BIOMOLECULES & THERAPEUTICS

Invited Review
Biomol Ther 25(1), 26-43 (2017)

Multifactorial Regulation of G Protein-Coupled Receptor Endocytosis

Xiaohan Zhang and Kyeong-Man Kim*

Pharmacology Laboratory, College of Pharmacy, Chonnam National University, Gwangju 61186, Republic of Korea

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, Ral, 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

Open Access **https://doi.org/10.4062/biomolther.2016.186**

Received Aug 18, 2016 **Revised** Nov 21, 2016 **Accepted** Nov 30, 2016
Published Online Jan 1, 2017

***Corresponding Author**
E-mail: kmkim@jnu.ac.kr
Tel: +82-62-530-2936, Fax: +82-62-530-2949

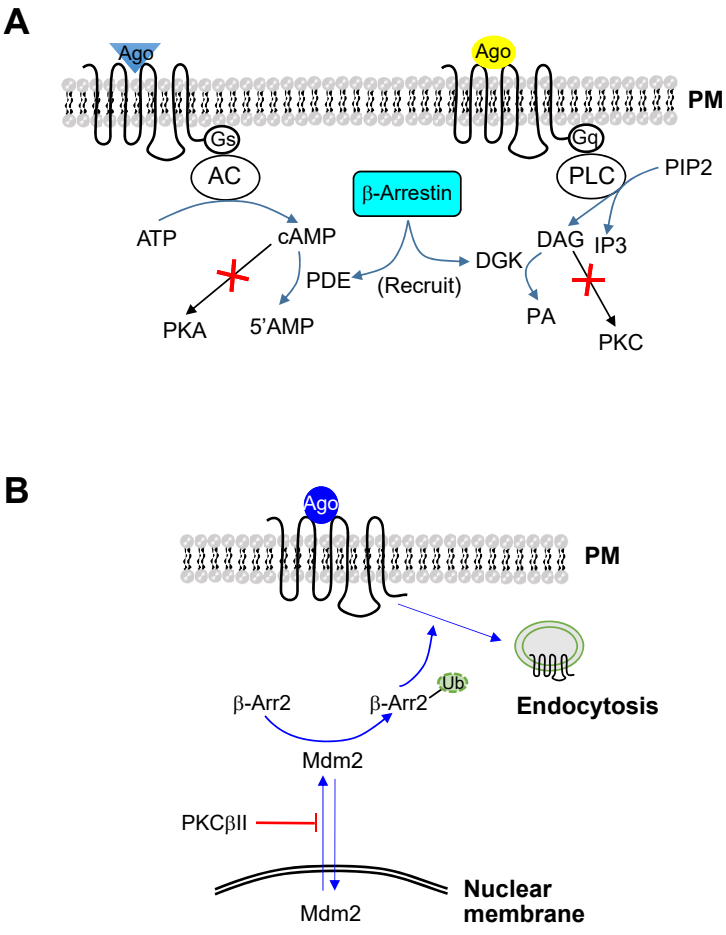

Fig. 1. Functional interactions between homologous and heterologous regulatory pathways of GPCRs. (A) Functional antagonism of β-arrestins on G_s- or G_q-coupled receptors downstream of receptor-G protein coupling. For G_s-coupled receptors, β-arrestins recruit phosphodiesterases to inhibit cAMP accumulation. For G_q-coupled receptors, β-arrestins recruit DGK to convert DAG to phosphatidic acid. (B) Effects of PKCβII on β-arrestin2 ubiquitination. PKCβII inhibits β-arrestin2 ubiquitination by interfering with the nuclear export of Mdm2, which occurs in response to agonist stimulation of GPCRs that have a tendency to undergo endocytosis. Ago, agonist; PM, plasma membrane; AC, adenylyl cyclase; PKA, protein kinase A; PDE, phosphodiesterase; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; DGK, diacylglycerol kinase; PA, phosphatidic acid; PKC, protein kinase C; β-Arr, β-arrestin; Ub, ubiquitin; Mdm2, Mouse double-minute-2 homolog; cAMP, cyclic adenosine monophosphate; GPCR, G protein-coupled receptor.

(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 β_2 adrenoceptor (β_2AR) 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 β-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-unoccupied 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

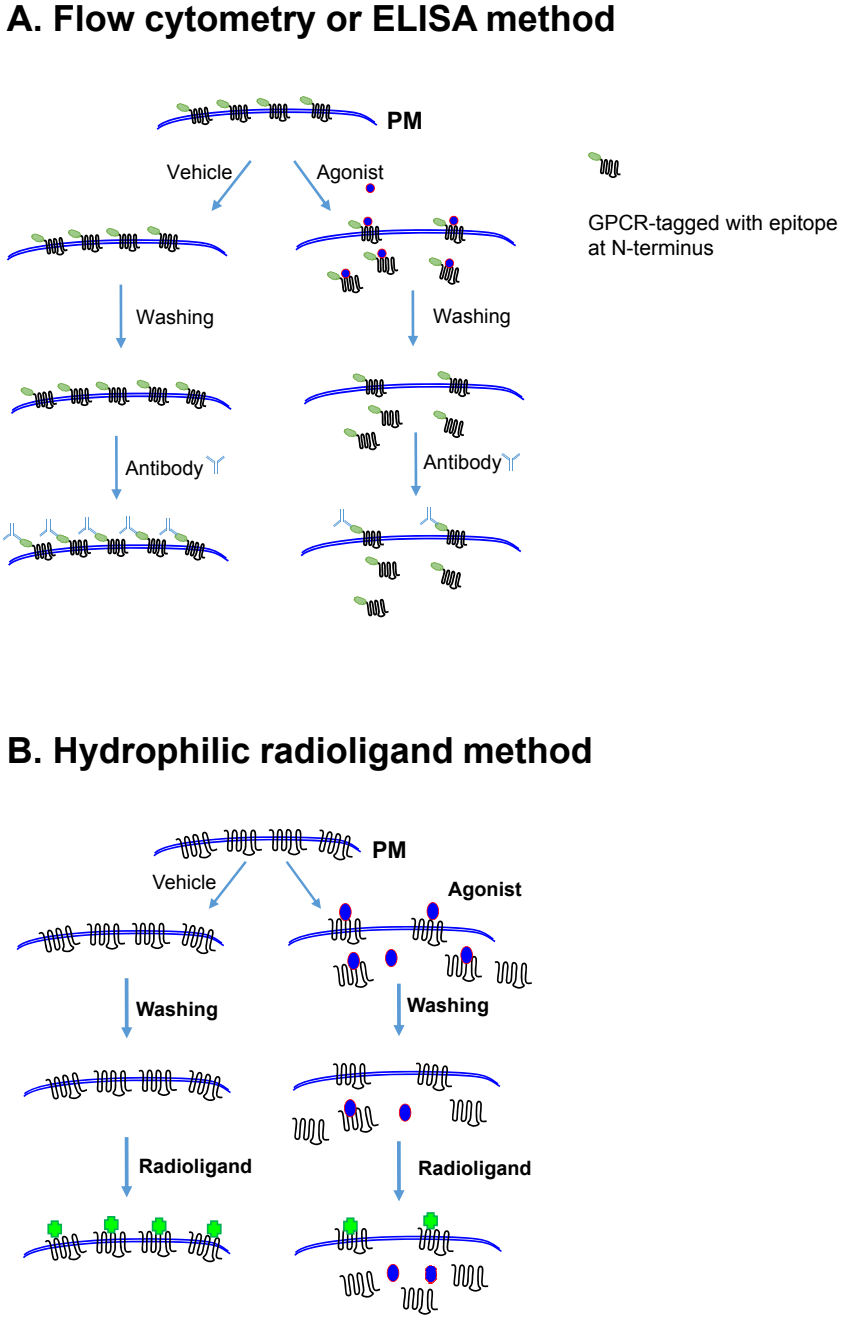

Fig. 2. Common methodologies used for determining GPCR endocytosis. (A) Determination of receptor endocytosis using epitope-tagged GPCRs at the N-terminus. This process involves induction of receptor internalization through agonist treatment for a desired period of time, washing, and labeling with primary and secondary antibodies. Receptors located on the cell surface are determined by flow cytometry or ELISA. According to the diagram, 60 percent of cell surface receptors are internalized. (B) Determination of receptor endocytosis using hydrophilic radioligands. This process involves induction of receptor endocytosis through agonist treatment for a desired period of time, thorough washing, and labeling with hydrophilic radioligands. Due to the hydrophilicity of radioligands, only receptors located on the cell surface are counted. According to the diagram, 60 percent of cell surface receptors are internalized. GPCR, G protein-coupled receptor; ELISA, enzyme-linked immunosorbent assay.

in heterologous regulatory processes remains elusive. Perhaps G_s- or G_q-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

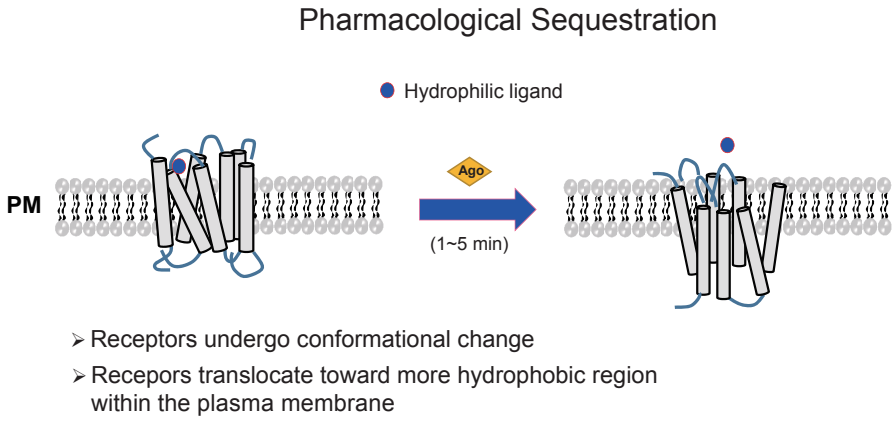

Fig. 3. Diagram showing the pharmacological sequestration of GPCRs. In pharmacological sequestration, receptors undergo conformational changes and translocate toward more hydrophobic regions within the plasma membrane. Pharmacologically sequestered receptors do not actually translocate to the cytosol, 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, GRK2/3-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 (Winstel *et al*., 1996; Cong *et al*., 2001). A subsequent study showed that GRK2 is phosphorylated by PKCα, PKCδ, and PKCδ *in vitro*, 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-arrestin2-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

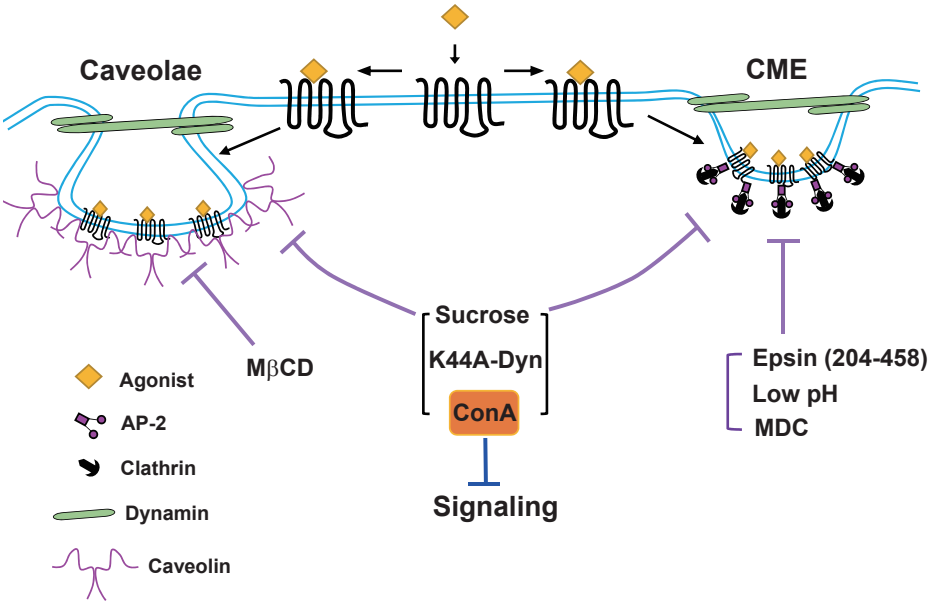

Fig. 4. Selectivity of endocytic inhibitors on clathrin-mediated and caveolae-dependent endocytosis. Epsin (204-458), low pH, and MDC can be used to block CME. MβCD is selective for caveolar endocytosis. Careful dosage adjustment is needed for MDC and MβCD. Sucrose, K44A-dynamin, and Con A do not show selectivity. Con A inhibits GPCR signaling independent of its effects on endocytosis. Epsin, epidermal growth factor receptor-pathway substrate 15-interacting protein; AP-2, adaptor protein 2; CME, clathrin-mediated endocytosis; Con A, concanavalin A; MDC, monodansylcadaverine; MβCD, methyl-β-cyclodextrin.

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 difficultly 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

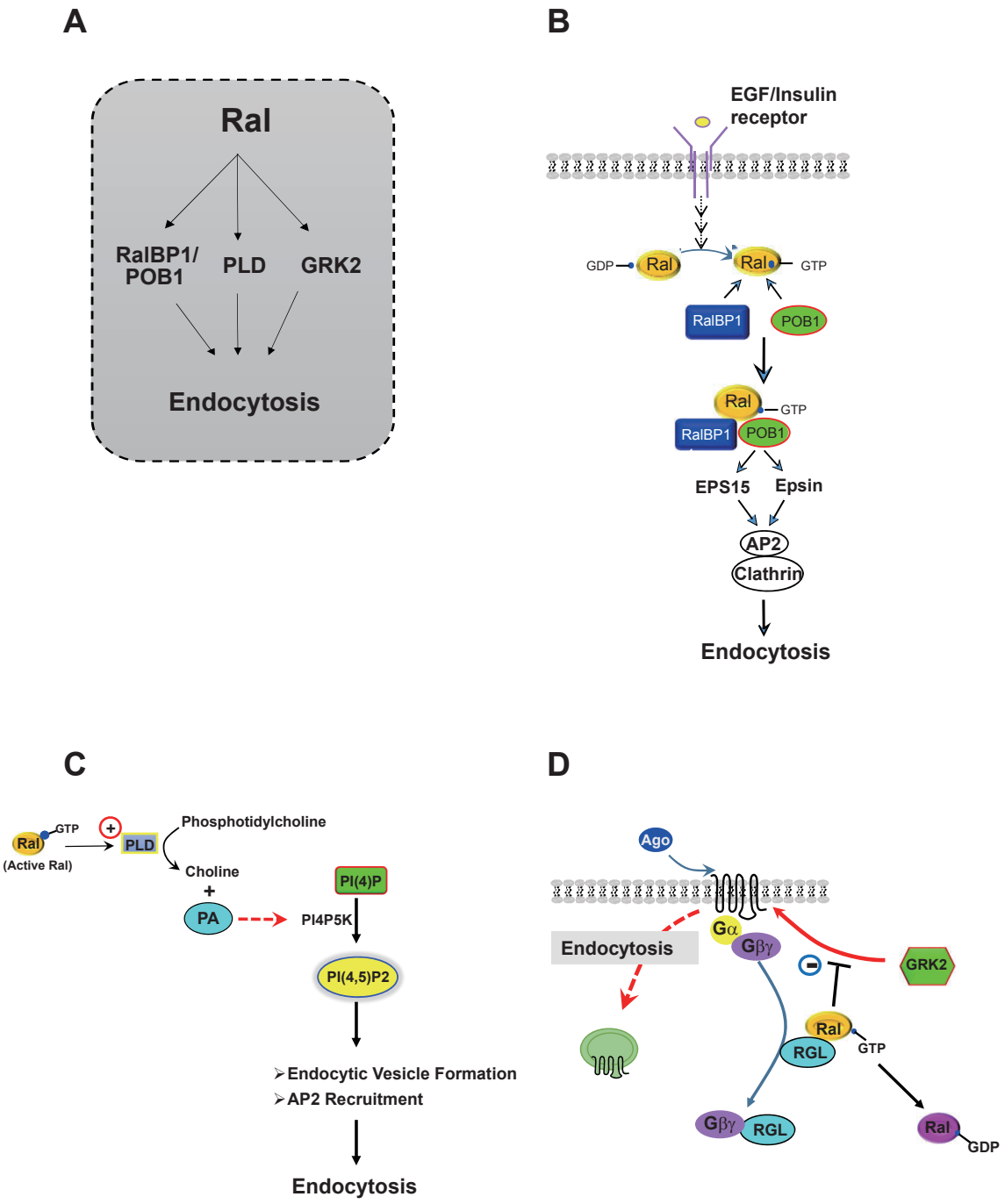

Fig. 5. Different modes of Ral regulation of receptor endocytosis. (A) Three downstream effectors of Ral in the regulation of receptor endocytosis. (B) RalBP1 (formerly known as RLIP76 or Ral-interacting protein) is involved in Ral-mediated regulation of endocytosis of receptors, such as EGF receptor 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. RalBP1, 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.

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 radioligands 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 D_3 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 (Claing *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 α_2 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 IC_{50} 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

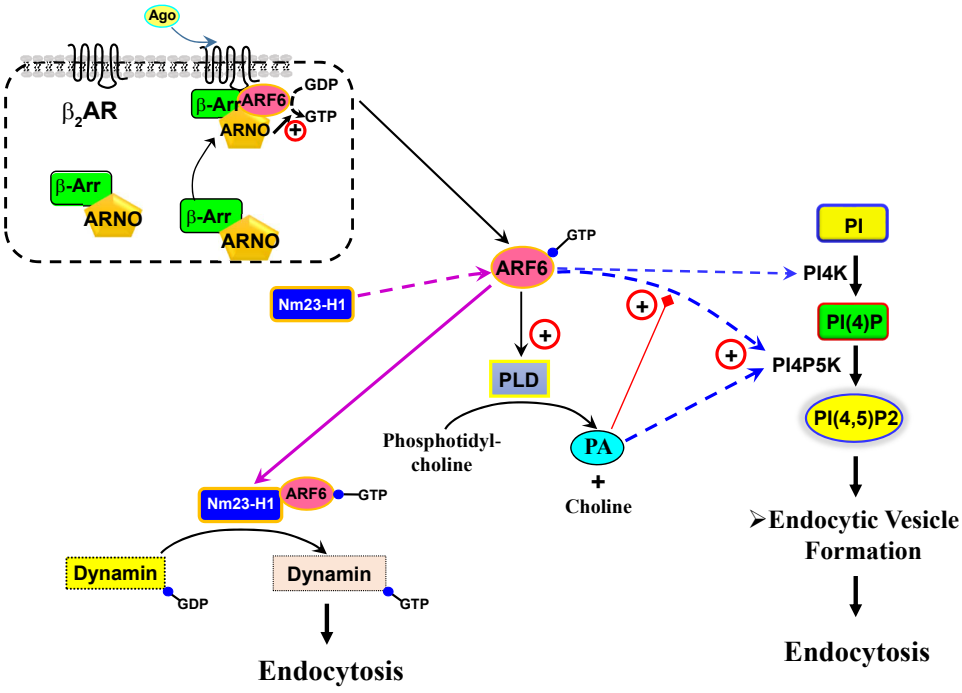

Fig. 6. Roles of Arf6 in regulating GPCR endocytosis. Studies of β_2AR indicated β-arrestin involvement in recruiting ARNO to convert GDP-bound Arf to the GTP-bound form. GTP-bound Arf6 regulates different routes of receptor endocytosis. For example, by recruiting NM23-H1, Arf6 converts GDP-dynamin to GTP-dynamin to enhance receptor endocytosis. GTP-Arf6 activates PI(4)P5K to convert PI(4)P to PI(4,5)P2. Arf6 protein also can activate PI4K to convert PI to PI(4)P. Additionally, GTP-Arf6 activates PLD2 to produce PA. ADP, adenosine diphosphate; Arf, ADP-ribosylation factor; ARNO, Arf nucleotide-binding-site opener; GDP, guanosine diphosphate; GTP, guanosine triphosphate; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4)P5K, phosphatidylinositol 4-phosphate 5-kinase; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI4K, phosphatidylinositol 4-kinase; NM23-H1, nucleoside diphosphate kinase; PA, phosphatidic acid; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; PLD, phospholipase D.

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 G_s, G_i, $G_{q/11}$, and $G_{12/13}$. 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 adenosine 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 (Chardin 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 Rho 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; Matsuzaki *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 [PI(4)P5K] (Jenkins *et al*., 1994; Antonescu *et al*., 2010). PI(4)P5K is a type I lipid kinase that generates the lipid second messenger phospholipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], which is critically important in clathrin-coated pit dynamics (Zoncu *et al*., 2007; Nakatsu *et*

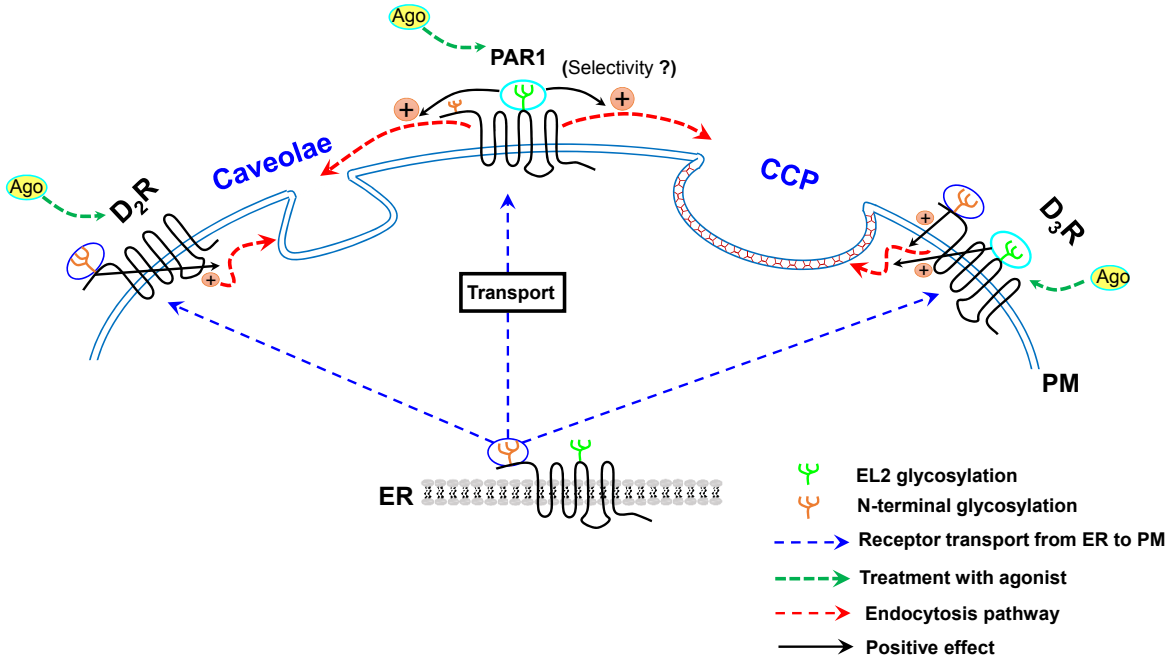

Fig. 7. Glycosylation in the regulation of receptor endocytosis. Glycosylation on the N-terminus of GPCRs is involved in GPCR localization on the plasma membrane. Studies on dopamine D_3 receptor and PARs suggest that glycosylation on different regions of the receptor is involved in the regulation of different receptor functions. Glycosylation on the N-terminus of D_2R, D_3R, or PAR1 is required for cell-membrane localization. Glycosylation on the N-terminus of D_2R and D_3R mediates their endocytosis within caveolae and CCP microdomains of the plasma membrane, respectively. Glycosylation within the second extracellular loop of D_3R and PAR1 is important for internalization. (Selectivity?) next to PAR1 represents that it is not known whether palmitoylation on the second extracellular loop of PAR1 mediates CME or caveolar endocytosis. GPCR, G protein-coupled receptor; D_3R, dopamine D_3 receptor; D_2R, dopamine D_2 receptor; PAR, protease activator receptor; CCP, clathrin-coated pit.

al., 2010). 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 Gβγ-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; Olkkonen 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 β_2AR (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 M_3 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 (D'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 application 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 (Houndolo *et al*., 2005). For example, in response to agonist stimulation of β_2AR, 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 β_2AR 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 phosphatidylcholine 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)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)P5K (Czech, 2000), and PI(4)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-ruffle 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 (Wenk *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; Adlanmerini *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; Lahaie *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 D_2 and D_3 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 urokinase 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 D_3 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 D_3R, 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 D_3 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; Adlanmerini *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 β_2AR (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 (Zheng *et al*., 2012). β_2AR 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 α_{2A}AR (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 β_2AR (Mumby *et al*., 1994) or 5-hydroxytryptamine$_{1A}$ 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 Gαs attachment to the membrane and signaling by controlling interactions with cognate receptors or G$\beta\gamma$ (Wedegaertner *et al*., 1993; Edgerton *et al*., 1994; Iiri *et al*., 1996; Sikarwar *et al*., 2014). A recent study involving Gαi 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 D_3 receptor (Zhang *et al*., 2016b). Second, palmitoylation has minimal or no effects on endocytosis of some GPCRs, such as β_2AR (Moffett *et al*., 1993), α_1AR (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 choriogonadotropin receptor (Kawate and Menon, 1994) and V_{1A} vasopressin receptor (Hawtin *et al*., 2001).

Interestingly, mutation of palmitoylation sites in the α_{2A}AR 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 β_2AR, 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 caveolae-dependent pathway (Liu *et al*., 2012). A recent study showed that β_2AR, 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; Booden *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 crosstalks 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 isotypes 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.

REFERENCES

Acconcia, F., Ascenzi, P., Bocedi, A., Spisni, E., Tomasi, V., Trentalance, A., Visca, P. and Marino, M. (2005) Palmitoylation-dependent estrogen receptor α membrane localization: regulation by 17β-estradiol. *Mol. Biol. Cell* **16**, 231-237.

Adachi, N., Hess, D. T., McLaughlin, P. and Stamler, J. S. (2016) S-palmitoylation of a novel site in the β2-adrenergic receptor associated with a novel intracellular itinerary. *J. Biol. Chem.* **291**, 20232-20246.

Adams, M. N., Christensen, M. E., He, Y., Waterhouse, N. J. and Hooper, J. D. (2011) The role of palmitoylation in signalling, cellular trafficking and plasma membrane localization of protease-activated receptor-2. *PLoS ONE* **6**, e28018.

Adlanmerini, M., Solinhac, R., Abot, A., Fabre, A., Raymond-Letron, I., Guihot, A. L., Boudou, F., Sautier, L., Vessières, E., Kim, S.H, Lière, P., Fontaine, C., Krust, A., Chambon, P., Katzenellenbogen, J. A., Gourdy, P., Shaul, P. W., Henrion, D., Arnal, J. F. and Lenfant, F. (2014) Mutation of the palmitoylation site of estrogen receptor α *in vivo* reveals tissue-specific roles for membrane versus nuclear actions. *Proc. Natl. Acad. Sci. U.S.A.* **111**, E283-E290.

Alaluf, S., Mulvihill, E. R. and McIlhinney, R. A. (1995) Palmitoylation of metabotropic glutamate receptor subtype 4 but not 1 α expressed

in permanently transfected BHK cells. *Biochem. Soc. Trans.* **23**, 87S.
Alvarez, R., López, D. J., Casas, J., Lladó, V., Higuera, M., Nagy, T., Barceló, M., Busquets, X. and Escribá, P. V. (2015) G protein-membrane interactions I: Gαi1 myristoyl and palmitoyl modifications in protein-lipid interactions and its implications in membrane microdomain localization. *Biochim. Biophys. Acta* **1851**, 1511-1520.
Anderson, R. G. (1998) The caveolae membrane system. *Annu. Rev. Biochem.* **67**, 199-225.
Antonescu, C. N., Danuser, G. and Schmid, S. L. (2010) Phosphatidic acid plays a regulatory role in clathrin-mediated endocytosis. *Mol. Biol. Cell* **21**, 2944-2952.
Arriza, J. L., Dawson, T. M., Simerly, R. B., Martin, L. J., Caron, M. G., Snyder, S. H. and Lefkowitz, R. J. (1992) The G-protein-coupled receptor kinases β ARK1 and β ARK2 are widely distributed at synapses in rat brain. *J. Neurosci.* **12**, 4045-4055.
Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G. and Lefkowitz, R. J. (1992) β-arrestin2, a novel member of the arrestin/β-arrestin gene family. *J. Biol. Chem.* **267**, 17882-17890.
Aziziyeh, A. I., Li, T. T., Pape, C., Pampillo, M., Chidiac, P., Possmayer, F., Babwah, A. V. and Bhattacharya, M. (2009) Dual regulation of lysophosphatidic acid (LPA1) receptor signalling by Ral and GRK. *Cell. Signal.* **21**, 1207-1217.
Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J. and Caron, M. G. (1994) A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β2-adrenergic receptor sequestration. *J. Biol. Chem.* **269**, 2790-2795.
Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G. and Lefkowitz, R. J. (1989) β-adrenergic receptor kinase: primary structure delineates a multigene family. *Science* **246**, 235-240.
Benovic, J. L., Kühn, H., Weyand, I., Codina, J., Caron, M. G. and Lefkowitz, R. J. (1987) Functional desensitization of the isolated β-adrenergic receptor by the β-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8879-8882.
Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M. G. and Lefkowitz, R. J. (1985) Phosphorylation of the mammalian β-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. *J. Biol. Chem.* **260**, 7094-7101.
Benovic, J. L., Strasser, R. H., Caron, M. G. and Lefkowitz, R. J. (1986) β-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2797-2801.
Bhattacharya, M., Babwah, A. V., Godin, C., Anborgh, P. H., Dale, L. B., Poulter, M. O. and Ferguson, S. S. (2004b) Ral and phospholipase D_2-dependent pathway for constitutive metabotropic glutamate receptor endocytosis. *J. Neurosci.* **24**, 8752-8761.
Bhattacharya, M., Babwah, A. V. and Ferguson, S. S. (2004a) Small GTP-binding protein-coupled receptors. *Biochem. Soc. Trans.* **32**, 1040-1044.
Bizzozero, O. A. (1997) The mechanism and functional roles of protein palmitoylation in the nervous system. *Neuropediatrics* **28**, 23-26.
Blanpain, C., Wittamer, V., Vanderwinden, J. M., Boom, A., Renneboog, B., Lee, B., Le Poul, E., El Asmar, L., Govaerts, C., Vassart, G., Doms, R. W. and Parmentier, M. (2001) Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. *J. Biol. Chem.* **276**, 23795-23804.
Böer, U., Neuschäfer-Rube, F., Möller, U. and Püschel, G. P. (2000) Requirement of N-glycosylation of the prostaglandin E2 receptor EP3β for correct sorting to the plasma membrane but not for correct folding. *Biochem. J.* **350 Pt 3**, 839-847.
Boname, J. M., Thomas, M., Stagg, H. R., Xu, P., Peng, J. and Lehner, P. J. (2010) Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. *Traffic* **11**, 210-220.
Booden, M. A., Eckert, L. B., Der, C. J. and Trejo, J. (2004) Persistent signaling by dysregulated thrombin receptor trafficking promotes breast carcinoma cell invasion. *Mol. Cell. Biol.* **24**, 1990-1999.
Bos, J. L. (1998) All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO J.* **17**, 6776-6782.
Boulware, M. I., Weick, J. P., Becklund, B. R., Kuo, S. P., Groth, R. D. and Mermelstein, P. G. (2005) Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. *J. Neurosci.* **25**, 5066-5078.
Bremnes, T., Paasche, J. D., Mehlum, A., Sandberg, C., Bremnes, B. and Attramadal, H. (2000) Regulation and intracellular trafficking pathways of the endothelin receptors. *J. Biol. Chem.* **275**, 17596-1604.
Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* **70**, 715-728.
Budd, D. C., Rae, A. and Tobin, A. B. (1999) Activation of the mitogen-activated protein kinase pathway by a Gq/11-coupled muscarinic receptor is independent of receptor internalization. *J. Biol. Chem.* **274**, 12355-12360.
Burgoyne, R. D. (1989) Small GTP-binding proteins. *Trends Biochem. Sci.* **14**, 394-396.
Cavenagh, M. M., Whitney, J. A., Carroll, K., Zhang, C. J., Boman, A. L., Rosenwald, A. G., Mellman, I. and Kahn, R. A. (1996) Intracellular distribution of Arf proteins in mammalian cells. Arf6 is uniquely localized to the plasma membrane. *J. Biol. Chem.* **271**, 21767-21774.
Chardin, P. and Tavitian, A. (1986) The ral gene: a new ras related gene isolated by the use of a synthetic probe. *EMBO J.* **5**, 2203-2208.
Chen, C. A. and Manning, D. R. (2000) Regulation of gα i palmitoylation by activation of the 5-hydroxytryptamine-1A receptor. *J. Biol. Chem.* **275**, 23516-23522.
Cho, D., Zheng, M., Min, C., Ma, L., Kurose, H., Park, J. H. and Kim, K. M. (2010) Agonist-induced endocytosis and receptor phosphorylation mediate resensitization of dopamine D_2 receptors. *Mol. Endocrinol.* **24**, 574-586.
Cho, D. I., Beom, S., Van Tol, H. H., Caron, M. G. and Kim, K. M. (2006) Characterization of the desensitization properties of five dopamine receptor subtypes and alternatively spliced variants of dopamine D_2 and D_4 receptors. *Biochem. Biophys. Res. Commun.* **350**, 634-640.
Chow, J. C., Condorelli, G. and Smith, R. J. (1998) Insulin-like growth factor-I receptor internalization regulates signaling via the Shc/mitogen-activated protein kinase pathway, but not the insulin receptor substrate-1 pathway. *J. Biol. Chem.* **273**, 4672-4680.
Claing, A., Chen, W., Miller, W. E., Vitale, N., Moss, J., Premont, R. T. and Lefkowitz, R. J. (2001) β-Arrestin-mediated ADP-ribosylation factor 6 activation and β 2-adrenergic receptor endocytosis. *J. Biol. Chem.* **276**, 42509-42513.
Claing, A., Laporte, S. A., Caron, M. G. and Lefkowitz, R. J. (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and β-arrestin proteins. *Prog. Neurobiol.* **66**, 61-79.
Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J. and Johnson, J. A. (1988) Activation of cAMP-dependent protein kinase is required for heterologous desensitization of adenylyl cyclase in S49 wild-type lymphoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1442-1446.
Conaway, R. C., Brower, C. S. and Conaway, J. W. (2002) Emerging roles of ubiquitin in transcription regulation. *Science* **296**, 1254-1258.
Cong, M., Perry, S. J., Lin, F. T., Fraser, I. D., Hu, L. A., Chen, W., Pitcher, J. A., Scott, J. D. and Lefkowitz, R. J. (2001) Regulation of membrane targeting of the G protein-coupled receptor kinase 2 by protein kinase A and its anchoring protein AKAP79. *J. Biol. Chem.* **276**, 15192-15199.
Connor, M., Osborne, P. B. and Christie, M. J. (2004) Mu-opioid receptor desensitization: is morphine different? *Br. J. Pharmacol.* **143**, 685-696.
Cosson, P., de Curtis, I., Pouysségur, J., Griffiths, G. and Davoust, J. (1989) Low cytoplasmic pH inhibits endocytosis and transport from the trans-Golgi network to the cell surface. *J. Cell Biol.* **108**, 377-387.

Czech, M. P. (2000) PIP2 and PIP3: complex roles at the cell surface. *Cell* **100**, 603-606.

Dang, V. C. and Christie, M. J. (2012) Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. *Br. J. Pharmacol.* **165**, 1704-1716.

Davies, P. J., Davies, D. R., Levitzki, A., Maxfield, F. R., Milhaud, P., Willingham, M. C. and Pastan, I. H. (1980) Transglutaminase is essential in receptor-mediated endocytosis of α2-macroglobulin and polypeptide hormones. *Nature* **283**, 162-167.

DeFea, K. A., Zalevsky, J., Thoma, M. S., Déry, O., Mullins, R. D. and Bunnett, N. W. (2000) β-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J. Cell Biol.* **148**, 1267-1281.

DeGraff, J. L., Gagnon, A. W., Benovic, J. L. and Orsini, M. J. (1999) Role of arrestins in endocytosis and signaling of α2-adrenergic receptor subtypes. *J. Biol. Chem.* **274**, 11253-11259.

Doherty, G. J. and McMahon, H. T. (2009) Mechanisms of endocytosis. *Annu. Rev. Biochem.* **78**, 857-902.

Donaldson, J. G., Cassel, D., Kahn, R. A. and Klausner, R. D. (1992) ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein β-COP to Golgi membranes. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6408-6412.

Dores, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., Meerloo, T. and Trejo, J. (2012) ALIX binds a YPX_3L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *J. Cell Biol.* **197**, 407-419.

Dores, M. R., Grimsey, N. J., Mendez, F. and Trejo, J. (2016) ALIX Regulates the Ubiquitin-Independent Lysosomal Sorting of the P2Y1 Purinergic Receptor via a YPX_3L Motif. *PLoS ONE* **11**, e0157587.

Dutta, D., Williamson, C. D., Cole, N. B. and Donaldson, J. G. (2012) Pitstop 2 is a potent inhibitor of clathrin-independent endocytosis. *PLoS ONE* **7**, e45799.

D'Souza-Schorey, C., Li, G., Colombo, M. I. and Stahl, P. D. (1995) A regulatory role for ARF6 in receptor-mediated endocytosis. *Science* **267**, 1175-1178.

Eason, M. G., Jacinto, M. T., Theiss, C. T. and Liggett, S. B. (1994) The palmitoylated cysteine of the cytoplasmic tail of α2A-adrenergic receptors confers subtype-specific agonist-promoted downregulation. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11178-11182.

Edgerton, M. D., Chabert, C., Chollet, A. and Arkinstall, S. (1994) Palmitoylation but not the extreme amino-terminus of Gq α is required for coupling to the NK2 receptor. *FEBS Lett.* **354**, 195-199.

Feig, L. A., Urano, T. and Cantor, S. (1996) Evidence for a Ras/Ral signaling cascade. *Trends Biochem. Sci.* **21**, 438-441.

Ferguson, S. S. (2007) Phosphorylation-independent attenuation of GPCR signalling. *Trends Pharmacol. Sci.* **28**, 173-179.

Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Ménard, L. and Caron, M. G. (1996) Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271**, 363-366.

Filipek, S., Stenkamp, R. E., Teller, D. C. and Palczewski, K. (2003) G protein-coupled receptor rhodopsin: a prospectus. *Annu. Rev. Physiol.* **65**, 851-879.

Fukata, M., Fukata, Y., Adesnik, H., Nicoll, R. A. and Bredt, D. S. (2004) Identification of PSD-95 palmitoylating enzymes. *Neuron* **44**, 987-996.

Funakoshi, Y., Hasegawa, H. and Kanaho, Y. (2011) Regulation of PIP5K activity by Arf6 and its physiological significance. *J. Cell. Physiol.* **226**, 888-895.

Gao, Z., Ni, Y., Szabo, G. and Linden, J. (1999) Palmitoylation of the recombinant human A1 adenosine receptor: enhanced proteolysis of palmitoylation-deficient mutant receptors. *Biochem. J.* **342**, 387-895.

Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**, 615-649.

Glickman, M. H. and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **82**, 373-428.

Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D. and De Matteis, M. A. (1999) ARF mediates recruitment of PtdIns-4-OH kinase-β and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. *Nat. Cell Biol.* **1**, 280-287.

Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H. and Benovic, J. L. (1996) β-arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor. *Nature* **383**, 447-450.

Greaves, J. and Chamberlain, L. H. (2011) DHHC palmitoyl transferases: substrate interactions and (patho) physiology. *Trends Biochem. Sci.* **36**, 245-253.

Groarke, D. A., Drmota, T., Bahia, D. S., Evans, N. A., Wilson, S. and Milligan, G. (2001) Analysis of the C-terminal tail of the rat thyrotropin-releasing hormone receptor-1 in interactions and cointernalization with beta-arrestin 1-green fluorescent protein. *Mol. Pharmacol.* **59**, 375-385.

Guo, S., Zhang, X., Zheng, M., Zhang, X., Min, C., Wang, Z., Cheon, S. H., Oak, M. H., Nah, S. Y. and Kim, K. M. (2015) Selectivity of commonly used inhibitors of clathrin-mediated and caveolae-dependent endocytosis of G protein-coupled receptors. *Biochim. Biophys. Acta* **1848**, 2101-2010.

Hanley, N. R. and Hensler, J. G. (2002) Mechanisms of ligand-induced desensitization of the 5-hydroxytryptamine(2A) receptor. *J. Pharmacol. Exp. Ther.* **300**, 468-477.

Hansen, S. H., Sandvig, K. and van Deurs, B. (1993) Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. *J. Cell Biol.* **121**, 61-72.

Hawtin, S. R., Tobin, A. B., Patel, S. and Wheatley, M. (2001) Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *J. Biol. Chem.* **276**, 38139-38146.

Hayashi, T., Thomas, G. M. and Huganir, R. L. (2009) Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. *Neuron* **64**, 213-226.

Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998) Dynamin-mediated internalization of caveolae. *J. Cell Biol.* **141**, 85-99.

Hicke, L. and Riezman, H. (1996) Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* **84**, 277-287.

Holtmann, M. H., Roettger, B. F., Pinon, D. I. and Miller, L. J. (1996) Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J. Biol. Chem.* **271**, 23566-23571.

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A. and Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521-532.

Houndolo, T., Boulay, P. L. and Claing, A. (2005) G protein-coupled receptor endocytosis in ADP-ribosylation factor 6-depleted cells. *J. Biol. Chem.* **280**, 5598-5604.

Huang, C. and Tai, H. H. (1998) Prostaglandin E2 receptor EP3α subtype: the role of N-glycosylation in ligand binding as revealed by site-directed mutagenesis. *Prostaglandins Leukot. Essent. Fatty Acids* **59**, 265-271.

Huang, J., Mahavadi, S., Sriwai, W., Grider, J. R. and Murthy, K. S. (2007) Cross-regulation of VPAC(2) receptor desensitization by M(3) receptors via PKC-mediated phosphorylation of RKIP and inhibition of GRK2. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G867-G874.

Hukovic, N., Panetta, R., Kumar, U., Rocheville, M. and Patel, Y. C. (1998) The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. *J. Biol. Chem.* **273**, 21416-21422.

Hutagalung, A. H. and Novick, P. J. (2011) Role of Rab GTPases in membrane traffic and cell physiology. *Physiol. Rev.* **91**, 119-149.

Iiri, T., Backlund, P. S., Jr., Jones, T. L., Wedegaertner, P. B. and Bourne, H. R. (1996) Reciprocal regulation of $G_s\alpha$ by palmitate and the βγ subunit. Proc. *Natl. Acad. Sci. U.S.A.* **93**, 14592-14597.

Itokawa, M., Toru, M., Ito, K., Tsuga, H., Kameyama, K., Haga, T., Arinami, T. and Hamaguchi, H. (1996) Sequestration of the short and long isoforms of dopamine D_2 receptors expressed in Chinese hamster ovary cells. *Mol. Pharmacol.* **49**, 560-566.

Ivanov, A. I. (2008) Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? *Methods Mol. Biol.* **440**, 15-33.
Jacob, C., Cottrell, G. S., Gehringer, D., Schmidlin, F., Grady, E. F. and Bunnett, N. W. (2005) c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. *J. Biol. Chem.* **280**, 16076-16087.
Jenkins, G. H., Fisette, P. L. and Anderson, R. A. (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.* **269**, 11547-11554.
Ji, S., Liu, X., Li, S., Shen, L., Li, F., Wang, J., Han, J. and Yao, L. (2003) PH domain of G protein-coupled receptor kinase-2 binds to protein kinase C (PKC) and negatively regulates activity of PKC kinase. *Front. Biosci.* **8**, a34-a39.
Jiang, H., Luo, J. Q., Urano, T., Frankel, P., Lu, Z., Foster, D. A. and Feig, L. A. (1995) Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* **378**, 409-412.
Jimenez-baranda, S., Gómez-Moutón, C., Rojas, A., Martínez-Prats, L., Mira, E., Ana Lacalle, R., Valencia, A., Dimitrov, D. S., Viola, A., Delgado, R., Martínez-A, C. and Mañes, S. (2007) Filamin-A regulates actin-dependent clustering of HIV receptors. *Nat. Cell Biol.* **9**, 838-846.
Jullien-Flores, V., Mahé, Y., Mirey, G., Leprince, C., Meunier-Bisceuil, B., Sorkin, A. and Camonis, J. H. (2000) RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. *J. Cell Sci.* **113**, 2837-2844.
Kawate, N. and Menon, K. M. (1994) Palmitoylation of luteinizing hormone/human choriogonadotropin receptors in transfected cells. Abolition of palmitoylation by mutation of Cys-621 and Cys-622 residues in the cytoplasmic tail increases ligand-induced internalization of the receptor. *J. Biol. Chem.* **269**, 30651-30658.
Kelly, B. T., Graham, S. C., Liska, N., Dannhauser, P. N., Höning, S., Ungewickell, E. J. and Owen, D. J. (2014) Clathrin adaptors. AP2 controls clathrin polymerization with a membrane-activated switch. *Science* **345**, 459-463.
Kelly, E., Bailey, C. P. and Henderson, G. (2008) Agonist-selective mechanisms of GPCR desensitization. *Br. J. Pharmacol.* **153 Suppl 1**, S379-S388.
Kennedy, M. E. and Limbird, L. E. (1993) Mutations of the α2A-adrenergic receptor that eliminate detectable palmitoylation do not perturb receptor-G-protein coupling. *J. Biol. Chem.* **268**, 8003-8011.
Kim, J., Ahn, S., Ren, X. R., Whalen, E. J., Reiter, E., Wei, H. and Lefkowitz, R. J. (2005) Functional antagonism of different G protein-coupled receptor kinases for β-arrestin-mediated angiotensin II receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1442-1447.
Kim, J. H., Cho, E. Y., Min, C., Park, J. H. and Kim, K. M. (2008) Characterization of functional roles of DRY motif in the 2nd intracellular loop of dopamine D2 and D3 receptors. *Arch. Pharm. Res.* **31**, 474-481.
Kim, J. H., Lee, S. D., Han, J. M., Lee, T. G., Kim, Y., Park, J. B., Lambeth, J. D., Suh, P. G. and Ryu, S. H. (1998) Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA. *FEBS Lett.* **430**, 231-235.
Kim, J. Y., Soede, R. D., Schaap, P., Valkema, R., Borleis, J. A., Van Haastert, P. J., Devreotes, P. N. and Hereld, D. (1997) Phosphorylation of chemoattractant receptors is not essential for chemotaxis or termination of G-protein-mediated responses. *J. Biol. Chem.* **272**, 27313-27318.
Kim, K. M., Pae, H. O., Zheng, M., Park, R., Kim, Y. M., Chung, H. T. (2007) Carbon monoxide induces heme oxygenase-1 via activation of protein kinase R-like endoplasmic reticulum kinase and inhibits endothelial cell apoptosis triggered by endoplasmic reticulum stress. *Circ. Res.* **101**, 919-927.
Kim, K. M., Valenzano, K. J., Robinson, S. R., Yao, W. D., Barak, L. S. and Caron, M. G. (2001) Differential regulation of the dopamine D2 and D3 receptors by G protein-coupled receptor kinases and β-arrestins. *J. Biol. Chem.* **276**, 37409-37414.
Koch, T., Brandenburg, L. O., Schulz, S., Liang, Y., Klein, J. and Hollt, V. (2003) ADP-ribosylation factor-dependent phospholipase D_2 activation is required for agonist-induced mu-opioid receptor endocytosis. *J. Biol. Chem.* **278**, 9979-9985.
Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M. and Lefkowitz, R. J. (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G$\beta\gamma$-mediated signaling. *J. Biol. Chem.* **269**, 6193-6197.
Kohno, T., Wada, A. and Igarashi, Y. (2002) N-glycans of sphingosine 1-phosphate receptor Edg-1 regulate ligand-induced receptor internalization. *FASEB J.* **16**, 983-992.
Kosugi, S. and Mori, T. (1996) Cysteine-699, a possible palmitoylation site of the thyrotropin receptor, is not crucial for cAMP or phosphoinositide signaling but is necessary for full surface expression. *Biochem. Biophys. Res. Commun.* **221**, 636-640.
Krasel, C., Dammeier, S., Winstel, R., Brockmann, J., Mischak, H. and Lohse, M. J. (2001) Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. *J. Biol. Chem.* **276**, 1911-1915.
Krauss, M., Kinuta, M., Wenk, M. R., De Camilli, P., Takei, K. and Haucke, V. (2003) ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Iγ. *J. Cell Biol.* **162**, 113-124.
Lahaie, N., Kralikova, M., Prézeau, L., Blahos, J. and Bouvier, M. (2016) Post-endocytotic deubiquitination and degradation of the metabotropic γ-aminobutyric acid receptor by the ubiquitin-specific protease 14. *J. Biol. Chem.* **291**, 7156-7170.
Lajoie, P. and Nabi, I. R. (2010) Lipid rafts, caveolae, and their endocytosis. *Int. Rev. Cell Mol. Biol.* **282**, 135-163.
Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G. and Barak, L. S. (1999) The β2-adrenergic receptor/βarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3712-3717.
Lawrence, J., Mundell, S. J., Yun, H., Kelly, E. and Venkateswarlu, K. (2005) Centaurin-α_1, an ADP-ribosylation factor 6 GTPase activating protein, inhibits β_2-adrenoceptor internalization. *Mol. Pharmacol.* **67**, 1822-1828.
Lefkowitz, R. J., Pierce, K. L. and Luttrell, L. M. (2002) Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Mol. Pharmacol.* **62**, 971-974.
Lichnerova, K., Kaniakova, M., Park, S. P., Skrenkova, K., Wang, Y. X., Petralia, R. S., Suh, Y. H. and Horak, M. (2015) Two N-glycosylation sites in the GluN1 subunit are essential for releasing N-methyl-d-aspartate (NMDA) receptors from the endoplasmic reticulum. *J. Biol. Chem.* **290**, 18379-18390.
Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, A. G. and Mumby, S. M. (1993) Lipid modifications of G proteins: α subunits are palmitoylated. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3675-3679.
Liu, R., Wang, D., Shi, Q., Fu, Q., Hizon, S. and Xiang, Y. K. (2012) Palmitoylation regulates intracellular trafficking of β2 adrenergic receptor/arrestin/phosphodiesterase 4D complexes in cardiomyocytes. *PLoS ONE* **7**, e42658.
Loerke, D., Mettlen, M., Yarar, D., Jaqaman, K., Jaqaman, H., Danuser, G. and Schmid, S. L. (2009) Cargo and dynamin regulate clathrin-coated pit maturation. *PLoS Biol.* **7**, e57.
Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G. and Lefkowitz, R. J. (1990) β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science* **248**, 1547-1550.
Lorenz, K., Lohse, M. J. and Quitterer, U. (2003) Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. *Nature* **426**, 574-579.
Luo, J. Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L. A., Morris, A. J., Kahn, R. A. and Foster, D. A. (1998) Functional association between Arf and RalA in active phospholipase D complex. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3632-3637.
Luttrell, L. M., Daaka, Y., Della Rocca, G. J. and Lefkowitz, R. J. (1997) G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *J. Biol. Chem.* **272**, 31648-31656.
Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L. and Lefkowitz, R. J. (2001) Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2449-2454.
Macia, E., Luton, F., Partisani, M., Cherfils, J., Chardin, P. and Franco,

M. (2004) The GDP-bound form of Arf6 is located at the plasma membrane. *J. Cell Sci.* **117**, 2389-2398.

Marchese, A., Chen, C., Kim, Y. M. and Benovic, J. L. (2003) The ins and outs of G protein-coupled receptor trafficking. *Trends Biochem. Sci.* **28**, 369-376.

Marotti, L. A., Jr., Newitt, R., Wang, Y., Aebersold, R. and Dohlman, H. G. (2002) Direct identification of a G protein ubiquitination site by mass spectrometry. *Biochemistry* **41**, 5067-5074.

Matsuzaki, T., Hanai, S., Kishi, H., Liu, Z., Bao, Y., Kikuchi, A., Tsuchida, K. and Sugino, H. (2002) Regulation of endocytosis of activin type II receptors by a novel PDZ protein through Ral/Ral-binding protein 1-dependent pathway. *J. Biol. Chem.* **277**, 19008-19018.

McCaffrey, P. G., Friedman, B. and Rosner, M. R. (1984) Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* **259**, 12502-12507.

McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J. and Lefkowitz, R. J. (2000) β-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**, 1574-1577.

McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M. and Smythe, E. (1998) A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. *Curr. Biol.* **8**, 34-45.

Meitzen, J., Luoma, J. I., Boulware, M. I., Hedges, V. L., Peterson, B. M., Tuomela, K., Britson, K. A. and Mermelstein, P. G. (2013) Palmitoylation of estrogen receptors is essential for neuronal membrane signaling. *Endocrinology* **154**, 4293-4304.

Menard, L., Ferguson, S. S., Barak, L. S., Bertrand, L., Premont, R. T., Colapietro, A. M., Lefkowitz, R. J. and Caron, M. G. (1996) Members of the G protein-coupled receptor kinase family that phosphorylate the β2-adrenergic receptor facilitate sequestration. *Biochemistry* **35**, 4155-4160.

Min, C., Zheng, M., Zhang, X., Caron, M. G. and Kim, K. M. (2013) Novel roles for β-arrestins in the regulation of pharmacological sequestration to predict agonist-induced desensitization of dopamine D3 receptors. *Br. J. Pharmacol.* **170**, 1112-1129.

Min, C., Zheng, M., Zhang, X., Guo, S., Kwon, K. J., Shin, C. Y., Kim, H. S., Cheon, S. H. and Kim, K. M. (2015) N-linked Glycosylation on the N-terminus of the dopamine D_2 and D3 receptors determines receptor association with specific microdomains in the plasma membrane. *Biochim. Biophys. Acta* **1853**, 41-51.

Mishra, S. and Murphy, L. J. (2004) Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. *J. Biol. Chem.* **279**, 23863-23868.

Mitchell, R., Robertson, D. N., Holland, P. J., Collins, D., Lutz, E. M. and Johnson, M. S. (2003) ADP-ribosylation factor-dependent phospholipase D activation by the M3 muscarinic receptor. *J. Biol. Chem.* **278**, 33818-33830.

Moffett, S., Adam, L., Bonin, H., Loisel, T. P., Bouvier, M. and Mouillac, B. (1996) Palmitoylated cysteine 341 modulates phosphorylation of the β2-adrenergic receptor by the cAMP-dependent protein kinase. *J. Biol. Chem.* **271**, 21490-21497.

Moffett, S., Mouillac, B., Bonin, H. and Bouvier, M. (1993) Altered phosphorylation and desensitization patterns of a human β2-adrenergic receptor lacking the palmitoylated Cys341. *EMBO J.* **12**, 349-356.

Moller, L. B., Pöllänen, J., Rønne, E., Pedersen, N. and Blasi, F. (1993) N-linked glycosylation of the ligand-binding domain of the human urokinase receptor contributes to the affinity for its ligand. *J. Biol. Chem.* **268**, 11152-11159.

Moore, C. A., Milano, S. K. and Benovic, J. L. (2007) Regulation of receptor trafficking by GRKs and arrestins. *Annu. Rev. Physiol.* **69**, 451-482.

Moss, J. and Vaughan, M. (1995) Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J. Biol. Chem.* **270**, 12327-12330.

Mostafapour, S., Kobilka, B. K. and von Zastrow, M. (1996) Pharmacological sequestration of a chimeric β3/β2 adrenergic receptor occurs without a corresponding amount of receptor internalization. *Recept. Signal Transduct.* **6**, 151-163.

Mott, H. R. and Owen, D. (2014) Structure and function of RLIP76 (RalBP1): an intersection point between Ras and Rho signalling. *Biochem. Soc. Trans.* **42**, 52-58.

Mukherjee, S., Ghosh, R. N. and Maxfield, F. R. (1997) Endocytosis. *Physiol. Rev.* **77**, 759-803.

Mumby, S. M., Kleuss, C. and Gilman, A. G. (1994) Receptor regulation of G-protein palmitoylation. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2800-2804.

Nabi, I. R. and Le, P. U. (2003) Caveolae/raft-dependent endocytosis. *J. Cell Biol.* **161**, 673-677.

Nakashima, S., Morinaka, K., Koyama, S., Ikeda, M., Kishida, M., Okawa, K., Iwamatsu, A., Kishida, S. and Kikuchi, A. (1999) Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. *EMBO J.* **18**, 3629-3642.

Nakatsu, F., Perera, R. M., Lucast, L., Zoncu, R., Domin, J., Gertler, F. B., Toomre, D. and De Camilli, P. (2010) The inositol 5-phosphatase SHIP2 regulates endocytic clathrin-coated pit dynamics. *J. Cell Biol.* **190**, 307-315.

Nelson, C. D., Perry, S. J., Regier, D. S., Prescott, S. M., Topham, M. K. and Lefkowitz, R. J. (2007) Targeting of diacylglycerol degradation to M1 muscarinic receptors by β-arrestins. *Science* **315**, 663-666.

Novick, P. and Zerial, M. (1997) The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.* **9**, 496-504.

Oddi, S., Dainese, E., Sandiford, S., Fezza, F., Lanuti, M., Chiurchiù, V., Totaro, A., Catanzaro, G., Barcaroli, D., De Laurenzi, V., Centonze, D., Mukhopadhyay, S., Selent, J., Howlett, A. C. and Maccarrone, M. (2012) Effects of palmitoylation of Cys(415) in helix 8 of the CB(1) cannabinoid receptor on membrane localization and signalling. *Br. J. Pharmacol.* **165**, 2635-2651.

O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. and Bouvier, M. (1989) Palmitoylation of the human β2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J. Biol. Chem.* **264**, 7564-7569.

O'Hayre, M., Degese, M. S. and Gutkind, J. S. (2014) Novel insights into G protein and G protein-coupled receptor signaling in cancer. *Curr. Opin. Cell Biol.* **27**, 126-135.

Okamoto, Y., Ninomiya, H., Tanioka, M., Sakamoto, A., Miwa, S. and Masaki, T. (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. *J. Biol. Chem.* **272**, 21589-21596.

Olkkonen, V. M. and Stenmark, H. (1997) Role of Rab GTPases in membrane traffic. *Int. Rev. Cytol.* **176**, 1-85.

Ong, S. T., Freeley, M., Skubis-Zegadło, J., Fazil, M. H., Kelleher, D., Fresser, F., Baier, G., Verma, N. K. and Long, A. (2014) Phosphorylation of Rab5a protein by protein kinase C is crucial for T-cell migration. *J. Biol. Chem.* **289**, 19420-19434.

Ooi, C. E., Dell'Angelica, E. C. and Bonifacino, J. S. (1998) ADP-Ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. *J. Cell Biol.* **142**, 391-402.

Orcl, L., Palmer, D. J., Amherdt, M. and Rothman, J. E. (1993) Coated vesicle assembly in the Golgi requires only coatomer and ARF proteins from the cytosol. *Nature* **364**, 732-734.

Palacios, F., Schweitzer, J. K., Boshans, R. L. and D'Souza-Schorey, C. (2002) ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. *Nat. Cell Biol.* **4**, 929-936.

Parenti, M., Vigano, M. A., Newman, C. M., Milligan, G. and Magee, A. I. (1993) A novel N-terminal motif for palmitoylation of G-protein α subunits. *Biochem. J.* **291**, 349-353.

Parton, R. G. and Simons, K. (2007) The multiple faces of caveolae. *Nat. Rev. Mol. Cell Biol.* **8**, 185-194.

Pearse, B. M. (1976) Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1255-1259.

Perry, S. J., Baillie, G. S., Kohout, T. A., McPhee, I., Magiera, M. M., Ang, K. L., Miller, W. E., McLean, A. J., Conti, M., Houslay, M. D. and Lefkowitz, R. J. (2002) Targeting of cyclic AMP degradation to β 2-adrenergic receptors by β-arrestins. *Science* **298**, 834-836.

Pfeffer, S. R. (1994) Rab GTPases: master regulators of membrane trafficking. *Curr. Opin. Cell Biol.* **6**, 522-526.

Pickart, C. M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **70**, 503-533.

Pierce, K. L., Maudsley, S., Daaka, Y., Luttrell, L. M. and Lefkowitz, R. J. (2000) Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1489-1494.

Pippig, S., Andexinger, S. and Lohse, M. J. (1995) Sequestration and recycling of β2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47**, 666-676.

Pitcher, J. A., Freedman, N. J. and Lefkowitz, R. J. (1998) G protein-coupled receptor kinases. *Annu. Rev. Biochem.* **67**, 653-692.

Poggi, M., Kara, I., Brunel, J. M., Landrier, J. F., Govers, R., Bonardo, B., Fluhrer, R., Haass, C., Alessi, M. C. and Peiretti, F. (2013) Palmitoylation of TNF α is involved in the regulation of TNF receptor 1 signalling. *Biochim. Biophys. Acta* **1833**, 602-612.

Pollok-Kopp, B., Hüttenrauch, F., Rethorn, S. and Oppermann, M. (2007) Dynamics of protein kinase C-mediated phosphorylation of the complement C5a receptor on serine 334. *J. Biol. Chem.* **282**, 4345-4353.

Posor, Y., Eichhorn-Grünig, M. and Haucke, V. (2015) Phosphoinositides in endocytosis. *Biochim. Biophys. Acta* **1851**, 794-804.

Qanbar, R. and Bouvier, M. (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol. Ther.* **97**, 1-33.

Rands, E., Candelore, M. R., Cheung, A. H., Hill, W. S., Strader, C. D. and Dixon, R. A. (1990) Mutational analysis of β-adrenergic receptor glycosylation. *J. Biol. Chem.* **265**, 10759-10764.

Rankovic, M., Jacob, L., Rankovic, V., Brandenburg, L. O., Schröder, H., Höllt, V. and Koch, T. (2009) ADP-ribosylation factor 6 regulates mu-opioid receptor trafficking and signaling via activation of phospholipase D_2. *Cell Signal.* **21**, 1784-1793.

Rastogi, R., Verma, J. K., Kapoor, A., Langsley, G. and Mukhopadhyay, A. (2016) Rab5 Isoforms Specifically Regulate Different Modes of Endocytosis in Leishmania. *J. Biol. Chem.* **291**, 14732-14746.

Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B. and Sandvig, K. (1999) Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell* **10**, 961-974.

Rohrer, J., Bénédetti, H., Zanolari, B. and Riezman, H. (1993) Identification of a novel sequence mediating regulated endocytosis of the G protein-coupled α-pheromone receptor in yeast. *Mol. Biol. Cell* **4**, 511-521.

Romer, W., Pontani, L. L., Sorre, B., Rentero, C., Berland, L., Chambon, V., Lamaze, C., Bassereau, P., Sykes, C., Gaus, K. and Johannes, L. (2010) Actin dynamics drive membrane reorganization and scission in clathrin-independent endocytosis. *Cell* **140**, 540-553.

Rosenthal, W., Antaramian, A., Gilbert, S. and Birnbaumer, M. (1993) Nephrogenic diabetes insipidus. A V2 vasopressin receptor unable to stimulate adenylyl cyclase. *J. Biol. Chem.* **268**, 13030-13033.

Ross, E. M. (1995) Protein modification. Palmitoylation in G-protein signaling pathways. *Curr. Biol.* **5**, 107-109.

Roth, B. L., Willins, D. L. and Kroeze, W. K. (1998) G protein-coupled receptor (GPCR) trafficking in the central nervous system: relevance for drugs of abuse. *Drug Alcohol Depend*. **51**, 73-85.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. and Anderson, R. G. (1992) Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673-682.

Sandvig, K., Olsnes, S., Petersen, O. W. and van Deurs, B. (1987) Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* **105**, 679-689.

Schmid, S. L. (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* **66**, 511-548.

Schmidlin, F., Dery, O., DeFea, K. O., Slice, L., Patierno, S., Sternini, C., Grady, E. F. and Bunnett, N. W. (2001) Dynamin and Rab5a-dependent trafficking and signaling of the neurokinin 1 receptor. *J. Biol. Chem.* **276**, 25427-25437.

Seachrist, J. L., Anborgh, P. H. and Ferguson, S. S. (2000) β 2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by rab GTPases. *J. Biol. Chem*. **275**, 27221-27228.

Shapiro, M. J., Weiss, E. J., Faruqi, T. R. and Coughlin, S. R. (2000) Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J. Biol. Chem.* **275**, 25216-25221.

Shen, Y., Xu, L. and Foster, D. A. (2001) Role for phospholipase D in receptor-mediated endocytosis. *Mol. Cell. Biol*. **21**, 595-602.

Shenoy, S. K., McDonald, P. H., Kohout, T. A. and Lefkowitz, R. J. (2001) Regulation of receptor fate by ubiquitination of activated β 2-adrenergic receptor and β-arrestin. *Science* **294**, 1307-1313.

Sibley, D. R., Benovic, J. L., Caron, M. G. and Lefkowitz, R. J. (1987) Regulation of transmembrane signaling by receptor phosphorylation. *Cell* **48**, 913-922.

Sibley, D. R. and Lefkowitz, R. J. (1985) Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature* **317**, 124-129.

Sikarwar, A. S., Hinton, M., Santhosh, K. T., Chelikani, P. and Dakshinamurti, S. (2014) Palmitoylation of Gαq determines its association with the thromboxane receptor in hypoxic pulmonary hypertension. *Am. J. Respir. Cell Mol. Biol.* **50**, 135-143.

Soto, A. G. and Trejo, J. (2010) N-linked glycosylation of protease-activated receptor-1 second extracellular loop: a critical determinant for ligand-induced receptor activation and internalization. *J. Biol. Chem.* **285**, 18781-18793.

Stadel, J. M., Nambi, P., Shorr, R. G., Sawyer, D. F., Caron, M. G. and Lefkowitz, R. J. (1983) Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3173-3177.

Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lütcke, A., Gruenberg, J. and Zerial, M. (1994) Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J.* **13**, 1287-1296.

Strasser, R. H., Sibley, D. R. and Lefkowitz, R. J. (1986) A novel catecholamine-activated adenosine cyclic 3',5'-phosphate independent pathway for β-adrenergic receptor phosphorylation in wild-type and mutant S49 lymphoma cells: mechanism of homologous desensitization of adenylate cyclase. *Biochemistry* **25**, 1371-1377.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H. and McGraw, T. E. (1999) Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6775-6780.

Takai, Y., Sasaki, T. and Matozaki, T. (2001) Small GTP-binding proteins. *Physiol. Rev.* **81**, 153-208.

Tanowitz, M. and Von Zastrow, M. (2002) Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes. *J. Biol. Chem.* **277**, 50219-50222.

Teis, D., Wunderlich, W. and Huber, L. A. (2002) Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. *Dev. Cell* **3**, 803-814.

Thompson, D. and Whistler, J. L. (2011) Dopamine D(3) receptors are down-regulated following heterologous endocytosis by a specific interaction with G protein-coupled receptor-associated sorting protein-1. *J. Biol. Chem.* **286**, 1598-1608.

Tran, T. M., Friedman, J., Qunaibi, E., Baameur, F., Moore, R. H. and Clark, R. B. (2004) Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the β2-adrenergic receptor using phosphoserine-specific antibodies. *Mol. Pharmacol.* **65**, 196-206.

Traub, L. M., Ostrom, J. A. and Kornfeld, S. (1993) Biochemical dissection of AP-1 recruitment onto Golgi membranes. *J. Cell Biol.* **123**, 561-573.

Ulloa-Aguirre, A., Timossi, C., Damián-Matsumura, P. and Dias, J. A. (1999) Role of glycosylation in function of follicle-stimulating hormone. *Endocrine* **11**, 205-215.

van Dam, E. M. and Robinson, P. J. (2006) Ral: mediator of membrane trafficking. *Int. J. Biochem. Cell Biol.* **38**, 1841-1847.

van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M. and Schmid, S. L. (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J. Cell Biol.* **122**, 553-563.

Vickery, R. G. and von Zastrow, M. (1999) Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J. Cell Biol.* **144**, 31-43.

Vieira, A. V., Lamaze, C. and Schmid, S. L. (1996) Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* **274**,

2086-2089.
von Kleist, L., Stahlschmidt, W., Bulut, H., Gromova, K., Puchkov, D., Robertson, M. J., MacGregor, K. A., Tomilin, N., Pechstein, A., Chau, N., Chircop, M., Sakoff, J., von Kries, J. P., Saenger, W., Kräusslich, H. G., Shupliakov, O., Robinson, P. J., McCluskey, A. and Haucke, V. (2011) Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* **146**, 471-484.
von Zastrow, M. (2001) Role of endocytosis in signalling and regulation of G-protein-coupled receptors. *Biochem. Soc. Trans.* **29**, 500-504.
Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J. and Bourne, H. R. (1993) Palmitoylation is required for signaling functions and membrane attachment of Gqα and Gsα. *J. Biol. Chem.* **268**, 25001-25008.
Weir, D. L., Laing, E. D., Smith, I. L., Wang, L. F. and Broder, C. C. (2014) Host cell virus entry mediated by Australian bat lyssavirus G envelope glycoprotein occurs through a clathrin-mediated endocytic pathway that requires actin and Rab5. *Virol. J.* **11**, 40.
Wenk, M. R., Pellegrini, L., Klenchin, V. A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T. F. and De Camilli, P. (2001) PIP kinase Iγ is the major PI(4,5)P(2) synthesizing enzyme at the synapse. *Neuron* **32**, 79-88.
West, M. A., Bright, N. A. and Robinson, M. S. (1997) The role of ADP-ribosylation factor and phospholipase D in adaptor recruitment. *J. Cell Biol.* **138**, 1239-1254.
Williams, A. M. and Enns, C. A. (1993) A region of the C-terminal portion of the human transferrin receptor contains an asparagine-linked glycosylation site critical for receptor structure and function. *J. Biol. Chem.* **268**, 12780-12786.
Winstel, R., Freund, S., Krasel, C., Hoppe, E. and Lohse, M. J. (1996) Protein kinase cross-talk: membrane targeting of the β-adrenergic receptor kinase by protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2105-2109.
Wolfe, B. L. and Trejo, J. (2007) Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. *Traffic* **8**, 462-470.
Yu, S. S., Lefkowitz, R. J. and Hausdorff, W. P. (1993) β-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J. Biol. Chem.* **268**, 337-341.
Yun, H. J., Kim, H., Ga, I., Oh, H., Ho, D. H., Kim, J., Seo, H., Son, I. and Seol, W. (2015) An early endosome regulator, Rab5b, is an LRRK2 kinase substrate. *J. Biochem.* **157**, 485-495.
Zhang, X., Choi, B. G. and Kim, K. M. (2016a) Roles of dopamine D_2 receptor subregions in interactions with β-Arrestin2. *Biomol. Ther. (Seoul)* **24**, 517-522.
Zhang, X., Le, H. T., Zhang, X., Zheng, M., Choi, B. G. and Kim, K. M. (2016b) Palmitoylation on the carboxyl terminus tail is required for the selective regulation of dopamine D_2 versus D3 receptors. *Biochim. Biophys. Acta* **1858**, 2152-2162.
Zhang, X., Sun, N., Zheng, M. and Kim, K. M. (2016c) Clathrin-mediated endocytosis is responsible for the lysosomal degradation of dopamine D3 receptor. *Biochem. Biophys. Res. Commun.* **476**, 245-251.
Zhang, X. and Kim, K. M. (2016) Palmitoylation of the carboxyl-terminal tail of dopamine D4 receptor is required for surface expression, endocytosis, and signaling. *Biochem. Biophys. Res. Commun.* **479**, 398-403.
Zhang, Z., Austin, S. C. and Smyth, E. M. (2001) Glycosylation of the human prostacyclin receptor: role in ligand binding and signal transduction. *Mol. Pharmacol.* **60**, 480-487.
Zheng, H., Chu, J., Qiu, Y., Loh, H. H. and Law, P. Y. (2008) Agonist-selective signaling is determined by the receptor location within the membrane domains. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 9421-9426.
Zheng, H., Loh, H. H. and Law, P. Y. (2013) Posttranslation modification of G protein-coupled receptor in relationship to biased agonism. *Meth. Enzymol.* **522**, 391-408.
Zheng, H., Pearsall, E. A., Hurst, D. P., Zhang, Y., Chu, J., Zhou, Y., Reggio, P. H., Loh, H. H. and Law, P. Y. (2012) Palmitoylation and membrane cholesterol stabilize mu-opioid receptor homodimerization and G protein coupling. *BMC Cell Biol.* **13**, 6.
Zheng, M., Zhang, X., Guo, S., Zhang, X., Choi, H. J., Lee, M. Y. and Kim, K. M. (2015) PKCβII inhibits the ubiquitination of β-arrestin2 in an autophosphorylation-dependent manner. *FEBS Lett.* **589**, 3929-3937.
Zheng, M., Zhang, X., Guo, S., Zhang, X., Min, C., Cheon, S. H., Oak, M. H., Kim, Y. R. and Kim, K. M. (2016) Agonist-induced changes in RalA activities allows the prediction of the endocytosis of G protein-coupled receptors. *Biochim. Biophys. Acta* **1863**, 77-90.
Zoncu, R., Perera, R. M., Sebastian, R., Nakatsu, F., Chen, H., Balla, T., Ayala, G., Toomre, D. and De Camilli, P. V. (2007) Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3793-3798.