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G Protein-coupled Receptor Kinase Regulates Dopamine D_3 Receptor Signaling by Modulating the Stability of a Receptor-Filamin-β-Arrestin Complex

A CASE OF AUTORECEPTOR REGULATION*

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In addition to its postsynaptic role, the dopamine D_3 receptor (D_3R) serves as a presynaptic autoreceptor, where it provides continuous feedback regulation of dopamine release at nerve terminals for processes as diverse as emotional tone and locomotion. D_3R signaling ability is supported by an association with filamin (actin-binding protein 280), which localizes the receptor with G proteins in plasma membrane lipid rafts but is not appreciably antagonized in a classical sense by the ligand-mediated activation of G protein-coupled receptor kinases (GRKs) and β-arrestins. In this study, we investigate GRK-mediated regulation of D_3R·filamin complex stability and its effect on D_3R·G protein signaling potential. Studies in HEK-293 cells show that in the absence of agonist the D_3R immunoprecipitates in a complex containing both filamin A and β-arrestin2. Moreover, the filamin directly interacts with β-arrestin2 as assessed by immunoprecipitation and yeast two-hybrid studies. With reductions in basal GRK2/3 activity, an increase in the basal association of filamin A and β-arrestin2 with D_3R is observed. Conversely, increases in the basal GRK2/3 activity result in a reduction in the interaction between the D_3R and filamin but a relative increase in the agonist-mediated interaction between β-arrestin2 and the D_3R. Our data suggest that the D_3R, filamin A, and β-arrestin form a signaling complex that is destabilized by agonist- or expression-mediated increases in GRK2/3 activity. These findings provide a novel GRK-based mechanism for regulating D_3R signaling potential and provide insight for interpreting D_3R autoreceptor behavior.

The D_3 dopamine receptor (D_3R),[1] in addition to serving in a classical postsynaptic sensory role, is also a presynaptic autoreceptor that is predominantly expressed in parts of the brain controlling behavior, including that involved with emotion and movement (1). The D_3R is homologous to four other dopamine receptor subtypes (2) but is closest in expression and function to the D_2R, (1, 3). The D_2 and D_3 autoreceptors have overlapping distributions in the brain (2, 4, 5), where they regulate presynaptic release of dopamine (DA) by at least three different mechanisms: by affecting rates of intracellular DA synthesis, by mediating vesicular secretion of DA at the plasma membrane, and by modulating reuptake of dopamine at plasma membrane DA transporters (1, 6). D_3R has a higher affinity for DA than D_2R, and experiments with D_3R knock-out mice suggest that in the striatum/nucleus acumbens the D_3R may regulate DA release at lower DA concentrations than D_2R by modulating DA secretion rates rather than DA synthesis or DA transporter activity (1).

The G protein-mediated signaling of G protein-coupled receptors (GPCR) in general and D_2 and D_3Rs in particular should terminate from arrestin protein binding to receptor intracellular loop or C-tail residues that are GPCR kinase (GRK)-phosphorylated (7–9). The subsequent trafficking of arrestin-receptor complexes to clathrin-rich regions of plasma membrane and clathrin-coated pits (CCPs) depends on the extent of GRK phosphorylation (10) and results in a sustained blockade of G protein coupling, the establishment of arrestin-dependent non-G protein-mediated signaling, and the endocytosis of arrestin-receptor complexes (7, 8, 11). CCP-initiated recycling of dephosphorylated receptors contributes to relief of the blockade in G protein signaling and a reinitiation of membrane signaling competence (12, 13). Autoreceptors that function as permanent sensors of extracellular neurotransmitter concentrations may require alternative mechanisms to regulate the transition between the classical G protein signaling and desensitized states observed for receptors with more conventional signaling responsibilities. In fact, although D_3Rs can be induced to internalize to a small degree, the large majority of D_3Rs remain at the plasma membrane and fail to internalize appreciably in CCPs after brief periods of DA stimulation (3).

The D_3R and D_2R undergo remarkably different degrees of arrestin-directed trafficking to CCPs (3). The molecular determinants including GRK phosphorylation sites underlying CCP association are confined to their relatively large third intracellular loops as demonstrated by experiments in which the loops were interchanged between receptors (3). In contrast to this post signaling behavior, the D_3R and D_2R share a requirement

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[1] The abbreviations used are: D_3R, dopamine D_3 receptor; CCP, clathrin-coated pit; D_2R, dopamine D_2 receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; DA, dopamine; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; β_2AR, β_2-adrenergic receptor; HA, hemagglutinin; GFP, green fluorescent protein.

for binding lipid raft-associated filamin (actin-binding protein 280) in order to couple efficiently to G proteins (14–16). Interestingly, these associations are also mediated by third intracellular loop residues (14–16). Filamin A also binds other GPCRs, including metabotropic glutamate receptors type 7 (17), calcitonin receptors (18), and μ-opioid receptors (19), suggesting that filamin may be an integral component for regulating the stability of receptor-G protein signaling complexes.

The small extent of DA-mediated D_3R endocytosis (3) suggests that the repetitive cycles of ligand-mediated GRK phosphorylation followed by intracellular dephosphorylation common to many other GPCRs are not critical for regulating D_3R signaling (13). However, GRK knock-out mice have revealed that receptors underlying dopaminergic-mediated locomotion do become supersensitive to ligand in the absence of some specific GRKs (20). Thus, significant GRK modulation of D_3R behavior may be highly likely despite the arrestin-trafficking results indicating otherwise. Here we show that D_3R, β-arrestin, and filamin form a basal signaling complex. The elevation of cellular GRK activity either by ligand addition or increases in the absolute amount of intracellular GRKs decreases the stability of this complex and results in a dramatic reduction in the G protein signaling potential of the D_3R. These findings suggest how the D_3R, as an autoreceptor, could rapidly desensitize and resensitize in order to provide fast feedback on short term fluctuations in DA concentration.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney cells (HEK-293) and COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture reagents were from Cellgro (Herndon, VA) or Invitrogen. A human melanoma cell line (M2) that does not express filamin A endogenously and the A7 cell line, an M_2 subclone that has been stably transfected with filamin A cDNA, are described in previous studies (21, 22). [^{32}P]Orthophosphate, [^{3}H]sulpiride, [^{3}H]adenine, [^{35}S]GTPγS, and [^{14}C]cAMP were from PerkinElmer Life Sciences, and [^{3}H]spiperone was from Amersham Biosciences. Anti-FLAG M2 antibody and horseradish peroxidase-labeled anti-mouse or anti-rabbit antibodies were from Sigma, anti-HA antibody was from Roche Applied Science, and anti-filamin A antibody was from Research Diagnostics Inc. (Flanders, NJ). Antibodies for β-arrestins and GRK2/GRK3 were provided by Drs. R. Lefkowitz and R. Premont (Duke University Medical Center), respectively.

Cell Culture and Transfection—HEK-293 cells or COS-7 cells were cultured in minimal essential medium or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin in a humidified atmosphere containing 5% CO_2. Transfections were carried out by the calcium phosphate precipitation method or using Lipofectamine (Invitrogen). Cells were used for the experiments 36 h following transfection to 60 h post-transfection for the cAMP assay. M2 and A7 cells were grown in minimal essential medium supplemented with 8% newborn calf serum and 2% fetal bovine serum. A7 cells were maintained in a medium containing G418 (500 μg/ml; Research Products International Corp., Mt. Prospect, IL). Transfections of M2 and A7 cells were conducted by electroporation at a setting of 1000 microfarads, 240–320 V using a Gene Pulser® (Bio-Rad) in a Cytomix buffer consisting of 25 mM HEPES, 120 mM KCl, 10 mM KH_2PO_4, 0.15 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM EGTA, pH 7.6.

Generation of Plasmid Constructs—Wild type human D_2R and D_3R in mammalian expression vector pCMV5 were previously described (23). Both short and long isoforms of dopamine D_2R ($D_{2S}R$ and $D_{2L}R$) were tagged at the amino terminus with the M2-FLAG epitope, and D_3R was tagged at the amino terminus with the HA epitope or with the M2-FLAG epitope (3). Expression constructs for GRKs 2 and 3 in pRK5, β-arrestins 1 and 2 in pCMV5, β-arrestin2-GFP in pEGFP, GRK2-K220R in pCDNA 1.1, and dynamin K44A in pRK5 have been described previously (24–26). D_3R-S309A was constructed by site-directed mutagenesis, and filamin A in pCDNA 3.0 was provided by Dr. Y. Ohta (Harvard University). Caine AC-V (adenylyl cyclase V) cDNA was from Dr. Y. Ishikawa (Columbia University).

Yeast Two-hybrid Assay—The yeast two-hybrid vectors and reagents were purchased from Clontech (Palo Alto, CA), and library screening was conducted as previously described (27). The amino terminus of β-arrestin2 (amino acids 1–80) subcloned into the yeast vector, pAS2-1, that contains the DNA-binding domain was used as bait. The human kidney cDNA library (100 μg) constructed in pACT2 and the bait plasmids were transformed into *Saccharomyces cerevisiae* strain Y187 and transformants were selected on artificial medium lacking tryptophan, leucine, and histidine. After a 4-day incubation at 30 °C, a β-galactosidase assay was conducted for His^+ clones to eliminate false positives, and resulting His^+/β-galactosidase$^+$ clones were sequenced with an automated ABI DNA sequencer.

Whole Cell Phosphorylation—HEK-293 cells were transfected with human β_2-adrenergic receptor tagged at the amino terminus with the HA epitope (HA-β_2AR), FLAG-D_2R, or HA-D_3R with or without GRKs. Cells were stimulated with 10 μM isoproterenol or DA for 5 min. Detailed procedures are described in our previous studies (3, 25). The amount of receptor in each sample was determined by saturation binding with 3 nM [^{3}H]spiperone. Protein concentration was assessed using the Bio-Rad DC protein assay. Phosphorylated receptors were quantitated using a PhosphorImager or were visualized by exposure to autoradiography film.

Photoaffinity Labeling—Photoaffinity labeling of the D_2R and D_3R was carried out as previously described (28).

Confocal Microscopy—For the β-arrestin translocation assays, HEK-293 cells were transfected with β-arrestin2-GFP (24) and D_3R with or without GRK3. One day after transfection, cells were seeded onto 35-mm dishes containing a centered, 1-cm well formed from a glass coverslip sealed hole in the plastic (confocal dishes) and allowed to recover 1 day. Cells were incubated with 2 ml of minimal essential medium containing 20 mM HEPES, pH 7.4, and viewed on a Zeiss laser-scanning confocal microscope.

Immunoprecipitation—COS-7 or HEK-293 cells were transfected using Lipofectamine or the calcium phosphate precipitation method, respectively. After 48 h, cells were lysed in radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) on the rotation wheel for 1 h at 4 °C. Supernatants were mixed with 35 μl of 50% slurry of FLAG-agarose beads (Sigma) between 2 and 3 h on the rotation wheel. Beads were washed with washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40) three times for 10 min each. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted.

GTPγS Binding—Membrane proteins were prepared in 20 mM HEPES, pH 7.4, 0.1 mM EDTA. For GTPγS binding, membrane proteins were incubated in 125 mM HEPES buffer containing 1 mM GDP and 100 nM [^{35}S]GTPγS, incubated for 2 h at 30 °C, filtered on a GF/B filter, and counted with a liquid scintillation counter. The coupling efficiency between receptor and G protein was determined as fmol of Gα accumulated for 2 h at 30 °C/fmol of receptor added. Receptor expression levels were closely matched between experimental groups.

Measurement of Cyclic AMP—Whole cell cyclic AMP accumulation was measured in HEK-293 cells by column chromatography as described in Ref. 23.

RESULTS

Dopamine D_3 Receptor Shows a Relatively Heavy Constitutive Phosphorylation—As shown in Fig. 1*A* (*right*), after radiolabeling of HEK-293 cells using [^{125}I]NAPS (*N*-(*p*-aminophenethy)spiperone), D_3Rs resolve into three different bands upon analysis by SDS-PAGE: a major band between 60 and 80 kDa, a minor band between 40 and 50 kDa, and a third band at 25 kDa, which may represent incompletely processed receptor protein. It is apparent from Fig. 1*B* that the total phosphorylation of the D_3R in HEK-293 cells does not appreciably increase after 5 min of agonist exposure (pre- and postexposure results varied by less than 5%). We have previously demonstrated a similar basal relative lack of change in phosphorylation of the D_3R to endogenous and overexpressed GRK2 in HEK cells at 10 min (3). In contrast, the basal phosphorylation of the D_3R was 5 times higher than the basal level of phosphorylation of a comparable amount of β_2AR protein, whose phosphorylation also increased by more than 3-fold in the presence of isoproterenol (Fig. 1*B*, *leftmost lanes*). Moreover, the relatively high basal phosphorylation of the D_3R and its lack of agonist-induced phosphorylation can be reversed by exchanging its third intracellular loop for the third intracellular loop of the D_2R (3). These results indicate that D_3R undergoes a marginal change in total phosphorylation

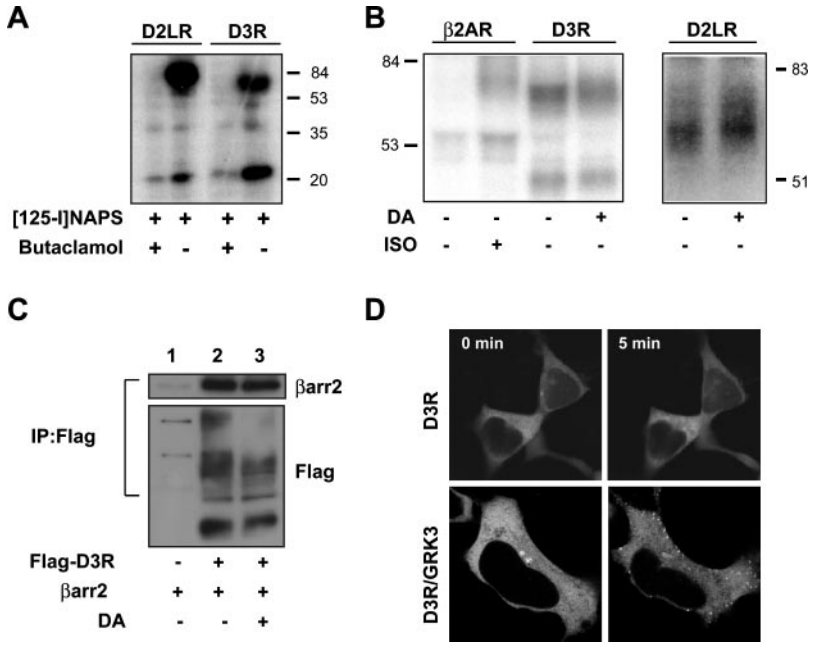

FIG. 1. **Characterization of the phosphorylation and β-arrestin binding of the dopamine D_3 receptor.** *A*, representative image of the photoaffinity-labeled long form of the D_2R and D_3R that were analyzed by SDS-PAGE. The receptor proteins were labeled with 500 pM [^{125}I]NAPS in the presence or absence of a 1 μM concentration of the competitive dopamine receptor ligand (+)-butaclamol (28). Molecular mass markers representing kDa (kDa) are to the *right* of the gel. *B*, SDS-PAGE of phosphorylated HA-β_2AR and HA-D_3Rs that were exposed to 10 μM isoproterenol (*ISO*) or DA for 5 min. Receptors were immunoprecipitated from HEK-293 cells transfected with 2 μg of receptor cDNAs in pCMV5. Receptor expression was determined by radioligand binding, and the same amount of receptor protein was loaded in each gel lane. Phosphorylated D_3R bands were not observed in mock-transfected HEK-293 cells (data not shown). *C*, association between FLAG-tagged D_3R and β-arrestin2 in COS-7 cells transfected with 3 μg of receptor cDNA in pCMV5 and with 2 μg of β-arrestin2 cDNA in pCMV5. Mock group is transfected with 4 μg of empty vector, pCMV5. Cells were treated with 1 μM dopamine for 15 min and lysed in radioimmune precipitation buffer. Immunoprecipitates obtained in radioimmune precipitation buffer were analyzed by SDS-PAGE with immunoblotting performed using antibodies to β-arrestin at a 1:4,000 dilution or using M2 FLAG antibodies at a 1:1,000 dilution. *IP*, immunoprecipitation. *D*, confocal microscopy images that demonstrate the extent of dopamine-mediated β-arrestin2-GFP translocation to the D_3R in the absence and presence of GRK3. HEK-293 cells were co-transfected with the D_3R (expressing ~5 pmol/mg protein) and with β-arrestin2-GFP with or without 2 μg of GRK3-pRK5. Cells in 2 ml of minimal essential medium containing 20 mM HEPES (pH 7.4) were stimulated with 10 μM DA for 5 min, and images were then obtained using a Zeiss laser-scanning confocal microscope (LSM-510).

in response to agonist stimulation of GRK activity as opposed to the 50% agonist-mediated increases in phosphorylation apparent for the $D_{2L}R$ (Fig. 1*B*, *right panel*), which was used as a positive control.

Dopamine D_3 Receptor Is Associated Basally with Arrestin—GRK-mediated receptor phosphorylation changes GPCR affinity for arrestins. Since the phosphorylation status of the D_3R in the absence of agonist was unexpectedly high compared with that of β_2AR or D_2R, the interaction between the D_3R and β-arrestin was assessed for the basal state. A Western blot for β-arrestins isolated from D_3R immunoprecipitates of COS-7 cells shows that the D_3R associates with β-arrestin2 under basal conditions (Fig. 1*C*, *lane 2*). Agonist exposure does not significantly change the degree of this association (Fig. 1*C*, *lanes 2* and *3*). A negligible agonist-induced D_3R phosphorylation is consistent with both the observed lack of change in the co-immunoprecipitation experiments (Fig. 1*C*, *lane 3*), and the absence of translocation of β-arrestins to D_3Rs at 5 min after agonist treatment (Fig. 1*D*, *upper panel*).

Exogenous GRK Expression Reduces the Basal Localization of the D_3R with β-Arrestin while Facilitating Agonist-induced β-Arrestin Recruitment—We have observed both in COS-7 and HEK-293 cells that DA treatment does not augment the basal association of D_3R with β-arrestin. However, an enhancement of GRK activity in these cells resulting from transfection of a plasmid encoding GRK noticeably increased the relative degree of agonist-dependent association of β-arrestins with D_3R in clathrin-coated pits as observed by microscopy (Fig. 1*D*, *lower panel*). A corresponding immunoprecipitation experiment to determine the association of β-arrestin with the D_3R in the

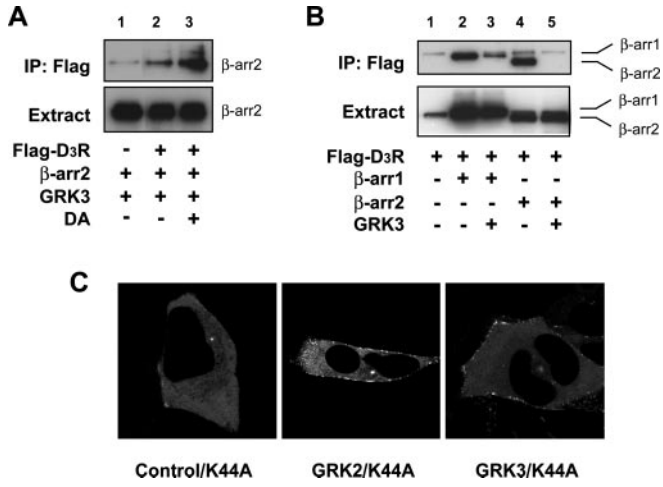

FIG. 2. **Effects of GRKs on the agonist-induced or basal association of β-arrestins with the dopamine D_3 receptor.** *A*, COS-7 cells were transfected with 3 μg of FLAG-tagged D_3R-pCMV5, 2 μg of β-arrestin2-pCMV5, and 2 μg of GRK3-pRK5. The mock group was transfected with 3 μg of pCMV5. Cells were treated with 1 μM dopamine for 5 min and lysed in radioimmune precipitation buffer. Immunoprecipitates were analyzed using SDS-PAGE followed by immunoblotting with antibodies to β-arrestin at a 1:4,000 dilution. *B*, COS-7 cells were transfected with FLAG-tagged D_3R (3 μg) and with either β-arrestin1 or -2 (2 μg) and with or without GRK3 (2 μg). Immunoblotting with β-arrestin antibodies was performed as in *A*. *C*, effects of GRK expression on the basal translocation of β-arrestin2 toward D_3R. HEK-293 cells were transfected with 3 μg of D_3R and dynamin I-K44A with 2 μg of empty pRK5, GRK2-pRK5, or GRK3-pRK5. Confocal images were obtained at 36 h post-transfection. *IP*, immunoprecipitation.

presence of transfected GRK3 and DA treatment is shown in Fig. 2*A* (*lanes 2* and *3*). The results are consistent with the microscopic observations showing that arrestin translocation is observable in coated pits under these conditions (Fig. 1*D*, *lower panels*).

The co-expression of GRKs appears to decrease the basal association of β-arrestins with D_3Rs. This is evident from the Western blots showing the amounts of β-arrestin1 and -2 that were immunoprecipitated with the FLAG-tagged D_3R in the absence of agonist (Fig. 2*B*, *lane 2 versus lane 3* and *lane 4 versus lane 5*). We observed above that agonist can drive D_3Rs to CCPs in the presence of elevated GRKs. To show that a similar behavior occurs under basal conditions, we trapped D_3Rs in CCPs using the endocytosis inhibitor dynamin1-K44A.

As shown in Fig. 2*C*, dynamin1-K44A alone did not have any effect on the subcellular localization of β-arrestin2-GFP. However, co-expression of GRK2 or GRK3 caused the accumulation of β-arrestin2-GFP at the plasma membrane. This accumulation of β-arrestin2-GFP is qualitatively similar to what we have previously reported for 10 μM dopamine-stimulated D3R in the presence of dynamin1-K44A (3). These results suggest that GRKs change the relative compartmentalization of the D_3R with β-arrestins when agonist is absent either by reducing the complement of β-arrestins prebound to the D_3R or shifting the equilibrium distribution of β-arrestin/D_3R toward CCPs. Consequently, other proteins that are also basally associated with D_3R may be affected by changes in GRK activity.

β-Arrestin2 Interacts with the D_3R-associated Filamin—The agonist-independent localization of β-arrestins with D_3Rs suggests that proteins basally binding D_3Rs are probable β-arrestin binding partners. This was tested in COS-7 cells for filamin A, which is localized in plasma membrane lipid rafts and is known to be important for the regulation of D_3R signaling in cells (15). COS-7 cells in comparison with other cell lines, such as HEK-293 cells, express relatively low levels of endogenous β-arrestins and thus provide a better background in which to assess transfected arrestin interactions by immunological methods (29). An association under basal conditions between β-arrestin2 and filamin A was confirmed by Western blot of COS-7 cell immunoprecipitates obtained using FLAG-tagged β-arrestin2 and anti-FLAG antibodies (Fig. 3*A*).

There are three human filamin isotypes (A, B, and C). They are ~70% homologous and structurally similar, consisting of multiple (twenty-four) Ig-like repeats that extend through their C termini (30). Their degree of homology and functional redundancy (30) suggests they all are potential β-arrestin binding partners. Indeed, a yeast two-hybrid library screen in an attempt to identify β-arrestin2-interacting proteins using the first 80 amino acids of the β-arrestin2 N terminus as bait yielded two colonies containing the distal third of human filamin C. The ability of full-length β-arrestin2 to also interact with this filamin segment is shown in Fig. 3*B*.

As observed above, the basal association of β-arrestin with filamin suggests that filamin may also be subject to GRK regulation. If so, D_3R signaling could be dramatically affected by changes in GRK activity directed toward destabilizing filamin/receptor association. To investigate this question, we began by studying the general relationship between filamin and D_3R signaling in cells that do not normally express filamin A.

Filamin Expression Regulates the Coupling of the D_3R to G Protein—The importance of filamin to D_3R second messenger signaling can be established using M2 cells, which normally do not express filamin A (15, 16). First, we are able to transfect M2 cells with quantitatively similar amounts of filamin A as contained in A7 cells (Fig. 4*A*). Although the total amounts of expressed immunoreactive filamin in the cell populations are

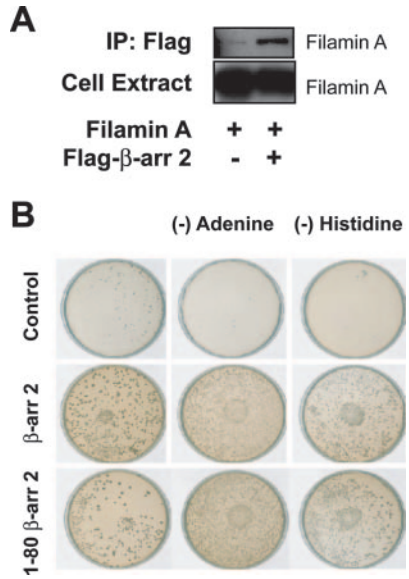

FIG. 3. **Interaction between β-arrestin2 and filamin A.** *A*, interactions between β-arrestin2 and filamin A are shown in COS-7 cells that were transfected with 2 μg of cDNA of M2-FLAG-tagged β-arrestin2 and/or filamin A in pCDNA3.0. Immunoprecipitation (*IP*) was conducted as described under "Experimental Procedures." Immunoprecipitates were analyzed by SDS-PAGE, and blots were probed with antibodies to filamin A at 1:1,000 dilutions. *B*, shown are images of plates demonstrating a protein interaction between β-arrestin and filamin C in yeast two-hybrid assay. *S. cerevisiae* strain Y187 was transfected with the amino terminus (amino acids 1–80) of β-arrestin2 in pAS2–1 (*lower row*) and the distal third of filamin C in pACT2 or between full-length β-arrestin2 and the distal third of filamin C (*middle row*). All of the plates lack tryptophan and leucine, and the *annotations above columns two* and *three* indicate the additional absence of adenine and histidine in the growth media. Plates were incubated for 4 days following yeast transformation.

similar, immunostaining and GFP co-transfection demonstrated that only one-third to one-half of the M2 cells are transfected and the efficiency of co-transfection, although appreciable, is less than 100% (data not shown).

We investigated the relationship between filamin A and G protein signaling of the D_3R in M2 cells by measuring GTPγS binding in the presence of increasing concentrations of DA (Fig. 4*B*). In order to improve the sensitivity of the assay, GTPγS binding was measured in M2 and control A7 cells transfected with the α subunit of G_o protein ($G\alpha_o$) in addition to D_3R. With coupling efficiency defined as the number of GTPγS molecules bound to the α subunit of G protein per receptor molecule, the basal G protein coupling of the D_3R was 9.4 ± 0.4% in M2 cells and 19.2 ± 1.7% in the control A7 cells, which express filamin A (Fig. 4*B*) (21). Co-expression of filamin A in M2 cells (*M2-Filamin A*) increased G protein coupling of D_3R to levels more nearly equivalent to those observed in A7 cells (Fig. 4*B*, *middle curve*). D_3R-S309A, a D_3R mutant that has a point mutation in an amino acid important for filamin A binding (15), exhibited reduced coupling efficiency (Fig. 4*C*). These results indicate a role for filamin A in D_3R-G protein signaling and suggest that alteration of filamin A/D_3R association would be one means to rapidly change D_3R signaling potential.

Agonist Treatment Reduces the Association between the D_3R and Filamin A—Filamin A interacts with a motif in the D_3R third cytoplasmic loop (14, 16), and a decrease in the cellular content of filamin A reduces, but does not totally prevent, D_3R signaling (Fig. 4, *B* and *C*). As such, receptors may retain the capacity to bind arrestins in the absence of filamin, and this is demonstrated by the immunoblot in Fig. 4*D*, where β-arrestin2 co-immunoprecipitated with FLAG-D3R in M2 cells. However, a dynamic reduction in the interaction between D_3R and fil-

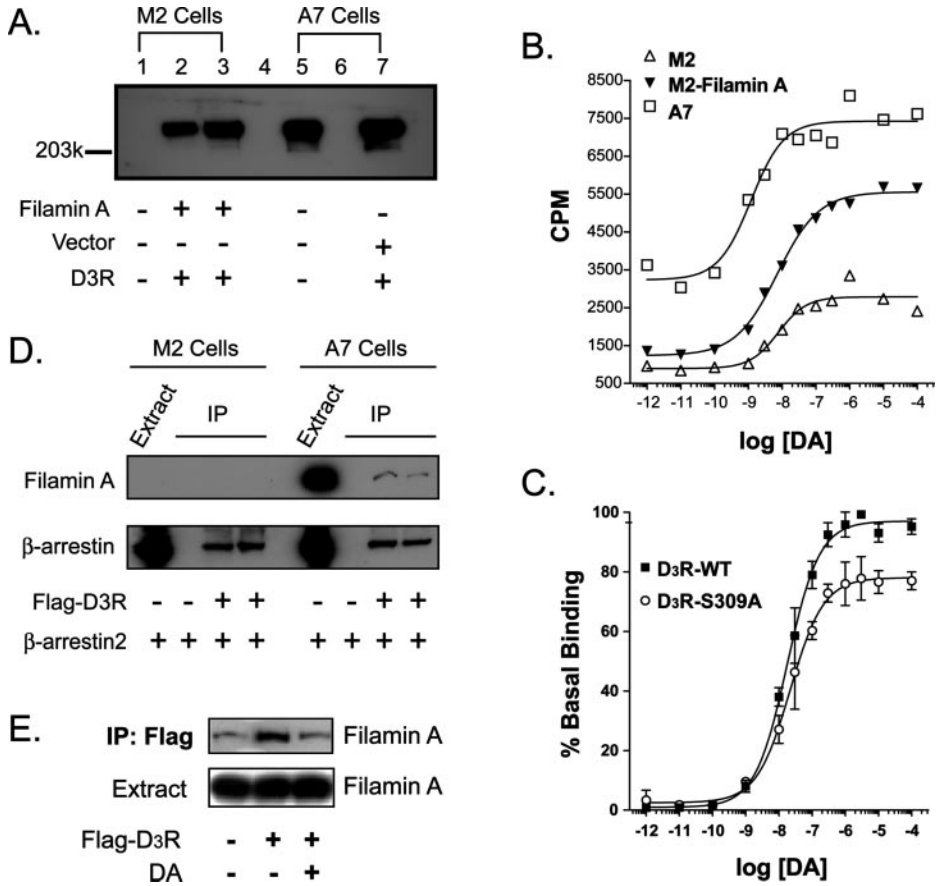

FIG. 4. **Effects of filamin A on D_3R-G protein coupling.** *A*, the ability to express filamin in M2 cells by electroporation was assessed 48 h post-transfection by immunoblotting using a monoclonal anti-filamin antibody. *Lanes 1* and *5* served as controls, and 5 μg of filamin A cDNA or control vector was electroporated in the presence of 10 μg of D3R cDNA at 300 V (*lane 2*), 320 V (*lane 3*), or 240 V (*lane 7*) and 1000 microfarads. Equal amounts of protein were added to each lane. Relative quantitation of each filamin band in the *lanes* after subtracting background was as follows: 0, 45, 59, 72, and 88 for *lanes 1*, *2*, *3*, *5*, and *7*, respectively. *B*, the effects of filamin A on GTPγs binding were evaluated in M2 and A7 cells over a range of dopamine concentrations. Cells were transfected with 10 μg of D_3R-pCMV5, 5 μg of $G\alpha_o$-pCMV5, with or without 5 μg of filamin A in pCDNA by electroporation (240 V/1000 microfarads for A7 cells and 320 V/1000 microfarads for M2 cells). The expression levels for M2-D_3R, M2-Fil-D_3R, and A7-D_3R were 1.09, 1.17, and 1.53 pmol/mg protein, respectively. Equal numbers of receptors were added per tube. Data represent results from two independent experiments with similar outcomes. *C*, HEK-293 cells were transfected with 3 μg of cDNAs encoding wild type D_3R or D_3R-S309A and 2 μg of $G\alpha_o$-pCMV5. The receptor expression level was 1.16 and 0.95 pmol/mg protein, respectively. Equal receptor amounts were added per tube. *D*, M2 and A7 cells transfected with β-arrestin2 and FLAG-tagged D3R were assessed for whether the two proteins could be co-immunoprecipitated using anti-FLAG antibody in the absence (M2 cells) and presence (A7 cells) of endogenous filamin. *E*, effects of agonist stimulation on the interaction between D_3R and filamin A were studied in COS-7 cells transfected with 3 μg of FLAG-D_3R and 2 μg of filamin A. After 48 h, cells were washed with prewarmed Dulbecco's modified Eagle's medium and treated with 30 nM dopamine dissolved in Dulbecco's modified Eagle's medium containing 10 μM sodium metabisulfite for 5 min. Immunoprecipitates were analyzed by SDS-PAGE, and immunoblots were probed with antibodies for filamin A at 1:1,000 dilutions. *IP*, immunoprecipitation.

amin A could provide a means to desensitize the receptor. We therefore tested whether agonist treatment disrupts the interaction between the D_3R and filamin A. As shown in Fig. 4*E* by immunoprecipitation of FLAG-tagged D_3R and immunoblotting for filamin A, the association of D_3R with filamin A is rapidly reduced at 5 min to 64.0 ± 15.7% of control by DA treatment (30 nM).

Exogenous GRK Expression Reduces the Basal Association between D_3R and Filamin A—GRK activity increases secondarily to agonist activation of GPCRs. Therefore, we tested whether the ability of agonists to dissociate filamin A-receptor complexes could be reproduced by enhancing GRK activity without exposing receptors to agonist. In COS-7 cells, the co-expression of GRK2 or GRK3 reduces the basal interaction between D_3R and filamin A (Fig. 5*A*, *upper panel*, compare *lane 3 versus lane 4* and both *lanes 3* and *4 versus lane 2*) as demonstrated by immunoprecipitation of the FLAG-tagged receptor and immunoblotting for filamin A. Additionally, as discussed in Fig. 2*B*, GRK co-expression reduces the basal association of D_3R with β-arrestins (Fig. 5*A*, *lower panel*, compare *lane 3* and *lane 4* and *lane 3 versus lane 2*). In contrast, a kinase-dead, dominant negative GRK2 (GRK2-K220R) enhances the basal association of the D_3R with filamin A and that of the D_3R with β-arrestin (Fig. 5*B*, compare *lane 2 versus lane 3*). These effects of GRK activity on the interactions between the D_3R, filamin A, and β-arrestin were not direct results of GRK2 phosphorylation of filamin A. In other words, purified filamin A was not phosphorylated by purified GRK2 (data not shown).

GRK Expression Reduces D_3R Signaling—Since an association between the D_3R and filamin A is needed for efficient G protein coupling, effects of GRK expression on the G protein coupling and the inhibition of cAMP production were tested. As expected from the results in Fig. 5, *A* and *B*, GRK overexpression significantly reduced both the basal and agonist-induced G protein coupling of the D_3R as assessed by GTPγS binding (Fig. 5*C*).

The GTPγS results indicate that second messenger production should be inhibited less well with excess GRK expression (*i.e.* the D_3R-mediated decrease in cellular cAMP content should be smaller). To test the effects of GRKs on the ability of the D_3R to inhibit cAMP production, the cellular cAMP was

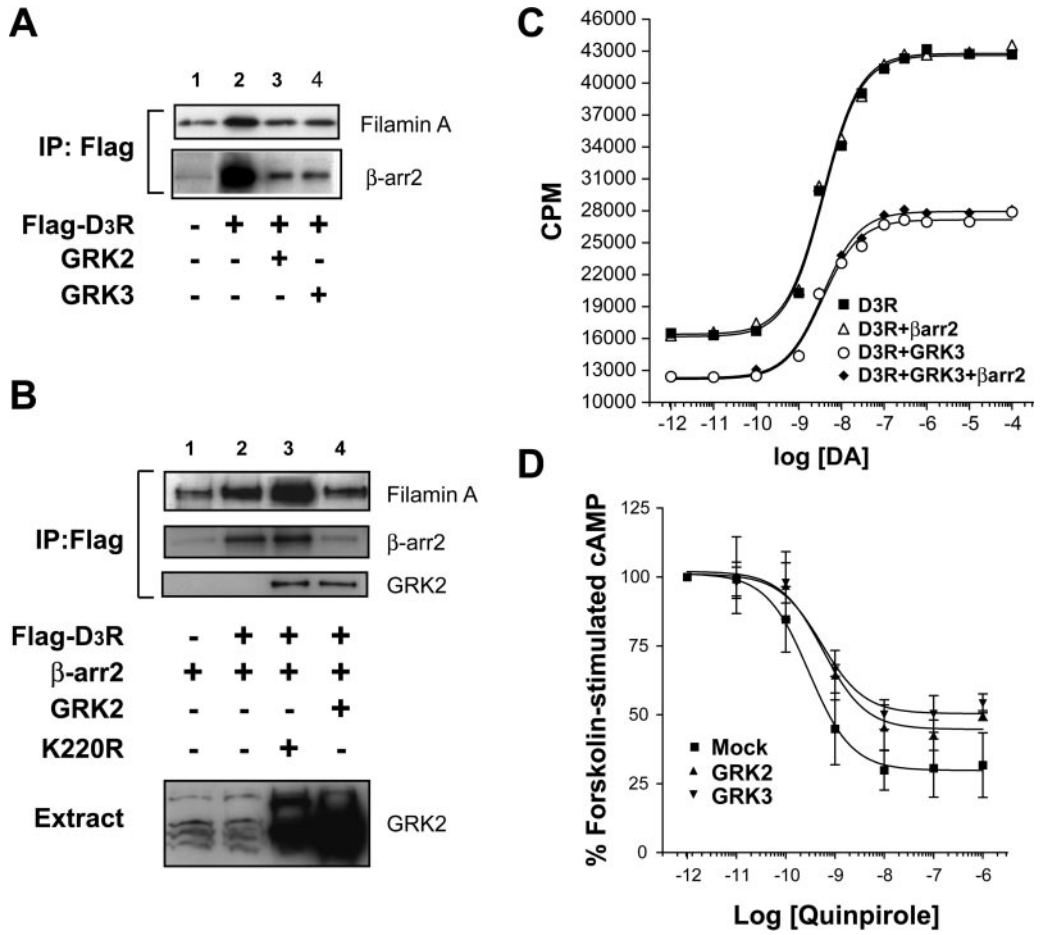

FIG. 5. **Effects of GRKs on the D_3R signaling and the assembly of signaling complex.** *A*, the association between D_3R and β-arrestin-filamin A was determined in COS-7 cells that were transfected with FLAG-D_3R (3 μg), β-arrestin2 in pCMV5 (2 μg), and GRK2 or GRK3 (2 μg each). *IP*, immunoprecipitation. *B*, effects of negative dominant mutant of GRK2 on the protein interaction between D_3R and β-arrestin or filamin A were studied. COS cells were transfected with 3 μg of FLAG-D_3R, 2 μg of β-arrestin2, and 2 μg of GRK2 or GRK2-K220R. Immunoblots were probed with antibodies for filamin A, β-arrestin, and GRK2. *C*, GTPγS binding was determined using HEK-293 cells that were transfected with D_3R (3 μg), GRK2 (2 μg), and β-arrestin2 (2 μg). The receptor expression level was adjusted to 2.4 pmol/mg protein. The graph is representative of two separate experiments with similar results. *D*, HEK-293 cells expressing D_3R/ACV were transfected with GRK2 or GRK3. Cells were treated with 100 nM quinpirole for 20 min. cAMP assay was carried out as described under "Experimental Procedures" in the presence of 1 μM forskolin and increasing concentrations of quinpirole. cAMP accumulation in the presence of 1 μM forskolin alone was normalized to 100%. The experiment was performed in triplicate, and *error bars* indicate S.D. values. *, $p < 0.05$ compared with the mock group.

first raised by forskolin stimulation, and the D_3R-mediated decrease in cellular cAMP was determined with increasing concentrations of quinpirole in cells transiently expressing GRK2 or GRK3. The dose-response curves in the presence of added kinase exhibit a decrease in V_{max} and an increase in IC_{50} (*i.e.* a shift of the curve to the right) (Fig. 5*D*). The IC_{50} (nM) and the maximal inhibition (%) for the control (0.31 ± 0.09 nM and 70 ± 7% ($p < 0.05$)) is shifted to 0.58 ± 0.15 nM and 55 ± 3% for GRK2 and to 0.53 ± 0.01 nM and 49 ± 3% ($p < 0.05$) for GRK3.

DISCUSSION

Recent studies on the distribution of D_3R protein in the brain show that it is expressed on virtually all dopaminergic neurons (4, 5), strongly supporting the idea of the D_3R as an autoreceptor. In agreement with this autoreceptor hypothesis, only a negligible fraction of plasma membrane D_3R internalizes as a result of persistent DA stimulation, even with increased GRK activity, so that the plasma membrane population of D_3Rs remains relatively constant (3). Therefore, GRK/β-arrestin-directed receptor internalization in CCPs plays a relatively minor role in the short term, agonist-mediated D_3R desensitization process, whereas GRK regulation of D_3R plasma membrane compartmentalization may play a more significant role.

Our studies indicate that GRKs serve as rheostats for adjusting the agonist-mediated G protein signaling potential of D_3Rs that are in basal conformations. The D_3R possesses an extensive third intracellular loop that contains multiple, putative GRK phosphorylation sites surrounding and dispersed throughout the filamin binding domain (14, 16). Our findings indicate that basal GRK phosphorylation of these sites potentially reduces D_3R binding to filamin and greatly reduces G protein-mediated signaling. Thus, basal GRK activity through phosphorylation of the D_3R third loop may simultaneously determine both the number of D_3R signaling complexes in filamin-rich lipid rafts and the extent of their agonist-mediated signaling, since agonist binding appears to shift receptors away from an association with filamin to one with β-arrestin.

In this novel GPCR desensitization model, basal GRK activity controls formation of D_3R·β-arrestin·filamin complexes, raising the question as to why arrestins need to co-localize with receptors and filamin at this stage. A general and perhaps the most basic explanation may be that arrestins are co-localized because this region is where signaling occurs and where homologous desensitization should occur most expeditiously. An explanation for the D_3R in particular may lie in the role D_3Rs presumably fill as autoreceptors. In order to provide continuous monitoring of ligand concentrations, D_3Rs should be more

resistant to removal from the cell surface than receptors without this functional requirement. As such, the D_3Rs exhibit relatively limited ability to remain associated with arrestins in comparison with other GPCRs, which is indicative of an intrinsically lower affinity. Therefore, from mass action considerations, the local concentration of arrestins plays a much greater role in sustaining D_3R desensitization than it would for a receptor with intrinsically greater arrestin affinity. Moreover, induced changes in the local concentration of β-arrestins with D_3Rs that are reciprocal to changes in GRK activity may provide a negative feedback for maintaining receptor signaling potential relatively constant. It would be interesting to test whether this novel model of GPCR desensitization will be applicable to other autoreceptor GPCRs that are involved in neurotransmitter concentration-dependent regulation of secretion.

Overall, D_3R signaling may be desensitized by at least two GRK-mediated mechanisms. Desensitization occurs with a reduction in G protein coupling due to an inability of filamin to localize receptors near G proteins. Alternatively, desensitization may occur from a potentially reversible, direct blockade of G protein coupling by arrestin/receptor binding. Only profound enhancements in GRK activity can marginally sustain a more classical β-arrestin/receptor desensitization picture involving clathrin-mediated receptor endocytosis.

An observation indicating that a modulation of basal GRK activity could be therapeutically useful for DA-related illness stems from studies of GRK6 knock-out mice. These animals become hyperresponsive to DA stimulation (31), indicating a role of GRK6 in regulation of postsynaptic D2-like receptors. Additionally, the GRK6 knock-out mouse striatal membranes display similarly increased GTPγS binding relative to wild type mouse controls, as do our control cells compared with cells expressing additional GRK2 or -3. Interestingly, we observed that GRK3-KO mice are hyposensitive to DA stimulations in locomotor assays (20), a finding that would be consistent with a specific role of this GRK in the regulation of DA autoreceptor function. Thus, if a genetic reduction of GRK activity can enhance dopamine receptor signaling *in vivo*, then a corresponding type-specific GRK inhibitor/activator might facilitate responsiveness to suboptimal levels of endogenous dopamine in the treatment of movement disorders like Parkinson's disease or to other monoaminergic neurotransmitters such as serotonin in the treatment of mood disorders like depression.

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