

Regulation of Dopamine D₁ Receptor Trafficking by Protein Kinase A-Dependent Phosphorylation

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ABSTRACT

The aim of this study was to use pharmacological inhibition of protein kinase A and mutation of potential protein kinase A phosphorylation sites to determine the role of protein kinase A-catalyzed phosphorylation of the dopamine D₁ receptor in agonist-stimulated desensitization and internalization of the receptor. To facilitate purification and imaging of the D₁ receptor, we attached a polyhistidine tag to the amino terminus and enhanced green fluorescent protein to the carboxyl terminus of the receptor (D₁-EGFP). D₁-EGFP was similar to the untagged D₁ receptor in terms of affinity for agonist and antagonist ligands, coupling to G proteins, and stimulation of cyclic AMP accumulation. D₁-EGFP and two mutants in which either Thr268 or Ser380 was replaced with Ala were stably expressed in NS20Y neuroblastoma cells. Pretreatment with the protein kinase A inhibitor H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-

isoquinolinesulfonamide) or substitution of Ala for Thr268 reduced agonist-stimulated phosphorylation of the receptor and resulted in diminished trafficking of the receptor to the perinuclear region of the cell. Substitution of Ala for Thr268 had no effect, however, on agonist-induced receptor sequestration or desensitization of cyclic AMP accumulation. Substitution of Ala for Ser380 had no effect on D₁ receptor phosphorylation, sequestration, desensitization, or trafficking to the perinuclear region. We conclude that protein kinase A-dependent phosphorylation of the D₁ receptor on Thr268 regulates a late step in the sorting of the receptor to the perinuclear region of the cell, but that phosphorylation of Thr268 is not required for receptor sequestration or maximal desensitization of cyclic AMP accumulation.

The dopamine D₁ receptor belongs to the superfamily of heptahelical receptors that modulate the activity of effectors such as adenylate cyclase by activation of specific heterotrimeric GTP-binding proteins (G proteins). For many G protein-coupled receptors (GPCRs), phosphorylation by protein kinases such as protein kinase A (PKA) or G protein-coupled receptor kinases (GRKs) is an early step in agonist-induced desensitization, the diminished responsiveness that occurs after continuous or repeated exposure of the receptors to agonist (Hausdorff et al., 1990). The mechanisms of desensitization and resensitization have been perhaps most thoroughly characterized for the β -adrenergic receptor, a G α_s -coupled receptor that is structurally and functionally homologous to the D₁ receptor. Occupation of the β -adrenergic

receptor by agonist stabilizes a conformation of the receptor that increases its phosphorylation by GRK, which in turn increases the affinity of the receptor for β -arrestin (Krupnick and Benovic, 1998). The coupling of the receptor to heterotrimeric G proteins is inhibited by the binding of β -arrestin, which is likely to be the immediate cause of rapid desensitization. β -Arrestin also acts as an adaptor protein to facilitate receptor endocytosis (Krupnick and Benovic, 1998). Once internalized the receptor is either resensitized by dephosphorylation and then recycled back to the cell surface (Pippig et al., 1995), or directed to an intracellular compartment for degradation (Tsao et al., 2001).

Although this model closely describes the regulation of some GPCRs, the regulation of other receptors differs significantly in ways that may be both cell- and receptor-dependent (Koenig and Edwardson, 1996). For example, GPCR desensitization and internalization can occur despite greatly reduced or undetectable agonist-induced phosphorylation of the receptor (Malecz et al., 1998; Sadeghi et al., 1998; Oli-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; PKA, cyclic AMP-dependent protein kinase; GRK, G protein-coupled receptor kinase; D₁-EGFP, D₁ receptor with enhanced green fluorescent protein attached to the C terminus and a polyhistidine tag at the amino terminus; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; GFP, green fluorescent protein; SCH 23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine.

vaes-Reyes et al., 2001), and dephosphorylation/resensitization can occur without internalization (Gardner et al., 2001).

Desensitization of the dopamine D₁ receptor has been demonstrated in a variety of tissue preparations, including brain slices and retina, primary neuronal culture, neuroblastoma, astrocytoma, or kidney cell lines expressing an endogenous D₁ receptor, and cell lines expressing a recombinant D₁ receptor (Memo et al., 1982; Barton and Sibley, 1990; Zhou et al., 1991; Ng et al., 1994; Sibley and Neve, 1997; Jiang and Sibley, 1999; Ventura and Sibley, 2000). The responsiveness of the D₁ receptor is probably regulated by PKA- and GRK-catalyzed phosphorylation of the receptor, because dopamine-induced desensitization is temporally associated with or preceded by receptor phosphorylation (Ng et al., 1994; Gardner et al., 2001), enhanced by activation of PKA or overexpression of GRK (Zhou et al., 1991; Tiberi et al., 1996), and reduced by inhibitors of PKA or GRK (Zhou et al., 1991). Furthermore, desensitization of the D₁ receptor is blunted in cells deficient in PKA (Ventura and Sibley, 2000), and mutation of a potential site of PKA phosphorylation of the D₁ receptor, Thr268, reduces the rate of agonist-induced desensitization (Jiang and Sibley, 1999). Ser380 has also been proposed to be a site of phosphorylation by PKA, because a peptide comprised of D₁ receptor amino acid residues 372 to 442 is phosphorylated by PKA on Ser380 (Zamanillo et al., 1995).

In this study, we assessed the role of two potential sites of phosphorylation by PKA, Thr268 and Ser380, in agonist-induced phosphorylation, desensitization, and internalization of the dopamine D₁ receptor. We created a D₁ receptor with enhanced green fluorescent protein (D₁-EGFP) at the carboxyl terminus and a hexa-histidine tag at the amino terminus. Wild-type D₁-EGFP and mutants in which an alanine residue was substituted for Thr268 (T268A) or Ser380 (S380A) were stably expressed in NS20Y neuroblastoma cells. We now report that mutation of Thr268, but not Ser380, prevented dopamine-induced phosphorylation of D₁-EGFP and redistribution of the receptor to the perinuclear region of NS20Y cells, without altering maximal desensitization of cyclic AMP accumulation or sequestration of the receptor away from the extracellular surface of the membrane.

Materials and Methods

Construction of Tagged and Mutant Receptors. We attached a polyhistidine tag to the N terminus of the rhesus macaque D₁ receptor (Machida et al., 1992) by using the polymerase chain reaction (PCR). The entire amplified portion of the gene was sequenced to confirm the absence of random mutations and cloned into pcDNA3. To place the EGFP to the C terminus, we used the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) to introduce a *Bam*HI site at Pro445 of the histidine-tagged D₁ receptor, which resulted in the loss of the terminal Thr residue (position 446), and the substitution of Asn for His444. A *Hind*III-*Bam*HI fragment containing the histidine-tagged D₁ receptor was cloned into pEGFP-N1 (CLONTECH, Palo Alto, CA), creating a histidine-tagged D₁ receptor with EGFP tethered by a six-residue linker at the C terminus to form the construct D₁-EGFP (Fig. 1). Mutants of the D₁-EGFP construct were made in which two potential sites of phosphorylation by PKA, Thr268 and Ser380, were changed to alanine, yielding the mutants T268A and S380A (Fig. 1). Untagged alanine substitution mutants of Thr268 and Ser380 for expression in C₆ glioma cells were constructed separately using the trans-PCR method described previously (Neve et al., 1991), except with *Pfu* thermostable DNA poly-

merase instead of *Taq*. The T268A mutant was cloned into pcDNA1, whereas the S380A mutant was cloned into pcDNA3. The PCR-amplified portion of each mutant was sequenced to confirm the presence of the desired mutation and to identify random errors introduced by PCR. The untagged T268A had two additional mutations that changed Ile34→Val in the first transmembrane domain and Gln436→His close to the C terminus.

Creation and Maintenance of Cell Lines. Both tagged and untagged wild-type and mutant receptors were stably expressed in NS20Y neuroblastoma cells by calcium phosphate coprecipitation, and after selection for G418-resistance, pooled populations of D₁-EGFP-, T268A-, and S380A-expressing cells were isolated using a flow cytometry system (BD FACS Vantage SE; BD Biosciences, San Jose, CA) with excitation at 488 nm. Cells were maintained at 37°C in a humidified atmosphere with 10% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical, St. Louis, MO) supplemented with 5% fetal bovine serum and 5% calf bovine serum, with 600 μg/ml G418 sulfate (Calbiochem, San Diego, CA). Clonal C₆ cell lines expressing untagged wild-type and mutant D₁ receptors were created by cotransfection with pBabe-Puro (Morgenstern and Land, 1990) and selection with puromycin (wild-type D₁ and T268A, 2 μg/ml) or by transfection (in pcDNA3) and selection with G418 (S380A, 600 μg/ml), exactly as described previously (Cox et al., 1995). C₆ cells expressing wild-type and mutant D₁ receptors were maintained in DMEM supplemented with 2% fetal bovine serum, 3% iron-supplemented calf bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, and either puromycin or G418.

[³H]SCH 23390 Saturation Binding. Confluent cells in 10 cm-diameter tissue culture plates were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na⁺-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10 to 15 min, the cells were scraped off the plate and centrifuged at 24,000g for 20 min. The crude membrane fraction was resuspended in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 155 mM NaCl) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 10 s. Aliquots of the membrane preparation (~30 μg of protein) were added to duplicate assay tubes containing 50 mM Tris-HCl, pH 7.4, 155 mM NaCl, 0.001% bovine serum albumin, and six concentrations of [³H]SCH 23390 (75.5 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) ranging from 0.05 to 2.0 nM in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 2 μM (+)-butaclamol. Incubations were carried out at 30°C for 60 min and terminated by

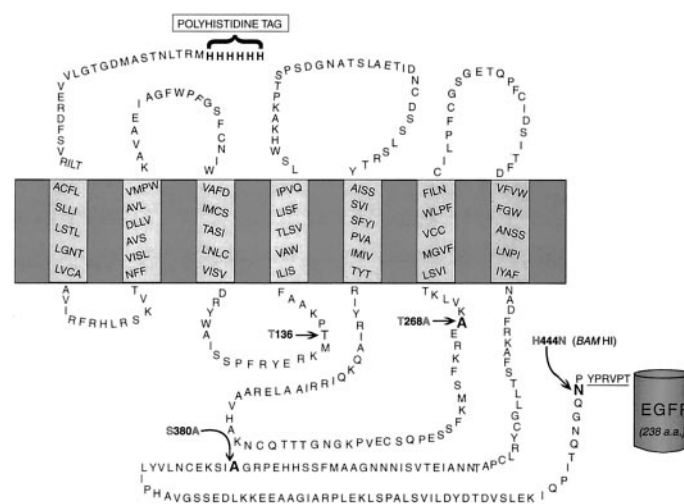


Fig. 1. Schematic diagram of the EGFP-tagged dopamine D₁ receptor. Residues of the receptor that were mutated to Ala to eliminate potential sites of phosphorylation are shown, as well as the mutation His444→Asn, which was the result of introducing a *Bam*HI site for in-frame cloning of the receptor into pEGFP-N1. The six residues that link the D₁ receptor and EGFP are indicated in the figure. A hexa-histidine tag was added to the N terminus of the receptor.

filtration with a 96-well Tomtec Mach II cell harvester (Tomtec, Orange, CT). Filters (BetaPlate filtermat A) were dried and 50 μ l of BetaPlate scintillation cocktail was added to each sample. Radioactivity on the filters was determined using an LKB/PerkinElmer Wallac BetaPlate 1205 scintillation counter (Gaithersburg, MD).

Agonist Binding. The crude membrane fraction was resuspended in competition binding assay buffer (20 mM HEPES, pH 7.5, 6 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol), incubated at 37°C for 15 to 20 min, centrifuged, and resuspended a second time in assay buffer. An aliquot of the membrane preparation was added to duplicate assay tubes containing assay buffer with 0.025% ascorbic acid, 0.001% bovine serum albumin, ~0.8 nM [³H]SCH 23390, and 24 concentrations of dopamine ranging from 1×10^{-12} to 1×10^{-3} M. Incubations were carried out and filtered as detailed above.

Cyclic AMP Accumulation Assay. NS20Y cells stably expressing wild-type or mutant D₁ receptors were plated out in 12-well tissue culture clusters and used to assess desensitization 36 to 48 h later, when they were at a density of ~150,000 cells/well, whereas C₆ cells stably expressing untagged wild-type and mutant D₁ receptors were plated in 48-well clusters at a density of 100,000 to 150,000 cells/well and used 3 to 4 days later. Cells were preincubated for 10 min with 200 μ l of assay buffer (Earle's balanced salt solution containing 0.02% ascorbic acid, 2% bovine calf serum, and 500 μ M 3-isobutyl-1-methylxanthine). The cells were then placed on ice and drugs added to triplicate wells as indicated. For measurement of dopamine-stimulated cyclic AMP accumulation in C₆ cells, 1 μ M propranolol was added to prevent potential stimulation of endogenous β -adrenergic receptors by dopamine. After incubation for 15 min at 37°C, the assay buffer was decanted and cells were lysed with 3% trichloroacetic acid (100 μ l/well). The cyclic AMP in each well was quantified using a competitive binding assay modified as described previously (Watts and Neve, 1996). Ten microliters of cell lysate was incubated in duplicate tubes containing 1.5 nM [³H]cyclic AMP and crude adrenal extract (~100 μ g of protein) in 500 μ l of cyclic AMP binding buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA) for 2 h on ice, and then harvested by filtration as described for radioligand binding assays, except using filtermat B. Unknown samples were plotted against a standard curve (0.1–100 pmol) to calculate the amount of cyclic AMP.

Metabolic Labeling. NS20Y neuroblastoma cells stably expressing the wild-type or mutant D₁-EGFP receptors were plated in six-well tissue culture clusters and used 36 to 48 h later, when they were at a density of ~320,000 cells/well. On the following day, plates were rinsed twice with 1 ml of phosphate-free DMEM, and then incubated for 1 h at 37°C with 200 μ Ci/ml of [³²P]PO₄ in 1 ml of phosphate-free DMEM containing 25 mM Na-HEPES, pH 7.4, and 0.02% ascorbic acid. Cells were then treated with 25 μ M dopamine or vehicle for 5 or 20 min, followed by cell lysis and affinity purification of D₁-EGFP.

Affinity Purification. The medium was aspirated off and replaced with ice-cold lysis buffer [10 mM Tris-HCl, pH 7.4, 10 mM NaF, 10 mM disodium pyrophosphate, and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)]. Cells were then loaded into 50-ml centrifuge tubes and centrifuged at 500 rpm for 5 min at 4°C. The supernatant was decanted, and cells were resuspended in lysis buffer before centrifugation at 40,000g at 4°C for 20 min. The supernatant was removed and the pellet resuspended in 3 ml of ice-cold solubilization buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% *n*-dodecylmaltose, 10 mM NaF, 10 mM disodium pyrophosphate, and 1 tablet of protease inhibitor cocktail/50 ml). The resuspended pellet was sonicated on ice for 20 s, and the contents were transferred to a 15-ml conical centrifuge tube and placed on a tilt shaker at 4°C for 1 h. After solubilization, the solution was centrifuged at 40,000g at 4°C to remove nonsolubilized material. The His-tagged receptor was then purified using nickel-charged Chelating Sepharose Fast Flow beads (Amersham Biosciences AB, Uppsala, Sweden) as described previously (Kobilka, 1995). The eluate (1 ml) from this procedure was collected in a 1.7-ml microfuge tube and

10 μ l of 1 M phosphate buffer, pH 4.5, was added for a final concentration of 10 mM. Next, 20 μ l of Protein G Sepharose 4 Fast Flow (Amersham Biosciences AB) and 1 μ g of monoclonal anti-autofluorescent protein (Quantum Biotechnologies Inc., Montreal, QC, Canada) were added. After incubating for 2 h at 4°C on a tilt rocker, the tube was centrifuged at 2000g and the supernatant removed by aspiration. The pellet was resuspended in 1 ml of radioimmunoprecipitation assay buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate) and centrifuged at 2000g; this step was repeated three times. The washed pellet was resuspended in 30 μ l of Laemmli sample buffer and incubated at 65°C for 20 min to dissociate the D₁-EGFP receptor from the protein G Sepharose. After centrifugation at 2000g for 5 min, the supernatant protein was separated by SDS-PAGE and transferred to a PVDF membrane. The D₁-EGFP receptor was detected by immunoblotting as described below using monoclonal anti-GFP (CLONTECH). Membranes were scanned using a Storm 840 imaging system (Molecular Dynamics, Sunnyvale, CA), and then juxtaposed to Kodak X-OMAT film for 24 to 48 h. The film was developed, and the resulting autoradiograph was digitally captured with a Hewlett Packard Scan Jet LP. The bands showing D₁-EGFP immunoreactivity were quantified by densitometry with ImageQuant software (Molecular Dynamics).

Immunoblotting. Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to PVDF membranes (Costar, Cambridge, MA). The membranes were blocked overnight with 5% nonfat dry milk, washed with Tris-buffered saline, and incubated with the indicated primary antibody for 2 h. The PVDF membranes were washed, incubated with secondary antibody (fluorescein-linked anti-mouse IgG; Amersham plc, Little Chalfont, Buckinghamshire, UK), and immunodetection was accomplished using an ECF Western blotting kit (Amersham plc). In some cases membranes were stripped and reblotted by using a Western blot recycling kit (Chemicon International, Temecula, CA) and a monoclonal antibody recognizing phosphothreonine (Santa Cruz Biotechnology, Santa Cruz, CA).

Fluorescence Microscopy. Sterile coverslips (Fisherbrand #1; Fisher Scientific, Fair Lawn, NJ) were placed in 12-well tissue culture dishes and seeded with cells stably expressing D₁-EGFP. Imaging of live cells was performed the next day by using either a Leica TCS SP scanning confocal microscope or a Nikon TE200 inverted fluorescent microscope with a CH350L camera. Images captured by the Nikon microscope were deconvolved using software by API Delta Vision (Applied Precision Co., Issaquah, WA). Cells were maintained at 37°C using a Delta T stage adapter (Biopetechs Co., Butler, PA) for the Nikon TE200 microscope and an RC 26 open bath imaging chamber on a PH-1 heater platform (Warner Instruments, Hamden, CT) for the Leica TCS SP. Image quantification was done using NIH Image (version 1.62B; <http://rsb.info.nih.gov/nih-image/>).

Biotinylation of Membrane Proteins. Cells grown to 80% confluence on 10-cm tissue culture plates were treated with 25 μ M dopamine or vehicle (0.02% ascorbic acid) in DMEM for 20 min, after which the medium was decanted and the plates were placed on ice. The remaining cell surface proteins were then biotinylated and separated from nonbiotinylated proteins by ImmunoPure immobilized streptavidin (Pierce Chemical, Rockford, IL) as described previously (Melikian and Buckley, 1999). Protein was eluted from the streptavidin beads with 30 μ l of Laemmli sample buffer and constant mixing for 20 min before separation by SDS-PAGE and transfer to PVDF membranes for Western blotting with monoclonal anti-GFP (CLONTECH). D₁-EGFP immunoreactivity was quantified by densitometry using ImageQuant software (Molecular Dynamics).

Data Analysis. Saturation isotherms, radioligand binding inhibition curves, and dose-response curves for cyclic AMP accumulation were analyzed by nonlinear regression with Prism 3.0 (GraphPad Software, San Diego, CA). *K_i*, *K_D*, and EC₅₀ values are geometric means from three or more experiments followed by the limits defined by the asymmetric standard error of the mean. For displacement of

radioligand binding by dopamine, curves were analyzed assuming the presence of one and two classes of binding sites. The assumption of two classes of binding sites was accepted when $p < 0.05$ for the improvement of the fit, determined using an F test. Statistical comparisons between cells expressing mutant and wild-type receptors or between treated and untreated cells were made using the Student's *t* test.

Results

Expression of D₁-EGFP Receptors in NS20Y Cells. We constructed a tagged dopamine D₁ receptor (D₁-EGFP) with a polyhistidine tag attached to the amino terminus and enhanced green fluorescent protein fused to the carboxyl terminus of the receptor (Fig. 1). To eliminate two potential sites of phosphorylation by PKA, alanine substitution mutants of D₁-EGFP were made for Thr268 (T268A) and Ser380 (S380A). The untagged D₁ receptor as well as tagged wild-type and mutant receptors were stably expressed in NS20Y mouse neuroblastoma cells. For wild-type or mutant D₁-EGFP receptors, fluorescence-activated cell sorting-enriched cell populations were selected that expressed each receptor at approximately 2 pmol/mg of membrane protein (Table 1). Because the cell line selected for untagged D₁ receptor expressed that receptor at a lower density (0.75 pmol/mg of protein) a second population of cells expressing D₁-EGFP at a lower density was also used for comparison. Mutant and wild-type receptors had similar affinities for the antagonist radioligand [³H]SCH 23390 ($K_D = 0.3$ – 0.6 nM; Table 1). Similarly, curves for inhibition of the binding of [³H]SCH 23390 by the agonist dopamine were best fit by assuming the presence of two classes of binding sites for each wild-type and mutant receptor, and the proportions of sites with high affinity for dopamine (24–37%) were similar among the receptors, as were the K_H and K_L values for dopamine. Thus, the antagonist and agonist binding properties of the D₁ receptor were not altered by tagging the receptor or by mutation of Thr268 or Ser380.

To investigate further the functional properties of the tagged and mutant receptors, stimulation of cyclic AMP accumulation by dopamine and the agonist 6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF 81297) was assessed. Maximal stimulation of cyclic AMP accumulation was similar in cells expressing wild-type D₁, D₁-EGFP, T268A, or S380A receptors (Table 2). In contrast, the mean EC₅₀ values for the tagged wild-type and mutant receptors were lower than the value for the untagged wild-

type receptor. This was particularly evident for dopamine, which was 3.5- to 7-fold more potent at the EGFP-tagged receptors than at the untagged D₁ receptor. The difference was not due to the higher level of expression of most of the EGFP-tagged receptors compared with the untagged receptor, because the difference was also observed for a cell line expressing D₁-EGFP at a lower density (Table 2). Thus, neither His- and EGFP-tagging nor mutation of Thr268 or Ser380 greatly altered ability of the D₁ receptor to maximally stimulate adenylate cyclase. Tagging the receptor caused a modest leftward shift in the potency of agonists that was similar for wild-type and mutant receptors.

Agonist-Induced Phosphorylation. To quantify agonist-induced incorporation of phosphate into the D₁-EGFP receptor, the receptor was purified by sequential nickel affinity chromatography and immunoprecipitation from NS20Y cells metabolically labeled with ³²P. As shown in the Western blot in Fig. 2A, the sequential purification procedure produced a cleaner receptor preparation than did nickel affinity chromatography alone. The incorporation of ³²P into the D₁-EGFP receptor was increased after treatment with 25 μM dopamine for 5 min ($76 \pm 30\%$) or 20 min ($137 \pm 46\%$) compared with untreated cells (Fig. 2, B and C). Dopamine treatment also increased the incorporation of ³²P into S380A, although neither the effect at 5 min ($p = 0.2$) nor the effect at 20 min ($p = 0.08$) was statistically significant. Collapsing the data for S380A across time resulted in a $73 \pm 31\%$ increase in dopamine-stimulated phosphorylation of the mutant ($p < 0.05$, $n = 24$). In contrast, dopamine treatment caused a slight reduction in the phosphorylation of T268A after 5 min, with no difference from untreated cells after 20 min of dopamine treatment (Fig. 2, B and C). Reprobing a subset of the filters with anti-phosphothreonine demonstrated that 20-min treatment with dopamine increased phosphothreonine immunoreactivity in D₁-EGFP by $41 \pm 15\%$, whereas the same treatment caused a nonsignificant decrease in phosphothreonine immunoreactivity in T268A (Fig. 3).

To address whether dephosphorylation might be occurring at the plasma membrane, we pretreated cells with 0.45 M sucrose to block receptor internalization. Sucrose pretreatment reduced basal receptor phosphorylation and also prevented dopamine-stimulated incorporation of phosphate (Fig. 4).

To determine whether the agonist-induced phosphorylation of the receptor was mediated by PKA, metabolically ³²P-labeled cells were pretreated with the PKA inhibitor

TABLE 1
Binding properties and density of D₁ receptors stably expressed in NS20Y neuroblastoma cells

Cell membranes were prepared, and saturation and competition binding assays were performed as described under *Materials and Methods*. Two populations of cells expressing D₁-EGFP were selected, a high receptor-density population (D₁-EGFP) that was used in subsequent studies of phosphorylation, internalization, and trafficking of the D₁ receptor, and a low receptor-density population [D₁-EGFP (L)] that was used in cyclic AMP accumulation experiments for comparison with the untagged D₁ receptor (Wt-D₁). K_D and B_{max} values for the binding of [³H]SCH 23390 were determined by saturation analysis. Dopamine affinity values were calculated from competition binding curves analyzed in terms of high (K_H) and low (K_L) affinity classes of binding sites. All affinity values are the geometric mean of 12 ([³H]SCH 23390) or three to six (dopamine) independent experiments followed by the limits defined by the asymmetric S.E.M.

	³ H[SCH 23390]		Dopamine		
	K_D	B_{max}	K_H	K_L	K_H
	nM	pmol/mg	nM		%
D ₁ -EGFP	0.34 (0.28–0.42)	2.20 ± 0.30	11 (9–16)	1320 (890–1950)	37 ± 9
T268A	0.29 (0.24–0.36)	1.75 ± 0.25	8 (4–17)	1040 (1000–1100)	34 ± 8
S380A	0.56 (0.53–0.59)	2.36 ± 0.39	3 (2–5)	1470 (1170–1850)	24 ± 10
D ₁ -EGFP (L)	0.33 (0.30–0.35)	0.50 ± 0.20	11 (6–22)	1070 (400–3020)	29 ± 7
Wt-D ₁	0.35 (0.28–0.43)	0.75 ± 0.23	9 (4–21)	1010 (740–1400)	36 ± 8

Characterization of Agonist-Induced Desensitization. Desensitization of the D₁ receptor was induced by

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D₁ receptor agonist stimulated cyclic AMP accumulation

Cyclic AMP accumulation was assessed in NS20Y cells stably expressing the indicated receptor construct. Potency (EC_{50}) and maximal stimulation (V_{max}) were calculated from nonlinear regression analysis of dose response curves for the indicated agonist. The EC_{50} values are geometric means from three to five independent experiments followed by the limits defined by the asymmetric S.E.M. Two populations of cells expressing D₁-EGFP were selected, a high receptor-density population (D₁-EGFP), which was used in subsequent studies of phosphorylation, internalization, and trafficking of the D₁ receptor, and a low receptor-density population [D₁-EGFP (L)], which was used for comparison with agonist-stimulated cyclic AMP accumulation by the untagged D₁ receptor (Wt-D₁).

Receptor	Dopamine		Cl-PB	
	EC ₅₀	V _{max}	EC ₅₀	V _{max}
	<i>nM</i>	<i>pmol cAMP/well</i>	<i>nM</i>	<i>pmol cAMP/well</i>
D ₁ -EGFP	6 (1–26)	90 ± 25	9 (6–14)	60 ± 34
T268A	5 (2–14)	74 ± 20	27 (21–36)	51 ± 23
S380A	4 (3–5)	100 ± 22	13 (10–18)	66 ± 4
D ₁ -EGFP (L)	14 (9–21)	39 ± 12	26 (13–54)	51 ± 11
Wt-D ₁	62 (23–170)	60 ± 30	77 (62–95)	63 ± 8

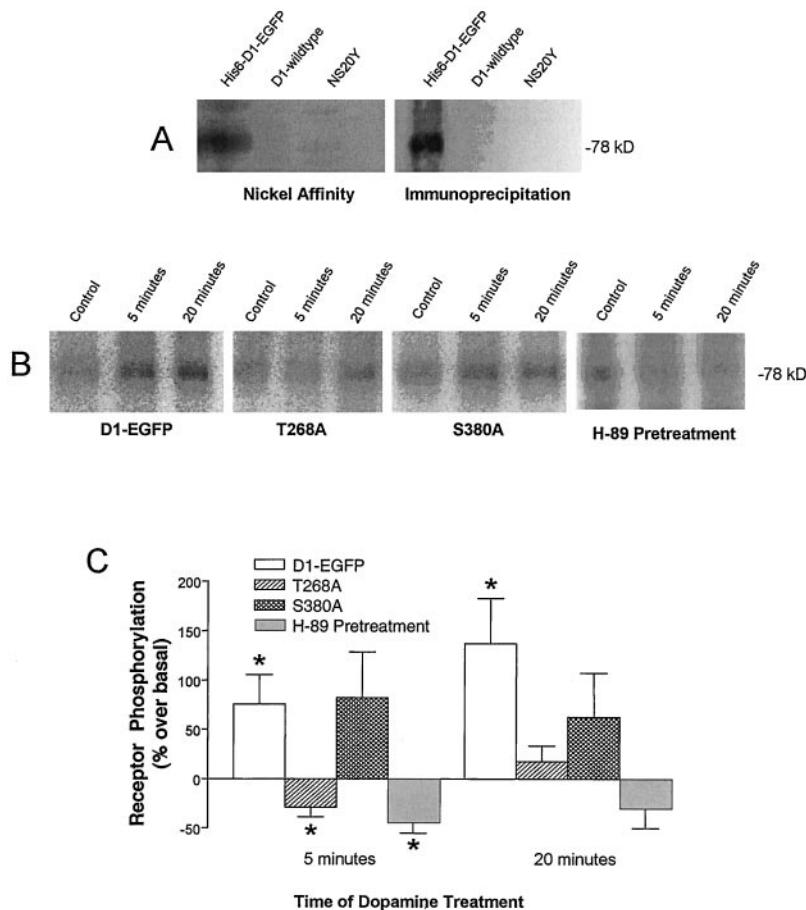


Fig. 2. Agonist-induced phosphorylation of D₁-EGFP and mutant receptors expressed in NS20Y neuroblastoma cells. A, immunoblot of preparations from untransfected NS20Y cells (NS20Y) or cells expressing D₁-EGFP (His6-D1-EGFP) or the untagged D₁ receptor (D1-wildtype). Receptors were purified by either nickel affinity resin alone (Nickel Affinity) or by nickel affinity resin followed by immunoprecipitation (Immunoprecipitation) with an anti-GFP monoclonal antibody (IgG2a). After SDS-PAGE and transfer to PVDF membranes, D₁-EGFP receptor protein was detected using a different anti-GFP monoclonal antibody (IgG1k). B, NS20Y cells were labeled with [³²P]H₃PO₄ then treated with 25 μ M dopamine for 5 or 20 min. In some experiments, cells were treated with the PKA inhibitor H-89 (5 nM, 30 min) before dopamine stimulation. D₁-EGFP, T268A, and S380A receptors were purified by sequential nickel affinity chromatography and immunoprecipitation before transfer of SDS-PAGE separated proteins to PVDF membranes and analysis by autoradiography. C, autoradiographs such as the representative image in B were quantified as described under *Materials and Methods*. Band optical density was determined, and data for dopamine-treated cells were expressed as the percentage increase over the density for vehicle-treated cells in the same experiment. Results are the mean \pm S.E. of 10 to 12 independent experiments. *, $p < 0.05$ using a paired Student's t test.

min (Fig. 5A; Table 3). Thus, tagging the D₁ receptor with His6 and EGFP did not alter acute desensitization of the receptor.

Because the T268A mutant was resistant to dopamine-induced phosphorylation, we determined whether acute desensitization of the mutant was altered. The magnitude of desensitization of T268A was similar to that of D₁-EGFP (Table 3; Fig. 5B), suggesting that PKA-catalyzed phosphorylation of the D₁ receptor on Thr268 is not required for maximal acute desensitization. The responsiveness of S380A was also decreased by ~50% within 5 min of dopamine pretreatment (Table 3; Fig. 5B).

Mutation of Thr268 has been reported to decrease the rate of desensitization of the D₁ receptor expressed in C₆ glioma cells (Jiang and Sibley, 1999), so it is possible that phosphorylation on this residue regulates desensitization in a cell type-dependent manner. In the present studies, C₆ cells expressing untagged D₁, T268A, or S380A receptors were treated for 1, 2, or 4 h with 10 nM, 100 nM, or 1 μ M dopamine before measuring dopamine-stimulated cyclic AMP accumulation. Pretreatment of C₆-D₁ cells with dopamine caused a

time- and concentration-dependent decrease in subsequent maximal stimulation of cyclic AMP accumulation by dopamine, with the greatest decrease of ~60% being evident after treatment with 1 μ M dopamine for 2 to 4 h (Fig. 6A). As observed for the tagged mutant receptors expressed in NS20Y cells, the time and dose dependence of desensitization of the untagged T268A and S380A receptors stably expressed in C₆ cells was similar to that of the D₁ receptor (Fig. 6, B and C).

Intracellular Trafficking. NS20Y cells expressing either D₁-EGFP or T268A were used to evaluate agonist-induced trafficking of the D₁ receptor. Cells were grown on coverslips that were placed in a heated (37°C) chamber on the microscope stage. The addition of 25 μ M dopamine to the medium markedly increased the intensity of fluorescence in the perinuclear region of cells expressing D₁-EGFP (Fig. 7A). The perinuclear and plasma membrane fluorescence was measured at the same coordinates of images captured every 2 min for 20 min, and the ratio of the two values was calculated for each time point. The ratio of perinuclear/membrane fluorescence increased linearly for up to 20 min of dopamine treatment of cells expressing D₁-EGFP, at which time the ratio had more than doubled (Fig. 7C). In contrast, although intracellular fluorescence increased in dopamine-treated cells expressing T268A (Fig. 7B), the accumulation of fluo-

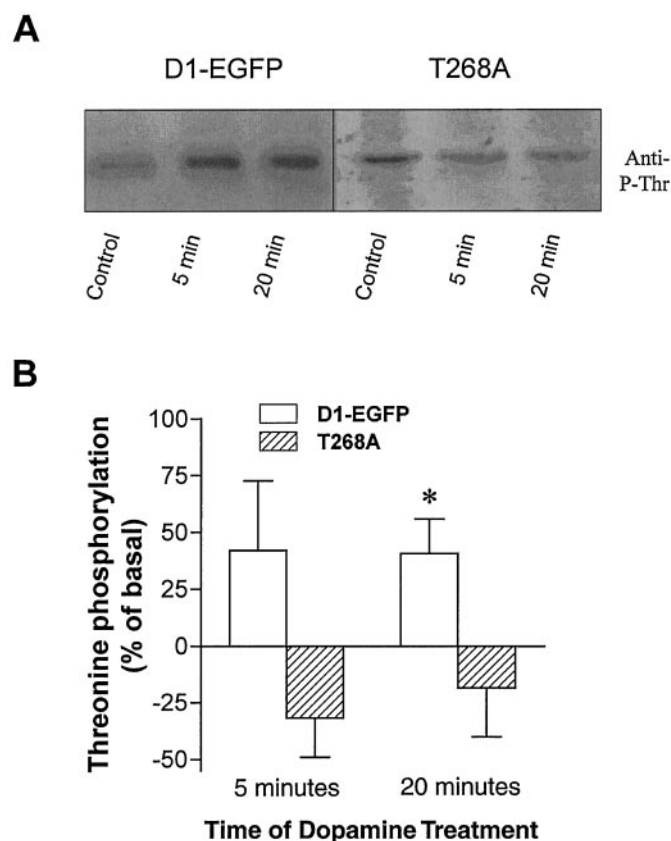


Fig. 3. Agonist-induced regulation of phosphothreonine immunoreactivity in D₁-EGFP and T268A receptors. PVDF membranes used for the analysis of incorporation of ³²P depicted in Fig. 2B were stripped and reprobed with anti-phosphothreonine. A, representative blot of membranes from cells expressing D₁-EGFP or T268A treated with vehicle or with 25 μ M dopamine for 5 or 20 min. B, images such as that depicted in A were quantified as described under *Materials and Methods*. Band optical density was determined, and data for dopamine-treated cells were expressed as the percentage of increase over the density for vehicle-treated cells in the same experiment. Specificity of the anti-phosphothreonine antibody was demonstrated by coincubation with phosphothreonine, which prevented detection of specific bands on the immunoblot (data not shown). Results are the mean \pm S.E. of three independent experiments. *, $p < 0.05$ using a paired Student's t test.

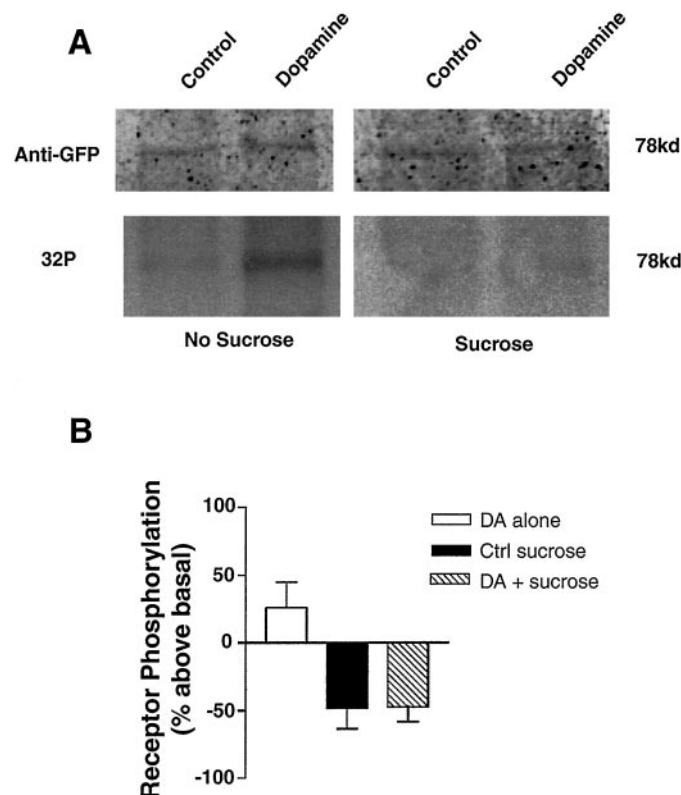


Fig. 4. Effect of sucrose on agonist-induced phosphorylation of D₁-EGFP receptors. NS20Y cells were labeled with [³²P]H₃PO₄ as described under *Materials and Methods*. Cells were pretreated with or without 0.45 M sucrose for 1 h, with 25 μ M dopamine added for the final 5 min. A, representative immunoblot of D₁-EGFP purified as described in the legend to Fig. 2 (top), and the corresponding autoradiograph (bottom). B, optical density of bands in the autoradiographs corresponding to D₁-EGFP immunoreactivity was quantified as described under *Materials and Methods*. Results are the mean \pm S.E. of three independent experiments.

rescence in the perinuclear region was not observed, and the ratio of perinuclear fluorescence to fluorescence in the plasma membrane was only slightly enhanced compared with untreated cells (Fig. 7C).

If the altered trafficking of T268A reflects a lack of phosphorylation by PKA, and then inhibition of PKA should similarly alter the trafficking of wild-type D₁-EGFP. Cells expressing wild-type D₁-EGFP were pretreated with the PKA inhibitor H-89 (5 nM) for 30 min, followed by the addition of

25 μ M dopamine. Inhibition of PKA prevented the dopamine-induced increase in perinuclear fluorescence (Fig. 7C).

Agonist-Induced Receptor Internalization. Biotinylation of cell surface proteins was used to quantify agonist-induced internalization of D₁ receptors. NS20Y neuroblastoma cells expressing either wild-type D₁-EGFP or T268A receptors were incubated with or without 25 μ M dopamine, and then placed on ice for biotinylation of the remaining surface receptors. Membranes were solubilized, and biotinylated proteins isolated by avidin gel matrix purification. The abundance of surface receptors before and after dopamine treatment was quantified by Western blot with an antibody directed against green fluorescent protein (Fig. 8A). Treatment with dopamine for 20 min reduced the abundance of D₁-EGFP receptors on the surface of the membrane by $29 \pm 11\%$ compared with cells not treated with dopamine (Fig. 8B). Similarly, dopamine decreased the abundance of T268A on the surface of the cell membrane by $32 \pm 12\%$, indicating that phosphorylation on Thr268 is not necessary for rapid receptor internalization.

To determine whether PKA regulates rapid internalization of the D₁ receptor, dopamine-induced internalization was assessed after 30-min pretreatment with 5 nM H-89. Under these conditions, dopamine treatment reduced the abundance of D₁-EGFP surface receptors by $27 \pm 9\%$, indicating that phosphorylation by PKA is not a requirement for internalization.

The lack of effect of the T268A mutation on desensitization of cyclic AMP accumulation and on receptor internalization suggested that phosphorylation by PKA regulates a late step in the endocytotic trafficking of the D₁ receptor to the perinuclear region, but that agonist-induced trafficking close to the cell membrane is not regulated by PKA-dependent phosphorylation of Thr268. The agonist-induced increase in T268A-containing endocytic vesicles shown in Fig. 7B suggested a similar conclusion. To evaluate this more closely, we tracked the appearance and disappearance of EGFP-containing vesicles in NS20Y cells expressing D₁-EGFP or T268A. Dopamine treatment of either wild-type (Fig. 9A) or mutant (Fig. 9B) receptors caused the formation of vesicles that remained close to the plasma membrane and seemed to re-merge with the membrane within several minutes of vesicle formation.

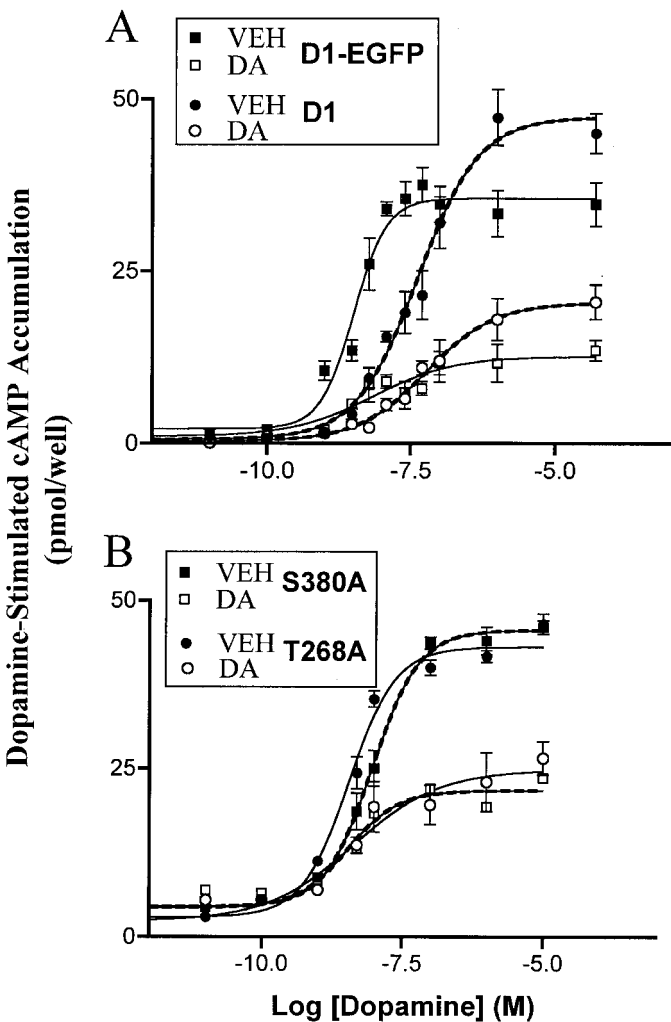


Fig. 5. Desensitization of dopamine-stimulated cyclic AMP accumulation by D₁ receptors stably expressed in NS20Y cells. Cells expressing the untagged D₁ receptor or D₁-EGFP (A), or T268A or S380A (B) were pretreated with dopamine (DA, 25 μ M) or vehicle (VEH) for 5 min, followed by washing and quantification of dopamine-stimulated cyclic AMP accumulation. The curves shown are the mean \pm S.E. of three independent experiments.

TABLE 3

Desensitization of D₁ receptor-stimulated cyclic AMP accumulation in NS20Y cells

NS20Y neuroblastoma cells expressing the indicated receptor were preincubated with vehicle or 25 μ M dopamine for the indicated time. Cells were extensively washed with DMEM on ice, and then dopamine stimulated cyclic AMP production was assessed as described under *Materials and Methods*. Data are expressed as the percentage reduction in maximal cyclic AMP accumulation compared with untreated cells. Each value is the mean \pm S.E. of three to six independent experiments.

Time	Receptor				
	Wild-Type	D ₁ -EGFP(L)	D ₁ -EGFP(H)	T268A	S380A
			%		
5 min	-43 \pm 18	-48 \pm 10	-52 \pm 11	-57 \pm 7	-54 \pm 5
60 min	-54 \pm 4	-43 \pm 3	-50 \pm 10	-63 \pm 9	-60 \pm 6

Discussion

The mechanisms of desensitization and resensitization of the dopamine D₁ receptor are not well understood. Although the high homology of the D₁ receptor with the β_2 -adrenergic receptor might indicate that similar mechanisms regulate the responsiveness of the receptors, even subtypes of β -adrenergic receptors differ substantially in this respect (Shiina

et al., 2000). For β -adrenergic receptors, a distinction has been made between desensitization that is homologous (resulting from stimulation of the same receptor) or heterologous (resulting from stimulation of a different receptor subtype, or nonreceptor-mediated activation of a second messenger) (Harden, 1983). A mechanism for heterologous desensitization is phosphorylation of the receptor by PKA (Clark et al., 1989; Hausdorff et al., 1989), whereas homologous desensitization of the β_2 -adrenergic receptor involves phosphorylation of the agonist-occupied receptor by both GRK (Benovic et al., 1986) and PKA (Post et al., 1996; Moffett et al., 2001). Heterologous desensitization of the β_2 -adrenergic receptor is mediated by PKA-dependent phosphorylation of Ser262, located in the carboxyl terminal portion of the third cytoplasmic loop, a position similar to that of Thr268 in

the D₁ receptor (Clark et al., 1989; Hausdorff et al., 1989; Yuan et al., 1994). In contrast, the PKA-dependent component of homologous (agonist-dependent) desensitization of the β_2 -adrenergic receptor involves phosphorylation of the PKA site Ser345,346 in the cytoplasmic tail of the receptor (Moffett et al., 2001). For the D₁ receptor, too, PKA and GRK may both contribute to homologous desensitization (Zhou et al., 1991). The aim of this study was to determine the role of PKA-catalyzed phosphorylation of the D₁ receptor in agonist-stimulated receptor desensitization and internalization.

To facilitate purification and imaging of the receptor, we attached a polyhistidine tag to the amino terminus and EGFP to the carboxyl terminus of the D₁ receptor. We chose NS20Y neuroblastoma cells for stable expression of the receptors because the line has some characteristics of striatal neurons (Amano et al., 1972), suggesting that it may be more physiologically relevant than non-neuronal cells for the characterization of a neuronal receptor such as the D₁ receptor. Although NS20Y cells have been reported to express endogenous dopamine D₁ receptors at low levels (Barton and Sibley, 1990), in our hands both D₁ receptors and dopamine-

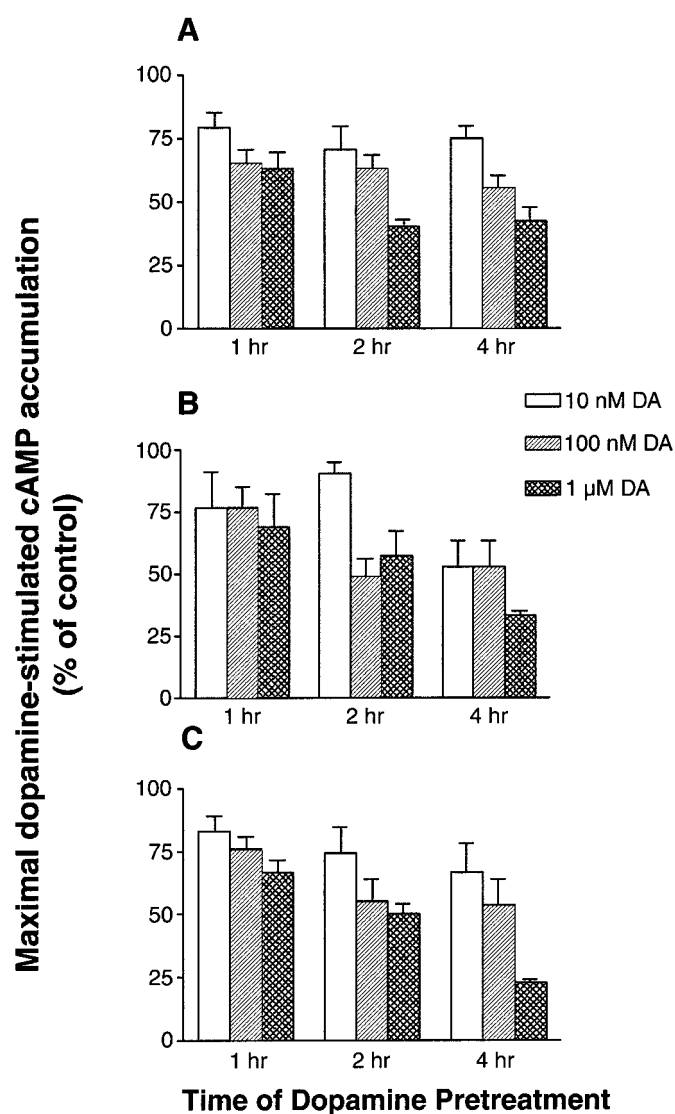


Fig. 6. Desensitization of maximal cyclic AMP accumulation by untagged D₁ receptors stably expressed in C₆ glioma cells. Cells expressing the D₁ receptor (A), T268A (B), or S380A (C) were treated with the indicated concentration of dopamine for 1, 2, or 4 h before determination of dopamine-stimulated cyclic AMP accumulation as described under *Materials and Methods*. Values for maximal stimulation were determined by non-linear regression of dose-response curves, and are expressed as the percentage of maximal stimulation in untreated cells. Results shown are the mean \pm S.E. of five to eight independent experiments.

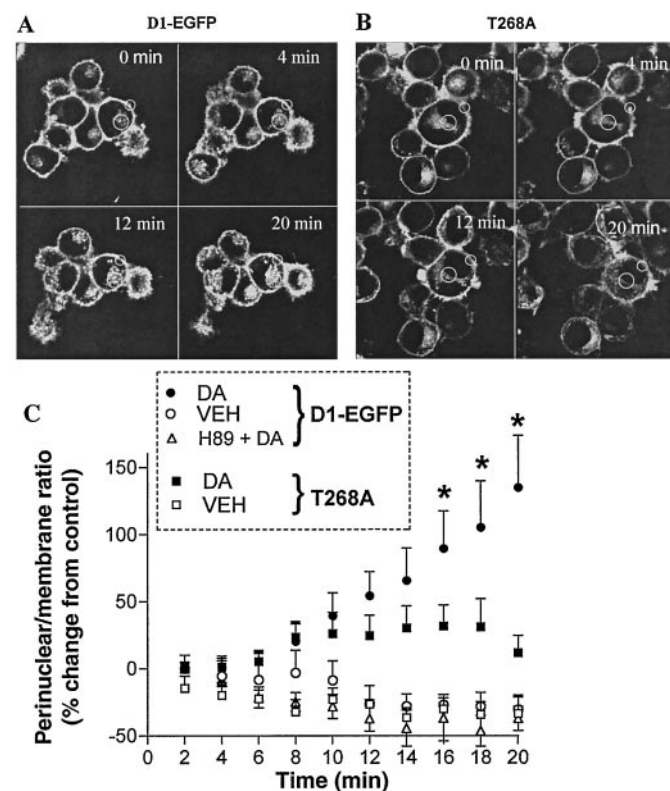


Fig. 7. Agonist-induced trafficking of D₁-EGFP receptors in NS20Y cells. NS20Y neuroblastoma cells expressing D₁-EGFP (A) or T268A (B) were treated with 25 μ M dopamine at 37°C. In some experiments, D₁-EGFP cells were pretreated for 30 min with 5 nM H-89 before incubation with dopamine. Cells were scanned at 2-min intervals; the time after the initiation of dopamine treatment is indicated in each frame. The fluorescence intensity within circles placed on the plasma membrane and on the region of perinuclear fluorescence was quantified and a ratio for each time point calculated as shown in C. The area and position of the circles for a given cell were held constant through consecutive scans of that cell. C, ratio of the fluorescence intensity in the perinuclear region to that in the membrane was calculated and expressed as the percentage of change from the ratio at time 0. Cells were treated with 25 μ M dopamine (DA) or vehicle for the indicated time. Results are the mean \pm S.E. of one cell from each of 19 independent experiments. *, $p < 0.05$ compared with T268A cells treated with dopamine, Student's t test.

stimulated cyclic AMP accumulation (data not shown) were barely detectable in untransfected cells.

The utility of any tagged molecule depends on the degree to which the tagged protein retains the functional characteristics of the wild-type protein. The polyhistidine- and EGFP-tagged D₁ receptor was similar to the untagged D₁ receptor in several respects. First, the receptors had similar affinities for the antagonist [³H]SCH 23390 and for the agonist dopamine. Second, the receptors had similar abilities to couple to G proteins, as indicated by the proportion of receptors with high affinity for dopamine in competition binding assays.

Third, the subcellular localization of D₁-EGFP in NS20Y cells was comparable with that determined in other cell types by using immunocytochemistry (Ng et al., 1994, 1995; Ariano et al., 1997; Vickery and von Zastrow, 1999). Finally, both receptors mediated agonist-stimulated cyclic AMP accumulation to a similar extent, although dopamine, in particular, was more potent at D₁-EGFP than at the untagged D₁ receptor.

Agonist-induced phosphorylation of the dopamine D₁ receptor has been demonstrated in Sf9 (Ng et al., 1994), human embryonic kidney 293 (Tiberi et al., 1996), and C₆ (Gardner et al., 2001) cells. We report herein that dopamine-induced phosphorylation of D₁-EGFP expressed in NS20Y neuroblastoma cells occurred within 5 min, and that dopamine treatment increased the phosphorylation of the receptor approximately 2-fold. The increased incorporation of phosphate was accompanied by increased phosphothreonine immunoreactivity. The primate D₁ receptor has three potential sites of PKA-catalyzed phosphorylation in cytoplasmic domains. Although the predicted rank order of preference of the sites for phosphorylation by PKA is Thr136 > Thr268 = Ser380 (Kennelly and Krebs, 1991), we mutated the two residues suggested by previous work to be involved in desensitization of the D₁ receptor (Thr268; Jiang and Sibley, 1999), or to be a site of PKA-catalyzed phosphorylation (Ser380; Zamanillo et al., 1995). Whereas mutation of Ser380 had no effect on dopamine-stimulated phosphorylation of the receptor, mutation of Thr268 prevented the dopamine-induced incorporation of phosphate. These data suggest strongly that occupancy of the D₁ receptor by dopamine stimulates phosphorylation of the receptor at Thr268. Pretreatment with the PKA inhibitor H-89 abolished dopamine-induced receptor phosphorylation, supporting the hypothesis that the phosphorylation was due to activation of PKA. Dopamine treatment tended to decrease the phosphorylation of T268A and of the D₁ receptor in the presence of H-89, particularly after short (5 min) dopamine treatments. It is possible that dopamine treatment enhances phosphatase activity, an effect that would be detectable only when dopamine-induced phosphorylation is prevented.

Although mutation of Thr268 prevented PKA-dependent phosphorylation of the D₁ receptor, the same mutation had no detectable effect on desensitization of dopamine-stimu-

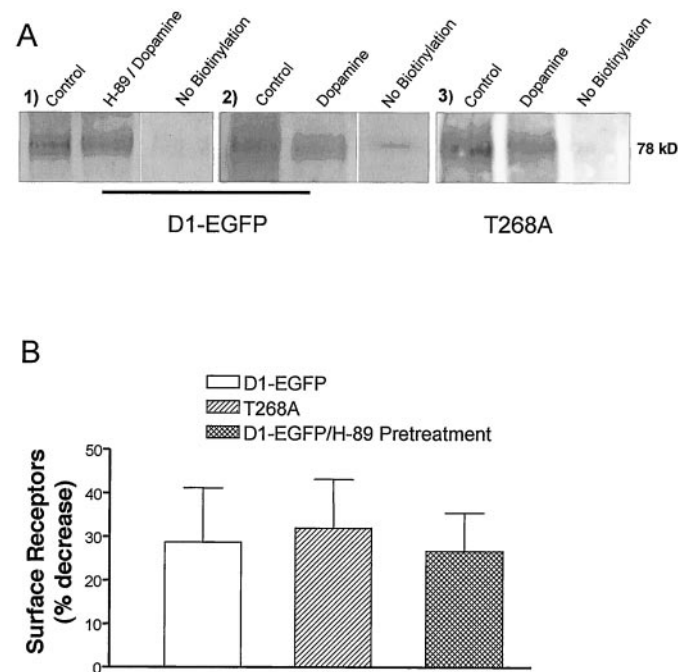


Fig. 8. Agonist-induced receptor sequestration in NS20Y cells. Biotinylation, avidin purification, SDS-PAGE separation, and immunoblotting with anti-GFP were carried out as described under *Materials and Methods*. A, representative immunoblots from cells expressing wild-type D₁-EGFP that were pretreated with 5 nM H-89 for 30 min before treatment with 25 μ M dopamine for 20 min (1), or cells expressing wild-type D₁-EGFP or T268A treated with 25 μ M dopamine for 20 min (2 and 3). B, band density was quantified and expressed as the percentage of decrease in optical density compared with cells not treated with dopamine. The results shown are the mean \pm S.E. from four to six experiments.

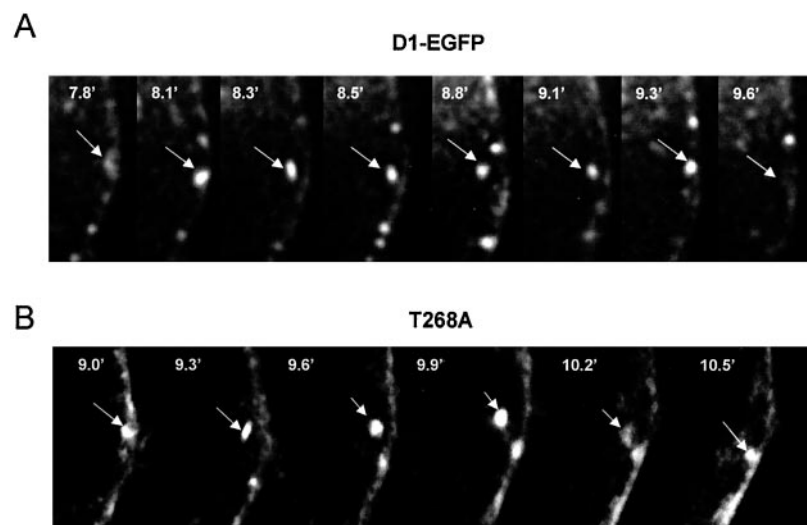


Fig. 9. Periplasmic recycling of endocytic vesicles containing the D₁-EGFP receptor. NS20Y neuroblastoma cells expressing D₁-EGFP or T268A were treated with 25 μ M dopamine at 37°C, and images were collected approximately every 15 s. In each, the arrow indicates a fluorescent vesicle that formed at the cell membrane, was internalized and then re-merged with the cell membrane. The time after the initiation of dopamine treatment is indicated in each frame. A, cells expressing D₁-EGFP. B, cells expressing the mutant T268A.

lated cyclic AMP accumulation in either NS20Y or C₆ cells, suggesting that desensitization in either cell line does not depend on PKA-dependent phosphorylation of this residue. The lack of effect of the PKA inhibitor H-89 on desensitization might suggest that PKA does not play any role in acute desensitization, but this result should be interpreted with caution because of the considerable evidence implicating PKA in homologous desensitization of the D₁ receptor (Zhou et al., 1991; Jiang and Sibley, 1999; Ventura and Sibley, 2000; Gardner et al., 2001). In particular, previous work has demonstrated that mutation of Thr268 reduces the rate more than the extent of desensitization of the D₁ receptor (Jiang and Sibley, 1999). It may be significant that D₁ receptor desensitization in C₆ cells occurred more slowly and to a lesser maximal extent in the present work than in previous work with this cell line (Lewis et al., 1998; Jiang and Sibley, 1999), which could explain the discrepancy in the observed role of Thr268 in receptor desensitization. Furthermore, because desensitization in NS20Y cells was maximal at the first time point studied, it is possible that prevention of PKA-dependent phosphorylation caused a reduction in the rate of desensitization that could not be detected by this experimental design. Species differences may also be important, because the rat D₁ receptor has several Ser/Thr residues in the C terminus and a potential PKA phosphorylation site at Ser229 that are not shared by the primate D₁ receptor.

It is surprising that inhibition of receptor phosphorylation by PKA, either by treatment with H-89 or by mutation of Thr268, prevented detectable agonist-induced phosphorylation of the D₁ receptor without preventing receptor desensitization. Work with the D₁ receptor (Tiberi et al., 1996; Gardner et al., 2001) and other closely related receptors suggests, on the one hand, that GRK-mediated phosphorylation would be detectable even when PKA is inhibited and, on the other hand, that the rate and extent of homologous desensitization would be greatly reduced in the complete absence of agonist-induced phosphorylation. One possibility is that the D₁ receptor is not phosphorylated by GRKs in our line of NS20Y cells. An alternative explanation for these results is that the GRK-phosphorylated D₁ receptor is rapidly dephosphorylated in NS20Y cells, so that our inability to detect agonist-induced phosphorylation when PKA was inhibited was a false negative result. This hypothesis is consistent with the observation that dephosphorylation of the D₁ receptor can occur in the plasma membrane, in the absence of receptor internalization (Gardner et al., 2001), and also with preliminary data indicating that sucrose-induced inhibition of receptor internalization greatly decreases basal and dopamine-induced phosphorylation of the D₁ receptor, and that phosphorylation of T268A or of the D₁ receptor in the presence of H-89 is enhanced by dopamine if cells are pretreated with the phosphatase inhibitor calyculin (J. N. Mason and K. A. Neve, unpublished observations). An alternative explanation is that phosphorylation by PKA is a prerequisite for phosphorylation by GRK, so that preventing the former also prevents the latter. This could be similar to the PKA-induced enhancement of β_2 -adrenergic receptor phosphorylation by GRK (Moffett et al., 2001), but would also suggest that agonist-induced phosphorylation is not necessary for D₁ receptor desensitization, as has been suggested for several other GPCR subtypes (Malecz et al., 1998; Sadeghi et al., 1998; Olivares-Reyes et al., 2001). Finally, as noted above, the

particular characteristics of desensitization observed in the present studies might have precluded detection of an effect of PKA inhibition on D₁ receptor desensitization.

In agreement with previous studies of the D₁ receptor (Ng et al., 1995; Ariano et al., 1997; Vickery and von Zastrow, 1999), agonist treatment caused a rapid accumulation of D₁-EGFP in the cytoplasm of NS20Y cells. In cells expressing the wild-type receptor, the accumulation was particularly evident in a perinuclear region that may represent an accumulation of recycling vesicles. Comparing agonist-induced cellular redistribution of the wild-type D₁-EGFP with that of the mutant T268A receptor, we found there to be significantly less accumulation of T268A in the perinuclear region. Consistent with the hypothesis that the effect of mutation of Thr268 was due to the loss of a PKA phosphorylation site, treatment of cells expressing wild-type D₁-EGFP with H-89 also prevented the accumulation of fluorescence in the perinuclear region.

Vesicles that form at the plasma membrane rapidly (<1 min) fuse to early endosomes, which are comprised of sorting endosomes and the recycling compartment (for review, see Mukherjee et al., 1997). After initial fusion with sorting endosomes, internalized receptors are thought to be sorted to the recycling compartment or to late endosomes, from which they are recycled to the membrane or shuttled to lysosomes, respectively. Depending on the cell type, the recycling compartment may be concentrated in the juxtanuclear region, or dispersed throughout the cell, whereas late endosomes are mainly perinuclear and close to the *trans*-Golgi network. Agonist-induced trafficking of D₁-EGFP to the perinuclear region could represent accumulation in the recycling compartment or sorting to late endosomes, suggesting that PKA-dependent phosphorylation of the D₁ receptor regulates trafficking into one of these compartments. The perinuclear accumulation of fluorescence showed little colocalization with fluorescent markers for lysosomes or the Golgi apparatus (data not shown), indicating that D₁-EGFP was not accumulating in either of those compartments.

Although trafficking of the D₁ receptor to the juxtanuclear region was inhibited by mutation of Thr268, the T268A mutant desensitized normally and seemed to accumulate intracellularly during treatment with dopamine. We used two approaches to evaluate internalization and recycling of the D₁-EGFP receptor. To quantify receptor internalization, cell surface receptors were biotinylated after dopamine treatment. Under these conditions, a decreased amount of biotinylated receptor is presumed to represent receptor that has been internalized by dopamine treatment. We found that dopamine treatment reduced the surface density of receptors by a similar amount in cells expressing D₁-EGFP or T268A. In the second approach, we used high-resolution fluorescence microscopy to confirm that, in cells expressing wild-type or T268A mutant receptors, EGFP-containing vesicles form and recycle to the membrane during treatment with dopamine. Thus, as suggested for the cholecystokinin receptor (Roettger et al., 1995), the D₁-EGFP receptor may be resensitized through rapid dephosphorylation in a vesicular compartment adjacent to the plasma membrane. On the other hand, because inhibition of endocytosis of the D₁ receptor in C₆ glioma cells does not prevent receptor dephosphorylation (Gardner et al., 2001), endocytosis may not be required for resensitization.

The signals that regulate the passage of internalized receptors to different endosomal compartments are not known, although receptor phosphorylation sites may contribute to this sorting. Phosphorylation of the epidermal growth factor receptor by protein kinase C shunts the receptor from the late endosome/lysosome pathway into a recycling pathway (Bao et al., 2000). *N*-Methyl-D-aspartate-induced internalization of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor is associated with dephosphorylation of a PKA site on the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor GluR1 subunit, and inhibition of PKA reduces the rate of reinsertion of the receptor into the cell membrane (Ehlers, 2000). Our data demonstrate that inhibiting PKA-catalyzed phosphorylation of the D₁-EGFP receptor pharmacologically or by mutation of Thr268 prevented agonist-induced trafficking of the receptor to a perinuclear region, without altering receptor internalization or desensitization. These results are consistent with a model in which phosphorylation of Thr268 of the D₁ receptor by PKA regulates a late step in the sorting of the receptor to the recycling compartment or to late endosomes.

References

- Amano T, Richelson E, and Nirenberg M (1972) Neurotransmitter synthesis by neuroblastoma clones. *Proc Natl Acad Sci USA* **69**:258–263.
- Ariano MA, Sortwell CE, Ray M, Altemus KL, Sibley DR, and Levine MS (1997) Agonist-induced morphologic decrease in cellular D_{1A} dopamine receptor staining. *Synapse* **27**:313–321.
- Bao J, Alroy I, Waterman H, Schejter ED, Brodie C, Gruenberg J, and Yarden Y (2000) Threonine phosphorylation diverts internalized epidermal growth factor receptors from a degradative pathway to the recycling endosome. *J Biol Chem* **275**:26178–26186.
- Barton AC and Sibley DR (1990) Agonist-induced desensitization of D₁-dopamine receptors linked to adenylyl cyclase activity in cultured NS20Y neuroblastoma cells. *Mol Pharmacol* **38**:531–541.
- Benovic JL, Strasser RH, Caron MG, and Lefkowitz RJ (1986) Beta-adrenergic kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc Natl Acad Sci USA* **83**:2797–2801.
- Clark RB, Friedman J, Dixon RAF, and Strader CD (1989) Identification of a specific site required for rapid heterologous desensitization of the β -adrenergic receptor by cAMP-dependent protein kinase. *Mol Pharmacol* **36**:343–348.
- Cox BA, Rosser MP, Kozlowski MR, Duwe KM, Neve RL, and Neve KA (1995) Regulation and functional characterization of a rat recombinant dopamine D3 receptor. *Synapse* **21**:1–9.
- Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* **28**:511–525.
- Gardner B, Liu ZF, Jiang D, and Sibley DR (2001) The role of phosphorylation/dephosphorylation in agonist-induced desensitization of D₁ dopamine receptor function: evidence for a novel pathway for receptor dephosphorylation. *Mol Pharmacol* **59**:310–321.
- Harden TK (1983) Agonist-induced desensitization of the β -adrenergic receptor-linked adenylyl cyclase. *Pharmacol Rev* **35**:5–32.
- Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG, and Lefkowitz RJ (1989) Phosphorylation sites on two domains of the β_2 -adrenergic receptor are involved in distinct pathways of receptor desensitization. *J Biol Chem* **264**:12657–12665.
- Hausdorff WP, Caron MG, and Lefkowitz RJ (1990) Turning off the signal: desensitization of β -adrenergic receptor function. *FASEB J* **4**:2881–2889.
- Jiang D and Sibley DR (1999) Regulation of D₁ dopamine receptors with mutations of protein kinase phosphorylation sites: attenuation of the rate of agonist-induced desensitization. *Mol Pharmacol* **56**:675–683.
- Kennelly PJ and Krebs EG (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem* **266**:15555–15558.
- Kobilka BK (1995) Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. *Anal Biochem* **231**:269–271.
- Koenig JA and Edwardson JM (1996) Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. *Mol Pharmacol* **49**:351–359.
- Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38**:289–319.
- Lewis MM, Watts VJ, Lawler CP, Nichols DE, and Mailman RB (1998) Homologous desensitization of the D_{1A} dopamine receptor: efficacy in causing desensitization dissociates from both receptor occupancy and functional potency. *J Pharmacol Exp Ther* **286**:345–353.
- Machida CA, Seales RP, Nipper V, Brown JA, Kozell LB, and Neve KA (1992) Molecular cloning and expression of the rhesus macaque D1 dopamine receptor gene. *Mol Pharmacol* **41**:652–659.
- Malecz N, Bambino T, Bencsik M, and Nissenson RA (1998) Identification of phosphorylation sites in the G protein-coupled receptor for parathyroid hormone. Receptor phosphorylation is not required for agonist-induced internalization. *Mol Endocrinol* **12**:1846–1856.
- Melikian HE and Buckley KM (1999) Membrane trafficking regulates the activity of the human dopamine transporter. *J Neurosci* **19**:7699–7710.
- Memo M, Lovenberg W, and Hanbauer I (1982) Agonist-induced subsensitivity of adenylyl cyclase coupled with a dopamine receptor in slices from rat corpus striatum. *Proc Natl Acad Sci USA* **79**:4456–4460.
- Moffett S, Rousseau G, Lagacé M, and Bouvier M (2001) The palmitoylation state of the β_2 -adrenergic receptor regulates the synergistic action of cyclic AMP-dependent protein kinase and β -adrenergic receptor kinase involved in its phosphorylation and desensitization. *J Neurochem* **76**:269–279.
- Morgenstern JP and Land H (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl Acids Res* **18**:3587–3596.
- Mukherjee S, Ghosh RN, and Maxfield FR (1997) Endocytosis. *Physiol Rev* **77**:759–803.
- Neve KA, Cox BA, Henningsen RA, Spanoyannis A, and Neve RL (1991) Pivotal role for aspartate-80 in the regulation of D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol Pharmacol* **39**:733–739.
- Ng GY, Mouillac B, George SR, Caron M, Dennis M, Bouvier M, and O'Dowd BF (1994) Desensitization, phosphorylation and palmitoylation of the human dopamine D₁ receptor. *Eur J Pharmacol Mol Pharmacol* **267**:7–19.
- Ng GYK, Trogadis J, Stevens J, Bouvier M, O'Dowd BF, and George SR (1995) Agonist-induced desensitization of dopamine D1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D1 receptor internalization. *Proc Natl Acad Sci USA* **92**:10157–10161.
- Olivares-Reyes JA, Smith RD, Hunyady L, Shah BH, and Catt KJ (2001) Agonist-induced signaling, desensitization, and internalization of a phosphorylation-deficient AT_{1A} angiotensin receptor. *J Biol Chem* **276**:37761–37768.
- Pippig S, Andexinger S, and Lohse MJ (1995) Sequestration and recycling of β_2 -adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**:666–676.
- Post SR, Aguila-Buhain O, and Insel PA (1996) A key role for protein kinase A in homologous desensitization of the β_2 -adrenergic receptor pathway in S49 lymphoma cells. *J Biol Chem* **271**:895–900.
- Roettger BF, Rentsch RU, Pinon D, Holicky E, Hadac E, Larkin JM, and Miller LJ (1995) Dual pathways of internalization of cholecystokinin receptor. *J Cell Biol* **128**:1029–1041.
- Sadeghi HM, Innamatori G, Esqueda E, and Birnbaumer M (1998) Processing and ligand-induced modifications of the V2 vasopressin receptor. *Adv Exp Med Biol* **449**:339–346.
- Shiina T, Kawasaki A, Nagao T, and Kurose H (2000) Interaction with β -arrestin determines the difference in internalization behavior between β_1 - and β_2 -adrenergic receptors. *J Biol Chem* **275**:29082–29090.
- Sibley DR and Neve KA (1997) Regulation of dopamine receptor function and expression, in *The Dopamine Receptors* (Neve KA and Neve RL eds) pp 383–424, Humana Press, Totowa, NJ.
- Tiberi M, Nash SR, Bertrand L, Lefkowitz RJ, and Caron MG (1996) Differential regulation of dopamine D1A receptor responsiveness by various G protein-coupled receptor kinases. *J Biol Chem* **271**:3771–3778.
- Tsao P, Cao T, and von Zastrow M (2001) Role of endocytosis in mediating down-regulation of G-protein-coupled receptors. *Trends Pharmacol Sci* **22**:91–96.
- Ventura ALM and Sibley DR (2000) Altered regulation of the D₁ dopamine receptor in mutant Chinese hamster ovary cells deficient in cyclic AMP-dependent protein kinase activity. *J Pharmacol Exp Ther* **293**:426–434.
- Vickery RG and von Zastrow M (1999) Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J Cell Biol* **144**:31–43.
- Watts VJ and Neve KA (1996) Sensitization of endogenous and recombinant adenylyl cyclase by activation of D₂ dopamine receptors. *Mol Pharmacol* **50**:966–976.
- Yuan N, Friedman J, Whaley BS, and Clark RB (1994) cAMP-dependent protein kinase and protein kinase C consensus site mutations of the beta-adrenergic receptor. Effect on desensitization and stimulation of adenylyl cyclase. *J Biol Chem* **269**:23032–23038.
- Zamanillo D, Casanova E, Alonso-Llamazares A, Ovalle S, Chinchetru MA, and Calvo P (1995) Identification of a cyclic adenosine 3',5'-monophosphate-dependent protein kinase phosphorylation site in the carboxy terminal tail of human D₁ dopamine receptor. *Neurosci Lett* **188**:183–186.
- Zhou X-M, Sidhu A, and Fishman PH (1991) Desensitization of the human D₁ dopamine receptor: evidence for involvement of both cyclic AMP-dependent and receptor-specific protein kinases. *Mol Cell Neurosci* **2**:464–472.

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