

Differential Regulation of Dopamine D1A Receptor Responsiveness by Various G Protein-coupled Receptor Kinases*

(Received for publication, November 14, 1995, and in revised form, December 7, 1995)

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The role of G protein-coupled receptor kinases (GRKs) in the regulation of dopamine D1A receptor responsiveness is poorly understood. To explore the potential role played by the GRKs in the regulation of the rat dopamine D1A receptor, we performed whole cell phosphorylation experiments and cAMP assays in 293 cells cotransfected with the receptor alone or with various GRKs (GRK2, GRK3, and GRK5). The agonist-dependent phosphorylation of the rat D1A receptor was substantially increased in cells overexpressing GRK2, GRK3, or GRK5. Moreover, we report that cAMP formation upon receptor activation was differentially regulated in cells overexpressing either GRK2, GRK3, and GRK5 under conditions that elicited similar levels of GRK-mediated receptor phosphorylation. Cells expressing the rat D1A receptor with GRK2 and GRK3 displayed a rightward shift of the dopamine dose-response curve with little effect on the maximal activation when compared with cells expressing the receptor alone. In contrast, cells expressing GRK5 displayed a rightward shift in the EC₅₀ value with an additional 40% reduction in the maximal activation when compared with cells expressing the receptor alone. Thus, we show that the dopamine D1A receptor can serve as a substrate for various GRKs and that GRK-phosphorylated D1A receptors display a differential reduction of functional coupling to adenylyl cyclase. These results suggest that the cellular complement of G protein-coupled receptor kinases may determine the properties and extent of agonist-mediated responsiveness and desensitization.

Phosphorylation is an important mechanism involved in the regulation of numerous cellular responses, notably the responsiveness of G protein-coupled receptors (1). This phosphorylation process is believed to be the triggering mechanism that leads to receptor desensitization. The cellular responses elicited upon activation of G protein-coupled receptors are regulated in a dynamic fashion by the action of two classes of serine/threonine kinases. The first class consists of the second messenger-dependent kinases such as protein kinase A and protein kinase C (1). The second class consists of receptor-

specific kinases that phosphorylate the agonist-occupied or activated form of the G protein-coupled receptors (1–3). These receptor kinases were originally described for rhodopsin (rhodopsin kinase) and the β_2 -adrenergic receptor (β -adrenergic receptor kinase) and are referred to as the G protein-coupled receptor kinases or GRKs¹ (1–3).

This large family of kinases includes six members (GRK1 to GRK6) whose activities are regulated by phospholipids, post-translational modifications, or G protein $\beta\gamma$ subunits (2–6). The GRKs are widely distributed in brain and periphery, suggesting an important role in the regulation of responsiveness of various G protein-coupled receptors (2, 7). Moreover, Arriaza *et al.* (7) have shown that β -adrenergic receptor kinase 1 (GRK2) and β -adrenergic receptor kinase 2 (GRK3) are found in pre-synaptic and postsynaptic localizations in various brain regions consistent with a general role for these kinases in the desensitization of neuronal G protein-coupled receptors and their putative role in the regulation of neuronal activity. However, little information exists as to the specificity of the various kinases and as to whether phosphorylation of a given receptor by different kinases results in the same attenuation of the biological signals.

The recent advent of molecular biology techniques has allowed a better understanding of the underlying mechanisms of the dopaminergic neurotransmission. So far, five distinct genes encoding at least six dopamine receptors have been isolated and characterized (8, 9). Dopamine receptors belong to the G protein-coupled receptor superfamily. These dopamine receptors are grouped into D1- and D2-like receptors based upon their similarity at the amino acid level and their ability to couple to the activation (D1A/D1 and D1B/D5) or inhibition (D2short, D2long, D3, and D4) of adenylyl cyclase (8, 9). Many of the neurophysiological effects of dopamine in retina and brain are thought to be mediated through the activation of dopamine D1A receptor subtype (10–15). However, the mechanisms involved in the regulation of the D1A receptor responsiveness are poorly understood. Upon exposure to dopamine, D1A receptors have been shown to undergo a desensitization process as evidenced in cellular systems expressing endogenous D1A receptors or heterologous expression systems (16–20). Furthermore, Zhou *et al.* (21) demonstrated using a protein kinase A inhibitor and a GRK inhibitor (heparin) that D1A receptors, expressed endogenously in SK-N-MC cells, could undergo both protein kinase A- and GRK-mediated desensitization. Although a recent study has shown that D1A receptor overexpressed in Sf9 cells can undergo agonist-dependent desensitization, which was associated with weak receptor phosphorylation (22), a convincing demonstration of a role for direct phosphorylation of the receptor in this process remains to be

* This work was supported in part by National Institutes of Health Grant NS-15976 and an Unrestricted Neurosciences Grant Award from Bristol-Myers Squibb (to M. G. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: GRK, G protein-coupled receptor kinase; HA, hemagglutinin.

clearly established. Most of the regulation studies performed previously in cellular systems have been limited by the low levels of receptor density (16–20). To avoid this difficulty, we have overexpressed the receptor alone or with various GRKs using a heterologous expression system to investigate the potential role of the GRK pathway in the phosphorylation and desensitization of the dopamine D1A receptor as well as to examine the biochemical and biological specificity of various GRKs. Our results indicate that the agonist-occupied form of the D1A receptor can serve as a substrate for a variety of GRKs. Moreover, we show that receptor phosphorylation by specific members of the GRK family leads to distinct desensitization patterns of dopamine responsiveness.

EXPERIMENTAL PROCEDURES

Expression Constructs—The dopamine rat D1A (rD1A) receptor was modified using oligo-directed mutagenesis to include the sequence for the hemagglutinin (HA) epitope recognized by a commercially available monoclonal antibody 12CA5 (23). The nine-residue sequence of the epitope (YPYDVPDYA) was inserted after Ala², and this residue was repeated after the epitope to increase the distance between the epitope and the putative N-linked glycosylation site to avoid potential steric interference. A 67-mer oligonucleotide containing the epitope sequence as well as the 5' regions of the rat D1A receptor was used to amplify a modified 5' end of the gene, which was spliced to the wild-type receptor gene 3' sequence using the internal *Bgl*/II site. The sequence of the modified gene was verified by using dideoxy sequencing method (U. S. Biochemical Corp.) and subcloned into the expression vector pCMV5. The HA-epitope tagged rat D1A receptor will be referred to as HA-rD1A receptor. The expression constructs for GRK2, GRK3, and GRK5 were described previously (7, 24). The β -galactosidase pCMV expression construct was purchased from Clontech.

Cell Culture and Plasmid Transfection—Human embryonic kidney cells (293 cells) were grown in minimal essential medium with Earle's salts supplemented with heat-inactivated fetal bovine serum (10% (v/v)) and gentamicin (100 μ g/ml) at 37°C in 5% CO₂ atmosphere. Cells seeded in 100-mm dishes (2.5 \times 10⁶ cells/dish) were transiently transfected by a modified calcium-phosphate method (25, 26). All experiments were performed with cells passaged 34–48 times.

Radioligand Binding—293 cells seeded in 100-mm dishes were transfected with 5–10 μ g of wild-type rD1A receptor or the HA-tagged receptor. Following transfection (18–24 h), cells were reseeded in 150-mm dishes and allowed to grow for an additional 32–48 h. Radioligand binding studies were performed on membranes expressing either the wild-type or HA-rD1A receptor as described previously (26). Protein concentrations were determined using the Bio-Rad assay kit with bovine serum albumin as standard.

Photoaffinity Cross-linking—Membranes from 293 cells expressing either the β -galactosidase alone, wild-type, or HA-rD1A receptor were resuspended in binding buffer (50 mM Tris-HCl, 120 mM NaCl, 5.0 mM KCl, 4.0 mM MgCl₂, 1.5 mM CaCl₂, and 1.0 mM EDTA, pH 7.4) containing protease inhibitors (20 μ g/ml phenylmethylsulfonyl fluoride; 10 μ g/ml benzamide, leupeptine, and soybean trypsin inhibitor; 5 μ g/ml aprotinin; 1 μ g/ml pepstatin A). Photoaffinity cross-linking was performed using SCH39111 (27), which was radioiodinated by procedures described by Amlaiky *et al.* (28). Membranes (20 μ g of proteins) were incubated with [¹²⁵I]-SCH39111 (3.0 nM) in the presence or absence of 10 μ M flupentixol in a final volume of 200 μ l at 25°C for 90 min. At the end of the incubation, membranes were washed by centrifugation (10,000 \times g for 15 min) 3 times with ice-cold 10 mM Na₂HPO₄, 100 mM NaCl (pH 7.4), and resuspended in 1 ml of the same buffer. Under dark conditions, 10 μ l of 2 mM SANPAH (freshly dissolved in dimethyl sulfoxide) were added to each sample, reacted for 15 min at 25°C, and stopped by the addition of 30 μ l of 1 M glycine. The reaction mixture was photolysed as described previously (29). Membranes were then pelleted by centrifugation and solubilized in 60 μ l of sample buffer (25 mM Tris-HCl (pH 6.5), 8% (v/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol). Photoaffinity cross-linking of proteins were resolved by SDS-polyacrylamide gel electrophoresis using 10% gels. Bands were visualized by autoradiography using Biomax films (Eastman Kodak Co.).

Biotinylation—293 cells transfected with 10 μ g of β -galactosidase, or HA-rD1A receptor were reseeded in 6-well dishes. Confluent cells were incubated in phosphate-buffered saline (pH 7.65) with 1 mg/ml of Biotin-XX, succinimidyl ester probe (dissolved in *N,N*-dimethylformamide) for 15 min at 37°C. At the end of the incubation, dishes were put on ice, and cells were washed three times with ice-cold phosphate-buffered

saline and processed as described by Freedman *et al.* (30). Cells were solubilized by adding 0.5 ml of RIPA+ buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM sodium fluoride, and 10 mM disodium pyrophosphate) containing protease inhibitors (as described above). Solubilized cell extracts were transferred to 1.5-ml conical tubes in a total volume of 0.8 ml, and solubilized for an additional hour at 4°C using an inversion wheel. Supernatant fractions were collected by centrifugation at 200,000 \times g for 15 min at 4°C in TLA centrifuge 100.2 rotor (Beckman), and 730 μ l of solubilized preparations were transferred to new tubes. Two aliquots (15 μ l) were taken for protein assay using bovine serum albumin as standard (Bio-Rad DC protein assay kit). Supernatant fractions (~700 μ l) were precleared by adding 100 μ l of 10% (v/v) protein A-Sepharose beads (Pharmacia Biotech Inc.) in 2% (w/v) bovine serum albumin and rotated for 1 h at 4°C, and transferred to new tubes containing 100 μ l of protein A-Sepharose beads and 20 μ g of purified monoclonal antibody 12CA5 (Babco). After 2 h of incubation at 4°C, beads were pelleted, and the supernatant discarded. The beads were then washed 3 times with 1 ml of RIPA+ buffer and dried by aspiration using a 28-gauge needle (30). Finally, 60 μ l of sample buffer was added to each tube, and immunocomplexes were dissociated at 25°C overnight. Samples were resolved by SDS-polyacrylamide gel electrophoresis using 10% gels. Proteins were transferred to nitrocellulose membrane (0.45 μ m pore size, Bio-Rad) and incubated with a 1:2000 dilution of streptavidin-horseradish peroxidase conjugate (Amersham Corp.), and chemiluminescence of immunocomplexes was detected using an ECL kit (Amersham Corp.).

Whole Cell Phosphorylation—293 cells were transfected with a total of 15 μ g of DNA. For control, GRK2, or GRK3 experimental conditions, 293 cells were cotransfected with 2.5 μ g of HA-rD1A receptor expression construct and 12.5 μ g of β -galactosidase (control), GRK2, or GRK3 expression construct. In the case of GRK5 experimental conditions, 293 cells were cotransfected with 5 μ g of HA-rD1A receptor expression construct and 10 μ g of GRK5 expression construct. Under these different experimental conditions, the expression levels for the HA-rD1A receptor were similar. A day prior to the assay, transfected cells were reseeded in 6-well dishes with 1–1.5 \times 10⁶ cells/well and grown for an additional 18 h. Cells were labeled for 90 min at 37°C, in 20 mM HEPES-buffered phosphate-free Dulbecco's modified Eagle's medium (pH 7.4), and gentamicin containing 0.2 mCi/ml of ³²P_i. At the end of the labeling period, cells were incubated in the presence or absence of 10 μ M dopamine. Solubilization and immunoprecipitation were carried out as described above. Phosphorylated receptors were resolved by SDS-polyacrylamide gel electrophoresis using 10% gels. Gel lanes were loaded with volumes giving equivalent amounts of HA-rD1A receptor as assessed by ligand binding. Gels were dried and exposed to Biomax film at room temperature for 4–24 h. The extent of receptor phosphorylation was quantitated with a PhosphorImager (Molecular Dynamics).

Immunoblotting—Transfected 293 cells reseeded in 6-well dishes (see above) were washed with phosphate-buffered saline, harvested in ice-cold lysis buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4), containing protease inhibitors, and homogenized with a plastic pestle. 20 μ g of proteins from total cell extracts were resolved by SDS-polyacrylamide gel electrophoresis using 10% gels. Moreover, 5 μ g of proteins from total Sf9 cell extract overexpressing GRK2, GRK3, or GRK5 were used as positive controls. Proteins were transferred to nitrocellulose membrane and incubated with a 1:2000 dilution of either GRK2/GRK3 or GRK5 polyclonal antibodies (7, 24), and the chemiluminescence of immunocomplexes was detected using an ECL kit (Amersham Corp.).

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed with gel used for whole cell phosphorylation experiments. Phosphorylated protein bands were identified by lining up the autoradiogram with the gel. The bands were excised from the gel, eluted in 50 mM NH₄HCO₃, 0.1% (v/v) SDS, 0.5% (v/v) 2-mercaptoethanol, precipitated 1 h on ice, and hydrolyzed in 50 μ l of 6 N HCl for 1 h at 110°C as described previously (31). Phosphoamino acids were resolved by two-dimensional electrophoresis on thin layer cellulose plates (Eastman Kodak Co.). The first dimension was carried out in pH 1.9 buffer (2.2% (v/v) formic acid, 1.38 M glacial acetic acid), and plates were run for 1.5 h at 900 V. The second dimension was carried out in pH 3.5 buffer (0.5% (v/v) pyridine, 0.87 M glacial acetic acid), and plates were run for 45 min at 900 V. Finally, thin layer cellulose plates were air-dried, stained with 1% (v/v) ninhydrin to visualize phosphoamino acid standards, and exposed on Biomax film for 48 h at –80°C.

cAMP Accumulation Assay—Following transfection (18–24 h), 293 cells were reseeded in 6-well dishes (wild-type *versus* HA time course experiments) or 12-well dishes (GRK experiments). The next day, culture medium was replaced by fresh minimum essential medium con-

taining 5% (v/v) fetal bovine serum, gentamicin, and labeled with [3 H]adenine (1.5 μ Ci/ml) for 18–22 h. Determinations of intracellular cAMP were assessed by incubating cells in 10 mM HEPES-buffered minimum essential medium (with no phosphodiesterase inhibitors) in the absence or presence of dopamine at 37°C for various periods of time (26). Assays were terminated by transferring dishes on ice, aspirating the medium, and adding 1 ml of stop solution (2.5% (v/v) perchloric acid, 100 μ M cAMP, and \sim 10,000 cpm of [3 H]cAMP). After 20–30 min in the cold, the acid-cell lysates were transferred to tubes containing 0.1 ml of neutralizing solution (4.2 M KOH). The salt precipitates were pelleted by centrifugation, and separation of [3 H]cAMP in the cell lysate supernatants was done using a sequential chromatography on Dowex and alumina columns. Data are presented as 1000 times the ratio [3 H]cAMP formed over the total uptake measured in the well.

Data Analysis—Radioligand binding and dose-response curves were analyzed using the curve-fitting programs ALLFIT and LIGAND (32, 33). Statistical analysis of data were performed using analysis of variance. Pairwise comparisons were assessed either by the *t* distribution test or GT2-method (34). Homogeneity of variances was assessed by the *F*_{max} test. The level of significance was established at 5% using one-tailed test. Results are expressed as the mean \pm S.E.

Materials—Human embryonic kidney cells (293) were obtained from American Tissue Culture Collection (CRL 1573). Tissue culture reagents were from Life Technologies, Inc. [3 H]Adenine, [3 H]cAMP, and 32 P_i were purchased from DuPont NEN. Dopamine-HCl and flupentixol-HCl were from Research Biochemical International. Protease inhibitors were obtained from Sigma. Nonidet P-40 was purchased from Calbiochem. SANPAH was purchased from Pierce. Biotin-XX succinimidyl ester probe was procured from Molecular Probes. SCH39111 was obtained from Schering-Plough Corp.

RESULTS

Molecular and Biochemical Characterization of the Hemagglutinin Epitope-tagged Dopamine D1A Receptor—Using polymerase chain reaction-based methodology, we have engineered the sequence coding for the hemagglutinin epitope (HA) in the amino terminus of the rat dopamine D1A (rD1A) receptor. To verify that the receptor function was not impaired by the insertion of the epitope, we characterize the binding and coupling properties of the HA-rD1A receptor. Saturation studies revealed that equilibrium dissociation constant (*K*_d) of the antagonist [125 I]-SCH23982 for 293 cells expressing either the wild-type or the HA-rD1A receptor was similar (0.51 ± 0.03 nM versus 0.46 ± 0.05 nM, respectively). We subsequently investigated the binding properties of agonists and antagonists. The affinity constants (*K*_a) of the agonist dopamine and the antagonist were identical at both the wild-type or HA-tagged receptor (data not shown). These results suggest that insertion of HA in the amino terminus does not affect the binding properties of agonists and antagonists displayed at the wild-type rD1A receptor.

The rD1A receptor has been shown to be coupled to the activation of adenylyl cyclase when expressed in 293 cells (26). To investigate the coupling properties of the HA-rD1A receptor, dose-response curves to dopamine were performed at different time points (2, 5, and 10 min) using a whole cell cAMP assay in the absence of phosphodiesterase inhibitors. The wild-type or HA-rD1A receptor display similar dose-response curves to dopamine at all time points investigated (Fig. 1). Over the time course studied, at similar receptor expression levels for both forms of the receptor, the maximal activation of adenylyl cyclase (*V*_{max}) increased to similar extent, while the basal activity was not statistically different. As depicted in Fig. 1, the effective concentration (EC₅₀) measured for the HA-rD1A receptor was not statistically different from the wild-type receptor (\sim 15 nM).

To determine the apparent molecular weight of the HA-rD1A receptor protein expressed in 293 cells, we performed photoaffinity cross-linking experiments using [125 I]-SCH39111 (27). As shown in Fig. 2A, the HA-rD1A receptor is expressed at the plasma membrane of 293 cells as a broad protein band of about

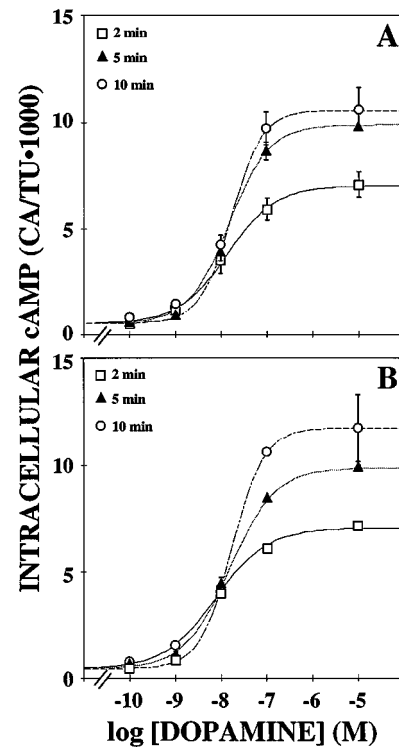


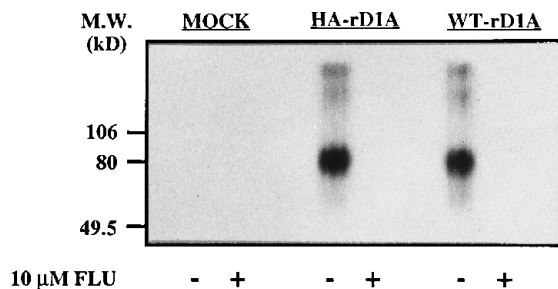
FIG. 1. Time course of dopamine-mediated activation of adenylyl cyclase in 293 cells transiently transfected with rat dopamine D1A receptor. A, Wild-type receptor; B, HA-tagged receptor. Whole cell cAMP assays were performed as described under "Experimental Procedures." Dose-response curves were performed with increasing concentrations of dopamine and exposed for 2, 5, and 10 min. Each point represents the mean of two independent experiments. Curves were fitted using the ALLFIT program (32). Determinations of EC₅₀ values at each stimulation times for either the wild type or HA-tagged rD1A receptor were not statistically different (Wild type, EC₅₀ = 15.5 ± 0.9 nM; HA, 15.6 ± 1.5 nM). Maximal activation values for the wild type (\square , 7.2 ± 0.2 ; \blacktriangle , 9.9 ± 0.2 ; \circ , 10.4 ± 0.2) and HA-tagged (\square , 7.6 ± 0.2 ; \blacktriangle , 9.9 ± 0.2 ; \circ , 11.6 ± 0.2) receptors were increased significantly over the time course studied. Expression levels for the wild type and HA-tagged were 10.6 and 11.1 pmol/mg of membrane protein, respectively.

80 kDa, an electrophoretic mobility similar to that of the wild-type rD1A receptor. These results suggest that insertion of the epitope in the amino terminus does not interfere with the glycosylation of the D1A receptor. This photoaffinity labeling was specific to the expression of the wild-type or tagged receptor, since no detectable labeling was observed in mock-transfected cells (Fig. 2A). The post-translational modification of the human homologue of the D1A receptor expressed in 293 cells was identical to its rat counterpart (data not shown).

To test the ability of the monoclonal antibody 12CA5 to immunoprecipitate the HA-rD1A receptor, 293 cells expressing the tagged receptor or pCMV β GAL (mock) were conjugated with a succinimidyl ester probe (biotin-XX), solubilized, and subjected to immunoprecipitation as described under "Experimental Procedures." Fig. 2B shows that the 12CA5 monoclonal antibody specifically immunoprecipitated protein, which can be visualized as a broad band of about 80 kDa. No such broad band could be detected by immunoprecipitation from mock transfected cells (Fig. 2B). Similar findings were obtained by photoaffinity cross-linking experiments using the [125 I]-SCH39111 (data not shown). Thus, we have shown that the molecular and biochemical characteristics of the HA-rD1A receptor are similar to that of the wild-type receptor.

Agonist-occupied D1A Receptor Is Phosphorylated by GRKs—To investigate the potential role of GRK-mediated phosphorylation in the regulation of the dopamine D1A recep-

A



B

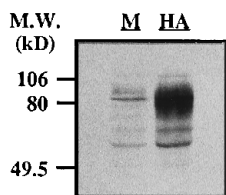


FIG. 2. Photoaffinity cross-linking and biotinylation of the HA-rD1A receptor expressed transiently in 293 cells. A, photoaffinity cross-linking experiments were performed in membranes prepared from 293 cells transfected with β -galactosidase alone (MOCK), HA-tagged rD1A (HA-rD1A) or wild-type receptor (WT-rD1A). Membranes were incubated with ^{125}I -SCH39111 in the absence or presence of $10\ \mu\text{M}$ flupentixol (FLU) as described under "Experimental Procedures." No detectable binding was measured in mock transfected cells, whereas cells harboring HA-rD1A or wild type receptor expressed 5.6 and 6.5 pmol/mg of membrane protein, respectively. Shown is a representative example of an experiment repeated 3 times. B, cells were transfected with β -galactosidase alone (M) or HA-tagged rD1A receptor (HA), biotinylated, solubilized, and immunoprecipitated using the purified monoclonal antibody 12CA5 as described under "Experimental Procedures." Immunocomplexes were resolved by SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and blotted with streptavidin-horseradish peroxidase conjugate. The expression level for cells harboring the HA-rD1A receptor was 9.4 pmol/mg of membrane protein.

tor responsiveness, 293 cells transfected with the HA-rD1A receptor alone or with either GRK2, GRK3 or GRK5 were exposed to $10\ \mu\text{M}$ dopamine for various times, and the agonist-dependent phosphorylation of the HA-rD1A receptor was monitored (Fig. 3). The time course of the GRK-mediated phosphorylation of the HA-rD1A receptor revealed that overexpression of the GRKs increase both the rate and extent of receptor phosphorylation when compared with cells overexpressing the receptor alone (Fig. 3). Maximal receptor phosphorylation was observed following 5 min of agonist exposure. Phosphorylation of HA-rD1A receptor by various GRKs upon exposure to dopamine for 5 min is illustrated in Fig. 4A. In cells expressing the receptor alone, the extent of agonist-dependent phosphorylation was approximately 150% above control basal conditions. In cells overexpressing either the GRK2, GRK3, and GRK5, the HA-rD1A receptor displayed an increase in agonist-dependent phosphorylation of approximately 350–450% (Fig. 4B). Increased agonist-dependent receptor phosphorylation could also be visualized by an upshift in the electrophoretic mobility of the broad 80 kDa band (Fig. 4A). Augmentation of agonist-dependent receptor phosphorylation was correlated with increased GRK expression as detected by Western blot analysis (Fig. 4C). Moreover, phosphoamino acid analysis showed that GRK-mediated receptor phosphorylation occurs

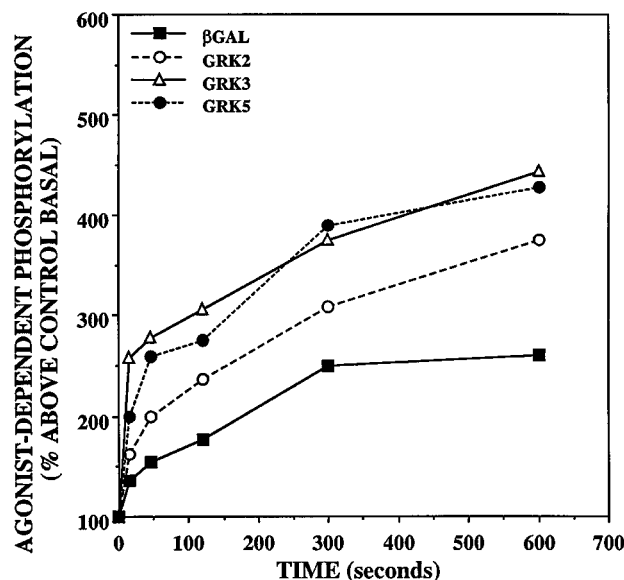


FIG. 3. Time course of agonist-dependent phosphorylation of HA-rD1A receptor overexpressed alone or with various GRKs in transiently transfected 293 cells. Cells transfected with the HA-rD1A receptor alone (β -galactosidase (β GAL) or with GRK2, GRK3, or GRK5 were labeled with $^{32}\text{P}_i$ (0.2 mCi/ml) for 90 min. The cells were then treated with or without $10\ \mu\text{M}$ dopamine for various periods of time. The phosphorylated receptors were solubilized and immunoprecipitated as described under "Experimental Procedures." The amount of receptor phosphorylation was quantitated by PhosphorImager, and data were expressed as percentage above control basal (as measured in cells transfected with HA-rD1A receptor and β GAL incubated in the absence of agonist). Each curve represents the mean of three to four independent experiments. The receptor expression obtained under the transfection conditions were similar and as follows: β -galactosidase, 11.2; GRK2, 11.6; GRK3, 10.0; and GRK5, 5.2 pmol/mg of membrane protein.

exclusively on serine residues (Fig. 4D). These data represent the first evidence for a direct role of GRK2, GRK3, and GRK5 in mediating dopamine D1A receptor phosphorylation.

Overexpression of GRKs Diminishes the Dopamine D1A Receptor Activation of Adenylyl Cyclase—To assess the functional importance of the GRK-mediated phosphorylation of the D1A receptor, we assessed the accumulation of cAMP in intact cells expressing the receptor alone or with different GRKs (Fig. 5). The basal adenylyl cyclase activity of cells harboring the receptor alone or co-transfected with different GRKs was not significantly modified under the same experimental conditions (Fig. 5A). However, a time course of intracellular cAMP accumulation reveals that activation of the D1A receptor by $10\ \text{nM}$ dopamine ($\sim\text{EC}_{50}$ dose) was dampened in cells overexpressing different GRKs at each time point studied (Fig. 5B). A time course of the intracellular cAMP formation elicited by stimulation of the HA-rD1A receptor using a maximal concentration of dopamine ($10\ \mu\text{M}$) was not modified in cells overexpressing GRK2 or GRK3 (Fig. 5C). Interestingly, however, cells overexpressing GRK5 display a significant blunting of the adenylyl cyclase response under these conditions (Fig. 5C). These results suggest that phosphorylation of the D1A receptor by various GRKs leads to functional uncoupling from the G_s protein as evidenced by a decrease in the rate and extent of adenylyl cyclase activation. Moreover, at a high concentration of dopamine, the rate and extent of adenylyl cyclase activation appears to be affected only in those cells overexpressing the GRK5.

To further elucidate the differences in the regulation of the dopamine D1A receptor responsiveness by GRK2, GRK3, and GRK5, cells expressing similar levels of the HA-rD1A receptor either alone or with these kinases were stimulated with increasing concentrations of dopamine for 5 min. Under these

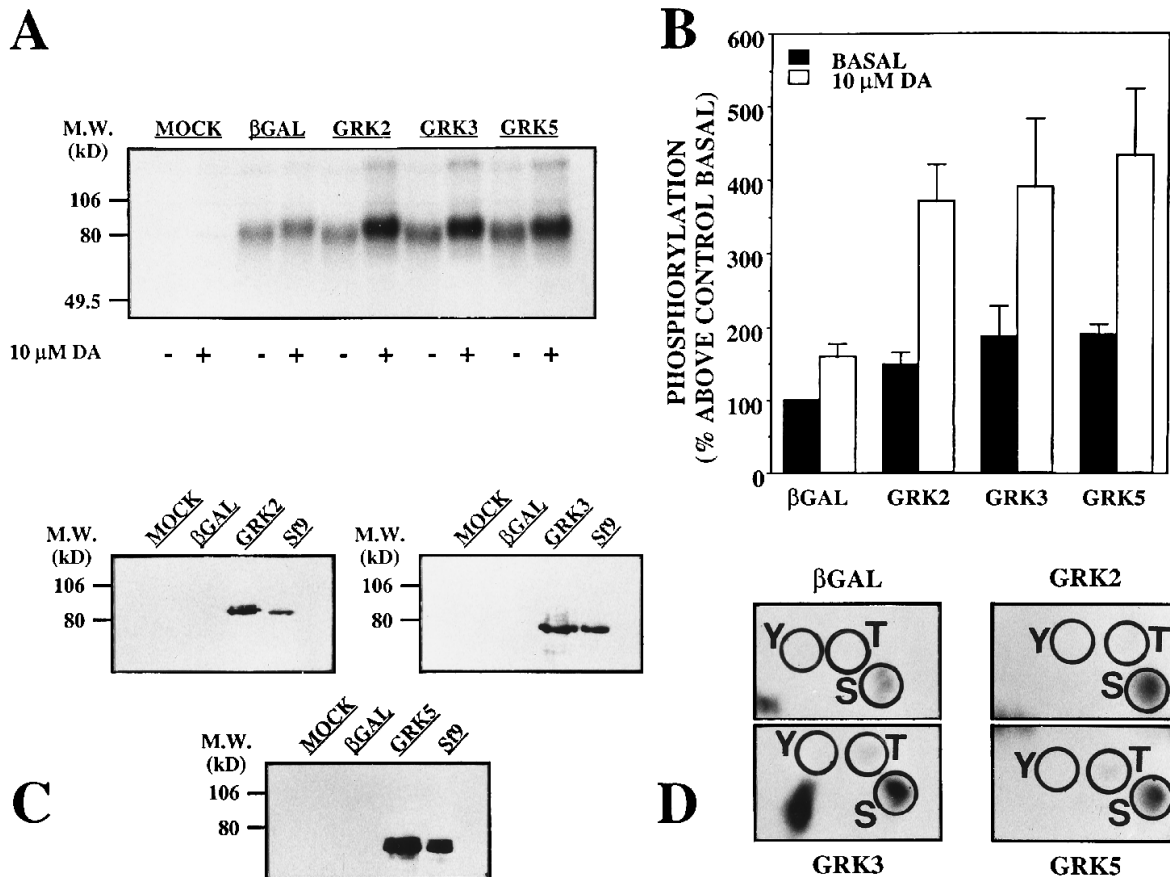


FIG. 4. Agonist-dependent phosphorylation and phosphoamino acid analysis of the HA-rD1A receptor expressed in 293 cells. *A*, cells transfected with β -galactosidase alone (*MOCK*), or transfected with the HA-rD1A receptor alone (β *GAL*) or with GRK2, GRK3, or GRK5 were treated with or without 10 μ M dopamine (*DA*) for 5 min were subjected to immunoprecipitation as described under "Experimental Procedures." The immunocomplexes were then resolved by SDS-polyacrylamide gel electrophoresis using 10% gels. The increase in receptor phosphorylation was visualized by autoradiography. Shown is a representative example of an experiment repeated 8–10 times. The receptor expression obtained under the different experimental conditions was similar and was as follows: β -galactosidase, 12.4; GRK2, 15.1; GRK3, 14.0; GRK5, 11.4 pmol/mg membrane protein. *B*, the receptor phosphorylation obtained under the experimental conditions described in *A* was quantitated by PhosphorImager. The results are expressed as the mean of 8–10 independent experiments. Data are presented as percentage above control basal phosphorylation (as measured in cells transfected with HA-rD1A and β -galactosidase in the absence of agonist). The receptor expression level obtained under the different experimental conditions were as follows: β -galactosidase, 14.8; GRK2, 15.2; GRK3, 13.3; and GRK5, 11.5 pmol/mg of membrane protein. *C*, cell lysates were prepared from 293 cells overexpressing the β -galactosidase only (*MOCK*), or the HA-rD1A receptor alone (β *GAL*) or with various GRKs. Cell lysates prepared from Sf9 cells overexpressing GRK2, GRK3, or GRK5 were used as controls. Immunoblotting was performed as described under "Experimental Procedures." *D*, phosphorylated bands obtained upon exposure to 10 μ M dopamine shown in *A* were excised, and the proteins were eluted and hydrolyzed with 6 N HCl. Phosphoamino acids were separated by two-dimensional electrophoresis on thin layer cellulose plates. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards are circled. The HA-rD1A receptor immunoprecipitated from cells coexpressing various GRKs were phosphorylated only on serine residues.

experimental conditions, dopamine elicited a dose-response curve in cells expressing the receptor alone with an EC_{50} of 23 nM with a maximal stimulation of 10–15 fold above basal activity (Fig. 6A). In cells expressing either GRK2 or GRK3, dose-response curves display a statistically significant 3- and 7-fold rightward shift in the EC_{50} with values corresponding to 68 nM and 157 nM for GRK2 and GRK3, respectively (Fig. 6A). Expression of GRK2 or GRK3, however, had no significant effect on either the -fold activation or V_{max} (Fig. 6A). In contrast to the GRK2 and GRK3 situation, cells overexpressing the GRK5 displayed the most striking change in the HA-D1A receptor responsiveness depicted by a marked decrease in the maximal stimulation of intracellular cAMP (Fig. 6A). A significant 2-fold rightward shift in the EC_{50} for dopamine was also observed (Fig. 6A). Concomitant to these dose-response curves, phosphorylation experiments were performed in the same transfected cells used for the whole cell cAMP assay. Interestingly, stimulation of the HA-rD1A receptor by 10 μ M dopamine for 5 min leads to a similar amount of the agonist-induced receptor phosphorylation by all the GRK isoforms utilized (Fig. 6B). This suggests that functional differences observed for the

D1A receptor responsiveness can not be explained by differences in the extent of receptor phosphorylation.

DISCUSSION

In this report we demonstrate that the agonist-occupied form of the D1A receptor can serve as a substrate for various GRKs. Phosphorylation of the D1A receptor by these GRKs leads to a diminished ability of the receptor to increase intracellular cAMP levels in response to dopamine. For an equivalent extent of phosphorylation of the D1A receptor by various kinases, the attenuation of responsiveness appears to be more pronounced following phosphorylation by GRK5 than GRK2 or GRK3. These results provide evidence that specificity of action can be demonstrated between G protein-coupled receptors and GRKs.

Direct Phosphorylation of Dopamine D1A Receptor by GRKs Regulates Signaling Function—Previous evidence for a regulatory role of phosphorylation in the function of the dopamine D1A receptor has come mostly through indirect means. Zhou *et al.* (21) used kinase inhibitors to imply a role for protein kinase A- and GRK-mediated phosphorylation of the D1A receptor in the process of agonist-mediated desensitization. In the latter

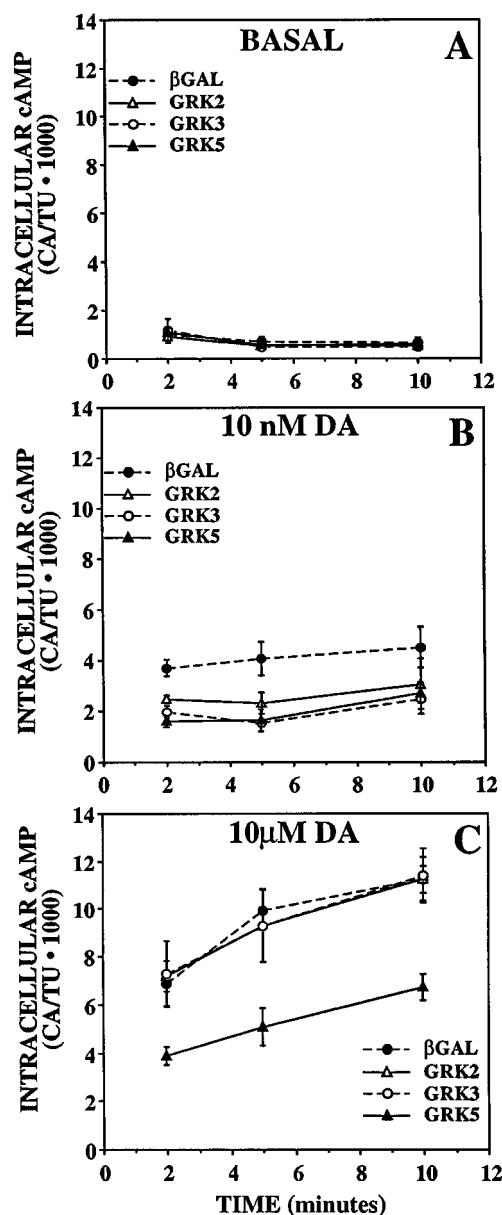


FIG. 5. Time course of dopamine-mediated adenylyl cyclase activation in 293 cells overexpressing the HA-rD1A receptor and various GRKs. A, basal; B, 10 nM dopamine; C, 10 μ M dopamine. Whole cell cAMP accumulation was measured following exposure to 0.1 mM ascorbate (*basal*) or dopamine (DA) for 2, 5, and 10 min. Data are presented as the mean of three independent experiments done in triplicate determinations. Receptor expression for each of the experimental procedures were as follows: β -galactosidase (β GAL), 6.7; GRK2, 5.6; GRK3, 5.2; and GRK5, 5.1 pmol/mg of membrane protein.

and other desensitization studies, direct phosphorylation of the D1A receptor could not be demonstrated presumably due to low levels of receptor expression in the various systems used (16–20). To circumvent these difficulties, we have overexpressed an epitope-tagged D1A receptor in 293 cells co-transfected with various GRKs.

In 293 cells, the HA-D1A receptor behaves identically to the wild-type receptor. In agreement with the observation that 293 cells contain low levels of endogenous GRKs (30), only a very weak agonist-mediated desensitization of the signal is observed in cells overexpressing the D1A receptors (data not shown). These findings correlate with the low agonist-mediated increase ($\sim 50\%$) in phosphorylation of the D1A receptor in the absence of exogenous kinases (Fig. 4C). In contrast, overexpres-

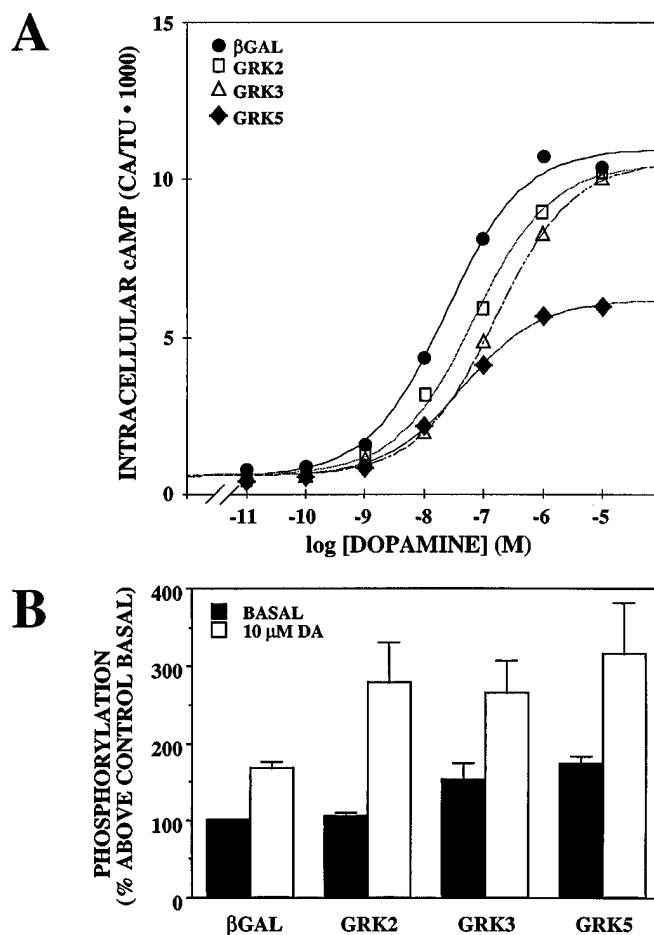


FIG. 6. Dose-response curves for dopamine-mediated adenylyl cyclase activation in 293 cells overexpressing the HA-rD1A receptor alone or with various GRKs. A, Whole cell cAMP assays were done as described under "Experimental Procedures." Dose-response curves were generated by stimulating cells with increasing concentrations of dopamine for 5 min. Each point represents the mean of four to five independent experiments done in duplicate determinations. Curves were fitted using the ALLFIT program. EC_{50} values \pm standard errors from the fits obtained for the different experimental procedures were as follows: β -galactosidase (β GAL), 22.9 ± 3.6 nM; GRK2, 67.9 ± 11.9 nM; GRK3, 157 ± 29.6 nM; and GRK5, 43.2 ± 12.8 nM. Dose-response curves obtained in cells overexpressing GRK2, GRK3, and GRK5 all display a significant rightward shift. The maximal activation obtained in cells overexpressing GRK5 (6.2 ± 0.2) was significantly reduced in comparison with the control curve (β -galactosidase; 11.0 ± 0.2) or GRK2 (10.5 ± 0.3) and GRK3 (10.6 ± 0.3) curves. B, whole cell phosphorylation were performed in the same transfected pool of cells used in (A) as described under "Experimental Procedures." Upon exposure to 10 μ M dopamine for 5 min, the extent of agonist-dependent phosphorylation obtained between the cells overexpressing the various GRKs was not statistically different but significantly higher than the one measured in control cells (HA-rD1A receptor and β -galactosidase). The receptor expressions were not statistically different between the four experimental conditions tested. Receptor levels were as follows: β -galactosidase, 9.6; GRK2, 9.3; GRK3, 8.5; and GRK5, 7.5 pmol/mg of membrane protein.

sion of various GRKs (>20 -fold as assessed by immunoblotting; Fig. 4C) increases both the rate and extent of the agonist-dependent phosphorylation of the transfected receptor, suggesting that this cellular system can be used to study the actions of the various GRKs. Indeed, for each transfected GRK, the extent of agonist-dependent phosphorylation is significantly greater than that observed as a result of the endogenous kinases only. Moreover, the D1A receptor undergoes a rapid loss of responsiveness, which is detectable as soon as 2 min after stimulation and remains relatively constant for at least 10 min. These results are consistent with the time course reported for GRK-mediated desensitization (35). This modification of the trans-

fects D1A receptor by the various GRKs supports a role of phosphorylation in regulating the functional state of the D1A receptor.

Distinct Modulation of D1A Receptor Responsiveness Suggests Specificity of GRK Actions—Phosphorylation of the D1A receptor by different GRKs resulted in attenuation of responsiveness with distinguishing characteristics. Indeed, phosphorylation of the D1A receptor by GRK5 afforded a dramatic attenuation of response with both a shift in the EC_{50} for dopamine and a marked 40% decrease in maximum response. In contrast, GRK2- and GRK3-mediated receptor phosphorylation led to significant rightward shifts of the dose-response curves for dopamine with no change in the maximal response (Fig. 6A). Therefore, GRK5 phosphorylation of the D1A receptor engenders a more profound desensitization than phosphorylation by either GRK2 or GRK3. These differences could not be attributed to differences in the extent of phosphorylation of the D1A receptor as similar levels of phosphorylation were achieved with all three kinases. Taken together, these results suggest that the D1A receptor responsiveness can be regulated differentially according to the GRK subtype expressed in a particular cell. What possible underlying mechanisms could explain such differential modulation of the D1A receptor function by the various GRKs?

First, these results might be explained by the existence of distinct GRK phosphorylation sites located within the cytoplasmic domains of the D1A receptor. Indeed, it is possible that phosphorylation of distinct GRK sites leads to different conformational changes of the phosphorylated D1A receptor, which may potentially display differences in their ability to activate adenylyl cyclase. Despite the large amount of evidence for the phosphorylation of G protein-coupled receptors by GRKs, very little is known about the exact nature of the phosphorylation sites for the various characterized GRKs. Distinct specificities have been demonstrated for β -adrenergic receptor kinase 1 (GRK2) and GRK5 using peptide substrates (36, 37). Thus, phosphorylation of distinct sites by various GRKs could result in different extent of attenuation of the response. It is interesting to note that, although GRKs are serine/threonine kinases, of the 22 serine and 14 threonine residues present in the cytoplasmic domains of the D1A receptor, only serine residues appear to be phosphorylated (Fig. 4D). Further studies using purified and GRK-phosphorylated D1A receptor will be required to determine the exact nature of the sites phosphorylated by GRK2, GRK3, and GRK5.

Second, the distinct modulation of D1A receptor responsiveness by the different GRKs might be explained by the potential role played by arrestin-like proteins, which have been demonstrated to be essential for the full extent of receptor desensitization for β -adrenergic receptor kinase 1 (GRK2) (3) and rhodopsin kinase (GRK1) (38). Meanwhile, no such data exist for GRK5-mediated desensitization. Under normal conditions, the levels of arrestin proteins are unlikely to be limiting (39); however, under conditions of overexpression of G protein-coupled receptors in heterologous systems, kinase and arrestin proteins may become limiting (40). Thus, the absence of a diminished V_{max} in cells overexpressing GRK2 and GRK3 in our studies might be explained by a limiting level of arrestin proteins. However, this raises the intriguing question about the potential role arrestin proteins play in GRK5-mediated desensitization of the D1A receptor. GRK5 belongs structurally to a different subfamily of kinases than GRK1, GRK2, and GRK3 (2, 41), and therefore receptor desensitization by GRK5 may be potentially elicited independent of the binding of arrestin proteins. In addition, several forms of arrestin proteins have been isolated (3), and it is interesting to speculate that

different phosphorylated sites may provide different interaction sites for the various arrestin proteins. Further studies are required to establish the precise role of arrestin proteins in the modulation of D1A receptor function upon its phosphorylation by GRKs. Finally, it is worth mentioning that these effects were observed in whole cell preparations, and therefore we cannot rule out that GRK5 also regulates a downstream effector important for D1A receptor signaling. Studies performed using added GRKs to membranes expressing D1A receptors may help to elucidate potentially these different effects (42). Regardless of the basis for the observed differences, our data document that under identical conditions, the effect of these various kinases can be significantly different (*i.e.* selectivity of action exists).

Previously, selectivity, or the lack thereof, in the ability of the different GRKs for phosphorylating different receptors has been documented. Thus, rhodopsin is a better substrate for GRK1 than GRK2 (43, 44); β_2 -adrenergic and m_2 -muscarinic receptors are better substrates for β -adrenergic receptor kinase 1 (GRK2) than GRK5 (37, 42), whereas the β_1 -adrenergic receptor appears to be as effectively phosphorylated by either GRK2, GRK3, or GRK5 (30). The dopamine D1A receptor represents yet a different type of selectivity in that the receptor appears to be covalently modified by the various kinases to a similar extent, but the biological consequence of that phosphorylation (*i.e.* desensitization) differs.

Physiological Relevance of D1A Receptor Phosphorylation—The demonstration of a role for the GRKs in regulating D1A receptor responsiveness in a heterologous mammalian expression system raises the issue of the physiological relevance of the differential modulation of D1A receptor function by GRK2, GRK3, and GRK5. Cellular co-localization of the D1A receptor (or any other G protein-coupled receptors) with different GRKs is currently unknown. However, *in situ* hybridization and immunohistochemistry studies have shown that GRK2 and GRK3 are expressed in brain regions that have been shown to contain D1A receptors (7). In addition, Arriaza *et al.* (7) have shown that GRK2 and GRK3 appear to be associated with presynaptic and more predominantly postsynaptic localizations in various brain regions, consistent with a putative role of these two GRKs in the desensitization of synaptic G protein-coupled receptors. Moreover, in support of a physiological relevance for D1A receptor regulation by GRK5, the GRK5 mRNA has been found in cortex and in retina that also express the D1A receptor (24, 45). Recently, it has been shown that exposure of D1A receptors to dopaminergic agonists leads to a greater desensitization of the D1A receptor in the retina than in the striatum (46). Thus, it would appear that regulation of D1A receptor function in these two tissues is different, and our results may provide the molecular and biochemical basis for this observation.

Studies using cellular systems have helped to delineate the molecular events involved in G protein-coupled receptor regulation (39). Several transgenic studies have now established the relevance of these mechanisms in various physiological situations (47, 48). Mice overexpressing carboxyl-terminal truncated rhodopsin, which lack the GRK1 phosphorylation sites, display abnormal prolonged flash responses, suggesting that phosphorylation of rhodopsin is essential for turnoff of the light signal *in vivo* (47). In addition, transgenic mice overexpressing GRK2 specifically in the heart display a reduced cardiac function as measured by a diminution of isoproterenol-stimulated left ventricular contractility, myocardial adenylyl cyclase activity, and decreased functional coupling of β -adrenergic receptors (48). Recent studies have also shown that levels and activities of GRKs can be modulated by physiological or pharmacological situations that modulate the levels of hor-

monal or neuronal input (40, 49). Since the D1A receptor mediates several behavioral paradigms and responses to psychostimulants, the regulation of its function by GRK-dependent events is a question of interest that will require further investigation of the underlying mechanisms possibly using genetically altered animals. The present study illustrates the functional importance the multiplicity of GRKs may play in regulating receptor responsiveness in these various physiological situations.

Acknowledgments—We thank Drs. Julie Pitcher and Neil Freedman for helpful discussions during the course of this study.

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