Dopamine signaling in mammals is mediated by five G protein-coupled receptor (GPCR) proteins divided into two groups based upon sequence homology, G protein coupling, signaling pathways, pharmacological profiles, and desensitization kinetics (Sibley and Monsma, 1992; Missale et al., 1998). The D1-like group consists of the D1 and D5 receptors that couple to Gαi/Gβγ to activate adenylyl cyclase. The D2-like group consists of the D2α, D2β, and D4 receptors that couple to Gαs/Gβγ to inhibit adenylyl cyclase and modulate voltage-gated K⁺ and Ca²⁺ channels.

The D1 Dopamine Receptor Is Constitutively Phosphorylated by G Protein-Coupled Receptor Kinase 4

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ABSTRACT

G protein-coupled receptor (GPCR) kinases (GRKs) phosphorylate agonist-activated GPCRs, initiating their homologous desensitization. In this article, we present data showing that GRK4 constitutively phosphorylates the D₁ receptor in the absence of agonist activation. This constitutive phosphorylation is mediated exclusively by the α isoform of GRK4; the β, γ, and δ isoforms are ineffective in this regard. Mutational analysis reveals that the constitutive phosphorylation mediated by GRK4α is restricted to the distal region of the carboxyl terminus of the receptor, specifically to residues Thr428 and Ser431. Phosphorylation of the D₁ receptor by GRK4α results in a decrease in cAMP accumulation, an increase in receptor internalization, and a decrease in total receptor number—all of which are abolished in a D₁ receptor mutant containing T428V and S431A. The increase in internalized D₁ receptors induced by GRK4α phosphorylation is due to enhanced receptor internalization rather than retarded trafficking of newly synthesized receptors to the cell surface. The constitutive phosphorylation of the D₁ receptor by GRK4α does not alter agonist-induced desensitization of the receptor because dopamine pretreatment produced a similar decrease in cAMP accumulation in control cells versus cells expressing GRK4α. These observations shift the attenuation of D₁ receptor signaling from a purely agonist-driven process to one that is additionally modulated by the complement of kinases that are coexpressed in the same cell. Furthermore, our data provide direct evidence that, in contrast to current dogma, GRKs can (at least in some instances) constitutively phosphorylate GPCRs in the absence of agonist activation resulting in constitutive desensitization.

Upon agonist activation, GPCRs undergo desensitization, a homeostatic process that results in a waning of receptor response under continued agonist stimulation (Ferguson et al., 1996; Gainetdinov et al., 2004). Desensitization involves phosphorylation of the receptor by GRKs and/or second messenger-activated kinases (cAMP-dependent protein kinase or protein kinase C). Homologous desensitization of GPCRs involves only activated receptors and is primarily mediated by GRKs. GRKs are serine/threonine-directed protein kinases composed of seven isoforms divided into three families (Penela et al., 2003). GRK1 and GRK7 compose the rhodopsin kinase/visual family, are expressed exclusively in retina, and participate in desensitization of opsins in rods and cones (Somers and Klein, 1984; Hisatomi et al., 1998; Weiss et al., 1998). GRK2 (βARK1) and GRK3 (βARK2) were originally identified as regulating the β-adrenergic receptor and com-
pose the βARK family. The GRK4 family consists of GRK4, GRK5, and GRK6. Four RNA splice variants have been identified for GRK4: α, β, γ, and δ (Premont et al., 1996). GRK4α represents the largest member; β, γ, and δ each represent different truncations of the α sequence. GRK-mediated phosphorylation has been shown to decrease receptor/G protein interactions and to initiate arrestin binding. Arrestin association further decreases G protein coupling and promotes endocytosis of the receptor. Once internalized, GPCRs can engage additional signaling pathways, be sorted for recycling to the plasma membrane, or targeted for degradation (Ferguson et al., 1996; Penela et al., 2003; Gainetdinov et al., 2004).

As with most GPCRs, the D1 dopamine receptor is known to undergo desensitization and internalization, and these events have been studied in a variety of systems. A preponderance of evidence indicates that desensitization/internalization of the D1 receptor is regulated by its phosphorylation state; this, in turn, is mediated by both second messenger-activated kinases as well as by GRKs. Phosphorylation of the D1 receptor by the cAMP-dependent protein kinase seems to occur on a single threonine residue, Thr268, within the 3rd cytoplasmic loop, and this modification regulates the rate of agonist-induced desensitization (Jiang and Sibley, 1999) and/or the intracellular trafficking of the receptor (Mason et al., 2002). In contrast, evidence concerning the number, number, and location of GRK phosphorylation sites within the D1 receptor is contradictory. Jackson et al. (2002) have suggested that the D1 receptor is phosphorylated by GRKs on multiple residues within its carboxyl terminus. Likewise, Lamey et al. (2002) have provided evidence that agonist-induced phosphorylation of the human D1 receptor is restricted to the carboxyl terminus; however, they report that only a single residue, Thr360, is involved. In contrast, Tiberi et al. (1996) observed that GRK-mediated phosphorylation of the D1 receptor takes place exclusively on serine residues. We have recently suggested that agonist-induced, GRK-mediated phosphorylation of the D1 receptor occurs on residues within both the carboxyl terminus and the third cytoplasmic loop and that the phosphorylation of these domains may take place in an ordered fashion (Kim et al., 2004). It is conceivable that these different results might be due to the expression of different complements of GRK isoforms in the various cell systems under study.

The D1 dopamine receptor seems to be a substrate for at least GRK2–5 (Tiberi et al., 1996; Felder et al., 2002). To investigate whether these different GRKs differentially phosphorylate the D1 receptor, we coexpressed each of the nonviral GRKs with the D1 receptor and examined its phosphorylation state in the absence or presence of agonist. We were surprised to find that GRK4 coexpression resulted in phosphorylation of the receptor in the basal state to a very high level that was only marginally increased upon addition of agonist. This constitutive phosphorylation of the D1 receptor was observed only with the α isoform of GRK4. This agonist-independent phosphorylation of the D1 receptor by GRK4α was found to result in a decrease in agonist-induced cAMP accumulation, an increase in basal receptor internalization, and a decrease in total receptor number. Our present results with GRK4 and the D1 receptor contrast with the existing dogma that GRKs phosphorylate only agonist-occupied/activated GPCRs and provide a novel mechanism whereby GRKs can regulate GPCR function.

Materials and Methods

Materials. HEK293-tesa201 (HEK293T) cells (Heinzel et al., 1998) were a gift from Dr. Vanitha Ramakrishnan. Rat D1-GFP was a gift from Dr. Qun-Yong Zhou (Bermak et al., 2002). GRK4 isoforms were obtained from Dr. Richard Premont. GRK4+ single-nucleotide polymorphisms (SNPs) were as described previously (Felder et al., 2002). GRK2 and GRK3 clones as well as the dominant-negative β- arrestin minigene construct (β-arrestin 319–418) were obtained from Dr. Jeffrey L. Benovic. [3HJSCH-23390 (86.00 Ci/mmol) and [32P]orthophosphate (carrier-free) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [3H]cAMP (31.4 Ci/mmol) was purchased from Diagnostic Products Corporation (Los Angeles, CA). Dopamine, Ro-20-1724, sodium metabisulfite, and anti-FLAG M2 affinity gel were purchased from Sigma (St. Louis, MO). (+)-Butaclamol was purchased from Research Biochemicals Inc. (Natick, MA). Cyclic AMP assay kits were obtained from Diagnostic Products Corp. (Los Angeles, CA). Calcium phosphate transfection kits were obtained from Clontech (Mountain View, CA). Minitest protease inhibitor cocktail was purchased from Roche Applied Science (Indianapolis, IN). QuikChange and QuikChange Multi site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA), and mutagenesis primers were synthesized by MWG Biotech Inc. (High Point, NC).

Cell culture media and reagents, NuPage gels, buffers, and InvitroLink 0.45-μm PVDF membranes were purchased from Invitrogen (Carlsbad, CA). Anti-GRK4 (H-70) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), peroxidase-conjugated goat anti-rabbit IgG secondary antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA), and SuperSignal West Dura extended duration substrate antibodies kit was purchased from Pierce (Rockford, IL).

Whole-Cell Phosphorylation Assay. These assays were performed as described previously (Gardner et al., 2001). In brief, 1 day before the experiment, transfected HEK293T cells were seeded at 1.5 × 106 cells per well of a six-well poly-D-lysine–coated plate, and 2 × 106 cells of the same transfection were seeded in a 100-mm culture dish and cultured overnight. Cells in the six-well plates were washed with EBSS and incubated for 1 h in phosphate-free DMEM. Media was then removed and replaced with 1 ml of fresh phosphate-free DMEM containing 106 μCi of [32P]H3PO4 and returned to the incubator for 45 min. This procedure has been shown previously to result in stoichiometric phosphorylation of the D1 receptor (Gardner et al., 2001). Cells were then challenged with 10 μM dopamine in the presence of 0.2 mM sodium metabisulfite for 15 min then placed on...
Cells were washed twice with ice-cold EBSS and solubilized for 1 h at 4°C in 1 ml of solubilization buffer (50 mM HEPES, 1 mM EDTA, 10% glycerol, 1% Triton X-100, pH 7.4, 50 mM NaF, 40 mM sodium pyrophosphate, and 150 mM NaCl) supplemented with Mini-Complete protease inhibitor cocktail. The samples were cleared by centrifugation and the protein concentration was determined using the BCA protein assay kit from Pierce. The specific activity of D1 receptor expression in each transfection group was determined by radioligand binding assays performed using cells in the 100-mm culture dishes described above. After quantifying the receptors in each transfection group, equal amounts of receptor protein were transferred to fresh tubes containing 50 μl of equilibrated anti-FLAG M2-affinity gel and incubated overnight with mixing at 4°C. The samples were washed three times with the following solutions in the order of 0.5 M NaCl solubilization buffer, 0.15 M NaCl solubilization buffer, and Tris-EDTA, pH 7.4, at 4°C. Proteins were eluted from the affinity gel by addition of 35 μl of 2× lithium dodecyl sulfate sample buffer containing reducing agent (Invitrogen) and incubated at 37°C for 1 h. Proteins were resolved on 4 to 12% NuPage Bis-Tris gradient gels run in MOPS SDS running buffer (Invitrogen), dried, and subjected to autoradiography.

Western Blotting. HEK293T cells were cultured, transfected, and seeded into six-well plates as described for the whole-cell phosphorylation assays above. Cells were washed twice with ice-cold EBSS and solubilized for 1 h at 4°C in 0.5 ml of solubilization buffer (50 mM HEPES, 1 mM EDTA, 10% glycerol, 1% Triton X-100, pH 7.4, 50 mM NaF, 40 mM sodium pyrophosphate, and 150 mM NaCl) supplemented with MiniComplete protease inhibitor cocktail. The samples were cleared by centrifugation and the protein concentration was determined using the BCA protein assay kit from Pierce. A concentrated stock of 4× lithium dodecyl sulfate sample buffer containing reducing agent (Invitrogen) was prepared and added to the cell lysate volume that corresponded to 45 μg of total protein per cell lysate and incubated at 37°C for 1 h. Proteins were resolved on 4 to 12% NuPage Bis-Tris gradient gels run in MOPS SDS running buffer (Invitrogen), transferred to Invitronol 0.45-μm PVDF membranes in NuPage transfer buffer containing methanol, according to the manufacturer's instructions, and blocked overnight at 4°C in a 1% BSA solution made in TBST. For identification of GRK4 proteins within the cell lysates, the PVDF membranes were incubated in a primary antibody solution of GRK4 (H-70) rabbit polyclonal antibody diluted 1:500 in 1% bovine serum albumin/TBST solution for 1 h at room temperature. The membranes were washed with TBST then incubated in a secondary antibody solution of peroxidase-conjugated goat anti-rabbit diluted 1:25,000 in 1% bovine serum albumin/TBST solution for 1 h at room temperature and washed with TBST. Western blots were developed using the SuperSignal West Dura extended duration substrate antibody kit from Pierce (Rockford, IL).

Radioligand Binding Assays. HEK293T cells were harvested by incubation with 5 mM EDTA in EBSS lacking CaCl2 and MgSO4 and collected by centrifugation at 300g for 10 min. The cells were resuspended in lysis buffer (5 mM Tris, pH 7.4, and 5 mM MgCl2) at 4°C and were disrupted using a Dounce homogenizer followed by centrifugation at 34,000g for 15 min. The resulting membrane pellet was resuspended in binding buffer (50 mM Tris, pH 7.4), and 100 μl of the membrane suspension was added to assay tubes containing [3H]SCH-23390 in a final volume of 1 ml. A portion of the membrane suspension was quantitated using the BCA protein assay (Pierce). (+)-Butaclamol was added at a final concentration of 3 μM to determine nonspecific binding. The assay tubes were incubated at room temperature for 1.5 h and the reaction was terminated by rapid filtration through GF/C filters pretreated with 0.6% polyethyleneimine. Radioactivity bound to filters was quantitated by liquid scintillation spectrometry at a counting efficiency of 47 to 60%.

Determination of cAMP Production. Transfected HEK293T cells were seeded into 24-well poly-d-lysine–coated plates (100,000 cells/well) and cultured for 1 day before experimentation. To assess desensitization, cells were pretreated with 10 μM dopamine with 0.2 mM sodium metabisulphite for 1 h and then washed three times with 200 μl of EBSS per well. Various concentrations of dopamine were added to each well in a final volume of 250 μl containing 50 μM Ro-20-1724 and 0.2 mM sodium metabisulphite and placed at 37°C for 20 min. The reaction was terminated by adding 200 μl of 3% perchloric acid and incubated on ice for 30 min before adding 80 μl of 15% K2CO3. The plates remained on ice for an additional 20 min and were centrifuged at 1300g for 10 min. Supernatant (50 μl) from each well was transferred to a 1.2-ml tube containing 150 μl of Tris-EDTA buffer, 50 μl of cAMP binding protein, and 50 μl of [3H]cAMP and incubated overnight at 4°C. Charcoal-dextran mix (1%; 250 μl) was added to each tube and gently vortexed. Tubes were incubated at 4°C for 10 min then centrifuged at 1300g for 20 min. Radioactivity in the supernatant from each tube was quantified by liquid scintillation spectroscopy at a counting efficiency of 47 to 60%. cAMP concentrations were calculated using a standard curve according to the protocol of the assay kit.

Confocal Microscopy. HEK293T cells (3×10⁴) were seeded in 100-mm culture dishes. The next day, the cells were transfected with 300 ng of D1-GFP in pEgFP-N1 vector with or without 4 to 5 μg of GRK4c and 3 μg of dominant-negative β-arrestin (319–418) then cultured for an additional 24 h before reseeding the transfections at 100,000 cells per poly-d-lysine–coated, glass-bottomed, 35-mm culture dish. Before stimulation, the media was replaced with OptiMEM I reduced serum medium. Confocal microscopy was performed on a Zeiss laser-scanning confocal microscope (LSM-510; Zeiss, Wesslyn Garden City, UK). Images were collected sequentially every 30 s for a total of 15 min after agonist stimulation with 20 μM dopamine using a single line excitation (488 nm).

Data Analysis. All phosphorylation assays were performed at least three times. Figures depict representative autoradiography obtained for each experimental condition. Relative intensities of the phosphorylated bands were determined by scanning the autoradiographs and analyzing the bands using LabWorks 4.0 (UVP, Inc., Upland, CA). Binding assays and cAMP experiments were performed three to four times. Radioligand binding parameters Kᵢ and Bₘₐₓ as well as EC₅₀ values for dopamine-stimulated cAMP production were calculated using the curve-fitting program Prism (version 3.02; GraphPad, San Diego, CA).

Results

GRK-Mediated Phosphorylation of the D₁ DAR in HEK293T Cells. To study GRK-mediated phosphorylation of the D₁ DAR, we transiently cotransfected HEK293T cells with the FLAG-tagged D₁ DAR and G protein-coupled receptor kinases GRK2, GRK3, and GRK4c. Empty expression vector was cotransfected as a control for the GRK cotransfection experiments. The transfected cells were then subjected to whole-cell phosphorylation assays to monitor phosphate incorporation into the D₁ DAR under basal conditions and in the presence of agonist. The D₁ DAR was phosphorylated under basal conditions, and this phosphorylation was increased upon cotransfection with various GRK subtypes (Fig. 1, A and B). Upon exposure to agonist (10 μM dopamine for 15 min), control cells expressing D₁ DAR alone displayed a 4-fold increase in receptor phosphate content, whereas cells coexpressing either GRK2 or GRK3 displayed a 7- to 12-fold increase in receptor phosphorylation. We were surprised to find that cells coexpressing D₁ DAR and GRK4c displayed a 4-fold increase in receptor phosphate content in the basal state compared with control, and this amount of phosphorylation was increased only marginally upon exposure to agonist. HEK293T cells cotransfected with the D₁ DAR and either GRK5 or GRK6 did not display phosphorylation pat-
terns (basal or agonist-stimulated) that differed significantly from control cells (data not shown). These data show that coexpression of GRK4α with D₁ DAR results in an increase in basal receptor phosphorylation that is comparable with control cells exposed to agonist.

**GRK4 Subtype-Specific Phosphorylation of the D₁ DAR.** The GRK4 subfamily displays a number of alternative RNA splice variants (Ambrose et al., 1992; Premont et al., 1996, 1999). GRK4α is the largest member of the GRK4 subgroup with alternative splicing resulting in the smaller β, γ, and δ splice variants (Fig. 2). To determine whether these shorter GRK4 splice variants could also mediate agonist-independent phosphorylation of the D₁ DAR, HEK293T cells were cotransfected with the D₁ DAR and each GRK4 splice variant and then subjected to whole-cell phosphorylation assays. Only the GRK4α splice variant displayed agonist-independent phosphorylation of the D₁ DAR whereas the GRK4β, -γ, and -δ subtypes had no effect (Fig. 3A). We also examined three SNPs of GRK4γ that have been reported to regulate desensitization of the D₁ DAR (Felder et al., 2002). We performed similar whole-cell phosphorylation experiments as shown in Fig. 3A with transfected HEK293T cells expressing D₁ DAR and three GRK4γ SNPs (γR65L, γA142V, and γA486V). None of the GRK4γ SNP's mediated phosphorylation of the D₁ DAR in a manner significantly different from

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**Fig. 1.** Effect of coexpressing individual GRK isoforms on D₁ receptor phosphorylation. HEK293T cells were transfected with wild-type (WT) FLAG-tagged D₁ receptor and empty vector (V) or GRK2 (+2), GRK3 (+3) or GRK4α (+4α). Transfected cells were metabolically labeled with [32P]H₃PO₄ for 45 min before stimulation with 10 μM dopamine (DA) for 15 min. Samples were then subjected to immunoprecipitation and resolved on 4 to 12% NuPage BT gels before autoradiography as described under Materials and Methods. Receptors were quantified in each transfection before gel electrophoresis to ensure equal amounts of D₁ receptor in each lane. A, autoradiogram of D₁ receptor immunoprecipitates from whole cell phosphorylation assays. D₁ DAR (1 pmol) was loaded in each lane. A single experiment representative of three is shown. B, the receptor phosphorylation obtained in A was quantified by scanning the autoradiographs followed by analysis with LabWorks software (UVP Inc.). Data are presented as the fold increase in phosphorylation above the WT basal condition and expressed as mean ± S.E.M. values from four independent experiments.

**Fig. 2.** Diagram of the human GRK4 splice variants. The diagram represents the amino acid sequence of the four GRK4 isoforms that result from alternative splicing of the GRK4 mRNA originally identified by (Ambrose et al., 1992; Premont et al., 1999). Boxes (clear/hatched) and lines (solid/dashed) represent identical amino acid sequences.

**Fig. 3.** Phosphorylation of the D₁ receptor coexpressed with individual GRK4 isoforms and SNPs. HEK293T cells were transfected with WT FLAG-tagged D₁ receptor and either empty V or various GRK4 constructs. Transfected cells were metabolically labeled with [32P]H₃PO₄ for 45 min before stimulation with 10 μM dopamine (DA) for 15 min. A, autoradiograms of D₁ receptor immunoprecipitates from whole-cell phosphorylation assays. HEK293T cells were transfected with WT D₁ receptor and empty V or human GRK4 splice variants (α, β, γ, and δ) or human GRK4γ SNPs (γR65L, γA142V, and γA486V). D₁ DAR (1 pmol) was loaded in each lane. A single experiment representative of four is shown. B, Western blot of cells transfected as in A with D₁ receptor and empty V or human GRK4 splice variants (α, β, γ, and δ). Cell lysate (45 μg) was loaded in each lane and visualized as described under Materials and Methods. The antibody used is directed to an epitope common to all isoforms. C, cells were treated as in A except that GRK4α and GRK4β isoforms were transfected using 2- or 4-fold the amount of DNA compared with empty vector or the GRK4α isoform. D₁ DAR (1 pmol) was loaded in each lane. A single experiment representative of three is shown.
that of control cells (Fig. 3A). To be sure that the inability of the shorter GRK4 subtypes to induce agonist-independent phosphorylation of the D₁ DAR was not due to less efficient expression of these constructs, Western blotting experiments were performed to monitor the level of GRK4 isoform expression. HEK293T cells were cotransfected with the four GRK4 splice variants as performed in Fig. 3A. These cells were subjected to Western blotting analysis using a primary antibody that detects all GRK4 splice variants. Cells transfected with GRK4 Δ3A displayed equally robust levels of GRK4 protein expression (Fig. 3B). To determine whether increasing GRK4β, -γ, or -δ, or GRK4γ SNP expression would result in an increase in basal D₁ DAR phosphorylation, HEK293T cells were cotransfected with 2- and 4-fold the amount of DNA used initially to boost the expression of the GRK4 variants. Increasing the amount of GRK4β, -γ, and -δ, or GRK4γ R65L, A142V, and A486V SNPs did not result in constitutive phosphorylation of the D₁ DAR (Fig. 3, C and D; data not shown for SNPs A142V and A486V). These data indicate that only the GRK4α subtype mediates constitutive phosphorylation of the D₁ DAR in the absence of agonist stimulation in HEK293T cells.

Identification of the GRK4α Phosphorylation Sites within the D₁ DAR. Two regions of the D₁ DAR have been implicated in mediating GRK phosphorylation—the third intracellular loop and the long carboxyl terminus (Jackson et al., 2002; Lamey et al., 2002; Kim et al., 2004). To determine the sites of GRK4α-mediated phosphorylation within the D₁ DAR, various receptor mutants were constructed using site-directed mutagenesis and coexpressed with GRK4α in HEK293T cells followed by whole-cell phosphorylation assays. Figure 4 depicts all of the receptor mutants that were used in this study.

We first examined four carboxyl terminal D₁ DAR truncation mutants that were constructed by inserting a stop codon after the amino acid positions 404, 394, 369, and 347, generating receptors with sequentially smaller carboxyl tail regions. Whole-cell phosphorylation assays were performed on cells expressing each D₁ DAR truncation construct and GRK4α. Figure 5A shows that truncation of the D₁ DAR at residue 404 reduced both basal and agonist-stimulated phosphorylation; more importantly, it completely eliminated the constitutive nature of the GRK4α phosphorylation. In this case, the T404 mutant exhibited a ~3-fold increase in phosphorylation in response to dopamine stimulation, as did the WT receptor (Fig. 5A). Further truncation of the D₁ DAR at position 394 did not produce results different from those with the T404 mutant, whereas truncation at residues 369 and 347 further reduced and eliminated receptor phosphorylation, as we have reported previously (Kim et al., 2004). Mutations were also made in previously identified (Kim et al., 2004) and other potential phosphorylation sites within the third intracellular loop of the D₁ DAR (Fig. 4) to determine whether these residues are involved in GRK4α-mediated phosphorylation. In no case did any third intracellular loop mutant alter the ability of GRK4α to phosphorylate the D₁ DAR in the absence or presence of dopamine stimulation (Fig. 5B).

The initial results with the truncation mutants suggest that the residues involved in the constitutive phosphorylation of the D₁ DAR by GRK4α are downstream of residue 404. Because truncation of the receptor at this residue might produce aberrant folding of the carboxyl terminus, resulting in altered receptor-GRK4α interactions, we created another

**Fig. 4.** Diagram of the rat D₁ receptor sequence. The wild-type receptor sequence is shown along with the various mutant receptor constructs used in this study. Four carboxyl terminal truncation mutants were generated by inserting a stop codon after amino acids 347, 369, 394, or 404 (T347, T369, T394, and T404). Shaded circles indicate serine or threonine residues mutated to alanine or valine, respectively, in various combinations. Third intracellular loop mutants include the individual serine point mutations (S2, S3, and S4), the S234 mutant that includes all three point mutants, S2, S3, and S4. In the 3rd TOT mutant, all of the serines and threonines in the third intracellular loop were simultaneously mutated. Individual point mutations in the distal carboxyl tail region include S417A, S421A, T428V, S431A, T439V, S441A, S445A, and T446V, whereas the mutT404 mutant contains all of these point mutations with in a single construct. D₁ DAR cluster mutant 1516 contains T428V and S431A point mutations, and cluster mutant 181920 contains S441A, S445A, and T446V point mutations as indicated.
mutant receptor in which all eight of the serine and threonine residues distal to residue 404 were mutated. This construct, mutT404, retains the region of the D₁ DAR between amino acid 404 and the end of the carboxyl terminus yet contains no GRK phosphorylation sites. Whole-cell phosphorylation assays performed with mutT404 and GRK4α displayed no difference in phosphorylation patterns compared with cells expressing the D₁ DAR truncated at residue 404, confirming that elimination of the serine and threonine residues in this region abolishes GRK4α-mediated, agonist-independent phosphorylation (Fig. 5C). These results indicate that GRK4α-mediated constitutive phosphorylation of the D₁ DAR occurs in the carboxyl tail region of the receptor between amino acid 404 and the end of the carboxyl terminus.

To further identify the specific D₁ DAR residues phosphorylated by GRK4α in the absence of agonist, each of the serine and threonine residues in the region between amino acid 404 and the carboxyl terminus of the receptor was individually mutated as depicted in Fig. 4. Preliminary experiments revealed that no single point mutation resulted in complete abolishment of GRK4α-mediated constitutive phosphorylation of the D₁ DAR; however, some point mutants seemed to be more effective than others. In particular, point mutants S417A, S421A, and T439V showed little or no reduction in constitutive phosphorylation by GRK4α; mutants T428V, S431A, S441A, S445A, and T446V, however, each displayed some degree of reduced constitutive phosphorylation (data not shown). To further investigate this effect, two D₁ DAR cluster mutants were created based on the location of the single point mutants that showed some reduction in GRK4α-mediated constitutive phosphorylation. D₁ DAR mutant “1516” contains the T428V and S431A mutations, and D₁ DAR mutant “181920” contains the S441A, S445A, and T446V mutations (Fig. 4). Each D₁ DAR cluster mutant was coexpressed with GRK4α and subjected to whole-cell phosphorylation assays in the absence and presence of dopamine (Fig. 6). As shown previously, dopamine-stimulation increases the phosphate content of the WT D₁ DAR by approximately 3-fold in the absence of GRK4α, whereas coexpression with GRK4α increases the basal phosphorylation and nearly eliminates the effect of dopamine stimulation. As can be seen, the two cluster mutants behave more similarly to the wild-type D₁ DAR (Fig. 6) and the mutant constructs T404 and mutT404 (Fig. 5C). This effect is particularly striking for the 1516 cluster mutant in which Thr428 and Ser431 are simultaneously mutated (Fig. 6B). These results suggest that five serine and threonine residues in the distal carboxyl terminus of the D₁ DAR contribute to the constitutive phosphorylation by GRK4α with a primary involvement of Thr428 and Ser431.

**Effect of GRK4α-Mediated Phosphorylation of D₁ DAR on cAMP Accumulation and Desensitization.** Having observed the unique nature of GRK4α-mediated phosphorylation of the D₁ DAR, we next studied its effects on dopamine-stimulated cAMP production. Coexpression of the wild-type D₁ DAR with GRK4α resulted in a significant decrease in dopamine-stimulated cAMP accumulation (Fig.
This reduction in D1 DAR-mediated cAMP accumulation by GRK4α was largely absent in similar experiments using the D1 DAR truncation mutant T404 and the corresponding mutT404 mutant (Fig. 7, B and C), neither of which exhibited constitutive GRK4α-mediated receptor phosphorylation. Furthermore, similar cAMP accumulation experiments performed with the D1 DAR cluster mutants 1516 and 181920 revealed that the GRK4α effect was attenuated in the 181920 mutant and abolished with the 1516 mutant (Fig. 8). These data show that constitutive phosphorylation of the D1 DAR by GRK4α results in a diminished ability of the receptor to stimulate cAMP production and that this effect is primarily mediated by phosphorylation of Thr428 and Ser431.

Because it seems that constitutive GRK4α-mediated phosphorylation of the D1 DAR produces desensitization of the receptor response, we wondered whether subsequent agonist treatment might produce even further desensitization. To test this, HEK293T cells were transiently transfected with the wild-type receptor, in the presence and absence of GRK4α, and then pretreated with 10 μM dopamine for 1 h. Figure 9A shows that dopamine pretreatment of the cells transfected with the D1 DAR and empty vector reduces the maximum cAMP response by 30 to 40%, in agreement with our previous findings (Kim et al., 2004). In cells cotransfected with GRK4α, the maximum cAMP response is already diminished by approximately 50%, as shown previously in Figs. 7A and 8A; however, dopamine pretreatment produces a further loss of 30 to 40% compared with untreated cells (Fig. 9A). Thus, the degree of agonist-induced desensitization is similar in the absence or presence of GRK4α (Fig. 9B). It is noteworthy that GRK4α coexpression does not completely eliminate agonist-induced D1 DAR phosphorylation (Fig. 1) suggesting that one or more of the functionally relevant sites phosphorylated by endogenous GRKs in these cells are not phosphorylated by GRK4α. HEK293T cells have been reported to...
express GRKs 2, 3, 5, and 6 (Iwata et al., 2005; Kim et al., 2005), and we have shown that GRK2 and GRK3 can promote phosphorylation of the D1 DAR (Fig. 1).

**Effect of GRK4α on D1 DAR Expression and Subcellular Location.** We were next interested in investigating whether GRK4α-mediated phosphorylation might affect the expression of the D1 DAR. Our first approach was to measure the total complement of cellular receptors using the D1-selective antagonist [3H]SCH-23390. Saturation binding assays were performed on membranes derived from HEK293T cells expressing the D1 DAR in the absence or presence of GRK4α and maximum binding capacities (B_{max}) were determined. Figure 10 shows that cotransfection of the wild-type D1 DAR with GRK4α results in a ~25% reduction in total D1 receptor expression (B_{max}). It is noteworthy that this effect by GRK4α was not observed with the 1516 mutant D1 DAR; however, the 181920 mutant produced results similar to those observed with the wild-type receptor. These results agree well with those obtained with the cAMP accumulation assays (Fig. 8) and support the notion that Thr428 and Ser431 (mutated in the 1516 construct) are the functionally relevant GRK4α phosphorylation sites.

Because the reduction of total receptor expression in response to GRK4α was modest (~25%) compared with the reduction in cAMP response (~50%), we sought to determine whether phosphorylation of the D1 DAR by GRK4α might additionally influence its location within the cell. Experiments were thus conducted using confocal fluorescence microscopy and a D1-GFP fusion construct that enables the intracellular visualization of the D1 DAR in real-time (Bermak et al., 2002). Figure 11, top, shows that under basal conditions, the D1 DAR is primarily located in the plasma membrane, although some intracellular fluorescence is observed. Short-term treatment of the cells with dopamine promotes internalization of the D1 DAR and an accumulation of intracellular fluorescence. Figure 11, bottom, shows that coexpression of GRK4α results in a substantial increase in the internalization of the D1 DAR under basal conditions. In this case, the only observable effect of dopamine treatment is to promote a small further decrease in cell surface receptor expression. These results suggest that constitutive phosphorylation of the D1 DAR by GRK4α promotes its sequestration from the cell surface under basal conditions.

To further test the notion that coexpression of GRK4α promotes receptor internalization rather than altering receptor maturation and insertion into the plasma membrane, we disrupted the internalization process through coexpression of a dominant-negative mutant of β-arrestin. This β-arrestin construct (319–418) consists of the C-terminal 100 amino acids that mediate clathrin binding (Krupnick et al., 1997) such that overexpression prevents endogenous arrestin-clathrin association. The D1 DAR has previously been shown to internalize via a mechanism mediated by β-arrestin and clathrin-coated pits (Vickery and von Zastrow, 1999; Macey...
zyme’s amino and carboxyl termini, respectively, that can cassette exons encoding 32 or 46 amino acids near the en-

regard. As shown in Fig. 2, the GRK4 role in D1 receptor phosphorylation. We were surprised to

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tions. In this regard, similar results have been reported by

Sallese et al. (1997), who found that only GRK4α, but not

GRK4β, γ, or δ, was able to phosphorylate light-activated rhodopsin using a reconstitution system. This activity was found to correlate with the ability of GRK4α to interact with calmodulin, a property not exhibited by the other GRK4 isoforms (Sallese et al., 1997). It will therefore be of interest to determine whether calmodulin, or a related interacting protein, is involved in GRK4α-mediated phosphorylation of the D1 receptor.

Mutational analysis revealed that constitutive phosphorylation of the D1 receptor by GRK4α was restricted to serine and threonine residues within the distal carboxyl terminus of the receptor. Two clusters of GRK4α phosphorylation sites were identified: the first consists of Thr428 and Ser431, whereas the second consists of Ser441, Ser445, and Thr446 (Fig. 4). Mutation of these residues—either singly, as clusters, all together, or via truncation of the distal end of the carboxyl terminus—reduced or eliminated constitutive phosphorylation by GRK4α. This effect was most pronounced for the Thr428/Ser431 mutant, suggesting that these two residues constitute the majority of the GRK4α phosphorylation.

Discussion

In this study, we have investigated the role and specificity of nonvisual GRK-mediated phosphorylation of the D1 dopamine receptor. In agreement with previous findings (Tiberi et al., 1996), we found that GRK2 and GRK3 both enhanced agonist-induced phosphorylation of the D1 receptor. In contrast, we did not observe consistent effects with either GRK5 or GRK6, suggesting that these GRKs may not play a major role in D1 receptor phosphorylation. We were surprised to find that expression of GRK4 with the D1 receptor resulted in enhanced receptor phosphorylation even in the absence of agonist activation. This constitutive phosphorylation of the receptor by GRK4 was found to be specific for the α isoform of the kinase—the β, γ, and δ isoforms were all inactive in this regard. As shown in Fig. 2, the GRK4 gene contains two cassette exons encoding 32 or 46 amino acids near the enzyme’s amino and carboxyl termini, respectively, that can undergo alternative RNA splicing. Only the α isoform of GRK4 contains both cassette exon sequences, suggesting that both regions are needed for constitutive phosphorylation of the D1 receptor. The mechanism by which this occurs is not clear, although possibilities include a higher enzymatic activity of the α isoform or specific interactions with a scaffold or adaptor protein that facilitates GRK4/D1 receptor interactions.

This experiment was performed three times with similar results.

Fig. 11. Confocal fluorescence microscopy of D1 receptor-GFP expression in the presence or absence of GRK4α. HEK293T cells were transfected with wild-type D1 receptor-GFP construct and either empty vector or GRK4α. Twenty-four hours after transfection, cells were plated on glass-bottomed 35-mm culture dishes, and confocal microscopy was performed using a Zeiss laser-scanning confocal microscope (LSM-510). Images were collected sequentially every 30 s after stimulation with 20 μM dopamine using single line excitation (488 nm). Shown are representative confocal microscopic images of D1 receptor-GFP fluorescence before dopamine stimulation (control) and after 720 s (12 min) of dopamine treatment. This experiment was performed three times with similar results.

Fig. 12. Effect of a dominant-negative mutant of β-arrestin on dopamine-induced D1-GFP internalization in the absence or presence of GRK4α. HEK293T cells were cotransfected with wild-type D1 receptor-GFP and a dominant-negative mutant of β-arrestin1 (Krupnick et al., 1997) and either empty vector or GRK4α. Twenty-four hours after transfection, cells were plated on 35 mm glass-bottom culture dishes, and confocal microscopy was performed on a Zeiss laser-scanning confocal microscope (LSM-510). Images were collected sequentially every 30 s after stimulation with 20 μM dopamine using single line excitation (488 nm). Shown are representative confocal microscopic images of D1 receptor-GFP fluorescence before dopamine stimulation (control) and after 720 s (12 min) of dopamine treatment. This experiment was performed three times with similar results.
sites. It is noteworthy that close inspection of the receptor sequence immediately surrounding these residues reveals the existence of several acidic amino acids that are typically found near residues that are phosphorylated by GRKs (Palczewski et al., 1989; Onorato et al., 1991; Fredericks et al., 1996).

Constitutive phosphorylation of the D1 receptor by GRK4α was found to be associated with desensitization of receptor-stimulated cAMP accumulation and internalization of the receptor in the absence of agonist stimulation. Simultaneous mutation of Thr428 and Ser431 abrogated the constitutive desensitization induced by GRK4α coexpression, supporting the notion that these residues comprise the functionally relevant GRK4α phosphorylation sites. These results suggest that phosphorylation by GRK4α may promote β-arrestin/D1 receptor association even in the absence of agonist activation. The fact that coexpression with a dominant-negative mutant of β-arrestin blocked the effects of GRK4α on D1 receptor internalization lends support to this hypothesis. It is conceivable that phosphorylation by GRK4α may also reduce D1 receptor/G protein coupling through conformational alterations of the carboxyl terminus, although this remains to be tested. It is interesting that constitutive phosphorylation by GRK4α was found to reduce the total cellular expression levels of the D1 receptor—an effect that was absent in the Thr428/Ser431 mutant. This observation is probably related to increased and prolonged internalization of the D1 receptor in the presence of GRK4α leading to enhanced receptor down-regulation. Lamey et al. (2002) found that mutation of Ser431 in the D1 receptor reduced agonist-induced down-regulation, supporting the notion that this residue is important for receptor trafficking.

Although constitutive phosphorylation of carboxyl terminal residues Thr428 and Ser431 by GRK4α promotes receptor desensitization and internalization, these are clearly not the sole GRK phosphorylation sites within the D1 receptor. It is noteworthy that agonist pretreatment in GRK4α transfected cells results in further desensitization of the cAMP response, an effect that is associated with a marginal enhancement of receptor phosphorylation. Furthermore, D1 receptor mutants that do not exhibit GRK4α-mediated phosphorylation exhibit a robust phosphorylation response when treated with agonists. Finally, our previous data suggested the presence of functionally relevant GRK phosphorylation sites within the 3rd cytoplasmic loop of the receptor (Kim et al., 2004). Taken together, these observations suggest that the residues constitutively phosphorylated by GRK4α may constitute a subset of the total number of GRK phosphorylation sites within the D1 receptor. Because we have shown that both GRK2 and GRK3 can phosphorylate the agonist-activated D1 receptor, and because both of these GRKs are endogenously expressed in HEK293 cells, it would seem that GRK2 and/or GRK3 exhibit broader specificity for phosphorylating D1 receptor residues than GRK4α. Further mapping of the functionally relevant GRK2/3 phosphorylation sites within the D1 receptor is currently in progress.

Our current results are highly relevant to recent research demonstrating that the GRK4 gene is linked to human essential hypertension (Felder et al., 2002; Zeng et al., 2004). Abnormalities in renal dopaminergic systems have long been suggested as precipitating mechanisms for hypertension (Zeng et al., 2004). For example, it has been shown that the D1 receptor in the kidney, which normally acts to regulate Na+ transport, is functionally diminished. This loss of activity does not seem to be due to any alteration in receptor sequence and is specific for D1 receptors that are expressed in renal tissue. Felder et al. (2002) have shown that GRK4 is expressed in the kidney and can promote D1 receptor desensitization. Using isolated renal proximal tubule cells, these investigators showed that both GRK4 and D1 receptors were coexpressed and that D1 receptor stimulation of cAMP accumulation was diminished in renal proximal tubule cells from hypertensive patients compared with those from normotensive controls subjects. These results suggest that GRK4 may tonically dampen D1 receptor signaling in renal tissue and that this effect may be increased in hypertensive subjects.

Indeed, the GRK4 gene locus, 4p16.3, is linked to hypertension, and it has been suggested that activating variants of GRK4 diminish D1 receptor signaling in the kidney leading to increased blood pressure (Zeng et al., 2004). Three SNPs leading to altered GRK4 sequences (R65L, A142V, and A486V) have been investigated within the context of the γ isoform and have indeed been found to promote enhanced D1 receptor desensitization (Felder et al., 2002). Although our current results strongly support a role for GRK4-mediated constitutive desensitization of the D1 receptor in hypertension, we observed no effects on D1 receptor phosphorylation by coexpressing the GRK4γ SNPs R65L, A142V, and A486V (Fig. 3). One possible explanation for these results is the observed specificity of the GRK4α isoform for constitutively phosphorylating the D1 receptor or possibly cell-type specificity for GRK4 isoform/D1 receptor interactions. Therefore, it will be important to reinvestigate the identified SNPs within the context of the GRK4α isoform for their ability to enhance D1 receptor phosphorylation.

Perhaps the most important observation of our present study is the demonstration of GRK-mediated phosphorylation of a GPCR in an agonist-independent fashion. Until now, GRK-mediated phosphorylation ofGPCRs has been suggested to be a strictly agonist-dependent process that links the process of receptor activation to desensitization (Ferguson et al., 1996; Penela et al., 2003; Gainetdinov et al., 2004). The only notable exceptions to this rule have been GPCRs harboring mutations that render them constitutively active (Pei et al., 1994); however, the wild-type D1 receptor does not exhibit constitutive activity (Charpentier et al., 1996). Our data thus expand the functionality of GRKs from kinases that phosphorylate only agonist-bound, activated receptors to kinases that are capable, under certain conditions, of phosphorylating additional receptor conformations (unoccupied, inactive receptors), resulting in profound modulation of the signaling potential for GPCRs. In the present example, tissue-specific (e.g., kidney) expression of GRK4 has the potential to dramatically alter the phosphorylation state, functionality and location of the unoccupied, inactive D1 receptor. These observations may provide a rationale for the presence of multiple GRK isoforms whereby the signaling potential for a given GPCR can be modulated, both in the presence and absence of agonist, by the particular repertoire of kinases coexpressed within a specific cellular environment.

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References


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