myristoylation, and the acylated cysteines occur within diverse sequences [137]. Palmitoylation of G α subunits is also a dynamic process, with depalmitoylation following formation of the active α_s -GTP complex resulting in release of α_s from the membrane, which may contribute to desensitization of G-protein signals. GRK6 is distinguished from other members of the GRK family by its mechanism of translocation to the plasma membrane upon palmitoylation [138]. Thus an interplay between different palmitoylated components of the signalling pathway of GPCRs, including deacylation of the G α subunits and the receptors concurrent with palmitoylation of GRK6, could contribute to desensitization.

RECEPTOR ENDOCYTOSIS

The ability of cells to respond to hormones and neurotransmitters requires the presence of receptors that are appropriately located at the plasma membrane, where they can interact with high affinity with agonists in the extracellular fluid. Thus the responsiveness of target cells is critically dependent on the subcellular distribution of receptors, and agonist-induced endocytosis of GPCRs is important in desensitization and resensitization of signalling (Figure 1c). Endocytosis of cell-surface receptors and subsequent intracellular sorting are critically important cellular processes, but the requirements for endocytosis, the endocytic mechanism, the fate of the internalized receptor and ligand, and the function of internalization differ between receptors (see [139] for a review). Most is known about the endocytosis of single-TMD receptors, such as the nutrient receptors and the tyrosine kinase receptors, but there has been recent interest in the mechanisms and function of endocytosis of the GPCRs.

Figure 6 Pathways of endocytosis and intracellular sorting of GPCRs

(a) Typical pathway for a neurotransmitter receptor, exemplified by the NK1-R or the β_2 -AR. Agonist binding, which is reversible, is followed by receptor phosphorylation by GRKs, interaction with β -arrestins and uncoupling from G-proteins, which mediate desensitization. The ligand–receptor complex is internalized via clathrin into vesicles that soon shed their clathrin coat and become early endosomes. Ligand and receptor dissociate in an acidified perinuclear compartment. Endosomal phosphatases may dephosphorylate the receptor, allowing dissociation of β -arrestins. The ligand is degraded, whereas the receptor is recycled to the plasma membrane, where it can interact with ligands with high affinity. Resensitization requires internalization, processing and recycling of receptors. (b) Typical pathway for a proteinase receptor, exemplified by the thrombin receptor or proteinase-activated receptor-2. Irreversible receptor cleavage is followed by receptor phosphorylation and uncoupling from G-proteins, which mediates desensitization. The receptor complex is internalized and rapidly traverses the endosomal compartment to the lysosomes. Resensitization requires mobilization of the large stores of receptors in the Golgi or synthesis of new receptor molecules.

Pathways of agonist-induced receptor endocytosis and trafficking

Agonist-mediated endocytosis has been observed for many GPCRs, including adrenergic receptors, muscarinic receptors, peptide receptors and proteinase receptors [140–145]. There has been considerable recent interest in endocytosis of the NK1-R for the peptide SP, which has been studied in transfected epithelial cells, in primary cultures of enteric neurons and in endothelial

cells of post-capillary venules and neurons of the central nervous system in the intact animal [142,146–151] (Figures 6a and 7a). SP and its receptor are internalized into early endosomes by a clathrin-mediated mechanism, and subsequent endosomal acidification permits dissociation of the ligand and its receptor and sorting into different pathways: SP is degraded in lysosomes and the NK1-R appears to be recycled. In support of receptor recycling is the observation that recovery of the receptor and of

Figure 7 Confocal images comparing the distributions of the NK1-R (a–f) and proteinase-activated receptor-2 (g–l) after agonist-induced endocytosis

Rat kidney epithelial cells were stably transfected with cDNA encoding epitope-tagged rat NK1-R or human proteinase-activated receptor-2. (a)–(f) Cells were incubated with 10 nM SP for 60 min at 4 °C, washed and incubated at 37 °C for 30 min (a–c) or 60 min (d–f), and fixed. In (a)–(c), cells were incubated with a rabbit antibody to the NK1-R and a mouse antibody to the transferrin receptor, followed by Texas Red-labelled goat anti-rabbit IgG and FITC-labelled goat anti-mouse IgG. An optical section through the base of these three cells shows that vesicles containing the NK1-R (a) also contain the transferrin receptor (b), as confirmed by superimposing these images (c), and are thus early endosomes. Yellow denotes co-localization (arrows). The NK1-R was co-localized with the transferrin receptor at all time points. In (d)–(f), cells were incubated with a rabbit antibody to the NK1-R and a mouse antibody to lysosomes, followed by rhodamine-labelled goat anti-rabbit IgG and FITC-labelled goat anti-mouse IgG. An optical section through these two cells shows that endosomes containing the NK1-R (d; small arrows) have a different size, shape and location from lysosomes (e; large arrows). The arrowheads in (d)–(f) show a rare instance of co-localization. (g)–(l) Cells were incubated with 10 nM trypsin for 10 min (g–i) or 60 min (j–l) at 37 °C, and fixed. In (g)–(i), cells were incubated with a rabbit antibody to proteinase-activated receptor-2 and mouse antibody to the transferrin receptor, followed by Texas Red-labelled goat anti-rabbit IgG and FITC-labelled goat anti-mouse IgG. An optical section through the centre of this cell shows that vesicles containing proteinase-activated receptor-2 (g) also contain the transferrin receptor (h), as confirmed by superimposing the images (i), and are thus early endosomes (arrowheads). At later times there was no co-localization of proteinase-activated receptor-2 and the transferrin receptor. In (j)–(l), cells were incubated with a rabbit antibody to proteinase-activated receptor-2 and a mouse antibody to lysosomes, followed by Texas Red-labelled goat anti-rabbit IgG and FITC-labelled goat anti-mouse IgG. An optical section shows that proteinase-activated receptor-2 (j) is found in lysosomes (k, l; arrowheads). Scale bars: (a)–(f), 10 μ m; (g)–(l), 2.5 μ m. Reproduced from *Molecular Biology of the Cell* (1995, volume 6, pp. 509–524), with permission of the American Society for Cell Biology.

agonist binding sites at the cell surface is prevented by inhibitors of endosomal acidification, which causes retention of the NK1-R within the cell and prevents degradation of SP. The β_2 -AR and the receptor for gastrin-releasing peptide are similarly recycled [140,143].

A distinctly different endocytic pathway is followed by the proteinase-activated receptors, such as the thrombin receptor and proteinase-activated receptor-2, which is activated by trypsin [144,145,152] (Figures 6b and 7b). Thrombin and trypsin cleave within the extracellular N-terminus of these receptors, exposing a tethered ligand domain that binds and activates the cleaved receptor molecules. Once activated, the cleaved receptors are internalized into early endosomes, but rapidly traverse this compartment and are sorted to lysosomes for degradation. Recovery of intact receptors at the cell surface requires either synthesis of new receptors or mobilization of intracellular pools of intact receptors. Indeed, in contrast with some of the neurotransmitter receptors such as the NK1-R, there are considerable stores of the proteinase receptors in the Golgi apparatus, and mobilization of these stores is required for the recovery of surface receptors. The mechanisms of this intracellular sorting of receptors have not been thoroughly examined. Recycling may be a default pathway for internalized receptors, since receptors and lipids are recycled at similar rates [153], whereas targeting to lysosomes may require specific sorting motifs [139]. The molecular motifs that specify this different trafficking of internalized receptors have not been identified, although they could be determined by analysis of chimaeric neurotransmitter and proteinase receptors.

The predominant pathway for agonist-induced internalization of GPCRs is via clathrin-coated pits. Thus the NK1-R, gastrin-releasing peptide receptor, β_2 -AR and thrombin receptor are internalized by a clathrin-dependent mechanism [140,142–144]. A caveolin-dependent mechanism has been also been described for the β_2 -AR, the endothelin-1 ET_A receptor and the cholecystokinin A receptor [154–156]. In pancreatic acinar cells, agonists induce migration of the cholecystokinin A receptor to a plasmalemma site, which differs from caveolae or clathrin-coated pits, where it becomes ‘insulated’ [157]. The functional implication of these alternative endocytic pathways is not known.

Domains involved in receptor endocytosis

Clathrin-mediated endocytosis requires the interaction of specific receptor domains with components of the endocytic machinery [139]. Endocytic motifs have been identified in the intracellular C-terminal tails of single-TMD receptors that are similar for different receptors and functionally interchangeable. Endocytic domains have also been identified for several GPCRs by receptor mutation experiments, but a common endocytic motif has not been identified, and it is unclear whether these motifs resemble those of the single-TMD receptors.

For some GPCRs, endocytic domains are found in the C-terminal tail. Thus truncation of the receptors for SP, gastrin-releasing peptide, thyrotropin-stimulating hormone, angiotensin II and parathyroid hormone reduces internalization [158–162]. However, this is not a universal theme, since removal of the C-terminal tail of the luteinizing-hormone/chorionic-gonadotropin receptor and the avian β_1 - or β_2 -ARs increases the rate of internalization, suggesting that the tails may contain motifs that impair endocytosis [163–165]. Serine/threonine-rich regions are critical for the agonist-induced internalization of several GPCRs [159,166]. However, this too is not the case for all receptors [158].

Tyrosine-containing endocytic motifs, in which tyrosine is of critical importance, have been identified for many single-TMD

proteins. They usually contain six residues forming an exposed β -turn. Positions 1, 3 and 6 are frequently occupied by aromatic or large hydrophobic residues, and the residues in positions 2, 4 and 5 tend to be polar and are often found in turns. The importance of these motifs for endocytosis has been established by demonstration that they interact with clathrin-associated proteins. Tyrosine-containing motifs in the C-terminal tails of the receptors for epidermal growth factor, asialoglycoproteins and polymeric immunoglobulins interact directly with the clathrin-associated adaptor protein complex AP-2 [167,168]. The μ 2-chain of AP-2 interacts with the Ser-Asp-Tyr-Gln-Arg-Leu motif of the C-terminal tail of the integral membrane protein TGN38 (TGN = *trans*-Golgi network), as well as with similar motifs from lamp-1, CD68, H2-Mb (histocompatibility Class 2 gene Mb) and the transferrin receptor [169]. The importance of this interaction has recently been questioned by the finding that deletion of the AP-2 binding site of the epidermal growth factor receptor abolishes AP-2 binding to the receptor without affecting internalization [170].

Equivalent tyrosine-containing endocytic motifs may exist for the GPCRs. Mutation of conserved tyrosine residues (positions 331, 341 and 349) within potential endocytic domains of the C-terminal tail of the NK1-R impairs agonist-induced endocytosis without substantially affecting agonist binding or signalling [158]. However, only the residues surrounding Tyr³³¹ fit the typical consensus sequence for tyrosine endocytic motifs, with aromatic or large hydrophobic residues in positions 3 and 6 (Gly-Asp³³¹-Tyr-Glu-Gly-Leu), whereas Tyr³⁴⁹ and Tyr³⁴¹ do not fit typical motifs. Tyrosine-containing motifs in the C-terminal tail of the angiotensin II receptor are also important for internalization [162]. In contrast, tyrosines in the C-terminal tails of the β_2 -AR and m2 muscarinic receptor are not critical for internalization [171,172].

A tyrosine-containing motif (Asn-Pro-Xaa₂₋₃-Tyr) in TMD VII is highly conserved within the GPCR family. It closely resembles endocytic motifs of the low-density lipoprotein and insulin receptors, which fit the six-residue consensus sequence for tyrosine-containing endocytic motifs. This led to speculation that this is a common endocytic motif for the GPCRs [173]. Mutation of Tyr³²⁶ of the Asn-Pro-Xaa₂₋₃-Tyr domain of the β_2 -AR to Ala abolishes internalization, reduces agonist-induced phosphorylation by β ARK1 and depresses activation of adenylate cyclase [173–175]. The more conservative Tyr³²⁶ → Phe mutation reduces internalization by only 25 %, and also depresses phosphorylation and activation of adenylate cyclase [174]. Similar mutations of the tyrosine residues of the NK1-R and the angiotensin II receptor also reduce internalization by ~ 25 % [158,176]. In contrast, an equivalent mutation does not affect endocytosis of the receptor for gastrin-releasing peptide [177]. Together, these observations suggest that the Asn-Pro-Xaa₂₋₃-Tyr sequence of TMD VII is not a universal endocytic motif, but it may be important for maintaining the appropriate conformation of the receptor to allow endocytosis or signalling to occur. Recent structural models of GPCRs suggest that the Asn-Pro-Xaa₂₋₃-Tyr motif is in an appropriate location to receive a signal from agonist-induced conformational changes in the ligand-binding region [178].

In summary, tyrosine-containing motifs may be important for endocytosis of some GPCRs, but they differ from the tyrosine-containing endocytic motifs of the single-TMD proteins. Studies involving mutation of surrounding residues and exchange of motifs between GPCRs and single-TMD receptors will be required in order to determine the extent to which these motifs resemble one another. The proteins that interact with the GPCRs and mediate endocytosis have not been identified.

Role of arrestins in receptor endocytosis

Recent evidence suggests that arrestins are involved in agonist-mediated receptor endocytosis [179]. Overexpression of β -arrestin-1/2 rescues agonist-mediated internalization of an internalization-defective mutant of the β_2 -AR, and overexpression of a dominant-negative β -arrestin mutant inhibits agonist-induced internalization of the β_2 -AR. Thus arrestins may function as adaptor molecules that may recruit cellular proteins that facilitate receptor endocytosis, or may directly mediate endocytosis themselves. Dynamin may be one protein that interacts with arrestins to enhance endocytosis of some receptors [180]. Dynamin is important for internalization of the β_2 -AR, since overexpression of a dynamin GTPase mutant inhibits agonist-induced endocytosis. In contrast, expression of the dynamin mutant or the dominant-negative β -arrestin mutant does not affect endocytosis of the angiotensin II receptor. However, overexpression of wild-type β -arrestin increases agonist-induced internalization of the angiotensin II receptor, an effect that is blocked by expression of mutant dynamin. Thus β -arrestins can target receptors for dynamin-dependent endocytosis.

Role of palmitoylation in endocytosis

There are contradictory reports of the role of palmitoylation in endocytosis. A role for palmitoylation in internalization was first indicated for the transferrin receptor, by the finding that fatty acid acylation decreased internalization [181]. Mutation of cysteine residues (positions 335 and 337) in the C-terminal tail of the thyrotropin-releasing-hormone receptor also inhibits endocytosis [161]. In contrast, mutation of cysteine residues (positions 621 and 622) in the luteinizing-hormone receptor, which abolishes palmitoylation, enhances agonist-stimulated internalization [130], and mutation of palmitoylation sites of the β_2 - and α_{2A} -adrenergic receptors has no effect on agonist-induced internalization [136,182]. The mechanism by which palmitoylation may affect endocytosis is not understood. Palmitoylation is postulated to be a determinant of the association of proteins with detergent-insoluble glycosphingolipid-enriched complexes, the precursors of caveolae [183]. Thus the localization of GPCRs to caveolae may depend on their acylation state.

Induction of agonist-induced receptor endocytosis: the endocytic switch

How does agonist binding induce receptor endocytosis? This is an important but largely unanswered question. Some receptors, exemplified by the transferrin receptor, are constitutively internalized without binding ligand, whereas others, such as the tyrosine kinase receptors and the GPCRs, are internalized after agonist binding. Tyrosine-containing motifs are common and interchangeable for constitutively internalized receptors and for the tyrosine kinase receptors that are internalized after agonist binding [184]. These motifs may be continuously exposed in the constitutively internalized receptors, but hidden in the resting state of the signalling receptors. Agonist binding could induce a conformational change or some form of receptor modification, such as phosphorylation, which may expose the endocytic motif and enable agonist-induced receptor endocytosis to occur.

The nature of the endocytic switch for agonist-induced internalization has been studied for the tyrosine kinase receptors. Endocytosis of the growth-factor receptors requires intrinsic tyrosine kinase activity [185,186]. Inactivation of the receptor kinase activity by a point mutation almost eliminates agonist-induced internalization of the epidermal-growth-factor receptor [187]. However, deletion of the entire tyrosine kinase domain,

leaving the remaining C-terminal tail with its endocytic motifs intact, results in constitutive receptor endocytosis. Since kinase activation alters receptor conformation, possibly resulting in the exposure of hidden endocytic domains, deletion of this domain may cause a similar conformational change resulting in constitutive internalization. Furthermore, the baseline endocytosis of unoccupied epidermal-growth-factor receptors is enhanced for receptors with the point mutation and for receptors lacking the entire tyrosine kinase domain. These observations lead to a two-step model for agonist-induced internalization. Firstly, agonist occupancy results in a conformational change that favours receptor interaction with the endocytic apparatus. Secondly, receptor kinase activity phosphorylates a target, which could bind to the receptor inducing further conformational changes, fully exposing the endocytic codes and amplifying the conformational change induced by ligand binding. For the GPCRs the nature of the switch resulting in ligand-induced internalization is unknown, but several possibilities exist.

Activation of G-proteins and second-messenger generation may be the basis of the endocytic switch, since antagonists do not usually induce internalization of GPCRs, and because internalization of partial agonists is correlated with their ability to activate G-proteins [188]. Indeed, many uncoupled mutant receptors are not internalized, and the degree of uncoupling often correlates with the decrease in internalization [189,190]. However, mutants of receptors that couple to G_s and G_q have been identified that either signal normally yet exhibit impaired internalization, or are deficient in G-protein coupling and yet are internalized normally [191,192]. Further evidence against this mechanism of endocytosis has been obtained from recent observations of the μ -opioid receptor in transfected cells and enteric neurons of the intact animal [193,194]. In these systems enkephalin and the agonist etorphine stimulate receptor endocytosis in the expected manner. Remarkably, the agonist morphine does not cause endocytosis, even at concentrations that strongly inhibit adenylate cyclase. Thus different agonists can induce different activated states of the receptor that similarly activate the G-protein, but which differ significantly in their intracellular trafficking. These differences may be of importance to our understanding of the pharmacological and therapeutic properties of opioid analogues and the basis of opioid tolerance and addiction. They also argue against the possibility that the same active conformation is required for both internalization and signal transduction, but rather support a model where the domains involved in internalization and G-protein coupling are in close proximity but distinct.

Receptor phosphorylation is also a candidate for inducing agonist-dependent internalization, since some GPCRs are phosphorylated rapidly in response to agonist binding and because serine/threonine-rich regions are known to be important for internalization [159,161,162,166,195,196]. However, internalization of the β_2 -AR is unaffected by deletion of phosphorylation sites for both GRKs and PKA [75,197]. Moreover, inhibitors of GRKs or PKA [82] and overexpression of a dominant-negative GRK mutant do not prevent receptor endocytosis [80]. Interest in this hypothesis has been rekindled by the findings that internalization of the m2 muscarinic receptor is facilitated by overexpression of β ARK1 and attenuated by overexpression of a dominant-negative mutant of β ARK1 [198], and overexpression of β ARK1 restores agonist-induced internalization of an internalization-defective mutant β_2 -AR [175]. β ARK may facilitate internalization by stabilizing a receptor conformation or promoting the interaction of the receptor with a cellular element (possibly β -arrestin) that mediates receptor internalization, even in the absence of GRK-induced receptor phosphorylation.

Function of agonist-induced endocytosis

The functional importance of the agonist-induced internalization of GPCRs has been examined by inhibiting internalization with drugs or by receptor mutagenesis. By depleting the plasma membrane of high-affinity receptors that are available to interact with hydrophilic hormones and neurotransmitters in the extracellular fluid, endocytosis may contribute to desensitization. However, inhibitors of endocytosis do not prevent homologous desensitization of the β_2 -AR or the NK1-R [199,200], and mutations of the β_2 -AR which prevent agonist-mediated endocytosis have no effect on desensitization [173]. Thus internalization is not the principal mechanism of desensitization of these receptors. However, when PKA and β ARK are inhibited, internalization does contribute to desensitization of the β_2 -AR [82].

Endocytosis is important for the resensitization of some receptors for hormones and neurotransmitters. Thus resensitization of cellular responses to agonists of the β_2 -AR and the NK1-R is blocked by inhibition of endocytosis by drugs or receptor mutation [173,199,200]. These findings imply that processing of the internalized receptor is necessary for resensitization (Figure 6a). This may include dissociation of the ligand in acidified endosomes, dephosphorylation of the receptor, dissociation of arrestins and recycling of the receptor to the plasma membrane. SP dissociates from the NK1-R in acidified perinuclear endosomes, and inhibitors of endosomal acidification inhibit receptor recycling and resensitization. Endosomes are enriched with phosphatase, and the β_2 -AR in endosomes is less phosphorylated than at the plasma membrane and is not resensitized if recycling is blocked [201,202]. Furthermore, the phosphatase inhibitor okadaic acid prevents resensitization of the NK1-R [200]. However, detailed studies of the phosphorylation state of receptors in endosomes and their association with agonists and arrestins are hampered by difficulties in endosome purification.

An alternative mechanism is responsible for resensitization of the thrombin receptor and proteinase-activated receptor-2, which are irreversibly activated by cleavage [145,152] (Figure 6b). GRKs and PKC phosphorylate these receptors and mediate desensitization, and the cleaved receptors are internalized and trafficked to lysosomes. Resensitization of cellular responses to proteinases requires mobilization of large intracellular pools and synthesis of new receptors, since it can be blocked by disruption of the Golgi apparatus with brefeldin A and by inhibition of new receptor synthesis with cycloheximide.

RECEPTOR DOWN-REGULATION

Down-regulation is characterized by a decrease in the total number of receptors in a cell, and is caused by long-term exposure to agonists for hours or days; recovery from down-regulation is similarly slow. There is some ambiguity in the use of the term 'down-regulation', since it is also used for growth-factor receptors and proteinase-activated GPCRs, which are degraded even after short-term exposure to agonists. We use down-regulation to refer to loss of receptors after long-term exposure to agonists. From a physiological viewpoint, it is probably rare that a cell is exposed continuously to hormones or neurotransmitters, since efficient mechanisms exist to remove them from the extracellular fluid (see above). However, down-regulation may occur under pathological circumstances, such as when there is continuous secretion of hormones and neurotransmitters from tumours. Down-regulation is also important during long-term administration of receptor agonists for therapeutic reasons, when it may be responsible for tolerance or

tachyphylaxis [203]. Although far less is known about receptor down-regulation than about desensitization, possible mechanisms include enhanced degradation and reduced synthesis of receptors (Figures 1a and 8) (for reviews see [42,204,205]).

Degradation of receptors

At least two signalling pathways contribute to down-regulation of the β_2 -AR, including an agonist-dependent PKA-independent pathway and a PKA-dependent heterologous pathway [206,207]. In support of the existence of an agonist-dependent pathway is the finding that mutant β_2 -ARs with defective receptor-G-protein coupling show significantly impaired agonist-induced down-regulation of receptor number, whereas cells with mutations downstream of the receptor and G-protein, in which there are defects in cAMP stimulation and PKA activation, nevertheless undergo near-normal agonist-induced down-regulation. However, cAMP analogues or forskolin, which directly activates adenylate cyclase, still promote receptor down-regulation, although at a lower rate compared with full agonists. A β_2 -AR mutant lacking potential PKA phosphorylation sites is down-regulated more slowly than the wild-type receptor, suggesting that PKA-dependent phosphorylation enhances degradation. In contrast, GRK-dependent phosphorylation does not appear to contribute to enhanced degradation, as several studies have reported that mutants lacking putative GRK phosphorylation sites are down-regulated normally [42,205,207]. Thus long-term agonist exposure and subsequent G-protein coupling may result in a distinctive phosphorylation pattern or in a particular receptor conformation that exposes lysosomal targeting sequences. These sequences may interact with the machinery in the sorting endosome and target the receptor away from the recycling pathway.

Lysosomal targeting

Most of the information about lysosomal targeting is derived from studies of lysosomal membrane proteins and growth-factor receptors. After synthesis, integral membrane proteins of lysosomes reach their destination either by an indirect route that involves trafficking to the plasma membrane, endocytosis and migration to the lysosomes, or directly from the *trans*-Golgi network. Tyrosine-containing motifs in the C-terminal tails are critical for lysosomal targeting of lysosome-associated membrane proteins I and II and of lysosomal acid phosphatase [208–210]. Deletion or mutation of the critical tyrosine results in accumulation of lysosomal acid phosphatase in the plasma membrane [208] and blocks delivery of lysosome-associated membrane protein I to the lysosome by the direct intracellular route [211]. This suggests that similar signals are recognized by sorting proteins in the plasma membrane and the *trans*-Golgi network. Lysosomal integral membrane protein II, which does not contain a tyrosine residue in its C-terminal tail, relies on a dileucine-related Leu-Ile motif for lysosomal targeting, since deletion of this motif inhibits endocytosis and results in accumulation of this protein at the cell surface [212].

Growth factor receptors are not constitutively targeted to the lysosomes, but endocytosis and transport to the lysosomes is dependent on agonist-induced receptor activation. Thus exposure to agonists depletes the cell of surface receptors and accelerates receptor degradation, reflected by a 8–10-fold decrease in half-life [185]. The importance of the endogenous tyrosine kinase domain for endocytosis and trafficking of the epidermal growth factor receptor is controversial, and has been a topic of recent investigation [185,187,213]. The unoccupied epidermal growth

Figure 8 Potential mechanisms of down-regulation of GPCRs by increased degradation and diminished stability of mRNA

Abbreviations: CREM, CRE modulator; β -ARB, β -adrenergic receptor mRNA binding protein.

factor receptor is internalized at a very low basal rate, and activation by agonist is necessary for rapid internalization and degradation. Mutant receptors lacking the tyrosine kinase domain but retaining the distal regulatory domains are constitutively internalized and are targeted to lysosomes in the absence of agonist, suggesting that the kinase domain is not required for lysosomal targeting [187]. Thus a conformational change induced by receptor activation may expose sterically hidden endocytic and lysosomal targeting domains. This conformation may be mimicked by the mutant with the deleted kinase domain [187]. However, receptors with a point mutation of the kinase domain that abolishes kinase activity are internalized at basal rates in the presence of agonist [213]. Although an active tyrosine kinase is required for efficient ligand-induced receptor recruitment into coated pits, autophosphorylation is not sufficient for ligand-induced recruitment of activated receptors into coated pits, suggesting that the kinase phosphorylates substrates, other than the receptor, that are required for efficient endocytosis [213].

Similar motifs may target receptors to different destinations. For example, tyrosine-based motifs direct the endocytosis of constitutively internalized receptors and are important for lysosomal targeting of lysosomal integral membrane proteins [139]. The dileucine motif also directs both endocytosis and lysosomal targeting of lysosomal proteins, the T-cell receptor and growth-factor receptors [212,214,215]. Thus the context in which a motif occurs may explain its differential functions. Another possibility is that tyrosine-based and dileucine motifs target proteins to the

sorting endosome, where an additional signal is required for lysosomal targeting, while proteins lacking this signal follow the default recycling pathway.

Far less is known about lysosomal targeting of GPCRs for hormones and neurotransmitters. After a brief exposure to agonists the β_2 -AR and the NK1-R are recycled efficiently and are rarely found in lysosomes [140,142]. It is not known if long-term exposure to agonists results in the targeting of these receptors to lysosomes. However, continuous exposure may cause multiple rounds of endocytosis and recycling, each of which would be accompanied by some mistargeting of a small proportion of receptors to lysosomes, which would eventually deplete the cell of receptors. Mutation of tyrosine residues in C-terminal tails of the β_2 -AR and the muscarinic m2 receptor impairs down-regulation without affecting internalization, suggesting that endocytosis and down-regulation are independent processes which rely on distinct signals [171,172].

Ubiquitin-mediated proteolysis

Ubiquitin is an abundant and highly conserved protein of 76 residues that is present in all eukaryotic cells. Ubiquitination of cellular proteins is a signal for the rapid degradation of many cytoplasmic, organelle-associated and membrane-bound proteins in proteasomes and lysosomes, and involves several steps (see [216,217] for reviews). Firstly, the ubiquitin-activating enzyme E1 catalyses the activation of the C-terminal glycine of ubiquitin

by ATP to give a thiol-ester intermediate. The ubiquitin carrier protein or ubiquitin-conjugating enzyme E2 then transfers activated ubiquitin from E1 to the ubiquitin-protein ligase E3. This enzyme catalyses the formation of an isopeptide bond between the activated glycine of ubiquitin and an ϵ -NH₂ group of a lysine residue on the target protein. This reaction involves specific binding of the substrate to E3 prior to the reaction with ubiquitin, suggesting that E3 plays a major role in substrate selection. In many cases E2 transfers activated ubiquitin directly to the substrate and therefore may also play an important role in specific substrate recognition. Once a protein has been tagged with ubiquitin, it must be delivered to the proteolytic machinery of the proteasome (a soluble multienzyme proteolytic complex) or the lysosome. Many of the proteins ubiquitinated by E2 are of the mono-ubiquitin type, while proteins targeted by E3 are typically multi-ubiquitinated. Multi-ubiquitination of substrates is generally required for recognition by the proteasome, while mono-ubiquitination may function as a signal for sorting to the lysosome.

Although ubiquitination is mostly associated with the degradation of intracellular proteins by the proteasome, cell-surface receptors are also modified by ubiquitin. For example, the T cell antigen receptor, the c-kit receptor and the platelet-derived growth factor β receptor undergo ligand-mediated ubiquitination within the C-terminal tail and subsequent degradation [217–221]. Ste2p, a GPCR in yeast for α pheromone, also undergoes ubiquitination [222]. Agonist binding induces internalization of Ste2p by a clathrin-dependent mechanism, and the internalized receptor is transported to the vacuole (the yeast equivalent of a lysosome), where it is degraded [223,224]. The sequence SINNDKSS within the C-terminal tail of Ste2p is necessary and sufficient for receptor endocytosis [225]. Ligand binding induces ubiquitination of the Ste2p C-terminal tail, which is required for endocytosis, since internalization is 5–15-fold slower in yeast mutants that lack multiple ubiquitin-conjugating enzymes [222]. Mutation of the lysine residue in the SINNDKSS motif abolishes ubiquitination, supporting the proposal that ubiquitination is an endocytosis signal for Ste2p. The mechanism by which ubiquitin induces receptor internalization is unknown. Ubiquitin may be recognized directly by the endocytic machinery or it may direct receptors into domains of the plasma membrane undergoing active endocytosis. It is not known whether ubiquitination is important for the internalization and degradation of GPCRs in mammalian cells. An EMKST motif in the C-terminal tail of the NK1-R is similar to the DAKSS core motif of Ste2p. Mutation of the lysine residue in the EMKST motif does not alter internalization of the mutated receptor, arguing against a role for this motif in the NK1-R [158].

Decreased receptor synthesis

The second component of receptor down-regulation is decreased receptor synthesis. This may be a result of reduced gene transcription or of a post-transcriptional event such as mRNA destabilization. Receptor mRNA destabilization is the prevailing mechanism and is responsible for the decline in receptor message after long-term agonist stimulation of the β_2 -AR, the thrombin receptor, the endothelin ET_B receptor and the muscarinic m1 receptor [206,226–228].

Short-term agonist stimulation of the β_2 -AR results in a transient increase in receptor mRNA levels through a direct increase in the rate of β_2 -adrenergic gene transcription. This is a cAMP-mediated event which relies on the binding of the transcription factor cAMP response element (CRE) binding protein (CREB) to the CRE which is present in the promoter region of

the β_2 -AR gene. The discovery of the CRE modulator (CREM), a CREB-related transcription factor which binds CREs in a dominant-negative fashion, suggests that under certain circumstances cAMP generation could also result in down-regulation of β_2 -AR gene transcription [229]. Indeed, nuclear run-off analysis indicates that agonist-induced down-regulation of the platelet-activating-factor receptor gene is due in part to reduced gene transcription [230].

Destabilization of mRNAs for GPCRs is strongly dependent on cAMP generation and PKA activation (Figure 8). The involvement of PKA suggests phosphorylation of a factor involved in the selective degradation of receptor mRNA or induction of transcription and translation of such a factor. A 35 kDa β -adrenergic-receptor mRNA binding protein which is induced by β -adrenergic agonists has been identified [231,232]. This protein binds to β_2 -AR mRNA, which undergoes dramatic down-regulation, but not to mRNA encoding the α_{1b} -, β_1 - or β_3 -adrenergic receptors, which show little or no down-regulation. β -adrenergic-receptor mRNA binding protein requires one or more AUUUA pentamers and flanking U-rich domains in the 3'-untranslated domain in order to recognize and bind the target mRNA. GenBank analysis has identified several candidate receptor mRNAs that may interact with this protein, including the thrombin receptor mRNA. The thrombin receptor undergoes agonist-induced down-regulation of mRNA via message destabilization, implying that members of the GPCR family may be similarly regulated [232].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The mechanisms that attenuate signalling by GPCRs are of considerable interest from several viewpoints. In the normal healthy organism they are important because they govern the ability of cells to respond to hormones and neurotransmitters, and thereby regulate intercellular signalling. Agonist removal from the extracellular fluid, receptor desensitization and receptor endocytosis prevent the uncontrolled stimulation of cells, and receptor resensitization is critical, for it allows cells to maintain their ability to respond to agonists over time. These regulatory mechanisms are also important from disease and therapeutic considerations. Receptor down-regulation may be important in disease states where there is continued secretion of an agonist, and during long-term therapy with receptor agonists. Defects in the attenuation mechanisms result in the uncontrolled stimulation of cells, which may lead to disease. For example, abnormalities in the 5-hydroxytryptamine transporter may be associated with chronic depression [233], and point mutations of the receptors for thyrotropin hormone and luteinizing hormone which result in constitutive activity of the receptors in the absence of ligand are associated with thyroid adenoma and a form of male precocious puberty respectively [234,235]. From a therapeutic standpoint, over half of all medicines used today exert their effects through signalling pathways that involve G-proteins. Thus many commonly used drugs act by interfering with mechanisms that remove neurotransmitters from the extracellular space, or are themselves agonists or antagonists of GPCRs. The discovery of new ways in which these signalling pathways are modulated could help to explain and perhaps counter the phenomenon of drug tolerance, i.e. the need for increasing doses to achieve a constant effect.

However, our knowledge of these mechanisms is far from complete. Much of the available information is derived from studies of reconstituted systems and transfected cell lines expressing abnormally large numbers of receptors, and many experiments use supra-physiological concentrations of agonists.

Far less is known about receptor regulation by physiological concentrations of agonists in highly differentiated cells, such as neurons, that naturally express GPCRs. Gene-deletion experiments have been particularly helpful in increasing our understanding of the biological importance of some of these regulatory processes, although tissue-specific gene deletion will be more informative. Furthermore, most studies have focused on the early stages of regulation, and far less is known about the long-term down-regulation of receptors, which is of particular importance in disease and therapy.

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