1521-0111/101/3/144–153$35.00
MOLECULAR PHARMACOLOGY

dx.doi.org/10.1124/molpharm.121.000429
Mol Pharmacol 101:144–153, March 2022

Minireview

Roles of Receptor Phosphorylation and Rab Proteins in G Protein-Coupled Receptor Function and Trafficking

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Received October 10, 2021; accepted December 22, 2021

ABSTRACT

The G protein-coupled receptors form the most abundant family of membrane proteins and are crucial physiologic players in the homeostatic equilibrium, which we define as health. They also participate in the pathogenesis of many diseases and are frequent targets of therapeutic intervention. Considering their importance, it is not surprising that different mechanisms regulate their function, including desensitization, resensitization, internalization, recycling to the plasma membrane, and degradation. These processes are modulated in a highly coordinated and specific way by protein kinases and phosphatases, ubiquitin ligases, protein adaptors, interaction with multifunctional complexes, molecular motors, phospholipid metabolism, and membrane distribution. This review describes significant advances in the study of the regulation of these receptors by phosphorylation and endosomal traffic (where signaling can take place); we revisited the bar code hypothesis and include two additional observations: 1) that different phosphorylation patterns seem to be associated with internalization and endosome sorting for recycling or degradation, and 2) that, surprisingly, phosphorylation of some G protein-coupled receptors appears to be required for proper receptor insertion into the plasma membrane.

SIGNIFICANCE STATEMENT

G protein-coupled receptor phosphorylation is an early event in desensitization/signaling switching, endosomal traffic, and internalization. These events seem crucial for receptor responsiveness, cellular localization, and fate (recycling/degradation) with important pharmacological/therapeutic implications. Phosphorylation sites vary depending on the cells in which they are expressed and on the stimulus that leads to such covalent modification. Surprisingly, evidence suggests that phosphorylation also seems to be required for proper insertion into the plasma membrane for some receptors.

Introduction

G protein-coupled receptors (GPCRs) are among the most abundant membrane protein families and comprise approximately 3–5% of protein-encoding genes in different sequenced genomes (Schiöth and Fredriksson, 2005). These receptors are structurally constituted of seven hydrophobic transmembrane α-helixes, connected by three intracellular loops and three extracellular loops; the amino terminus is located in the extracellular side, whereas the carboxyl terminus is located intracellularly (Kobilka, 2013; Lefkowitz, 2013). An amphipathic α-helix is found in the carboxyl terminus of many GPCRs and is frequently denominated helix 8; it has been shown that it allows maintaining the surface expression of these receptors and promotes their intracellular traffic (Huynh et al., 2009; Kirchberg et al., 2011; Zhu et al., 2015).

These receptors exert most of their best-characterized effects through interaction with G proteins, which are heterotrimeric GTPases constituted of α, β, and γ subunits, although interaction with other signaling entities, particularly β-arrestins, is known to occur (discussed ahead). Active GPCRs function as guanine nucleotide exchange factors, i.e., GPCR-agonist interaction sparks conformational changes that permit the intimate interaction of these receptors with heterotrimeric G proteins (Kobilka, 2013), leading to the exchange of GDP for GTP in the α subunits and the dissociation of the β/γ dimers (active state). The GTP-loaded G protein α subunits and the β/γ dimers modulate the activity of effector proteins such as enzymes (i.e., adenylyl cyclase, phospholipase C, or cyclic GMP

This work was partially supported by a grant from Consejo Nacional de Ciencia y Tecnologia (Fronteras 6676) and DGAPA [IN201221].
dx.doi.org/10.1124/molpharm.121.000429.

ABBREVIATIONS: ERK 1/2, extracellular signal-regulated kinases 1/2; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase.

phosphodiesterase, among others) and ion channels, modifying the intracellular concentrations of second messengers and ions, initiating and amplifying the intracellular propagation of the signal

GPCR Desensitization and Posttranslational Modifications Involved

GPCR signaling is modulated by different mechanisms, including the desensitization and resensitization processes (Ferguson, 2001; Hausdorff et al., 1990; Lefkowitz, 1998; Zhao et al., 2021). Desensitization is generally defined as a diminished cellular response to continuous or repetitive exposure to an agent. This is usually associated with pharmacological stimulation by synthetic agonists. Nonetheless, it is a physiologic process that occurs continually in organisms. Consider, for example, the adjustment of our senses, such as vision, smell, or taste, and how they rapidly and dynamically adapt to different illuminations, the presence of odors in the environment, or tastants in our foods. Desensitization involves many processes with different time courses, from rapid (minutes) diminutions of responsiveness to much more prologued effects (hours or days). This review will be focused mainly on the initial phase of this process. Some aspects of the acute and long-term phases of desensitization have been recently reviewed (Rajagopal and Shenoy, 2018). Operationally, this process has been divided into two different types: homologous, i.e., that induced by ligand activation of the desensitized receptor, and heterologous, i.e., that caused by agents unrelated to the affected receptor (i.e., activation of distinct receptors or signaling pathways; in other words, taking place in an unoccupied, agonist-free, receptor). Resensitization is the return to baseline conditions involving different processes with distinct mechanisms and time courses.

GPCRs are subject to different posttranslational modifications, including, among others, N- and O-glycosylation, tyrosine sulfation, proteolysis, SUMOylation, ubiquitination, palmitoylation, and phosphorylation [reviewed in (Cottrell, 2013; Goth et al., 2020; Han and Jiang, 2022; Patwardhan et al., 2021; Trudel et al., 2016; Xu et al., 2019)]. Of these modifications, three have been studied in some detail due to their reversibility and the possibility of regulating these receptors: palmitoylation, ubiquitination, and phosphorylation. Palmitoylation frequently occurs by forming thioester bonds with two adjacent cysteines in the GPCR carboxyl terminus, possibly creating a fourth intracellular loop. Although palmitoylation has been studied in various GPCRs, its functional roles are far from entirely known and vary among different receptors. Some authors have observed little effect of agonists on GPCR palmitoylation using mass spectrometry (Trester-Zedlitz et al., 2005). Nevertheless, there is evidence indicating that palmitoylation is essential for the proper insertion of some receptors into the plasma membrane and their signaling and could have distinct effects on receptor internalization (Charest and Bouvier, 2003; Chini and Parenti, 2009; Ohno et al., 2009; Patwardhan et al., 2021; Qanbar and Bouvier, 2003). Ubiquitin is a polypeptide of $\approx$ 8 kDa transferred by a complex of three enzymatic activities (E1, E2, and E3) to the ε-amino groups of lysine residues in proteins, including GPCRs. There is clear evidence indicating that ubiquitination is involved in GPCR trafficking and degradation, although this appears to vary in different receptors and the cellular context in which they are expressed (Jean-Charles et al., 2016b; Patwardhan et al., 2021; Sarker et al., 2011; Shenoy et al., 2001). In addition, the possibility that this modification could be involved in signaling, particularly in endosomal signaling, has also been suggested [(Burton and Grimsey, 2019; Sarker et al., 2011; Skieterska et al., 2017) and references therein]. Ubiquitination seems to take place after receptor phosphorylation and receptor-β-arrestins association occurs (Jean-Charles et al., 2016a). It is noteworthy that crucial players in GPCR phosphorylation [i.e., G protein receptor kinase (GRK) 2 (Salcedo et al., 2006)] and signaling [i.e., β-arrestin, itself (Jean-Charles et al., 2016a)] are also subjected to ubiquitination.

Receptor Phosphorylation, the Barcode Hypothesis, and β-Arrestins

GPCR phosphorylation is an early event in receptor desensitization. It seems to be the main factor in desensitization (or signaling switching, i.e., G protein-mediated to G protein-independent pathways) and GPCR internalization. Since the early 1980s, phosphorylation of GPCRs (Stadel et al., 1983) was associated with this process. Different groups have extensively studied this using distinct GPCRs and methodologies, and it continues to be considered the earliest posttranslational modification related to receptor desensitization and internalization. Nevertheless, it should be mentioned that desensitization can occur in the absence of GPCR phosphorylation (Ferguson, 2007).

GPCRs are phosphorylated by a variety of protein kinases. It is generally accepted that during homologous desensitization, GPCRs are phosphorylated by a family of protein kinases known as GRKs, comprising seven members (GRK1–7). Some of these are expressed predominantly in specific tissues, such as those present in the retina [visual GRK (i.e., GRK1 and GRK7)], or GRK4, which is found mainly in the testis, whereas others (GRK2, -3, -5, and -6) are ubiquitously expressed. These protein kinases contain a central conserved catalytic domain and variable amino and carboxyl termini, which appears to confer on them selectivity in their action and to participate in their regulation (Gurevich and Gurevich, 2019a; Moore et al., 2007; Ribas et al., 2007; Sterne-Marr et al., 2004; Watari et al., 2014).

During heterologous desensitization, receptor phosphorylation is catalyzed by protein kinases, many of these belonging to the AGC family, which includes more than 60 different members, including second messenger-activated kinases (such as protein kinases A and C isoforms), and many others that are also involved in signaling (Arencibia et al., 2013; Pearce et al., 2010). It should be mentioned that considering homologous desensitization as exclusively mediated by GRK and heterologous desensitization as mediated only by other protein kinases is likely a gross oversimplification. There is intense crosstalk between different protein kinases (Elorza et al., 2000; Ribas et al., 2007), and there is evidence that even in agonist-induced receptor phosphorylation, other protein kinases and even transactivation of a different type of receptor could be involved (see for example Casas-González and García-Sáinz, 2006; García-Sáinz et al., 2011).

It has been shown that GPCR phosphorylation favors association with β-arrestins; receptor interactions with these interesting proteins are involved in different roles in signaling and internalization. It soon became apparent that receptor binding to β-arrestins plays two initial functions: 1) that of sterically impeding productive receptor–G protein interaction [exceptions exist; see below (Thomsen et al., 2016)], and 2) that of recruiting the endocytic machinery via association with clathrin and the clathrin adaptor, AP2 (Fessart et al., 2005; Goodman et al., 1996; Laporte et al., 2000; Laporte et al., 1999), as depicted in Fig. 1. Many GPCRs internalize, with or without the ligand, into clathrin-coated vesicles, where they interact with different types of endosomes and are subjected to complex traffic and eventually are recycled back to the plasma membrane or proteolyzed in the lysosomes or by the proteasome (Dores and Trejo, 2019; Ferguson, 2001; Goodman et al., 1996; Jean-Charles et al., 2016b; Lefkowitz, 1998; Lefkowitz, 2013; Penela et al., 2001; Rajagopal and Shenoy, 2018).

Interestingly, the role of β-arrestin is not limited to these two functions. Studies by the group of Lefkowitz demonstrated that β-adrenoceptor activation stimulates the mitogen-activated protein kinases, such as extracellular signal-regulated kinase 1/2 (ERK 1/2), and that this effect requires receptor endocytosis, as evidenced by the participation of β-arrestins and dynamin (reviewed in Lefkowitz, 1998). These provocative data indicated that GPCR association with β-arrestin not only desensitizes (arrests) G protein-mediated signaling but also induces a "signaling switch" turning on distinct pathways, i.e., the β-arrestin-mediated actions (Fig. 1).

It should be mentioned that it was observed that GPCR–β-arrestin interaction showed at least two variations: 1) some receptors (named Class A, such as β_2-adrenergic, μ opioid, endothelin type A, dopamine D_{1A}, and α_{1B}-adrenergic, among many others) bind β-arrestins transiently and internalize, but recycle to the plasma membrane and resensitize rapidly; 2) in contrast, other receptors (named Class B, such as the angiotensin AT_{1a} and the vasopressin V_2 receptors, among others) bind β-arrestins for more extended periods, also internalize, but recycle and resensitize slower (Oakley et al., 1999; Oakley et al., 2000; Tohgo et al., 2003). Interestingly, the sequences that define the interaction stability with the β-arrestins are present in the carboxyl terminus of these receptors (Oakley et al., 1999; Oakley et al., 2000; Tohgo et al., 2003). Studies on other receptors have confirmed these results, observing that phosphorylated residues and acidic amino acids participate in such interactions. In a study employing the crystal structure of the rhodopsin-arrestin complex, the authors validated a series of phosphorylated codes that could represent a common mechanism for recruitment of β-arrestin by GPCRs (Zhou et al., 2017).

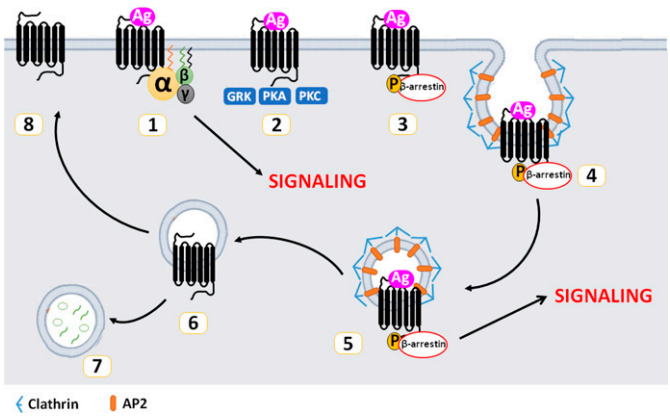

Fig. 1. GPCR desensitization. (1) GPCR activation by agonist (Ag) leads to G protein activation and signaling. (2) Subsequently, GPCR phosphorylation by distinct GPCR kinases (GRK) or protein kinases A (PKA) or C (PKC) takes place. (3) Phosphorylated receptors interact with β-arrestins, leading to (4) the endocytic machinery's recruitment and receptor endocytosis. (5) The GPCRs in endosomes signal through β-arrestins. (6) GPCRs can be (7) degraded in the lysosomes and/or the proteasome or (8) recycled back to the plasma membrane.

Interestingly, these authors found that phosphorylation codes that putatively promote β-arrestin binding exist in GPCR subfamilies mainly in the carboxyl terminus but also in the intracellular loop 3; many of those GPCRs contain more than one of these putative binding sites for β-arrestins, indicating that intimate multisite interactions can exist (Zhou et al., 2017). It has been shown that a single receptor can simultaneously bind both through its core region and its carboxyl terminus to G proteins; in these cases, GPCR binding to β-arrestins does not impede G protein interaction, which provides a potential physical basis for a newly appreciated sustained G protein signaling from internalized GPCRs (Thomsen et al., 2016). Interestingly, recent evidence suggests that such internalized signaling complexes might also include effectors such as some isoforms of adenylyl cyclase (Lazar et al., 2020). These authors showed trafficking of adenylyl cyclase from the plasma membrane to endosomes; this was apparently selective for isoform 9 because isoform 1 remains in the plasma membrane. (Lazar et al., 2020), Adenylyl cyclase 9 trafficking was triggered by ligand-induced activation of Gs-coupled GPCRs, and they transit through a similar dynamin-dependent early endocytic pathway; however, unlike GPCR traffic, which requires β-arrestin but not Gs, adenylyl cyclase 9 traffic requires Gs but not β-arrestin (Lazar et al., 2020).

Arrestins are ≈45 kDa proteins that participate in many functions (Gurevich and Gurevich, 2019b). They can interact with many different proteins and are, in many cases, associated with gene expression, proliferation, or differentiation (Gurevich and Gurevich, 2019b and references therein). Four subtypes of these proteins are present in the majority of vertebrates, two visual arrestins (arrestins 1 and 4) and the non-visual arrestins, frequently named β-arrestins 1 and 2 (or arrestins 2 and 3, respectively) (reviewed in Gurevich and Gurevich, 2019a; Gurevich and Gurevich, 2019b; Smith and Rajagopal, 2016). Much effort has been devoted to differentiating GPCR actions mediated through G proteins from those mediated through β-arrestins (Gurevich and Gurevich, 2019a; Gurevich and Gurevich, 2019b; Gurevich and Gurevich, 2020). Based on such a dichotomy, efforts have been made to explain GPCR-biased agonism (i.e., the ability of some agonists to preferentially exert some effects over others). However, results have been controversial and challenging to interpret. For example, in a very elegant paper (Alvarez-Curto et al., 2016) employing cells with targeted elimination of Gq/11 proteins and arrestins, the authors detected a new interplay of signaling pathways, where receptor phosphorylation can impact ERK 1/2 signaling through a mechanism apparently independent of arrestins. It should be considered that under the action of agonists, GPCRs adopt different conformations (Kahsai et al., 2011; Kobilka, 2013), which might affect their interaction with distinct G proteins,

exposing or hiding phosphorylatable residues. Such phosphorylations at separate sites might affect β-arrestin binding affinity and conformation, both of which seem to be very versatile (Yang et al., 2015; Zhou et al., 2017); such conformational changes could lead to distinct functional outcomes (Gurevich and Gurevich, 2018; Gurevich and Gurevich, 2019a; Gurevich and Gurevich, 2020). Therefore, it seems likely that biased agonism might result from different interrelated signaling events.

As indicated, β-arrestin binding to phosphorylated GPCRs facilitates interaction with the AP-2 adaptor, receptor integration in clathrin-coated vesicles, and receptor internalization. Evidence suggests that some GPCRs signal through β-arrestins from both the plasma membrane and endosomes and that the relative magnitudes of such different processes vary among receptors (Eichel and von Zastrow, 2018). This raises the question of whether more than one mechanism of endosomal G protein activation by GPCRs exists and the functional consequences of altering the location and timing of specific receptor-mediated signaling reactions (Eichel and von Zastrow, 2018; Lobingier and von Zastrow, 2019; Wright et al., 2021). It should be indicated that these processes do not involve only the β-arrestin-ERK 1/2 signaling pathway. In an exciting contribution by Vilardaga's group (White et al., 2021), the authors showed that parathyroid hormone receptor-induced cyclic AMP production encodes distinct biologic outcomes. They engineered a biased parathyroid ligand that elicits cyclic AMP production at the plasma membrane but not at endosomes by impairing β-arrestin coupling to the receptor, i.e., by altering receptor endocytosis. Despite inducing a robust and sustained cyclic AMP response at the plasma membrane, the biased parathyroid ligand was unable to increase the formation of active vitamin D. These data showed that endosomal signaling was essential to elicit the complete physiologic response to activation of the parathyroid hormone receptor. As indicated by the authors: "These results unveil subcellular signaling location as a means to achieve specificity in parathyroid hormone receptor-mediated biological outcomes and raise the prospect of rational drug design based upon spatiotemporal manipulation of GPCR signaling" (White et al., 2021). A fascinating example of how GPCR internalization affects the biologic outcome (also mentioned in a later section) is the sphingosine 1-phosphate "agonist/functional antagonist", fingolimod (FTY720), which is biased toward receptor degradation inducing downregulation of the sphingosine 1-phosphate ($S1P_1$) receptor (Brinkmann et al., 2010).

The bar code hypothesis still stands by underlining that different phosphorylation patterns direct receptor function. It should likely include the spatiotemporal characteristics of signaling imposed by receptor internalization and signaling from compartments besides the plasma membrane. In other words, to comprehend signaling at an integral cell physiology level, time and cellular localization seem to be very relevant.

Initial studies using different GPCRs evidenced that these receptors could be phosphorylated by at least two groups of protein kinases, GRKs and second messenger-activated protein kinases (Fig. 1), frequently at different residues; the possibility that phosphorylation at distinct residues could have signaling consequences was suggested (see for example Kim et al., 2005, reviewed in Tobin, 2008; Tobin et al., 2008). A breakthrough took place with the demonstration by the group of Tobin that the M_3 muscarinic receptor expressed in distinct cells was phosphorylated by casein kinase 2 but also by other protein kinases, resulting in differential phosphorylation of the same receptor and that the signaling outcome also differs (Torrecilla et al., 2007) (Fig. 2). Evidence also suggested that interaction with different β-arrestins took place and that such association was not just the consequence of the interplay with the anionic charges of the phosphate groups, all of which was required, but that the location of such ionic charges, i.e., their physical distance and the relationship with surrounding residues, is critical. This was the formal emergence of a conceptual framework on which more defined ideas on GPCR signaling and regulation have been developed and has been colloquially denominated the "phosphorylation barcode hypothesis" (Kim et al., 2005; Prihandoko et al., 2015; Tobin, 2008; Tobin et al., 2008; Torrecilla et al., 2007) (Fig. 2). Many laboratories joined the effort to characterize which receptor sites are phosphorylated in cellulo, using mass spectrometry, and under which conditions. This includes, among many others, the β_2-adrenoceptor (reviewed in Lefkowitz, 2013, but see also Latorraca et al., 2020; Nobles et al., 2011; Zindel et al., 2015); the M_3 muscarinic cholinergic receptor (Bradley et al., 2016; Butcher et al., 2011; Tobin, 2008; Tobin et al., 2008; Torrecilla et al., 2007), the free fatty acid receptor (FFA) 4 receptor (Alfonzo-Méndez et al., 2016; Butcher et al., 2014; Prihandoko et al., 2016); the α_1-adrenoceptor subtypes (Alcántara-Hernández et al., 2017; Alfonzo-Méndez et al., 2018; Carmona-Rosas et al., 2019; Hernández-Espinosa et al., 2019); the sphingosine 1-phosphate receptor 1 (Oo et al., 2011); and the ghrelin receptor, GHSR1a (Bouzo-Lorenzo et al., 2016). From these studies (and others not cited, due to space limitations, and to whose authors we apologize), there is clearly significant variation in the phosphorylated sites among GPCRs. From our perspective, the phosphorylation code remains a challenging enigma to be broken. Despite the difficulties, it appears to be a promising path to better understand how GPCR actions (signaling) and fate (recycling/proteolysis) are decided and how the internal machinery of the cells "senses" this and defines the pathways to activate and the vesicular traffic "routes" through which the receptors are required to transit. It also should be

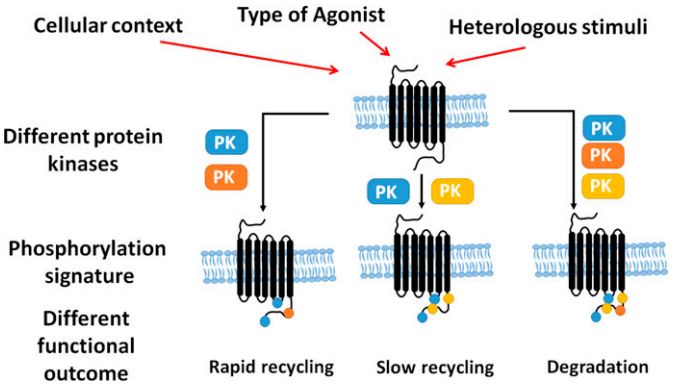

Fig. 2. Phosphorylation barcode hypothesis. The cellular context and the different stimuli modulate receptor phosphorylation and functional outcomes. A GPCR could be phosphorylated by distinct protein kinases (PK, different colors) at the carboxyl terminus and the third intracellular loop sites, resulting in different phosphorylation signatures. These signatures translate to phosphorylation codes that direct the GPCR actions and fates (recycling/degradation). Modified from Tobin et al., 2008.

considered that GPCRs with distinct phosphorylation signatures coexist in a single cell (Shen et al., 2018). Structural studies have also been employed to determine the interaction of GPCR domains at atomic resolution, such as the fully phosphorylated carboxyl terminus of the vasopressin V_2 receptor, with β-arrestin-1 (Shukla et al., 2013). We are endlessly astonished at how a single phosphorylation site in the human vasopressin V_2 receptor possesses a decisive contribution to β-arrestin recruitment; its mutation results in strong G protein bias (Dwivedi-Agnihotri et al., 2020). Similarly, a spatially positioned double-phosphorylation-site cluster in the bradykinin receptor B_2, analogous to one present in the vasopressin V_2 receptor, reverses the contribution of β-arrestin in ERK1/2 activation from inhibitory to promotive (Baidya et al., 2020).

Does GPCR Phosphorylation Always Result in Internalization?

There is compelling evidence indicating that this is frequently the case. We assume that baseline phosphorylation was due to the activity of protein kinases in unstimulated cells and could be a result of some GPCR constitutive activity. However, caution needs to be exercised, and the idea that receptor phosphorylation might participate in defining the localization of receptors in the plasma membrane should be considered. During our work on clarifying the phosphorylation sites present in the α_{1D}-adrenoceptor, we were surprised to find that in the case of some mutants, a large proportion of the receptors were localized in intracellular vesicles (Alfonzo-Méndez et al., 2018). Further studies suggested that the phosphorylation of a distal cluster in the carboxyl terminus (T507, S515, S516, and S518) favors α_{1D}-AR localization at the plasma membrane. Summarizing the findings, substituting these residues for non-phosphorylatable amino acids resulted in the intracellular localization of the receptors, whereas phospho-mimetic substitution (i.e., substitutions by aspartates) allowed for α_{1D}-adrenoceptor plasma membrane localization (Carmona-Rosas et al., 2019). When this paper was in press, an elegant study was published indicating that the Frizzled 6 receptor is phosphorylated at S648 by casein kinase 1 ε and that this phosphorylation is critical for proper membrane localization of this receptor, at the apical portion of the plasma membrane, in epithelial cells (Strakova et al., 2018). The previously mentioned data indicated that these GPCRs need to be phosphorylated during their transportation from the rough endoplasmic reticulum to reach the plasma membrane (Fig. 3). More recent work showed that protein kinase C phosphorylation of the μ opioid receptor at serine 363, on the carboxyl terminus, is required and sufficient for receptor recycling to the plasma membrane after agonist stimulation (Kunselman et al., 2019) (Fig. 3).

As already noted in a previous publication (Carmona-Rosas et al., 2019), there is evidence that the phosphorylation of channel receptor subunits, such as those of quisqualate receptors or γ-aminobutyric acid A receptors, increases their membrane localization (Abramian et al., 2014; Comenencia-Ortiz et al., 2014; Kibaly et al., 2016; Lin et al., 2009). It is currently unknown how general such a receptor phosphorylation requirement is for GPCR plasma membrane localization. Similarly, it is unknown whether such phosphorylation

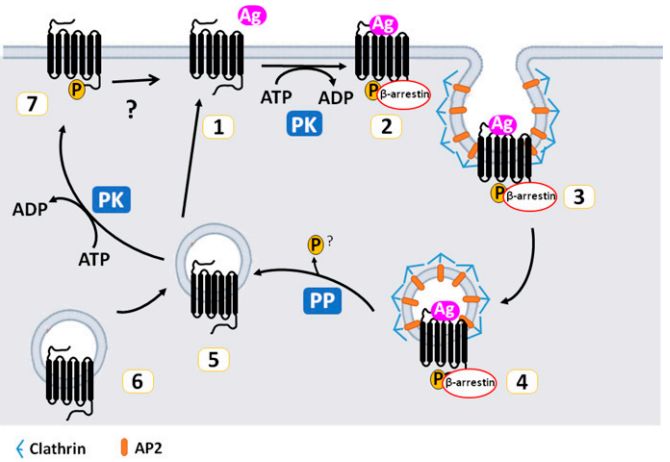

Fig. 3. GPCR phosphorylation might regulate receptor recycling. (1) GPCR agonist (Ag) activation leads to (2) GPCR phosphorylated by protein kinases (PK), which facilitates interaction with β-arrestins (3) and to receptor endocytosis (4). The GPCR is dephosphorylated (5) and recycled back to the plasma membrane or (7) phosphorylated to be subsequently recycled back to the plasma membrane. GPCR in their anterograde transport (6) could be phosphorylated or not to be inserted into the plasma membrane.

remains covalently bound to the receptor or removed once the GPCR is located in the plasma membrane. Indeed, much clarification is needed about this process; however, we think this possibility should be considered within the phosphorylation barcode hypothesis.

Rab Proteins

GPCRs are in constant traffic. They move from their site of synthesis in the rough endoplasmic reticulum to the plasma membrane through the anterograde vesicular traffic. Still, they do not remain there but are subjected to internalization, fast and slow recycling, and degradation, through intricate retrograde vesicular traffic. Eukaryotic cells possess a complex organized membrane system that enables the GPCRs to signal, desensitize, and resensitize, and these events occur in different membrane compartments. Many steps in these processes are regulated by the Rab family of small GTPases. Like the heterotrimeric G proteins (Homma et al., 2021; Hutagalung and Novick, 2011), Rab proteins cycle between two states, an active (GTP-loaded) state and an inactive (GDP-loaded) state; this cycling is modulated by guanine nucleotide exchange factors and GTPase-activating proteins (Homma et al., 2021; Hutagalung and Novick, 2011). When activated by a guanine nucleotide exchange factor, Rabs localize via their prenylated (or doubly prenylated) carboxyl terminal group to specific membranes, such as the endoplasmic reticulum, Golgi apparatus, secretory vesicles, early endosomes, late endosomes, or lysosomes; GTPase-activating proteins terminate these processes. Additionally, guanosine dissociation inhibitors regulate Rab proteins by preventing their insertion into specific membranes (Homma et al., 2021).

Approximately 60 Rab proteins have been identified in mammals (Homma et al., 2021; Hutagalung and Novick, 2011). The role of Rab proteins in GPCR traffic from the rough endoplasmic reticulum to the plasma membrane (anterograde) has been studied in detail by different groups, particularly that of Guangyu Wu, and authoritative reviews

have been published (see Wang et al., 2018; Wang and Wu, 2012; Zhang and Wu, 2019, and references therein). Here, we will focus on the roles of Rab protein on internalization (retrograde transport) and recycling, particularly Rab5, Rab4, Rab11, Rab7, and Rab9, which play critical roles in these processes.

Rab5 proteins (Fig. 4) are fundamental in the targeting of GPCRs from the plasma membrane to early endosomes (Seachrist et al., 2000; Yuan and Song, 2020), as they control the formation of clathrin-coated vesicles (Zhu et al., 2004) and the endosome motility on microtubules (Nielsen et al., 1999), which is fundamental for vesicular traffic. Early endosomes contain other effectors and regulatory proteins, such as the early endosome antigen 1, rabaptin 5, and rabenosyn-5, among others, which are essential in endosome fusion (Somsel Rodman and Wandinger-Ness, 2000) (Fig. 4). Rab5 is known to participate in the internalization of a large number of different GPCRs, including, among many others, the following: dopamine D_2 receptors (De Vries et al., 2019; Iwata et al., 1999): angiotensin II AT_1 receptors (Dale et al., 2004; Esseltine et al., 2011; Li et al., 2010; Szakadati et al., 2015); α_{1A}- (de-Los-Santos-Cocotle et al., 2020), α_{1B}- (Alfonzo-Méndez et al., 2017; Castillo-Badillo et al., 2015; Hernández-Espinosa et al., 2020), β_1- (Gardner et al., 2011), and β_2-adrenoceptors; the cannabinoid receptor 2 (Grimsey et al., 2011); the FFA1 (Qian et al., 2014) and FFA4 receptors (Flores-Espinoza et al., 2020); the sphingosine 1-phosphate $S1P_1$ receptor (Martínez-Morales et al., 2018), and the bradykinin B2 receptors (Charest-Morin et al., 2013).

Receptors in early endosomes can have various cell destinations via different vesicular trafficking pathways. A direct rapid recycling pathway for receptors to the plasma membrane depends on Rab4. It should be considered that Rab5, Rab11, and Rab4 can be found simultaneously in a single endosome but apparently distributed in distinct specific domains. The presence of a particular Rab protein does not exclude that of other Rab proteins in the same endosome, although they are apparently segregated; this phenomenon is known as Rab protein microdomains (Sönnichsen et al., 2000).

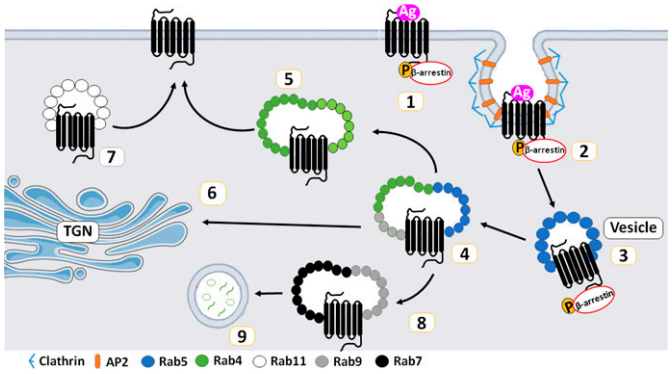

Fig. 4. Intracellular location of different Rab GTPases. GPCR phosphorylation facilitates interaction with β-arrestins (1) recruiting the endocytic machinery that initiates receptor endocytosis (2). Rab5 controls clathrin-coated vesicle formation, endocytosis, and vesicle fusion with early endosomes (3). Rab4, Rab5, and Rab9 are found in different endosomes where they can colocalize (4). Rab4 regulates fast recycling (5). Rab 9 favors the sorting into the Trans Golgi Network (TGN) (6). Rab11 regulates slow recycling to the plasma membrane (7). Rab9 and Rab7 are found in late endosomes (8) and favor receptor degradation (lysosomes) (9).

It has been observed that Rab4 (Fig. 4) participates in the rapid recycling of different receptors, including GPCRs (van der Sluijs et al., 1992), and a few examples will be discussed next. On studying the roles of Rab5 and Rab4 on β_2-adrenoceptor internalization and recycling, it was observed that the dominant-negative Rab4-N121I mutant blocked β_2-adrenoceptor resensitization by blocking receptor recycling from endosomes back to the cell surface, and, interestingly, it was observed that changes in the adrenoceptor phosphorylation state took place, suggesting that adrenoceptor dephosphorylation occurs as the receptor transits between the Rab5- and Rab4-positive compartments (Seachrist et al., 2000; Seachrist et al., 2002; Shenoy et al., 2008). Rapid and pronounced Rab4-dependent β_2-adrenoceptor recycling to the plasma membrane was observed after agonist removal by taking advantage of a pH-sensitive green fluorescent protein that permitted the detection of receptors in the plasma membrane but not when located in intracellular acidic compartments (Yudowski et al., 2009). It is also noteworthy that the site of interaction of Rab proteins with GPCRs has been defined for the angiotensin II AT_1 receptor (Esseltine et al., 2011). It was observed that several Rab proteins (Rab4, Rab7, and Rab11) bind to the last 10 amino acid residues of the AT_1 receptor (Esseltine et al., 2011).

Rab11 GTPase is mainly associated with slow receptor recycling (Fig. 4). It is localized at the trans-Golgi network, post-Golgi vesicles, and the recycling endosome, placing it at the intersection between the endocytic and exocytic trafficking pathways (Welz et al., 2014). Interestingly, Rab11a and some of its binding partners play a prominent role in recycling the human β_1-adrenoceptor (Gardner et al., 2011). Likewise, it has been observed that Rab11a and the unconventional myosin Vb regulate M_4 muscarinic acetylcholine receptors (Volpicelli et al., 2002).

The Rab9 GTPase directs vesicles to late endosomes, slow recycling, and transport to the Golgi apparatus (Barbero et al., 2002; Kloer et al., 2010; Ng et al., 2012) (Fig. 4). Proteins destined for degradation (downregulation) and Rab7 and Rab9 are found in late endosomes delivering cargo to lysosomes (Barbero et al., 2002). Consistent with this, it has been observed that Rab7 silencing prevents μ-opioid receptor lysosomal targeting and rescues opioid responsiveness (Mousa et al., 2013). Overexpression of Rab7 is associated with increased angiotensin II AT_1 receptor degradation (Dale et al., 2004). Evidence suggests that Rab7 also plays a role in lysosomal biogenesis (Bucci et al., 2000). Nevertheless, it is worth mentioning that the proteasome α-subunit, XAPC7, interacts specifically with Rab7 and late endosomes, indicating that Rab7 might participate in both the proteasomal and lysosomal degradation of GPCRs.

Studies on the interaction of α_{1B}-adrenoceptors with Rab proteins under homologous (noradrenaline) and heterologous (sphingosine 1-phosphate or phorbol myristate acetate) desensitization, using Förster resonance energy transfer indicated that these receptors are directed to different endocytic vesicles depending on the desensitization type. Agonist-stimulated α_{1B}-adrenoceptors interacted with proteins present in early endosomes, such as the early endosomes antigen 1, Rab 5, Rab 4, and Rab 11, but not with late endosome markers such as Rab 9 and Rab 7. In marked contrast, $S1P_1$ stimulation with sphingosine 1-phosphate or direct pharmacological activation of protein kinase C, with active phorbol esters, induced

a rapid but relatively small and transient α_{1B}-adrenoceptor interaction with Rab 5 and a more pronounced and sustained one with late endosomal markers such as Rab 9 and Rab 7 (Alfonzo-Méndez et al., 2017; Castillo-Badillo et al., 2015). α_{1B}-Adrenoceptor phosphorylation sites differ in cells stimulated by noradrenaline and phorbol esters (Hernández-Espinosa et al., 2019).

The very drastic pattern of α_{1B}-adrenoceptor–Rab protein interactions, from one extreme or the other, became more gradual or diffuse when different receptors were studied. Pharmacodynamic differences exist between agonists acting on α_{1A}-adrenoceptors; in particular, oxymetazoline appears to be an internalization-biased agonist as compared with noradrenaline (Akinaga et al., 2013; Alcántara-Hernández et al., 2017; da Silva et al., 2017; Quaresma et al., 2019). They also induce different receptor phosphorylation patterns (Akinaga et al., 2013; Alcántara-Hernández et al., 2017). Noradrenaline and methoxamine increased α_{1A}-adrenoceptor interaction with Rab5 and Rab7 but did not modify that with Rab9. In contrast, oxymetazoline induced adrenoceptor interaction with Rab5 and Rab9 and only an insignificant increase in the receptor Rab7 signal. Phorbol myristate acetate increased α_{1A}-adrenoceptor interaction with Rab5 and Rab9 but did not modify it with Rab7. The data suggested that cell stimulation with phorbol myristate acetate induced α_{1A}-adrenoceptor interaction with the late endosomes, suggesting that these receptors exhibit slow recycling to the plasma membrane after they have transited to the trans-Golgi network. In contrast, noradrenaline and methoxamine likely induce faster recycling and might direct some adrenoceptors toward degradation and/or to very slow recycling to the plasma membrane. Oxymetazoline produced a mixed interaction pattern with the Rab proteins (de-Los-Santos-Cocotle et al., 2020).

We also studied the sphingosine 1-phosphate receptor, $S1P_1$, interaction with Rab proteins. This receptor is of particular interest because it regulates lymphocyte egress from the lymph nodes, which impacts immunity, particularly in some autoimmune diseases (Pérez-Jeldres et al., 2021; Rivera et al., 2008). The prodrug FTY720 is phosphorylated in the organism to generate the actual agonist, FTY720-phosphate, and is currently accepted for treating relapsing multiple sclerosis (Brinkmann et al., 2010). In addition, $S1P_1$ phosphorylation and ubiquitination have been studied (Oo et al., 2011). Data indicate that sphingosine 1-phosphate, FTY720-phosphate, and the protein kinase C activator, phorbol myristate acetate, induce interaction with early endosomes, but the natural agonist, sphingosine 1-phosphate, induced rapid receptor recycling. In contrast, the phorbol ester favored interaction with the late and slow-recycling endosomes, and FTY720-phosphate triggered receptor interaction with vesicles associated with proteasomal/lysosomal degradation (Rab7) (Martínez-Morales et al., 2018).

The FFA4 receptor is phosphorylated in response to agonists, phorbol esters, and the activation of receptor tyrosine kinases (such as the insulin receptor) (Burns and Moniri, 2010; Burns et al., 2014; Butcher et al., 2014; Flores-Espinoza et al., 2020; Sánchez-Reyes et al., 2014; Senatorov et al., 2020; Villegas-Comonfort et al., 2019; Villegas-Comonfort et al., 2017). FFA4 agonist-activation (docosahexaenoic acid) induced an association with early endosomes (as suggested by interaction with Rab5) and rapid recycling to the plasma membrane (as indicated by receptor interaction with Rab4). Sustained agonist stimulation also appears to allow the FFA4 receptors to interact with late endosomes (interaction with Rab9), slow recycling (interaction with Rab 11), and target them to degradation (Rab7). Previous work did not observe rapid recycling but detected receptor targeting to lysosomal compartments (Watson et al., 2012). Phorbol myristate acetate triggered a fast association with early endosomes (Rab5), slow recycling to the plasma membrane (Rab11), and some receptor degradation (Rab7). Insulin-induced FFA4 receptor internalization included interaction with early endosomes (Rab5) and late endosomes (Rab9) and fast and slow recycling to the plasma membrane (Rab4 and Rab11, respectively) (Flores-Espinoza et al., 2020). The findings with the FFA4 and $S1P_1$ receptors reveal similarities, but also differences, in terms of what takes place when studying α_1-adrenoceptor subtypes. Therefore, no general internalization patterns can be defined, suggesting that additional studies with different GPCRs are required.

It is well established that receptor phosphorylation plays a role in GPCR–β-arrestin interaction, favoring association with the AP-2 adaptor complex, clathrin, and GPCR internalization. Similarly, receptor internalization can take different "routes", leading to distinct functional outcomes (endosomal signaling, rapid or slow recycling, or degradation). Studies from many laboratories suggest that the selection of such routes is not stochastic but seems to follow precise patterns. Besides, the Förster resonance energy transfer analysis of GPCR–Rab protein interaction indicates very close proximity between the GPCRs and these small GTPases. In addition, as carefully studied by Ferguson and collaborators, the carboxyl terminus of GPCRs seems to be critical in GPCR interaction with Rab proteins (Esseltine et al., 2011; Esseltine and Ferguson, 2013; Esseltine et al., 2012). Rab5 also contributes to the formation and/or budding of clathrin-coated vesicles (Seachrist et al., 2000), leading to the homotypic fusion of endocytic vesicles. These observations suggest that vesicular cargo proteins, such as some GPCRs, may control their targeting between intracellular compartments by directly regulating the activity of components of the intracellular trafficking machinery such as Rab5a (Seachrist and Ferguson, 2003; Seachrist et al., 2002). Critical knowledge gaps on how Rab proteins are regulated have been pointed out (Lachance et al., 2014). In our opinion, a crucial question that remains unanswered is the relationship between GPCR phosphorylation (the receptor phosphorylation barcode), internalization, and traffic to distinct destinations (rapid recycling, slow recycling, and degradation, for example). There is, indeed, a complex but fascinating pathway ahead.

Acknowledgments

The authors express their gratitude to Nadia Teresa Cedillo Romero and Maggie Brunner, M.A., for editorial/style corrections.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Martínez-Morales, Romero-Ávila, Reyes-Cruz, García-Sáinz.

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