Biochimica et Biophysica Acta 1863 (2016) 77–90

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Agonist-induced changes in RalA activities allows the prediction of the endocytosis of G protein-coupled receptors

Mei Zheng [a,1], Xiaohan Zhang [a,1], Shuohan Guo [a], Xiaowei Zhang [a], Chengchun Min [a], Seung Hoon Cheon [b], Min-Ho Oak [c], Young Ran Kim [a], Kyeong-Man Kim [a,*]

[a] Department of Pharmacology, College of Pharmacy, Chonnam National University, Gwang-Ju 500-757, Republic of Korea
[b] Department of Medicinal Chemistry, College of Pharmacy, Chonnam National University, Gwang-Ju 500-757, Republic of Korea
[c] College of Pharmacy, Mokpo National University, Muan-gun, Jeollanamdo 534-729, Republic of Korea

ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 10 October 2015
Accepted 13 October 2015
Available online 22 October 2015

Keywords:
Epsin
Angiotensin
LPA
β-Arrestin
β-Adrenoceptor

ABSTRACT

GTP binding proteins are classified into two families: heterotrimeric large G proteins which are composed of three subunits, and one subunit of small G proteins. Roles of small G proteins in the intracellular trafficking of G protein-coupled receptors (GPCRs) were studied. Among various small G proteins tested, GTP-bound form (G23V) of RalA inhibited the internalization of dopamine D_2 receptor independently of the previously reported downstream effectors of RalA, such as Ral-binding protein 1 and PLD. With high affinity for GRK2, active RalA inhibited the GPCR endocytosis by sequestering the GRK2 from receptors. When it was tested for several GPCRs including an endogenous GPCR, lysophosphatidic acid receptor 1, agonist-induced conversion of GTP-bound to GDP-bound RalA, which presumably releases the sequestered GRK2, was observed selectively with the GPCRs which have tendency to undergo endocytosis. Conversion of RalA from active to inactive state occurred by translocation of RGL, a guanine nucleotide exchange factor, from the plasma membrane to cytosol as a complex with Gβγ. These results suggest that agonist-induced Gβγ-mediated conversion of RalA from the GTP-bound form to the GDP-bound form could be a mechanism to facilitate agonist-induced internalization of GPCRs.

1. Introduction

Agonist binding induces conformational changes of G protein-coupled receptors (GPCRs) and stimulates the dissociation of Gα from Gβγ, leading to the control of different effectors and regulation of receptor responsiveness. According to the current paradigm of GPCR regulation, dissociated Gβγ mediates the recruitment of GPCR kinase2 or 3 (GRK2 or GRK3) which possesses PH domain to the receptors to phosphorylate them [1,2]. Phosphorylated receptors display increased affinity for β-arrestins, and β-arrestins are translocated from cytosol to the plasma membrane where agonist-occupied receptors are located [3]. Within minutes of agonist exposure, activated receptors undergo the process of endocytosis. Several key cellular elements have been reported to be involved in the process of receptor endocytosis, for example, AP2 and epsins which are adaptors and sorting proteins, respectively [4].

GTP binding proteins are classified into two families: heterotrimeric large G proteins which are composed of three subunits (α, β, and γ), and one subunit of small G proteins. In contrast to the heterotrimeric G protein family, which contains only several members having relatively conserved functions, much larger numbers of small G proteins with highly variable functions have been reported [5,6].

Ral belongs to small GTPases of the Ras super-family, and has multiple regulatory roles such as gene transcription, cytoskeletal regulation, cell differentiation and migration [5,7–9]. In addition, several lines of evidence have implicated the roles of Ral in the endocytosis of receptor proteins [10]. For example, Ral inhibits the clathrin-dependent endocytosis of transferrin, activin, insulin, and EGF receptors *via* the recruitment of Ral-binding protein 1 (RalBP1) [11–13].

Among several small G proteins tested in our preliminary study, active RalA showed inhibitory activities on D_2R endocytosis. In this study, we wanted to understand the relationship between the activities of RalA and endocytosis of D_2R. We also questioned whether the mechanism obtained from D_2R could be extended to other GPCRs as a principle to explain the endocytosis of other GPCRs.

2. Materials and methods

2.1. Materials

Dopamine (DA), isoproterenol (ISO), anti-FLAG M2 antibodies, anti-FLAG-conjugated agarose beads, antibodies to actin and green fluorescence protein (GFP), horseradish peroxidase (HRP)-labeled secondary antibodies, and glutathione beads were purchased from Sigma/Aldrich

* Corresponding author at: Department of Pharmacology, College of Pharmacy, Chonnam National University, Gwang-Ju 500-757, Republic of Korea.
E-mail address: kmkim@jnu.ac.kr (K.-M. Kim).
[1] These authors equally contributed to this work.

http://dx.doi.org/10.1016/j.bbamcr.2015.10.007
0167-4889/

Chemical (St Louis, MO, USA). VU 0155069 (PLD inhibitor) was purchased from Cayman Chemical (Ann Arbor, MI, USA). [^{3}H]-Sulpride (87 Ci/mmol) and [^{3}H]-CGP-12,177 (41.7 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Antibodies to LPAR1 were purchased from Abcam (Cambridge, MA, USA). Antibodies to HA epitope, RalA, phosphoserine, MDM2 and GRK2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to β-arrestins were provided by Dr. Robert Lefkowitz (Duke University, Durham, NC, USA).

2.2. DNA constructs

Wild-type (WT) human D_2R and D_3R in pCMV5, D_2R, D_3R and MDM2 tagged at the N-terminus with the M2-FLAG epitope, and β-arrestin2 constructs were previously described [14,15]. Flag-tagged human β_2AR and AT1R were prepared by polymerase chain reactions. HA-tagged LPAR1 was provided by Dr. S. Na (Konkuk University, Korea). GRK2-CT was previously described [16]. Yellow fluorescence protein (YFP)-Gβ1 [17], RalBP1 and POB1constructs [11], and Ral constructs [18,19], were reported previously. Small hairpin RNA (shRNA) of GRK2 [20], β-arrestin1/β-arrestin2 [21], and MDM2 [22] was described previously. ΔN11-G23V-RalA and GFP-RGL were prepared by site-directed mutagenesis or by polymerase chain reactions.

2.3. Endocytosis assay

Endocytosis of D_2R and β_2AR was measured based on the hydrophilic properties of [^{3}H]-sulpiride and [^{3}H]-CGP-12,177 [14,23]. HEK-293 cells expressing D_2R or β_2AR were seeded 1 day after transfection at a density of 1.5 x 10^5 cells/well in 24-well plates. The following day, the cells were rinsed once and pre-incubated for 15 min with 0.5 ml of pre-warmed serum-free medium containing 10 mM HEPES, pH 7.4, at 37 °C. The cells were stimulated with 10 μM DA or ISO for 0–60 min as indicated. The cells were then incubated with 250 μl of [^{3}H]-sulpiride (final concentration 2.2 nM) or [^{3}H]-CGP-12,177 (final concentration 10 nM) at 4 °C for 150 min in the absence and presence of unlabeled competitive inhibitor (10 μM haloperidol or propranolol). The cells were washed three times with the same medium and 1% SDS was added. The samples were mixed with 2 ml Lefkofluor scintillation fluid and counted on a liquid scintillation analyzer. The endocytosis of D_3R was determined by ELISA method or flow cytometry as described previously [14].

2.4. Whole cell phosphorylation

HEK-293 cells were seeded one day after transfection at a density of ~1 x 10^6 cells/25-mm well. Cells were stimulated with 10 μM DA for 5 min. The [^{32}P]-labeled phosphorylated receptors were assessed by autoradiography. The detailed procedures are described elsewhere [14]. For the normalization of the loading volumes, the amount of receptor in each sample was determined by saturation binding with 3 nM [^{3}H]-spiperone.

2.5. Immunoprecipitation

Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 10% glycerol) on a rotation wheel for 4 h at 4 °C. Supernatant was combined with 25 μl of a 50% slurry of agarose beads coated with anti-FLAG antibodies for 2–3 h on a rotation wheel. Beads were washed with washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40) three times for 5 min each. The resulting immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane. The membrane was blotted with antibodies to corresponding target proteins.

2.6. Measurement of GTP-bound RalA

Activation of Ral was measured by a glutathione-S-transferase (GST)-pull down assay using an activation-specific probe, the Ral-binding domain (397–518 amino acid residues) of RalBP (RBD) [24]. For this, RBD was bacterially expressed as a fusion protein with glutathione-S-transferase (GST-RBD). Cell lysates obtained from the HEK-293 cells transfected with corresponding GPCRs and GFP-RalA cDNA were added to the column containing GST-RBD pre-coupled to glutathione-agarose beads and incubated for 45 min with continuous shaking at 4 °C. Beads were washed four times with GST-binding buffer and then treated with Laemmli sample buffer. For the measurement of endogenous RalA activities, eluents of GST pull-down were blotted with antibodies against RalA.

2.7. Immunocytochemistry

The HEK-293 cells were plated on cover slips, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature. Cells were incubated with PBS containing 3% fetal bovine serum and 1% bovine serum albumin for 1 h and then incubated with FLAG, GRK2, and HA antibodies at 1:1000 dilutions for 1 h at room temperature. For the detection of endogenous LPAR1, antibodies against LPAR1 were used at 1:50 dilutions. After three washes, cells were incubated with Alexa 555- conjugated anti-mouse antibodies or Alexa 647-conjugated anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA) at 1:500 dilutions. After three washes with washing buffer, the cells were mounted in a Vectashield (Vector Laboratories, Burlingame, CA, USA) and viewed by a laser scanning confocal microscope (TCS SP5/ABOS/Tandem, Germany).

2.8. Data analyses

The values are expressed as mean ± SEM for the number of independent experiments indicated in the figure legends. Student's *t*-test was used to compare results. A p-value <0.05 was considered significant. Comparisons among experimental groups were performed using ANOVA and Tukey's simultaneous test.

3. Results

3.1. RalA regulates the internalization of dopamine D_2 receptor and β_2 adrenergic receptor in an activity-dependent manner

In order to study the roles of small G proteins on the intracellular trafficking of GPCRs, effects of small G proteins, such as Rab5, Rab23, ARF1, ARF6, and RalA were tested for the internalization of dopamine D_2 receptor (D_2R). These small G proteins are known to be involved in various processes such as vesicle formation, scission, targeting, and fusion formation [25,26]. Among these small G proteins, an active RalA mutant (GTP hydrolysis-resistant form, G23V) but not an inactive RalA mutant (GDP bound form, S28N) [8,9] inhibited the endocytosis of D_2R in HEK-293 cells (Fig. 1A) and SH-SY5Y dopaminergic neuroblatoma cell lines (Fig. 1B), which are physiologically relevant cells for dopaminergic functions. WT-RalA also inhibited the endocytosis of D_2R but at lower extent.

Essentially the same results were observed with β_2 adrenergic receptor (β_2AR). As shown in the left panel of Fig. 1C, agonist stimulation of HEK-293 cells expressing β_2AR-GFP (green fluorescence protein) resulted in the endocytosis of β_2AR in the endocytic vesicles, which was clearly inhibited in the cells expressing G23V-RalA (3rd panel).

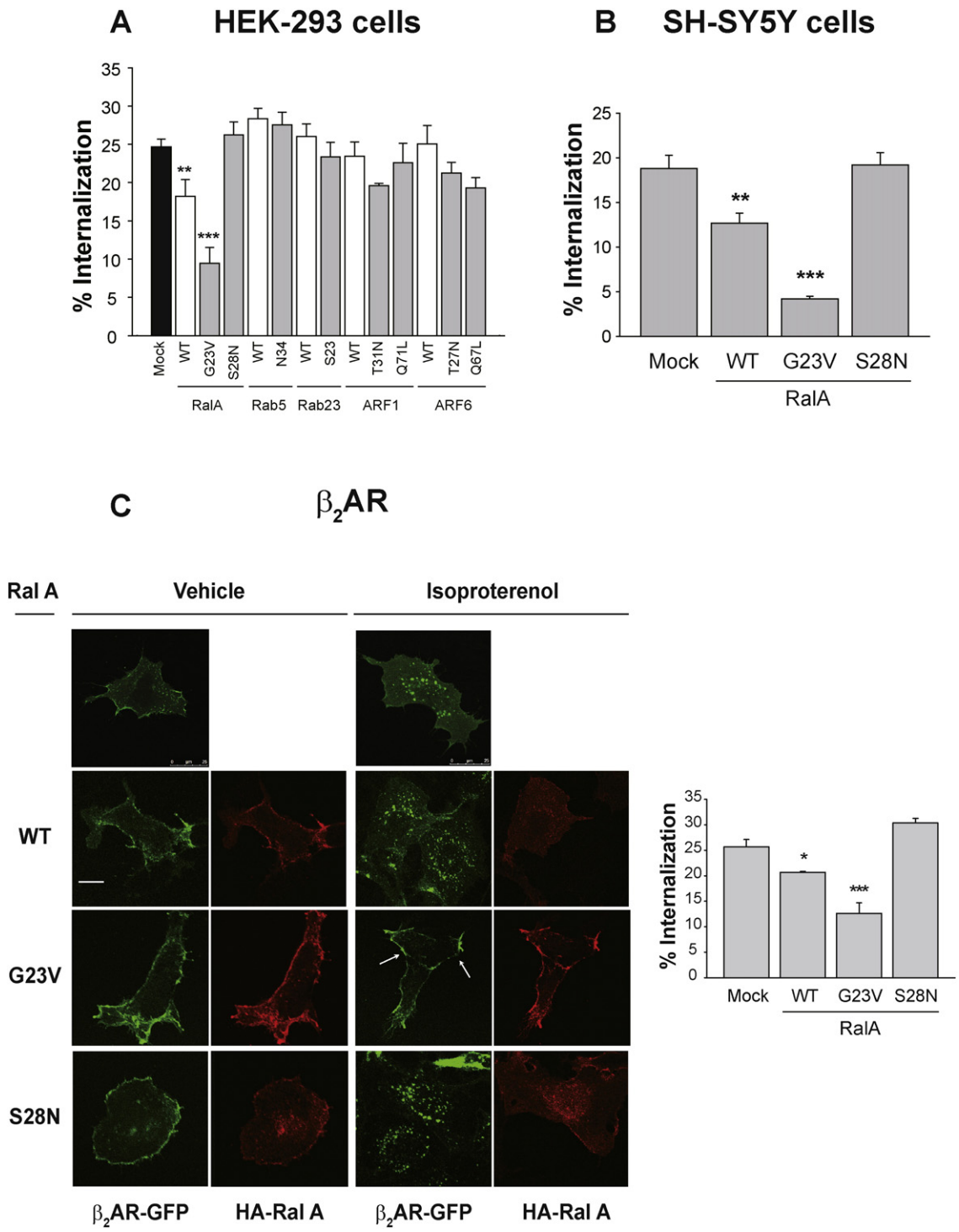


Fig. 1. RalA inhibits the endocytosis of dopamine D_2 receptor and β_2 adrenergic receptor in an activity-dependent manner. (A) Roles of small GTP binding proteins in the endocytosis of D_2R. HEK-293 cells expressing D_2R were transfected with WT, active (G23V), and inactive (S28N) RalA; WT and dominant negative (N34) Rab5; WT and dominant negative (S23N) Rab23; WT, dominant negative (T31N), and constitutively active (Q71L) ARF1; WT, inactive (T27N), and active (Q67L) ARF6. Expression levels of D_2R were maintained between 1.1 and 1.4 pmol/mg protein. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with Mock-transfected group. (B) Effects of RalA on the endocytosis of D_2R in SH-SY5Y cells. Cells expressing D_2R (0.85 pmol/mg protein) were transfected with WT-, G23V-, or S28N-RalA. Endocytosis assay was conducted as described in Materials and Methods. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the Mock or S28N-RalA group. $P < 0.001$ when G23V-RalA group was compared with WT-RalA group. (C) Effects of RalA on the endocytosis of β_2AR. (Left panel) GFP-tagged β_2AR was co-transfected with either Mock vector or HA-tagged RalA constructs. Cells were treated with 1 μM isoproterenol (ISO) for 20 min and labeled with antibodies to HA. The horizontal bar represents 10 μm. Arrows represent β_2AR located on the plasma membrane. Data represent results from two independent experiments with similar outcomes. (Right panel) Effects of RalA on the endocytosis of β_2AR measured by the radioligand method. Cells expressing β_2AR (1.8 pmol/mg protein) were transfected with RalA constructs. Cells were treated with either the vehicle or 1 μM ISO for 20 min. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with the WT- or S28N-RalA group. Each data point represents mean ± SEM (n = 6).

3.2. Active RalA inhibits endocytosis of dopamine D_2 receptor by sequestering GRK2 from the receptors

RalBP1 (RalA-binding protein 1) is a downstream target of RalA [27] that interacts with POB1, a partner of RalBP1 [28], and is known to be involved in the endocytosis of EGF and insulin receptor [11]. Unlike EGF receptor (Figure S1), the deletion mutants of RalBP1 and POB1 did not affect the endocytosis of D_2R (Figure S2A). PLD is another well-known effector protein of Ral [10]. PLD inhibitors block agonist-stimulated endocytosis of EGF, δ-opioid, and angiotensin type 1A receptor [29–33]. However, treatment of cells with the PLD inhibitor VU 0155069 did not have any effect on the endocytosis of D_2R (Figures S2B). In agreement with these results, ΔN11-G23V-RalA (a RalA mutant with 11 amino acids deleted from its N-terminus, which does not couple to PLD) inhibited the endocytosis of D_2R to a similar extent as G23V-RalA (Figure S2C). These results collectively suggested that RalBP1, POB1, and PLD do not mediate the RalA-mediated inhibition of D_2R endocytosis.

GRK2 and β-arrestins are established cellular components that drive the endocytosis of GPCRs. To test whether GRK2 and β-arrestins are involved in RalA-mediated inhibition of D_2R endocytosis, biochemical interactions between them were examined. As shown in Fig. 2A, interaction of GRK2 with active form RalA but not with WT-RalA or inactive form RalA increased in response to agonist stimulation of D_2R. The same results were obtained from the studies of β_2AR (Fig. 2B). These results were also confirmed with endogenous GRK2 (Figure S3). To locate the subregions of GRK2 that interact with G23V-RalA, GST fusion proteins were prepared for RGS homology (RH), catalytic (Cat), and Pleckstrin homology (PH) domain of GRK2. As shown in Fig. 2C, the catalytic domain of GRK2 interacted with G23V-RalA. Since the GTP-bound status of G23V-RalA is fixed, it is likely that the increased enzymatic activity of GRK2 which is mediated by activated D_2R is the determining factor that enhances the association between the two proteins. Indeed, active RalA was phosphorylated on the serine residue(s) when it was associated with GRK2 (Fig. 2D). In addition, the interaction between K220R-GRK2, an enzymatic inactive mutant [34], and G23V-RalA did not change in response to D_2R activation (Fig. 2E). Since G23V-RalA sequestered GRK2 from receptor, it was expected that D_2R phosphorylation will be inhibited by G23V-RalA. As shown in Fig. 2F, agonist-induced phosphorylation of D_2R was inhibited when G23V-RalA was co-expressed. These results indicate that GRK2 binds to RalA in a GTP-selective manner, leading to decreases in association of GRK2 and D_2R, which results in the inhibition of phosphorylation and endocytosis of D_2R.

Since interaction between GRK2 and active RalA increased in response to activation of D_2R or β_2AR, and because the phosphorylation of D_2R was blocked by active RalA, interaction between D_2R and GRK2 was expected to be decreased by G23V-RalA. As shown in Fig. 3A, interaction between D_2R and GRK2 increased in response to agonist stimulation and this increase was abolished when G23V-RalA was co-expressed. Next we determined the effects of RalA on the interaction between D_2R and β-arrestin2. Since GRK2 mediates agonist-induced receptor phosphorylation which facilitates the recruitment of β-arrestins to the plasma membrane where receptors are located, inhibition of the interaction between receptor and GRK2 will indirectly inhibit the interaction between receptor and β-arrestin. Interaction between D_2R and β-arrestin2 was determined by β-arrestin translocation assay [14]. As shown in the top panel of Figure S4, β-arrestin2 translocated toward plasma membrane in response to agonist treatment. The translocation was significantly inhibited in the cells expressing G23V-RalA (3rd panel) but a less extent in the cells expressing WT-RalA (2nd panel).

To determine whether RalA affects the interaction between receptor and β-arrestin2 independently of receptor phosphorylation, we utilized D_2R-IC23. In D_2R-IC23, all of the 15 serine and 12 threonine residues located within the 2nd and 3rd intracellular loops were mutated to alanine and valine residues, respectively. D_2R-IC23 has been used to study receptor phosphorylation-independent processes [35]. Thus, effects of RalA on the interaction between D_2R-IC23 and β-arrestin2 exclude the effects of RalA on the GRK2-mediated facilitation of β-arrestin recruitment. As shown in Fig. 3B, G23V-RalA did not have any effect on the interaction between D_2R-IC23 and β-arrestin2. Thus, it could be postulated that active RalA selectively interferes with the interaction between D_2R and GRK2.

In accordance with these results, inhibitory activities of RalA on D_2R endocytosis occurred similarly in control and β-arrestin1/2-KD cells but were significantly reduced in GRK2-knockdown (GRK2-KD) cells (Fig. 3C & D). On the other hand, knockdown of GRK5, another GRK which increases the endocytosis of D_2R in an agonist-dependent manner [14], did not affect the inhibitory activities of G23V-RalA on the endocytosis of D_2R (Fig. 3E).

3.3. Gβγ-mediated conversion of GTP-RalA to GDP-RalA is required for the endocytosis of GPCRs

Our results from HEK-293 and SH-SY5Y cells suggested that receptor endocytosis would be constitutively being inhibited by active RalA both for D_2R and β_2AR. Thus, it could be postulated that agonist-induced conversion of GTP-RalA to GDP-RalA is needed for receptor endocytosis to occur. To test this hypothesis, we selected two GPCRs that readily internalize (D_2R, β_2AR) and two GPCRs that rarely internalize (D_3R, β_1AR) [14,36,37]. As shown in Fig. 4A and B, agonist stimulation of D_2R or β_2AR resulted in inhibition of RalA (about 95 and 70%, respectively). In contrast, stimulation of D_3R or β_1AR did not influence the RalA activities. Essentially the same results were obtained for the endogenous RalA in SH-SY5Y cells in which D_2R or D_3R were stably expressed (Fig. 4C). Thus, it could be hypothesized that activation of the GPCRs which have tendency to undergo endocytosis mediate a decrement of RalA activity. This hypothesis was corroborated by the studies of a chimeric receptor between D_3R and D_2R, D_3R-(IC2/3-D_2R), in which the 2nd and 3rd intracellular loops of D_3R were replaced with those of D_2R. In D_3R-(IC2/3-D_2R), signaling properties remained the same but the endocytic properties became opposite (Fig. 4D, lower panel) [14]. In agreement with the results from Fig. 4A–C, agonist stimulation of D_3R-(IC2/3-D_2R), but not D_3R, resulted in an inhibition of RalA (Fig. 4D, upper panel).

Next, we determined whether signaling through G protein is required for the inhibition of RalA. For this, three GPCRs, which commonly undergo endocytosis but have different signaling pathways, were selected (Fig. 4E). Pertussis toxin (PTX) treatment completely and partly blocked the RalA inhibition mediated by D_2R and β_2AR, respectively (Fig. 4E; compare 2/3 *vs.* 4/5 lanes in the upper panel of the GST-pull down part). Since D_2R and β_2AR are coupled to Gi/o and Gs/i [38], respectively, and AT_1R to Gq, it is unlikely that a specific Gα subtype was involved in the conversion of GTP-RalA to GDP-RalA. On the other hand, GRK2-CT, which sequesters the Gβγ subunit, blocked the RalA inhibition mediated by all of the three receptors, suggesting a common involvement of Gβγ in the inhibition of RalA. AT_1R belongs to endocytic class B GPCRs which form stable intracellular receptor/β-arrestin complexes after receptor endocytosis [39,40]. Agonist stimulation of vasopressin type 2 receptor (V_2R), another endocytic class B GPCR also showed the same pattern of RalA inhibition (Figure S5). These results suggest that Gβγ-mediated RalA inhibition could be a common cellular mechanism to regulate receptor endocytosis regardless of signaling pathways and endocytic properties.

3.4. Characterization of D_2R-mediated inhibition of RalA

Since inhibition of RalA is suggested to be related to the endocytosis of GPCRs, we wanted to determine whether receptor endocytosis mediates the inhibition of RalA or whether inhibition of RalA is required for the receptor endocytosis to occur. As shown in Fig. 5A, blockade of the endocytosis of D_2R by pretreatment with sucrose did not affect the

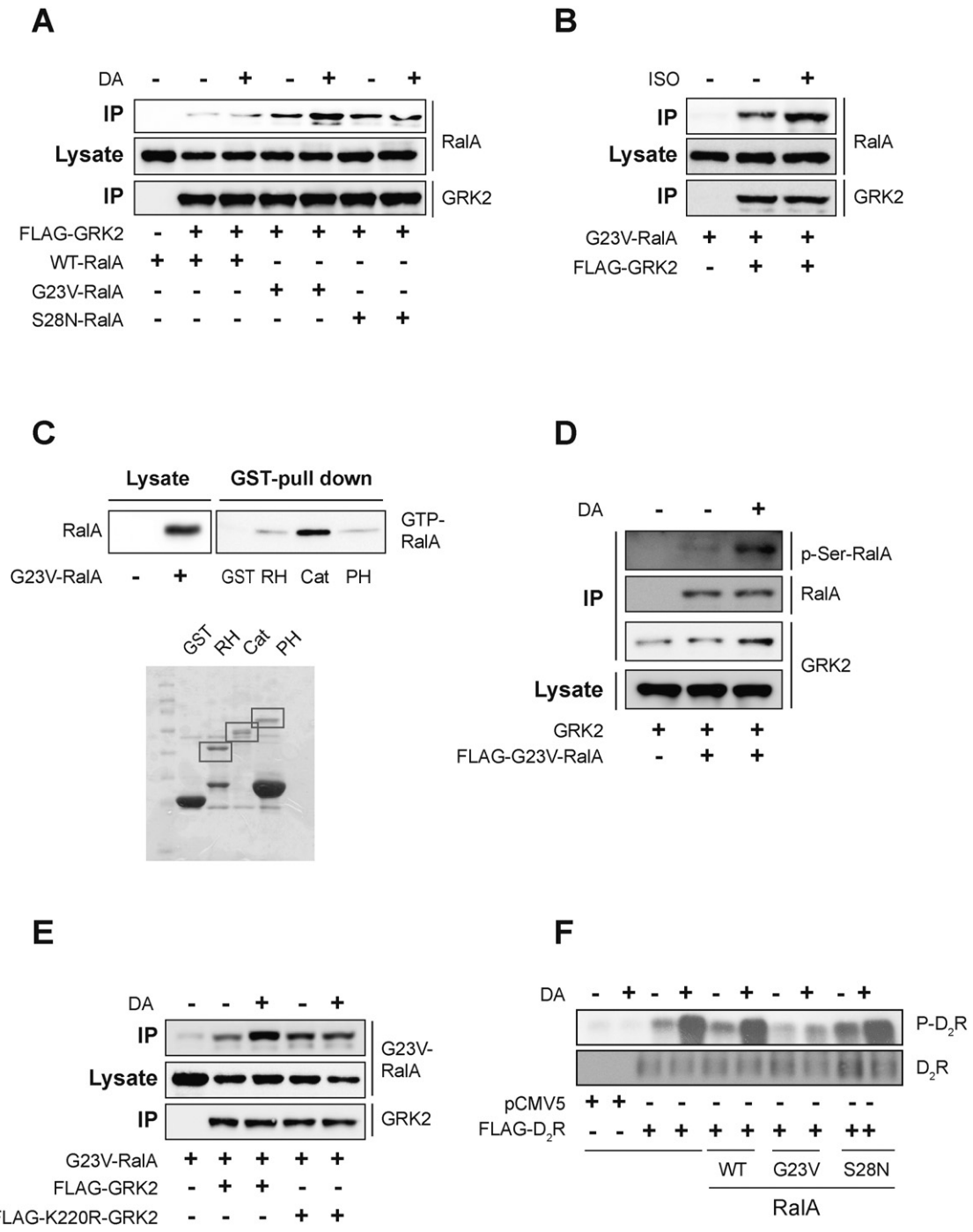
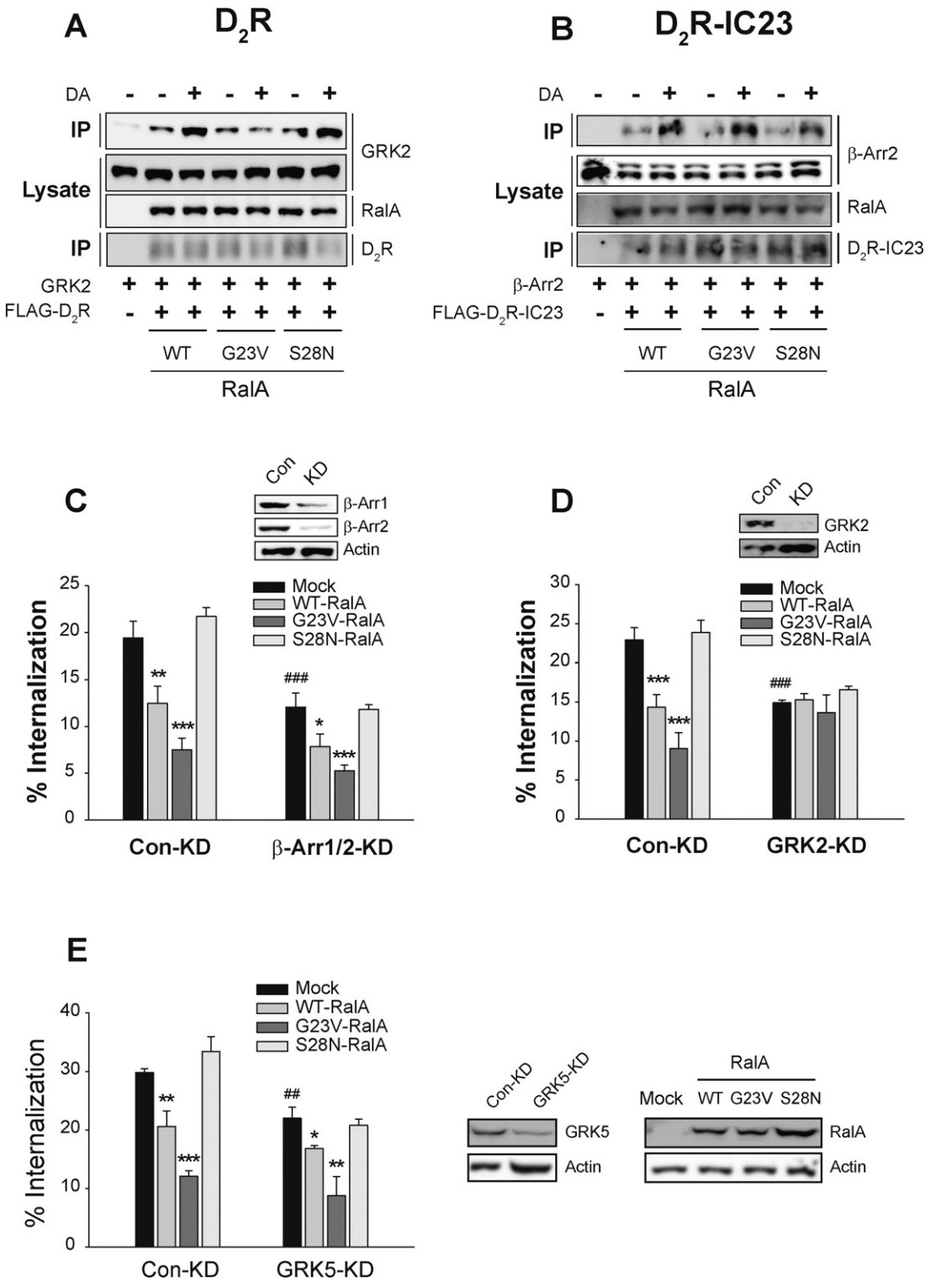


Fig. 2. Functional interaction between RalA and GRK2. (A) GTP-dependent interaction between RalA and GRK2. Cells expressing D_2R (1.7 pmol/mg protein) were transfected with FLAG-GRK2, along with RalA constructs, and were treated with 10 μM DA for 1 min. Cell lysates were immunoprecipitated with FLAG beads and blotted with antibodies to RalA and FLAG. Fold of increase of immunoprecipitation was as follows: 1.67 ± 0.38 (WT), 3.93 ± 1.12 (G23V), 1.03 ± 0.32 (S28N). G23V group was significantly different from WT and S28N group ($p < 0.05$, $n = 3$). (B) Interaction between GRK2 and active RalA in response to β_2AR stimulation. Cells expressing β_2AR (1.2 pmol/mg protein) were transfected with FLAG-GRK2 and G23V-RalA, and stimulated with 1 μM ISO for 1 min. Cell lysates were immunoprecipitated with FLAG beads and blotted with antibodies to RalA and FLAG. Fold of increase was 3.77 ± 1.22. (C) Identification of the GRK2 subdomains that interact with active RalA. GST fusion proteins were prepared for RH, and the catalytic and PH domains of GRK2. Cell lysates expressing G23V-RalA were incubated with GST fusion proteins. Eluents were immunoblotted with antibodies to RalA. Squares in the lower panel represent GST fusion proteins of GRK2 subdomains. Data represent results from two independent experiments with similar outcomes. (D) GRK2-mediated phosphorylation of active RalA in response to agonist stimulation of D_2R. HEK-293 cells expressing D_2R (1.7 pmol/mg protein) were transfected with FLAG-tagged G23V-RalA and GRK2 in pCMV5. Cells were stimulated with 10 μM DA for 1 min. Cell lysates were immunoprecipitated with FLAG beads and immunoprecipitates were blotted with antibodies to FLAG, GRK2, and phosphoserine. Data represent results from four independent experiments with similar outcomes. (E) Effects of enzymatic activities of GRK2 on the interaction with active RalA. HEK-293 cells stably expressing D_2R (1.7 pmol/mg protein) were transfected with G23V-RalA along with FLAG-tagged WT-GRK2 or K220R-GRK2. Cells were treated with 10 μM DA for 1 min. Cell lysates were immunoprecipitated with FLAG beads, and blotted with antibodies to RalA and FLAG. Fold of increase for WT-GRK2 and K220R-GRK2 was 3.33 ± 0.90 and 1.03 ± 0.32, respectively ($p < 0.05$, $n = 3$). (F) Effects of RalA on agonist-induced phosphorylation of D_2R. HEK-293 cells were transfected with FLAG-D_2R (2.1 pmol/mg protein) along with RalA constructs. Receptor phosphorylation was determined after 5 min of stimulation with 10 μM DA. Cell lysates were immunoprecipitated with FLAG beads. The phosphorylated receptors were visualized by autoradiography and the total receptors were detected with antibodies to rabbit FLAG antibodies (lower panel). Data represent results from two independent experiments with similar outcomes.

D_2R-mediated inhibition of RalA. A similar result was obtained when clathrin-mediated receptor endocytosis was blocked by co-expression of epsin (204–458), a dominant negative mutant of epsin [11] (Fig. 5B). On the other hand, when D_2R is mutated so that it cannot undergo endocytosis through exchange of the 2nd and 3rd intracellular loops with those of D_3R, D_2R-(IC2/3-D_3R), the ability to inhibit RalA was abrogated (Fig. 5C). These results suggested that RalA would still be inhibited as far as the receptor have the propensity to internalize, even if receptor endocytosis was blocked by external treatments acting downstream of the endocytic cascade. These results suggested that agonist-induced RalA inhibition would be an upstream event for receptor endocytosis, rather than a post-endocytic event.

GRK2-mediated receptor phosphorylation is a well-established cellular event that initiates receptor endocytosis. A mutant of D_2R, D_2R-IC23 which was used in Fig. 3B was employed to determine the relationship between RalA inhibition and receptor phosphorylation. D_2R-IC23 is phosphorylation deficient, but still undergoes endocytosis [35]. As shown in Fig. 5D, RalA was inhibited when either D_2R or D_2R-



IC23 was activated, suggesting that RalA would act on certain cellular processes located upstream of receptor phosphorylation.

3.5. EGF inhibits GPCR endocytosis by activating RalA

HEK-293 cells endogenously express various plasma membrane receptors and related signaling components [22]. We wanted to determine whether the principles obtained from the over-expressed proteins could be applied to endogenous receptors such as EGFR and LPA1R. When cells were treated with LPA, LPA1R underwent endocytosis (Fig. 6A) and RalA was inhibited at 1 min and returned near basal level at 15 min (Fig. 6B).

When cells were treated with EGF, RalA activities were elevated (Fig. 6C). As expected from the inhibitory activities of active RalA on receptor endocytosis, endocytosis of D_2R was inhibited when cells were treated with EGF (Fig. 6D, compare Mock groups of Veh-treated and EGF-treated cells). Also the inhibitory activities WT-RalA became similar to those of G23V-RalA when cells were treated with EGF probably because WT-RalA was switched to active RalA. In addition, inhibitory activities effects of EGF on the endocytosis of D_2R were abolished in RalA-KD cells (Fig. 6E), suggesting that inhibitory activities of EGF treatment on the D_2R endocytosis occurred through activation of RalA.

As expected from the stimulatory effects of EGF on RalA activites and from the inhibitory effects of active RalA on GPCR endocytosis, pre-treatment with EGF inhibited LPA-induced endocytosis of LPA1R (Fig. 7A). Also EGF treatment increased the interaction of endogenous GRK2 with endogenous RalA (Fig. 7B) but inhibited the interaction of GRK2 with LPA1R (Fig. 7C).

3.6. Interaction between RalA and RGL, a guanine nucleotide exchange factor, is inhibited in response to the activation of GPCRs which have tendency to undergo endocytosis

Ral GDP dissociation stimulator-like (RGL) is one of the guanine nucleotide exchange factors (GEFs) of Ral [41,42]. Presently, RGL stimulated the GDP → GTP exchange and increased the GTP-bound state of RalA (Figure S6A) and RGL was tested for D_2R endocytosis. As shown in Figure S6B, RGL inhibited the endocytosis of D_2R and its inhibitory activities were significantly reduced when endogenous RalA was knocked down. In agreement with the results from RalA, inhibitory activities of RGL on D_2R endocytosis were abolished in GRK2-KD cells but were intact in β-arrestins-KD cells (Figures S6C and S6D).

Since RalA was inhibited in response to agonist stimulation of the GPCRs which have tendency to undergo endocytosis in a Gβγ-dependent manner, we hypothesized that the inhibition of RalA was achieved by Gβγ-mediated dissociation of RGL from RalA. As shown in Fig. 8A, RGL dissociated from RalA in response to D_2R stimulation (compare the 2nd & 3rd lane) in a Gβγ-dependent manner (compare lanes 4 and 5). Virtually the same results were obtained with the stimulation of β_2AR, but not D_3R (Figure S7A). On the other hand, D_2R-mediated interaction between Gβγ and RGL increased (Fig. 8B) and the time course of their association coincided with that of RalA inhibition (Fig. 8C). These results were confirmed by immunocytochemical studies. At the basal state, Gβγ, RGL, and RalA co-localized on the plasma membrane of the cells which stably express D_2R (Fig. 8D, left panel). When the cells were treated with DA (Fig. 8D, right panel), interaction between Gβγ and RGL became evident, and they translocated to the cytosolic region and colocalized. As a result, they were separated from the RalA which was still associated with the plasma membrane (indicated by arrows). Translocation of RGL was less clear in the cells expressing D_3R, which does not undergo endocytosis, and large proportion of RalA and RGL remained co-localized on the plasma membrane both before and after agonist stimulation (Figure S7B).

4. Discussion

The present study reveals a novel regulatory mechanism involved in the endocytosis of GPCRs. As shown in the diagram of Fig. 9, conversion of GTP-RalA to GDP-RalA is a critical cellular event that allows receptor-mediated endocytosis to occur. The most notable finding in this work is that it provides a principle that could predict the occurrence of GPCR endocytosis regardless of their signaling pathways (Gs, Gi/o, Gq) or endocytic properties (class A or class B). Active (GTP-bound) RalA has high affinity to GRK2 and inhibits the endocytosis of GPCRs by sequestering active GRK2 from its effectors including the agonist-primed receptors. RalA is selectively inhibited by agonist stimulation of the GPCRs which have tendency to undergo endocytosis and inhibition of RalA occurs as long as the GPCR possesses the propensity to undergo endocytosis. Conversion of active RalA to inactive RalA which has low affinity to GRK2 allows interaction between receptor and GRK2. These results suggest that the endocytosis of certain GPCRs is regulated by the ability of each receptor to inhibit RalA activities.

The diagram in Fig. 9 was proposed based on following results. Gβγ and GRK2 is constitutively associated near (or on) the plasma membrane (Fig. S8A, Veh-treated), and they dissociate in response to agonist stimulation (Fig. S8A. ISO-treated & Fig. S8B). When cells are treated with agonist, Gβγ transiently interacts with receptor (Fig. S8C) and translocates to cytosol (Fig. 8D). RGL dissociates from active RalA in a Gβγ-dependent manner (Fig. 8A), interacts with Gβγ (Fig. 8B), and translocates toward the cytosol in association with Gβγ (Fig. 8D). The GRK2 which is dissociated from Gβγ interacts with GTP-RalA though catalytic domain (Fig. 2A, B, C). As RGL dissociates from active RalA and translocates to the cytosol as a complex with Gβγ (Fig. 8A, B, D), GTP-RalA is converted to GDP-RalA which has low affinity for GRK2. GRK2 is now free and is recruited to the plasma membrane (Fig. S8A) and interacts with receptor (Fig. 3A).

According to the well-established paradigm of GRK functions on GPCR internalization, GRKs phosphorylate the GPCR, and then the phosphorylated receptor recruits β-arrestins that target the receptor to the

Fig. 3. GRK2 is involved in the RalA-mediated inhibition of D_2R endocytosis. (A) Effects of RalA on the interaction between D_2R and GRK2. HEK-293 cells were transfected with GRK2, FLAG-D_2R, along with RalA constructs. Cells were treated with 10 μM DA for 1 min. Cell lysates were incubated with 25 μl FLAG beads, and eluents were immunoblotted with antibodies to GRK2 (80 kDa) and RalA. Fold of increase of immunoprecipitation was as follows: 4.27 ± 1.10 (WT), 1.07 ± 0.64 (G23V), 4.17 ± 1.23 (S28N). WT group was significantly different from G23V and S28N group ($p < 0.05$, n = 3). (**B**) Effects of RalA on the interaction between non-phosphorylatable D_2R and β-arrestin2. HEK-293 cells were transfected with β-arrestin2, FLAG-D_2R-IC23, along with RalA constructs. Cells were treated with 10 μM DA for 1 min. Cell lysates were incubated with 25 μl FLAG beads, and eluents were immunoblotted with antibodies to β-arrestin2 (45 kDa) and RalA. Fold of increase of immunoprecipitation was as follows: 3.67 ± 1.55 (WT), 3.77 ± 1.40 (G23V), 3.27 ± 1.46 (S28N, n = 3). (**C**) Roles of β-arrestins in the RalA-mediated inhibition of D_2R endocytosis. HEK-293 cells stably expressing control shRNA or β-arrestin1/2 shRNA were transfected with D_2R along with RalA constructs. Receptor expression levels were maintained around 1.4 pmol/mg protein. About 85% and 70% of cellular β-arrestin1 and β-arrestin2 was knocked down, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with each Mock group (n = 6). ### $p < 0.001$ compared with Con-KD/Mock group. $P < 0.001$ when WT- or G23V-RalA group was compared with S28N-RalA group. $P < 0.05$ when G23V-RalA group was compared with WT-RalA group. (D) Roles of GRK2 in the RalA-mediated inhibition of D_2R endocytosis. HEK-293 cells stably expressing control shRNA or GRK2 shRNA constructs were transfected with D_2R along with RalA constructs. Receptor expression levels were maintained around 1.8 pmol/mg protein. About 90% of GRK2 was knocked down. $^{***}p < 0.001$ compared with the Mock group, ### $p < 0.001$ compared with Con-KD/Mock group (n = 6). $P < 0.001$ when WT-RalA group was compared with S28N-RalA group in Con-KD cells. $P < 0.001$ when G23V-RalA group was compared with S28N-RalA group in Con-KD cells. (E) Roles of GRK5 in the RalA-mediated inhibition of D_2R endocytosis. HEK-293 cells stably expressing control shRNA or GRK5 shRNA constructs were transfected with D_2R along with RalA constructs. Receptor expression levels were maintained around 1.7 pmol/mg protein. About 70% of GRK5 was knocked down. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the Mock group, $^{\#\#}p < 0.01$ compared with Con-KD/Mock group (n = 6). $P < 0.05$ when WT-RalA group was compared with S28N-RalA group in Con-KD cells. $P < 0.001$ and $p < 0.01$when G23V-RalA group was compared with S28N-RalA group in Con-KD cells and GRK5-KD cells, respectively.

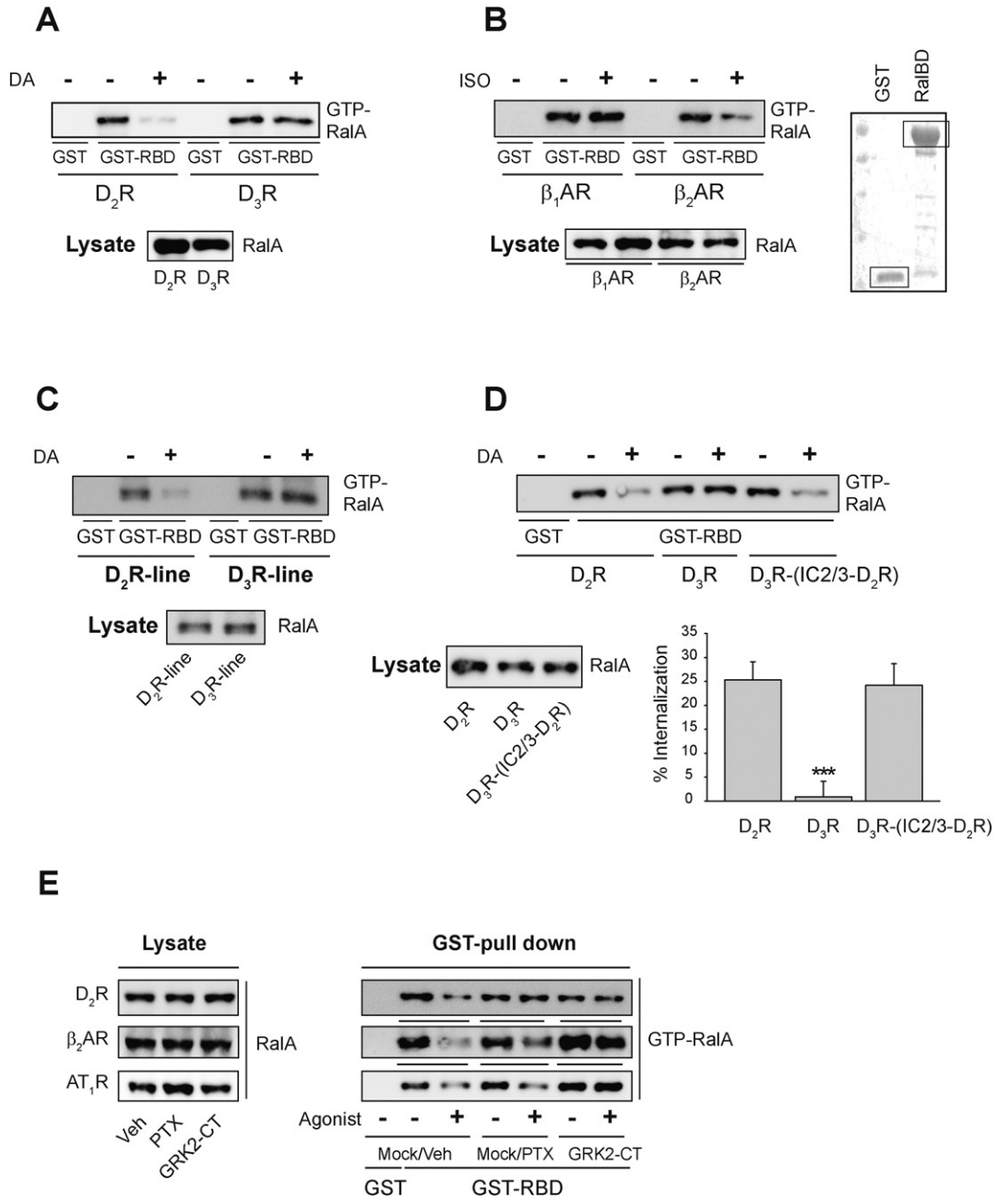


Fig. 4. Selective regulation of RalA activities through the GPCRs which have tendency to undergo endocytosis. (A) Effects of stimulation of D_2R and D_3R on the RalA activities. HEK-293 cells were transfected with RalA together with D_2R or D_3R in pCMV5, and were stimulated with 10 μM DA for 1 min. Bacterial lysates containing the GST fusion proteins of RBD were mixed with the cell lysates. The eluents were analyzed with SDS-PAGE gel and the blots were probed with antibodies to RalA (28 kDa). About 95% of GTP-RalA was decreased by DA treatment. Receptor expression levels of D_2R and D_3R were 1.2 and 1.7 pmol/mg protein, respectively. Data represent results from two independent experiments with similar outcomes. (B) Effects of stimulation of β_1AR and β_2AR on the RalA activities. Cells were transfected with RalA together with β_1AR or β_2AR in pCMV5, and stimulated with 1 μM ISO for 1 min. About 70% of GTP-RalA was decreased by ISO treatment in the cells expressing β_2AR. Receptor expression levels of β_1AR and β_2AR were 1.4 and 0.95 pmol/mg protein, respectively. Data represent results from two independent experiments with similar outcomes. (C) Regulation of endogenous RalA activities in response to agonist stimulation of D_2R or D_3R. SH-SY5Y cells which stably express D_2R (1.2 pmol/mg protein) or D_3R (1.4 pmol/mg protein) were stimulated with 10 μM DA for 1 min. About 95% of GTP-RalA was decreased by DA treatment in the cells expressing D_2R. Data represent results from two independent experiments with similar outcomes. (D) Effects of altering endocytic properties between D_2R and D_3R on the inhibition of RalA activities. Cells were transfected with RalA along with D_2R, D_3R, or D_3R-(IC2/3-D_2R), and stimulated with 10 μM DA for 1 min. About 70–80% of GTP-RalA was decreased by DA treatment in the cells expressing D_2R or D_3R-(IC2/3-D_2R). Data represent results from two independent experiments with similar outcomes. ***: $p < 0.001$ compared with D_2R and D_3R-(IC2/3-D_2R) group. (E) Roles of Gα and Gβγ subunits in the RalA inhibitions through the receptors which have tendency to undergo endocytosis. Cells were transfected with RalA together with 1.5 μg D_2R, β_2AR, or angiotensin type 1 receptor (AT_1R) in pCMV5, and treated with 10 μM DA, 1 μM ISO, or 1 μM angiotensin II for 1 min. Cells were either treated with PTX (100 ng/ml) or transfected with 4 μg of the carboxy-terminal domain of GRK2 (GRK2-CT) in pRK5 in a 100 mm culture dish. About 60–90% of GTP-RalA was decreased by treatment with an agonist for each receptor in Mock/Vehicle condition. Similar RalA inhibitions were observed by stimulation of AT_1R in the presence and absence of PTX treatment. Data represent results from four independent experiments with similar outcomes.

clathrin-mediated endocytic machinery. Based on this paradigm, it is quite hard to understand how G23V-RalA (or even WT-RalA) blocks D_2R endocytosis in the absence of β-arrestins. Previously we have reported that D_2R undergoes both clathrin-mediated endocytosis (CME) and caveolar endocytosis, and that only phosphorylated receptors undergo CME [43]. We also showed that D_2R-IC23, a non-phosphorylatable D_2R, undergoes non-CME process (caveolar or unidentified endocytosis) [43]. As shown in Fig.S9A, GRK2 still increases

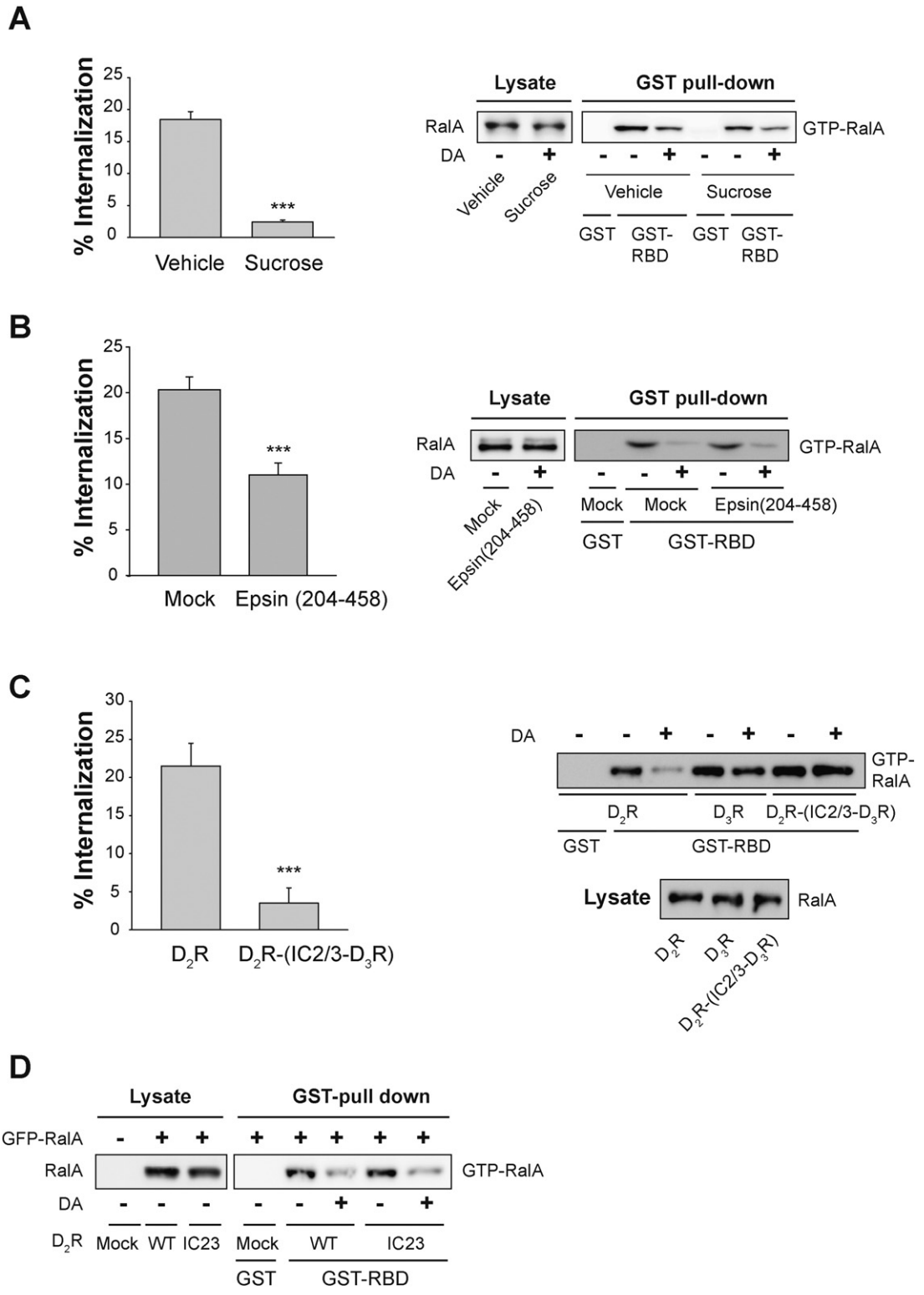


Fig. 5. Roles of receptor endocytosis and phosphorylation on the regulation of RalA activities. (A) Relationship between receptor endocytosis and regulation of RalA. HEK-293 cells expressing D_2R were treated with 0.45 M sucrose for 20 min, followed by 10 μM DA for 1 h. Receptor expression levels were maintained around 0.85 pmol/mg protein. ***: $p < 0.001$ compared with the vehicle group ($n = 3$). For the determination of changes in RalA activities, cells expressing D_2R were transfected with RalA, and treated with 10 μM DA for 1 min after pretreatment with vehicle or sucrose. DA-induced decrease in GTP-RalA was 63.5 ± 12.5 and 72.4 ± 15.7% ($n = 3$) in vehicle- and DA-treated group, respectively. (B) Relationship between clathrin-mediated receptor endocytosis and regulation of RalA HEK-293 cells were transfected D_2R with or without Epsin (204–458). Receptor expression levels were maintained around 1.8 pmol/mg protein. ***$p < 0.01$ compared with the Mock group ($n = 3$). For the determination of changes in RalA activities, cells were treated with 10 μM DA for 1 min, and levels of active RalA were determined. GTP-RalA was decreased about 90–95% by DA treatment in the cells transfected with either Mock vector or epsin (204–458). Data represent results from two independent experiments with similar outcomes. (C) Relationship between endocytic properties of D_2R and RalA activities. HEK-293 cells were transfected with D_2R or D_2R-(IC2/3-D_3R) and receptor expression levels were adjusted about 1.4–1.7 pmol/mg protein. ***$p < 0.01$ compared with D_2R group ($n = 3$). About 90% of GTP-RalA was decreased by DA treatment in the cells expressing D_2R. Data represent results from two independent experiments with similar outcomes. (D) Roles of receptor phosphorylation on the inhibition of RalA. HEK-293 cells were transfected with RalA along with D_2R or D_2R-IC23, and treated with 10 μM DA for 1 min. Receptor expression levels were maintained around 1.7 pmol/mg protein. About 80–85% of GTP-RalA was decreased by DA treatment in the cells expressing D_2R or D_2R-IC23. Data represent results from two independent experiments with similar outcomes.

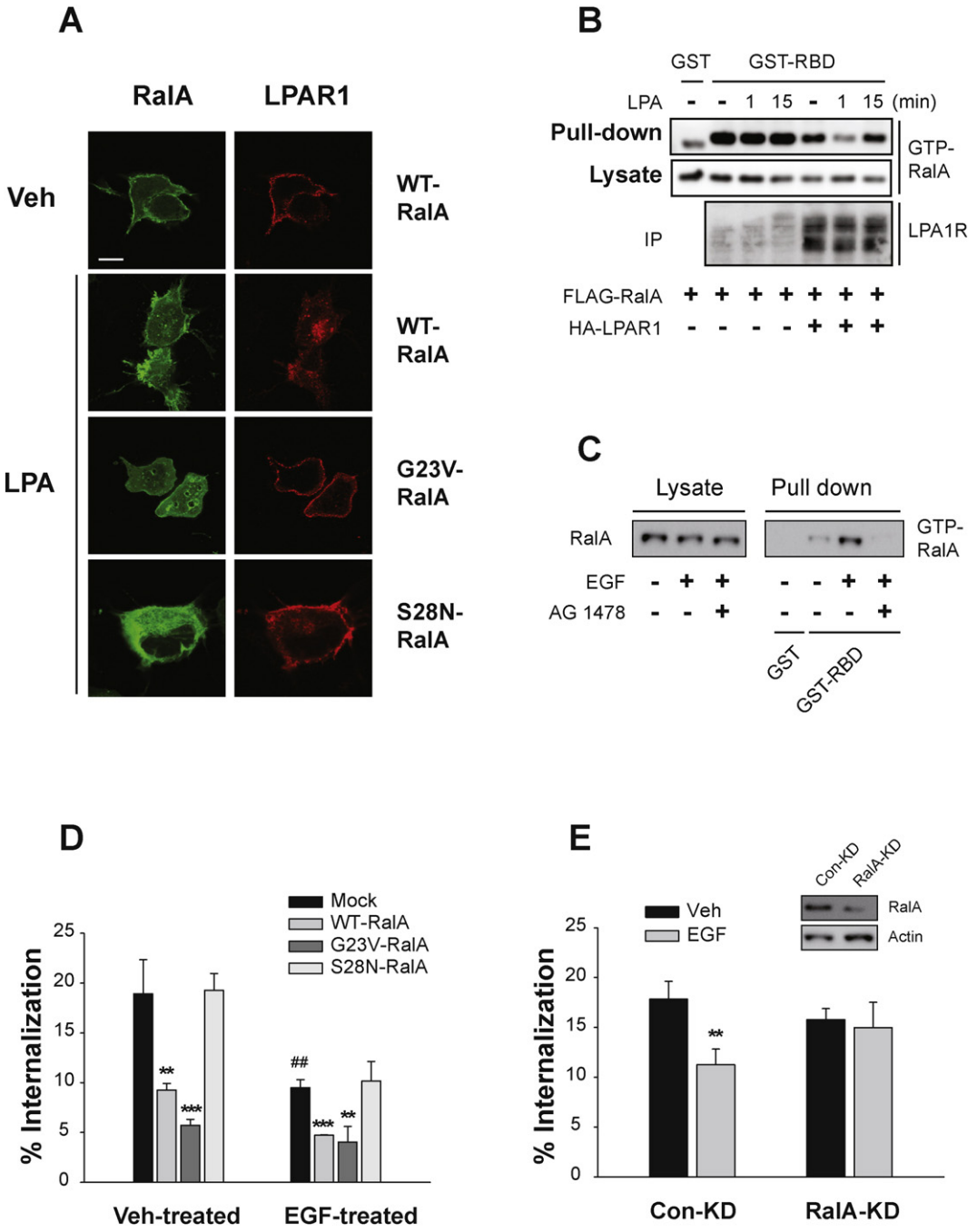


Fig. 6. Functional interactions among RalA, LPAR1, and EGFR. (A) Role of RalA activity in the LPAR1 endocytosis. HEK-293 cells were co-transfected with HA-tagged human LPAR1 and GFP-tagged WT-, G23V- or S28N-RalA. Cells were stimulated with 10 μM lysophosphatidic acid (LPA) for 20 min. The scale bar represents 10 μm. Representative images were selected from each experimental group. (B) Effect of LPAR1 stimulation on RalA activity. HEK-293 cells expressing HA-tagged human LPAR1 were stimulated with 10 μM LPA for 1–15 min. Between 70 and 80% of GTP-RalA was decreased when cells were treated with LPA for 1 min. Data represent results from two independent experiments with similar outcomes. (C) Effects of EGFR stimulation on RalA activity. HEK-293 cells were treated with 100 ng/ml EGF for 20 min with or without pretreatment with 1 μM AG1478 for 30 min. Data represent results from two independent experiments with similar outcomes. (D) Effects of EGF treatment on the RalA-mediated regulation of D_2R internalization. Cells expressing D_2R (1.4 pmol/mg protein) and different RalA constructs were treated with vehicle or 100 ng/ml EGF for 20 min, followed by treatment with 10 μM DA for 1 h. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with each Mock group. $^{\#\#}p < 0.01$ when compared with Mock/vehicle-treated group. (E) RalA is required for the inhibitory effects of EGF treatment on the internalization of D_2R. Cells expressing D_2R (1.7 pmol/mg protein) were pretreated either with vehicle or 100 nM EGF for 20 min, followed by treatment with 10 μM DA for 1 h. About 70% of cellular RalA was decreased in RalA-KD cells. $^{**}p < 0.01$ compared with Con-KD/vehicle-treated group.

the endocytosis of D_2R-IC23 in clathrin-KD cells in which only non-CME processes are available. However, effects of GRK2 on the internalization of D_2R-IC23 are abolished in Cav1-KD cells in which both CME and caveolar endocytosis are absent, suggesting that GRK2 could mediate both CME and caveolar endocytosis. In addition, interaction between GRK2 and caveolin1 is increased in response to agonist stimulation of β_2AR (Fig.S9B).

It is noticeable that RalA does not affect the endocytosis which is mediated by β-arrestins, another critical cellular component responsible for GPCR endocytosis. A recent study has shown that both β_2AR

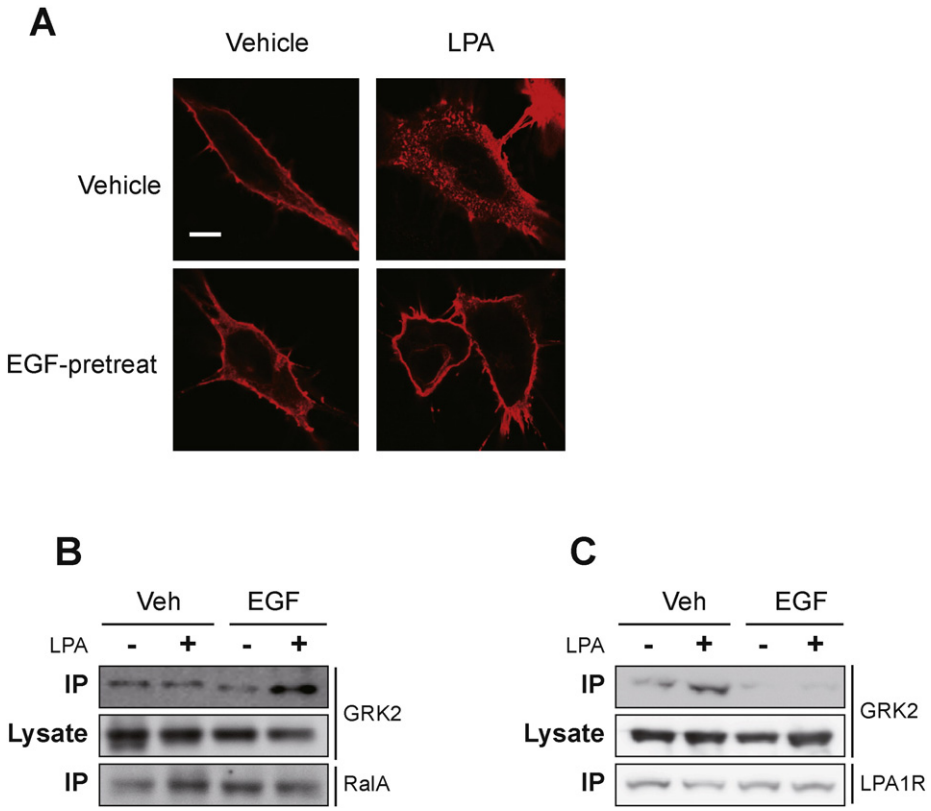


Fig. 7. EGFR-mediated activation of RalA sequesters GRK2 from LPAR1. (A)Effects of EGFR activation on the endocytosis of LPAR1. Cells expressing HA-tagged LPAR1 were treated with either vehicle or 100 ng/ml EGF for 20 min, followed by 10 μM LPA for 20 min. Cells were labeled with anti-HA antibodies at a dilution of 1:500 for 1 h at room temperature, followed by labeling with rabbit Alexa-594 at a 1:500 dilution. Horizontal bar represent 10 μM. (B)Effects of EGFR activation on the interaction between endogenous RalA and GRK2. HEK-293 cells were treated with either vehicle or 100 ng/ml EGF for 20 min. Cell lysates were immunoprecipitated with antibodies against RalA, and immunoprecipitates were blotted with antibodies to GRK2. Data represent results from two independent experiments with similar outcomes. (C) Effects of EGFR activation on the interaction between endogenous LPAR1 and GRK2. Cells were treated with either vehicle or 100 ng/ml EGF for 20 min. Cell lysates were immunoprecipitated with antibodies to LPAR1, and immunoprecipitates were blotted with antibodies to GRK2. Data represent results from two independent experiments with similar outcomes.

and D_2R undergo endocytosis through multiple endocytic routes including clathrin-mediated, caveolar as well as unidentified endocytic pathway(s) [43]. In order to understand the relationship between RalA-mediated regulation of GPCR endocytosis and the specific microdomains involved, functional roles of GRK2 and β-arrestins in each endocytic pathway are needed to be clarified.

It is generally accepted that binding of Gβγ and lipids such as PIP2 to the PH domain of GRK2 mediates receptor phosphorylation and membrane association of the kinase [44–48]. However, detailed molecular mechanisms of the intermediate steps, such as intracellular trafficking properties of Gβγ before and after interaction with GRK2 or involvement of intermediate cellular components which affect their interactions, are still unclear. Our results might provide some clue regarding the intermediate mechanisms that regulate the interactions among receptor, Gβγ, and GRK2.

GPCRs are classified into class A and class B, depending on their endocytic properties [39]. Endocytic class A GPCRs, such as β_2AR and D_2R, establish a transient interaction with β-arrestin and dissociate from β-arrestin when they enter the cytosol as endocytic vesicles. Endocytic class B GPCRs, such as AT_{1A} receptor and vasopressin type 2 receptor, establish a stable interaction with β-arrestin and co-localize in endosomes after being internalized. Presently, both endocytic class A and class B GPCRs inhibited RalA as far as they have tendency to undergo endocytosis, suggesting that the principles established in this study can be applied regardless of the post-endocytic fate of the receptor.

Our results from D_2R and β_2AR show that GRK2, rather than RalBP1, is involved in the regulation of receptor endocytosis. GRK2 preferentially binds to active RalA rather than to the receptors, resulting in diminished receptor endocytosis. Similar to our results, a recent study showed that RalA interacts with GRK2 and that their interaction is prompted by activation of the LPA1 receptor [49]. Thus, it could be speculated that single transmembrane receptors and the seven transmembrane receptors employ RalBP1 and GRK2, respectively, to induce receptor proteins to connect to endocytosis adaptors such as AP-2.

In summary, present study reveals a novel regulatory mechanism involved in the endocytosis of GPCRs. Conversion of GTP-RalA to GDP-RalA is a critical cellular event that allows receptor-mediated endocytosis to occur. The importance of this work is that it provides a principle that could predict the occurrence of GPCR endocytosis.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

We thank the Korea Basic Science Institute for technical support. This work was funded by KRF-2014R1A2A2A01002547.

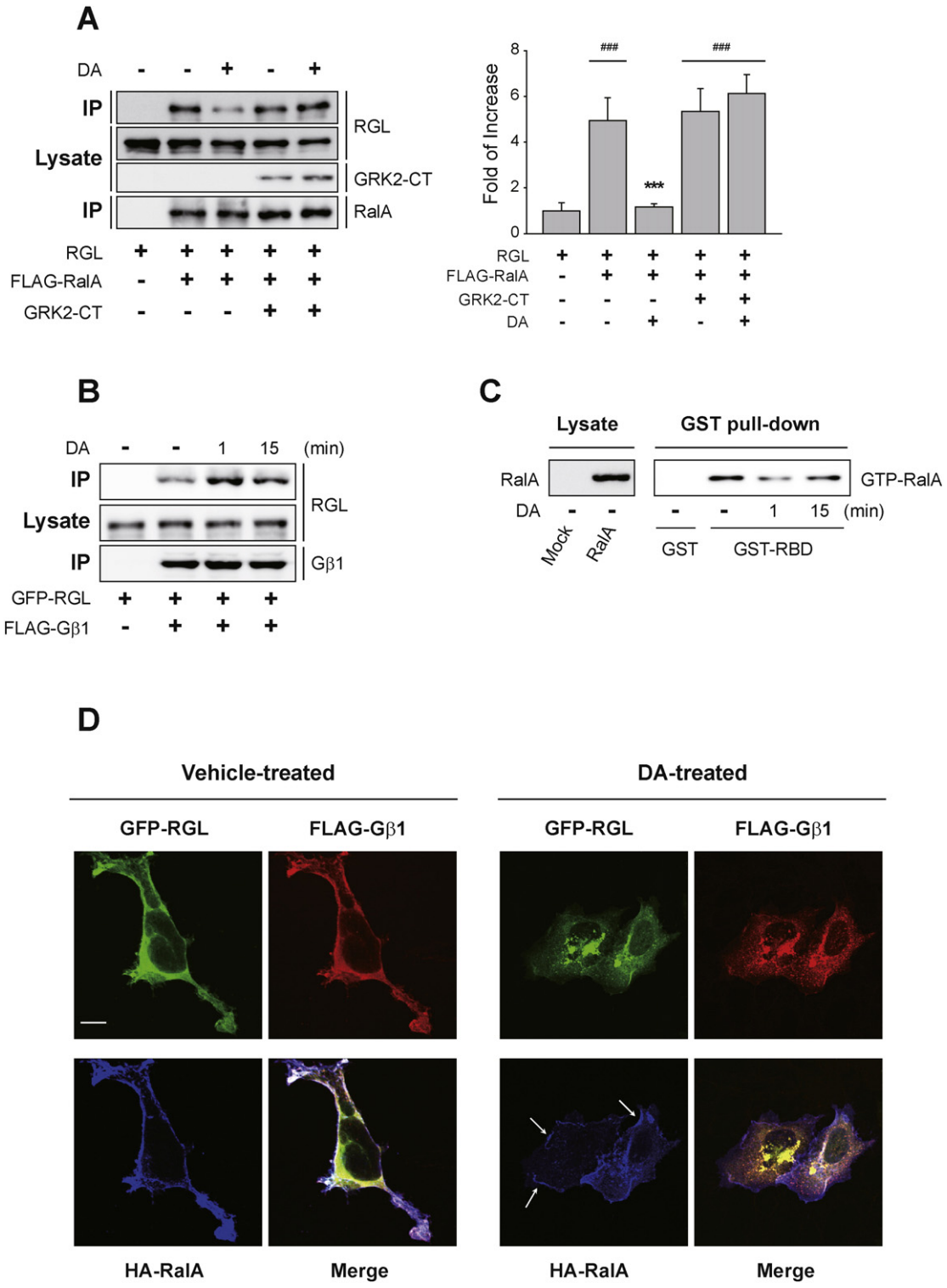


Fig. 8. Molecular mechanisms involved in the D_2R-mediated inhibition of RalA. (A) Involvement of the Gβγ subunit in the D_2R-mediated dissociation of RGL from RalA. Cells expressing D_2R (1.7 pmol/mg protein) were transfected with GFP-RGL (105 kDa), FLAG-RalA, and/or GRK2-CT (20 kDa). Cells were treated with 10 μM DA for 1 min. Cell lysates were immunoprecipitated with FLAG beads and blotted with antibodies to GFP, GRK2, and FLAG. $^{***}p < 0.001$ compared with other RGL/RalA groups. $^{\#\#\#}p < 0.001$ compared with RGL or RGL/RalA/DA group. (B) Time course of the interaction between RGL and Gβ1 (36 kDa). Cells stably expressing D_2R were transfected with GFP-RGL along with FLAG-Gβ1. Cells were treated with vehicle or with 10 μM DA for 0-15 min. Cell lysates were immunoprecipitated with FLAG beads and blotted with antibodies to GFP. Interaction between RGL and Gβ1 increased 4.2 ± 1.2 (1 min) and 2.9 ± 1.1 (15 min) folds in response to agonist stimulation of D_2R ($p < 0.05$, n = 3). (C) Time course of RalA inhibition. The cells which stably express D_2R were transfected with GFP-RalA. Cells were treated with 10 μM DA for 0–15 min. Level of GTP-RalA was 100 ± 21.8 (0 min), 38.7 ± 11.2 (1 min), and 66.7 ± 12.6 (15 min). There was statistical differences between '1 min' group and other groups ($p < 0.05$, n = 3). (D) Dissociation between RGL and RalA by agonist stimulation of D_2R determined by immunocytochemistry. Cells stably expressing D_2R were transfected with FLAG-Gβ1 along with GFP-RGL and HA-RalA. Cells were treated with vehicle (left panel) or with 10 μM DA for 5 min (right panel). Cells were labeled with FLAG and HA antibodies at 1:1000 dilutions, followed by Alexa 555- and Alexa 647- conjugated secondary antibodies at 1:500 dilutions. The horizontal bar represents 10 μm. Arrows represent RalA on the plasma membrane.

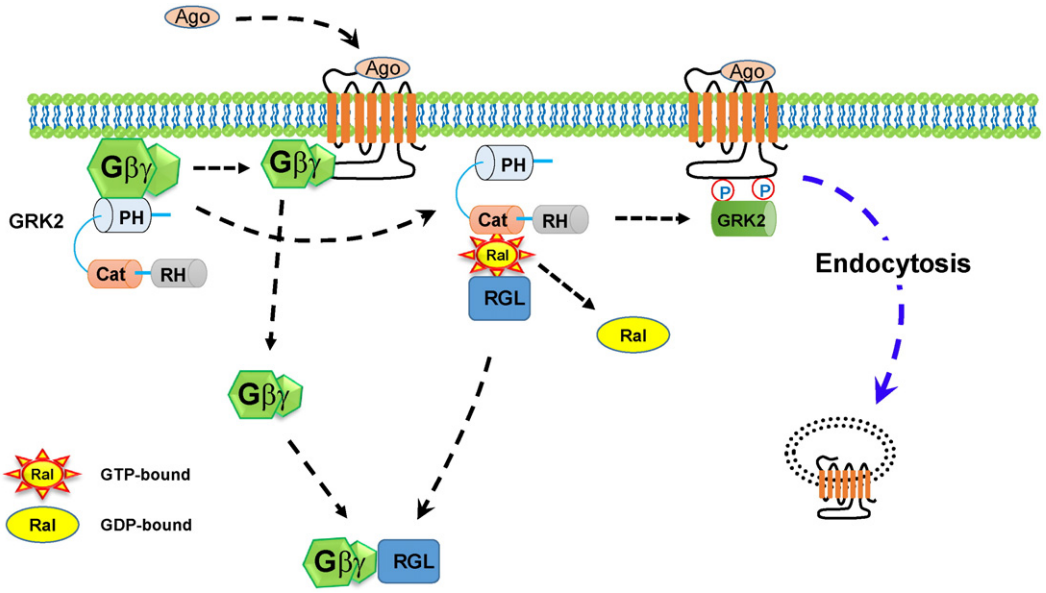


Fig. 9. Diagram showing Gβγ-mediated conversion of GTP-bound to GDP-bound RalA in response to agonist stimulation of internalizing GPCRs. GRK2 is bound to Gβγ through PH domain. In response to agonist stimulation, Gβγ dissociates from GRK2 and translocates toward cytosol as a complex with RGL, resulting in the dissociation of RGL from RalA. GRK2 which is dissociated from Gβγ interacts with GTP-RalA which is associated with RGL through the catalytic domain of GRK2. As RGL translocates to cytosol as a complex with Gβγ, GTP-RalA is converted to GDP-RalA to which GRK2 has low affinity. GRK2 which is released in the cascade of RalA inhibition is ready for the interaction with receptor or other endocytic machineries.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.10.007.

References

[1] J.L. Benovic, A. DeBlasi, W.C. Stone, M.G. Caron, R.J. Lefkowitz, Beta-adrenergic receptor kinase: primary structure delineates a multigene family, Science 246 (1989) 235–240.
[2] J.L. Benovic, R.H. Strasser, M.G. Caron, R.J. Lefkowitz, Beta-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 (1986) 2797–2801.
[3] L.S. Barak, S.S. Ferguson, J. Zhang, M.G. Caron, A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation, J. Biol. Chem. 272 (1997) 27497–27500.
[4] A. Claing, S.A. Laporte, M.G. Caron, R.J. Lefkowitz, Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins, Prog. Neurobiol. 66 (2002) 61–79.
[5] Y. Takai, T. Sasaki, T. Matozaki, Small GTP-binding proteins, Physiol. Rev. 81 (2001) 153–208.
[6] R.D. Burgoyne, Small GTP-binding proteins, Trends Biochem. Sci. 14 (1989) 394–396.
[7] P. Chardin, A. Tavitian, The ral gene: a new ras related gene isolated by the use of a synthetic probe, EMBO J. 5 (1986) 2203–2208.
[8] L.A. Feig, T. Urano, S. Cantor, Evidence for a Ras/Ral signaling cascade, Trends Biochem. Sci. 21 (1996) 438–441.
[9] J.L. Bos, All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral, EMBO J. 17 (1998) 6776–6782.
[10] E.M. van Dam, P.J. Robinson, Ral: mediator of membrane trafficking, Int. J. Biochem. Cell Biol. 38 (2006) 1841–1847.
[11] S. Nakashima, K. Morinaka, S. Koyama, M. Ikeda, M. Kishida, K. Okawa, A. Iwamatsu, S. Kishida, A. Kikuchi, Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors, EMBO J. 18 (1999) 3629–3642.
[12] V. Jullien-Flores, Y. Mahe, G. Mirey, C. Leprince, B. Meunier-Bisceuil, A. Sorkin, J.H. Camonis, RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis, J. Cell Sci. 113 (Pt 16) (2000) 2837–2844.
[13] T. Matsuzaki, S. Hanai, H. Kishi, Z. Liu, Y. Bao, A. Kikuchi, K. Tsuchida, H. Sugino, 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 (2002) 19008–19018.
[14] K.M. Kim, K.J. Valenzano, S.R. Robinson, W.D. Yao, L.S. Barak, M.G. Caron, Differential regulation of the dopamine D2 and D3 receptors by G protein-coupled receptor kinases and beta-arrestins, J. Biol. Chem. 276 (2001) 37409–37414.
[15] R. St-Arnaud, J.G. Chabot, G. Pelletier, F. Labrie, P. Walker, Epidermal growth factor: structure, location, phosphorylation and regulation of its receptor, Biochimie 66 (1984) 515–530.
[16] S. Beom, D. Cheong, G. Torres, M.G. Caron, K.M. Kim, Comparative studies of molecular mechanisms of dopamine D2 and D3 receptors for the activation of extracellular signal-regulated kinase, J. Biol. Chem. 279 (2004) 28304–28314.
[17] P.R. O'Neill, W.K. Karunarathne, V. Kalyanaraman, J.R. Silvius, N. Gautam, G-protein signaling leverages subunit-dependent membrane affinity to differentially control betagamma translocation to intracellular membranes, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E3568–E3577.
[18] M. Shipitsin, L.A. Feig, RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells, Mol. Cell. Biol. 24 (2004) 5746–5756.
[19] K.M. de Bruyn, J. de Rooij, R.M. Wolthuis, H. Rehmann, J. Wesenbeek, R.H. Cool, A.H. Wittinghofer, J.L. Bos, RalGEF2, a pleckstrin homology domain containing guanine nucleotide exchange factor for Ral, J. Biol. Chem. 275 (2000) 29761–29766.
[20] Q. Guo, H. Subramanian, K. Gupta, H. Ali, Regulation of C3a receptor signaling in human mast cells by G protein coupled receptor kinases, PLoS One 6 (2011), e22559.
[21] X. Zhang, F. Wang, X. Chen, Y. Chen, L. Ma, Post-endocytic fates of delta-opioid receptor are regulated by GRK2-mediated receptor phosphorylation and distinct beta-arrestin isoforms, J. Neurochem. 106 (2008) 781–792.
[22] B.K. Atwood, J. Lopez, J. Wager-Miller, K. Mackie, A. Straiker, Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis, BMC Genomics 12 (2011) 14.
[23] M. Staehelin, P. Simons, K. Jaeggi, N. Wigger, Cgp-12177. A hydrophilic beta-adrenergic receptor radioligand reveals high affinity binding of agonists to intact cells, J. Biol. Chem. 258 (1983) 3496–3502.
[24] M. van Triest, J. de Rooij, J.L. Bos, Measurement of GTP-bound Ras-like GTPases by activation-specific probes, Methods Enzymol. 333 (2001) 343–348.
[25] A.C. Magalhaes, H. Dunn, S.S. Ferguson, Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins, Br. J. Pharmacol. 165 (2012) 1717–1736.
[26] N. Segev, GTPases in intracellular trafficking: an overview, Semin. Cell Dev. Biol. 22 (2011) 1–2.
[27] S.B. Cantor, T. Urano, L.A. Feig, Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases, Mol. Cell. Biol. 15 (1995) 4578–4584.
[28] M. Ikeda, O. Ishida, T. Hinoi, S. Kishida, A. Kikuchi, Identification and characterization of a novel protein interacting with Ral-binding protein 1, a putative effector protein of Ral, J. Biol. Chem. 273 (1998) 814–821.
[29] R. Slaaby, T. Jensen, H.S. Hansen, M.A. Frohman, K. Seedorf, PLD2 complexes with the EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist stimulation, J. Biol. Chem. 273 (1998) 33722–33727.
[30] Y. Shen, L. Xu, D.A. Foster, Role for phospholipase D in receptor-mediated endocytosis, Mol. Cell. Biol. 21 (2001) 595–602.
[31] T. Koch, L.O. Brandenburg, S. Schulz, Y. Liang, J. Klein, V. Hollt, ADP-ribosylation factor-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis, J. Biol. Chem. 278 (2003) 9979–9985.
[32] T. Koch, L.O. Brandenburg, Y. Liang, S. Schulz, A. Beyer, H. Schroder, V. Hollt, Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization, J. Neurochem. 88 (2004) 680–688.
[33] G. Du, P. Huang, B.T. Liang, M.A. Frohman, Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis, Mol. Biol. Cell 15 (2004) 1024–1030.

[34] G. Kong, R. Penn, J.L. Benovic, A beta-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the beta 2-adrenergic receptor, J. Biol. Chem. 269 (1994) 13084–13087.
[35] D. Cho, M. Zheng, C. Min, L. Ma, H. Kurose, J.H. Park, K.M. Kim, Agonist-induced endocytosis and receptor phosphorylation mediate resensitization of dopamine D(2) receptors, Mol. Endocrinol. 24 (2010) 574–586.
[36] S.S. Yu, R.J. Lefkowitz, W.P. Hausdorff, Beta-adrenergic receptor sequestration. A potential mechanism of receptor resensitization, J. Biol. Chem. 268 (1993) 337–341.
[37] T. Shiina, A. Kawasaki, T. Nagao, H. Kurose, Interaction with beta-arrestin determines the difference in internalization behavor between beta1- and beta2-adrenergic receptors, J. Biol. Chem. 275 (2000) 29082–29090.
[38] K.L. Pierce, L.M. Luttrell, R.J. Lefkowitz, New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades, Oncogene 20 (2001) 1532–1539.
[39] R.H. Oakley, S.A. Laporte, J.A. Holt, M.G. Caron, L.S. Barak, Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors, J. Biol. Chem. 275 (2000) 17201–17210.
[40] R.H. Oakley, S.A. Laporte, J.A. Holt, L.S. Barak, M.G. Caron, Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*, J. Biol. Chem. 276 (2001) 19452–19460.
[41] A. Kikuchi, S.D. Demo, Z.H. Ye, Y.W. Chen, L.T. Williams, RalGDS family members interact with the effector loop of ras p21, Mol. Cell. Biol. 14 (1994) 7483–7491.
[42] H. Murai, M. Ikeda, S. Kishida, O. Ishida, M. Okazaki-Kishida, Y. Matsuura, A. Kikuchi, Characterization of Ral GDP dissociation stimulator-like (RGL) activities to regulate c-fos promoter and the GDP/GTP exchange of Ral, J. Biol. Chem. 272 (1997) 10483–10490.
[43] S. Guo, X. Zhang, M. Zheng, X. Zhang, C. Min, Z. Wang, S.H. Cheon, M.H. Oak, S.Y. Nah, K.M. Kim, Selectivity of commonly used inhibitors of clathrin-mediated and caveolae-dependent endocytosis of G protein-coupled receptors, Biochim. Biophys. Acta 1848 (2015) 2101–2110.
[44] J.A. Pitcher, J. Inglese, J.B. Higgins, J.L. Arriza, P.J. Casey, C. Kim, J.L. Benovic, M.M. Kwatra, M.G. Caron, R.J. Lefkowitz, Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors, Science 257 (1992) 1264–1267.
[45] Y. Daaka, J.A. Pitcher, M. Richardson, R.H. Stoffel, J.D. Robishaw, R.J. Lefkowitz, Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 2180–2185.
[46] R. Sterne-Marr, P.A. Leahey, J.E. Bresee, H.M. Dickson, W. Ho, M.J. Ragusa, R.M. Donnelly, S.M. Amie, J.A. Krywy, E.D. Brookins-Danz, S.C. Orakwue, M.J. Carr, K. Yoshino-Koh, Q. Li, J.J. Tesmer, GRK2 activation by receptors: role of the kinase large lobe and carboxyl-terminal tail, Biochemistry 48 (2009) 4285–4293.
[47] S.K. DebBurman, J. Ptasienski, J.L. Benovic, M.M. Hosey, G protein-coupled receptor kinase GRK2 is a phospholipid-dependent enzyme that can be conditionally activated by G protein betagamma subunits, J. Biol. Chem. 271 (1996) 22552–22562.
[48] J.A. Pitcher, Z.L. Fredericks, W.C. Stone, R.T. Premont, R.H. Stoffel, W.J. Koch, R.J. Lefkowitz, Phosphatidylinositol 4,5-bisphosphate (PIP2)-enhanced G protein-coupled receptor kinase (GRK) activity. Location, structure, and regulation of the PIP2 binding site distinguishes the GRK subfamilies, J. Biol. Chem. 271 (1996) 24907–24913.
[49] A.I. Aziziyeh, T.T. Li, C. Pape, M. Pampillo, P. Chidiac, F. Possmayer, A.V. Babwah, M. Bhattacharya, Dual regulation of lysophosphatidic acid (LPA1) receptor signalling by Ral and GRK, Cell. Signal. 21 (2009) 1207–1217.