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# β-Arrestin2 Plays Permissive Roles in the Inhibitory Activities of RGS9-2 on G Protein-Coupled Receptors by Maintaining RGS9-2 in the Open Conformation▽

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**Together with G protein-coupled receptor (GPCR) kinases (GRKs) and β-arrestins, RGS proteins are the major family of molecules that control the signaling of GPCRs. The expression pattern of one of these RGS family members, RGS9-2, coincides with that of the dopamine $D_3$ receptor ($D_3R$) in the brain, and *in vivo* studies have shown that RGS9-2 regulates the signaling of D2-like receptors. In this study, β-arrestin2 was found to be required for scaffolding of the intricate interactions among the dishevelled-EGL10-pleckstrin (DEP) domain of RGS9-2, Gβ5, R7-binding protein (R7BP), and $D_3R$. The DEP domain of RGS9-2, under the permission of β-arrestin2, inhibited the signaling of $D_3R$ in collaboration with Gβ5. β-Arrestin2 competed with R7BP and Gβ5 so that RGS9-2 is placed in the cytosolic region in an open conformation which is able to inhibit the signaling of GPCRs. The affinity of the receptor protein for β-arrestin2 was a critical factor that determined the selectivity of RGS9-2 for the receptor it regulates. These results show that β-arrestins function not only as mediators of receptor-G protein uncoupling and initiators of receptor endocytosis but also as scaffolding proteins that control and coordinate the inhibitory effects of RGS proteins on the signaling of certain GPCRs.**

The regulation of G protein-coupled receptors (GPCRs) involves various cellular events in different time frames, and the detailed regulatory mechanism can be unique for each receptor type and the signal it mediates. Much of our knowledge concerning the molecular basis of homologous desensitization of GPCRs is derived from studies of the $\beta_2$-adrenergic receptor ($\beta_2AR$), in which GPCR kinases (GRKs) and β-arrestins play central roles. According to this working model, GRK-mediated receptor phosphorylation, followed by the association of β-arrestin, causes uncoupling of the GPCR from the G protein (16, 18, 30). However, the detailed molecular mechanism of this uncoupling of receptors from the G protein is unclear, aside from the simple idea that β-arrestins could physically interfere with the interaction between the receptor and G protein.

Upon agonist binding, GPCRs stimulate the conversion of the inactive heterotrimeric GTP-binding protein GDP-Gαβγ to GTP-Gα and Gβγ. The duration of the active state of the G protein, GTP-Gα, is regulated by two different cellular components, the weak GTPase activity of Gα itself and the catalytic activity of GTPase-activating proteins (GAPs). Regulators of G protein signaling (RGS) act as GAPs for the heterotrimeric G protein α subunit (49). More than 30 RGS proteins have been discovered over the last decade, and they are divided into 8 subfamilies (11, 26, 52).

Among these RGS proteins, RGS2, RGS4, and RGS9-2 are known to be mutually related to the dopaminergic nervous system. It is known that the expression of the genes for RGS2 and RGS4 changes in response to dopaminergic stimulation (43, 44); however, the roles of RGS2 and RGS4 in the signaling and intracellular trafficking of $D_2R$ and $D_3R$ have not been reported. RGS9-2 is highly enriched in the striatum and nucleus accumbens, where D2-like receptors ($D_2R$, $D_3R$, $D_4R$) exert their major physiological actions. It was reported that D2-like receptors are functionally regulated by RGS9-2. For example, viral expression of RGS9-2 in the nucleus accumbens or dialysis of RGS9-2 proteins into striatal cholinergic interneurons reduced the behavioral or electrophysiological response to stimulation of the D2-like receptor (6, 35). On the other hand, knockout of RGS9-2 enhanced behavioral responses to the activation of D2-like receptors (28, 35). The specific subtype of D2-like receptors was not identified in these studies.

RGS9-2 belongs to the R7 RGS subfamily and contains the RGS, dishevelled-EGL10-pleckstrin (DEP) homology, and G-gamma-like (GGL) domains. The RGS domain binds to the Gα subunit and mediates GAP activity; the DEP domain is a protein module of ~90 amino acids that was first discovered in three proteins, dishevelled, EGL-10, and pleckstrin; and the GGL domain confers protein stability by dimerization with Gβ5 (8, 32, 48).

The molecular mechanism of R7 RGS protein has been extensively characterized for the regulatory actions of RGS7 on the signaling of the M3 muscarinic receptor. The DEP domain, which exists as a complex with Gβ5 (inactive, closed state), is converted to another conformation in response to activation of the M3 muscarinic receptor. In this new conformation (active, open state), the DEP domain is dissociated from Gβ5 but bound to a third-party binding protein and able to inhibit the signaling of the M3 muscarinic receptor (33). A key question remaining is what controls the conversion from the inactive to the active conformation? Although previous

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studies have suggested that the interaction between the intracellular receptor regions of the M3 muscarinic receptor and the DEP domain of RGS7 contribute to the conversion between the open and closed states (38, 39), it is still not clear whether there is a common intermediate cellular component that connects the DEP domain and the receptor regions.

In this study, we found that RGS9-2, which interacted with both $D_2R$ and $D_3R$, exerted inhibitory regulation on the signaling of $D_3R$ exclusively and that this selectivity was achieved by the differences in affinity between the receptor and β-arrestin2. β-Arrestin2 mediated the assembly of a regulatory complex consisting of the DEP domain of RGS9-2 and Gβ5, which was required for the regulation of $D_3R$ functions. These findings represent a novel regulatory mechanism for GPCRs in which β-arrestins scaffold RGS9-2 and Gβ5 to the signaling pathway of certain receptor proteins.

## MATERIALS AND METHODS

**Materials.** Human embryonic kidney (HEK293) cells, rat glioma C6 cells, and mouse neuroblastoma neuro 2a cells were obtained from the American Type Culture Collection (Manassas, VA). [$^3$H]sulpiride and [$^3$H]spiperone were purchased from NEN (Boston, MA). Dopamine, (−)quinpirole, forskolin, mouse FLAG antibodies, mouse FLAG antibody-conjugated agarose beads, rabbit FLAG antibodies, glutathione *S*-transferase (GST)-conjugated agarose beads, antibodies to actin and RGS9-2, and horseradish peroxidase-labeled secondary antibodies were obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO). Antibodies to green fluorescent protein (GFP) were obtained from Clontech (Mountain View, CA). Antibodies to β-arrestins were provided by R. J. Lefkowitz (Duke University). Anti-mouse antibody–-Alexa Fluor 555, anti-rabbit antibody–Alexa Fluor 647, and anti-rabbit antibody–Alexa Fluor 594 were purchased from Molecular Probes (Invitrogen, Carlsbad, CA).

**Plasmid constructs.** Detailed information about the human $D_2R$ and $D_3R$ and rat β-arrestins are described elsewhere (5, 24, 25). Chimeric receptors consisting of $D_2R$ and $D_3R$ in which the second and third intracellular loops were exchanged were described previously (25, 36). The human $D_4$ receptor, with 4-fold repeats of a 48-bp sequence within the third intracellular loop ($D_{4\text{-}4}$), was described previously (47). Full-length RGS9-2, RGS9-2 lacking the DEP domain (DEPless), and the DEP domain fused with enhanced GFP (EGFP) as described previously (28) were provided by J. Schwarz (California Institute of Technology). The DEP domain was also tagged with the M2-FLAG epitope at the N-terminal tail in the pCMV5 vector or fused to GST. RGS2 and RGS4 were tagged at the C terminus with EGFP. EGFP-Gβ5 was provided by T. E. Hébert (McGill University, Montreal, Quebec, Canada), and R7-binding protein (R7BP) constructs were provided by K. A. Martemyanov (University of Minnesota).

**Immunoprecipitation.** The cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) on a rotation wheel for 1 h at 4°C. The supernatants were mixed with 35 μl of a 50% slurry of anti-FLAG-agarose beads for 2 to 3 h on a rotation wheel. The beads were washed with washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40) three times for 10 min each.

***In vitro* binding studies.** To determine the interaction between the third intracellular loop of $D_2R$ or $D_3R$ with RGS9-2 or β-arrestin2, the third cytoplasmic loop of $D_2R$ or $D_3R$ was bacterially expressed as a fusion protein with GST. Since the whole third loop of $D_2R$ was not expressed as a soluble protein (data not shown), the third loop of $D_2R$ was divided into two regions ($I_3D_2$-N and $I_3D_2$-C). $I_3D_2$-N covers R-227 to I-304, and $I_3D_2$-C covers E-250 to K-342. A GST fusion protein with the third loop of rat $D_3R$ ($I_3D_3$) was described previously (10). BL21 bacterial cells expressing fusion proteins were treated with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h, lysed, and centrifuged, and the resulting supernatant was aliquoted and stored at −70°C until use. Lysates of HEK293 cells expressing RGS9-2–EGFP or β-arrestin2 were incubated with glutathione agarose beads which had been bound to GST fusion proteins. Agarose beads were washed and retained proteins were eluted with SDS sample buffer.

To determine the region of β-arrestin2 involved in the interaction with DEP, full-length β-arrestin2 (β-arr2-FL), the N domain of β-arrestin2 (β-arr2-N), the C domain of β-arrestin2 (β-arr2-C), and the C domain plus the carboxy tail of β-arrestin2 (β-arr2-C-CT) were expressed as GST fusion proteins. Bacterial lysates containing the GST fusion proteins of various constructs of β-arrestin2 were mixed with lysates of HEK293 cells transfected with DEP-EGFP. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed by SDS-PAGE and immunoblotted with antibodies to GFP or β-arrestins.

**Whole-cell cAMP assays.** Cellular cyclic AMP (cAMP) was measured by an indirect method using reporter gene induction. This method had been used for the determination of $D_2R$ signaling (19, 20). Cells were transfected with $D_2R$ or $D_3R$ together with a reporter plasmid containing the firefly luciferase gene under the transcriptional control of multiple cAMP-responsive elements (CRE) or a vector control. Cells were stimulated with 1 to 3 μM forskolin with or without the dopamine agonist quinpirole for 4 h, and then relative luciferase activity was measured using a dual-luciferase assay kit (Promega, Madison, WI).

**Internalization assay.** Internalization of $D_2R$ and $D_3R$ was measured using the hydrophilic properties of sulpiride as described previously (25). Briefly, HEK293 cells expressing $D_2R$ or $D_3R$ were seeded 1 day after transfection at a density of $1.5 \times 10^5$ cells/well in 24-well plates. The following day, cells were rinsed once and preincubated for 15 min with 0.5 ml of prewarmed, serum-free medium containing 10 mM HEPES, pH 7.4, at 37°C. Cells were stimulated with 10 μM dopamine or 1 μM phorbol myristate acetate (PMA) for 30 to 60 min as indicated. Stimulation was terminated by quickly cooling the plates on ice and washing the cells three times with ice-cold serum-free medium containing 20 mM HEPES, pH 7.4. Cells were incubated with 250 μl of [$^3$H]sulpiride (final concentrations, 2.2 nM for $D_2R$ and 7.2 nM for $D_3R$) at 4°C for 150 min in the absence or presence of an unlabeled competitive inhibitor (10 μM haloperidol). Cells were washed two times with the same medium, and 1% SDS was added. Samples were mixed with 3 ml of Lefkofluor scintillation fluid and counted on a liquid scintillation analyzer.

**shRNAs of β-arrestins.** HEK293 cells were stably expressed with small hairpin RNA (shRNA) constructs of GFP, β-arrestin1, and/or β-arrestin2. Levels of endogenous β-arrestins were detected by immunoblotting. Detailed information about the shRNA plasmids was published previously (51).

**Statistics.** All of the results are expressed as the mean ± the standard error of the mean. Comparisons between groups were performed using analysis of variance. For some results, a Student *t* test was used.

## RESULTS

**RGS9-2 exerts selective inhibitory effects on the signaling of $D_3R$ out of three D2-like receptor subtypes.** RGS2, RGS4, and RGS9-2 are known to be closely related to the dopaminergic nervous system and drug addiction (45). RGS9-2 is highly enriched in the striatum and nucleus accumbens, where it regulates the functional responses of D2-like receptors *in vivo* (6, 28, 35). Regulatory roles for RGS2 and RGS4 in the signaling of D2-like receptors have not been reported. Also, the specific D2-like receptor subtypes that are regulated by RGS9-2 have not yet been identified, even though several *in vivo* studies have shown that the brain functions of D2-like receptors are inhibited by RGS9-2.

Effects of RGS2, RGS4, and RGS9-2 on the signaling of D2-like receptors were determined. The signaling of $D_2R$, $D_3R$, and $D_4R$ was measured by determining the dose-dependent inhibition of cAMP production. Cellular cAMP levels were determined either by direct column chromatography or by an indirect reporter gene assay as described previously (9). RGS2 did not have any effect (data not shown), but RGS4 inhibited the signaling of all three D2-like receptors (data not shown). Under the same experimental conditions, RGS9-2 selectively inhibited the signaling of $D_3R$ (Fig. 1A to C). RGS9-2 alone showed moderate but statistically significant inhibition of $D_3R$ signaling (Fig. 1B, open squares, dotted line, shows that the maximum inhibition of cAMP production decreased from 57% to 49%). Gβ5, which is known to bind to the GGL domain of RGS proteins (32), did not have a noticeable effect by itself but showed synergistic activity with RGS9-2 (Fig. 1B). As re-

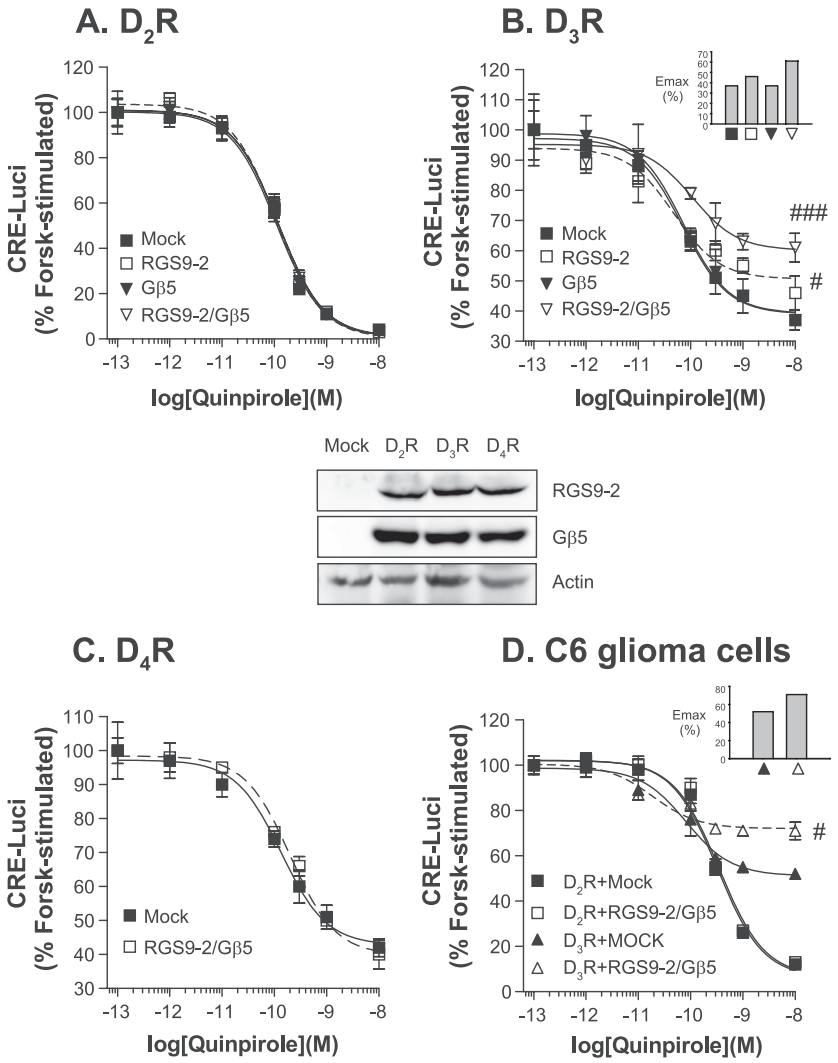


FIG. 1. Effects of RGS9-2 on the signaling of D2-like receptors. (A) Effects of RGS9-2 on the signaling of $D_2R$ were determined in cells expressing $D_2R$ together with EGFP-Gβ5 (0.6 μg) and/or RGS9-2–EGFP (3 μg). Cellular cAMP was measured using the CRE-Luci (luciferase) reporter gene as described in Materials and Methods. The receptor expression level was maintained at around 1.2 pmol/mg protein. (B) Effects of RGS9-2 on the signaling of $D_3R$ were determined in cells expressing $D_3R$ as in panel A. The receptor expression level was maintained at around 1.5 pmol/mg protein. #, $P < 0.05$ for the RGS9-2 group versus the mock-treated group. ###, $P < 0.001$ for the RGS9-2+Gβ5 group versus the mock-treated group. (C) Effects of RGS9-2 on the signaling of $D_4R$ were determined in cells expressing $D_4R$ together with EGFP-Gβ5 and RGS9-2–EGFP. The receptor expression level was maintained at around 1.2 pmol/mg protein. Levels of RGS9-2 and Gβ5 expression were determined in the cells used in panels A to C. Cell lysates from each experimental group were separated by SDS-PAGE and blotted with antibodies to GFP and actin. (D) Effects of RGS9-2 on the signaling of $D_3R$ and $D_2R$ were determined in brain-derived C6 glioma cells expressing $D_3R$ or $D_2R$ together with Gβ5 and RGS9-2 as described for panels A and B. Receptor expression levels were around 0.7 pmol/mg protein. #, $P < 0.05$ for the $D_3R$+RGS9-2+Gβ5 group versus the $D_3R$+Mock group. Emax, maximum effect.

ported previously (37), the role of Gβ5 was more than stabilization of RGS9-2. Gβ5 had a synergistic effect on the signaling of $D_3R$ (data not shown).

The $IC_{50}$ of quinpirole increased from 57.2 to 107.5 pM, and the maximum inhibition of cAMP production was decreased from 57 to 40% by coexpression of Gβ5 and RGS9-2. The selectivity of RGS9-2/Gβ5 for the signaling of $D_3R$ was also confirmed in brain-derived C6 glioma cells. As in HEK293 cells, the signaling of $D_3R$, but not that of $D_2R$, was inhibited by coexpression of RGS9-2 and Gβ5 in C6 glioma cells (Fig. 1D). These results show that only RGS9-2 exerts selective regulatory activity on the signaling of a specific subtype of D2-like receptors.

**RGS9-2 interacts with and inhibits the internalization and signaling of $D_2R$ and $D_3R$, respectively.** As a first step in understanding the molecular mechanisms involved in the inhibition of $D_3R$ signaling by RGS9-2, receptor regions which mediate the functional interaction with RGS9-2 were determined. When the interactions between RGS9-2 and $D_2R$/$D_3R$ were determined by immunoprecipitation, RGS9-2 interacted more abundantly with $D_3R$ than with$D_2R$ (Fig. 2A) ($P < 0.01$). When quantified, the immunoprecipitation of RGS9-2 in-

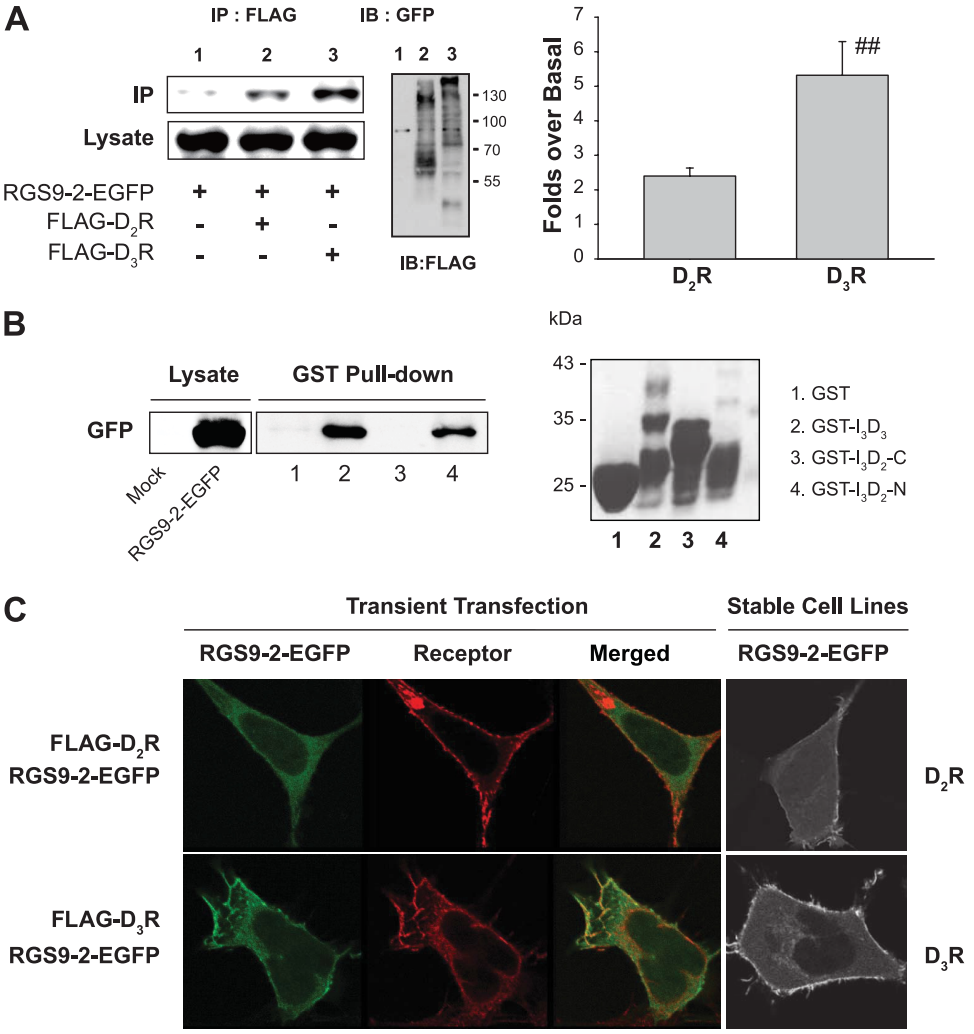


FIG. 2. Dopamine $D_2R$ and $D_3R$ differently interact with RGS9-2. (A) Interaction between RGS9-2 and $D_2R$ or $D_3R$ was determined by immunoprecipitation. HEK293 cells were transfected with RGS9-2–EGFP together with FLAG-tagged $D_2R$ or $D_3R$ in pCMV5. Cell lysates were immunoprecipitated (IP) with FLAG beads and immunoblotted (IB) with antibodies to GFP. The receptor expression level was maintained at around 1.2 pmol/mg protein. Receptor proteins were immunoprecipitated with mouse monoclonal antibody FLAG beads, and the resulting immunoprecipitates were blotted with antibodies to rabbit FLAG antibodies. Both $D_2R$ and $D_3R$ are known to be resolved into 2 or 3 bands by SDS-PAGE (24). The data represent results of three independent experiments with similar outcomes. (B) Determination of interaction between RGS9-2 and $D_2R$ or $D_3R$ by GST pulldown assay. Bacterial lysates containing the GST fusion proteins of the third intracellular loop of $D_2R$ ($I_3D_2$-N, the N-terminal part; $I_3D_2$-C, the C-terminal part) or $D_3R$ ($I_3D_3$) were mixed with lysates of HEK293 cells transfected with RGS9-2–EGFP. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed by SDS-PAGE and blotted with antibodies to GFP (GST pulldown part). A blot of HEK293 cell lysates is shown in the lysate part. On the right is an SDS-PAGE analysis of the afterwash of bacterial cell lysates. (C) Colocalization of RGS9-2 and $D_2R$ or $D_3R$ was determined by immunocytochemistry. Cells were transfected with FLAG-$D_2R$ or $D_3R$ along with RGS9-2–EGFP (left two panels). Cells were labeled with antibodies to FLAG, followed by Alexa Fluor 594-conjugated anti-rabbit secondary antibodies. In the right panel, cells stably expressing $D_2R$ (1.2 pmol/mg protein) or $D_3R$ (0.9 pmol/mg protein) were transfected with RGS9-2–EGFP.

creased 2.4-fold ± 0.232-fold and 5.32-fold ± 0.973-fold over the background in cells expressing similar levels of $D_2R$ and $D_3R$, respectively. Similar results were obtained in a GST pulldown assay. As shown in Fig. 2B, the third intracellular loop of $D_3R$ interacts more abundantly with RGS9-2 than $D_2R$ does. In the case of $D_2R$, the third intracellular loop was divided into two regions to increase the solubility of GST fusion protein and it was found that the N-terminal region, but not the C-terminal region, interacts with RGS9-2. Immunocytochemical studies showed that colocalization with RGS9-2 was more evident in cells expressing $D_3R$ than in cells expressing $D_2R$ (Fig. 2C, left two panels). In accordance with this, a larger fraction of RGS9-2 was found on the plasma membrane of cells stably expressing $D_3R$ than on the plasma membrane of cells stably expressing $D_2R$ (Fig. 2C, right panel). These results are consistent with the more abundant coimmunoprecipitation of RGS9-2 with $D_3R$ than with $D_2R$ (Fig. 2A and B).

A recent study showed that RGS9-2 inhibits the internalization of $D_2R$ (7). In agreement with these results, the combination of RGS9-2 and Gβ5 significantly inhibited the agonist-induced internalization of $D_2R$ (data not shown). However, this could not be tested for $D_3R$ since agonist-induced inter-

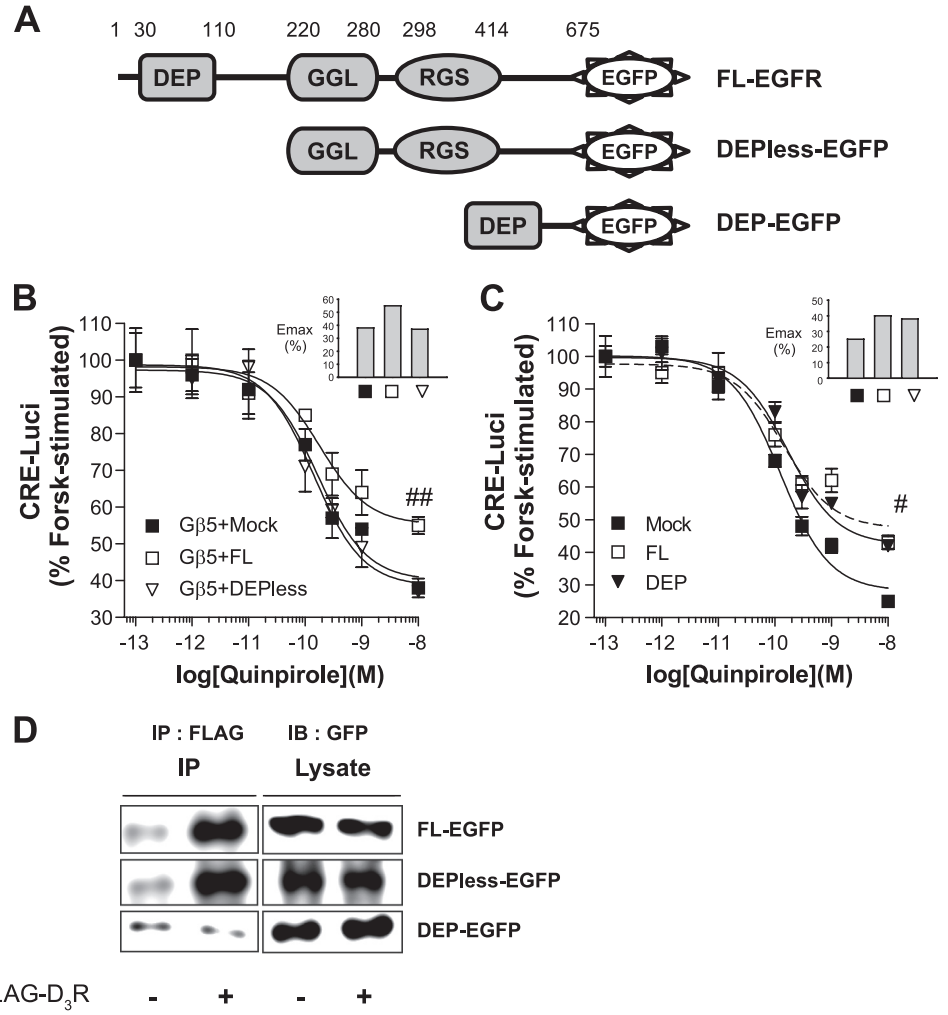


FIG. 3. Roles of specific subdomains of RGS9-2 in the regulation of $D_3R$ signaling. (A) Schematic diagram of the RGS9-2 constructs (28). The numbers at the top of the diagram indicate the positions of the domains in the original wild-type protein starting from the first Arg residue. (B and C) Role of the DEP domain in the regulation of $D_3R$ signaling by RGS9-2. (B) Cells were transfected with 3 μg of the FL-RGS9-2–EGFP or DEPless-EGFP construct together with Gβ5 in pCMV5. Receptor expression levels were equalized for each experimental group (around 1.7 pmol/mg protein). ##, $P < 0.01$ for the Gβ5+FL group versus the Gβ5+Mock group. (C) Cells were transfected with 3 μg FL-RGS9-2–EGFP and DEP-EGFP. Receptor expression levels were adjusted to around 2.0 pmol/mg protein. #, $P < 0.05$ for the FL or DEP group versus the mock-treated group. (D) Interactions between the $D_3R$ and RGS9-2 proteins were determined by immunoprecipitation from cell lysates expressing FLAG-$D_3R$ along with FL-RGS9-2–EGFP, DEPless–RGS9-2–EGFP, and the DEP domain of RGS9-2–EGFP. Immunoprecipitation (IP) and immunoblotting (IB) were conducted with FLAG beads and antibodies to GFP, respectively. Receptor expression levels were adjusted to around 1.7 pmol/mg protein. The data represent results of three independent experiments with similar outcomes. Emax, maximum effect.

nalization of $D_3R$ does not occur (25). Instead, the effect of RGS9-2/Gβ5 on the PMA-induced internalization of $D_3R$ was tested and it was found to have no effect (data not shown). These results show that RGS9-2/Gβ5 interacts with both $D_2R$ and $D_3R$ and selectively inhibits the internalization and signaling of $D_2R$ and $D_3R$, respectively.

**The DEP domain is involved in the inhibition of $D_3R$ signaling by RGS9-2.** Next, the specific regions of RGS9-2 that mediate the inhibitory activities against the signaling of $D_3R$ were determined. RGS9-2 contains three well-known structural domains, the DEP, GGL, and RGS domains. A previous study showed that the DEP domain of a yeast RGS, namely, Sst2, interacts with the C-terminal tail of the yeast GPCR Ste2, suggesting a role for DEP domains in the selective targeting of RGS proteins to specific GPCRs (3).

The role of the DEP domain in the regulation of $D_3R$ signaling was tested using an RGS9-2 construct lacking the DEP domain (DEPless) and the isolated DEP domain (Fig. 3A). As shown in Fig. 3B, signaling of $D_3R$ was inhibited by full-length RGS9-2 (FL-RGS9-2) but not by the DEPless form of RGS9-2. The effects of the DEP domain itself on the signaling of $D_3R$ were comparable to those of FL-RGS9-2 in the absence of Gβ5 (Fig. 3C). Signaling was compared in the absence of Gβ5 because the isolated DEP domain does not contain the GGL domain which binds to Gβ5.

To understand the molecular mechanisms involved in the regulation of $D_3R$ signaling by RGS9-2, the interaction between the $D_3R$ and RGS9-2 proteins was tested. As shown in Fig. 3D, $D_3R$ was immunoprecipitated with FL-RGS9-2 (4.28-fold ± 1.17-fold over background binding) and even better

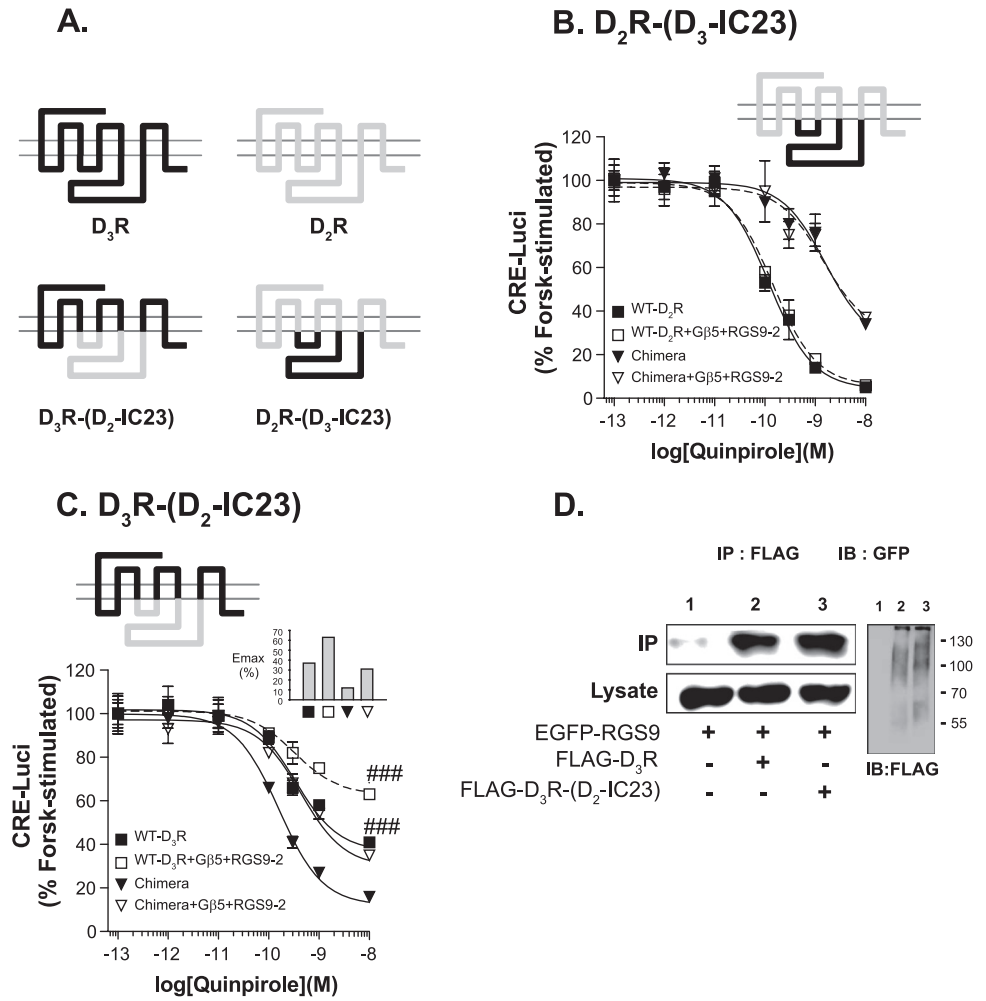


FIG. 4. Determination of receptor regions responsible for RGS9-2-mediated inhibition of $D_3R$ signaling. (A) Schematic representation of chimeric receptors consisting of $D_2R$ and $D_3R$, whose second and third intracellular loops were switched. Signaling of the chimeric receptors consisting of $D_2R$ and $D_3R$ was compared with that of the corresponding wild-type (WT) receptor: $D_2R$ versus $D_2R$-($D_3$-IC23) (B) or $D_3R$ versus $D_3R$-($D_2$-IC23) (C) Receptor expression levels were adjusted to around 1.5 to 1.7 pmol/mg protein. ###, $P < 0.001$ for the WT-$D_3R$+RGS9-2+Gβ5 group versus the WT-$D_3R$ group or for the Chimera+RGS9-2+Gβ5 group versus the Chimera group. (D) Profiles of RGS9-2 interactions with chimeric receptors were determined by immunoprecipitation (IP) with FLAG beads and immunoblotting (IB) with antibodies to GFP or FLAG. Receptor expression levels were adjusted to around 2.1 pmol/mg protein. Receptor proteins were immunoprecipitated and immunoblotted as described in the legend to Fig. 2A. The data represent results of three independent experiments with similar outcomes. Emax, maximum effect.

with DEPless-RGS9-2 (5.08-fold ± 1.38-fold over background binding), which lacked regulatory activity on $D_3R$ signaling. The DEP domain which inhibited $D_3R$, however, did not show noticeable binding to $D_3R$ (1.32-fold ± 1.28-fold over background binding). The results in Fig. 3B to D together suggest that the DEP domain plays a central role in the inhibition of $D_3R$ signaling, but in contrast to the yeast analog of RGS, SSt, the activity profile of RGS9-2 does not correlate with its binding to $D_3R$. In accordance with these results, the crystal structure of the Gβ5-RGS9 complex shows that the DEP domain is positioned at a location remote from the plasma membrane where GPCR and RGS9-2 form a complex (8). Similar findings on the functional role of the DEP domain were reported for inhibition of the endocytosis of the μ opioid receptor by RGS9-2 (34) and inhibition of the signaling of the M3 muscarinic receptor by RGS7 (38), a member of the R7 family of RGS proteins to which RGS9-2 belongs. As in these studies, the DEP domain, rather than the catalytic activity of GTPase activation through the RGS domain, seems to determine the inhibitory effect of RGS9-2 on the signaling of $D_3R$.

**Intermediate components could be involved in the inhibitory effect of RGS9-2 on the signaling of $D_3R$.** Results in Fig. 1 and 2 show that RGS9-2 interacts with the third intracellular loop of $D_2R$ and $D_3R$; however, the signaling of $D_3R$ is selectively inhibited by RGS9-2. These results suggest that the interaction between the intracellular loop and RGS9-2 might not be a critical factor that determines the selectivity of RGS9-2 for the signaling of $D_3R$. To confirm this, two chimeric receptors consisting of $D_2R$ and $D_3R$, in which the second and third intracellular loops were exchanged (36) (Fig. 4A), were utilized. $D_2R$ and $D_3R$ possess long third cytoplasmic loops but very short C-terminal tails; therefore, the second and third intracellular loops were reported to be involved in the signaling and intracellular trafficking of $D_2R$ and $D_3R$ (25, 36). Unex-

pectedly, exchange of these loops did not alter the regulatory effect of RGS9-2 on the signaling of these two receptors. As shown in Fig. 4B, RGS9-2 did not affect the signaling of $D_2R$-($D_3$-IC23), the $D_2R$ chimera that contained the second and third loops of $D_3R$. On the contrary, RGS9-2 exerted similar inhibitory effects on the signaling of wild-type $D_3R$ and $D_3R$-($D_2$-IC23), the $D_3R$ chimera that contained the second and third intracellular loops of $D_2R$ (Fig. 4C). As expected, RGS9-2 showed similar interactions with wild-type $D_3R$ (4.5-fold ± 1.1-fold over background binding) and $D_3R$-($D_2$-IC23) (4.78-fold ± 1.5-fold over background binding) (Fig. 4D).

The results of the GST pulldown assay (Fig. 2B) and functional studies using chimeric receptors (Fig. 4) show that RGS9-2 has higher affinity and functional selectivity for $D_3R$. Considering that the second and third intracellular loops are involved in the G protein coupling (36) and intracellular trafficking (25) of $D_2R$ and $D_3R$, it was unexpected that the specificity of RGS9-2 for the inhibition of $D_3R$ signaling was not altered when the intracellular loops were switched with each other. These results suggest that certain cellular components are likely to be involved in mediating the specificity of RGS9-2 for $D_3R$.

**β-Arrestin2 is required for the inhibition of $D_3R$ signaling by RGS9-2.** Results in Fig. 2 to 4 suggest that the inhibitory effect of RGS9-2 on the signaling of $D_3R$ could be mediated by a certain cellular element which interacts with both the DEP domain of RGS9-2 and $D_3R$. A previous study with *Xenopus* oocytes showed that the DEP domain is involved in the interaction with β-arrestins (22). Also, a study with PC12 cells showed that β-arrestin2 is immunoprecipitated with RGS9-2 and that this interaction was increased with activation of the μ opioid receptor (34). Interestingly, it was reported that $D_3R$, but not $D_2R$, is constitutively bound to β-arrestins in the absence of receptor activation (24), suggesting that β-arrestins could be the cellular element which mediates the inhibitory effect of RGS9-2 on the signaling of $D_3R$. As shown in Fig. 5A (upper panel), β-arrestin2 interacted much more weakly with DEPless-RGS9-2 than with FL-RGS9-2 or the DEP domain alone (FL, 3.77-fold ± 0.92-fold over background binding; DEPless, 1.66-fold ± 0.35-fold over background binding; DEP, 4.12-fold ± 1.05-fold over background binding; $P < 0.05$ for the DEPless-RGS9-2 group versus the FL-RGS9-2 or DEP domain group), suggesting that the DEP domain of RGS9-2 is involved in their interaction. The profile of binding between β-arrestin2 and RGS9-2 variants agrees with the profile of inhibition of the signaling of $D_3R$ by RGS9-2 variants (Fig. 3B and C). Interaction with RGS9-2 was specific for β-arrestin2 (Fig. 5A, lower panel).

To understand the functional meaning of the interaction between RGS9-2 and β-arrestin2, endogenous β-arrestin2 was knocked down by the stable expression of β-arrestin2 shRNA (data not shown). The inhibitory activity of RGS9-2 against the signaling of $D_3R$ was abolished in β-arrestin2 knockdown (KD) cells (Fig. 5B). The 50% inhibitory concentration ($IC_{50}$) was increased from 62 to 236 pM and from 57 to 84 pM by the coexpression of RGS9-2 in control KD cells and β-arrestin2 KD cells, respectively. The interaction between $D_3R$ and FL-RGS9-2 was also abolished in β-arrestin2 KD cells (Fig. 5C) (FL, 0.9-fold over background binding; DEP, 1.08-fold over background binding). These results were also confirmed by a GST pull-down assay. Interaction between RGS9-2 and the third intracellular loop of $D_3R$ decreased when cellular β-arrestin2 was knocked down (Fig. 5D). The interaction of $D_3R$ with DEPless was inhibited to a much lesser extent than that with wild-type RGS9-2 when endogenous β-arrestin2 was knocked down (Fig. 5C). This was expected because RGS9-2 binds to β-arrestin2 via the DEP domain, which is absent from the DEPless form. These results show that the regulatory activity of RGS9-2 on $D_3R$ signaling is correlated with the interaction between β-arrestin2 and the DEP domain.

The region of β-arrestin2 which interacts with the DEP domain of RGS9-2 was determined by a GST pulldown assay. For this, β-arrestin2 was divided into N domain, C domain, and CT (C terminus) regions as described in Materials and Methods. As shown in Fig. 5E, the DEP domain of RGS9-2 was found to interact with β-arrestin2 mainly through the N domain.

**Affinity of $D_3R$ for β-arrestin2 determines the selectivity of RGS9-2 for $D_3R$ signaling.** Since β-arrestin2 provides the selectivity of the functional and biochemical interactions between RGS9-2 and $D_3R$, the profile of binding between β-arrestin2 and $D_3R$ was compared with that of binding between β-arrestin2 and $D_2R$. When GST pulldown assays were conducted with β-arrestin2 and the third intracellular loop of $D_2R$ or $D_3R$, heavier binding of β-arrestin2 with the third intracellular loop of $D_2R$ was observed (Fig. 6A), and this result is in agreement with a previous report (29). The same study had shown that the second intracellular loop behaves in a similar way. However, when their interaction was determined by coimmunoprecipitation of the whole receptor protein molecule and β-arrestin2, β-arrestin2 was more abundantly immunoprecipitated with $D_3R$ than with $D_2R$, as reported previously (23, 24). $D_3R$ was constitutively bound to β-arrestin2 in the absence of agonist treatment (Fig. 6B), and this was also corroborated by microscopic images showing that $D_3R$ and β-arrestin2 colocalized on the plasma membrane (data not shown). These results show that β-arrestin2 interacts with the third intracellular loop of $D_2R$ and $D_3R$; however, their interaction is differently modulated by the receptor regions of $D_3R$ outside the intracellular loops. In support of this assumption, $D_3R$-($D_2$-IC23), the $D_3R$ chimera which contains the second and third intracellular loops of $D_2R$, showed the same β-arrestin2 interaction properties as wild-type $D_3R$ (Fig. 6C). As expected, $D_3R$, β-arrestin2, and RGS9-2 colocalized on the plasma membrane (data not shown).

These results suggest that the receptor regions which control binding to β-arrestin2 likely coincide with those involved in the regulation of RGS9-2 activity for the inhibition of $D_3R$ signaling. For example, $D_3R$-($D_2$-IC23) and $D_3R$ showed similar functional interactions with RGS9-2 (Fig. 4C) and similar binding with RGS9-2 (Fig. 4D) and β-arrestin2 (Fig. 6C). Further, the inhibitory effect of RGS9-2 on the signaling of $D_3R$-($D_2$-IC23) and $D_3R$ was abolished in β-arrestin2 KD cells (Fig. 6D). Therefore, it is suggested that the higher affinity between $D_3R$ and β-arrestin2 than between $D_2R$ and β-arrestin2 might determine the inhibitory activity of RGS9-2. It is not the amino acid sequence of the second and third loops of $D_3R$ which determines the constitutive interaction with β-arrestins, but the overall conformation of $D_3R$ may be such that it weakly

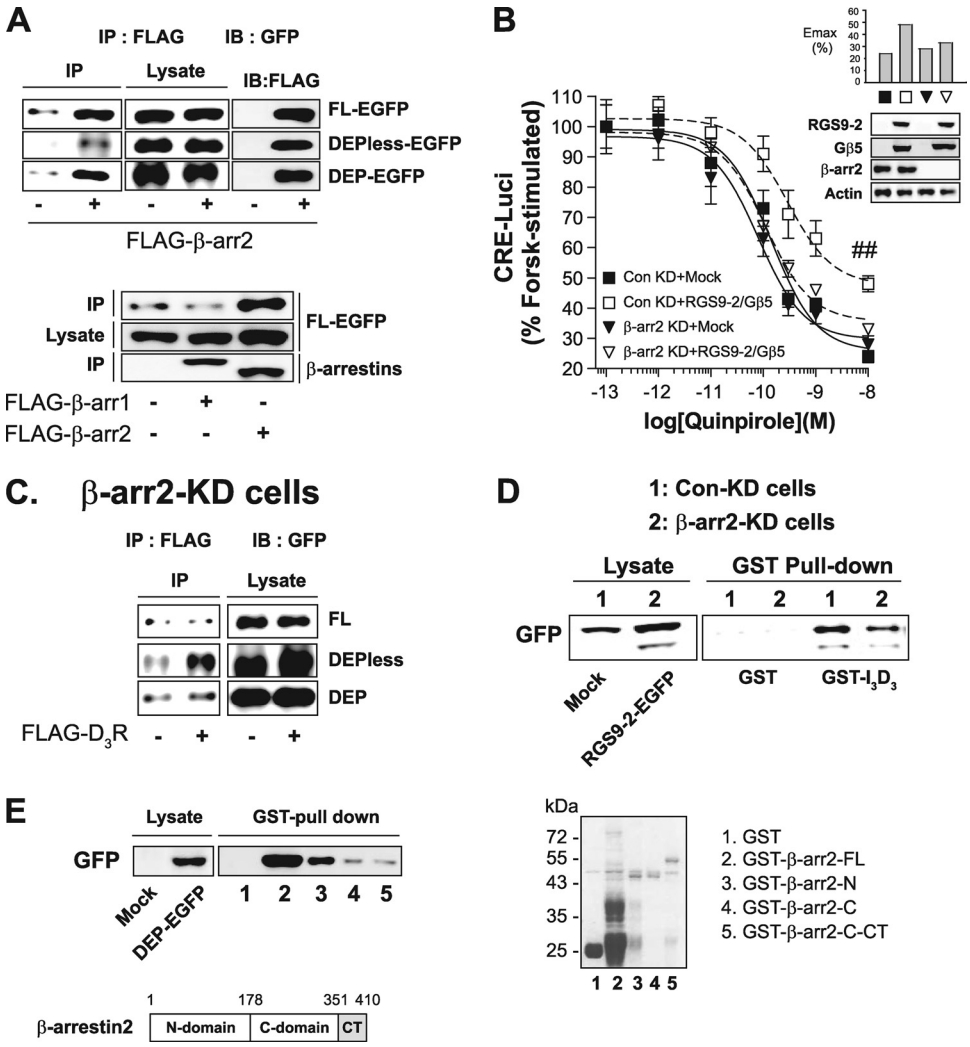


FIG. 5. Roles of β-arrestins in the regulatory effects of RGS9-2 on the signaling of $D_3R$. (A) Interactions between β-arrestin2 and RGS9-2 proteins were assessed by immunoprecipitation in cells expressing FLAG–β-arrestin2 along with FL-RGS9-2–EGFP, DEPless–RGS9-2–EGFP, and the DEP domain of RGS9-2–EGFP. Cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with FLAG beads and antibodies to GFP or FLAG, respectively (upper panel). In the lower panel, cells were transfected with RGS9-2–EGFP along with FLAG–β-arrestin1 or FLAG–β-arrestin2. Cell lysates were immunoprecipitated with FLAG beads, and the immunoprecipitates were immunoblotted with GFP or FLAG. The data represent results of three independent experiments with similar outcomes. (B and C) Roles of β-arrestin2 in the regulatory activity of RGS9-2 were determined in β-arrestin2 KD cells. (B) Role of β-arrestin2 in the inhibitory effect of RGS9-2 on the signaling of $D_3R$. Receptor expression levels were maintained at around 1.8 to 2.0 pmol/mg protein. ##, $P < 0.01$ for the Con-KD+RGS9-2/Gβ5 group versus the Con-KD+Mock or β-arr2-KD+Mock group. Expression of endogenous β-arrestin2 was inhibited through β-arrestin2 shRNA expression in plasmid pcDNA3.0(Neo) (data not shown). Cell lysates were immunoblotted with antibodies to GFP (RGS9-2 and Gβ5), β-arrestin2, and actin. Con, control. (C) Role of β-arrestin2 in the interaction between the $D_3R$ and RGS9-2 proteins. Immunoprecipitations from β-arr2-KD cells expressing FLAG-$D_3R$ along with FL-RGS9-2–EGFP, DEPless RGS9-2–EGFP, and the DEP domain of RGS9-2–EGFP were conducted. Cell lysates were immunoprecipitated and immunoblotted with FLAG beads and antibodies to GFP, respectively. Receptor expression levels were maintained at around 1.9 pmol/mg protein. The data represent results of three independent experiments with similar outcomes. (D) The role of β-arrestin2 in the interaction between RGS9-2 and $D_3R$ was determined with a GST pulldown assay. Bacterial lysates containing the GST fusion proteins of the third intracellular loops of $D_3R$ ($I_3D_3$) were mixed with lysates of Con-KD or β-arr2-KD cells, which were transfected with RGS9-2–EGFP. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed by SDS-PAGE and blotted with antibodies to GFP (GST pulldown part). A blot of HEK293 cell lysates is shown in the lysate part. (E) Interaction between β-arrestin2 and the DEP domain was determined in a GST pulldown assay. Bacterial lysates containing the GST fusion proteins of the FL protein (GST-β-arr2-FL), the N domain (GST-β-arr2-N), the C domain (GST-β-arr2-C), or the C domain plus the carboxy tail of rat β-arrestin2 (GST-β-arr2-C-CT) were mixed with lysates of HEK293 cells transfected with DEP-EGFP. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed by SDS-PAGE and blotted with antibodies to GFP (GST pulldown part). A blot of HEK293 cell lysates is shown in the lysate part. On the right is an SDS-PAGE analysis of the afterwash of bacterial cell lysates. Emax, maximum effect.

interacts with G protein (36) but abundantly interacts with β-arrestins.

As β-arrestin2 binds tightly to $D_3R$ (Fig. 6B), it was expected that KD of β-arrestin2 would increase the signaling efficiency of $D_3R$. However, our results show that the inhibitory effect of quinpirole on forskolin-stimulated cAMP accumulation remained the same regardless of the KD of endogenous β-arrestin2 (Fig. 5B). One possibility is that the remaining β-arrestin1

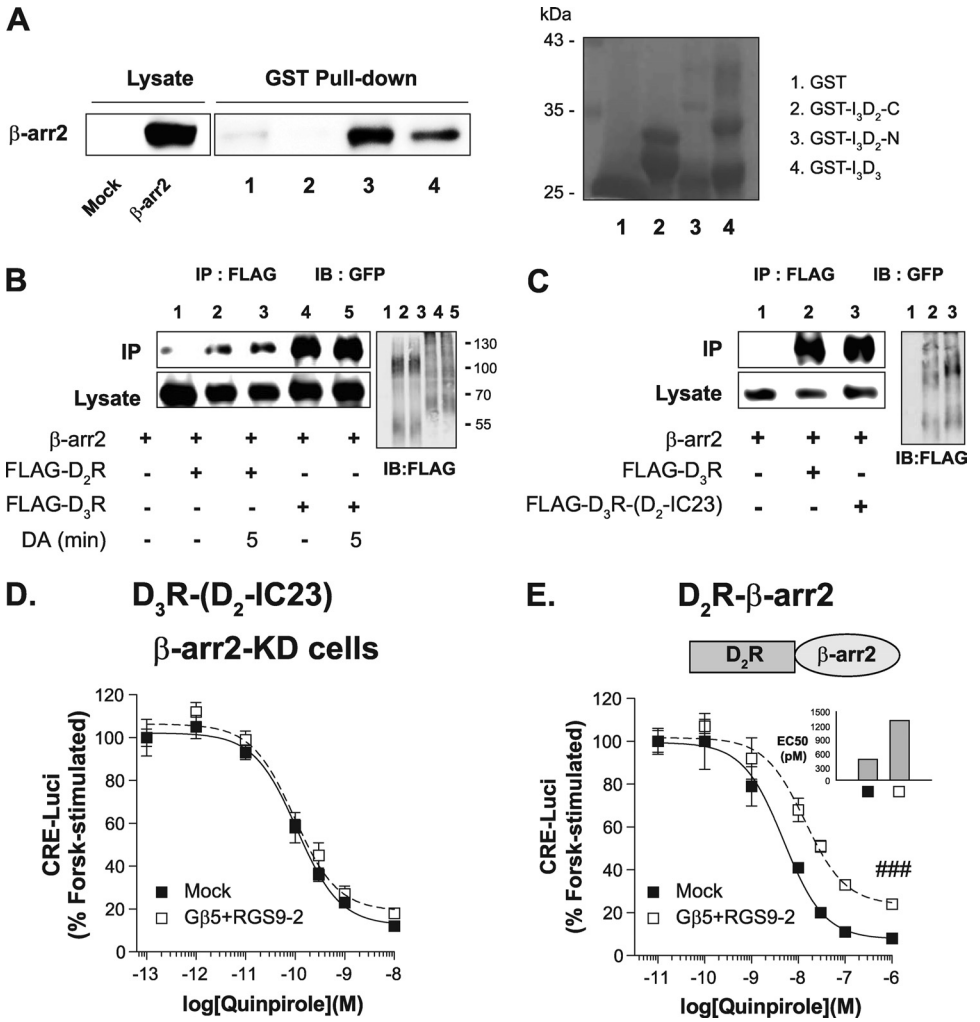


FIG. 6. Role of affinity for β-arrestin2 in the regulation of $D_3R$ signaling through RGS9-2. (A) Interaction between β-arrestin2 and $D_3R$ was determined by GST pulldown assay. Bacterial lysates containing the GST fusion proteins of the third intracellular loop of $D_2R$ or $D_3R$ were mixed with lysates of HEK293 cells transfected with β-arrestin2. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed by SDS-PAGE and blotted with antibodies to GFP (GST pulldown part). A blot of HEK293 cell lysates is shown in the lysate part. On the right is an SDS-PAGE analysis of the afterwash of bacterial cell lysates. (B and C) Comparison of the profiles of β-arrestin2 interactions with $D_2R$ and $D_3R$ in cells expressing β-arrestin2 and FLAG-$D_2R$ or FLAG-$D_3R$ (B) or with $D_3R$ and $D_3R$-($D_2$-IC23) in cells expressing β-arrestin2 and FLAG-$D_3R$ or FLAG–$D_3R$-($D_2$-IC23) (C). Cells were treated with 10 μM dopamine for 5 min and blotted with antibodies to β-arrestin2. Receptor expression levels were maintained at around 1.5 to 1.7 pmol/mg protein. Receptor proteins were immunoprecipitated (IP) and immunoblotted (IB) as described in the legend to Fig. 2A. The data represent results of three independent experiments with similar outcomes. (D) Role of β-arrestin2 in the regulatory effect of RGS9-2 on the signaling of $D_3R$-($D_2$-IC23) as determined in β-arrestin2 KD cells in comparison with that in control cells (Fig. 4C). Receptor expression levels were around 1.7 pmol/mg protein. (E) Role of affinity for β-arrestin2 in the regulatory effects of RGS9-2 on the signaling of $D_2R$ as determined in cells expressing $D_2R$–β-arr2, a fusion of $D_2R$ and β-arrestin2. Receptor expression levels were around 0.6 pmol/mg protein. ###, $P < 0.001$ for the Gβ5+RGS9-2 group versus the mock-treated group. $EC_{50}$, 50% effective concentration.

compensated for the decrease in cellular β-arrestin2. To test this, we also knocked down β-arrestin1 but found that the dose-response curve was only slightly shifted to the left (data not shown). Another possibility is that β-arrestins need RGS9-2 to exert inhibitory effects on the signaling of $D_3R$, as shown in Fig. 5B. If this is the case, coexpression or KD of β-arrestins would influence the signaling of $D_3R$ only in cells expressing RGS9-2. Indeed, coexpression of β-arrestin2 inhibited the signaling of $D_3R$ in a dose-dependent manner in neuro2a cells where RGS9-2 is expressed, but not in HEK293 cells, which do not express RGS9-2 (data not shown).

To corroborate that the affinity between receptor protein and β-arrestins determines the selectivity of RGS9-2 for $D_2R$ and $D_3R$, the affinity of $D_2R$ for β-arrestin2 was increased by fusing the two proteins ($D_2R$–β-arr2). The ligand-binding and intracellular trafficking properties of this fusion protein were similar to those of wild-type $D_2R$ and were described previously (9). As shown in Fig. 1A and 6E, the inhibitory effect of RGS9-2 on receptor signaling became evident as the affinity between the receptor protein and β-arrestin2 was increased. Therefore, the results in Fig. 6 suggest that the affinity of $D_3R$ for β-arrestins determine the selectivity of RGS9-2 for $D_3R$.

**Selectivity of RGS9-2 for $D_3R$ is mediated by β-arrestin2 as an intermediate which connects to Gβ5.** A subgroup of the RGS family, namely, RGS9, RGS11, RGS7, and RGS6, commonly possesses a GGL domain that binds the fifth member of the heterotrimeric G protein β subunit (Gβ5) (40). Gβ5 is known to mediate the inhibitory effects of RGS9-2 (37). Since β-arrestins and RGS proteins are well established for the inhibition of GPCR signaling, role of each regulatory component in the signaling of $D_3R$ was determined. When exogenous β-arrestin2 or RGS9-2/Gβ5 was individually added back into β-arrestin2 KD cells, β-arrestin2 or RGS9-2/Gβ5 did not have significant inhibitory activity on the signaling of $D_3R$. When they were added back together, β-arrestin2 and RGS9-2/Gβ5 synergistically increased the inhibitory effects against $D_3R$ signaling (Fig. 7A). However, β-arrestin2 and Gβ5 failed to show synergistic activity under the same experimental conditions (data not shown), suggesting that β-arrestin2, Gβ5, and RGS9-2 together are needed for maximal regulatory activity.

Since our results (Fig. 3, 5, and 7) show that β-arrestin2, Gβ5, and the DEP domain of RGS9-2 are needed for the maximal regulatory activity of RGS9-2 on the signaling of $D_3R$, protein interactions among these cellular components were tested. Gβ5 interacted with both β-arrestin2 (Fig. 7B, 4.82-fold over background binding) and $D_3R$ (Fig. 7C, 3.08-fold over background binding) but not with $D_2R$, suggesting that the selectivity of RGS9-2 for $D_3R$ might result from its selective association with the regulatory complex composed of Gβ5 and β-arrestin2.

To understand the molecular details involved in the functional interactions among the DEP domain, Gβ5, and β-arrestin2, the interactions between these proteins themselves were determined. First, our results showed a direct interaction between Gβ5 and β-arrestin2 in a GST pulldown assay (data not shown). As reported previously (33, 38), Gβ5 interacted with the DEP domain and this interaction was β-arrestin2 dependent (Fig. 7D). The same results were obtained in a GST pull-down assay (data not shown). The interaction between the GST-DEP domain and Gβ5 was almost completely abolished when cellular β-arrestin2 was knocked down. The interaction between $D_3R$ and Gβ5 was also β-arrestin2 dependent (Fig. 7E), suggesting that β-arrestin2 acts as a scaffold connecting Gβ5 to $D_3R$ and the DEP domain. Coexpression of Gβ5 inhibited the interaction between the isolated DEP domain and β-arrestin2 (Fig. 7F), suggesting that the isolated DEP domain and Gβ5 competitively bind to β-arrestin2. Considering that both the DEP domain and Gβ5 are needed for the inhibitory activity of RGS9-2/β-arrestin2, the balance between RGS9-2 and Gβ5 could be important. Indeed, a previous study with RGS7 emphasized that the ratio of RGS7 to Gβ5 is important for the inhibitory activity on the signaling of the muscarinic M3 receptor protein (38). These results overall indicate that the DEP domain of RGS9-2 and Gβ5 collaboratively regulate the signaling of $D_3R$, in which β-arrestin2 plays a permissive role.

**β-Arrestin2 scaffolds multiple cellular components, enabling functional regulation and proper subcellular localization of RGS9-2.** RGS9-2 was diffusely distributed in the cytosolic region of HEK293 cells (Fig. 8A, first panel) but was found mainly on the plasma membrane when the endogenous β-arrestins were knocked down (Fig. 8A, second panel). Under these experimental conditions, $D_3R$ and RGS9-2 were found on the plasma membrane (data not shown) even though they do not physically contact each other (Fig. 5C). Coexpression of β-arrestin2 resulted in the redirection of RGS9-2 in the cytosol (data not shown). These results suggest that β-arrestins somehow force RGS9-2 to be localized in the cytosol.

In the striatum, RGS9-2 is complexed with Gβ5 and a R9AP-like protein called R7BP (31). R7BP is a neuronal protein that anchors RGS proteins of the R7 family as a complex with Gβ5 (12). Previously it was reported that R7BP interacts with RGS9-2 in the DEP domain and renders its localization on the plasma membrane (42). As shown in Fig. 8B, RGS9-2 was found on the plasma membrane when it was coexpressed with R7BP. Therefore, it is conceivable that the cytosolic pool of RGS9-2/Gβ5 exists in the R7BP-free form, either in a dimeric form or in association with another protein, presumably β-arrestin2. If this is the case, β-arrestin2 is expected to compete with R7BP for interaction with the DEP domain, confining the localization of RGS9-2 to cytosolic regions. Figure 8C shows that β-arrestin2 makes a complex with RGS9-2, and their interaction was disrupted by the introduction of exogenous R7BP. The same results were obtained with the DEP domain of RGS9-2 (Fig. 8D), and we corroborated these results by showing that β-arrestin2 competes with R7BP for binding with the DEP domain (Fig. 8E). Therefore, depending on the expression levels of β-arrestin2, the subcellular localization of RGS9-2 will be changed, resulting in the differential regulation of cellular processes in the plasma membrane and cytoplasmic regions. It is interesting that RGS9-2 does not interact with $D_3R$ and cannot inhibit the signaling of $D_3R$ in the absence of β-arrestins, even though more of two proteins appears to be localized on the plasma membrane. Unexpectedly, R7BP, which plays a critical role in the stabilization and anchoring of RGS9-2 on the plasma membrane, showed antagonistic effects on the regulatory actions of RGS9-2/β-arrestin2 on the signaling of $D_3R$ (Fig. 8F).

Overall the results show that β-arrestin could work as a scaffolding protein around RGS9-2. β-Arrestin determines the subcellular localization of RGS9-2 and controls the conformation of RGS9-2 between the open and closed states. Through protein-protein interactions, it acts as a switch to turn protein interactions on and off around $D_3R$, as well as the regulatory effects against $D_3R$ signaling.

## DISCUSSION

This study was conducted to assess the regulatory mechanisms of RGS proteins that are known to be associated with the brain dopaminergic nervous system. Among the RGS proteins tested, RGS9-2 showed selective inhibitory effects on the signaling of $D_3R$ mediated by Gi/o proteins. According to a recent publication of the crystal structure of the RGS9-2 and Gβ5 complex (8), direct contact between two proteins occurs only in the DHEX linker. The interaction between the DEP domain and Gβ5 suggested by a biochemical study (32) is likely to be mediated by certain intermediates, probably β-arrestin2. Based on our results and a recent publication (32), we propose a working model (Fig. 9) in which β-arrestin2 competes with R7BP to locate RGS9-2 in the cytosolic region and converts the conformation of RGS9-2 to the open (active) state by acting as a binding site for the DEP domain and Gβ5. The

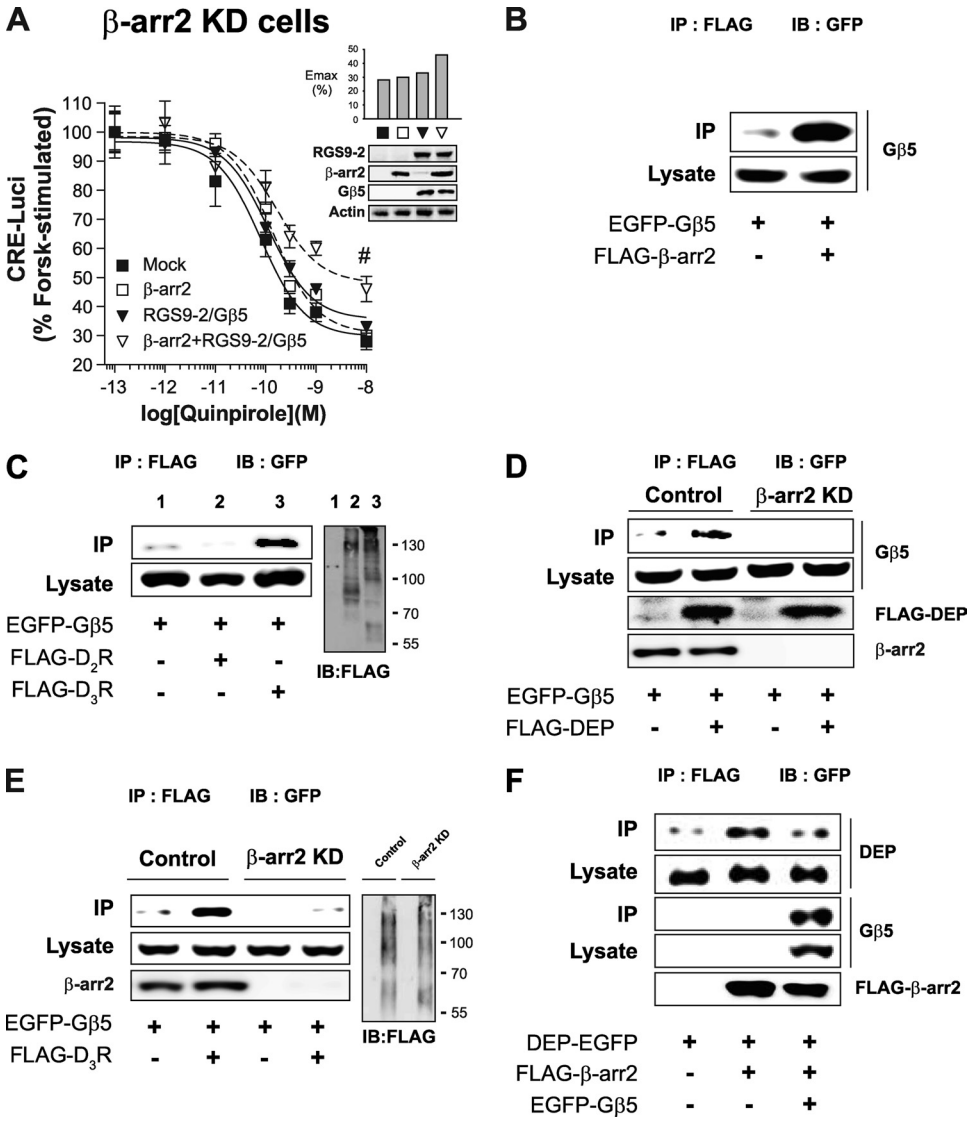


FIG. 7. Role of β-arrestin2 in the interaction of Gβ5 with adjacent proteins. (A) Cooperative activities of β-arrestin2 and RGS9-2 in the inhibition of $D_3R$ signaling. Cells were transfected with low doses of β-arrestin2 (1 μg) and RGS9-2 (1 μg). Receptor expression levels were maintained at around 1.9 pmol/mg protein. Cell lysates were immunoblotted with antibodies to GFP, β-arrestin2, and actin. #, $P < 0.05$ for the β-arr2+RGS9-2/Gβ5 group versus the β-arr2 or RGS9-2/Gβ5 group. (B) Interaction between β-arrestin2 and Gβ5 was determined by immunoprecipitation. Lysates of cells transfected with EGFP-Gβ5 and/or FLAG–β-arrestin2 were immunoprecipitated (IP) with antibodies to FLAG and immunoblotted (IB) with antibodies to GFP. (C) Selective interaction between Gβ5 and $D_3R$. Lysates of cells transfected with EGFP-Gβ5 and FLAG-$D_2R$ or FLAG-$D_3R$ were immunoprecipitated with antibodies to FLAG and immunoblotted with antibodies to GFP. Receptor proteins were immunoprecipitated and immunoblotted as described in the legend to Fig. 2A. (D) Effects of β-arrestin2 on the interaction between the DEP domain and Gβ5 were determined in cells stably expressing control shRNA or β-arrestin2 shRNA. The cells were additionally transfected with EGFP-Gβ5 and FLAG-DEP. The data represent results of three independent experiments with similar outcomes. (E) Effects of β-arrestin2 on the interaction between $D_3R$ and Gβ5 were determined in cells expressing FLAG-$D_3R$ and EGFP-Gβ5. Immunoprecipitation and immunoblotting were conducted with FLAG beads and antibodies to GFP, respectively. Receptor expression levels were maintained at around 1.9 pmol/mg protein by [$^3$H]sulpiride binding. Immunoprecipitated receptor proteins were immunoblotted as described in the legend to Fig. 2A. The data represent results of two independent experiments with similar outcomes. (F) Competitive binding of β-arrestin2 by the DEP domain and Gβ5. Cells expressing FLAG–β-arrestin2 and DEP-EGFP were transfected with EGFP-Gβ5. Cell lysates were immunoprecipitated with FLAG beads and blotted with GFP or FLAG antibodies. The data represent results of three independent experiments with similar outcomes. Emax, maximum effect.

results in Fig. 7 and 8 consistently show that β-arrestin2 recruits Gβ5 and RGS9-2 to $D_3R$ using the DEP domain as a bridge. This regulatory complex is created by the scaffolding activities of β-arrestin2, and this explains the molecular mechanism for stabilizing the open conformation of the R7 RGS family, which is functionally active for the inhibition of GPCR signaling.

Through this study, three major outcomes for research on the regulation of GPCRs were achieved. First, the subtype of D2-like receptors regulated by RGS9-2 was identified. Second,

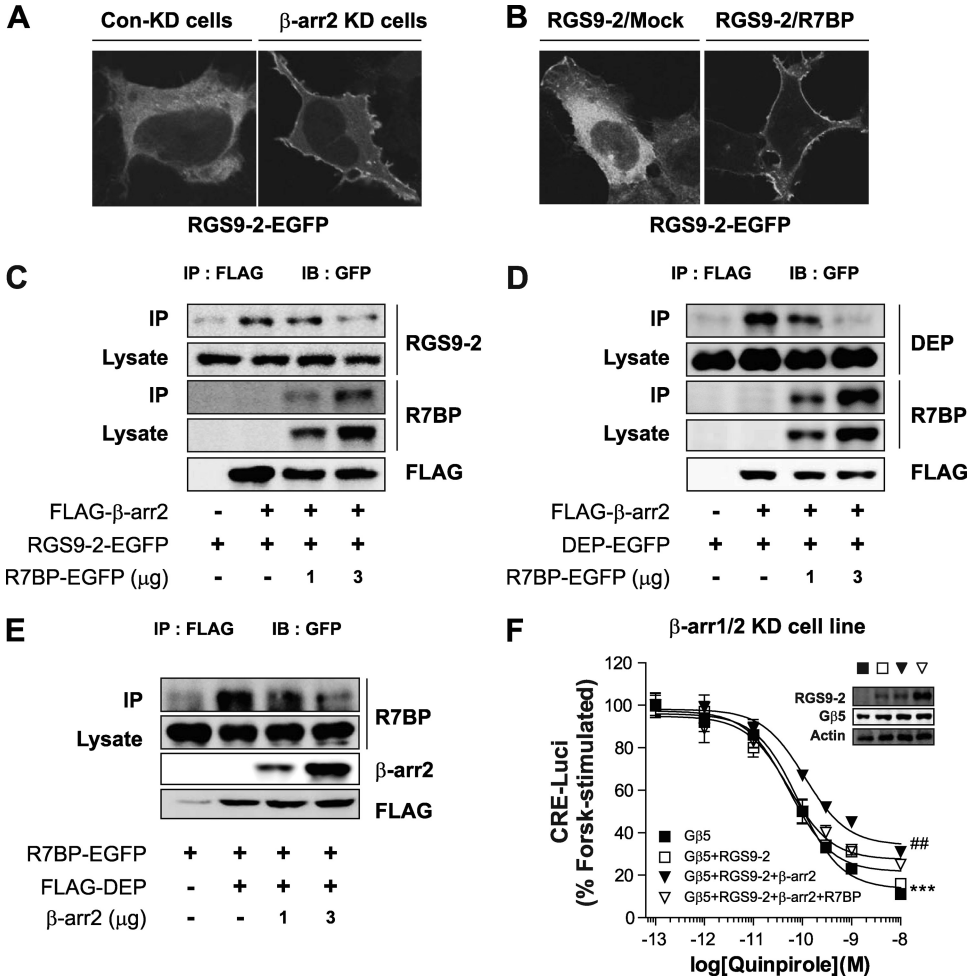


FIG. 8. Roles of β-arrestin2 in the subcellular localization of RGS9-2. (A) Effects of KD of endogenous β-arrestins on the subcellular localization of RGS9-2. Con-KD and β-arr2-KD cells were transfected with RGS9-2–EGFP. (B) Effects of R7BP on the subcellular localization of RGS9-2. HEK293 cells were transfected with RGS9-2–EGFP and/or R7BP. (C) Competitive binding of RGS9-2 and R7BP to β-arrestin2. Cells expressing FLAG–β-arrestin2 and RGS9-2–EGFP were transfected with increasing amounts of R7BP-EGFP. Cell lysates were immunoprecipitated (IP) with antibodies to the FLAG epitope and immunoblotted (IB) with antibodies to GFP and FLAG. The data represent results of three independent experiments with similar outcomes. (D) Competitive binding of the DEP domain and R7BP to β-arrestin2. Cells expressing FLAG–β-arrestin2 and DEP-EGFP were transfected with increasing amounts of R7BP-EGFP. Cell lysates were immunoprecipitated with antibodies to the FLAG epitope and immunoblotted with antibodies to GFP and FLAG. (E) Competitive binding of β-arrestin2 and R7BP to the DEP domain of RGS9-2. Cells expressing FLAG-DEP and RGS9-2–EGFP were transfected with increasing amounts of β-arrestin2. Cell lysates were immunoprecipitated with FLAG beads and immunoblotted with antibodies to GFP and FLAG. The data represent results of three independent experiments with similar outcomes. (F) Roles of β-arrestins and R7BP in the regulatory effects of RGS9-2 on the signaling of $D_3R$. HEK293 cells in which β-arrestin1 and β-arrestin2 were simultaneously knocked down (data not shown) were transfected with $D_3R$ and different combinations of RGS9-2, Gβ5, β-arrestin2, and R7BP. Cellular cAMP levels were determined as described in the legend to Fig. 1, and the levels of RGS9-2 expression were determined by immunoblotting with antibodies to GFP and actin. ***, $P < 0.001$ for the Gβ5 group versus the Gβ5+RGS9-2+β-arr2 group; ##, $P < 0.01$ for the Gβ5+RGS9-2+β-arr2 group versus the Gβ5+RGS9-2 or Gβ5+RGS9-2+β-arr2+R7BP group.

new functional aspects of β-arrestins for the regulation of GPCRs are elucidated. Third, some of the molecular details involved in the regulation of GPCRs through mutual interaction with the R7 family RGS proteins were clarified.

Considering that previous studies of the roles of RGS9-2 in the functional regulation of the dopaminergic nervous system were conducted with brain tissues in response to an agonist for D2-like receptors, this is the first to determine the specific dopamine receptor subtypes which are functionally associated with RGS9-2. Our results show that RGS9-2/Gβ5 inhibits the internalization and signaling of $D_2R$ and $D_3R$, respectively.

β-Arrestins are one of the protein families involved in the regulation GPCRs. It is well established that β-arrestins bind to the receptor proteins phosphorylated by GRKs with high affinity and prevent further association with G proteins. However, it is still not clear how β-arrestins interfere with the signaling of GPCRs, for example, whether they inhibit G protein coupling alone or in collaboration with other cellular components. Especially, it was expected that KD of β-arrestins which were tightly bound to $D_3R$ would shift the dose-response curve to the left, but our results show that the removal of β-arrestin2 (Fig. 5B) or β-arrestin1/2 (data not shown) does

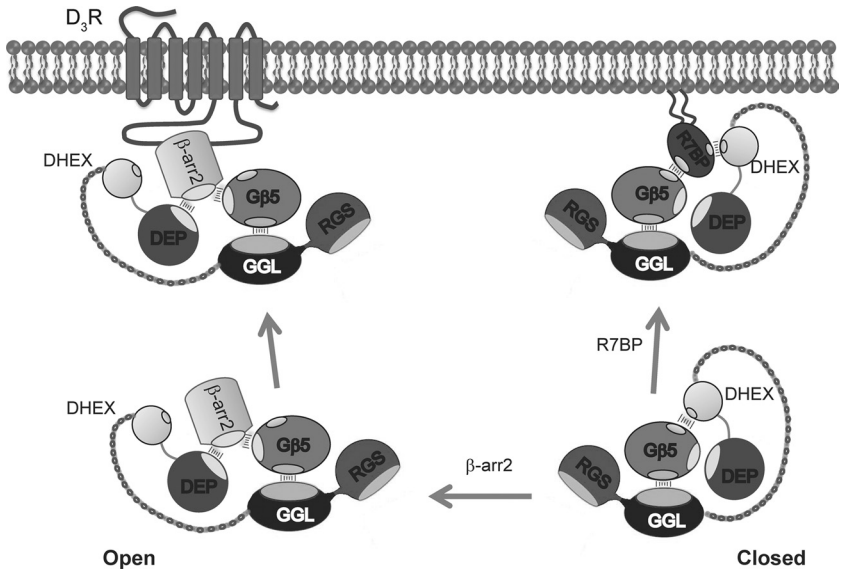


FIG. 9. Proposed working model of the $D_3R$ regulatory complex. When Gβ5 and the DEP domain of the RGS9 protein are associated, RGS9-2 is in the inactive (closed) conformation and cannot regulate G protein cycling. If the interaction between Gβ5 and the DEP domain is disrupted, the conformation of the RGS protein is converted to the active (open) conformation (33). RGS9-2 forms a stable complex with Gβ5 via its GGL domain (1). The DHEX linker, but not the DEP domain, directly interacts with Gβ5 (8), and β-arrestin mediates the interaction between the DEP domain and Gβ5 (Fig. 7D). The opposite surface of Gβ5 makes transient/dynamic contacts with the DHEX linker (direct interaction) and the DEP domain (mediated by β-arrestin2) (32) (Fig. 7D and F). This transient contact is disrupted when R7BP interacts with the RGS-Gβ5 complex in the cleft formed between Gβ5 and the DHEX linker region (32). R7BP anchors the RGS9-2/Gβ5 complex on the plasma membrane and stabilizes RGS9-2. R7BP competes with β-arrestin2 for binding with the DEP domain (Fig. 8E) and functionally antagonizes the scaffolding activity of β-arrestin2 for the regulation of $D_3R$ signaling (Fig. 8F). Overall, β-arrestin2 converts the RGS9-2/Gβ5 complex to the active conformation by binding to the DEP domain and Gβ5. Also, β-arrestin2 binds with the DEP domain in competition with R7BP, rendering RGS9-2 in the active (open) conformation in the cytosol.

not affect the signaling of $D_3R$. In this sense, it is noticeable that β-arrestin2, which does not have inhibitory activity by itself (Fig. 5B), is required for the inhibitory effect of RGS9-2 on the signaling of $D_3R$ by altering the conformation of RGS9-2 from the closed to the open state (Fig. 8 and 9). A key goal was to identify the cellular determinants that confer the selectivity of RGS9-2 for the receptor protein it regulates. Our study suggests that the affinity between the receptor protein and β-arrestin2, which is presumably determined by the overall receptor conformation rather than by the specific amino acid sequence within the intracellular loops, would determine this selectivity (Fig. 6). It was unexpected but interesting that the interaction between the DEP domain of RGS9-2 with β-arrestin2 rather than $D_3R$ correlates with the inhibitory activity of RGS9-2 on the signaling of $D_3R$ (Fig. 3 and 5). Therefore, it can be speculated that the interaction between the DEP domain and β-arrestin2 somehow evokes cellular effects which are directed to $D_3R$ through its high affinity for β-arrestin2. In this sense, our study allows new interpretations of the long-held hypothesis of receptor-G protein uncoupling by β-arrestins. Rather than simply blocking the physical interaction between the receptor and G proteins, β-arrestins seem to more sophisticatedly regulate the signaling of some GPCRs by scaffolding RGS9-2.

In addition to the identification of the D2-like receptor subtype regulated by RGS9-2 and the new regulatory roles of β-arrestins in $D_3R$ signaling, our study also provides critical information to explain current controversial issues within the R7 RGS family research area. As suggested in a recent review (2), there are several unanswered questions regarding the molecular mechanisms of the regulation of GPCRs by the R7 RGS family.

First, does the inhibitory activity of RGS9-2 require GAP activity or can it be explained by direct association with receptors? In the case of the inhibition of the μ opioid receptor internalization by RGS9-2 or the signaling of the M3 muscarinic receptor by RGS7, the latter regulatory mechanism seems to be working. The evidence for this was that the isolated DEP domain itself was enough to explain the inhibitory effects of RGS9-2 and RGS7. Our study of the regulatory activity of RGS9-2 on the signaling of $D_3R$ also suggests that GAP activity might not be an important factor, but the DEP domain, which does not contain enzymatic activity, plays a central role. In addition to these confirmatory data on the current issue, our study also reveals critical molecular details about the regulatory activity of RGS9-2: the interaction of RGS9-2 with β-arrestin through the DEP domain is required for the inhibitory activity of RGS9-2 on the signaling of $D_3R$.

Second, does RGS9-2 specifically regulate a selected receptor or does it function as a universal regulator of several GPCRs in neurons? All of our results consistently show that the affinity of the receptor protein for β-arrestins in the resting state is the critical factor that determines the regulatory selectivity of RGS9-2 for $D_3R$. Notably, the binding of $D_3R$ with β-arrestins is much stronger than that of $D_2R$ in the resting state and this property is determined by the structural features outside the second and third intracellular loops. On the other hand, agonist-induced β-arrestin translocation is evident only

with $D_2R$, but not $D_3R$, and this property depends entirely on the amino acid sequence of the second and third intracellular loops (25). These results show that distinct receptor regions are involved in the regulation of receptor signaling through interaction with β-arrestin2/RGS9-2 and agonist-induced intracellular trafficking of receptor proteins.

Third, what is the cellular component or event which determines the conversion of the conformational state of the R7 family of RGS proteins from a closed state to an open state? This question has been extensively pursued for the regulatory actions of RGS7 on the signaling of the M3 muscarinic receptor. The DEP domain, which exists as a complex with Gβ5 (closed conformation, inactive), is converted to another conformation in response to activation of the M3 muscarinic receptor. In this new conformation (open conformation, active), the DEP domain is dissociated from Gβ5 but bound to a third-party binding protein and is able to inhibit the signaling of the M3 muscarinic receptor (33). Although previous studies have suggested that the interaction between the intracellular receptor regions of the M3 muscarinic receptor and the DEP domain of RGS7 contribute to the conversion between the open and closed states (38, 39), it is still not clear whether there is a common cellular component that disrupts the direct interaction between the DEP domain and Gβ5. Our results show that β-arrestin2 prevents the direct interaction between the DEP domain and Gβ5, suggesting that RGS9-2 will be in the closed conformation in the absence of β-arrestin2 and will be shifted to the open state as the cellular levels of β-arrestin2 are elevated.

Both $D_3R$ and RGS9-2 are anatomically and functionally related to drug addiction. They are densely expressed in the limbic area, which includes the striatum, nucleus accumbens, and olfactory tubercle (14, 15, 41), the major brain areas closely related to drug addiction (27). In the striatum, RGS9-2 is upregulated by intermittent cocaine administration or by cocaine self-administration (35, 46). Studies with $D_3R$ KO mice (1, 21, 50) have shown that $D_3R$ is involved in reward-related behavior and $D_3R$ selective partial agonists were found to inhibit cocaine-seeking behavior (17). Therefore, the results of this study could be applied to the understanding of and therapeutic applications for drug addiction.

Overall, our study provides a novel molecular interpretation involved in the inhibitory effects of β-arrestins on the signaling of certain GPCRs. In addition to their roles as mediators of GPCR desensitization (4) and endocytosis (13), β-arrestins are involved in the formation of a regulatory complex via intricate protein interactions with $D_3R$, RGS9-2, Gβ5, and R7BP and act as a molecular switch to allow regulation of the signaling of $D_3R$. These results suggest that β-arrestins and RGS9-2 could play important pathophysiological roles in various brain diseases in which $D_3R$ is the main cellular target, for example, in the negative symptoms of schizophrenia, Parkinson's disease, or drug addiction.

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