Endocytosis and recycling of G protein-coupled receptors

Jennifer A. Koenig and J. Michael Edwardson

Agonist stimulation of G protein-coupled receptors causes a dramatic reallocation of their intracellular distribution. Activation of receptors triggers receptor endocytosis and, since receptors recycle back to the cell surface continuously, a new steady state is reached where a significant proportion of receptors is located intracellularly. Although this movement of receptors is remarkable, its role has been enigmatic. Recent developments have provided insights into the compartments through which the receptors move, the nature of the signals that trigger receptor translocation, and the significance of receptor cycling for cell function. In this article, Jennifer Koenig and Michael Edwardson review recent progress in this field and place receptor movement within a mathematical framework that reveals the extent and rate of intracellular receptor movement.

The intracellular distribution of G protein-coupled receptors is determined by four main pathways of movement of receptors within cells, which are shown diagrammatically in Fig. 1. Newly synthesized receptors are delivered to the cell surface from the Golgi complex. In unstimulated cells there is a relatively slow endocytosis of receptors from the surface into endosomes. In the presence of agonist, the rate of endocytosis is increased dramatically. Once the receptors reach endosomes, they can either be recycled back to the plasma membrane, the predominant pathway, or routed to lysosomes for degradation. This review describes the techniques that can be used to quantify the rate and extent of receptor movement within cells. The routes taken by receptors and the signals involved in initiating endocytosis will then be discussed. Finally, the functional role that receptor cycling may play in the regulation of receptor activity will be considered. To avoid any possible confusion, the terms that will be used here to describe the intracellular movement of receptors are defined in Box 1.

Measurement of the internalization of receptors and ligands

The methodology used to determine changes in intracellular receptor distribution varies with the receptor type and the available experimental tools. For receptors where membrane-impermeant antagonist radioligands are available, changes in surface receptor number can be measured through changes in the radioligand binding site density. In these experiments (Fig. 2a), the cells are treated with agonist drug for varying times at 37°C, washed thoroughly, and then incubated at low temperatures (usually 4–16°C) with radioligand. Physiological temperatures are required to observe rapid endocytosis; low temperature is required to prevent further endocytosis and recycling of the receptors during the quantitation step. If required, a membrane-permeant antagonist radioligand can be used to measure total receptor number. An alternative approach is to take advantage of the availability of receptors which are epitope-tagged on their extracellular terminus. In this case, immunostaining with fluorescently tagged antibodies followed by fluorescence microscopy can provide qualitative information on the redistribution of receptors. Flow cytometry or enzyme-linked immunosorbent assay can be used for quantitative analysis of changes in surface receptor number. After incubation with agonist at 37°C, the cells are fixed with formaldehyde to prevent further movement of receptors. Subcellular fractionation is also sometimes used to distinguish between receptors in ‘plasma membrane’ and ‘light vesicle’ fractions. The ‘light vesicle’ fractions are not well characterized but do contain endosomes among other organelles. Changes in receptor density are determined by immunoblotting.

Endocytosis of peptide receptors is commonly measured by monitoring the uptake of radiolabelled or fluorescent agonist ligands into the cell (Fig. 2b). This method relies on the assumption that the ligand is endocytosed with its receptor before the ligand–receptor complex dissociates. Because most peptide agonists have a high affinity for their receptor, this assumption is usually justified. In practice, the cells are incubated with ligand to allow surface binding and endocytosis. A rapid wash to remove free ligand will leave cells containing both surface-bound and internalized ligand. The cells can then be washed in a low pH buffer at reduced temperature to accelerate the dissociation of surface-bound ligand, leaving internalized ligand inside the cell. It is often implicitly assumed that one receptor is endocytosed with each peptide ligand that is endocytosed. However, this ratio will depend on the rates of ligand–receptor dissociation and receptor endocytosis. It is likely that, when the agonist binds, the receptor is activated and becomes predisposed to endocytosis. If the ligand dissociates, the receptor may still be associated with the endocytic machinery and be endocytosed without ligand. So the presence of agonist enhances the rate of receptor endocytosis but the amount of agonist endocytosed depends on both its dissociation rate and the receptor endocytosis rate. Indeed, for the lower affinity ligands, such as the muscarinic acetylcholine receptor agonists, it is unlikely that they are internalized at all.

Endocytosis and recycling of G protein-coupled receptors are both energy and temperature dependent; these two processes are almost entirely prevented below 16°C (Refs 1, 2; Fig. 2b). Other
**Fig. 1.** Diagrammatic view of receptor transport pathways. Terms are defined in Box 1.

**Box 1. Definition of terms**

- **Endocytosis**: movement of receptor or ligand from the cell surface to an internal compartment. Endocytosis usually occurs within minutes of agonist treatment. In the case of receptors there is a basal rate of endocytosis (usually slow, occurs without agonist ligand and is therefore constitutive) and an agonist-stimulated rate.

- **Recycling**: movement of receptor or ligand from an internal compartment to the cell surface. Recycling is assumed to be constitutive.

- **Internalization**: loss of surface receptor number determined by a combination of the effects of endocytosis and recycling.

- **Sequestration**: synonymous with internalization.

- **Receptor cycling**: continual agonist-stimulated endocytosis and constitutive recycling of receptors.

- **Downregulation**: loss of total receptor number due to agonist-induced endocytosis and subsequent degradation. Can become significant after an hour or more of agonist treatment.

- **Desensitization**: loss of functional response. Can be short-term (seconds-minutes) or long-term (hours).

- **Resensitization**: recovery of functional response after desensitization.
experimental manipulations that have been shown to inhibit receptor endocytosis include increased extracellular hyperosmolarity (usually achieved with 0.45M sucrose), depletion of intracellular K⁺ and acidification of the cytosol⁶,⁷. Treatment with phenylarsine oxide also inhibits endocytosis⁸, although it is known to have many effects on cellular function through interactions with sulphhydryl groups⁹. The properties described above are characteristic of endocytosis via clathrin-coated pits, and in some cases agonist-stimulated accumulation of G protein-coupled receptors in these pits, and their budding from the cell surface to form clathrin-coated vesicles, have been directly visualized (Fig. 3). There have also been reports of endocytosis via uncoated pits. The nature of these structures remains unclear, however, and the suggestion that caveolae might be responsible for nonclathrin-dependent endocytosis now seems unlikely¹⁰.

Receptor recycling is constitutive (i.e. unaffected by the presence of agonist) and is inhibited by agents such as monensin and nigericin that raise the pH within endosomes (normally pH 5–6; Ref. 11). Receptor recycling is normally measured through the recovery of surface receptor number after washout of agonist. Such experiments should be performed in the presence of cycloheximide to prevent delivery of receptors to the cell surface due to new synthesis. In some cases, the availability of irreversible antagonists allows receptors remaining at the cell surface to be irreversibly alkylated so that only receptors arriving at the surface will be detected by ligand binding. This approach makes it possible to

---

Fig. 3. Immunogold electron microscopy of gold-conjugated cholecystokinin (CCK) interacting with CCK receptors in clathrin-coated pits and vesicles in Chinese hamster ovary cells. After 5 min of incubation at 37°C, gold label has clustered near the plasma membrane (a and c). A higher magnification of these structures shows gold localized to coated pits (b) and tubulovesicular endosomes (d). After 30 min of incubation, the gold label was observed near the nucleus (e) in multivesicular bodies (f). Bar, 200 μm. inset, 100 μm. N, nucleus; PM, plasma membrane. Reproduced from Ref. 6, with permission.
distinguish between delivery due to new synthesis and delivery due to recycling\(^\text{1,2}\) (Fig. 4). The fate of endocytosed agonist peptide ligands is less clear, possibly because of the complication of ligand degradation by extracellular proteases. Significant recycling of a stable analogue of somatostatin in a neuroblastoma cell line has been demonstrated and shown to be the factor limiting the amount of radiolabelled agonist retained inside the cell\(^{\text{5}}\).

**Quantitative analysis of receptor cycling**

**Two-compartment model**

It is possible to estimate the number of receptors cycling to and from the plasma membrane using kinetic modelling based on a simple two-compartment model. The modelling makes no assumptions about whether the ligand is endocytosed with the receptor – it is assumed only that application of agonist dramatically and instantaneously increases the rate of endocytosis of the receptor. It can be shown mathematically (see Box 2) that the number of surface receptors and the number of endosomal receptors at any time are determined by both the rate constant for endocytosis and the rate constant for recycling. The rates of endocytosis and recycling can be determined by nonlinear curve fitting of experimental data to Eqn 2. Table 1 shows some rate constants that have been derived for different receptors.

Although only the change in the number of surface receptors is measured, it is possible to derive the instantaneous rates of endocytosis and recycling. For example, the rate of endocytosis is \(k_3 \times R_s\), and since from Eqn 2, \(R_i\) is known at any time, the rate of movement of receptors from the surface to endosomes is also known at any time. Figure 5 shows rate estimations for the movement of muscarinic M\(_3\) receptors in SH-SY5Y cells, that have been derived from the data shown in Fig. 2a. It can be seen that almost a third of the surface receptors are endocytosed in the first 7 min of agonist treatment. Because the rate of endocytosis depends on the surface receptor number, it will fall as internalization reaches steady state. Summation of the rate of movement with time gives the turnover time. In the case of Fig. 5, all of the surface receptors are endocytosed in the first 7 min of agonist treatment. In contrast, the proportion internalized is only 68%, since many of the endocytosed receptors are recycled. It is important to note that the rate and extent of internalization, and the time taken to cycle from the cell surface to endosomes and back again depends on both the rates of endocytosis and recycling. The mathematical formulation of these ideas is given in Box 2.

Mathematical modelling and the explicit statement of the assumptions involved lead to a number of predictions which can be directly tested by experiment. Two of these predictions – the dependence of endocytosis on agonist concentration and the effects of inverse agonists – will be considered here. The extent of internalization, that is the proportion of receptors inside the cell at steady state, is determined by both the rates of endocytosis and recycling (Box 2):

\[
\text{% internalized} = 100 \times \frac{k_2}{k_2 + k_3}
\]

Since the rate of recycling is constitutive, only the rate of endocytosis is likely to depend on agonist concentration. The relationship between the proportion of receptors internalized and the rate constant for endocytosis is clearly not linear (Fig. 6c). As yet, the relationship between the rate of endocytosis and agonist concentration is unknown. Understanding this relationship may yield clues regarding underlying biochemical mechanisms: it will be interesting to see, for example, whether the rate of endocytosis depends simply upon occupancy of the receptor with ligand, or whether there is a significant efficacy component.

The same relationship can be used to predict the internalization of constitutively active receptors. If constitutively active receptors have a similar rate constant for endocytosis as agonist-occupied receptors and a similar rate constant for recycling as wild-type receptors, then the proportion of receptors inside the cell will be high (as \(k_3 > k_2\)) and it should be possible to observe a large intracellular pool. If a proportion of endocytosed receptors is routed to lysosomes rather than being recycled (see Box 3), then constitutively active receptors should have a higher rate of degradation than wild-type receptors. Unless new synthesis is faster for constitutively active receptors than wild-type receptors, then the total

---

**Figure 4.** Delivery of muscarinic acetylcholine M\(_4\) receptors to the surface of NG108 cells. Cells were treated with vehicle (unstimulated cells) or carbachol (20\(\mu\)M) to induce receptor endocytosis. After thorough washing, the surface receptors were inactivated by incubation with the irreversible antagonist prop-hexylcholine mustard. The cells were warmed to 37°C for varying times to allow receptors to be delivered to the cell surface and the number of surface receptors was quantified by the binding of a membrane-impermeant antagonist radioligand. The green line represents the delivery of receptors to the cell surface in unstimulated cells and is due to new synthesis. The red line represents delivery of receptors to the cell surface in carbachol-treated cells in the presence of the protein synthesis inhibitor cycloheximide to block new synthesis. Here the delivery of receptors is due to recycling from endosomes. The blue line is the sum of new synthesis and recycling. Within the first two minutes after washout of agonist, most of the delivery of receptors to the cell surface is due to recycling from endosomes, while after two hours, the contribution of delivery of newly synthesized receptors becomes important. Data from Ref. 1.

---

**Table 1.** Some rate constants that have been derived for different receptors.

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Rate Constant</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscarinic M(_3)</td>
<td>(k_3)</td>
<td>Endocytosis rate constant</td>
</tr>
<tr>
<td></td>
<td>(k_2)</td>
<td>Recycling rate constant</td>
</tr>
</tbody>
</table>

---

**Box 2.** Mathematical formulation of ideas.

---

**Box 3.** Roles of proteasomes in receptor degradation.

---

**Box 4.** Kinetic mechanisms of receptor cycling.
In a two-compartment model, receptors can only cycle between the cell surface and endosomes (Fig. 1). The rate of movement of receptors from the surface to endosomes depends on the number of receptors at the surface ($R_s$) and the rate constant for endocytosis ($k_e$).

rate of endocytosis = $k_e \times R_s$

Similarly, the rate of recycling depends on the number of receptors in endosomes ($R_e$) and the recycling rate constant ($k_r$).

rate of recycling = $k_r \times R_e$

The rate of change of the number of surface receptors

$$\frac{dR_s}{dt} = k_e R_e - k_r R_s$$

is the rate of receptors arriving by recycling less the rate leaving by endocytosis.

The total number of receptors, $R_s + R_e$, is equal to the number of receptors at time $t = 0$, i.e., $R_s + R_e = R_{ss}$.

Substituting $R_s = R_{ss} - R_e$, Eqn 1 can be rewritten as:

$$\frac{dR_e}{dt} = k_e (R_{ss} + R_e) - (k_e + k_r) R_e$$

which is now of the form

$$\frac{dR_e}{dt} = b - ay$$

This integrates to $ay - b = Ce^{-at}$ where $b = k_e (R_{ss} + R_e)$; $\alpha = k_e + k_r$ and $C$ is the constant of integration. A little algebra leads to an equation which describes the number of surface receptors as a function of time:

$$R_s = \frac{1}{k_e + k_r} [k_e (R_{ss} + R_e) + (k_e R_{ss} - k_e R_e) e^{-(k_e + k_r)t}]$$

(2)

Note that this is a more general form of the equation derived for GLUT4 trafficking\(^{\dagger}\) where it is assumed that $R_{ss} = 0$.

Figure 2a shows some theoretical predictions from this equation for an experiment where drug is added at $t = 0$ and the number of surface receptors is monitored with time. The different lines correspond to different values for $k_e$. The other parameters remain constant ($k_r = 0.1 \text{ min}^{-1}, R_{ss} = 100\%, R_e = 0\%)$. This could represent a series of experiments with receptors with different rates of endocytosis.

It is important to note that the rate and extent of internalization and the time taken to cycle from the cell surface to endosomes and back again depend on both the rates of endocytosis and recycling. This idea is illustrated in Fig. 2b where the endocytic rate constant is the same in each case. The faster the recycling rate, the smaller the proportion of receptors that are internalized at steady state. Therefore it follows that for a series of, for example, mutant receptors, differences in the proportion of internalized receptors may be due to changes in the rate of recycling rather than, or as well as, changes in the rate of endocytosis. This can be described more formally by defining the following relationships which can be used to compare the rate and extent of internalization and receptor cycling between receptors with different endocytosis and recycling rate constants:

- The steady-state number of surface receptors ($R_{ss}$) and endosomal receptors ($R_{ss}$) in the presence of agonist is:

$$R_{ss} = \frac{k_e (R_{ss} + R_e)}{k_e + k_r}$$

(3)
be estimated from the rate of delivery to the plasma membrane. In agonist-treated cells, delivery of receptors to the surface occurs both along the synthetic route and by recycling from endosomes. In neuroblastoma cells most of the muscarinic receptors delivered to the surface in the first 30 min after carbachol stimulation come from the recycling pathway. However, 2 h after removal of agonist, half of the receptors delivered to the surface come from the new synthesis pathway.

Receptor degradation should also feature in any attempt to model intracellular receptor movement, especially as the rate of degradation increases dramatically in the presence of agonist. Receptor density often decreases after agonist concentrations have been elevated for some time, and this change represents an important component of the response to chronic drug treatment. Taking both new synthesis and degradation into account obviously makes the modelling more complex. However, the availability of powerful personal computers and numerical simulation software has made analysis of multicompartment models much easier.
synthesis and degradation into account, the model is still likely to be too simplistic. For example, only a single endocytotic compartment is considered, when it is already clear that a finite time is required for receptors to move through coated vesicles and endosomes en route back to the plasma membrane. The introduction of further complexity at this point, however, is probably premature, since it is not yet possible to distinguish these compartments experimentally.

Control of endocytosis and recycling

Endocytosis motifs

Receptor endocytosis has been best characterized for receptors for polypeptides such as low density lipoprotein (LDL) and transferrin. Here, the purpose of endocytosis is to capture the ligand for subsequent use by the cell. The receptors cycle continuously between the cell surface and endosomes, binding ligand at the cell surface and depositing it in endosomes. Mutational analysis of the endocytosis of these receptors has led to the proposition that aromatic residues in two sequence motifs in the cytoplasmic domains, YXXZ and NXXY, where Z is a hydrophobic residue, act as recognition sequences for endocytosis. Presumably, this sequence remains exposed in these receptors, thus allowing continual interaction with the endocytotic apparatus.

In contrast to this ‘conveyor belt’ mode of operation, G protein-coupled receptors reside predominantly at the plasma membrane and are endocytosed only in response to agonist stimulation. Presumably, any endocytosis motif in these receptors must become exposed after activation of the receptor with agonist. The similarity of the sequence NPXY, present at the cytoplasmic end of the seventh transmembrane domain of many members of the G protein-coupled receptor superfamily, to the NXXY motif of a number of other receptors such as LDL receptors led to the suggestion that this motif may be important in receptor endocytosis. However, the story does not seem to be quite so straightforward. First, the NPLIY sequence in the β2-adrenoceptor seems to be involved in G protein coupling, agonist affinity, phosphorylation and downregulation, as well as endocytosis. The second effect of the mutation can be rescued by increased phosphorylation (see below), and third, mutations in this motif do not affect the endocytosis of at least two members of the superfamily (the gastrin-releasing peptide receptor and the angiotensin AT1 receptor).

Agonist-dependent phosphorylation

The paradigm for the modulation of G protein-coupled receptor function in response to agonist stimulation has been the β2-adrenoceptor. It is well known that stimulation of this receptor causes phosphorylation at different intracellular sites by protein kinase A and β-adrenoceptor kinase (βARK), a member of a family of G protein receptor kinases (GRKs). Phosphorylation by βARK, on the carboxy-terminal tail, causes the recruitment of the cytosolic protein β-arrestin, which binds to the third intracellular loop and the carboxy tail of the receptor and uncouples it from the G protein, Gs (Ref. 21). This phosphorylation event is crucially involved in receptor desensitization, which is seen as a rapidly developing (seconds–minutes) reduction in the ability of the receptor to generate second messenger in response to continued agonist stimulation. The role of phosphorylation in G protein-coupled receptor endocytosis has been more controversial. Early studies using mutant β2-adrenoceptors that lacked phosphorylation sites seemed to indicate that phosphorylation was not required for endocytosis. However, when cells expressing endocytosis-deficient receptors in which the Y in NPLIY had been mutated to an A (the Y226A mutant), were co-transfected with βARKs, it was found that the receptor was phosphorylated and its endocytosis was rescued, indicating that phosphorylation might in fact act as an endocytosis signal. Rescue was prevented by deletion of a putative βARK phosphorylation sites, and both phosphorylation and endocytosis of wild-type β2-adrenoceptor could be reduced by

---

Table 1. Rate constants for endocytosis and recycling of receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell type</th>
<th>Endocytotic rate constant (min⁻¹)</th>
<th>Recycling rate constant (min⁻¹)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>CHO</td>
<td>0.03–0.3</td>
<td>0.058</td>
<td>58</td>
</tr>
<tr>
<td>EGF</td>
<td>NG108-15</td>
<td>0.03</td>
<td>0.06</td>
<td>11</td>
</tr>
<tr>
<td>Muscarinic M1</td>
<td>SH-SVY</td>
<td>0.28</td>
<td>0.074</td>
<td>13</td>
</tr>
<tr>
<td>Muscarinic M3</td>
<td>CHO</td>
<td>0.12</td>
<td>nd</td>
<td>59</td>
</tr>
<tr>
<td>β2-Adrenoceptor</td>
<td>CHO</td>
<td>0.09</td>
<td>0.11</td>
<td>17</td>
</tr>
<tr>
<td>β2-Adrenoceptor</td>
<td>CHO</td>
<td>0.09</td>
<td>0.11</td>
<td>17</td>
</tr>
</tbody>
</table>
| CHO, Chinese hamster ovary; EGF, epidermal growth factor; nd, not determined.
overexpression of a dominant-negative mutant of bARK1. Together, this work suggests that mutation of this tyrosine motif affects the conformation of the activated receptor such that it is less able to interact with GRKs or intracellular proteins in the endocytic machinery. Furthermore, it is likely, in common with the epidermal growth factor (EGF) receptor, that phosphorylation could cause a conformational change in the receptor which either enhances interaction with intracellular adaptor proteins, or exposes cryptic motifs which interact with intracellular adaptors.

In addition to GRKs, β-arrestin has also been implicated in endocytosis. For example, overexpression of β-arrestin rescues the endocytosis of mutants of the β2-adrenoceptor defective in this function, and this effect is enhanced by co-overexpression of bARK (Ref. 26). Furthermore, it has recently been shown that β-arrestin is involved in mediating the interaction between the β2-adrenoceptor and clathrin27. As β-arrestins do not bind to phosphorylated, unactivated β2-adrenoceptor, although not as well as to phosphorylated, activated receptor, it is possible that phosphorylation itself can cause recruitment of β-arrestin and hence endocytosis, but that agonist stimulation is required for maximal endocytosis. This suggests that different mutations can affect endocytosis to different degrees. When the receptor is activated by agonist and has all the appropriate phosphorylation sites and intracellular binding motifs, then it is endocytosed efficiently, but if it loses any of these sites, endocytosis is not turned off, it is just less efficient. This is underlined by the observation that phosphorylation by GRK2 facilitates the endocytosis of the muscarinic M2 receptor by decreasing the concentration of agonist required for maximal endocytosis, having no effect at saturating concentrations28.

A number of other G protein-coupled receptors act as substrates for various GRKs, both in vitro and in vivo, although phosphorylation may occur not only on the C-terminal domain but also on the second or third intracellular loop29,30. For example, phosphorylation of muscarinic receptors is thought to be involved in desensitization31,32 but the role of phosphorylation by GRKs in endocytosis of the muscarinic receptor is still unclear, and reports are sometimes directly contradictory29,33.

Other determinants of endocytosis

The endocytosis of different receptor subtypes is often different. In transfected Chinese hamster ovary (CHO) cells, for example, agonist stimulation triggers endocytosis of M3 and M4 subtypes (which inhibit adenylate cyclase) but endocytosis of the M1 and M2 subtypes (which stimulate phospholipase C) is much less efficient33. Hence, the signals for endocytosis might be different for subtypes that couple to different second messenger systems. Alternatively, the second messengers themselves may be involved. Although membrane transport steps are known to be modulated through the action of second messengers34, there is no direct evidence that the intracellular transport of G protein-coupled receptors is controlled in this way. Indeed, in the case of the M3 receptor, it has been shown that the second messenger response is not required for endocytosis, although coupling of the receptor with the G protein does seem to be important35.

The fact that M3 receptors endocytose poorly in transfected CHO cells but efficiently in SH-SY5Y cells, where they are expressed naturally, suggests that the CHO cell may lack a component of the signalling system that is present in SH-SY5Y cells35. Some of the conflict between different studies might be a consequence of the use of different cell types, which might show quantitative and qualitative differences in their expression of protein kinases and other components of the endocytic machinery. The effect of receptor density has not been systematically examined for G protein-coupled receptors although it might be expected to be important since a high level of EGF receptor expression in A431 cells does lead to saturation of the endocytic apparatus36. Having said this, M3 receptors overexpressed in CHO cells still undergo endocytosis37.

Recent work has pointed to the involvement of ubiquitination in endocytosis and degradation of receptors. Ste2p is a G protein-coupled receptor for the hormone α-factor in yeast. The receptor becomes ubiquitinated on its cytoplasmic tail rapidly in response to ligand binding and a single Lys→Arg mutation eliminates both ubiquitination and endocytosis of the receptor38. It is thought that the ubiquitination signals the direction of the receptor towards the lysosomal degradation pathway. The platelet-derived growth factor β receptor also undergoes polyubiquitination following ligand binding39, and it will be interesting to see whether mammalian G protein-coupled receptors also undergo this form of regulation.
Box 3. Analysis of receptor cycling taking into account receptor synthesis

The rate of change of total receptor number \( (R_T) \) is

\[
\frac{dR_T}{dt} = k_x - k_d
\]

which integrates to

\[
R_T = (k_x - k_d)t + R_0 + R_p.
\]

The rate of change of surface receptor number is now the same as in Box 2 but the delivery due to new synthesis must be added:

\[
\frac{dR_s}{dt} = k_x R_e - k_e R_s + k_d.
\]

Using the same methods as before leads to an equation of the form

\[
\frac{dR_s}{dt} = a - bR_s - cy
\]

which integrates to

\[
y = cy + \frac{a}{C} - \frac{b}{C} e^{-Ct}
\]

where

\[
c = k_x + a(R_e + R_p), b = k_e(k_x - k_d), c = k_e + k_x \text{ and } C \text{ is the constant of integration. This gives Eqn 9:}
\]

\[
R_s(t) = \frac{1}{k_x + k_e} \left[ \frac{k_e + k_x R_e + \left( \frac{k_e}{k_x} - 1 \right)}{\left( k_x + k_e \right) \left( k_x + k_e \right)} \right] e^{-k_x t} + \frac{k_e}{k_x + k_e} R_s(0)
\]

When \( t \) becomes large, the exponential term approaches zero and the equation reduces to a line with slope

\[
-\frac{R_e}{k_x + k_d} \left( \frac{b}{k_x} + k_e \right)
\]

Determinants of the fate of endocytosed receptors

After endocytosis, receptors enter a tubulo-vesicular endosomal compartment. The nature of this endosomal compartment has recently been investigated using immunofluorescence microscopy. G protein-coupled receptors appear to share the same endosomal pathway as the transferrin receptor which is characterized by the presence of the small GTP-binding protein rab5. Once in the endosome, G protein-coupled receptors may either be recycled to the plasma membrane or directed to other destinations on the endocytic pathway, such as the lysosome. The different extents of recycling and degradation for different G protein-coupled receptors suggest that signals operate in early endosomes to determine the subsequent fate of the endocytosed receptor, with recycling to the plasma membrane being the default pathway. The phosphate groups added to the \( \beta \)-adrenoceptor in response to agonist stimulation do not appear to have any signalling function in the endosome, as calyculin A, which blocks dephosphorylation of the receptor, does not affect its recycling to the cell surface. It remains a possibility, however, that the activation state of the receptor when it reaches the endosome has some bearing on its fate. The behaviour of the thrombin receptor, which is activated irreversibly by a unique proteolytic mechanism, provides direct evidence in favour of this idea. Thrombin cleaves the receptor’s amino terminus to reveal a new terminus, which then acts as a tethered peptide ligand. Activated receptors are rapidly endocytosed and delivered to lysosomes for degradation. Receptors at the plasma membrane are eventually replenished by delivery of receptors from an intracellular pool, which is labelled by Golgi markers. For G protein-coupled receptors where agonists are generally of low affinity (i.e. micromolar), such as the \( \beta \)-adrenoceptor and the muscarinic receptor, it is unlikely that sufficient agonist will be endocytosed to cause significant receptor activation in endosomes. Peptides, however, often have higher affinity for their respective G protein-coupled receptors (i.e. nanomolar to picomolar) and it is therefore possible that the concentration of the agonist in the lumen of the endosome may be high enough to cause receptor activation. If continued activation does somehow target G protein-coupled receptors to lysosomes, then one would expect the fate of the receptor to depend on agonist affinity. This idea remains to be tested.
Is it necessary to take new synthesis and degradation into account? There are two reasons to use a model with more than two compartments: (1) after 30 min of treatment with agonist, a large proportion of receptors delivered to the surface come from new synthesis rather than recycling; (2) it is possible to determine the proportion of receptors which are recycled rather than degraded. If endosomes are considered as a sorting compartment, then a ratio of recycling and degradation rate constants gives an indication of the relative sorting out of endosomes to either lysosomes or the cell surface. To be able to do this, degradation must be treated as an exponential process in the same way as recycling, i.e. the rate of degradation is proportional to the number of receptors in endosomes = \( k_e \times R \). The advantage of using Eqn 5 is that the data can be fitted using non-linear curve-fitting techniques. Many software packages such as Fityk, GraphPad Prism and SigmaPlot allow equations defined by the user. However, Eqn 5 estimates the degradation rate by assuming that it follows linear kinetics. To compare recycling and degradation rate constants, it is necessary to treat degradation as an exponential process. The most straightforward way to do this is to take an iterative approach which entails setting a simple program or designing a spreadsheet to perform simulations of the data. Alternatively, the program Modemaker (Cherwell Scientific) has been designed to tackle just this sort of problem. This allows estimation of the best fit parameters from experimental data as well as simulating the model under various conditions. The data from Fig. 2a (main text) were analysed in this way and are plotted in Fig. 2 with the calculated number of receptors in endosomes and lysosomes. This analysis gave a very similar value for \( k_e \) (0.31 min\(^{-1}\)) compared to the value obtained for nonlinear curve fitting of the data to Eqn 5 (0.28 min\(^{-1}\)), but now the degradation rate \( k_e \) is 0.008 ± 0.003 min\(^{-1}\) instead of 0.63% min\(^{-1}\). The comparison of \( k_e \) (0.074 min\(^{-1}\)) and \( k_e \) (0.008 min\(^{-1}\)) suggests that ~10% of receptors that reach endosomes are not recycled. Because the internalization rate is so high, many receptors reach endosomes and degradation becomes significant within 1 h. Note that degradation appears linear in this instance.

**Functional significance of receptor recycling**

**Desensitization and resensitization**

It might seem reasonable to propose that receptor internalization plays a role in desensitization, with a fall in the numbers of G protein-coupled receptors at the plasma membrane causing a reduction in the cellular response to agonist. However, loss of receptors from the surface is usually too slow to account for the rapid onset of desensitization and, there are cases where the drop in surface receptor number occurs without desensitization, and vice versa. Finally, inhibition of endocytosis often does not affect desensitization, and the effects of mutations such as those described above on endocytosis and desensitization are often disparate. Although there may be cases where endocytosis does play a role in desensitization, it is also possible that receptor cycling is involved in resensitization. For instance, agonist stimulation of the \( \beta_2 \)-adrenoceptor results in receptor phosphorylation, which in turn causes uncoupling of the receptor from its G protein and subsequent endocytosis. The receptor is translocated away from the agonist into an endosomal compartment where the low intraluminal pH enhances phosphatase activity. There it is dephosphorylated and recycled to the plasma membrane where it initiates another response. In support of this idea, it has been shown that inhibition of \( \beta_2 \)-adrenoceptor endocytosis by concanavalin A or hyperosmolar sucrose, or recycling by monensin or of dephosphorylation by calyculin A all inhibit receptor resensitization.

In contrast to these results for the \( \beta_2 \)-adrenoceptor, endocytosis delays the resensitization of \( \mu \) receptors. This could be explained by the postulation of a slow process of reactivation after recycling of newly recycled receptors is of a similar value to that for recycling or endocytosis, a considerable proportion of receptors at the surface would be refractory to stimulation. It is important to think of the internalization and recycling of receptors as a dynamic system where the receptors are constantly cycling to and from the surface. Perhaps we should also think of desensitization as a dynamic balance between desensitization and resensitization. If resensitization is indeed affected by constant cycling of receptors to and from the cell surface and if the net amount of desensitization is a balance between the rates of desensitization and resensitization, then we should be thinking about the role of receptor cycling rather than endocytosis in desensitization.
**Signal transduction**

For some G protein-coupled receptors, endocytosis may be crucial to the generation of a cellular response. Several peptide hormones, such as neurotransmitters, interleukin-1 and angiotensin II, are known to affect neuronal function via regulation of gene expression, and stimulation of the target cell is followed by delivery of both ligand and receptor into the perinuclear region, and even into the nucleus itself. Extensive intracellular transport has been demonstrated in vitro; for example, neurotransin injected into the rat caudate nucleus was shown to be captured by receptor-mediated endocytosis and transported to the substantia nigra by retrograde axonal transport.

**Concluding remarks**

At present, the G protein-coupled receptor superfamily has over 200 members. Although all of these receptors have a common membrane topology, there is little conservation of specific amino acid sequence across the whole superfamily and the variety of cellular responses initiated by the receptors is enormous. Much of the information available at present concerning the nature of the signals specifying endocytosis, and the consequences of receptor cycling for cell function, is derived from studies of a single receptor type (the β2-adrenoceptor). The biggest challenge now is to determine how much of this information is applicable to other receptors, so that general principles can be formulated. It is known that the second messengers generated by activation of a given receptor depend on the presence of particular types of G proteins and effector proteins. Furthermore, the efficiency of endocytosis will also depend on the nature of the endocytotic machinery, which may itself vary with cell type. It is important to bear these points in mind.

**Selected references**

International Union of Pharmacology

Forthcoming meetings

1997

Advances in Serotonin Receptor Research
In collaboration with the Serotonin Club
San Francisco, USA
8–10 October 1997

1998

1st IUPHAR Conference on Receptor Mechanisms:
Principles of Agonism
Merano, Italy
23–25 July 1998

XIIIth International Congress of Pharmacology
Munich, Germany
26–31 July 1998

Programme details and registration information for these meetings

can be obtained from:

IUPHAR Media
68 Half Moon Lane
London
UK SE24 9JE

And via the IUPHAR WWW site at