

Using green fluorescent protein to understand the mechanisms of G-protein-coupled receptor regulation

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Abstract

G protein-coupled receptor (GPCR) activation is followed rapidly by adaptive changes that serve to diminish the responsiveness of a cell to further stimulation. This process, termed desensitization, is the consequence of receptor phosphorylation, arrestin binding, sequestration and down-regulation. GPCR phosphorylation is initiated within seconds to minutes of receptor activation and is mediated by both second messenger-dependent protein kinases and receptor-specific G protein-coupled receptor kinases (GRKs). Desensitization in response to GRK-mediated phosphorylation involves the binding of arrestin proteins that serve to sterically uncouple the receptor from its G protein. GPCR sequestration, the endocytosis of receptors to endosomes, not only contributes to the temporal desensitization of GPCRs, but plays a critical role in GPCR resensitization. GPCR down-regulation, a loss of the total cellular complement of receptors, is the consequence of both increased lysosomal degradation and decreased mRNA synthesis of GPCRs. While each of these agonist-mediated desensitization processes are initiated within a temporally dissociable time frame, recent data suggest that they are intimately related to one another. The use of green fluorescent protein from the jellyfish *Aequora victoria* as an epitope tag with intrinsic fluorescence has facilitated our understanding of the relative relationship between GRK phosphorylation, arrestin binding, receptor sequestration and down-regulation.

Key words

- Arrestin
- Endocytosis
- Green fluorescent protein
- G protein-coupled receptors
- G protein-coupled receptor kinases
- Resensitization

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Introduction

G protein-coupled receptors (GPCRs) form the largest family of integral membrane receptors. These receptors are seven trans-membrane-spanning proteins that respond to a wide variety of stimuli including light, odour, taste, hormones and neurotransmitters. Activation of a GPCR results in the modulation of intracellular second messenger levels and/or ionic conductances via the coupling of receptors to a wide variety of effector systems via heterotrimeric guanine

nucleotide-binding proteins (G proteins). Agonist activation of a GPCR induces the isomerization of the receptor to a "high-affinity" agonist-binding conformation catalyzing the exchange of GDP for GTP on the G protein α -subunit (1). This exchange of GDP for GTP allows the dissociation of the G_{α} -subunit from the $G_{\beta\gamma}$ -subunits, which when dissociated from one another regulate the activity of effector systems such as adenylyl cyclase and potassium channels.

Shortly following exposure to an agonist, GPCR responsiveness wanes as the conse-

quence of receptor desensitization. Receptor desensitization plays an important physiological role by preventing overstimulation of GPCR signaling cascades which in the case of constitutively active mutant receptors leads to diseases such as retinal degeneration, precocious puberty and thyroid tumors (2-4). Moreover, receptor desensitization provides a mechanism by which GPCR-mediated signals from multiple cellular or hormonal inputs can be filtered into meaningful information. However, the up-regulation of proteins associated with GPCR desensitization has also been associated with diseases such as hypertension, congestive heart failure and drug dependence and tolerance (5-8). Consequently, a firm understanding of how the processes of receptor activation, desensitization and resensitization are related to one another is critical to the development of strategies aimed at ameliorating GPCR-related diseases. The present report overviews the current understanding of GPCR regulatory mechanisms and how green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is facilitating this understanding.

Receptor desensitization

Phosphorylation

The desensitization of GPCR responsiveness results as the culmination of several events including: the uncoupling of receptors from their heterotrimeric G protein as the consequence of receptor phosphorylation, sequestration (endocytosis) of receptors to endosomes and down-regulation of the total cellular complement of receptors. While each of these processes is initiated by the exposure of receptors to agonist, they proceed at different rates and can be dissociated on this basis. Receptor/G protein uncoupling occurs in response to phosphorylation by both second messenger-dependent protein kinases (e.g., cAMP-dependent protein kinase, PKA) and receptor-specific G protein-coupled receptor

kinases (GRKs) (9-11). GRK-phosphorylation of GPCRs occurs within seconds to minutes following the activation of a receptor by the agonist and promotes the binding of arrestin proteins. Arrestins when bound to receptors sterically interdict receptor/G protein interactions (12-15). Second messenger-dependent protein kinase-mediated phosphorylation of GPCRs does not promote arrestin protein binding; rather, it is the second messenger-dependent protein kinase-mediated phosphorylation event itself that results in receptor desensitization. For example, in the case of the β_2 -adrenergic receptor (β_2 AR), phosphorylation of a PKA consensus site in the third intracellular loop of this receptor is thought to sterically prevent the interactions between the receptor and its G protein (9). Both second messenger-dependent protein kinases and GRKs contribute to the agonist-dependent desensitization of GPCRs. However, unlike GRKs, second messenger-dependent protein kinases exhibit the capacity (when activated) to indiscriminately phosphorylate receptors that have not been exposed to the agonist (9-11).

The GRK protein family of Ser/Thr kinases consists of six members which can be subclassified according to both sequence homology and functional similarity (9-11,16): 1) rhodopsin kinase (GRK1), 2) β -adrenergic receptor kinase 1 (β ARK1 or GRK2) and β ARK2 (GRK3), 3) the GRK4 subfamily, comprised of GRK4, GRK5 and GRK6. In unstimulated cells GRK1, GRK2 and GRK3 are primarily localized to the cytosol and translocate to their agonist-activated plasma membrane-bound receptor targets. In contrast, GRK4, GRK5 and GRK6 exhibit substantial membrane localization in the absence of agonist activation of GPCRs. GRKs phosphorylate GPCRs at serine and threonine residues residing within the carboxyl-terminal tail (e.g., β_2 AR) or third intracellular loop (e.g., m2 muscarinic acetylcholine receptor, m2 mAChR). The GRK protein structure can be subdivided loosely into three

functional domains: an amino-terminal domain that is thought to be important for substrate recognition, a central catalytic domain and a variable carboxyl terminal domain critical for plasma membrane targeting (9,16). Several distinct mechanisms for membrane targeting are utilized by GRKs. These mechanisms include farnesylation of a carboxyl-terminal CAAX motif (GRK1), pleckstrin homology (PH) domain interactions with G protein β_γ -subunits (GRK2 and GRK3), palmitoylation of cysteine residues (GRK4 and GRK6) and electrostatic interactions between highly basic amino-terminal amino acids and membrane phospholipids (17-20). Recently, a combination of biochemical techniques used in conjunction with a GFP-tagged GRK2 has identified tubulin as a novel GRK substrate (21).

Sequestration

Receptor sequestration is initiated within the same time-course as phosphorylation but proceeds somewhat slower. The agonist-promoted sequestration of GPCRs to a "light-vesicular" or "endosomal" pool of membranes is also thought to contribute to the temporal desensitization of GPCRs by removing receptors to a compartment inaccessible to the agonist. However, the sequestration of receptors is not absolutely required for the manifestation of GPCR desensitization since treatments that block the endocytosis of GPCRs do not prevent GPCR desensitization (22,23). Rather, receptor sequestration appears to play a more important role in the resensitization of GPCR responsiveness. This idea arose from the following observations: 1) after agonist stimulation β_2 ARs isolated from the light vesicular pool of membranes exhibit lower stoichiometry of phosphorylation (24), 2) pharmacological treatments that block receptor endocytosis and recycling also block receptor resensitization (22,23) and 3) sequestration-defective mutants that desensitize but do not

resensitize have been identified (25). Consequently, receptor sequestration is best described as a process involving the internalization of GPCRs to an endosomal compartment where they become dephosphorylated and recycled back to the plasma membrane in their pre-ligand exposed state. The precise endocytic mechanism by which GPCRs endocytose remains controversial as both clathrin-dependent and -independent mechanisms have been described. However, in the case of the β_2 AR, the prominent endocytic mechanism in many cell lines appears to be via clathrin-coated vesicles (26).

While the molecular mechanisms involved in the phosphorylation-dependent desensitization of GPCR responsiveness have been fairly well delineated, until recently, the identification of the molecular intermediates involved in GPCR endocytosis and resensitization has been less forthcoming. Surprisingly, it was found that the same proteins contributing to the desensitization of GPCR responsiveness, GRKs and β -arrestins, also contribute to GPCR sequestration (26-32). The involvement of GRK-mediated phosphorylation in GPCR sequestration was first demonstrated for the m2 mAChR (27). These results initially conflicted with earlier data obtained using β_2 ARs lacking their putative sites for both GRK- and PKA-mediated phosphorylation which sequestered normally in Chinese hamster ovary cells (33). However, using a β_2 AR-Y326A mutant, which neither sequestered nor resensitized, a role for GRK-mediated phosphorylation in the sequestration of the β_2 AR was also established (28). Moreover, it was shown that in human embryonic kidney (HEK 293) cells the sequestration of a β_2 AR lacking putative sites for GRK- but not PKA-mediated phosphorylation was impaired by 50% (28). Nonetheless, the observation that β_2 ARs lacking putative sites for phosphorylation still exhibited a capacity for sequestration indicated that, while GRK-mediated phosphorylation facilitated the sequestration of the β_2 AR, it

was not absolutely required. Rather, GRK-mediated phosphorylation promoted the binding of β -arrestins which are absolutely required for β_2 AR sequestration (29). β -Arrestins bind to GPCRs, uncouple them from their cognate heterotrimeric G protein and target them specifically for dynamin-dependent clathrin-coated vesicle-mediated endocytosis (26). The targeting of β -arrestin-bound receptors to coated pits appears to involve an interaction between the carboxyl-terminal domains of β -arrestin1 and β -arrestin2 with the globular head of the clathrin-heavy chain (34-36). The other two members of the arrestin family, visual arrestin and cone arrestin, appear to lack the clathrin-binding motif and therefore do not support GPCR endocytosis (34).

Down-regulation

Receptor down-regulation involves a loss in the total cellular complement of particular GPCR in response to prolonged or repeated agonist stimulation. Down-regulation occurs as the consequence of both increased lysosomal degradation of pre-existing receptors and reduced mRNA and protein synthesis (37-40). PKA not only contributes to GPCR desensitization by phosphorylating receptors, but in the case of β_2 AR, PKA activation leads to a decrease in steady-state β_2 AR mRNA levels (38). It is uncertain whether receptor sequestration represents the initial step in the lysosomal degradation of GPCRs following long-term agonist exposure. It is possible that, while the majority of sequestered receptors are recycled back to the plasma membrane, a small fraction of the internalized receptors are sorted in endosomes for degradation in lysosomes (41). This hypothesis is supported by a recent study indicating that the overexpression of β -arrestin sequestration dominant-negatives reduces the rate of β_2 AR down-regulation (42). Alternatively, GPCRs are targeted for degradation via a distinct endocytic path-

way. Evidence for a distinct pathway comes from the mutation studies where receptor mutants have been identified which do not sequester, but down-regulate more efficiently (25) as well as receptor mutants that do not down-regulate but sequester normally (43). Moreover, tyrosine residues located in the carboxyl-terminal tail of the β_2 AR have been implicated in receptor down-regulation but not sequestration (37).

Use of green fluorescent protein to study GPCR regulation

A great deal of knowledge has accumulated concerning the mechanisms by which GPCRs signal and desensitize. However, by virtue of the nature of the membrane-delimited environment in which GPCRs reside, their study has required laborious biochemical techniques. The introduction of foreign epitope tags into the cDNAs encoding GPCRs has facilitated the study of both GPCR phosphorylation and endocytosis. This has been particularly important for receptor systems for which good radio-ligands and antibodies have not been available or are prohibitively expensive. The recent introduction of the jellyfish *Aequora victoria* GFP for use as an autofluorescent epitope tag (44) has allowed the visualization of GPCRs and their regulatory proteins in real-time with a precision not afforded by fluorescent antibody staining of fixed cells. The ability of GFP to allow real-time visualization of proteins in living cells now allows us to effectively assess how both agonist and/or the overexpression of dominant-negative proteins affect changes in the dynamic distribution of both GPCRs and their regulatory proteins.

Receptor-GFP conjugates

To date GFP has been used to epitope tag three distinct GPCRs, the cAMP chemoattractant protein receptor (CAR1) in *Dictyostelium discoideum*, the β_2 AR and the chole-

cystokinin type A receptor (CCKAR) (45-48). These receptor-GFP conjugates have been used to begin to answer several questions that cannot be adequately addressed by immunofluorescence in fixed cell preparations. Xiao et al. (45) examined the distribution of CAR1 in living cells undergoing cAMP-directed migration. Time-lapse imaging of individual cells showed that CAR1 remains evenly distributed on the cell surface of a cell and all its projections during chemotaxis. Moreover, pseudopods at migration fronts showed transiently reduced fluorescent signals suggesting that the movement of the receptors into these zones lagged the protrusion process. The addition of GFP to the carboxyl-terminal tail of the β_2 AR results in a receptor that is fully functional with respect to all its measurable parameters (e.g., G protein-coupling, receptor sequestration and agonist-dependent phosphorylation) and in fact appears to be better coupled to adenylyl cyclase than the wild-type receptor (46). Using the GFP-tagged β_2 AR, we showed that the diffusion rate for the β_2 AR is consistent with the free diffusion rates found for other membrane proteins as the low density lipoproteins, indicating that their diffusibility following agonist-mediated β -arrestin desensitization would not be a limiting step in receptor endocytosis (46). Moreover, it was found that in living cells the β_2 AR-GFP conjugate internalized to endosomes and that within 15 min could be found in more complicated structures formed from the fusion of multiple endosomes. Perhaps the most interesting data generated using a GFP-tagged GPCR come from studies of a CCKAR-GFP conjugate (49). When expressed in different cell lines this receptor exhibited different properties of endocytosis. In CHO, COS-1 and HeLa cells the CCKAR-GFP internalizes in an agonist-dependent manner, whereas in NIH3T3 the receptor constitutively endocytoses. The constitutive endocytosis of the receptor could be prevented by antagonist treatment of the cells. Interest-

ingly, unlike the β_2 AR, the CCKAR-GFP internalizes to endosomes but is not targeted to lysosomes for degradation (46-48). The apparent differences in the compartmentalization of the CCKAR and the β_2 AR highlight the utility of GFP as a tool to pinpoint receptor-specific differences in GPCR regulation.

GPCR regulatory protein-GFP conjugates

In addition to coupling GFP to GPCRs, it has been possible to couple GFP to both GRK2 and β -arrestin2 without affecting their normal function as GPCR regulatory proteins (21,49). The conjugation of GFP to the carboxyl-terminus of β -arrestin2 has greatly facilitated our ability to assess both the normal distribution of β -arrestin2 in cells and the redistribution of β -arrestin2 to the plasma membrane in response to agonist activation (49). β -arrestin2 GFP not only supported β_2 AR endocytosis, but was easily observed to redistribute in a time- and agonist dose-dependent manner consistent with the pharmacological characteristics of β_2 AR sequestration (49; Ferguson SSG and Caron MG, unpublished observations). Moreover, β -arrestin2 GFP translocation was GRK-dependent (49). Because both GRK-mediated phosphorylation and β -arrestin binding to GPCRs represent a divergent step in the GPCR signaling and desensitization cascades, this β -arrestin2 GFP translocation assay represents a potentially powerful universal methodology for screening orphan receptors, GRK inhibitors and novel GPCR ligands. An additional advantage of β -arrestin2 GFP is that this technique offers the potential to examine: 1) the agonist specificity of receptor β -arrestin/receptor interactions, 2) whether β -arrestins endocytose with receptors in endocytic vesicles, 3) how drug treatments affect β -arrestin/receptor interactions and 4) the mechanisms regulating the dissociation of the GPCR/ β -arrestin endocytic complex.

Conclusion

The dual function of GRKs and arrestins in the regulation of both the desensitization and resensitization of GPCRs highlights the complexity of the GPCR signal transduction system. It is now clear that GPCR regulatory processes that were traditionally considered to be mediated by distinct mechanisms have now been shown to exhibit considerable overlap. This is particularly true of β -

arrestins which when overexpressed re-establish the resensitization phenotype of mutant receptors impaired in their ability to resensitize (50). The use of both GPCR- and β -arrestin-GFP constructs to examine the effects of both agonist and antagonists on the activity and intracellular processing of GPCRs is expected to contribute to the development of new strategies to treat diseases associated with perturbations in GPCR function.

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