Multifactorial Regulation of G Protein-Coupled Receptor Endocytosis

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Abstract
Endocytosis is a process by which cells absorb extracellular materials via the inward budding of vesicles formed from the plasma membrane. Receptor-mediated endocytosis is a highly selective process where receptors with specific binding sites for extracellular molecules internalize via vesicles. G protein-coupled receptors (GPCRs) are the largest single family of plasma-membrane receptors with more than 1000 family members. But the molecular mechanisms involved in the regulation of GPCRs are believed to be highly conserved. For example, receptor phosphorylation in collaboration with β-arrestins plays major roles in desensitization and endocytosis of most GPCRs. Nevertheless, a number of subsequent studies showed that GPCR regulation, such as that by endocytosis, occurs through various pathways with a multitude of cellular components and processes. This review focused on i) functional interactions between homologous and heterologous pathways, ii) methodologies applied for determining receptor endocytosis, iii) experimental tools to determine specific endocytic routes, iv) roles of small guanosine triphosphate-binding proteins in GPCR endocytosis, and v) role of post-translational modification of the receptors in endocytosis.

Key Words: G protein-coupled receptor, Endocytosis, Rap, ARF6, Glycosylation, Palmitoylation

INTRODUCTION

Cells are able to take up materials from their environment through endocytic processes. In receptor-mediated endocytosis, the engulfed materials (cargo; ligand and receptor) move to a specialized region of the plasma membrane, such as clathrin-coated pits (Mukherjee et al., 1997). In the clathrin-coated pits, the cargo is wrapped by the plasma membrane, which is coated with clathrins, leading to the formation of nascent vesicle buds (Pearse, 1976). As newly formed vesicles mature, they separate from the plasma membrane to form intracellular vesicles (van der Bliek et al., 1993). Receptor-mediated endocytosis is characterized by the highly selective sorting of molecules to be ingested by the cell. This selectivity results from the specific interaction between a receptor on the plasma membrane and its extracellular ligand. The functional role of receptor-mediated endocytosis is interpreted as the regulation of receptor functions rather than ingestion of ligands from the cell exterior.

Following agonistic stimulation, G protein-coupled receptors (GPCRs) undergo conformational changes that allow binding to G proteins (Gilman, 1987), leading to the activation of various signaling pathways and initiation of intracellular trafficking. After agonist stimulation, the receptor is phosphorylated by GPCR kinases (GRKs) (Pitcher et al., 1998), enhancing the binding of β-arrestins, which connect to adaptors, such as adaptor protein (AP)-2 and clathrin (Ferguson et al., 1996; Goodman et al., 1996; Laporte et al., 1999). These cellular processes are classified as homologous (agonist-induced, GRK-mediated) regulation of receptor responsiveness. By contrast, heterologous regulation of receptor responsiveness occurs regardless of agonists occupying the receptor. Receptor phosphorylation mediated by second-messenger-mediated kinases [protein kinase A (PKA) or protein kinase C (PKC)] is a key cellular event that leads to heterologous regulation.

The molecular mechanisms involved in GPCR endocytosis are highly conserved. GRKs/β-arrestins and PKA/PKCs are two protein families that mediate homologous and heterologous regulation, respectively. However, as described in the following sections, various cellular environments and components are involved in the regulation of GPCR endocytosis. Among these multiple factors, this review focused on the functional interactions between homologous and heterologous pathways and the roles of small guanosine triphosphate-binding proteins.
(GTP)-binding proteins and receptor post-translational modification on the regulation of receptor endocytosis.

**FUNCTIONAL CROSSTALK BETWEEN HOMOLOGOUS AND HETEROLOGOUS REGULATION OF GPCRs**

Although not mandatory, a major mechanism underlying GPCR regulation is receptor phosphorylation (Stadel et al., 1983; Barak et al., 1994; Ferguson, 2007; Cho et al., 2010). Second-messenger-dependent protein kinases, such as PKA and PKC, are responsible for agonist-nonspecific heterologous regulation. Alternatively, GRK2/3 is responsible for agonist-specific homologous regulation. Initially, GPCR phosphorylation by PKA or PKC was regarded as the sole mechanism of GPCR regulation (Benovic et al., 1985); however, it was found that $\beta_2$ adrenoceptor ($\beta_2$AR) could be phosphorylated in S49 lymphoma cells, which lack functional PKA (Strasser et al., 1986), suggesting the existence of additional protein kinases capable of phosphorylating GPCRs and leading to identification of the novel protein kinase family of GRKs (Benovic et al., 1986, 1989). Subsequent studies showed that $\beta$-arrestins (Lohse et al., 1990; Attramadal et al., 1992), analogues of visual arrestin, were required for receptor desensitization (Benovic et al., 1987) and potentiation of receptor endocytosis (Ferguson et al., 1996; Goodman et al., 1996).

Second-messenger-dependent protein kinases such as PKA and PKC can phosphorylate GPCRs at consensus phosphorylation sites and interfere with receptor-G protein coupling. Since PKA and PKC can phosphorylate agonist-occupied receptors, as well as agonist-occupied receptors, they inhibit not only ongoing signaling, but also prevent subsequent signaling activation. A complete understanding of the selective involvement of second-messenger-dependent protein kinases in the regulation of GPCRs.
in heterologous regulatory processes remains elusive. Perhaps $G_\alpha$- or $G_\beta$-coupled receptors, which are phosphorylated by GRK2/3, could be subjected to feedback regulation by activated PKA or PKC, suggesting that receptor phosphorylation mediated by second-messenger-dependent protein kinases could also contribute to homologous desensitization (Clark et al., 1988; Kelly et al., 2008).

Along with ligand selectivity, the main difference between homologous and heterologous regulation is the concentration of agonist required to induce receptor phosphorylation. Agonists...
Pharmacological Sequestration

- Hydrophilic ligand

PM

(1–5 min)

Fig. 3. Diagram showing the pharmacological sequestration of GPCRs. In pharmacological sequestration, receptors undergo conformational changes and translocate toward more hydrophobic regions within the plasma membrane. Pharmacologically sequestered receptors do not actually translocate to the cytosol, but cannot bind to hydrophilic ligands, because they are located within more hydrophobic regions of the plasma membrane. GPCR, G protein-coupled receptor; PM, plasma membrane.

in the nanomolar range are sufficient for PKA/PKC-mediated GPCR phosphorylation and desensitization (Jimenez-Baranda et al., 2007; Kim et al., 2008). In the case of PKA/PKC-mediated receptor phosphorylation, the signaling cascade initiated from the agonist-activated receptor is strongly amplified i.e., G protein—cyclic adenosine monophosphate (cAMP)/diacylglycerol (DAG)—PKA/PKC. By contrast, GRK2-mediated receptor phosphorylation, which depends upon agonist occupancy, requires higher concentrations of agonist as compared with those required for PKA/PKC-mediated phosphorylation. Additionally, higher levels of receptor expression are needed to facilitate β-arrestin translocation to mediate receptor desensitization and endocytosis. Therefore, GRK2/3-mediated receptor phosphorylation plays important roles in synaptic nerve terminals where high concentrations of neurotransmitters are attainable. By contrast, PKA/PKC-mediated receptor phosphorylation might affect tissues with low concentrations of circulating agonists (Arriza et al., 1992; Tran et al., 2004; Pollok-Kopp et al., 2007; Kelly et al., 2008).

Understanding the functional interaction between homologous and heterologous pathways of GPCR regulation is an important field of study. Reports implicated the involvement of GRK2 and β-arrestins on PKC/PKA-mediated regulatory pathways. For example, activated GRK2 binds to PKCβ through its pleckstrin homology domain, resulting in PKC inhibition (Ji et al., 2003). β-Arrestins inhibit PKC-mediated regulatory pathways by activating DAG kinase, which subsequently mediates conversion of DAG to phosphatidic acid (PA) (Nelson et al., 2007). Another study showed that β-arrestins recruit cAMP phosphodiesterases to ligand-activated receptors, promoting the degradation of cAMP and thus inhibiting PKA activation (Perry et al., 2002) (Fig. 1A).

There have been various and sometimes contradictory reports regarding the effects of PKA or PKC on the homologous regulatory pathway. For example, PKA or PKC activates GRK2 by enhancing its translocation to the plasma membrane (W instel et al., 1996; Cong et al., 2001). A subsequent study showed that GRK2 is phosphorylated by PKCo, PKCδ, and PKCitro, relieving the tonic inhibition by calmodulin (Krasel et al., 2001). By contrast, other studies showed that Raf-kinase-inhibitor protein is phosphorylated on Ser153 by PKC activation, leading to its association with GRK2 and the inhibition of GRK2 activity (Lorenz et al., 2003; Huang et al., 2007). Furthermore, a recent study showed that PKCβII inhibits the homologous regulatory pathway by inhibiting β-arrestin-2 ubiquitination (Zheng et al., 2015) (Fig. 1B). Ubiquitination involves addition of ubiquitin, a small (8.5 kDa) regulatory protein and is a frequent cue for the degradation of the acceptor protein by the proteasome (Glickman and Ciechanover, 2002). In addition to protein degradation, ubiquitination is required for other functions, including β-arrestin-2-mediated endocytic activities (Shenoy et al., 2001).

Therefore, it is suggested that functional interactions between homologous and heterologous pathways occur in two layers: between the major players of each endocytic pathway (PKA/PKC, GRK2/β-arrestins) or through manipulation of second-messenger levels. Further studies are needed to clarify the contradictory reports regarding the functional roles of PKC in the homologous regulation of GPCRs.

METHODOLOGIES USED FOR DETERMINATION OF RECEPTOR ENDOCYTOSIS

The terms ‘endocytosis’, ‘internalization’, and ‘sequestration’ are usually used interchangeably to describe the inward movement of extracellular material across the plasma membrane through endocytic processes. Strictly speaking, sequestration describes the isolation of ligands from receptors located on the cell surface; thus, it does not necessarily represent inward movement of materials from the exterior of the cell into the cytosol. Various experimental approaches have been utilized to assess GPCR internalization (endocytosis) or sequestration.

In the case of receptor endocytosis or internalization, the most universally utilized methodology is tagging of receptors at the N-terminus with specific epitopes, usually hemagglutinin (HA) or FLAG (Kim et al., 2001; Zhang and Kim, 2016) (Fig. 2A). Antibody labeled receptors on the cell surface can be detected using either enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). For the ligand-binding methodology, radiolabeled hydrophilic ligands
are commonly used to directly label receptors on the cell surface (Fig. 2B). Radiolabeled hydrophobic ligands are sometimes combined with hydrophilic ligands (to compete with surface binding of hydrophobic radioligands) to measure total receptor levels, as well as intracellular receptor levels (Itokawa et al., 1996; Kim et al., 2001; Zhang et al., 2016b). In our personal experience, the radioligand-binding assay is more convenient and accurate when compared with other methodologies. Receptor internalization can also be measured by fluorescence (Thompson and Whistler, 2011) or cell-surface biotinylation (Vickery and von Zastrow, 1999); however, these two methods can result in either difficulty in selectively quantifying receptors on the cell membrane and in the cytosol or the necessity to supplement with immunoprecipitation and immunoblot assays, which could introduce large variations in the measurements. When receptor sequestration accompanies conformational changes and short-distance trafficking within the plasma membrane, resulting in failure to bind hydrophilic ligands (pharmacological sequestration; Fig. 3), hydrophilic radioligands are used to label receptors on the cell surface.

**FACS analysis**

Detection of GPCR endocytosis by FACS requires an N-terminal epitope-tagged GPCR. Because the location of the N-terminus changes from the exterior to the interior of the cell following GPCR internalization, FACS can be used to quantify the decrease in GPCR localization at the exterior surface of the plasma membrane (Kim et al., 2001). In this procedure, cells are transfected with a GPCR containing an epitope-tagged N-terminus. Cells are distributed in 6-well plates, stimulated with a vehicle or agonist for a desired period, and labeled with antibodies specific to the corresponding epitope. The mean cell-surface fluorescence and the number of fluorescent cells are then determined by FACS, and the percentage of receptor endocytosis is calculated from the FACS values of vehicle- or agonist-treated cells [i.e., (vehicle-treated−agonist-treated)/(vehicle-treated)].

**ELISA**

The principle behind determining receptor internalization by ELISA is essentially the same as that for FACS analysis: presence of an N-terminal epitope tag on the receptor (Zhang et al., 2016a); the epitope is no longer recognized by the cognate antibody once the receptor is internalized. For ELISA, cells are transfected with a receptor tagged at the N-terminus with a specific epitope, stimulated with agonist for a period of time, and labeled with antibodies corresponding to the specific epitope, followed by labeling with horseradish peroxidase-conjugated secondary antibodies. o-Phenylenediamine, a horseradish-peroxidase substrate, is then added, and the optical density (OD) of the supernatants is read using an ELISA reader. The background reading obtained from mock-transfected cells is subtracted to calculate the percentage of internalization. It is important to have a sufficient margin between the OD values of the cells transfected with receptor cDNA and mock plasmid. The percentage of receptor endocytosis is calculated from the OD values of vehicle- and agonist-treated cells [i.e., (vehicle-treated−agonist-treated)/(vehicle-treated)].

**Radioligand binding**

Hydrophilic radiolabeled ligands can be used to selectively label receptors expressed on the cell surface (Kim et al., 2001; Zheng et al., 2016). Cells are transfected with a GPCR, distributed in a 24-well plate, and stimulated with an agonist for a desired period of time. Cells need to be thoroughly washed with ice-cold serum-free media or low-pH buffer to stop further
Fig. 5. Different modes of Ral regulation of receptor endocytosis. (A) Three downstream effectors of Ral in the regulation of receptor endocytosis. (B) RalBP1 (formerly known as RLIP76 or Ral-interacting protein) is involved in Ral-mediated regulation of endocytosis of receptors, such as EGF receptor and insulin receptor. Agonist-activated receptors transmit signals through Ral, RalBP1, and POB1 to Epsin and Eps15. Epsin and Eps15 bind to the AP-2/clathrin complex, leading to formation of clathrin-coated vesicles, which contain transmembrane receptors. This diagram was modified based on a previous publication (Nakashima et al., 1999). (C) Ral-mediated activation of PLD is involved in the endocytosis of EGF receptor and metabotropic glutamate receptor. Pi(4,5)P2 is synthesized from Pi(4)P by Pi(4)P5K, which is activated by PA, a product of PLD. (D) RalA regulates GPCR endocytosis in an activity dependent manner. GTP-bound RalA sequesters GRK2 from binding to its receptor, resulting in the inhibition of receptor endocytosis. When cells are treated with agonist, Gβγ translocates to the cytosol as a complex with RGL, resulting in the dissociation of RGL from RalA and conversion of GTP-RalA to GDP-RalA, to which GRK2 has low affinity. GRK2 dissociated from GTP-RalA is the prepared for interaction with a receptor or other endocytic regulators. Ral-BP1, Ral-binding protein; EGF, epidermal growth factor receptor, Eps15, EGF-pathway substrate 15; Epsin, Eps15-interacting protein; AP-2, adaptor protein 2; PLD, phospholipase D; Pi(4,5)P2, phosphatidylinositol 4,5-bisphosphate; Pi(4)P, phosphatidylinositol 4-phosphate; Pi(4)P5K, phosphatidylinositol 4-phosphate 5-kinase; PA, phosphatidic acid; GPCR, G protein-coupled receptor; GRK, GPCR kinase; GDP, guanosine diphosphate; RGL, Ral-GDP-dissociation-stimulator-like protein; GTP, guanosine triphosphate.
in intracellular trafficking and to completely remove the agonist bound to the receptors. During the initial stage of the assay protocol, it is important to confirm that all of the agonists are removed under washing conditions. Cells are labeled with a hydrophilic radioligand on ice, during which time, further intracellular trafficking is blocked. Half of the experimental group needs to be labeled with a radioligand along with an excess concentration of non-labeled ligand to determine non-specific binding. Cells are washed thoroughly, lysed with detergent, and counted using a liquid-scintillation counter. The percentage of receptor endocytosis is calculated from the specific binding values of vehicle- and agonist-treated cells [i.e., (vehicle-treated–agonist-treated)/(vehicle-treated)].

**Pharmacological sequestration**

Agonist-induced intracellular trafficking of GPCRs typically involves the actual movement of receptors from the exterior surface of the plasma membrane into the cytosol (Moore et al., 2007). Both the radioligand-binding method and epitope-tagged receptor approaches can be applied to measure this internalization. By contrast, pharmacological sequestration involves conformational changes of the receptors accompanied by a shift toward more hydrophobic domains within the plasma membrane without movement into other intracellular compartments (Mostafapour et al., 1996). A recent study showed that pharmacological sequestration can be performed to predict the acute tolerance (desensitization) of the dopamine D3 receptor (Min et al., 2013). Since pharmacological sequestration does not accompany actual translocation of receptors across the plasma membrane, the ligand-binding method, but not flow cytometry or ELISA, can be applied to determine the extent of pharmacological sequestration (failure of hydrophilic ligand binding). In this procedure, cells are transfected with a GPCR and stimulated with vehicle or agonist for 1 to 5 min, during which time, typical receptor endocytosis does not occur. The reaction is stopped by placing the cells on ice, followed by washing with ice-cold low-pH buffer to completely remove all of the agonist bound to the receptor on the cell surface. Cells are then incubated with hydrophilic radioligands on ice in the absence or presence of excess unlabeled ligand. The cells are washed and lysed, and the remaining radioactivity is counted using a liquid scintillation counter. The percentage of pharmacological sequestration is calculated from the binding values of vehicle-treated and agonist-treated cells [i.e., (vehicle-treated–agonist-treated)/(vehicle-treated)].

**SELECTIVE REGULATION OF CLATHRIN-MEDIATED AND CAVEOLAE-DEPENDENT ENDOCYTIC PATHWAYS**

Clathrin-mediated and caveolae-dependent pathways are the best-characterized internalization routes of GPCRs (Hansen et al., 1993; Doherty and McMahon, 2009; Guo et al., 2015). Clathrin-mediated endocytosis (CME) is initiated by the formation of specialized membrane regions called clathrin-coated pits, into which cell-surface receptors concentrate and form clusters. Through a series of highly regulated steps, the pits bud off to form clathrin-coated vesicles with the help of dynamin, a protein that separates the newly-formed vesicles from the plasma membrane (Schmid, 1997; Marchese et al., 2003; Cho et al., 2006). A number of adaptor and accessory molecules are involved in this process, including the AP-2 complex, amphiphysin, GRK2/3, and β-arrestins that phosphorylate and connect receptors to clathrin and the AP-2 complex (Claiog et al., 2002; Wolfe and Trejo, 2007; Ivanov, 2008; Romer et al., 2010).

Caveolae are flask-shaped invaginations of the plasma membrane and contain caveolin1 as a main component (Rothberg et al., 1992; Anderson, 1998; Parton and Simons, 2007). Caveolae are involved in mediating receptor signaling (Parton and Simons, 2007) and clathrin-independent, raft-dependent receptor endocytosis (Nabi and Le, 2003; Lajoie and Nabi, 2010). Caveolar endocytosis is sensitive to cholesterol depletion and requires dynamin (Henley et al., 1998; Rodal et al., 1999).

A number of molecular biological tools and pharmacological agents have been used to selectively inhibit CME and caveolar endocytosis. Molecular biology approaches include the use of dominant-negative mutants or RNA-interference technology to compete with or downregulate the expression of endogenous proteins involved in CME. With the stipulations that they do not exhibit serious cytotoxicity and that their selectivity be established, pharmacological agents might be more convenient than molecular biological approaches, because they are easy to use and influence the entire cell population equally (Ivanov, 2008).

There have been a number of reports suggesting selectivity of various cellular environments and chemical inhibitors of CME. For example, clathrin-coated pits can be blocked by decreasing cytosolic pH (Sandvig et al., 1987; Cosson et al., 1989). Monodansylcadaverine (MDC), an inhibitor of tissue transglutaminase (Mishra and Murphy, 2004), was used to block CME of insulin-like growth factor-1 and the α2 adrenergic receptor (Chow et al., 1998; Pierce et al., 2000). Transglutaminase is involved in protein cross-linking (Davies et al., 1980), which mediates clathrin clustering and internalization (Budd et al., 1999). Pitstop2 (N-[5-(4-bromobenzylidene)-4-oxo-4,5-dihydro-1,3-thiazol-2-yl]naphthalene-1-sulfonamide) is a recently developed CME inhibitor of transferrin, with an IC50 value of 12 µM to 15 µM. Pitstop2 inhibits the association between the terminal domain of clathrin and amphiphysin (von Kleist et al., 2011). However, the selectivity of these inhibitors was not clearly established, because criteria used to evaluate their selectivity were not properly implemented.

In a recent study, clathrin heavy chain- or caveolin1-knockdown cells were employed to determine the specificity of various chemical and molecular biological tools for CME and caveolar endocytosis (Guo et al., 2015). The study showed that sucrose, concanavalin A (Con A), and dominant-negative mutants of dynamin blocked other endocytic pathways, as well as the clathrin-mediated pathway. In particular, Con A non-specifically interfered with the signaling of several GPCRs tested in the study. Decreased pH, MDC, and dominant-negative epidermal growth factor (EGF)-receptor-pathway substrate 15 (Eps15)-interacting protein (Epsin) mutants are specific for CME when used properly, whereas Pitstop2 is marginally selective for CME (Dutta et al., 2012; Guo et al., 2015). These results are summarized in Fig. 4.

**ROLES OF SMALL G PROTEINS IN GPCR ENDOCYTOSIS**

GTP-binding proteins (GTPases) are classified into two
families: heterotrimeric large G proteins, which are composed of three subunits (α, β, and γ), and small G proteins. Based on the amino acid sequence of the Gα subunit, heterotrimeric G proteins are divided into Gαs, Gαi, Gαq/11, and Gα12/13. The small G-protein superfamily is generally classified into five subfamilies: the Ras family (Ras, Rap, and Ral), the Rho family (Rho, Rac, and cdc42), the adeosine diphosphate ribosylation factor (Arf) family (Arf1-6, Arl1-Arl7, and Sar), the Rab family, and the Ran family (Burgoyne, 1989; Takai et al., 2001). All of these small GTPases control cell function by cycling between a GDP-bound ‘inactive’ state and a GTP bound ‘active’ state (Bos, 1998; Takai et al., 2001). An increasing number of studies show that GPCRs crosstalk with small G proteins. For example, small G proteins and related regulators [e.g., RhoA, Rabs, Arfs, and Arf-guanine-nucleotide-exchange factors (GEFs)] can associate directly with GPCRs, and GPCRs may also function as GEFS for small GTPases (Bhattacharya et al., 2004a). In this review, we focused on the roles of Ral, Rab5, and Arf in GPCR endocytosis.

Effects of Ral on GPCR endocytosis

Ral, a member of the Ras family, possesses multiple regulatory roles, such as gene transcription, cytoskeletal regulation, and cell differentiation and migration (Charind and Tavitian, 1986; Feig et al., 1996; Bos, 1998; Takai et al., 2001). Additionally, Ral is implicated in the endocytosis of receptors containing single transmembrane domains (van Dam and Robinson, 2006). Three downstream components of Ral have been reported: Ral-binding protein 1 (RalBP1) or Ral-interacting protein-76 kDa, phospholipase D (PLD), and GRK2 (Fig. 5A). First, Ral may regulate receptor endocytosis through RalBP1. RRalBP1 contains a GTPase-activating protein domain for the Ras family proteins and a Ral-binding domain. Thus, RalBP1 could play a role in connecting Rho- and Ras-family signaling (Mott and Owen, 2014). Active Ral and a dominant-negative mutant of RalBP1 inhibit endocytosis of insulin receptor, transferrin receptor, EGF receptor, and activin type II receptor (Nakashima et al., 1999; Jullien-Flores et al., 2000; Matsuoka et al., 2002). RalBP1 associates with POB1, which forms a complex with Eps15, Epsin, AP-2, and clathrin to regulate endocytosis of transmembrane receptors (Fig. 5B). Second, Ral appears to regulate receptor endocytosis through PLD activation (Jiang et al., 1995; Kim et al., 1998) (Fig. 5C). For example, endocytosis of EGF receptor requires Ral-dependent PLD activation (Shen et al., 2001), and both metabotropic glutamate receptor (mGlur)-1a and -5a can be internalized constitutively by a Ral/PLD2-mediated endocytic mechanism (Bhattacharya et al., 2004b), which requires PLD2-dependent phosphatidic acid (PA) formation. Additionally, PA plays a regulatory role in clathrin-coated vesicle formation and receptor-mediated endocytosis by activating phosphatidylinositol 4-phosphate 5-kinase [Pl(4)PSK] (Jenkins et al., 1994; Antonescu et al., 2010). Pl(4)PSK is a type I lipid kinase that generates the lipid second messenger phosphoatidylphosphatidylinositol 4,5-bisphosphate [Pl(4,5)P2], which is critically important in clathrin-coated pit dynamics (Zoncu et al., 2007; Nakatsu et al., 2007).
Furthermore, AP-2 can bind clathrin in collaboration with PI(4,5)P2 (Kelly et al., 2014). Third, Ral might regulate receptor endocytosis through a functional interaction with GRK2 (Fig. 5D). Ral regulates the signaling or endocytosis of lysophosphatidic acid receptor-1 by modulating the interaction between the receptor and GRK2 (Aziziyeh et al., 2009). Zheng et al. (2016) recently showed that GTP-bound RalA inhibits the GPCR endocytosis by sequestering GRK2 from the activated receptors. Agonist-induced conversion of GTP-to GDP-bound RalA, which presumably releases sequestered GRK2, was observed selectively with the GPCRs, which have a tendency to undergo endocytosis. According to this study, agonist-induced Gγ-mediated conversion of GTP-RalA to GDP-RalA is suggested as a critical cellular event that allows receptor-mediated endocytosis to occur.

**Effects of Rab5 on GPCR endocytosis**

The Rab GTPase family regulates multiple steps of vesicular-membrane trafficking, including vesicle budding, docking, and fusion. Thus, Rab is referred to as a master regulator of intracellular transport (Pfeffer, 1994; Ollikonen and Stenmark, 1997; Hutagalung and Novick, 2011).

Rab5 is associated with the plasma membrane and early endosomes, and regulates multiple steps involved in vesicular trafficking (Bucci et al., 1992; Novick and Zerial, 1997). Evidence for the roles of Rab5 were obtained using the Rab5 mutants Rab5-S34N and Rab5-Q79L, a dominant-negative mutant and a constitutively active mutant, respectively. Rab5 mediates the formation of clathrin-coated vesicles at the cell surface. Rab5 is a component of clathrin-coated vesicles, and a complex of Rab5 and Rab-guanine nucleotide-dissociation inhibitor is necessary for the invagination of clathrin-coated pits (Bucci et al., 1992; McLauchlan et al., 1998; Seachrist et al., 2000; Weir et al., 2014). A possible isotype-specific interaction of Rab5 with clathrin was recently reported in Leishmania donovani (Rastogi et al., 2016).

Furthermore, Rab5 mediates the transport and fusion of endocytic vesicles with early endosomes. The Rab5-Q79L mutant stimulates endosome fusion and endocytosis, whereas the Rab5-S34N mutant blocks these processes. These effects are observed with transferrin receptor, endothelin receptors, neurokinin-1 receptor, and β3AR (Stenmark et al., 1994; Bremnes et al., 2000; Seachrist et al., 2000; Schmidlin et al., 2001). Rab5 was also reported to be phosphorylated by protein kinases, such as leucine-rich repeat kinase 2 or PKCε (Ong et al., 2014; Yun et al., 2015), suggesting intricate functional interactions with other signaling pathways.

**Effects of Arf in GPCR endocytosis**

Mammalian Arfs are divided into three classes: class I (Arf1-3), class II (Arf4-5), and class III (Arf6) (Moss and Vaughan, 1995). Arf1 and Arf6 are the best-characterized Arf subtype in terms of their roles in the intracellular trafficking of membrane proteins. Arf1 can be recruited to the plasma membrane on activation of some GPCRs (Mitchell et al., 2003), and Arf1 activation promotes the recruitment of components.
needed for the formation of trafficking vesicles, such as coats for non-clathrin (coatomer for COP1 vesicles) (Donaldson et al., 1992; Orcl et al., 1993) and clathrin (AP-1 and AP-3) (Traub et al., 1993; Ooi et al., 1998). Additionally, Arf1-mediated PLD activation is required for endocytosis of M₁ muscarinic receptors and µ-opioid receptor (Luo et al., 1998; Koch et al., 2003; Mitchell et al., 2003).

Arf6 is mainly found on the plasma membrane, but not within clathrin-coated vesicles (O’Souza-Schorey et al., 1995; Cavenagh et al., 1996). A previous study proposed that the GDP-bound form of Arf6 localizes to the plasma membrane, the GDP-GTP cycle of Arf6 occurs at the plasma membrane, and activated Arf6 triggers clathrin translocation onto the membrane (Macia et al., 2004). Two Arf6 mutants (Q67L and T27N), despite unclear activation of T27N (Macia et al., 2004), are considered to mimic the GTP- and GDP-bound forms of Arf6, respectively, and have been used extensively to elucidate Arf6 localization and function.

Arf6 plays an essential role in the internalization of several GPCRs, regardless of their endocytic route (Houdolou et al., 2005). For example, in response to agonist stimulation of β₂AR, β-arrestins recruit Arf nucleotide-binding-site opener (ARNO; Arf6-GEF) to a position near the plasma membrane to form a complex comprising β-arrestin, Arf6, and ARNO. These processes result in Arf6 activation, which is essential for β₂AR internalization (Claing et al., 2001; Lawrence et al., 2005) (Fig. 6, upper panel). Similar to Arf1, Arf6 regulates GPCR endocytosis via PLD2 activation, which leads to the hydrolysis of phosphatidylincholine to PA and choline (West et al., 1997; Rankovic et al., 2009). Additionally, Arf6 is capable of increasing PI(4,5)P₂ concentration and facilitating clathrin-coated pit assembly (Krauss et al., 2003; Posor et al., 2015). PI(4,5)P5K, which catalyzes the conversion of PI(4)P to PI(4,5)P₂, is a downstream effector of Arf6 (Godi et al., 1999; Honda et al., 1999). Arf6 requires PA, the product of PLD, to activate PI(4,5)P5K (Czech, 2000), and PI(4,5)P5K can also be directly activated by PA. PI(4,5)P2 plays a regulatory role in a wide variety of cellular functions, including exocytosis, endocytosis, endosomal recycling, and membrane-ripple formation (Honda et al., 1999; Funakoshi et al., 2011). PI(4,5)P2 is also required for the initial targeting of AP-2 to the plasma membrane, as well as for cargo recognition, which stabilize nascent coated pits during clathrin-mediated endocytosis (Wen et al., 2001; Loerke et al., 2009).

Alternatively, Arf6-GTP might recruit other proteins, such as Nm23-H1, a nucleoside diphosphate kinase that provides a source of GTP for dynamin-dependent fission of coated vesicles (Palacios et al., 2002). These hypotheses are summarized in Fig. 6.

**ROLES OF POST-TRANSLATIONAL MODIFICATIONS IN GPCR ENDOCYTOSIS**

GPCRs are post-translationally modified in a number of ways, including phosphorylation, ubiquitination, glycosylation, and palmitoylation. Receptor phosphorylation has been extensively investigated, and its roles in receptor endocytosis are well established (McCaffrey et al., 1984; Sibley et al., 1987), whereas the roles of ubiquitination in receptor endocytosis are clear only for yeast GPCRs (Hicke and Riezman, 1996; Shenoy et al., 2001; Zhang et al., 2016c). In this review, we focused on the roles of ubiquitination, glycosylation, and palmitoylation in receptor endocytosis.

**Roles of ubiquitination in receptor endocytosis**

During ubiquitination, ubiquitin, a small (8.5 kDa) regulatory protein, is added to a substrate protein. Ubiquitination requires three enzymes: activating, conjugating, and ubiquitin ligases (E1, E2, and E3, respectively). The carboxyl group of Gly76, the terminal amino acid of ubiquitin, is bound to the epsilon group of the target lysine residue on the substrate (Pickart, 2001; Marotti et al., 2002). Of the several lysine residues located within the ubiquitin molecule, Lys48 is involved in the formation of polyubiquitin chains that signal proteins for proteasomal processing. Alternatively, Lys63-linked chains are involved in endocytic processes (Boname et al., 2010). While the major role of ubiquitination involves mediation of protein degradation (Glickman and Ciechanover, 2002), ubiquitination also mediates various other cellular functions, such as gene transcription, cell division, differentiation, signal transduction, protein trafficking, and protein interactions (Alaluf et al., 1995; Conaway et al., 2002; Adlammerini et al., 2014).

A role of ubiquitination in GPCR trafficking was originally reported from studies in yeast α-mating factor pheromone receptor (Ste2p). In 1993, it was found that Lys337 is required for Ste2p endocytosis (Rohrer et al., 1993). Using a yeast mutant lacking multiple ubiquitin-conjugating enzymes, it was shown that ubiquitination is required for Ste2p endocytosis and subsequent lysosomal degradation (Hicke and Riezman, 1996; Kim et al., 2005).

Several studies demonstrated diverse roles for ubiquitination in the regulation of mammalian GPCR trafficking. An essential role of ubiquitination was proposed in post-endocytic lysosomal sorting rather than in endocytosis (Koch et al., 1994; Holtmann et al., 1996; Jacob et al., 2005; Kim et al., 2007; Lahaye et al., 2016; Zhang et al., 2016c). By contrast, some GPCRs, including δ-opioid receptor, protease-activated receptor 1 (PAR1), and the P2Y1 purinergic receptor, are delivered to the lysosome independent of receptor ubiquitination (Tanowitz and Von Zastrow, 2002; Dores et al., 2012, 2016).

**Roles of glycosylation in receptor endocytosis**

N-linked glycosylation is a highly conserved post-translational modification that occurs on the Asn-X-Ser/Thr motif on GPCR extracellular domains (Kim et al., 1997; Ulloa-Aguirre et al., 1999; Filipek et al., 2003). Glycosylation aids the association of proteins with specific plasma-membrane microdomains (Rands et al., 1990; Boer et al., 2000; Kohno et al., 2002; Lichnerova et al., 2015). A recent study showed that N-linked glycosylation of the N-terminus of dopamine D₂ and D₃ receptors determines the endocytic pathways used by these receptors via their interactions with specific microdomains (Min et al., 2015).

Glycosylation also plays an important role in the ligand-binding affinity of many receptors, such as the EP₃α receptor (Huang and Tai, 1998), human urikase receptor (Moller et al., 1993), and human transferrin receptor (Williams and Enns, 1993). Deglycosylation of these receptors causes significant decreases in ligand-binding affinity.

Additionally, the roles of glycosylation could be specific to the receptor region where glycosylation occurs. For example, N-linked glycosylation of the D₃ receptor on the N-terminus is responsible for surface expression, desensitization, and inter-
nalization, whereas N-linked glycosylation within the second extracellular loop is exclusively responsible for internalization (Min et al., 2015). As in D2R, glycosylation on the N-terminus and within the second extracellular loop of PAR1 is important for its transport to the cell surface and internalization, respectively (Soto and Trejo, 2010) (Fig. 7). A similar effect was reported for the prostacyclin receptor (Zhang et al., 2001).

The extent of glycosylation and its influence on receptor function seem to vary according to receptor type and cellular environment. Therefore, carefully controlled experiments are necessary to understand the broader functional roles of glycosylation and the underlying regulatory mechanisms.

**Roles of palmitoylation in receptor endocytosis**

Palmitoylation is mediated by palmitoyl transferase, which contains a characteristic Cys-rich Asp-His-His-Cys domain (Fukata et al., 2004; Greaves and Chamberlain, 2011). Palmitoylation is reversible and usually occurs on either Cys residues of membrane proteins or, less frequently, on Ser and Thr residues (Bizzozero, 1997; Qanbar and Bouvier, 2003). Palmitoylation exhibits various effects on receptor localization on the plasma membrane. For example, palmitoylation is needed for the proper localization of receptors, such as dopamine D1 receptor (Zhang et al., 2016b), CB1 cannabinoid receptor (Oddi et al., 2012), and PAR2 (Adams et al., 2011). Palmitoylation of the NR2 subunit of the N-methyl-D-aspartate receptor in the C-terminal region differentially affects surface expression, depending on the location of specific palmitoylation sites (Hayashi et al., 2009). Plasma membrane versus nuclear localization of estrogen receptors is also controlled by differential palmitoylation, which may promote the interaction between these estrogen receptors and caveolins (Acconcia et al., 2005; Boulware et al., 2005; Meitzen et al., 2013; Adlam- merini et al., 2014). In caveolae, estrogen receptors associate with mGluRs and activate them (Meitzen et al. 2013).

Palmitoylation regulates both G proteins and their receptors (Wedegaertner et al., 1993; Ross, 1995), and is required for efficient signaling by most GPCRs, including β2AR (O’Dowd et al., 1989; Moffett et al., 1993), endothelin receptor type B (Okamoto et al., 1997), CB1 cannabinoid receptor (Oddi et al., 2012), PAR 2 (Adams et al., 2011), and µ-opioid receptor (Zeng et al., 2012). β2AR palmitoylation on Cys341 inhibits PKA access, allowing for more efficient coupling with G proteins (Moffett et al., 1996). By contrast, palmitoylation is not required for normal signaling by some GPCRs, such as α2bAR (Kennedy and Limbird, 1993; Eason et al., 1994) and thyrotropin receptor (Kosugi and Mori, 1996). In the case of tumor necrosis factor (TNF)α receptor, a member of the cytokine-receptor family, the affinity of the receptor for TNF decreases when the TNF ligand is palmitoylated (Poggi et al., 2013), suggesting that palmitoylation of ligand rather than receptor could regulate signaling. Palmitoylation-mediated redistribution of GPCRs between lipid raft and non-raft microdomains on the plasma membrane indirectly implicates palmitoylation in biased signaling (Zheng et al., 2008, 2013). The concept of biased signaling involves the agonists of one particular receptor activating downstream signaling pathways with different efficacies. The µ-opioid receptor can activate extracellular signal-regulated kinase (ERK) phosphorylation through either G protein- or β-arrestin-dependent pathways, depending on the association of the receptor with lipid raft or non-lipid raft microdomains, respectively (Zheng et al., 2008).

Similar to GPCRs, the α subunits of G proteins are palmitoylated (Linder et al., 1993; Parenti et al., 1993), with palmitoylation regulated by agonist stimulation of GPCRs, such as β2AR (Mumbey et al., 1994) or 5-hydroxytryptamine4 receptor (Chen and Manning, 2000). Palmitoylation also influences membrane association, subcellular localization, and protein-protein interactions of Gα subunits. For example, palmitoylation regulates Gαq and Gα12/13 attachment to the membrane and signaling by controlling interactions with cognate receptors or Goβγ (Wedegaertner et al., 1993; Edgerton et al., 1994; Iiri et al., 1996; Sikarwar et al., 2014). A recent study involving Gα1 showed that palmitoylation regulates selective association with membrane microdomains having different compositions of fatty acids (Alvarez et al., 2015).

Palmitoylation exhibits various effects on receptor endocytosis. First, palmitoylation is required for the endocytosis of thyrotropin-releasing hormone receptor (Groarke et al., 2001), somatostatin receptor 5 (Hukovic et al., 1998), PAR2 (Adams et al., 2011), and dopamine D3 receptor (Zhang et al., 2016b). Second, palmitoylation has minimal or no effects on endocytosis of some GPCRs, such as β2AR (Moffett et al., 1993), α2AR (Gao et al., 1999), and C-C chemokine receptor type 5 (Blanpain et al., 2001). Third, palmitoylation has inhibitory effects on the endocytosis of luteinizing hormone/human chorionic gonadotropin receptor (Kawate and Menon, 1994) and V1a vasopressin receptor (Hawtin et al., 2001).

Interestingly, mutation of palmitoylation sites in the α2bAR does not affect receptor endocytosis, but completely inhibits agonist-induced downregulation (Eason et al., 1994). More diverse functional roles and palmitoylation sites were reported for β2AR, including mutation of the previously established palmitoylation site Cys341, which does not affect receptor endocytosis, but alters the endocytic route to a β-arrestin-independent and caveola-dependent pathway (Liu et al., 2012). A recent study showed that β2AR, in response to agonist treatment, is palmitoylated at Cys265 via palmitate transferase, which is localized within the Golgi complex (Adachi et al., 2016).

As discussed, GPCR post-translational modifications affect various receptor functions, including cell-surface expression, signaling, endocytosis, and agonist affinity. Caution is needed when interpreting the functional consequences of post-translational modifications, given the possibility that the effects of palmitoylation could be indirect and secondary to those on receptor surface expression.

**FUNCTIONAL ROLES OF GPCR ENDOCYTOSIS**

Defects in GPCR signaling have been implicated in multiple human diseases, including autoimmunity, vascular diseases, and cancer (Rosenthal et al., 1993; O’Hayre et al., 2014). Because receptor trafficking is crucial for the temporal and spatial control of GPCR signaling, GPCR endocytosis could exhibit various degrees of physiological and pathological importance (Roth et al., 1998; Shapiro et al., 2000; von Zastrow, 2001; Boedden et al., 2004).

Receptor endocytosis, which results in a decreased number of receptors on the cell surface, can be perceived as a mechanism of negative feedback to protect cells from agonistic overstimulation (Sibley and Lefkowitz, 1985). It is generally accepted that receptors are desensitized (uncoupled with
effectors) within seconds to minutes after agonist stimulation via receptor phosphorylation and association with β-arrestins (Sibley et al., 1987; Dang and Christie, 2012). According to this molecular paradigm, most receptors undergoing endocytosis are already desensitized (phosphorylated and bound to β-arrestins), and are dephosphorylated by protein phosphatase type A2 in acidic environments. Thereafter, receptors recycle back to the cell surface in a resensitized state or are degraded in the lysosome (Menard et al., 1996; McDonald et al., 2000). Therefore, combined with receptor recycling, the main functional role of receptor endocytosis is considered to be a restoration of receptor responsiveness rather than a decrement in signaling (Yu et al., 1993; Pippig et al., 1995; Cho et al., 2010). However, endocytosis/recycling might not be mandatory for the resensitization of receptor responsiveness, because receptors can be rapidly dephosphorylated on the cell surface without the need for endocytosis and recycling (Nelson et al., 2007). Thus, interpretations of experimental results related to receptor endocytosis need to be handled with precaution.

The uncertainty in the consequences of receptor endocytosis might arise from limitations in experimental design (Connor et al., 2004), especially when experiments involve receptor overexpression or employ assays in which intense amplification of signaling processes occurs. Under these conditions, the effects of a decrease in the number of receptors on the cell surface would be underestimated. Another problem is employment of endocytic inhibitors or mutant receptors to make conclusions about the functional meaning of receptor endocytosis without their endocytic properties having been fully characterized (Yu et al., 1993; Pippig et al., 1995; Hanley and Hensler, 2002; Cho et al., 2010).

Although it is difficult to completely overcome the obstacles in selective regulation of specific endocytic routes, several points need to be carefully considered. First, the selectivity of inhibitors for specific endocytic routes (e.g., CME or caveolar endocytosis) is always relative (Guo et al., 2015), and at the same time there could be mutual interactions between endocytic pathways (Rodal et al., 1999; Subtil et al., 1999). Second, pharmacological and molecular biological blockade of endocytic processes can inhibit endocytosis of a GPCR, but simultaneously affect other membrane proteins. For example, it was initially concluded that ERK activation requires GPCR endocytosis (Luttrell et al., 1997). Later, it was suggested that the endocytosis of epidermal growth factor receptor (EGFR), which crossesstalks with GPCRs, is required for ERK activation (Vieira et al., 1996; DeGraff et al., 1999; Pierce et al., 2000). There still exist some conflicting issues. For example, studies on protease-activated receptor (PAR)1, the neurokinin-1 receptor, and the angiotensin 1A receptor showed that internalized GPCRs form complexes on internal membranes via β-arrestin, with downstream components of the mitogen-activated protein kinase-signaling pathway, including Raf1, meiosis-specific serine/threonine-protein kinase (MEK)1, and ERK2 (DeFea et al., 2000; Luttrell et al., 2001; Teis et al., 2002). Based on these observations, the authors suggested that endocytosis of other signaling components, such as phosphorylated MEK rather than activated GPCRs or EGFRs might be required for ERK activation. Third, some experiments inevitably need to be conducted using indirect approaches that may alter protein function. For example, determination of roles associated with receptor phosphorylation through site-directed mutagenesis of potential and consensus phosphorylation sites or through abolishment of endogenous protein kinases. The introduction of point mutations could affect other aspects of receptor function in conjunction with receptor phosphorylation. Moreover, knockdown or knockout of endogenous protein kinases not only affects receptor phosphorylation, but also other cellular proteins, which could directly or indirectly affect receptor functions. Finally, studies using HEK-293 cells suggested that heterogeneity within the same cell line or among different cells can introduce response diversity and add to the complexity of the regulatory mechanisms of each receptor (Lefkowitz et al., 2002).

**CONCLUSIONS**

GPCRs are the largest group of cell membrane receptors, with as many as 800-1000 different human genes predicted to encode GPCRs, resulting in highly variable structural features, signaling mechanisms, and tissue distribution. Nevertheless, the molecular mechanisms involved in the regulation of GPCR functions are perceived as being highly conserved, with only several protein kinases and a couple of arrestin isoforms playing central roles in receptor endocytosis. However, as described here, complicated regulatory mechanisms are involved in GPCR endocytosis through a multitude of cellular components and processes, including heterogeneity of endocytic routes, functional interactions between different endocytic processes and pathways, post-translational modifications of receptor and endocytic vehicles, as well as other regulatory components, such as small G proteins. Beside the intrinsic complexity of endocytic processes, a primary obstacle in this research area involves the lack of powerful experimental tools and techniques to dissect the critical events associated with endocytosis.

**ACKNOWLEDGMENTS**

This work was funded by KRF-2014R1A2A2A01002547.

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