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The D_1 Dopamine Receptor is Constitutively Phosphorylated by G Protein-Coupled Receptor Kinase 4

Evidence for a Novel Mechanism of Regulation

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Running Title: **GRK4-Mediated D_1 Receptor Phosphorylation**

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Abbreviations: GRK, G-protein-coupled receptor kinase; GPCR, G protein-coupled receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; AC, adenylyl cyclase; cAMP, adenosine 3',5'-cyclic monophosphate; βARK, β-adrenergic receptor kinase; DAR, dopamine receptor; SNP, single-nucleotide polymorphism; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethyleneeiaminetetraacetic acid; DMEM, Dulbecco's modified essential medium; FCS, fetal calf serum; HBS, HEPES buffered saline; EBSS, Earle's Balanced Salt Solution; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline containing 0.05% Tween 20.

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ABSTRACT

G protein-coupled receptor (GPCR) kinases (GRKs) phosphorylate agonist-activated GPCRs, initiating their homologous desensitization. Here, we present data showing that GRK4 constitutively phosphorylates the D_1 receptor in the absence of agonist activation. This constitutive phosphorylation is mediated exclusively by the α isoform of GRK4, as 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 T428 and S431. Phosphorylation of the D_1 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 D_1 receptor mutant containing T428V and S431A. The increase in internalized D_1 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 D_1 receptor by GRK4α does not alter agonist-induced desensitization of the receptor since dopamine pretreatment produced a similar decrease in cAMP accumulation in control cells versus cells expressing GRK4α. These observations shift the attenuation of D_1 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.

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INTRODUCTION

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 (Missale et al., 1998; Sibley and Monsma, 1992). The D_1-like group consists of the D_1 and D_5 receptors that couple to $G\alpha_s/G\alpha_{olf}$ to activate adenylyl cyclase (AC). The D_2-like group consists of the D_2, D_3, and D_4 receptors that couple to $G_{\alpha i}/G_{\alpha o}$ to inhibit AC and modulate voltage-gated K^+ and Ca^{2+} channels.

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 (PKA or PKC). Homologous desensitization of GPCRs involves only activated receptors and is primarily mediated by GRKs. GRKs are serine/threonine-directed protein kinases comprised of seven isoforms divided into three families (Penela et al., 2003). GRK1 and GRK7 comprise the rhodopsin kinase/visual family, are expressed exclusively in retina, and participate in desensitization of opsins in rods and cones (Hisatomi et al., 1998; Somers and Klein, 1984; Weiss et al., 1998). GRK2 (βARK1) and GRK3 (βARK2) were originally identified as regulating the β-adrenergic receptor and comprise the βARK family. The GRK4 family consists of GRK4, GRK5, and GRK6. There are four RNA splice variants identified for GRK4: α, β, γ, and δ (Premont et al., 1996). GRK4α represents the largest member while 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 initiate arrestin binding. Arrestin association further decreases G protein coupling and promotes

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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; Gainetdinov et al., 2004; Penela et al., 2003).

As with most GPCRs, the D_1 dopamine receptor is known to undergo desensitization and internalization and these events have been studied in variety of systems. A preponderance of evidence indicates that desensitization/internalization of the D_1 receptor is regulated by its phosphorylation state and this, in turn, is mediated by both second messenger-activated kinases as well as by GRKs. Phosphorylation of the D_1 receptor by the cAMP-dependent protein kinase (PKA) appears 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, there is conflicting evidence concerning the nature, number and location of GRK phosphorylation sites within the D_1 receptor. Jackson et al. (2002) have suggested that the D_1 receptor is phosphorylated by GRKs on multiple residues within its carboxyl terminus. Similarly, Lamey et al. (2002) have provided evidence that agonist-induced phosphorylation of the human D_1 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 D_1 receptor takes place exclusively on serine residues. We have recently suggested that agonist-induced, GRK-mediated phosphorylation of the D_1 receptor occurs on residues within both the carboxyl terminus and 3rd cytoplasmic loop and that the phosphorylation of these domains may take place in an ordered fashion (Kim et al., 2004). Conceivably, these different results might be due to a different complement of GRK isoforms being expressed in the various cell systems under study.

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The D_1 dopamine receptor appears to be a substrate for at least GRK2-5 (Felder et al., 2002; Tiberi et al., 1996). In order to investigate whether the D_1 receptor is differentially phosphorylated by these different GRKs, we co-expressed each of the non-visual GRKs with the D_1 receptor and examined its phosphorylation state in the absence or presence of agonist. Surprisingly, we found that GRK4 co-expression 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 D_1 receptor was only observed with the α isoform of GRK4. This agonist-independent phosphorylation of the D_1 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 D_1 receptor contrast with the existing dogma that GRKs only phosphorylate agonist-occupied/activated GPCRs and provide a novel mechanism where GRKs can regulate GPCR function.

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MATERIALS AND METHODS

Materials. HEK293-tsa201 (HEK293T) cells (Heinzel et al., 1988) were a gift from Dr. Vanitha Ramakrishnan. Rat D_1-GFP was a gift from Dr. Qun-Yong Zhou (Bermak et al., 2002). GRK4 isotypes were obtained from Dr. Richard Premont. GRK4γ SNPs were as previously described (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. [^{3}H]SCH-23390 (86.00 Ci/mmol) and [^{32}P]orthophosphate (carrier-free) were obtained from Perkin Elmer Life and Analytical Sciences (Boston, MA). [^{3}H]cAMP (31.4 Ci/mmol) was purchased from Diagnostic Products Corporation (Los Angeles, CA). Dopamine, 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (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). Cell culture media and reagents were purchased from GIBCO (Grand Island, NY). Calcium phosphate transfection kits were obtained from Clontech BD Biosciences (Palo Alto, CA). MiniComplete™ 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). NuPage gels, buffers, and Invitrolon 0.45 μm PVDF membranes were purchased from Invitrogen (San Diego, 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).

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Cell Culture and Transfection. HEK293T cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10 μg/mL gentamycin. Cells were grown at 37°C in 5% CO_2 and 90% humidity. An amino terminal FLAG epitope-tagged construct for the rat D_1 receptor (Monsma et al., 1990) was created from $pSF\beta_2$, an expression construct containing a FLAG-tagged β_2-adrenergic receptor (Guan et al., 1992) to create the wild-type $pSFD_1$ as previously reported (Gardner et al., 2001). This construct, and mutants thereof, were used for all experiments except for the confocal fluorescence microscopy experiments where the D_1-GFP construct was used. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kits. For phosphorylation mutants, serine residues were mutated to alanine residues and threonine residues were mutated to valine residues. All mutant FLAG-D_1 constructs were verified by DNA sequence analysis prior to use. HEK293T cells were transfected using the calcium phosphate precipitation method (Clontech, CA). After 24 hr, the transfected cells were divided and reseeded for subsequent experiments the following day.

Whole Cell Phosphorylation Assay. These assays were performed as previously described (Gardner et al., 2001). Briefly, one day prior to the experiment, transfected HEK293T cells were seeded at 1.5 x 10^6 cells per well of a 6-well poly-D-lysine coated plate and 2 x 10^6 cells of the same transfection were seeded in a 100 mm culture dish and cultured overnight. Cells in the 6-well plates were washed with EBSS and incubated for 1 hr in phosphate-free DMEM. Media was then removed and replaced with 1 mL of fresh phosphate-free DMEM containing 106 μCi of $[^{32}P]H_3PO_4$ and returned to the incubator for 45 minutes. This procedure has previously been shown to result in stoichiometric phosphorylation of the D_1 receptor (Gardner et al., 2001). Cells were then challenged with 10 μM dopamine in the presence of 0.2

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mM sodium metabisulfite for 15 minutes then placed on ice. Cells were washed twice with cold EBSS and solubilized for 1 hour 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 MiniComplete™ protease inhibitor cocktail. The samples were cleared by centrifugation and the protein concentration was determined using the BCA protein assay kit from Pierce (Rockford, IL). The specific activity of D_1 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 TE (Tris-EDTA, pH 7.4) at 4°C. Proteins were eluted from the affinity gel by addition of 35 μL of 2X LDS sample buffer containing reducing agent (Invitrogen) and incubated at 37°C for 1 hr. Proteins were resolved on 4-12% 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 6-well plates as described for the whole cell phosphorylation assays above. Cells were washed twice with cold EBSS and solubilized for 1 hour 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 (Rockford, IL). A concentrated stock of

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4X LDS 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 hr. Proteins were resolved on 4-12% NuPage Bis-Tris gradient gels run in MOPS SDS running buffer (Invitrogen), transferred to Invitrolon 0.45 μm PVDF membranes in NuPage transfer buffer containing methanol according to the Invitrogen manual, 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% BSA/TBST solution for 1 hour 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% BSA/TBST solution for 1 hour at room temperature and washed with TBST. Western blots were developed using the Super Signal 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 $CaCl_2$ and $MgSO_4$ and collected by centrifugation at 300 x g for 10 min. The cells were resuspended in lysis buffer (5 mM Tris, pH 7.4 and 5 mM $MgCl_2$) at 4°C and were disrupted using a dounce homogenizer followed by centrifugation at 34,000 x g 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 [^{3}H]SCH-23390 in a final volume of 1 mL and 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 non-specific binding. The assay tubes were incubated at room temperature for 1.5 hr and the reaction was terminated by rapid filtration through GC/F filters pretreated with 0.6%

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polyethyleneimine. Radioactivity bound to filters was quantitated by liquid scintillation spectroscopy at a counting efficiency of 47-60%.

Determination of cAMP Production. Transfected HEK293T cells were seeded into 24-well poly-D-lysine coated plates (100,000 cells/well) and cultured for one day prior to experimentation. To assess desensitization, cells were pretreated with 10 μM dopamine with 0.2 mM sodium metabisulfite for 1 hr and then washed three times with 200 μL 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 metabisulfite 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 prior to adding 80 μL of 15% $KHCO_3$. The plates remained on ice for an additional 20 min and were centrifuged at 1,300 x g for 10 min. 50 μL of the supernatant 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 [^{3}H]cAMP and incubated overnight at 4°C. 250 μL of 1% charcoal-dextran mix was added to each tube and gently vortexed. Tubes were incubated at 4°C for 10 min then centrifuged at 1,300 x g for 20 min. Radioactivity in the supernatant from each tube was quantified by liquid scintillation spectroscopy at a counting efficiency of 47-60%. cAMP concentrations were calculated using a standard curve according to the protocol of the assay kit.

Confocal Microscopy. 300,000 HEK293T cells were seeded in 100 mm culture dishes. The next day, the cells were transfected with 300 ng of D_1-GFP in pEGFP-N1 vector with or without 4-5 μg of GRK4α and 3 μg of dominant negative β-arrestin (319-418) then cultured for an additional 24 hr prior to reseeding the transfections at 100,000 cells per poly-D-lysine-coated, glass-bottom 35 mm culture dish. Prior to stimulation, the media was replaced with Opti-MEM I reduced serum medium. Confocal microscopy was performed on a Zeiss laser-scanning confocal

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microscope (LSM-510). Images were collected sequentially every 30 seconds 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, K_D and B_{max}, as well as EC_{50} values for dopamine-stimulated cAMP production were calculated using GraphPad Prizm 3.02 curve-fitting program GraphPad Prizm Inc. (San Diego, CA).

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RESULTS

GRK-mediated phosphorylation of the D_1 DAR in HEK293T cells. To study GRK-mediated phosphorylation of the D_1 DAR, we transiently cotransfected HEK293T cells with the FLAG-tagged D_1 DAR and G protein-coupled receptor kinases GRK2, GRK3, and GRK4α. 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 D_1 DAR under basal conditions and in the presence of agonist. The D_1 DAR was phosphorylated under basal conditions and this phosphorylation was increased upon cotransfection with various GRK subtypes (Fig. 1A and 1B). Upon exposure to agonist (10 μM dopamine for 15 min), control cells expressing D_1 DAR alone displayed a four-fold increase in receptor phosphate content, while cells coexpressing either GRK2 or GRK3 displayed a seven to twelve-fold increase in receptor phosphorylation. Surprisingly, cells coexpressing D_1 DAR and GRK4α displayed a four-fold increase in receptor phosphate content in the basal state, as compared to control, and this amount of phosphorylation was only marginally increased upon exposure to agonist. HEK293T cells cotransfected with the D_1 DAR and either GRK5 or GRK6 did not display phosphorylation patterns (basal or agonist-stimulated) that differed significantly from control cells (data not shown). These data show that coexpression of GRK4α with D_1 DAR results in an increase in basal receptor phosphorylation that is comparable to control cells exposed to agonist.

GRK4 subtype-specific phosphorylation of the D_1 DAR. The GRK4 subfamily displays a number of alternative RNA splice variants (Ambrose et al., 1992; Premont et al., 1999; Premont et al., 1996). 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 if

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these shorter GRK4 splice variants could also mediate agonist-independent phosphorylation of the D_1 DAR, HEK293T cells were cotransfected with the D_1 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 D_1 DAR whereas the GRK4β, γ, and δ subtypes had no effect (Fig. 3A). We also examined three single-nucleotide polymorphisms (SNPs) of GRK4γ that have been reported to regulate desensitization of the D_1 DAR (Felder et al., 2002). We performed similar whole cell phosphorylation experiments as shown in Fig. 3A with transfected HEK293T cells expressing D_1 DAR and three GRK4γ SNPs (γR65L, γA142V, and γA486V). None of the GRK4γ SNPs mediated phosphorylation of the D_1 DAR in a manner that differed significantly from control cells (Fig 3A). In order to be sure that the inability of the shorter GRK4 subtypes to induce agonist-independent phosphorylation of the D_1 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 GRK4α, β, γ, or δ all displayed equally robust levels of GRK4 protein expression (Fig. 3B). To determine if increasing GRK4β, γ, δ or GRK4γ SNP expression would result in an increase in basal D_1 DAR phosphorylation, HEK293T cells were cotransfected with double (2X) and four-fold (4X) the amount of DNA used initially in order 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 D_1 DAR (Fig. 3C and 3D; data not shown for SNPs A142V and A486V). These data indicate that only the

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GRK4α subtype mediates constitutive phosphorylation of the D_1 DAR in the absence of agonist stimulation in HEK293T cells.

Identification of the GRK4α phosphorylation sites within the D_1 DAR. Two regions of the D_1 DAR have been implicated in mediating GRK phosphorylation - the third intracellular loop and the long carboxyl terminus (Jackson et al., 2002; Kim et al., 2004; Lamey et al., 2002). To determine the sites of GRK4α-mediated phosphorylation within the D_1 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.

Initially, we examined four carboxyl terminal D_1 DAR truncation mutants that were constructed by inserting a stop codon after the following 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 D_1 DAR truncation construct and GRK4α. Fig. 5A shows that truncation of the D_1 DAR at residue 404 reduced both basal and agonist-stimulated phosphorylation, but more importantly, completely eliminated the constitutive nature of the GRK4α phosphorylation. In this case, the T404 mutant exhibited about a 3-fold increase in phosphorylation in response to dopamine stimulation, as did the WT receptor (Fig. 5A). Further truncation of the D_1 DAR at position 394 did not produce results differing from those with the T404 mutant, whereas truncation at residues 369 and 347 further reduced and eliminated receptor phosphorylation, as we have previously reported (Kim et al., 2004). Mutations were also made in previously identified (Kim et al., 2004) and other potential phosphorylation sites within the 3rd intracellular loop of the D_1 DAR (cf. Fig. 4) to determine if these residues are involved in GRK4α-mediated phosphorylation. In no case did any third

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intracellular loop mutant alter the ability of GRK4α to phosphorylate the D_1 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 D_1 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 mutant receptor where all eight of the serine and threonine residues distal to residue 404 were mutated. This construct, mutT404, retains the region of the D_1 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 as compared to cells expressing the D_1 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 D_1 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 D_1 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 were 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 D_1 DAR, however, some point mutants appeared to be more effective than others. Specifically, point mutants S417A, S421A, and T439V showed little or no reduction in constitutive phosphorylation by GRK4α; however, mutants T428V, S431A, S441A, S445A, and T446V each displayed some degree of reduced

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constitutive phosphorylation (data not shown). To further investigate this, two D_1 DAR cluster mutants were created based on the location of the single point mutants that showed some reduction in GRK4α-mediated constitutive phosphorylation. D_1 DAR mutant "1516" contains the T428V and S431A mutations and D_1 DAR mutant "181920" contains the S441A, S445A, and T446V mutations (cf. Fig. 4). Each D_1 DAR cluster mutant was coexpressed with GRK4α and subjected to whole cell phosphorylation assays in the absence and presence of dopamine (Fig. 6). As previously shown, dopamine-stimulation increases the phosphate content of the WT D_1 DAR by about 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 D_1 DAR (Fig. 6) and the mutant constructs T404 and mutT404 (cf. Fig. 5C). This effect is particularly striking for the 1516 cluster mutant in which Thr-428 and Ser-431 are simultaneously mutated (Fig. 6B). These results suggest that five serine and threonine residues in the distal carboxyl terminus of the D_1 DAR contribute to the constitutive phosphorylation by GRK4α with a primary involvement of Thr-428 and Ser-431.

Effect of GRK4α-mediated phosphorylation of D_1 DAR on cAMP accumulation and desensitization. Having observed the unique nature of GRK4α-mediated phosphorylation of the D_1 DAR, we next studied its effects on dopamine-stimulated cAMP production. Coexpression of the wild-type D_1 DAR with GRK4α resulted in a significant decrease in dopamine-stimulated cAMP accumulation (Fig. 7A). This reduction in D_1 DAR-mediated cAMP accumulation by GRK4α was largely absent in similar experiments using the D_1 DAR truncation mutant T404 and the corresponding mutT404 mutant (Fig. 7B and 7C), neither of which exhibit constitutive GRK4α-mediated receptor phosphorylation. Furthermore, similar cAMP accumulation

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experiments performed with the D_1 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 D_1 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 Thr-428 and Ser-431.

Since it appears that constitutive GRK4α-mediated phosphorylation of the D_1 DAR produces desensitization of the receptor response, we wondered if subsequent agonist treatment might produce even further desensitization. In order 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 hr. Fig. 9A shows that dopamine pretreatment of the cells transfected with the D_1 DAR and empty vector reduces the maximum cAMP response by 30-40%, in agreement with our previous findings (Kim et al., 2004). In cells cotransfected with GRK4α, the maximum cAMP response is already diminished by about 50%, as previously shown in Figs. 7A and 8A, however, dopamine pretreatment produces a further 30-40% loss when compared to untreated cells (Fig. 9A). Thus, the degree of agonist-induced desensitization is similar in the absence or presence of GRK4α (Fig. 9B). Notably, GRK4α coexpression does not completely eliminate agonist-induced D_1 DAR phosphorylation (see above) suggesting that one or more of the functionally relevant sites phosphorylated by endogenous GRKs in these cells are not phosphorylated by GRK4α. Interestingly, 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 D_1 DAR (Fig. 1).

Effect of GRK4α on D_1 DAR expression and subcellular location. We were next interested in investigating if GRK4α-mediated phosphorylation might affect the expression of

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the D_1 DAR. Our first approach was to measure the total complement of cellular receptors using the D_1-selective antagonist [^{3}H]-SCH23390. Saturation binding assays were performed on membranes derived from HEK293T cells expressing the D_1 DAR in the absence or presence of GRK4α and maximum binding capacities (B_{MAX}) were determined. Fig. 10 shows that co-transfection of the wild-type D_1 DAR with GRK4α results in a ~25% reduction in total D_1 receptor expression (B_{MAX}). Interestingly, this effect by GRK4α was not observed with the 1516 mutant D_1 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 Thr-428 and Ser-431 (mutated in the 1516 construct) are the functionally relevant GRK4α phosphorylation sites.

Since the reduction of total receptor expression in response to GRK4α was modest (~25%) compared to the reduction in cAMP response (~50%), we sought to determine if phosphorylation of the D_1 DAR by GRK4α might additionally influence its location within the cell. Experiments were thus conducted using confocal fluorescence microscopy and a D_1-GFP fusion construct that enables the intracellular visualization of the D_1 DAR in real-time (Bermak et al., 2002). Fig 11 (top) shows that under basal conditions, the D_1 DAR is primarily located in the plasma membrane, although some intracellular fluorescence is observed. Acute treatment of the cells with dopamine promotes internalization of the D_1 DAR and an accumulation of intracellular fluorescence. Fig 11 (bottom) shows that co-expression of GRK4α results in a substantial increase in the internalization of the D_1 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 D_1 DAR by GRK4α promotes its sequestration from the cell surface under basal conditions.

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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 which mediate clathrin binding (Krupnick et al., 1997) such that over-expression prevents endogenous arrestin-clathrin association. The D_1 DAR has previously been shown to internalize via a β-arrestin and clathrin-coated pit mediated mechanism (Macey et al., 2005; Vickery and von Zastrow, 1999). Consistent with previous observations, coexpression of D_1-GFP with the dominant-negative mutant of β-arrestin completely inhibited agonist-induced receptor internalization (Fig. 12 top). Importantly, the β-arrestin mutant also disrupted the GRK4α-induced intracellular accumulation of the D_1-GFP receptor under basal and agonist-stimulated conditions (Fig. 12 bottom). This result suggests that GRK4α does not affect the initial transport of the D_1 DAR to the cell surface. Taken together (Figs. 11 and 12), these data indicate that phosphorylation of the D_1 DAR by GRK4α induces the translocation of the receptor from the plasma membrane to intracellular locations in the absence of agonist stimulation.

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DISCUSSION

In this study, we have investigated the role and specificity of non-visual GRK-mediated phosphorylation of the D_1 dopamine receptor. In agreement with previous findings (Tiberi et al., 1996) we found that GRK2 and GRK3 both enhanced agonist-induced phosphorylation of the D_1 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 D_1 receptor phosphorylation. Surprisingly, we found that expression of GRK4 with the D_1 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, which 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 D_1 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/D_1 receptor interactions. 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). Consequently, it will be of interest to determine if calmodulin, or a related interacting protein, is involved in GRK4α–mediated phosphorylation of the D_1 receptor.

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Mutational analysis revealed that constitutive phosphorylation of the D_1 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 (cf. Fig. 4). Mutation of these residues, either singly, as clusters, altogether, 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 sites. Interestingly, close inspection of the receptor sequence immediately surrounding these residues reveals the existence of several acidic amino acids that are typically found in close proximity to residues that are phosphorylated by GRKs (Fredericks et al., 1996; Onorato et al., 1991; Palczewski et al., 1989).

Constitutive phosphorylation of the D_1 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 co-expression supporting the notion that these residues comprise the functionally relevant GRK4α phosphorylation sites. These results suggest that phosphorylation by GRK4α may promote β-arrestin/D_1 receptor association even in the absence of agonist-activation. The fact that co-expression with a dominant-negative mutant of β-arrestin blocked the effects of GRK4α on D_1 receptor internalization lends support to this hypothesis. Conceivably, phosphorylation by GRK4α may also reduce D_1 receptor/G protein coupling through conformational alterations of the carboxyl terminus, although this remains to be tested. Interestingly, constitutive phosphorylation by GRK4α was found to reduce the total cellular expression levels of the D_1 receptor - an effect that

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was absent in the Thr428/Ser431 mutant. This observation is probably related to increased and prolonged internalization of the D_1 receptor in the presence of GRK4α leading to enhanced receptor down-regulation. Previously, Lamey et al. (2002) found that mutation of Ser431 in the D_1 receptor reduced agonist-induced down-regulation supporting the notion that this residue is important for receptor trafficking.

While 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 D_1 receptor. Notably, 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. Further, D_1 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 D_1 receptor. Since we have shown that both GRK2 and GRK3 can phosphorylate the agonist-activated D_1 receptor, and both of these GRKs are endogenously expressed in HEK293 cells, it would appear that GRK2 and/or GRK3 exhibit broader specificity for phosphorylating D_1 receptor residues than GRK4α. Further mapping of the functionally relevant GRK2/3 phosphorylation sites within the D_1 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

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mechanisms for hypertension (Zeng et al., 2004). Specifically, it has been shown that the D_1 receptor in the kidney, which normally acts to regulate Na^+ transport, is functionally diminished. This loss of activity does not appear to be due to any alterations in receptor sequence and is specific for D_1 receptors that are expressed in renal tissue. Felder et al. (2002) have recently shown that GRK4 is expressed in the kidney and can promote D_1 receptor desensitization. Using isolated renal proximal tubule (RPT) cells, these investigators showed that both GRK4 and D_1 receptors were co-expressed and that D_1 receptor stimulation of cAMP accumulation was diminished in RPT cells from hypertensive patients compared to those from normotensive controls. These results suggest that GRK4 may tonically dampen D_1 receptor signaling in renal tissue and that this effect may be increased in hypertensive individuals.

Indeed, the GRK4 gene locus, 4p16.3, is linked to hypertension and it has been suggested that activating variants of GRK4 diminish D_1 receptor signaling in the kidney leading to increased blood pressure (Zeng et al., 2004). Three single nucleotide polymorphisms (SNPs) leading to altered GRK4 sequences (R65L, A142V, and A486V) have been investigated within the context of the γ isoform and indeed found to promote enhanced D_1 receptor desensitization (Felder et al., 2002). While our current results strongly support a role for GRK4-mediated constitutive desensitization of the D_1 receptor in hypertension, we did not observe any effects on D_1 receptor phosphorylation by co-expressing 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 D_1 receptor, or possibly cell-type specificity for GRK4 isoform/D_1 receptor interactions. Consequently, it will be important to reinvestigate the identified SNPs within the context of the GRK4α isoform for their ability to enhance D_1 receptor phosphorylation.

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Perhaps the most important observation of our present study is the demonstration of GRK-mediated phosphorylation of a GPCR in an agonist-independent fashion. Hitherto, GRK-mediated phosphorylation of GPCRs has been suggested to be a strictly agonist-dependent process that links the process of receptor activation to desensitization (Ferguson et al., 1996; Gainetdinov et al., 2004; Penela et al., 2003). 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 D_1 receptor does not exhibit constitutive activity (Charpentier et al., 1996). Our data thus expand the functionality of GRKs from kinases that only phosphorylate agonist-bound, activated receptors to kinases that, under certain conditions, are capable 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 D_1 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 co-expressed within a specific cellular environment.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. **Effect of co-expressing individual GRK isoforms on D_1 receptor phosphorylation.** HEK293T cells were transfected with wildtype (WT) FLAG-tagged D_1 receptor and empty vector (V) or GRK2 (+2), GRK3 (+3) or GRK4α (+4α). Transfected cells were metabolically labeled with [^{32}P]H_3PO_4 for 45 min prior to stimulation with 10 μM dopamine (DA) for 15 min. Samples were then subjected to immunoprecipitation and resolved on 4-12% NuPage BT gels prior to autoradiography as described in "Materials and Methods." Receptors were quantified in each transfection prior to gel electrophoresis to ensure equal amounts of D_1 receptor in each lane. **A**, Autoradiogram of D_1 receptor immunoprecipitates from whole cell phosphorylation assays. 1 pmole of D_1 DAR was loaded in each lane. A representative experiment (performed 3-5) times is shown. **B**, The receptor phosphorylation obtained in **A** was quantified by scanning the autoradiographs followed by analysis with Labworks™ software (UVP Inc., Upland CA). Data are presented as the fold-increase in phosphorylation above the WT basal condition and expressed as mean ± SEM 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 D_1 receptor co-expressed with individual GRK4 isoforms and SNPs.** HEK293T cells were transfected with wildtype (WT) FLAG-tagged D_1 receptor and either empty vector (V) or various GRK4 constructs. Transfected cells were metabolically

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labeled with [^{32}P]H_3PO_4 for 45 min prior to stimulation with 10 μM dopamine (DA) for 15 min. **A**, Autoradiograms of D_1 receptor immunoprecipitates from whole cell phosphorylation assays. HEK293T cells were transfected with wildtype (WT) D_1 receptor and empty vector (V) or human GRK4 splice variants (α, β, δ, and γ) or human GRK4γ single-nucleotide polymorphisms (SNPs γR65L, γA142V, and γA486V). 1 pmole of D_1 DAR was loaded in each lane. A single experiment representative of four is shown. **B**, Western blot of cells transfected as in **A** with D_1 receptor and empty vector (V) or human GRK4 splice variants (α, β, δ, and γ). 45 μg of cell lysate was loaded per lane and visualized as described in "Materials and Methods". The antibody used is directed to an epitope common to all isoforms. **C**, Cells were treated as in **A** except that GRK4β and GRK4δ isoforms were transfected using twice (2X) or four times (4X) as much DNA as empty vector or the GRK4α isoform. 1 pmole of D_1 DAR was loaded in each lane. A single experiment representative of three is shown. **D**, Cells were treated as in **C** except increasing amounts of GRK4γ or the GRK4γ R65L SNP were employed. 1 pmole of D_1 DAR was loaded in each lane. A single experiment representative of three is shown.

Fig. 4. **Diagram of the rat D_1 receptor sequence**. The wildtype receptor sequence is shown along with the various mutant receptor constructs utilized 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 are simultaneoulsy mutated. Individual point mutations in the distal

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carboxyl tail region include S417A, S421A, T428V, S431A, T439V, S441A, S445A, and T446V, while the mutT404 mutant contains all of these point mutations with in a single construct. D_1 DAR cluster mutant 1516 contains T428V and S431A point mutations, and cluster mutant 181920 contains S441A, S445A, and T446V point mutations as indicated.

Fig. 5. **Effect of GRK4α on phosphorylation of wild-type and mutant D_1 receptors.** HEK293T cells were transfected with either wildtype (WT) or mutant D_1 receptors and either empty vector (V) or GRK4α (+4α). Transfected cells were metabolically labeled with [^{32}P]H_3PO_4 for 45 min prior to stimulation with 10 μM dopamine (DA) for 15 min. In each case, (WT) D_1 receptor cotransfected with either empty vector or GRK4α are included as controls. **A**, Autoradiogram of D_1 receptor immunoprecipitates from whole cell phosphorylation assays using the carboxyl terminal truncation mutants (see Fig. 4 for nomenclature). 0.5 pmole of D_1 DAR was loaded in each lane. A single experiment representative of three is shown. **B**, Autoradiogram of D_1 receptor immunoprecipitates from whole cell phosphorylation assays using the third intracellular loop mutants (see Fig. 4 for nomenclature). 1.5 pmoles of D_1 DAR was loaded in each lane. A single experiment representative of three is shown. **C**, Autoradiogram of D_1 receptor immunoprecipitates from whole cell phosphorylation assays using mutT404 and T404. 2 pmoles of D_1 DAR was loaded in each lane. A single experiment representative of three is shown.

Fig. 6. **Effect of GRK4α on phosphorylation of wild-type and D_1 DAR cluster mutants.** HEK293T cells were transfected with either wildtype (WT) or cluster mutant (1516 or 181920) D_1 receptor constructs (see Fig. 4) and either empty vector (V) or GRK4α (+4α). Transfected

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cells were metabolically labeled with [^{32}P]H_3PO_4 for 45 min prior to stimulation with 10 μM dopamine for 15 min. **A**, Autoradiogram of D_1 receptor immunoprecipitates from whole cell phosphorylation assays. 1.5 pmole of D_1 DAR was loaded in each lane. A representative experiment (performed 4 times) is shown. **B**, The receptor phosphorylation (intensity) obtained for each transfection was quantified by scanning the autoradiograms followed by analysis with Labworks™ software (UVP Inc., Upland CA). The fold increase in phosphorylation in response to dopamine stimulation was calculated by dividing the intensity of the phosphorylation in the dopamine-stimulated groups by the phosphorylation seen in non-treated groups (basal). An experimental result showing no increase of phosphorylation over basal would yield a value of 1.0. The data are expressed as mean ± SEM values from four independent experiments.

Fig. 7. **Effect of GRK4α on D_1 receptor-stimulated cAMP accumulation.** HEK293T Cells were cotransfected with wild-type (WT), T404 carboxyl truncation mutant, or mutT404 D_1 receptor constructs (see Figure 4 for nomenclature) with or without GRK4α. Two days post-transfection, the cells were challenged with the indicated doses of dopamine for 15 min and cAMP accumulation assays were performed as described in "Experimental Procedures." The data are expressed as a percentage of the maximum cAMP response produced by the indicated receptor construct coexpressed with empty vector. **A**, wild-type (WT) D_1 receptor was coexpressed with empty vector or GRK4α. A single experiment is shown, representative of four individual experiments. **B**, D_1 receptor truncation T404 was coexpressed with empty vector or GRK4α. A single experiment is shown, representative of four individual experiments. **C**, D_1 receptor mutant mutT404 was coexpressed with empty vector or GRK4α. A single experiment is shown, representative of four individual experiments.

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Fig. 8. **Effect of cluster mutations on GRK4α-mediated regulation of D_1 receptor-stimulated cAMP accumulation.** Cells were cotransfected with wild-type (WT) or cluster mutant (1516 or 181920) D_1 receptor constructs (see Figure 4 for nomenclature) with or without GRK4α as described in figure 7. The data are expressed as a percentage of the maximum cAMP response produced by the indicated receptor construct coexpressed with empty vector. **A**, wild-type (WT) D_1 receptor was coexpressed with empty vector or GRK4α. A single experiment is shown, representative of four individual experiments. **B**, D_1 receptor cluster mutant 1516 was coexpressed with empty vector or GRK4α. A single experiment is shown, representative of four individual experiments. **C**, D_1 receptor cluster mutant 181920 was coexpressed with empty vector or GRK4α. A single experiment is shown, representative of four individual experiments.

Fig. 9. **Agonist-induced desensitization of wild-type D_1 receptor in the presence and absence of GRK4α.** HEK293T cells were transfected with wild-type (WT) D_1 receptor in the absence (empty vector V) or presence of GRK4α. Agonist-desensitization (Des) was assessed by incubating cells with 10 μM dopamine for 1 hour, washing, and rechallenging the cells with the indicated doses of dopamine for 15 min. **A**, cAMP accumulation assay performed on cells transfected as indicated and performed as described in "Materials and Methods." A representative experiment performed five times with similar results is shown. **B**, Percent desensitization for each transfection group was calculated by dividing the maximum cAMP produced by the desensitized cells (dashed lines in **A**) by the maximum cAMP produced by the corresponding control cells (solid lines in **A**), then subtracting this ratio from 1 and converting to

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a percentage. Percentages for each transfection group were calculated for five independent experiments and the average % desensitization ± SEM are reported.

Fig. 10. **Effect of GRK4α on the expression of the D_1 receptor in HEK293T cells.** The cells were transiently transfected with wild-type (WT) D_1 receptor or the cluster mutants 1516 or 181920 (see Fig. 4 for nomenclature) in the absence (empty vector, V) or presence of GRK4α (+4α). After two days, the cells were harvested, membranes prepared, and [^{3}H]SCH-23390 saturation binding assays were performed and B_{MAX} values determined as described under "Materials and Methods." Since transiently transfected cells were used to express each construct, the receptor binding data (B_{MAX} values) are reported as percentage of control due to the variability in exact receptor expression from experiment to experiment. Cells were transfected with each combination of expression constructs indicated for each individual experiment. Control receptor expression and affinities for each construct were not significantly different within an individual experiment. Ranges for all experiments are as follows: B_{MAX} = 6-12 pmol receptor/mg protein, and K_D = 0.075-0.32 nM for [^{3}H]SCH23390. Maximum binding for wild-type (WT) and cluster mutant 181920 constructs coexpressed with GRK4α were found to be significantly reduced as compared to corresponding control cells ("*" = $p < 0.05$, Student's t test, paired).

Fig. 11. **Confocal fluorescence microscopy of D_1 receptor-GFP expression in the presence or absence of GRK4α.** HEK293T cells were transfected with wild-type D_1 receptor-GFP construct and either empty vector or GRK4α. Twenty-four hours post-transfection, cells were plated on 35 mm glass-bottom culture dishes, and confocal microscopy was performed using a

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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 D_1 receptor-GFP fluorescence prior to 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 D_1-GFP internalization in the absence or presence of GRK4α.** HEK293T cells were cotransfected with wild-type D_1 receptor-GFP and a dominant-negative mutant of β-arrestin1 (Krupnick et al., 1997) and either empty vector or GRK4α. Twenty-four hours post-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 D_1 receptor-GFP fluorescence prior to dopamine stimulation (control) and after 720 s (12 min) of dopamine treatment. This experiment was performed three times with similar results.

Figure 1

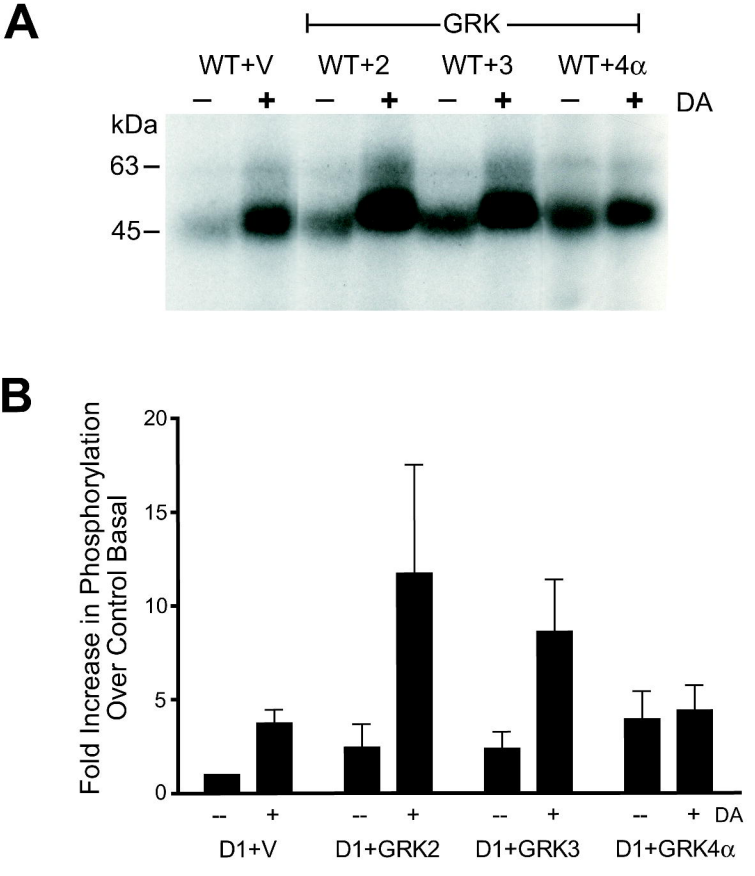

A
GRK
WT+V
WT+2
WT+3
WT+4α
– + – + – + – + DA
kDa
63
45
B
Fold Increase in Phosphorylation Over Control Basal
20
15
10
5
0
– + – + – + – + DA
D1+V
D1+GRK2
D1+GRK3
D1+GRK4α

Figure 2

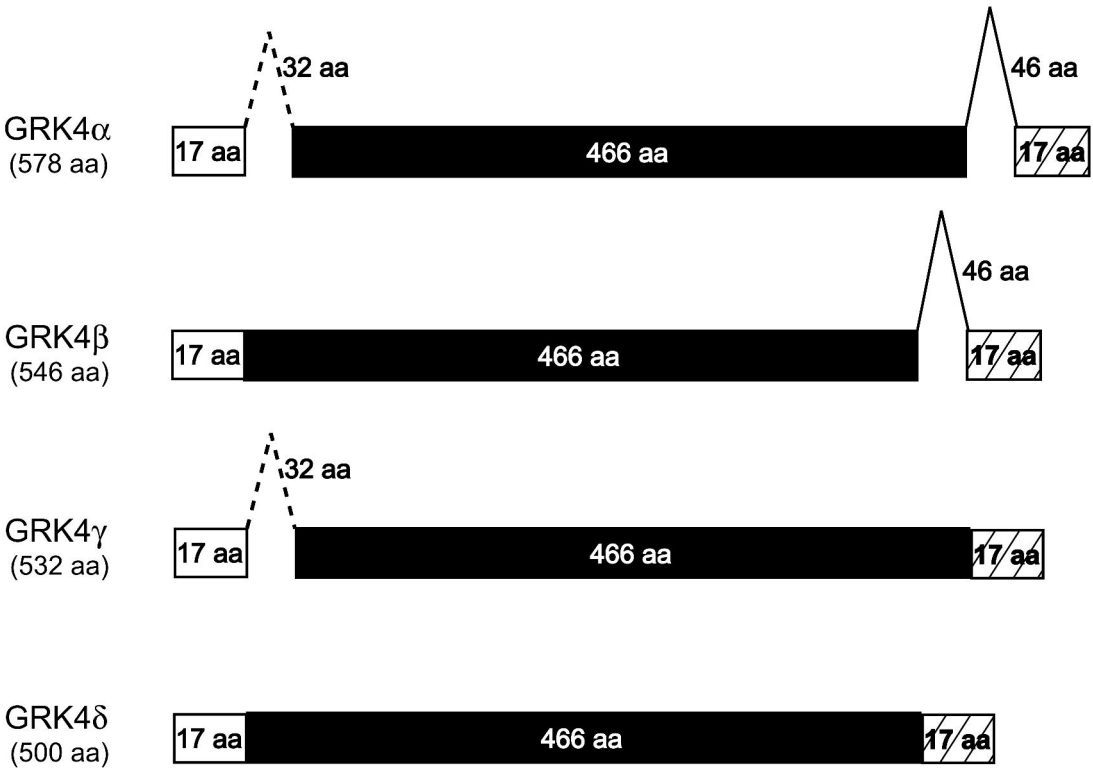

GRK4α
(578 aa)
32 aa
46 aa
17 aa
466 aa
17 aa
GRK4β
(546 aa)
46 aa
17 aa
466 aa
17 aa
GRK4γ
(532 aa)
32 aa
17 aa
466 aa
17 aa
GRK4δ
(500 aa)
17 aa
466 aa
17 aa

Figure 3

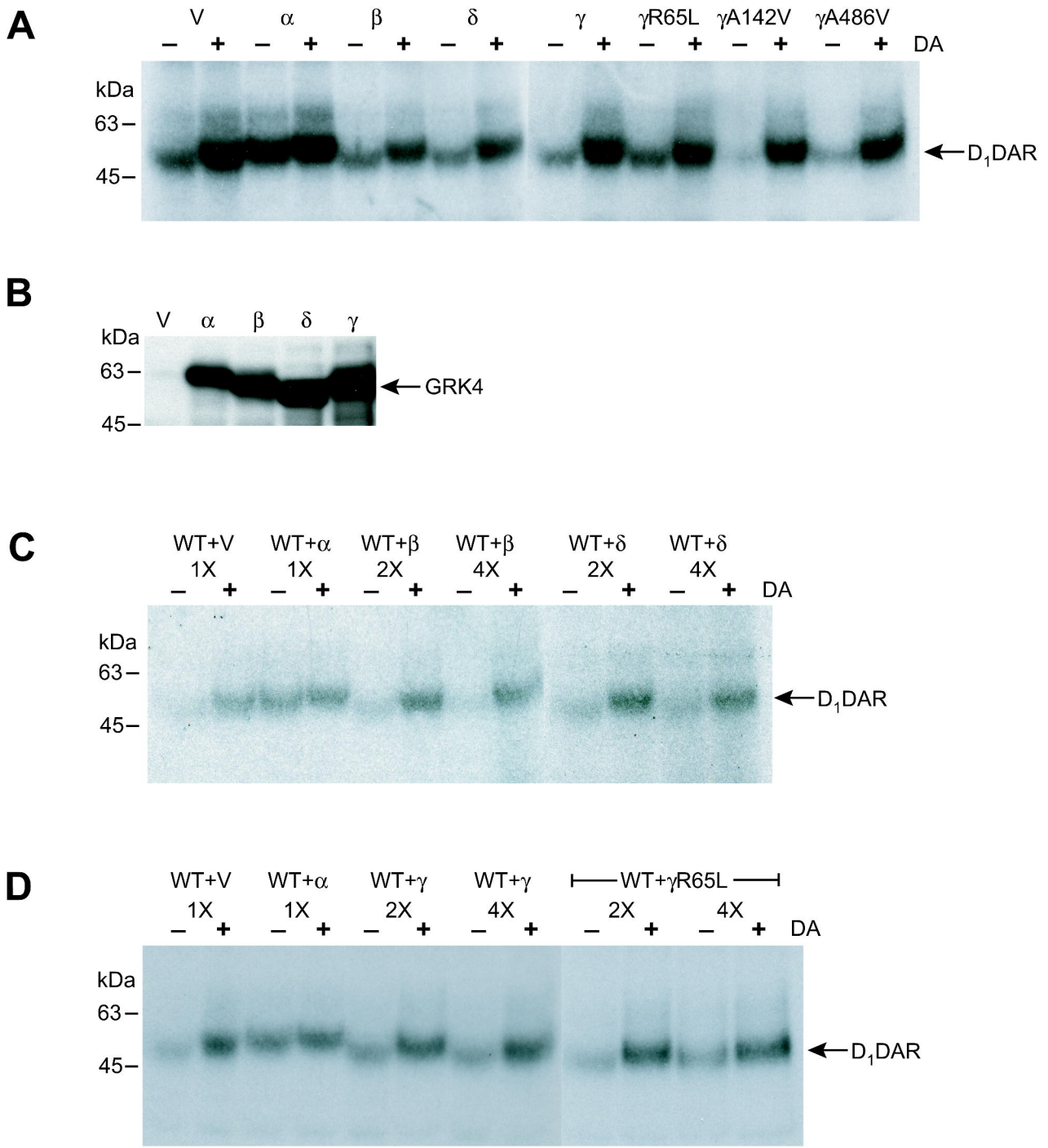

A
V
α
β
δ
γ
γR65L
γA142V
γA486V
– + – + – + – + – + – + – + – + DA
kDa
63
45
D1DAR
B
V α β δ γ
kDa
63
45
GRK4
C
WT+V 1X
WT+α 1X
WT+β 2X
WT+β 4X
WT+δ 2X
WT+δ 4X
– + – + – + – + – + – + DA
kDa
63
45
D1DAR
D
WT+V 1X
WT+α 1X
WT+γ 2X
WT+γ 4X
WT+γR65L
2X
4X
– + – + – + – + – + – + DA
kDa
63
45
D1DAR

Figure 4

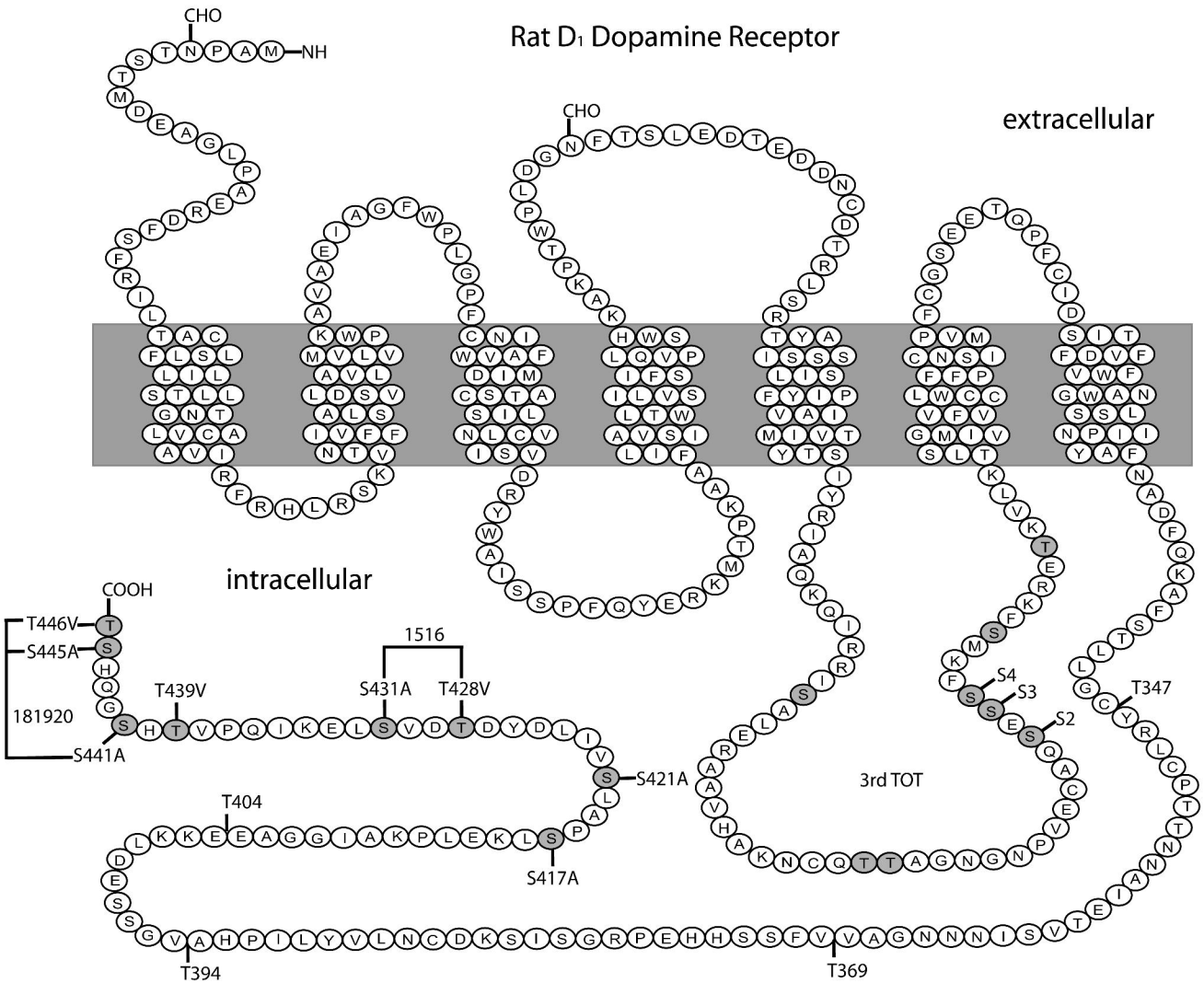

Rat D1 Dopamine Receptor
CHO
NH
extracellular
intracellular
COOH
T446V
S445A
181920
S441A
T439V
1516
S431A
T428V
S421A
3rd TOT
S4
S3
S2
T347
T404
S417A
T394
T369

Figure 5

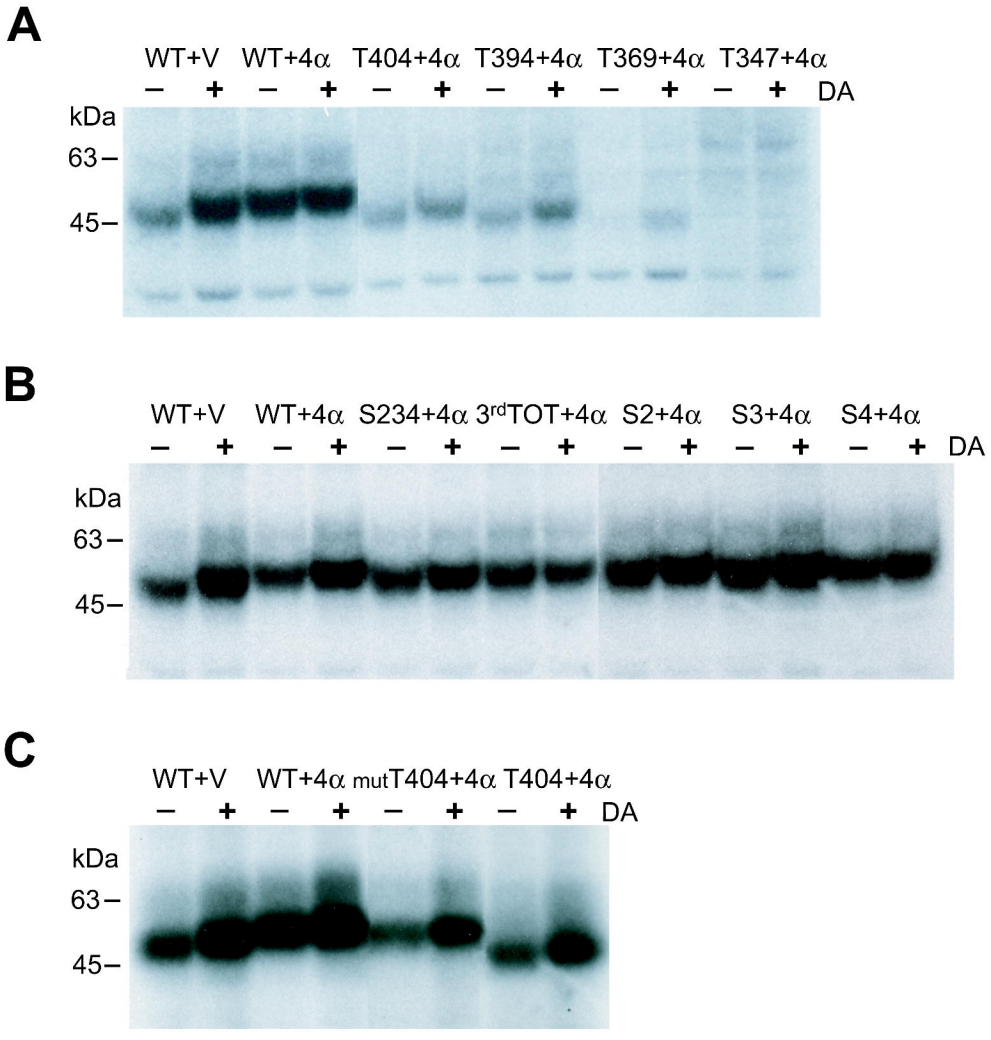

A
WT+V
WT+4α
T404+4α
T394+4α
T369+4α
T347+4α
– + – + – + – + – + – + DA
kDa
63–
45–
B
WT+V
WT+4α
S234+4α
3rdTOT+4α
S2+4α
S3+4α
S4+4α
– + – + – + – + – + – + – + DA
kDa
63–
45–
C
WT+V
WT+4α
mutT404+4α
T404+4α
– + – + – + – + DA
kDa
63–
45–

Figure 6

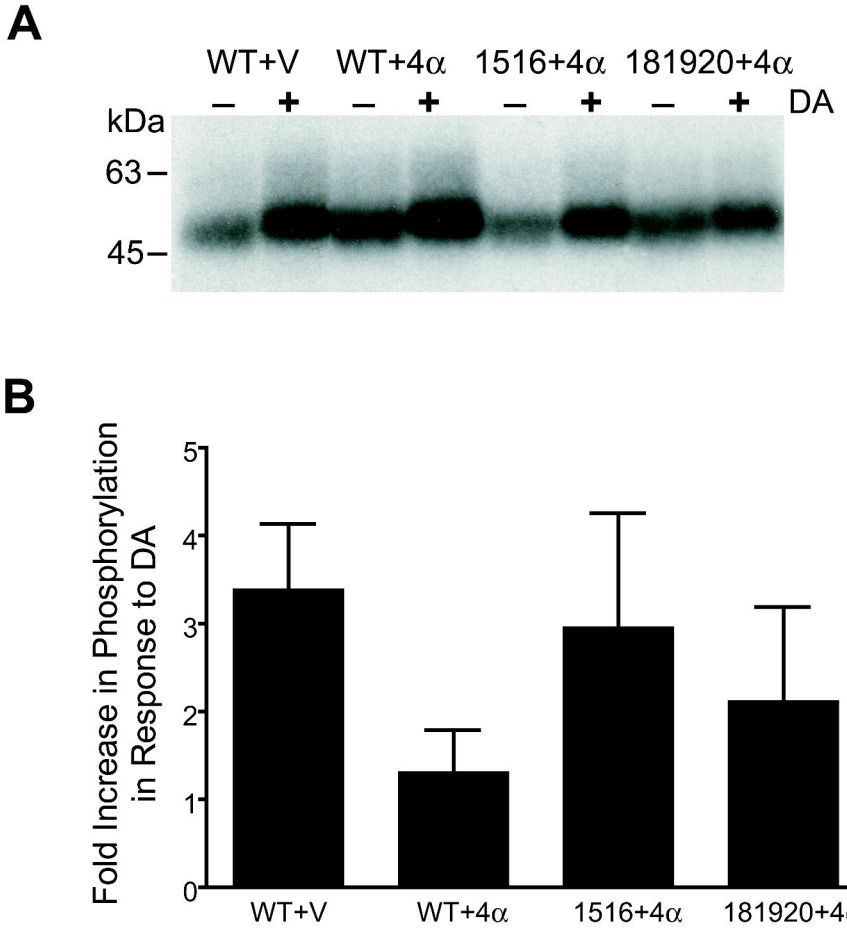

A
WT+V
WT+4α
1516+4α
181920+4α
– + – + – + – + DA
kDa
63
45
B
Fold Increase in Phosphorylation in Response to DA
0
1
2
3
4
5
WT+V
WT+4α
1516+4α
181920+4α

Figure 7

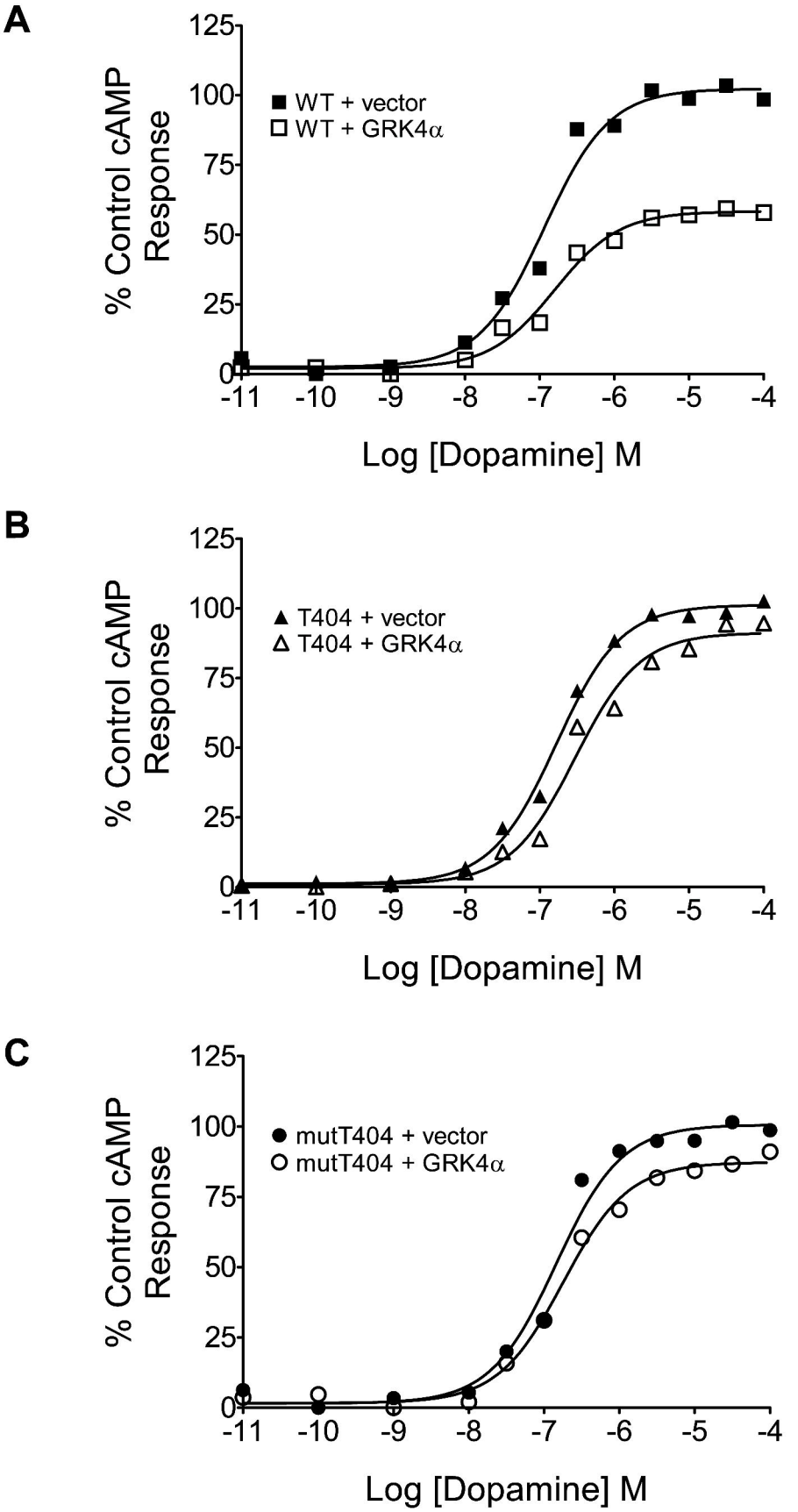

A
125
100
75
50
25
0
% Control cAMP Response
WT + vector
WT + GRK4α
-11
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M
B
125
100
75
50
25
0
% Control cAMP Response
T404 + vector
T404 + GRK4α
-11
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M
C
125
100
75
50
25
0
% Control cAMP Response
mutT404 + vector
mutT404 + GRK4α
-11
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M

Figure 8

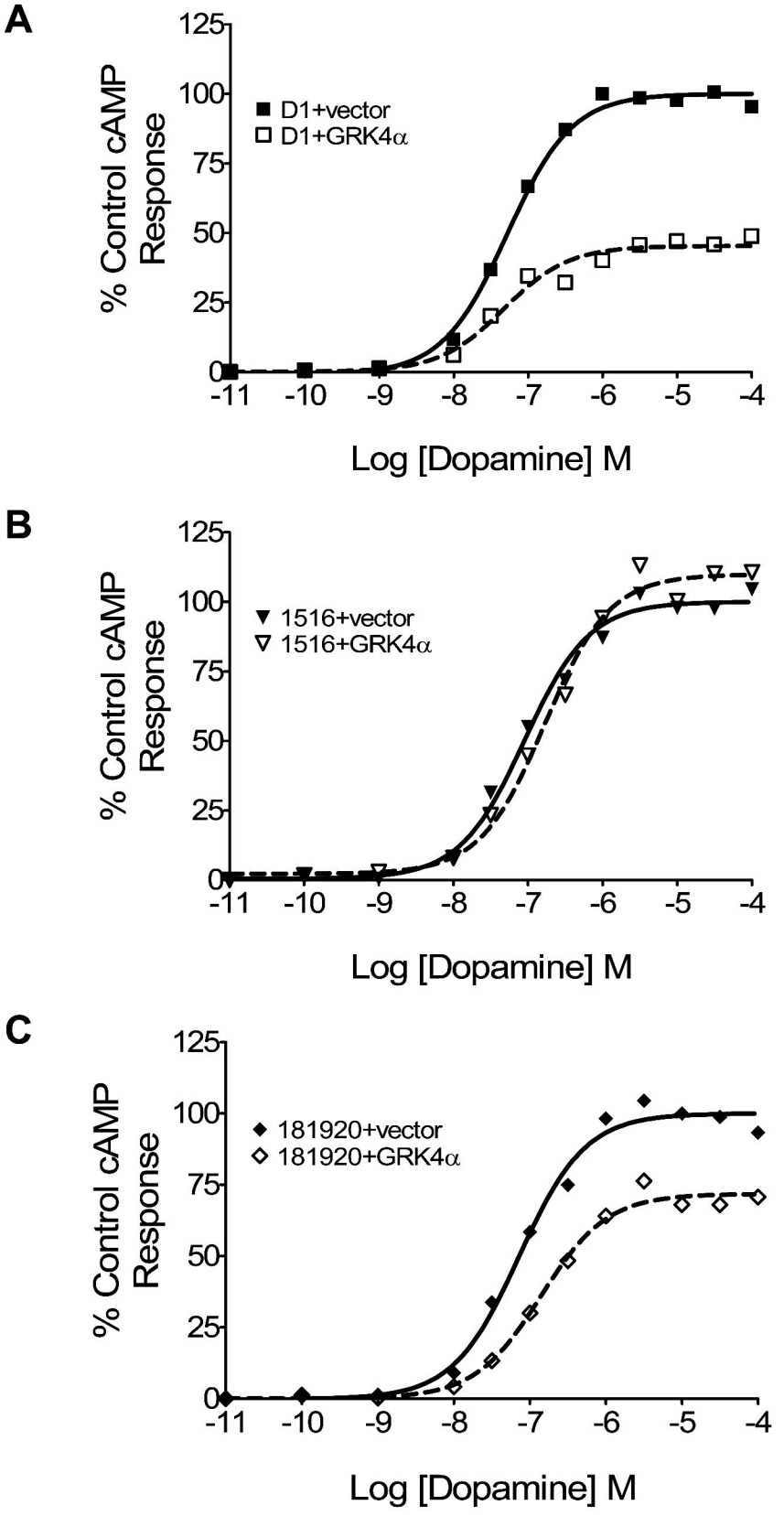
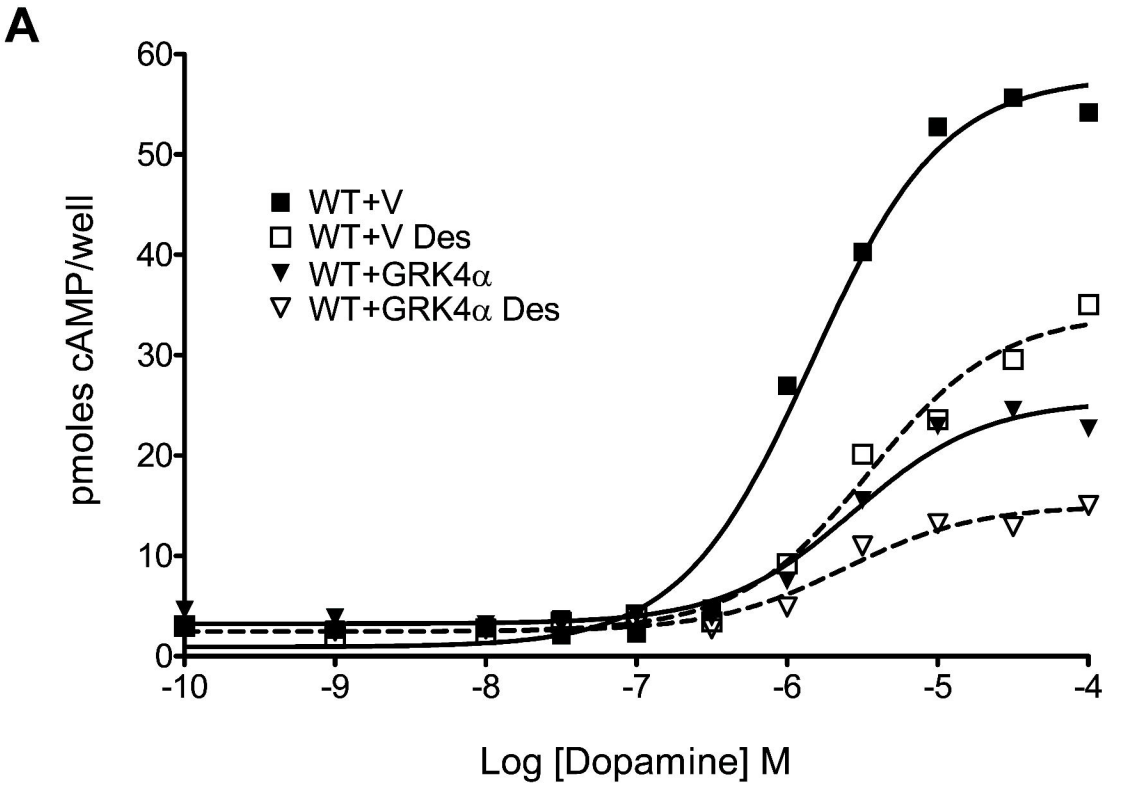

A
125
100
75
50
25
0
% Control cAMP Response
D1+vector
D1+GRK4α
-11
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M
B
125
100
75
50
25
0
% Control cAMP Response
1516+vector
1516+GRK4α
-11
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M
C
125
100
75
50
25
0
% Control cAMP Response
181920+vector
181920+GRK4α
-11
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M

Figure 9


A
60
50
40
30
20
10
0
pmoles cAMP/well
WT+V
WT+V Des
WT+GRK4α
WT+GRK4α Des
-10
-9
-8
-7
-6
-5
-4
Log [Dopamine] M

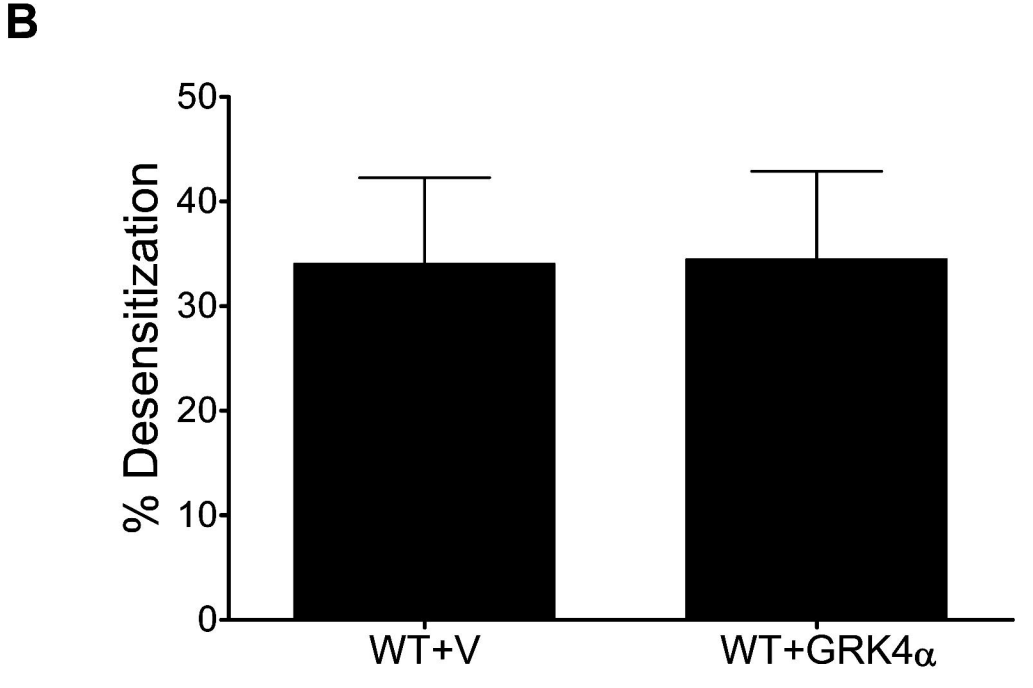

B
50
40
30
20
10
0
% Desensitization
WT+V
WT+GRK4α

Figure 10

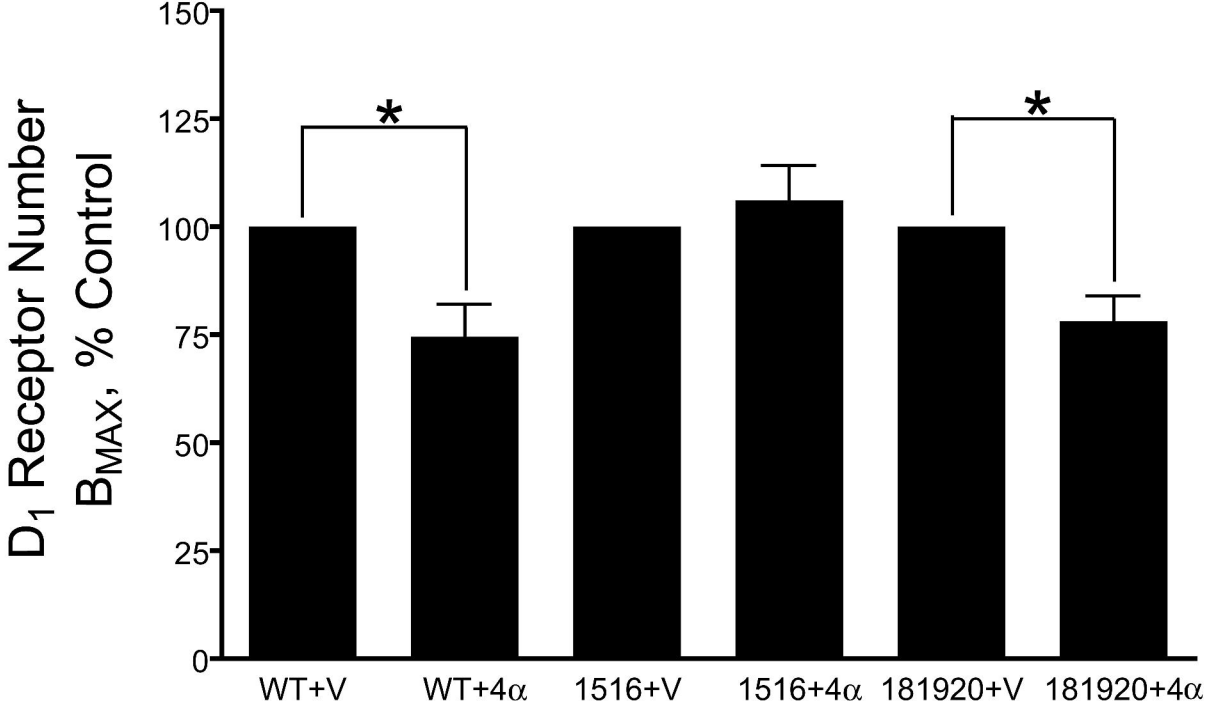

D1 Receptor Number
BMAX, % Control
150
125
100
75
50
25
0
*
*
WT+V
WT+4α
1516+V
1516+4α
181920+V
181920+4α

Figure 11

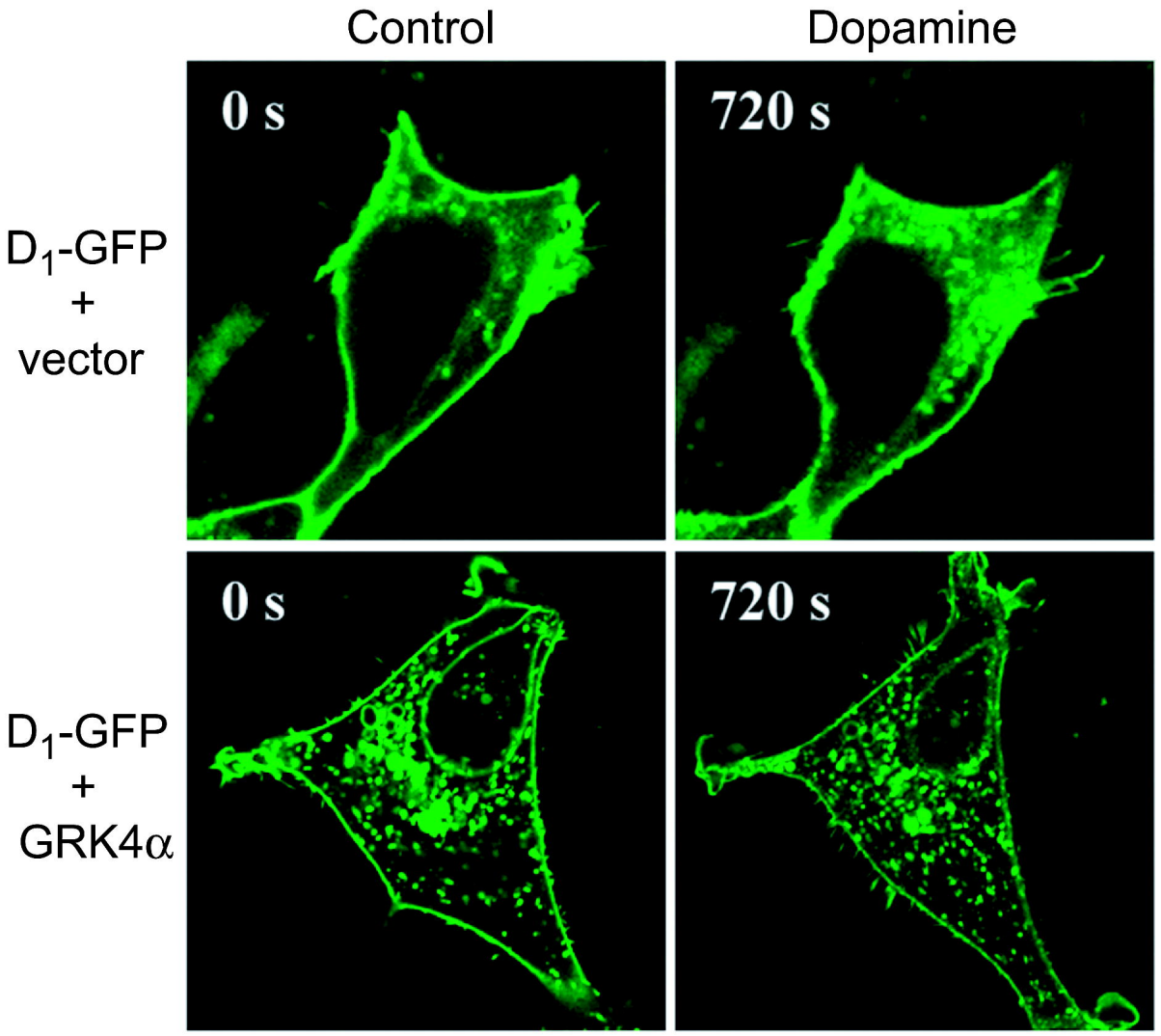
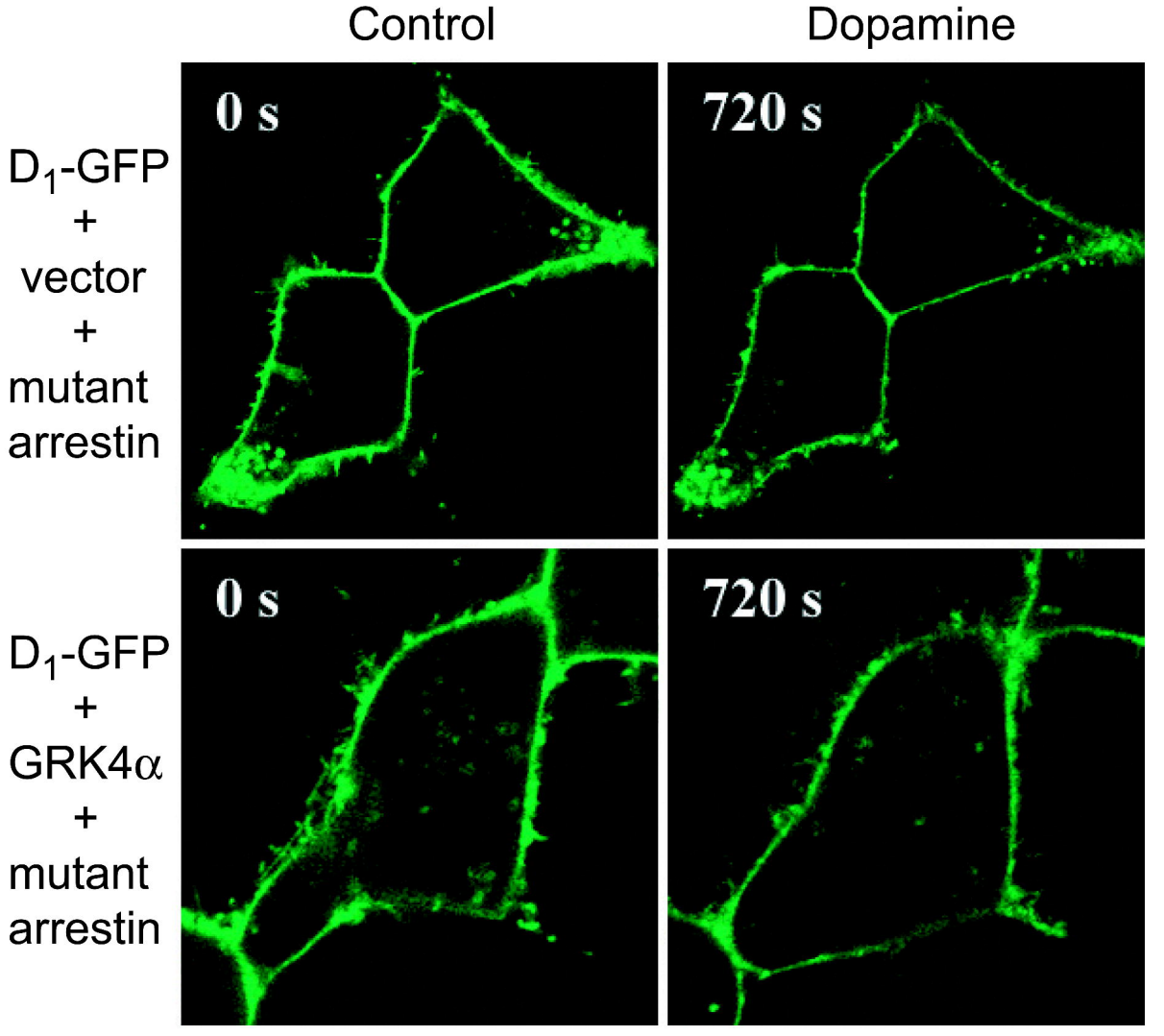


Figure 12


Control
Dopamine
0 s
720 s
D1-GFP + vector + mutant arrestin
0 s
720 s
D1-GFP + GRK4α + mutant arrestin