

The Role of Phosphorylation/Dephosphorylation in Agonist-Induced Desensitization of D₁ Dopamine Receptor Function: Evidence for a Novel Pathway for Receptor Dephosphorylation

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ABSTRACT

Exposure of D₁ dopamine receptors to agonists results in rapid desensitization of the receptor-stimulated accumulation of cAMP. It is believed that agonist-induced phosphorylation of the receptor plays a critical role in the processes that underlie this phenomenon. To investigate the role of agonist-induced receptor phosphorylation, a FLAG epitope was added to the amino terminus of the rat D₁ dopamine receptor and this construct was stably expressed in C6 glioma cells. It was found that the D₁ receptor was stoichiometrically phosphorylated under basal conditions and that its phosphorylation state was increased by 2- to 3-fold upon exposure of the cells to dopamine for 10 min. The dopamine-induced receptor phosphorylation could be blocked by D₁-selective antagonists but was unaffected by inhibitors of either protein kinase A or protein kinase C. The incorporation of phosphate into the receptor was

rapid but transient, despite the continued presence of dopamine. A comparison of the rates of receptor phosphorylation ($t_{1/2} < 1$ min) and dopamine-induced desensitization ($t_{1/2} \sim 7$ min) revealed that receptor phosphorylation was not the rate limiting step for receptor desensitization. Upon removal of dopamine, the receptor was rapidly dephosphorylated ($t_{1/2} \sim 10$ min) and this was not blocked by agents (i.e., concanavalin A or hypertonic sucrose) that inhibit D₁ receptor internalization. Using specific inhibitors, the phosphatase involved in D₁ receptor dephosphorylation was shown not to correlate with the recently identified "G protein-coupled receptor phosphatase" (*Proc Natl Acad Sci USA* 92:8343-8347, 1995). These results suggest that the phosphorylated D₁ receptor is processed through a novel recovery pathway and that internalization is not required for receptor dephosphorylation.

Dopamine receptors (DARs) belong to the large G protein-coupled receptor superfamily and molecular cloning studies have revealed the existence of five structurally distinct subtypes (Sibley and Monsma, 1992; Civelli et al., 1993; Neve and Neve, 1997). These can be divided into two subgroups on the basis of their amino acid sequences as well as their pharmacological and transductional properties. The first subgroup comprises the D₁ and D₅ DARs and is termed "D₁-like". When expressed in mammalian cells, activated D₁-like receptors stimulate adenylyl cyclase and raise intracellular levels of cAMP (Robinson and Caron, 1997). The second DAR subgroup includes the D₂, D₃, and D₄ receptors and is termed "D₂-like". The D₂-like DARs are coupled to the inhibition of adenylyl cyclase as well as the modulation of potassium and calcium ion channels (Huff, 1997). As with other G protein-coupled receptors, DARs are subject to a wide variety of regulatory mechanisms, which can either positively or negatively modulate their expression and functional activity (Sibley and Neve, 1997).

One of the most important forms of regulatory mechanisms that modulate signaling by G protein-coupled receptors is that of agonist-induced desensitization—defined as the tendency of receptor-mediated responses to wane over time despite continued agonist stimulation. Recent studies with β -adrenergic receptor systems have suggested a general paradigm for agonist-induced desensitization (Krupnick and Benovic, 1998; Lefkowitz, 1998). This involves phosphorylation of the receptors by a member of the G protein-coupled receptor kinase (GRK) family leading to the binding of an Arrestin-like protein ultimately resulting in uncoupling of the receptor from its cognate G protein and decreased functional activity. The binding of an Arrestin molecule also promotes internalization of the receptor through clathrin-coated pits into an endosomal compartment, where it may be dephosphorylated by a G protein-coupled receptor phosphatase (Pitcher et al., 1995) and recycled to the cell surface or degraded via a lysosomal pathway. Although this desensitization paradigm has in some instances been shown to be

ABBREVIATIONS: DAR, dopamine receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; PMA, phorbol 12-myristate 13-acetate; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; DMEM, Dulbecco's modified essential medium; EBSS, Earle's balanced salt solution; PAGE, polyacrylamide gel electrophoresis; TPBS, Tween PBS; BSA, bovine serum albumin; TBS, Tris-buffered saline; PKA, protein kinase A; PKC, protein kinase C; GRP, G protein-coupled receptor phosphatase; DA, dopamine.

operative for other G protein-coupled receptors, recent studies have suggested that there may be significant exceptions and widespread variations to this general scheme (Innamorati et al., 1998; Oakley et al., 1999; Vickery and von Zastrow, 1999; Walker et al., 1999; Zhang et al., 1999).

Previous investigations of agonist-induced regulation of DARs have revealed great variability among the subtypes. For instance, agonist-induced desensitization is not always observed with the D₂ DAR and, in some instances, agonist occupancy of this subtype results in increased receptor expression (Sibley and Neve, 1997). In contrast, the D₁ DAR has been shown to exhibit agonist-induced refractoriness in both endogenous and recombinant/heterologous cellular expression systems (reviewed in Sibley and Neve, 1997; Lewis et al., 1998; Jiang and Sibley, 1999). Recent data have also provided support for a phosphorylation pathway underlying agonist-induced desensitization of the D₁ DAR. For instance, studies using intracellular inhibitors of protein kinases (Zhou et al., 1991) or elimination of phosphate acceptor sites in the receptor via site-directed mutagenesis (Jiang and Sibley, 1999) have indirectly implicated a role for phosphorylation in D₁ DAR desensitization. Moreover, studies involving the expression of the D₁ DAR in Sf9 (Ng et al., 1994) or HEK-293 cells (Tiberi et al., 1996), have shown that the receptor undergoes agonist-induced phosphorylation and that in the HEK-293 cells, this phosphorylation is enhanced by coexpression of GRKs. Neither of these latter studies, however, directly addressed the role of receptor phosphorylation in the agonist-induced desensitization process.

We now demonstrate that the D₁ DAR, when expressed in C6 glioma cells, is stoichiometrically phosphorylated in response to agonist activation and that this phosphorylation is both rapid and transient. The transient nature of this phosphorylation was investigated further; it was found that, unlike β -adrenergic receptors, internalization is not required for D₁ DAR receptor dephosphorylation to take place. Our current data significantly characterizes the role that phosphorylation plays in agonist-induced regulation of D₁ DAR function and further suggest that the phosphorylated D₁ receptor is processed through a novel recovery pathway.

Experimental Procedures

Materials. C6 Glioma cells were purchased from American Type Culture Collection (Manassas, VA). [³H]SCH-23390 (70–71.3 Ci/mmol) and [³H]cAMP (31.4 Ci/mmol), were obtained from DuPont/NEN (Boston, MA). [³²P]Orthophosphate (carrier-free) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Dopamine, forskolin, Ro-20-1724, (\pm)-propranolol, (\pm)-isoproterenol, (\pm)-butaclamol, and anti-D₁ DAR monoclonal antibody were purchased from Research Biochemicals Inc. (Natick, MA). cAMP assay kits were from Diagnostic Products Corp. (Los Angeles, CA). Cell culture media and reagents were from Life Technologies (Grand Island, NY). Fetal calf serum was purchased from Summit Biotechnology (Purchase, CO). Calcium phosphate transfection kits were from Invitrogen (San Diego, CA). Concanavalin A and MiniComplete protease inhibitor cocktail were purchased from Roche Diagnostics (Nutley, NJ). Bisindolylmaleimide-1, H-89, KT5720, CPT-cAMP, PMA, okadaic acid, and calyculin A were purchased from Calbiochem (San Diego, CA). Western-Star immunoblotting kits were supplied by Tropix (Bedford, MA). M2-affinity gel and all other reagents were purchased from Sigma (St. Louis, MO).

Cell Culture and Transfections. C6 glioma cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented

with 10% fetal calf serum, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cell cultures were grown at 37°C in 5% CO₂ and 90% humidity. An amino-terminal FLAG epitope-tagged construct of the rat D₁ DAR (Monsma et al., 1990) was created from pSF β_2 , an expression construct containing a FLAG-tagged β_2 -adrenergic receptor (Guan et al., 1992). The β_2 -adrenergic receptor sequence was excised using *NcoI* and *SalI* and, after *NcoI/SalI* digestion of the rat D₁ DAR sequence, the D₁ DAR was inserted in-frame 3' to the FLAG epitope sequence to create pSFD₁. The pSFD₁ receptor construct (30 μ g) was then cotransfected with the pMAM-neo plasmid DNA (3 μ g) into C6 glioma cells using the calcium phosphate precipitation method (transfection kit; Invitrogen). In brief, cells were seeded in 150-mm² plates and transfection was carried out after 30 to 40% confluence was achieved. DNA and 60 μ l of 2 M CaCl₂ were mixed in H₂O in a total volume of 500 μ l, which was then slowly mixed with 500 μ l of HEPES-buffered saline. The reaction mixture was incubated at the room temperature for 30 min and then evenly added to the cell culture dish containing 15 ml of fresh media. After overnight incubation at 37°C, the transfection media was replaced by 25 ml of standard media. The cultures were split after another 2 to 3 days and G418 (700 μ g/ml) was added to the media. G418-resistant clones were selected after 2 weeks, expanded, and further screened and characterized by a radioligand binding assay.

Radioligand Binding Assays. Cells were harvested by incubation with 5 mM EDTA in Earle's balanced salt solution (EBSS) and collected by centrifugation at 300g for 10 min. The cells were resuspended in lysis buffer (5 mM Tris, pH 7.4 at 4°C; 5 mM MgCl₂) and were disrupted using a Dounce homogenizer followed by centrifugation at 34,000g for 10 min. The resulting membrane pellet was resuspended in binding buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂, 120 mM NaCl). The membrane suspension (final protein concentration, 50 μ g/tube) was then added to assay tubes containing [³H]SCH-23390 in a final volume of 0.5 ml. (+)-Butaclamol was added at the final concentration of 1 μ M to determine nonspecific binding. The assay tubes were incubated at room temperature for 1 h and the reaction was terminated by rapid filtration through GF/C filters pretreated with 0.3% polyethylenimine. Radioactivity bound to the filters was quantified by liquid scintillation spectroscopy at a counting efficiency of 47%.

Determination of cAMP Production. C6 glioma cells were seeded into 96-well plates (50,000 cells per well) and cultured for 1 day before the experiment. To assess desensitization, the cultures were first preincubated for the indicated time periods in the absence or presence of dopamine with 0.1 mM L-ascorbic acid and 5 μ M (\pm)-propranolol (to block endogenous β -adrenergic receptors) and in 20 mM HEPES-buffered DMEM (pH 7.4 at 37°C). Subsequently, the cells were washed four times with 200 μ l of EBSS (37°C) and were further incubated with various concentrations of dopamine in a total volume of 100 μ l at 37°C for 15 min in the presence of 30 μ M Ro-20-1724, 100 μ M L-ascorbic acid, and 5 μ M (\pm)-propranolol. The reaction was terminated by discarding the supernatant and adding 100 μ l of 3% perchloric acid per well. After incubating on ice for 30 min, 40 μ l of 15% KHCO₃ was added to the wells and the plates were further incubated for 10 min. The plates were then centrifuged for 10 min at 1,300g and 50 μ l of the supernatant from each well was subsequently transferred to a 1.2-ml tube containing 250 μ l of reaction mixture (150 μ l of Tris-EDTA buffer, 50 μ l of cAMP binding protein, and 50 μ l of [³H]cAMP). After incubation at 4°C overnight, 250 μ l of charcoal-dextran mix (1%) was added to each tube followed by incubation at 4°C for 15 min then centrifugation for 15 min at 1,300g. Radioactivity in the supernatant from each tube was quantified by liquid scintillation spectroscopy at a counting efficiency of 47%. cAMP concentrations were calculated using a standard curve according to the protocol of the assay kit.

Whole-Cell Phosphorylation Assays. One day before the experiment, cells were seeded at 1×10^6 per well of a 6-well plate and cultured overnight. Cells were then washed with EBSS and incubated for 1 h in phosphate-free DMEM. Media was then removed and

replaced with 2 ml of fresh media supplemented with 200 $\mu\text{Ci/ml}$ [^{32}P]H₃PO₄. After 90 min at 37°C, the cells were then challenged with dopamine or other agents in media supplemented with 100 μM L-ascorbic acid for the times and concentrations described in the text. Cells were then transferred to ice, washed twice with ice-cold EBSS, and solubilized for 1 h at 4°C in 1 ml of solubilization buffer (50 mM HEPES, 1 mM EDTA, 10% glycerol, 1% Triton X-100, pH 7.4 at 4°C) + 150 mM NaCl supplemented with MiniComplete protease cocktail, 0.1 mM phenylmethylsulfonyl fluoride and phosphatase inhibitors (5 mM sodium pyrophosphate, 50 mM NaF). The samples were cleared by centrifugation in a Microfuge and the protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein were then transferred to fresh tubes with 50 μl of washed M2-affinity gel and incubated overnight with mixing at 4°C. The samples were then washed once with solubilization buffer and 500 mM NaCl, once with solubilization buffer and 150 mM NaCl, and once with Tris-EDTA, pH 7.4 at 4°C. Samples were then incubated in 2 \times SDS-PAGE loading buffer for 1 h at 37°C before being resolved by 8% SDS-PAGE. The gels were dried and subjected to autoradiography. To study receptor dephosphorylation, after challenge with 10 μM dopamine for 10 min, cells were washed twice with EBSS then fresh media were added and incubated at 37°C for various times as indicated. When antagonists and other agents were used, cells were preincubated with the appropriate agent for the stated times before challenge with dopamine. The agent concentrations were then maintained until the samples were processed for immunoprecipitation. All assays included cells challenged with vehicle as an internal control.

Receptor Stoichiometry Measurements. The amount of receptor protein used in each immunoprecipitation was determined by radioligand binding and bicinchoninic acid protein assay. The specific activity of the receptor protein was calculated assuming the immunoprecipitation was 100% efficient. After developing the autoradiograph, the appropriate region of the gel was excised and radioactivity was measured by liquid scintillation counting. The specific activity of [γ - ^{32}P]ATP was determined by the method of Hawkins et al. (1983) as described previously (Carter, 1995). ATP concentrations were calculated from parallel unlabeled samples using a luciferin-luciferase assay (Sigma).

Western Blotting. C6 glioma cell membranes, or samples that had been subjected to immunoprecipitation, were resolved by 8% SDS-PAGE and transferred to nitrocellulose. Blots were blocked in 0.1% Tween PBS (TPBS) + 5% nonfat dried milk for 1 h at room temperature. The blots were then incubated overnight at 4°C with TPBS and 5% nonfat milk plus anti-D₁ DAR monoclonal antibody (1:500). Blots were washed in TPBS and incubated with secondary antibody (goat-anti rat IgG-alkaline phosphatase conjugated; 1:10,000 dilution) for 1 h at room temperature. Blots were washed and visualized with the use of WesternStar chemiluminescence (Tropix, Bedford, MA).

Immunohistochemistry and Confocal Microscopy. The C6-FD₁ cells were grown on cover slips for 1 to 2 days before antibody staining. The cultures were treated with or without dopamine for 30 min and then washed three times with 1 \times ice-cold TBS, pH 7.4. Before staining, the cells were treated with blocking solution (3% normal goat serum, 2% horse serum, and 1% BSA). The primary antibody, anti-FLAG M2 monoclonal antibody, was diluted with 1 \times TBS containing 3% BSA. Live cells were incubated with the M2 antibody (30 $\mu\text{g/ml}$) at 4°C for 1 to 2 h, washed three times with ice-cold TBS, and fixed with 2% paraformaldehyde for 30 min. After three washes with TBS, the fixed cells were stained with Cy3 conjugated donkey anti-mouse IgG (1:200 dilution in TBS) for 45 min at room temperature, followed by a final three washes of TBS. The coverslips were mounted with Vectashield mounting medium and subjected to confocal microscopy. All fluorescent images were viewed under a Zeiss LSM 510 inverted confocal microscope (Zeiss, Oberkochen, Germany).

Data Analysis. All binding assays were routinely performed in triplicate and were repeated three to four times. cAMP experiments were performed in duplicate and were repeated three to four times. Estimations of the radioligand binding parameters K_D and B_{max} , as well as the EC₅₀ values for dopamine-stimulation of cAMP production, were calculated using the GraphPad Prism curve-fitting program. The curves presented throughout this manuscript, representing the best fits to the data, were generated using this software program as well. The relative intensities of phosphorylated bands were determined by scanning the autoradiographs and analyzing using the software program NIH Image.

Results

Immunoprecipitation of Phosphorylated D₁ Dopamine Receptors. To characterize phosphorylation of the D₁ DAR in intact cells, we engineered an epitope-tagged construct of the receptor by placing the FLAG peptide sequence (Guan et al. 1992) at its amino terminus. This allows for purification of the receptor from cellular homogenates via immunoprecipitation using antibodies directed to the FLAG sequence. We stably transfected this construct in C6 glioma cells and selected a clonal line (C6-FD₁) which expressed about 1.3 pmol/mg of protein of receptor binding activity. Preliminary characterization of these cells indicated that the FLAG-tagged D₁ receptor exhibited normal ligand binding characteristics and stimulated cAMP levels in a fashion identical to that of wild-type receptors (data not shown).

Before attempting immunoprecipitation of metabolically labeled D₁ receptors, we first determined the apparent molecular mass of the D₁ DAR using a monoclonal antibody directed to the D₁ receptor (Levey et al., 1993) to immunoblot membranes from transfected C6 glioma cells (Fig. 1A). A broad band was observed in membranes from C6-FD₁ cells that had an apparent molecular mass of 55 to 60 kDa (Fig. 1A, lane 2). This band was not present in membranes from untransfected cells (Fig. 1A, lane 1). The D₁ DAR has a predicted molecular mass of about 49 kDa and contains two consensus sites for N-linked glycosylation (Monsma et al., 1990). Thus, the apparent larger molecular mass is consistent with a glycosylated receptor protein.

We next examined immunoprecipitation of the D₁ DAR from the transfected C6 cells using anti-FLAG antisera conjugated to an affinity gel as described under *Experimental Procedures*. The precipitated samples were then analyzed by SDS-PAGE and immunoblotted using the monoclonal anti-D₁DAR antibody. A broad band with an apparent molecular mass of 55 to 60 kDa was observed (Fig. 1B, lane 1). This was similar to that observed in membranes from C6-FD₁ cells (Fig. 1A, lane 2). Again, this band was specific to the transfected cells. As an aside, we found that, whereas the anti-FLAG antibodies were useful for immunoprecipitation, they were not useful for immunoblotting. Conversely, whereas the D₁ DAR monoclonal antibody was useful for immunoblotting, it was not useful for immunoprecipitation. Consequently, all further immunoprecipitations were performed with the affinity gel-conjugated anti-FLAG antibodies. When C6-FD₁ cells were pretreated with 10 μM dopamine for 10 min before immunoprecipitation, a notable decrease in the mobility of the D₁ DAR was observed (Fig. 1B, lane 2) and the receptor exhibited an apparent molecular mass of 60 to 66 kDa. Although this mobility shift was not examined intensively in

this study, it should be noted that a decrease in SDS-PAGE mobility is commonly observed with phosphorylated proteins.

To directly examine the phosphorylation status of the D₁ DAR, the C6 glioma cells were metabolically labeled with [³²P]H₃PO₄ to isotopically label the intracellular ATP pool followed by immunoprecipitation of the receptor. Figure 2A shows an autoradiogram of immunoprecipitates from metabolically labeled untransfected and transfected cells. As can be seen in Fig. 2A, lane 2, there is a phosphorylated protein with a molecular mass of about 55 to 60 kDa, which was not observed in untransfected cells (Fig. 2A, lane 1). This protein band corresponds with the D₁ DAR, which was identified via the immunoblotting experiments shown in Fig. 1. When the C6-FD₁ cells were pretreated with 10 μ M dopamine for 10 min, the phosphorylation state of the D₁ DAR was increased by 2- to 3-fold and the mobility of the receptor was decreased (Fig. 2A, lane 3). Figure 2B shows the results of densitometric scanning of multiple experiments of the type shown in Fig. 2A. As can be seen, treatment of the cells with 10 μ M dopamine for 10 min results in a 2.5-fold increase in the phosphorylation of the D₁ DAR.

To assess the stoichiometry of D₁ DAR phosphorylation, we determined the specific activity of the cellular [γ -³²P]ATP by the method of Hawkins et al. (1983) as described previously (Carter, 1995). Using this approach, we estimated a stoichiometry of 1 mol of phosphate/mol of receptor in the basal state with an increase to 2.7 mol of phosphate/mol of receptor after challenging the cells with 10 μ M dopamine for 10 min.

These values are likely to be somewhat under-estimated because our calculation assumes complete immunoprecipitation of the solubilized receptors, which is probably not the case. Nonetheless, this analysis establishes that the D₁ receptor is *stoichiometrically* phosphorylated in the C6 glioma cells and that its phosphate content is increased by 2- to 3-fold upon agonist activation.

The pharmacology of the D₁ DAR phosphorylation process was next determined (Fig. 3). When the β -adrenergic agonist isoproterenol was substituted for dopamine, no increase in D₁ receptor phosphorylation was observed. It should be noted that C6 cells express functional β -adrenergic receptors. This demonstrates that receptor occupancy by dopamine is required to observe stimulation of D₁ DAR phosphorylation.

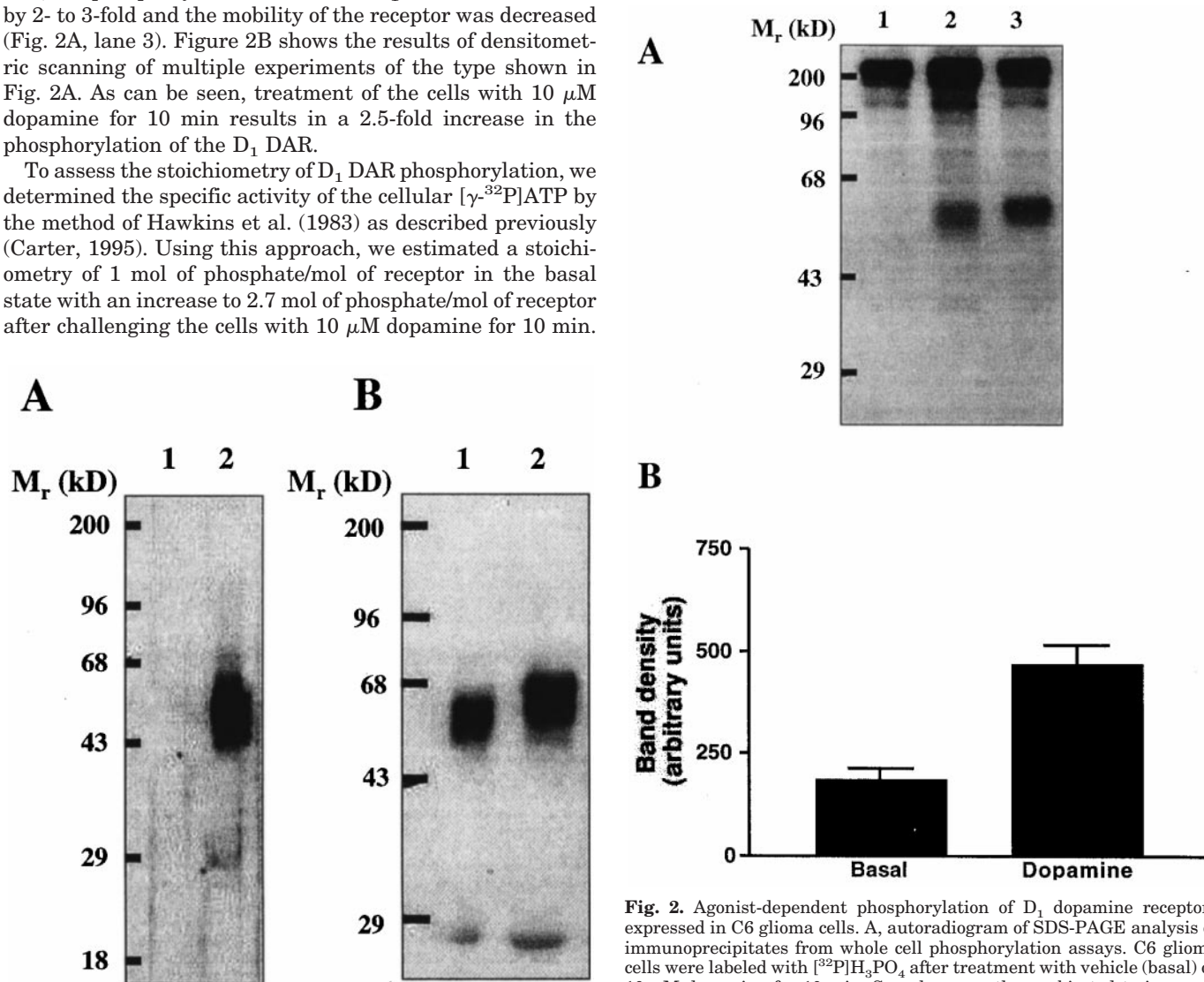


Fig. 1. Immunoblot of membrane preparations and immunoprecipitated samples from untransfected C6 glioma cells and transfected C6-FD₁ cells. A, blot showing membranes (10 μ g of protein) from untransfected C6 glioma cells (lane 1) or transfected C6-FD₁ cells (lane 2). B, immunoprecipitated samples from C6-FD₁ cells that were pretreated with vehicle (lane 1) or 10 μ M dopamine for 10 min (lane 2). Cell treatments, membrane preparation and solubilization, immunoprecipitation, SDS-PAGE and immunoblotting were performed as described under *Experimental Procedures*. Positions of prestained molecular mass (M_r) markers are indicated in kilodaltons. A single representative experiment is shown that was performed three times with similar results.

Fig. 2. Agonist-dependent phosphorylation of D₁ dopamine receptors expressed in C6 glioma cells. A, autoradiogram of SDS-PAGE analysis of immunoprecipitates from whole cell phosphorylation assays. C6 glioma cells were labeled with [³²P]H₃PO₄ after treatment with vehicle (basal) or 10 μ M dopamine for 10 min. Samples were then subjected to immunoprecipitation as described under *Experimental Procedures* and resolved by 8% SDS-PAGE. The extent of receptor phosphorylation was visualized by autoradiography. Lane 1, dopamine-treated untransfected C6 cells. Lane 2, transfected C6 cells treated with vehicle. Lane 3, transfected C6 cells treated with dopamine. A representative experiment is shown with average basal and dopamine-stimulated data presented in B. B, the receptor phosphorylation obtained in A was quantified by scanning the autographs followed by analysis with the software package NIH Image. Data are the mean values of band density (arbitrary units) from 27 independent experiments. The mean values \pm S.E.M. were 189 \pm 24 (basal) and 470 \pm 44 (dopamine-stimulated).

This was demonstrated further in that the dopamine-stimulated increase in receptor phosphorylation could be inhibited by the D_1 -selective antagonist SCH-23390 and by the nonselective dopamine antagonist (+)-butaclamol. The inactive isomer (-)-butaclamol did not inhibit dopamine-stimulated receptor phosphorylation. The agonist-induced D_1 DAR phosphorylation in the C6 cells thus seems to be homologous in nature and pharmacologically specific.

Characterization of Protein Kinases Involved in D_1 Dopamine Receptor Phosphorylation. We next examined the contribution of the second messenger protein kinases, PKA and PKC, in dopamine-stimulated phosphorylation of the D_1 DAR. C6-FD $_1$ cells were incubated with various kinase inhibitors or activators before dopamine challenge as described under *Experimental Procedures*. Neither PKA nor PKC seem to be largely involved in the dopamine-stimulated increase in receptor phosphorylation (Fig. 4). This was demonstrated by the relative lack of effect of the PKC inhibitor bisindolylmaleimide-1 or the PKA inhibitors H-89 and KT5720 on dopamine-stimulated D_1 DAR phosphorylation (Fig. 4). Furthermore, direct activation of PKA using forsko-

lin or CPT-cAMP, both in the presence of the phosphodiesterase inhibitor Ro-20-1724, does not stimulate D_1 DAR phosphorylation (Fig. 4). Interestingly, it was observed that direct activation of PKC using the phorbol ester PMA resulted in a small increase in the phosphorylation state of the D_1 DAR (Fig. 4). This response could be blocked with the inclusion of bisindolylmaleimide-1, but not when H-89 was used, further indicating a PKC-mediated response (Fig. 4). Overall, these results suggest that the dopamine-stimulated receptor phosphorylation probably occurs for the most part via a GRK-mediated pathway.

Relationship of Receptor Phosphorylation to Dopamine-Induced Desensitization. To investigate the role that receptor phosphorylation plays in agonist-induced desensitization, we initially examined the dose-response relationships for these processes (Fig. 5). The dopamine-stimulated receptor phosphorylation was found to be dose-dependent, as demonstrated using C6 cells that were challenged with increasing concentrations of dopamine for 10 min (Fig. 5, A and B). It was observed that 10 μ M dopamine was a maximally effective concentration and that the EC_{50}

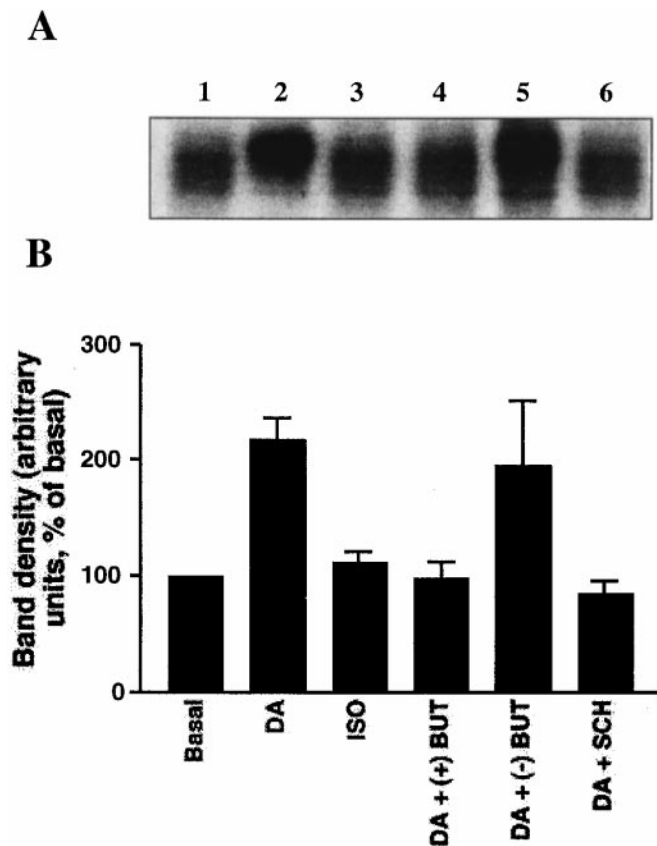


Fig. 3. Pharmacological characterization of dopamine-stimulated D_1 receptor phosphorylation in C6 glioma cells. Whole-cell phosphorylation assays were performed as described in Fig. 2. A, autoradiogram in which the cells were pretreated for 10 min with the following drugs: lane 1, vehicle (basal); lane 2, 10 μ M dopamine (DA); lane 3, 10 μ M isoproterenol; lane 4, 10 μ M dopamine + 3 μ M (+)-butaclamol; lane 5, 10 μ M dopamine + 3 μ M (-)-butaclamol; lane 6, 10 μ M dopamine + 3 μ M SCH-23390. The samples were then subjected to immunoprecipitation and resolved by 8% SDS-PAGE as described under *Experimental Procedures*. The autoradiograph shown is representative of three experiments. B, the receptor phosphorylation results obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. Data are presented as a percentage above basal phosphorylation.

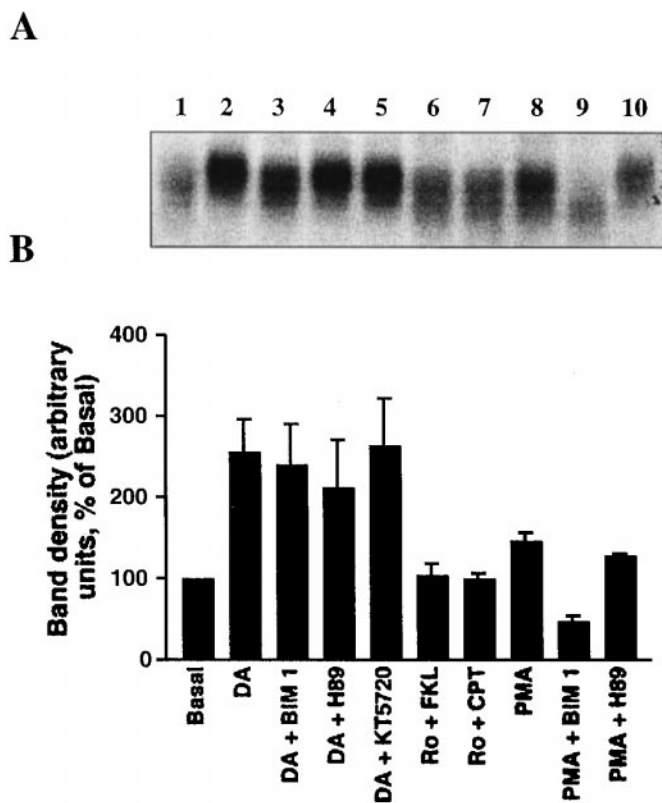


Fig. 4. Characterization of the role of protein kinases A and C in the phosphorylation of D_1 dopamine receptors expressed in C6 glioma cells. Whole-cell phosphorylation assays were performed as described in Fig. 2. A, autoradiogram in which the cells were pretreated in the absence or presence of dopamine or the indicated drugs: lane 1, vehicle (basal); lane 2, 10 μ M dopamine (DA); lane 3, DA + 10 μ M bisindolylmaleimide (BIM 1); lane 4, DA + 10 μ M H89; lane 5, DA + 10 μ M KT5720; lane 6, 100 μ M Ro-20-1724 + 10 μ M forskolin; lane 7, 100 μ M Ro-20-1724 + 1 mM CPT-cAMP; lane 8, 1 μ M PMA; lane 9, PMA + BIM 1; lane 10, PMA + H89. The samples were then subjected to immunoprecipitation and resolved by 8% SDS-PAGE as described under *Experimental Procedures*. The autoradiograph shown is representative of three experiments. B, the receptor phosphorylation results obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. Data are presented as a percentage above basal phosphorylation.

for this response was 200 to 300 nM. The desensitization of the cAMP accumulation response by dopamine was investigated by preincubating the cells with increasing concentrations of dopamine for 10 min. The cells were then extensively washed and rechallenge with a single dose of dopamine (10 μ M). It was observed that dopamine exhibited an EC₅₀ value of 150 to 200 nM for inducing desensitization and that 10 μ M was a maximally effective dose (Fig. 5C). Thus, the agonist-induced receptor phosphorylation and desensitization responses exhibit similar dose-response relationships.

We next wished to compare the time courses of dopamine-

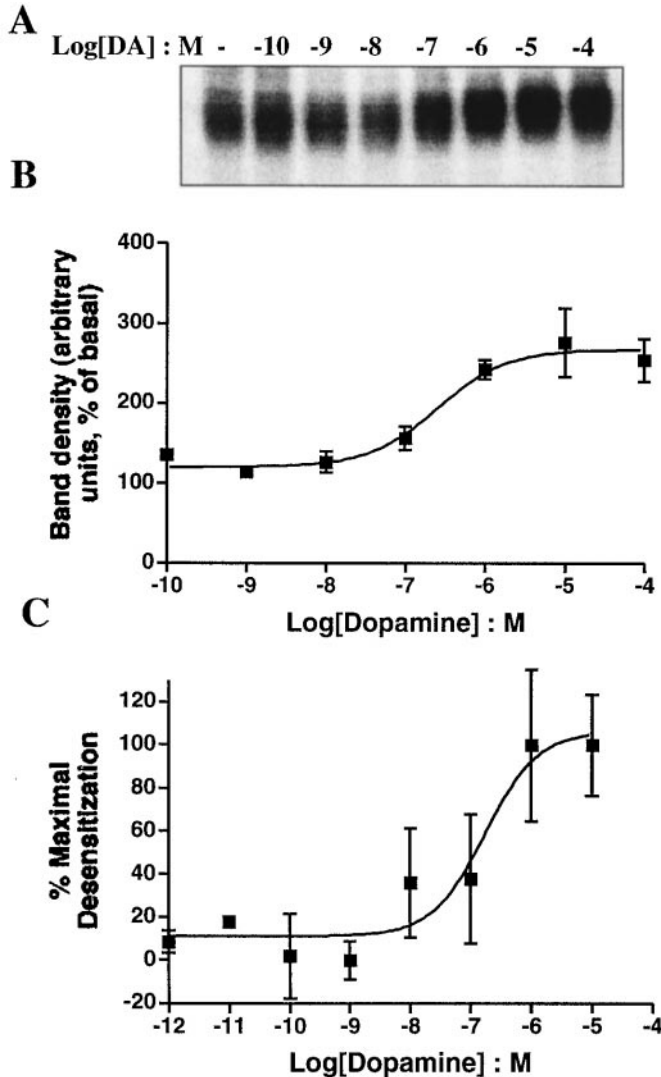


Fig. 5. Dose-response curves for dopamine-induced D₁ dopamine receptor phosphorylation and desensitization in C6 glioma cells. Whole-cell phosphorylation assays were performed as described in Fig. 2. A, autoradiogram in which the cells were pretreated in the absence (basal) or presence of the indicated concentrations of dopamine. The experiment shown is representative of three independent experiments. B, the receptor phosphorylation results obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. Data are presented as a percentage above basal phosphorylation. C, C6 cells were pretreated with vehicle or the indicated concentrations of dopamine for 10 min followed by extensive washing with EBSS at 37°C. Dopamine-stimulated cAMP levels were then assessed as described under *Experimental Procedures*. The percentage desensitization was calculated by dividing the desensitization observed at each dopamine concentration by that observed at 10 μ M dopamine. The data represent the mean values from three independent experiments.

stimulated receptor phosphorylation and desensitization. The time course for receptor phosphorylation is shown in Fig. 6. It was observed that receptor phosphorylation was rapid reaching a peak within 1 to 3 min after agonist exposure. The extent of receptor phosphorylation was then seen to decrease until, after about 60 min, it approached the background level observed in the absence of dopamine (basal). In contrast, the time course of agonist-induced desensitization of the cAMP response was slower and was characterized by both a decrease in maximum response and an increase in the EC₅₀ value for dopamine (Fig. 7A). More detailed time course data, which examined desensitization of the maximum response, showed that the rate of desensitization exhibited a $t_{1/2}$ of about 7 min with maximum desensitization not being achieved until about 30 min (Fig. 7B). The agonist-induced receptor phosphorylation thus seems to significantly precede the desensitization response (compare Figs. 6 and 7).

Characterization of Receptor Dephosphorylation and Resensitization. We next wished to examine the rates of receptor dephosphorylation and resensitization of the cAMP response, especially given that the agonist-induced receptor phosphorylation seemed transient in nature (Fig. 6). In an initial series of experiments, whole-cell phosphorylation assays were performed on C6-FD₁ cells that had been incubated with 10 μ M dopamine for 10 min. Cells were then

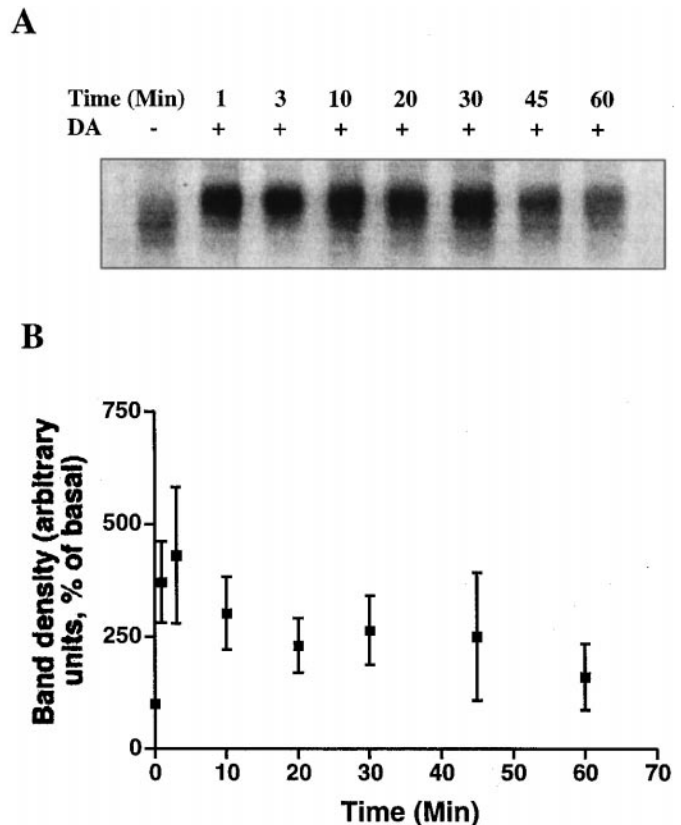


Fig. 6. Time course of dopamine-stimulated phosphorylation of D₁ dopamine receptors in C6 glioma cells. Whole-cell phosphorylation assays were performed as described in Fig. 2 and under *Experimental Procedures*. A, autoradiogram in which the cells were pretreated in the absence (basal) or presence of 10 μ M dopamine for the indicated time periods. The experiment shown is representative of three independent experiments. B, the receptor phosphorylation results obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. Data are presented as arbitrary density units.

extensively washed with EBSS and further incubated in the absence of dopamine in fresh medium for various periods of time before being subjected to immunoprecipitation. It was observed that, after the removal of dopamine, the agonist-stimulated receptor phosphorylation was rapidly reversed with a $t_{1/2}$ of 5 to 10 min and was back to basal levels within 30 min (Fig. 8A and B). A similar experimental design was used to investigate the resensitization of the D_1 DAR that had previously been desensitized with dopamine. C6-FD₁ cells were challenged with 10 μ M dopamine for 60 min then washed extensively and incubated in fresh buffer without dopamine for increasing periods. After this, cAMP accumulation assays were performed using a 10 μ M test dose of dopamine (Fig. 8C). Interestingly, in contrast to the rapid

rate of receptor dephosphorylation, resensitization of the D_1 DAR-mediated cAMP response occurred slowly, not returning to control levels until after 5 to 6 h in culture (Fig. 8C).

Given the results in Fig. 8, we decided to investigate the process of receptor dephosphorylation in greater detail. Our initial hypothesis was that the phosphorylated D_1 DAR might be processed in a manner similar to that of the β_2 -adrenergic receptor. Detailed studies have shown that, after receptor phosphorylation, the β_2 -adrenergic receptor undergoes rapid sequestration via β -Arrestin- and clathrin-mediated endocytosis followed by dephosphorylation within an

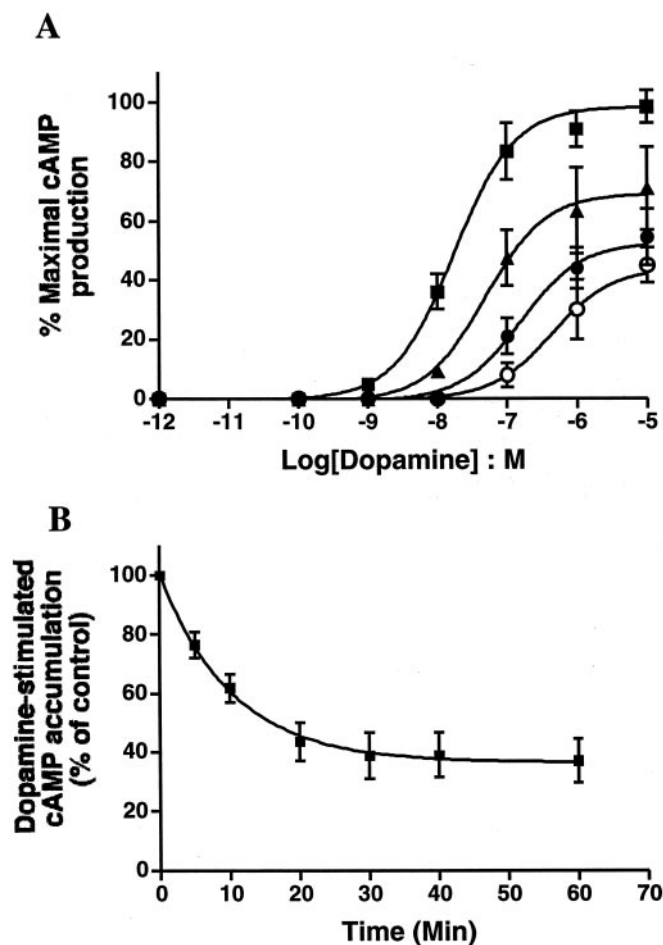


Fig. 7. Time course of dopamine-induced desensitization of D_1 dopamine receptor mediated cAMP accumulation in C6 glioma cells. C6 glioma cells were preincubated with vehicle or 10 μ M dopamine for increasing periods of time. Cells were then extensively washed with EBSS (37°C) and then dopamine-stimulated cAMP levels were assessed as described under *Experimental Procedures*. A, dose-response curves for dopamine-stimulated cAMP production after pretreatment with 10 μ M dopamine for the indicated times: control (■); 10 min (▲); 30 min (●); and 60 min (○). The following EC_{50} and V_{max} values (percentage of control) were calculated from the curves: control, EC_{50} = 18 nM, V_{max} = 100%; 10 min DA, EC_{50} = 49 nM, V_{max} = 69%; 30 min DA, EC_{50} = 160 nM, V_{max} = 53%; 60 min DA, EC_{50} = 440 nM, V_{max} = 44%. B, after pretreatment with 10 μ M dopamine for the indicated times, the cells were washed and dopamine-stimulated cAMP production was assessed using a single concentration (10 μ M) of dopamine. The data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. The data represent the mean \pm S.E.M. values from three independent experiments.

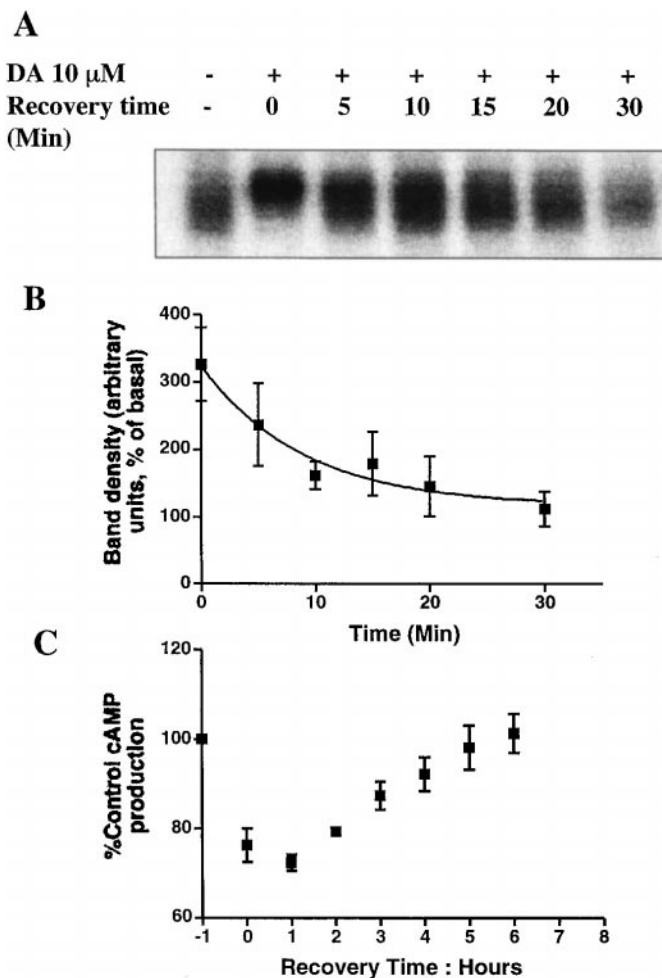


Fig. 8. Time courses for dephosphorylation and resensitization of D_1 dopamine receptors in C6 glioma cells. Whole-cell phosphorylation experiments were carried out as described under *Experimental Procedures*. C6 glioma cells were treated with either vehicle (basal) or 10 μ M dopamine for 10 min. To examine the recovery of phosphorylation to basal levels, the cells were subsequently washed twice with EBSS (37°C) and further incubated in fresh medium without dopamine for the times indicated. The samples were then subjected to immunoprecipitation and resolved by 8% SDS-PAGE. A, the autoradiogram shown is representative of three experiments. B, the receptor phosphorylation obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. Data are presented as percentage above basal phosphorylation. C, C6 cells were pretreated with 1 μ M dopamine for 60 min and subsequently washed twice with EBSS (37°C) and further incubated in fresh medium without dopamine for the times indicated. Dopamine-stimulated cAMP production was next assessed using a single concentration (10 μ M) of dopamine. The data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. The data represent the mean \pm S.E.M. values from three independent experiments and are presented as the percentage of cAMP accumulated (■) relative to cAMP accumulated in an untreated control.

acidified vesicular compartment by a novel protein phosphatase termed "GRP" for G protein-coupled receptor phosphatase (Pitcher et al., 1995). After dephosphorylation, the receptor can recycle to the plasma membrane and resensitization is achieved. It has been found that if β_2 -adrenergic receptor endocytosis is blocked, then receptor dephosphorylation is likewise inhibited and receptor resensitization does not occur (Yu et al., 1993; Pippig et al., 1995; Krueger et al., 1997; Zhang et al., 1997).

Our first approach was to examine the effect of inhibiting internalization of the receptor on the dephosphorylation process. Previously, treatment of cells with the plant lectin concanavalin A or hypertonic sucrose was shown to inhibit D₁ receptor internalization (Ng et al., 1995; Ariano et al., 1997); however, this has not yet been demonstrated using C6 glioma cells. We thus performed the experiment shown in Fig. 9. In this study, we used antibodies directed against the FLAG epitope on the D₁ DAR to immunostain intact C6-FD₁ cells

and visualize the receptor using confocal fluorescence microscopy. Because the FLAG epitope is located on an extracellular region of the receptor and the cells are intact and not permeabilized, the observed fluorescence is extracellular in nature. In the absence of dopamine, intense fluorescence is observed around the cellular exterior suggesting that the receptor is predominantly localized to the plasma membrane (Fig. 9). Under basal conditions, concanavalin A or hypertonic sucrose treatments do not seem to affect the D₁ DAR distribution (Fig. 9). Under control conditions, treatment of the cells with 50 μ M dopamine for 30 min induces a nearly complete loss of cellular fluorescence, suggesting that internalization of the receptor has taken place (Fig. 9). In contrast, the extracellular fluorescence is retained after dopamine treatment if the cells have been co-treated with either concanavalin A or hypertonic sucrose (Fig. 9). In the latter cases, however, the fluorescence is more punctate in appearance, suggesting an agonist-induced clustering of the receptors, but no internalization.

Based on the results in Fig. 9, we next treated the cells with concanavalin A or hypertonic sucrose to see what effect this would have on the dephosphorylation of the receptor (Fig. 10). The design for these experiments was similar to

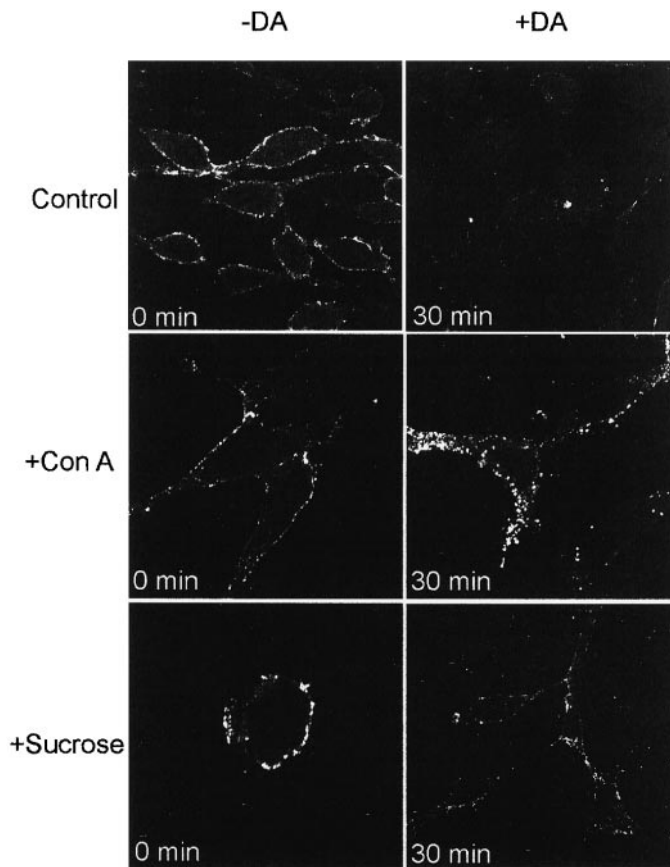


Fig. 9. The effect of concanavalin A or hypertonic sucrose on the internalization of D₁ dopamine receptors. The cells were treated at 37°C in the absence (0 min) or presence of 50 μ M dopamine for 30 min in the absence (control) or presence of 0.25 mg/ml concanavalin A (+ Con A) or 0.45 M sucrose (+ Sucrose) and then washed three times with TBS. The primary antibody, anti-FLAG M2 monoclonal antibody, was diluted with 1 \times TBS containing 3% BSA as described under *Experimental Procedures*. Live intact cells were incubated with the M2 antibody (30 μ g/ml) at 4°C for 1 to 2 h, washed three times with ice-cold TBS, and fixed with 2% paraformaldehyde for 30 min. After three washes with TBS, the fixed cells were stained with Cy3 conjugated donkey anti-mouse IgG (1:200 dilution in TBS) for 45 min at room temperature, followed by a final three washes of TBS. The coverslips were mounted with Vectashield medium and subjected to confocal microscopy. All fluorescent images were viewed under a Zeiss LSM 510 inverted confocal microscope. Representative fields are shown from experiments that were performed on three independent occasions.

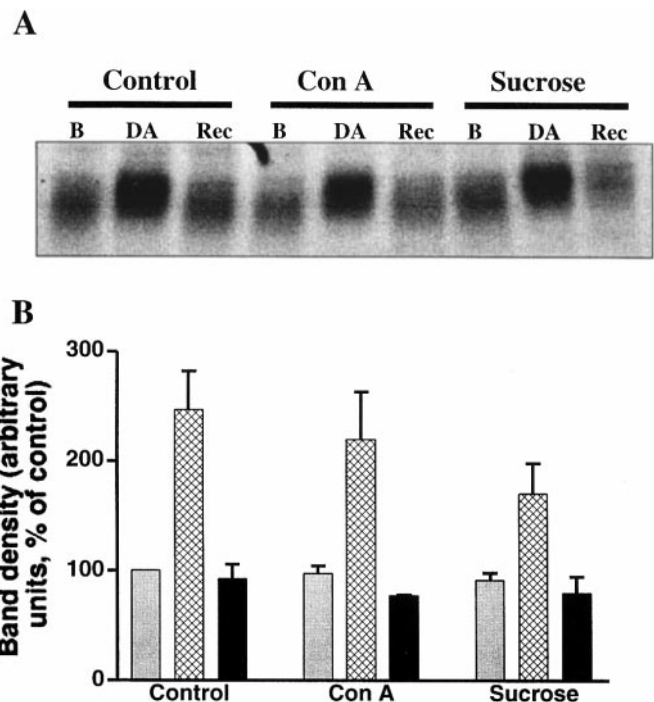


Fig. 10. The effect of internalization inhibitors on the dephosphorylation of D₁ dopamine receptors after pretreatment with dopamine. Whole-cell phosphorylation experiments were performed as described under *Experimental Procedures*. Before dopamine treatment, the cells were incubated with either vehicle (control), 0.25 mg/ml concanavalin A, or 0.45 M sucrose for 20 min. The cells were next treated with either vehicle or 10 μ M dopamine for 10 min. One sample from each group was then washed twice with EBSS (37°C) and further incubated in fresh medium without dopamine for 20 min. After treatment, the samples were subjected to immunoprecipitation and resolved by 8% SDS-PAGE. A, representative autoradiogram showing receptor phosphorylation in basal (B), dopamine-stimulated (DA), and dopamine-stimulated followed by a 20 min recovery period (Rec) sample groups. B, the receptor phosphorylation obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. In each treatment group, (▨, DA-treated; ■, "recovered"), the phosphorylation data are presented as percentage above basal phosphorylation (□).

that used in Fig. 8 except that a fixed recovery period of 20 min was used after removal of dopamine. As can be seen, after a 20-min recovery period, the level of receptor phosphorylation had returned to near basal levels in the control treatment group, similar to that previously observed in Fig. 8. Surprisingly, pretreatment of the cells with hypertonic sucrose or concanavalin A did not have any effect on the D₁ receptor dephosphorylation after agonist removal (Fig. 10). These results suggest that receptor internalization is not required for D₁ receptor dephosphorylation to occur and that the phosphatase responsible for this action may reside in a different cellular compartment than the previously identified G protein-coupled receptor phosphatase.

To further characterize the dephosphorylation pathway of the D₁ receptor, we examined the effects of two protein phosphatase inhibitors, okadaic acid and calyculin A, which have been shown to inhibit the G protein-coupled receptor phosphatase and/or resensitization of the β_2 -adrenergic receptor (Pitcher et al., 1995; Pippig et al., 1995; Krueger et al. 1997). As indicated in Fig. 11, however, neither of these inhibitors blocked D₁ receptor dephosphorylation and, after 20 min of recovery, all treatment groups exhibited receptor phosphorylation states that were near basal levels. These results,

although preliminary in nature, would seem to suggest that the previously identified "G protein-coupled receptor phosphatase" (Pitcher et al., 1995) is not involved in D₁ receptor dephosphorylation.

Discussion

In this report, we have investigated the role that phosphorylation plays in regulating D₁ DAR function. Previous investigations have provided support for a phosphorylation pathway underlying agonist-induced desensitization of the D₁ DAR. Studies using intracellular inhibitors of protein kinases (Zhou et al. 1991) or elimination of phosphate acceptor sites in the receptor protein (Jiang and Sibley, 1999) have implicated a role for phosphorylation in D₁ DAR desensitization. Also, studies involving the expression of the D₁ DAR in Sf9 (Ng et al., 1994) or HEK-293 cells (Tiberi et al., 1996), have shown that the D₁ receptor is phosphorylated and that, in HEK-293 cells, this phosphorylation is enhanced by coexpression of GRKs. Here we demonstrate that the D₁ DAR, when expressed in C6 glioma cells, is phosphorylated in response to agonist activation and that this phosphorylation is dose-dependent, rapid and transient. Our estimation of phosphate/receptor stoichiometries suggest that, under basal conditions, there is at least 1 mol of phosphate incorporated into the D₁ DAR and that the receptor phosphate content increases by up to 3-fold upon maximal dopamine activation. Although the exact number and location of phosphate acceptor sites on the D₁ DAR is unclear at present, the observation that the agonist-induced phosphorylation is stoichiometric in nature lends support to the hypothesis that this is a physiologically relevant process.

Our initial characterization of D₁ DAR phosphorylation in C6 glioma cells revealed this to be strictly dependent upon agonist occupancy/activation of the receptor. The increase in receptor phosphorylation was not mimicked by antagonist occupancy, although D₁-selective antagonists could block the phosphorylation increase observed with dopamine treatment. Moreover, treatment of the C6 cells with a β -adrenergic agonist had no effect on D₁ DAR phosphorylation, despite the fact that this treatment results in increased intracellular levels of cAMP and activation of PKA. This suggests an absence of heterologous "cross talk" in the C6 cells and that the agonist-induced D₁ DAR phosphorylation is homologous in nature, at least for relatively short periods of agonist exposure.

Our investigation of the biochemical pathway of D₁ DAR phosphorylation indicates that this process is not predominantly mediated by either PKA or PKC. This was suggested by the relative lack of effect of intracellular activators of PKA or PKC on D₁ DAR phosphorylation or by PKA/PKC inhibitors on agonist-induced receptor phosphorylation. It should be noted, however, that cellular treatment with PMA, which directly activates PKC, resulted in a slight (~50%) but consistent increase in D₁ DAR phosphorylation. Interestingly, we have previously observed that PMA treatment of C6 glioma cells results in partial desensitization of the D₁ DAR-mediated cAMP response (Jiang and Sibley, 1997). This is unlikely to be related to the D₁ agonist-induced effect; however, because D₁ DAR stimulation is not linked to activation of the PKC system and the PKC inhibitor bisindolylmaleimide-1 had no effect on agonist-induced receptor phosphoryla-

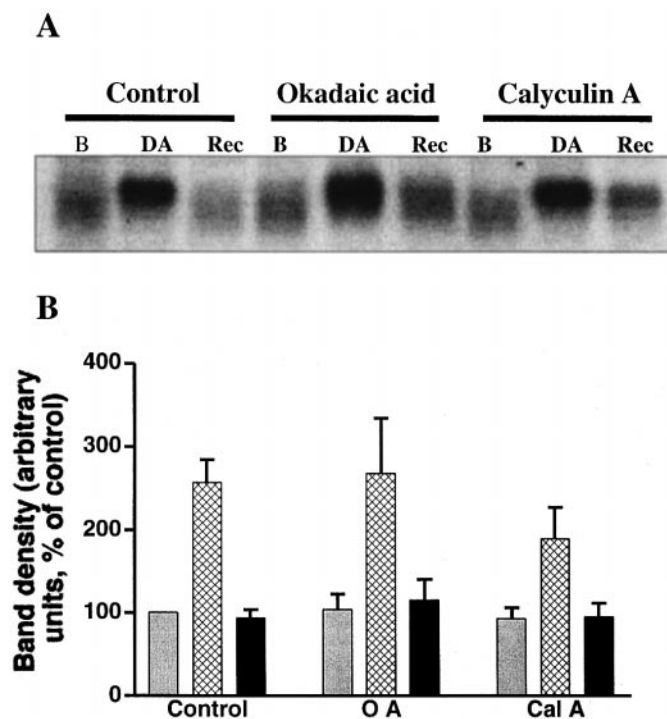


Fig. 11. The effect of protein phosphatase inhibitors on the dephosphorylation of D₁ dopamine receptors after pretreatment with dopamine. Whole-cell phosphorylation experiments were performed as described under *Experimental Procedures*. Before dopamine treatment, the cells were incubated with either vehicle (control), 1 μ M okadaic acid, or 10 nM calyculin A for 20 min. The cells were next treated with either vehicle or 10 μ M dopamine for 10 min. One sample from each group was then washed twice with EBSS (37°C) and further incubated in fresh medium without dopamine for 20 min. After treatment the samples were subjected to immunoprecipitation and resolved by 8% SDS-PAGE. A, representative autoradiogram showing receptor phosphorylation in basal (B), dopamine-stimulated (DA), and dopamine-stimulated followed by a 20-min recovery period (Rec) sample groups. B, the receptor phosphorylation obtained under the experimental conditions described in A were expressed as the mean \pm S.E.M. of three independent experiments. In each treatment group (■, DA-treated; ■, "recovered"), the phosphorylation data are presented as percentage above basal phosphorylation (■).

tion. Although the default hypothesis is that the observed D₁ DAR phosphorylation is predominantly mediated by one or more GRKs, additional experimentation will be required to directly demonstrate GRK involvement in this biochemical process.

The lack of effect of PKA activators and inhibitors on the D₁ DAR phosphorylation was especially interesting given that, in other cellular systems, intracellular activation of PKA has been shown to result in desensitization of D₁ mediated responses (Bates et al., 1991; Black et al., 1994). Also, Zhou et al. (1991) have shown that inhibition of PKA could partially attenuate agonist-induced desensitization of the D₁ DAR. In addition, we have recently observed that mutagenesis of Thr-268, which resides within a PKA recognition motif in the rat D₁ DAR, results in an attenuation of the rate, but not extent, of agonist-induced receptor desensitization (Jiang and Sibley, 1999). These effects of PKA, however, are not universally observed; a recent study using monkey D₁ DAR expressed in C6 glioma cells reported no effect of treatment with cAMP analogs on D₁ DAR responsiveness (Lewis et al., 1998). One possible explanation for these disparate results is that PKA may not directly phosphorylate the D₁ DAR and that the observed effects of cAMP analogs (Bates et al., 1991; Black et al., 1994) are caused by phosphorylation of downstream components. In this scenario, Thr-268 may be a substrate for a kinase other than PKA. Another possibility is that PKA does directly phosphorylate the D₁ DAR (on Thr-268 or elsewhere) but that agonist occupancy of the receptor is absolutely required and this is not mimicked by cAMP analogs. One would then predict that treatment of the cells with PKA inhibitors should diminish the dopamine-induced phosphorylation, but this was, in fact, not observed (Fig. 4). However, if the number of residues phosphorylated by GRKs greatly exceeds those that phosphorylated by PKA, then it might be difficult to detect a small reduction of phosphate content in the receptor through PKA inhibition. Obviously, final resolution of this issue will require the complete delineation of all the phosphorylation sites on the D₁ DAR using mutagenesis and other approaches.

To further investigate the role that phosphorylation plays in agonist-induced desensitization, we compared the dose-response and time course relationships for these two regulatory events. It was found that the potency ($EC_{50} \sim 200$ nM) of dopamine for inducing receptor phosphorylation was identical with that for inducing desensitization suggesting that these events are tightly linked. Interestingly, the potency of dopamine for inducing phosphorylation/desensitization was about 10-fold less than that for stimulating cAMP accumulation in the C6 glioma cells ($EC_{50} = \sim 20$ nM; see Fig. 7). This suggests that the phosphorylation/desensitization processes are less correlated with cAMP generation and are more highly correlated with the degree of receptor occupancy by agonists, which agrees well with previous findings on GRK-mediated phosphorylation reactions (Krupnick and Benovic, 1998; Lefkowitz, 1998).

A comparison of the rate of receptor phosphorylation with the rate of desensitization clearly indicates that phosphorylation of the D₁ DAR precedes desensitization. In our experiments, agonist-induced receptor phosphorylation was near maximal at the earliest measurable time point (1 min) whereas the $t_{1/2}$ for attenuation of the D₁ DAR mediated cAMP response was approximately 7 to 10 min. Clearly,

phosphorylation of the D₁ DAR is not the rate-limiting step in its desensitization. Presumably, subsequent events, such as the binding of an Arrestin protein to the phosphorylated receptor and/or the physical translocation of the receptor from its cognate G protein must be rate limiting. Surprisingly, the dopamine-induced receptor phosphorylation was transient in nature despite the continued presence of the agonist. Subsequent to maximal receptor phosphorylation (at 1–3 min of agonist exposure), the phosphate content of the D₁ DAR gradually declines to basal levels by 60 min with a $t_{1/2}$ of ~ 20 to 30 min. We investigated this in more detail using wash-out experiments and found that, after maximal phosphorylation and agonist removal, the phosphate content of the receptor returned to basal levels with a $t_{1/2}$ of about 10 min. Interestingly, the time course for receptor dephosphorylation seems to be similar to, or lag slightly behind, that for the on-set of desensitization. Surprisingly, after agonist washout, the dopamine-stimulated cAMP response recovers slowly, not attaining control levels of activity until after several hours of washout. This suggests that, despite its rapid dephosphorylation, the D₁ DAR does not recycle and recover in a rapid, simple fashion in C6 glioma cells.

Recent studies have shown that, after receptor phosphorylation, the β_2 -adrenergic receptor undergoes rapid endocytosis followed by dephosphorylation within an acidified vesicular compartment by a GRP that exhibits characteristics of the PP2A family of protein phosphatases (Pitcher et al., 1995). Given these observations, we predicted that inhibition of receptor internalization should prevent the rapid dephosphorylation of the D₁ DAR. In contrast, exposure of the cells to the plant lectin concanavalin A or hypertonic sucrose, treatments which were shown to block D₁ DAR internalization, did not block the rapid dephosphorylation of the D₁ DAR. This observation indicates that the D₁ DAR need not be internalized to be dephosphorylated. To further investigate the nature of the dephosphorylation reaction, we tested the effects of okadaic acid, a potent inhibitor of the GRP, and the phosphatase inhibitor calyculin A, both of which have been shown to inhibit the dephosphorylation of the β_2 -adrenergic receptor (Pitcher et al., 1995; Pippig et al., 1995; Krueger et al., 1997). Neither of these agents had any effect on the D₁ DAR dephosphorylation process suggesting the existence of a novel pathway which does not involve the recently identified GRP.

Based on all of these observations, we would like to propose the following hypothetical pathway for D₁ DAR phosphorylation/dephosphorylation in C6 glioma cells. Agonist occupancy of the D₁ DAR results in its rapid (seconds to minutes) phosphorylation by one or more GRKs. Phosphorylation of the D₁ DAR by PKA may also occur, although this is not a predominant reaction. After receptor phosphorylation, the D₁ DAR associates with an Arrestin protein that serves to uncouple the receptor and target it for internalization. The Arrestin-binding/receptor-translocation processes take place within minutes and represent the rate-limiting steps for D₁ DAR desensitization. Thus, although D₁ receptor phosphorylation is necessary, it is not sufficient for desensitization to take place. Subsequently, through a novel pathway or mechanism, the D₁ DAR is dephosphorylated during the translocation process before its removal from the cell surface and entry into internal endosomal compartments. The phosphatase responsible for this reaction is not the recently identified

GRP but may be a known or novel protein phosphatase and is most likely associated with the plasma membrane as opposed to intracellular domains. Once internalized, the D₁ DAR may recycle to the cell surface, but this is not a rapid event and represents the rate-limiting step for the receptor resensitization process. Although not investigated in the present study, prolonged (hr) agonist exposure may result in an additional down-regulation of D₁ DAR expression.

Recently, in an elegant series of studies (Zhang et al., 1997 and 1999; Oakley et al., 1999; Walker et al., 1999; Zhang et al., 1997 and 1999), Caron and colleagues have suggested that, for several G protein-coupled receptors, the rate-limiting step for receptor resensitization is their dephosphorylation. Moreover, it was suggested that the rate of receptor dephosphorylation is dependent on the rate of dissociation of an Arrestin protein from the phosphorylated receptor (Oakley et al., 1999). Thus, the β_2 -adrenergic receptor, which resensitizes quickly, exhibits rapid dissociation of β -Arrestin such that β -Arrestin is not internalized along with the receptor in HEK-293 cells (Oakley et al., 1999). Conversely, the V2 vasopressin receptor, which re-sensitizes slowly, exhibits delayed dissociation of β -Arrestin such that the receptor- β -Arrestin complex is cointernalized (Oakley et al., 1999). Interestingly, the D₁ DAR was shown to exhibit rapid β -Arrestin dissociation; however, neither the phosphorylation status of the D₁ DAR nor its rate of resensitization was examined in this previous study (Zhang et al., 1999). Obviously, given our current data, the rate of D₁ DAR re-sensitization in C6 cells seems to be more correlated with its rate of endosomal trafficking and recycling than with its rate of dephosphorylation. This suggests that the model of Caron and colleagues (Oakley et al., 1999) may not be applicable to all G protein-coupled receptors or to all cell types. An important issue that we are currently attempting to address is to examine directly the intracellular trafficking of the D₁ DAR in response to agonist treatment of the C6 cells to follow the receptor's fate and re-cycling once it undergoes internalization.

A review of the literature indicates that several other G protein-coupled receptors exhibit similar phenomena as we have observed here for the D₁ DAR. For instance, both the bradykinin B2 receptor expressed in human fibroblasts (Blaukat et al., 1996) and the vasopressin V1a receptor expressed in HEK-293 cells (Innamorati et al., 1998) exhibit rapid but transient phosphorylation in response to agonist treatment. Interestingly, as we have observed here with the D₁ DAR, okadaic acid was reported not to affect the dephosphorylation of the V1a vasopressin receptor (Innamorati et al., 1998). Similarly, the CCK receptor has been reported to undergo transient agonist-induced phosphorylation as well as a complex internalization process in acinar or CHO cells (Klueppelberg et al., 1991; Roettger et al., 1995). It was further suggested that the CCK receptor may be dephosphorylated in a smooth vesicular compartment adjacent to the plasma membrane. All of these observations suggest that our current findings with the D₁ DAR may have wide-spread applicability to many G protein-coupled receptors and different cell types.

References

- 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.
- Bates MD, Caron MG and Raymond JR (1991) Desensitization of DA₁ dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. *Am J Physiol* **260**:F937–F945.
- Black LE, Smyk-Randall EM and Sibley DR (1994) Cyclic-AMP-mediated desensitization of D₁ dopamine receptor-coupled adenylyl cyclase in NS20Y neuroblastoma cells. *Mol Cell Neurosci* **5**:567–575.
- Blaukat A, Alla SA, Loshe MJ and Muller-Esterl W (1996) Ligand-induced phosphorylation dephosphorylation of the endogenous bradykinin B2 receptor from human fibroblasts. *J Biol Chem* **271**:32366–32374.
- Carter AN (1995) Post-translational modification: Phosphorylation and phosphatases, in *Current Protocols in Protein Science* (Coligan JE, Dunn BM, Ploegh KL, Speicher DW and Einfeldt PT eds) pp. 13.8.11–13.8.15, John Wiley & Son, Inc., New York.
- Civelli O, Bunzow JR and Grandy DK (1993) Molecular diversity of the dopamine receptor. *Annu Rev Pharmacol Toxicol* **33**:281–307.
- Guan XM, Kobilka TS and Kobilka BK (1992) Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide. *J Biol Chem* **267**:21995–21998.
- Hawkins PT, Michell RH and Kirk CJ (1983) A simple assay method for determination of the specific radioactivity of the γ -phosphate group of ³²P-labelled ATP. *Biochem J* **210**:717–720.
- Huff RM (1997) Signaling pathways modulated by dopamine receptors in *The Dopamine Receptors* (Neve KA and Neve RL eds) pp. 167–192, Humana Press, Totowa, NJ.
- Innamorati G, Sadeghi H and Birnbaumer M (1998) Transient phosphorylation of the V1a vasopressin receptor. *J Biol Chem* **273**:7155–7161.
- Jiang D and Sibley DR (1997) Investigation of protein kinase A and C regulation of the D₁ dopamine receptor using site-directed mutagenesis techniques (Abstract). *Soc Neurosci Abstr* **23**:1773.
- Jiang D and Sibley DR (1999) Regulation of D₁ dopamine receptors with mutations of protein kinase phosphorylation sites: Attenuation of agonist-induced desensitization. *Mol Pharmacol* **56**:675–683.
- Klueppelberg UG, Gates LK, Gorelick FS and Miller LJ (1991) Agonist-regulated phosphorylation of the pancreatic cholecystokinin receptor. *J Biol Chem* **266**:2403–2408.
- Krueger KM, Daaka Y, Pitcher JA and Lefkowitz RJ (1997) The role of sequestration in G protein-coupled receptor resensitization: Regulation of β_2 adrenergic receptor dephosphorylation by vesicular acidification. *J Biol Chem* **272**:5–8.
- 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.
- Lefkowitz RJ (1998) G protein coupled receptors: New roles for receptor kinases and β -arrestins in receptor signaling and desensitization. *J Biol Chem* **273**:18677–18680.
- Levey AI, Hersch SM, Rye DB, Sunahara RK, Niznik HB, Kitt CA, Price DL, Maggio R, Brann MR and Ciliax BJ (1993) Localization of D₁ and D₂ dopamine receptors in brain with subtype-specific antibodies. *Proc Natl Acad Sci USA* **90**:8861–8865.
- 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.
- Monsma FJ, Mahan LC, McVittie LD, Gerfen CR and Sibley DR (1990) Molecular cloning and expression of a D₁ dopamine receptor linked to adenylyl cyclase activation. *Proc Natl Acad Sci USA* **87**:6723–6727.
- Neve KA and Neve RL (1997) Molecular biology of dopamine receptors, in *The Dopamine Receptors* (Neve KA and Neve RL eds) pp. 27–76, Humana Press, Totowa, NJ.
- Ng GYK, 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* **267**:7–19.
- Ng GYK, Trogadis J, Stevens J, Bouvier M, O'Dowd BF and George SR (1995) Agonist-induced desensitization of D₁ receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D₁ receptor internalization. *Proc Natl Acad Sci USA* **92**:10157–10161.
- Oakley RH, Laporte SA, Holt JA, Barak LS and Caron MG (1999) Association of β -arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem* **274**:32248–32257.
- Pippig S, Andexinger S and Lohse MJ (1995) Sequestration and recycling of β_2 adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**:666–676.
- Pitcher JA, Payne ES, Csontos C, Depauli-roach AA and Lefkowitz RJ (1995) The G protein-coupled receptor phosphatase: A protein phosphatase type 2A with a distinct subcellular-distribution and substrate-specificity. *Proc Natl Acad Sci USA* **92**:8343–8347.
- Robinson SW and Caron MG (1997) Interaction of dopamine receptors with G proteins, in *The Dopamine Receptors* (Neve KA and Neve RL eds) pp. 137–165, Humana Press, Totowa, NJ.
- Roettger BF, Rentsch RU, Pinon D, Holicky E, Hadac E, Larkin JM and Miller LJ (1995) Dual pathways of internalization of the cholecystokinin receptor. *J Cell Biol* **128**:1029–1041.
- Sibley DR and Monsma FJ (1992) Molecular biology of dopamine receptors. *Trends Pharmacol Sci* **13**:61–69.
- 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 and Caron, MG (1996) Differential regulation of the dopamine D_{1A} receptor responsiveness by various G protein-coupled receptor kinases. *J Biol Chem* **271**:3771–2778.
- 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.
- Walker JKL, Premont RT, Barak LS, Caron MG and Shetzline MA (1999) Properties of secretin receptor internalization differ from those of the β_2 adrenergic receptor. *J Biol Chem* **274**:31515–31523.
- Yu SS, Lefkowitz RJ and Hausdorff WP (1993) β adrenergic receptor sequestration: A potential mechanism of receptor resensitization. *J Biol Chem* **268**:337–341.
- Zhang J, Barak LS, Anborgh PH, Laporte SA, Caron MG and Ferguson SSG (1999) Cellular trafficking of G protein-coupled receptor/ β -arrestin endocytic complexes. *J Biol Chem* **274**:10999–11006.
- Zhang J, Barak LS, Winkler KE, Caron MG and Ferguson SSG (1997) A central role for β -arrestins and clathrin-coated vesicle-mediated endocytosis in β_2 adrenergic receptor resensitization. *J Biol Chem* **272**:27005–27014.
- Zhou X, 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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