Minireview

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G Protein-coupled Receptors

III. NEW ROLES FOR RECEPTOR KINASES AND β-ARRESTINS IN RECEPTOR SIGNALING AND DESENSITIZATION*

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Of the many forms of GPCR[1] regulation none has received as much attention as the process of receptor desensitization, *i.e.* the waning responsiveness of the receptors in the face of persistent stimulation (1, 2). Numerous mechanisms have been discovered, including those that operate at the transcriptional, translational, and protein levels. The latter category in turn includes mechanisms that regulate the rate of degradation of the receptors. Finally, there are mechanisms for the covalent modification of the receptors as well as for the regulation of their association with other proteins and their subcellular localization (1–3). This brief review is concerned with this last group of mechanisms, which appears to be most important with respect to the rapid (seconds–minutes as opposed to hours or days) control of receptor function.

Traditionally, receptor desensitization has been viewed as a process antithetical to receptor activation, one which terminates or attenuates receptor signaling. Three families of regulatory molecules have been found to participate in desensitization of heptahelical receptors: second messenger-regulated kinases (*e.g.* PKA and PKC), GRKs (*e.g.* βARK, rhodopsin kinase), and the arrestins (visual and non-visual). After briefly reviewing the well established paradigms for regulation of GPCRs by these three families of molecules, I will attempt to demonstrate how newly acquired insights into the function of these receptor regulatory molecules are reshaping understanding of the classical dichotomy between receptor activation and desensitization. The new information suggests that receptor signaling and desensitization are in reality two intimately linked aspects of receptor function and that mechanisms previously viewed as "desensitizing" with respect to one signaling pathway may be "activating" with respect to another.

Established Paradigms

Second Messenger Kinases—One well established mechanism for desensitizing GPCRs is via feedback regulation by the second messenger-stimulated kinases, which they activate. Both PKA, activated by G_s-coupled receptors, and PKC, activated by G_q-coupled receptors, participate in such regulation (Refs. 1 and 4, and references therein). As first documented for the β_2-adrenergic receptor, phosphorylation occurs on serine residues located in the third cytoplasmic loop or C-terminal tail of the receptors (5, 6). Phosphorylation directly alters receptor conformation such that interaction with the G protein is impaired. This type of receptor regulation generally mediates "heterologous" or non-"agonist-specific" desensitization because any stimulant that elevates cAMP (or diacylglycerol in the case of PKC) has the potential to cause the phosphorylation and desensitization of any GPCR containing an appropriate PKA and/or PKC consensus phosphorylation site.

GRKs and β-Arrestins—The major cellular mechanism mediating rapid, agonist-specific, or homologous desensitization of G protein-coupled receptors consists of a two-step process in which the agonist-occupied receptors are phosphorylated by a GRK and then bind an arrestin protein, which sterically interdicts signaling to the G protein. These mechanisms have been extensively reviewed elsewhere (1, 2, 7–10). The family of GRKs currently includes six members (GRKs 1–6), of which the most thoroughly investigated are rhodopsin kinase (GRK1) and βARK1 (GRK2).

The arrestin family includes at least six members, several of which undergo alternative splicing (2, 10). Some forms are found in the brain and many other tissues (*e.g.* β-arrestins 1 and 2), whereas others are confined to the retina (*e.g.* visual arrestins, cone arrestin). Direct binding studies have demonstrated that GRK-catalyzed phosphorylation of β_2-adrenergic receptors increases affinity of β-arrestin binding 10–30-fold whereas agonist occupancy of the receptors has a much less significant effect on the affinity of this interaction (11, 12). The regions on the arrestins that bind to the intracellular loops of the receptors have been mapped (13).

Regulation of GRKs—An allosteric mechanism for activating GRKs mediated by binding their substrates, the activated or agonist-occupied receptors, has been explicitly demonstrated for both GRK1 and -2 (14, 15). Other factors regulating activity include PKC (16, 17), lipids (18, 19), and calcium-binding proteins such as recoverin (20) and calmodulin (21, 22) (all reviewed in Ref. 7).

Several mechanisms for targeting GRKs to their membrane-bound receptor substrates have been discovered. GRK2 and -3 appear to be largely cytosolic enzymes. When an agonist stimulates a GPCR it causes the receptor to interact with a heterotrimeric G protein leading to dissociation into its α and βγ dimer subunits (23). The βγ subunit complex, which is prenylated with a geranylgeranyl group at the C terminus of γ (farnesyl in the case of transducin) is membrane-bound (23). In a coordinated process, free Gβγ and membrane phosphatidylinositol bisphosphate appear to bind to a C-terminal domain of GRK2 or -3, termed a pleckstrin homology domain (19, 24). Interaction of these ligands with the pleckstrin homology domain translocates or targets the kinase to the membrane-bound, agonist-occupied receptor, where it is then appropriately situated to interact with its substrate. Different Gβγ isoform combinations have preferential affinity for either GRK2 or -3, perhaps providing a basis for specificity in GRK-receptor interactions (25). Distinct mechanisms appear to explain the membrane targeting of other members of the GRK family (see Ref. 7).

GPCR Endocytosis—Many GPCRs undergo agonist-promoted endocytosis, internalization, or sequestration (3). Depending on the receptor this may utilize the classical clathrin-coated vesicle machinery or non-coated vesicle pathways (3). The functional significance and mechanisms involved in GPCR sequestration have been the subject of intense study and some controversy over the years. Initially, it was thought that internalization of the receptors might contribute to rapid desensitization. Later it was shown that rapid functional uncoupling of the receptors did not require this process (reviewed in Ref. 1). Subsequently, receptor sequestration was viewed as an early step in the so-called "down-regulation" of receptors, which occurs after prolonged (hours–days) agonist stimulation and which ultimately ends in degradation within lysosomes. However, this relationship remains unproven. Two other roles for receptor sequestration have recently received experimental support-receptor resensitization and receptor signaling. These are discussed below.

New Paradigms

Role of β-Arrestins and GRKs in Receptor Endocytosis—Several lines of evidence now indicate that GRK-catalyzed phosphorylation of GPCRs followed by β-arrestin binding are crucial steps in the

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C; βARK, β-adrenergic receptor kinase; GRK, G protein-coupled receptor kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAPK/ERK kinase.

internalization of several heptahelical receptors (reviewed in Ref. 2). For example, in the case of the m2-muscarinic cholinergic receptor, GRK2 overexpression enhances sequestration, whereas a dominant negative form of βARK1 retards it in COS cells (26). Similar observations have been made with the β_2-adrenergic receptor. In particular, a mutated β_2-adrenergic receptor (Y326A), which is a poor substrate for β-adrenergic receptor kinase, is not sequestered. Sequestration of this receptor can be restored by overexpression of βARK (27).

The role of GRK phosphorylation of the receptors in the sequestration process is to facilitate β-arrestin binding. Thus, β-arrestin overexpression also restores sequestration of the β_2-adrenergic receptor Y326A mutant and can promote the sequestration of β_2-adrenergic receptor mutants, which lack the GRK phosphorylation sites (28). Further confirming the crucial role of β-arrestins in the internalization process is the finding that "dominant negative" forms of β-arrestin (*e.g.* β-arrestin1-V53D or S412D) strikingly impair receptor sequestration (28, 29). Supporting the generality of these mechanisms is the finding that removal of the C-terminal tails (containing the likely sites of GRK phosphorylation) from a number of different GPCRs impairs their sequestration (2).

How does β-arrestin function to promote the internalization of GPCRs? Recently it has been demonstrated, using purified proteins, that β-arrestins 1 and 2 (but not visual arrestin) bind directly, stoichiometrically, and with high affinity to clathrin (30). β-Arrestin/arrestin chimeras, which are defective in either β_2-adrenergic receptor or clathrin binding, are impaired in their ability to promote receptor endocytosis. Using immunofluorescence microscopy of intact cells, agonist stimulation of β_2-adrenergic receptors was shown to promote the co-localization of the receptors and β-arrestin with clathrin. These results suggest that β-arrestin functions in some way as an adaptor in the clathrin-coated vesicle-mediated endocytosis of G protein-coupled receptors (30). Whether, as it occurs in cells, this is via a direct interaction with clathrin or with some other protein(s) remains to be determined.

As noted above, however, not all GPCR endocytosis proceeds via clathrin-coated vesicle pathways (2, 3). For example, internalization of angiotensin II 1A receptors does not ordinarily utilize this pathway as demonstrated by the lack of dependence of angiotensin II 1A receptor sequestration on either β-arrestin or the GTPase dynamin (31). However, when β-arrestin is overexpressed a fraction of the angiotensin II 1A receptors can be made to engage the clathrin-mediated pathway (31).

The function of β-arrestin 1 in GPCR sequestration is regulated by phosphorylation/dephosphorylation of the β-arrestin molecule (29). Cytoplasmic β-arrestin 1 is constitutively phosphorylated on Ser-412, a C-terminal site. When it is recruited to the plasma membrane by agonist stimulation of the β_2-adrenergic receptors, β-arrestin 1 becomes rapidly dephosphorylated. This dephosphorylation is required for its function in the pathway of receptor endocytosis but not for receptor binding and desensitization. Neither the kinase(s) nor phosphatase(s) that participates in this regulatory process has as yet been identified. This regulation of the endocytic function of β-arrestin 1 by dephosphorylation at the plasma membrane is reminiscent of that previously demonstrated for the classical endocytic adaptor protein complex AP2 (32). Interestingly, Ser-412 is not present in other members of the arrestin family. Hence, the other arrestins must be regulated by phosphorylation at other sites or by entirely different mechanisms.

Receptor Endocytosis and Resensitization—The process of rapid agonist-induced desensitization is also generally rapidly reversible. Thus after removal of the agonist isoproterenol from contact with cells, adenylyl cyclase responsiveness to agonist generally returns to normal within 15–30 min. As early as 1986, Sibley *et al.* (33) suggested that β_2-adrenergic receptor internalization played a role in receptor dephosphorylation and resensitization. It was observed that after agonist stimulation, receptors in internalized vesicles contained less phosphate than those in plasma membrane fractions. Moreover, the vesicle fraction was enriched in a phosphatase activity capable of dephosphorylating the receptor. Later it was demonstrated that if β_2-adrenergic receptor sequestration was blocked either by treatment of cells with sucrose or by creating a sequestration-defective β_2-adrenergic receptor mutant, the recovery from desensitization normally observed within 20 min of removal of agonist was blocked (34). The lectin concanavalin A, another reagent that blocks receptor sequestration, also blocked resensitization (34), as do dominant negative mutants of β-arrestin or dynamin, which inhibit sequestration (35). β-Arrestin or GRK2 overexpression rescues resensitization of a sequestration-impaired β_2-adrenergic receptor mutant (35).

The phosphatase responsible, at least for β_2-adrenergic receptor dephosphorylation, is a membrane-associated phosphatase of the PP-2A family. It has been termed the GPCR phosphatase and, at least *in vitro*, is active against not only the GRK-phosphorylated β_2-adrenergic receptor but also the α_{2a}-adrenergic receptor and rhodopsin (36). It is not active against the PKA-phosphorylated β_2-adrenergic receptor. *In vitro*, the phosphatase is active on the phosphorylated β_2-adrenergic receptor only at acidic pH (37). In intact cells, dephosphorylation of the receptors proceeds only after conformational changes in the receptor, which are apparently induced by the low pH uniquely present in the sequestered vesicles into which they are internalized after agonist stimulation (Fig. 1). If the acidic pH normally found in endosomal vesicles is disrupted by treatment of cells with NH_4Cl (37) or other reagents (38), receptor-phosphatase association is blocked and receptor dephosphorylation does not occur. Such treatments also block receptor resensitization (38). Taken altogether these findings indicate that the very same molecules that initiate receptor desensitization (β-arrestin and GRKs) also initiate the process of internalization into acidified endosomal vesicles, which is required for receptor dephosphorylation and resensitization. To date the processes involved in receptor recycling to the plasma membrane after dephosphorylation are not well understood.

Receptor Endocytosis and Mitogenic Signaling—A wide variety of GPCRs are able to activate MAP kinases such as Erk1 and -2 and in some cases to thereby effect mitogenic responses (39, 40). The mechanisms by which GPCR-mediated signals stimulate the Erks have been extensively studied. G_i-, G_q-, and G_o-mediated pathways have been described (39). The G_i- mediated signals are generally carried by the $G\beta\gamma$ subunits (39, 40), which lead to the activation of Src (or closely related tyrosine kinases) and subsequent tyrosine phosphorylation of several adaptor (*e.g.* Shc) or "scaffold" (*e.g.* epidermal growth factor receptor) proteins. This in turn leads to the recruitment of the Ras nucleotide exchange complex Grb2-mSOS to the plasma membrane. Sequential activation of Ras, Raf, MEK, and the Erks follows (reviewed in Refs. 39 and 40, and references therein).

Very recently, it has been discovered that activation of this pathway by GPCRs requires their endocytosis (41, 42). Expression of dominant negative mutants of β-arrestin or dynamin, which blocks receptor endocytosis, blocks activation of MAP kinase (42). Other mechanistically distinct inhibitors of clathrin-mediated endocytosis such as concanavalin A, hypertonic sucrose, depletion of intracellular potassium, low temperature, or monodansylcadaverine all have similar effects (41). The site of the block has been localized to the activation of MEK by Raf. Thus, in the presence of the endocytosis inhibitors, GPCR-stimulated Shc tyrosine phosphorylation and Raf activation proceed normally, yet Erk1 and -2 are not phosphorylated (42). The requirement for GPCR internalization for Erk1 and -2 activation is in striking contrast to well established paradigms for classical plasma membrane-delimited, second messenger-generating signaling pathways, such as those involving adenylyl cyclase and phospholipase C. These pathways do not require GPCR endocytosis and are unaffected by dominant negative forms of β-arrestin and dynamin (42).

Because the requirement for GPCR endocytosis is relatively downstream in the pathway leading to Erk1 and -2 activation why might internalization of the receptor itself be required? One hypothesis is that the agonist-occupied receptor organizes the assembly of a multiprotein signaling complex at the plasma membrane including some or all of the components in the pathway up to and including Raf. Activation of MEK, however, presumably requires internalization of active Raf, as part of a complex signaling particle stabilized in some way by the receptor. Although such a mechanism is conjectural at this point, what does seem clear is that GRK-catalyzed phosphorylation of the receptor and subsequent β-arrestin-mediated internalization via clathrin-coated pits are required for transduction of the MAP kinase activation signal. Interestingly,

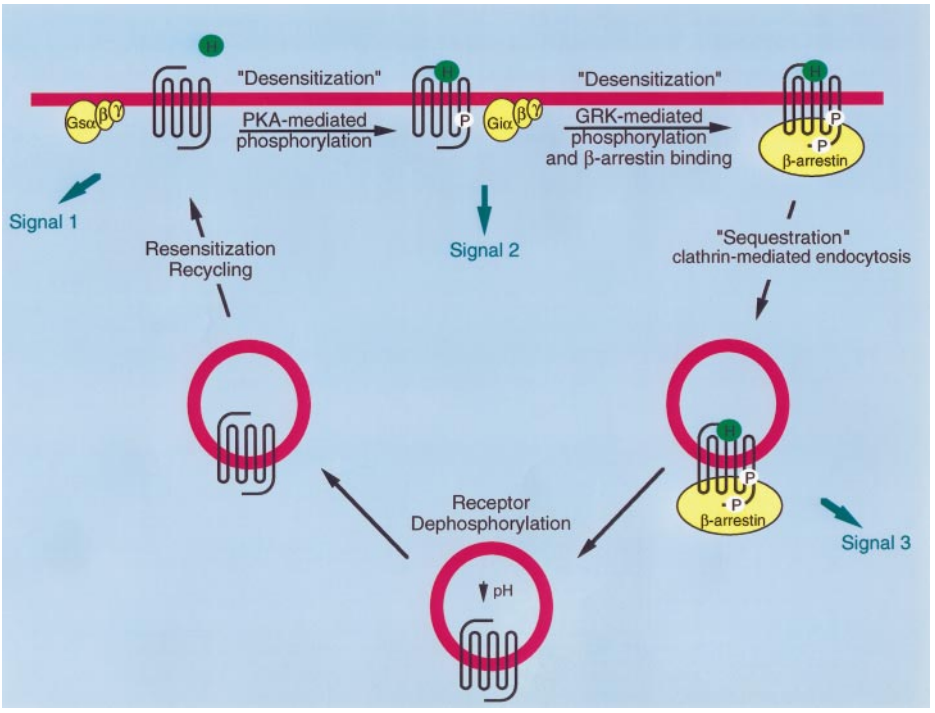

FIG. 1. **Multiple signaling roles of a GPCR illustrated for the β_2-adrenergic receptor.** Agonist occupancy of the receptor leads to activation of G_s and adenylyl cyclase (signal 1). PKA phosphorylation of the receptor uncouples it from G_s and facilitates its coupling to G_i, which inhibits adenylyl cyclase (signal 2). GRK phosphorylation of the receptor and subsequent β-arrestin binding further desensitize the receptor. β-Arrestin also mediates internalization of the receptor via clathrin-coated pits and vesicles. Internalization of the receptors is required for activation of Erk1 and -2 (signal 3) as well as for dephosphorylation and resensitization of the receptors (see text for details). Other as yet unknown signals might be generated as the receptor recycles to the plasma membrane.

recent evidence indicates that MAP kinase activation by the tyrosine kinase epidermal growth factor receptor also requires endocytosis of the receptor (43). This represents an emerging analogy between GPCR-mediated and tyrosine kinase receptor-mediated mitogenic signaling mechanisms (39). It should be stressed that the involvement of GRKs and β-arrestins in GPCR-mediated mitogenic signaling places these two "desensitizing" proteins squarely in the role of important "signaling" molecules (Fig. 1).

The cAMP-dependent Protein Kinase and Signal Switching—As described above, second messenger-activated kinases such as PKA and PKC have been shown to desensitize GPCRs in a feedback regulatory fashion. Recent findings, however, suggest that this view may be too limited and that in some cases "signal switching" rather than signal desensitization may be a more appropriate description. It has long been appreciated that many GPCRs can couple to multiple G proteins, although often one pathway predominates over another. In the case of the β_2-adrenergic receptor, for example, most of its actions appear to be mediated by coupling to G_s and activation of adenylyl cyclase. However, in some settings such as the heart, β_2-adrenergic receptors have been shown to interact with G_i as well (44). What had not been appreciated until quite recently, however, is the role of PKA-catalyzed phosphorylation of the receptors in controlling the specificity of G protein coupling.

When expressed in HEK 293 cells, β_2-adrenergic receptors activate Erk1 and -2 by the $G_i\beta\gamma$-mediated, Ras-dependent pathway described above (45). The response is blocked by pertussis toxin or reagents that sequester $G\beta\gamma$. However, in contrast with the actions of more "classical" G_i-coupled receptors such as that for lysophosphatidic acid, β-adrenergic stimulation of the Erks is also blocked by the PKA inhibitor H89 (45). Moreover, a mutated β_2-adrenergic receptor in which the two consensus PKA phosphorylation sites have been removed fails to activate MAP kinase, although it is normal with respect to mediating activation of adenylyl cyclase. In a direct assay of receptor-G protein interaction, the ability of agonist-occupied β_2-adrenergic receptors to catalyze GTP-GDP exchange on G_i was blocked by H89. These findings indicate that in order for the receptors to couple productively to G_i they must first interact with G_s, thereby inducing PKA activation and phosphorylation of the receptors (45). The findings are entirely consistent with earlier observations of Nishimoto and co-workers (46). They found that a small peptide derived from the third cytoplasmic loop of the β_2-adrenergic receptor (which contains the PKA phosphorylation site) could directly activate G_s but not G_i *in vitro*. When phosphorylated by PKA, coupling to G_s was diminished and that to G_i increased. Because coupling of β_2-adrenergic receptors to G_i leads to inhibition of adenylyl cyclase, this switching mechanism can be viewed as yet another aspect of the overall desensitization process that is engaged to blunt the G_s-mediated stimulation of adenylyl cyclase. However, given the ability of G_i to couple the receptor to entirely distinct pathways (*e.g.* MAP kinase activation), it is perhaps appropriate to frame this as a more general mechanism for switching the coupling of receptors from one G protein to another (Fig. 1). Whether this mechanism also operates in PKC-mediated signaling pathways remains to be determined.

Novel Substrates for GRKs—It has been thought that the only substrates for GRKs are the GPCRs themselves. However, quite recently it was discovered that tubulin is an excellent substrate as well (47). *In vitro*, K_m and V_{max} for tubulin phosphorylation by GRK2 are essentially equivalent to the values obtained for the agonist-occupied β_2-adrenergic receptor. In fact, GRK2 appears to account for most of the endogenous "tubulin kinase" present in tissues. In cells, stimulation of GPCRs leads to increased tubulin association with GRK2 and increased tubulin phosphorylation. GRK2 can also be observed to decorate cellular microtubules as assessed by immunofluorescence microscopy (47). At present, the functional consequences of this phosphorylation are unknown.

It seems likely that there may well be other non-receptor substrates for GRKs. A provocative ramification of the existence of such substrates relates to an interesting feature of the regulation of GRKs. As noted above, it has been demonstrated for both GRK1 and GRK2 that interaction of the GRK with its activated receptor substrate leads to allosteric activation of the kinase (14, 15). Thus, once having bound to a receptor, the kinase is activated as well with respect to other substrates, *e.g.* tubulin. Although completely speculative at present, these considerations raise the possibility that GRKs might function directly as signaling elements representing essentially agonist-activated kinases. The downstream components of such putative signaling cascades remain to be determined.

Future Perspectives and Speculation—To date the short list of proteins shown to interact directly with GPCRs, in an agonist-dependent fashion, includes heterotrimeric G proteins, β-arrestins, and GRKs. One might speculate that the ability of these proteins to interact with the receptors in a fashion directly controlled by agonists situates them ideally to function as signaling molecules. In the case of GRKs, as noted above, this simply requires that there be non-receptor GRK substrates, such as tubulin. In the case of β-arrestin, perhaps they function as adaptors linking the agonist-occu-

pied receptors to downstream signaling elements. The recently discovered role of β-arrestins in receptor internalization demonstrates how the β-arrestins can act as bifunctional molecules linking the receptors to other cellular molecules. It will be interesting to see what other such adaptor functions might be identified for the β-arrestins.

Taken together, the information summarized here suggests that current concepts of GPCR signaling and desensitization are continuing to evolve. Molecules previously viewed as being exclusively involved in receptor desensitization turn out to play crucial roles in receptor signaling (Fig. 1). Increasingly, the various processes included under the umbrella of "receptor desensitization" are revealed instead to function as coordinated molecular switches turning on new signaling pathways even as they turn off others.

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