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Regulation of D_1 Dopamine Receptors with Mutations of Protein Kinase Phosphorylation Sites: Attenuation of the Rate of Agonist-Induced Desensitization

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ABSTRACT

Investigations of D_1 receptor regulation have suggested a role for cAMP-dependent protein kinase (PKA) in agonist-induced desensitization and down-regulation of receptor expression. Given the presence of at least four possible consensus recognition sites for PKA on the D_1 receptor protein, a reasonable hypothesis is that some of these PKA-mediated effects are caused by phosphorylation of the receptor. As an initial test of this hypothesis, we used site-directed mutagenesis to create a mutant D_1 receptor with substitutions at each of its four potential PKA phosphorylation sites. The modified amino acids are as follows: Thr135 to Val, Ser229 to Ala, Thr268 to Val, and Ser380 to Ala. Characterization of the wild-type and mutant receptors stably expressed in C6 glioma cells suggests that the mutations have no effect on receptor expression, antagonist or agonist affinities, or on functional coupling with respect to cAMP generation. Similarly, dopamine preincubation of the stably transfected C6 cells expressing either the wild-type or mutated D_1 receptors results in an agonist-induced loss of ligand binding activity (down-regulation) in an identical fashion. In contrast, the time of onset of dopamine-induced desensitization is greatly attenuated in the quadruple mutant receptor. After 1 h of dopamine pretreatment, the wild-type receptor exhibits ~80% desensitization of the cAMP response, whereas the mutant receptor is desensitized by only ~20%. Further analyses of single mutated receptors, in which only one of the four putative phosphorylation sites is modified, reveals that Thr268 in the third cytoplasmic loop of the receptor protein is primarily responsible for regulating the desensitization kinetics. These results are consistent with the hypothesis that phosphorylation of the D_1 receptor on Thr268 is important for rapid agonist-induced homologous desensitization.

Dopamine receptors belong to a large family of receptor proteins whose actions are mediated through the activation of heterotrimeric G proteins. Thus far, five distinct genes encoding different dopamine receptor proteins have been isolated and characterized (Neve and Neve, 1997). The protein products of these genes are structurally and pharmacologically distinct but can be divided into two major subfamilies, referred to as D_1- and D_2-like receptors. The D_1-like receptor subfamily is composed of two members, the D_1 and D_5 receptors, also known as the D_{1A} and D_{1B} subtypes. In contrast, the D_2 subfamily consists of three receptors, the D_2, D_3, and D_4 subtypes. In addition to their structural and pharmacological dissimilarities, the D_1- and D_2-like subfamilies differ in their G protein coupling and transductional properties (Huff, 1997; Robinson and Caron, 1997). The D_1-like receptors generally couple to G_s, resulting in stimulation of adenylyl cyclase activity and increased levels of the second messenger cAMP. In contrast, the D_2-like receptors exhibit coupling to $G_{i/o}$-like proteins resulting in modulation of various ion channels and/or depression of adenylyl cyclase activity. Like other G protein-coupled receptors, dopamine receptors are subject to a variety of regulatory mechanisms that modulate their expression, functional activity, and G protein coupling (Sibley and Neve, 1997).

Regulatory mechanisms that modulate signaling by G protein-coupled receptors have been extensively studied for the β-adrenergic receptor systems (Hausdorff et al., 1989; Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). An emerging concept from these studies is that protein phosphorylation plays a pivotal role in controlling the functional activity of the receptor proteins. In general, two major classes of protein kinases have been shown to be important for mediating this phosphorylation. These include the G protein-coupled receptor kinases (GRKs), which phosphorylate only the agonist occupied or activated form of the receptor protein and are believed to be critical for homologous, or agonist-specific, forms of desensitization (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). In addition, there are second messenger-activated protein kinases, such as the cAMP-dependent protein kinase (PKA), which can phosphor-

ABBREVIATIONS: GRK, G protein-coupled receptor kinase; PKA, cAMP-dependent protein kinase; EBSS, Earle's balanced salt solution.

ylate G protein-coupled receptors in an agonist-independent fashion (Hausdorff et al., 1989). Although initially thought to be important in only heterologous or nonspecific forms of receptor desensitization, recent data has suggested that second messenger-activated protein kinases may play important roles in homologous, or agonist-specific forms, of receptor desensitization (Chuang et al., 1996; Post et al., 1996). In this case, receptor phosphorylation by the second messenger-activated kinase would constitute a typical negative feedback loop.

The role of protein phosphorylation in agonist-induced desensitization of dopamine receptors is only beginning to be addressed. Among the various subtypes, perhaps the most information has been generated on the D_1 receptor. This receptor has been shown to exhibit agonist-induced refractoriness in both endogenous and recombinant/heterologous cellular expression systems (see Sibley and Neve, 1997 for review). Previous studies have shown that intracellular activation of PKA can partially mimic agonist-induced desensitization of D_1 receptors, thereby suggesting a role for this kinase in D_1 receptor desensitization (Bates et al., 1991; Black et al., 1994). Furthermore, Zhou et al. (1991) have found that intracellular inhibitors of both PKA and GRKs could attenuate D_1 receptor desensitization, thus implying a role for both kinase systems. In contrast, Bates et al. (1993) and Lewis et al. (1998) have provided data arguing that PKA is not important for agonist-induced D_1 receptor desensitization. More recent studies, involving the expression of D_1 receptors in Sf9 (Ng et al., 1994) or human embryonic kidney 293 cells (Tiberi et al., 1996), have shown that the D_1 receptor undergoes agonist-induced phosphorylation and that in the human embryonic kidney 293 cells, this phosphorylation is enhanced by coexpression of GRKs 2, 3, and 5. Taken together, all of these studies imply a role for both PKA- and GRK-mediated phosphorylation events in D_1 receptor desensitization, although the relative importance of each remains to be determined.

Although previous studies of D_1 receptor regulation have suggested a role for PKA in agonist-induced desensitization, the mechanism by which PKA contributes to this process remains to be established. Given the presence of at least four potential consensus recognition sites for PKA on the D_1 receptor protein, a reasonable hypothesis is that some of the PKA-mediated effects are caused by direct phosphorylation of the receptor. As a first approach to investigating this possibility, we have created mutant D_1 dopamine receptors with substitutions at each of the four potential PKA phosphorylation sites using site-directed mutagenesis techniques. We now show that these mutations substantially attenuate the rate of agonist-induced desensitization of the D_1 receptor. These results are consistent with the hypothesis that direct phosphorylation of the D_1 receptor is important for rapid agonist-induced homologous desensitization.

Experimental Procedures

Materials. C6 Glioma cells were purchased from American Type Culture Collection (Rockville, MD). [^{3}H]SCH-23390 (70 to 71.3Ci/mmol; *R*(+)-6-chloro-7,8-dihydroxy-3-allyl-1-phenly-2,3,4,5-tetrahydro-1*H*-3-benzazepine) and [^{3}H]cAMP (31.4 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). Dopamine, forskolin, RO-201724 (4-[(butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone), (−)-propranolol and (+)-butaclamol were purchased from Research Biochemicals Inc. (Natick, MA). cAMP assay kits were obtained from Diagnostic Products Corp. (Los Angeles, CA). Cell culture media and reagents were purchased from Life Technologies (Grand Island, NY). Fetal calf serum was purchased from Summit Biotechnology (Purchase, CO) and calcium phosphate transfection kits were from In-Vitrogen (San Diego, CA). All other reagents were of highest quality available and obtained from commercial suppliers.

Cell Cultures. C6 glioma cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cell cultures were grown at 37°C in 5% CO_2. For radioligand binding assays, the transfected C6 cells expressing either wild-type or mutant D_1 receptors were plated on 150- × 20-mm culture dishes until achieving 90% confluence. On the day of the assay, the cells were pretreated with 10 μM dopamine in the media containing 0.1 mM sodium metabisulfite for the indicated time periods at 37°C. The cells were washed three times with Earle's balanced salt solution (EBSS; pH 7.4) and scraped off for membrane preparation.

DNA Constructs and Plasmid Transfection. The rat D_1 receptor cDNA was mutagenized at amino acid positions Thr135, Ser229, Thr268, and/or Ser380 by a site-directed mutagenesis technique using the Transformer Site-Directed Mutagenesis Kit from Clontech (Palo Alto, CA). The resulting receptor constructs were subcloned into the *Not*I site of the mammalian expression vector pCD-SRα (Takebe et al., 1988) and the complete D_1 receptor sequences were confirmed by DNA sequencing. The wild-type and mutant D_1 receptor constructs (30 μg) were then cotransfected with the pMAM-neo plasmid DNA (3 μg) into C6 glioma cells using the calcium phosphate precipitation method (calcium phosphate transfection kit; Invitrogen). In brief, cells were seeded in 150-mm^2 plates. Transfection was carried out after 30 to 40% confluence was achieved. DNA and 60 μl of 2 M $CaCl_2$ were mixed in water in a total volume of 500 μl, which was then slowly mixed with 500 μl of HEPES-buffered saline. The reaction mixture was incubated at 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 μg/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 Assay. Cells were harvested by incubation with 5 mM EDTA in EBSS and collected by centrifugation at 300*g* for 10 min. The cells were resuspended in lysis buffer (5 mM Tris, pH 7.4, at 4°C and 5 mM $MgCl_2$) and were disrupted using a Dounce homogenizer, followed by centrifugation at 34,000*g* 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 $CaCl_2$, 4 mM $MgCl_2$, 120 mM NaCl). The membrane suspension (final protein concentration, 50 μg/tube) was then added to assay tubes containing 0.015 to 2 nM [^{3}H]SCH-23390 in a final volume of 0.5 ml. (+)-Butaclamol was added at the final concentration of 1 μM 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 quantitated by liquid scintillation spectroscopy at a counting efficiency of 47%.

Determination of cAMP Production. C6 glioma cells expressing either the wild-type or the mutant D_1 receptors were seeded into 96-well plates (50,000 to 60,000 cells/well) and cultured using charcoal-treated fetal calf serum for 1 day before the experiment. To assess desensitization, the cultures were first preincubated in the absence or presence of dopamine or forskolin with 0.5 mM L-ascorbic acid for the indicated time periods. Subsequently, the cells were washed three times with 200 μl of ice-cold EBSS and were further incubated with various concentrations of dopamine in a total volume of 100 μl at 37°C for 15 min in the presence of 0.1 mM RO-021724, 1

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mM L-ascorbic acid, and 1 μM (−)-propranolol. The reaction was terminated by discarding the supernatant and adding 100 μl of 3% perchloric acid per well. After incubating on ice for 15 min, 40 μl of 15% $KHCO_3$ was added to the wells and the plates were further incubated for 5 min. The plates were then centrifuged for 10 min at 1300*g* and 50 μl of the supernatant from each well was subsequently transferred to a 1.2-ml tube containing 250 μl of reaction mixture (150 μl of Tris-EDTA buffer, 50 μl of cAMP binding protein, and 50 μl of [^{3}H]cAMP). After incubation at 4°C overnight, 250 μl of charcoal-dextran mix (1%) was added to each tube, which was then incubated at 4°C for 15 min followed by centrifugation for 15 min at 1300*g*. 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 the protocol of the assay kit.

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. Estimation of the radioligand binding parameters, K_d, B_{max}, and EC_{50} values for dopamine-stimulation of cAMP production were calculated using the GraphPad Prizm curve-fitting program (GraphPad Software, San Diego, CA). The curves presented throughout this manuscript, representing the best fits to the data, were generated using this software program as well.

Results

Generation of Mutant D_1 Receptor Constructs. As an initial approach, we examined the predicted cytoplasmic regions of the rat D_1 dopamine receptor for potential consensus recognition sequences for PKA-mediated phosphorylation. A survey (Kennelly and Krebs, 1991) of PKA-mediated phosphorylation sites indicates that the presence of basic amino acids, particularly arginine, in the amino terminus of the phosphoacceptor serine or threonine is a key factor in the substrate recognition of PKA. A rank order of preferred consensus sequences for PKA has been suggested to consist of R-R/K-X-S/T > R-X_2-S/T = R-X-S/T, which describes most of the sequences that have been surveyed. Scanning of the cytoplasmic regions of the D_1 receptor reveals four such serine or threonine residues within the context of a consensus recognition sequence for PKA (Fig. 1). One of these residues (Thr135) is found in the second intracellular loop of the receptor, two others (Ser229 and Thr268) are present in the third cytoplasmic loop, and the fourth residue (Ser380) is in the long carboxyl terminus of the receptor protein. Using site-directed mutagenesis methods, we modified these phosphoacceptor sites by changing the serines to alanines and the threonines to valines (amino acids of comparable volume) so as to preclude potential phosphorylation of these sites. One construct was created in which all four of these residues were simultaneously mutated (the quadruple mutant or Mut Q) whereas four other constructs were created, each of which contained only a single amino acid mutation (Muts I-IV).

Expression of the Wild-Type and Mutant D_1 Receptors in C6 Glioma Cells. All of the mutated D_1 receptors, along with the wild-type receptor, were stably expressed in C6 glioma cells for further characterization and analysis. We initially examined the ligand binding properties of the mutant and wild-type receptors. Figure 2 shows Scatchard plots of saturation-binding isotherms for the wild-type and quadruple mutant D_1 receptors using membranes prepared from the transfected C6 glioma calls. As can be seen, both of these constructs bind the D_1 selective radioligand, [^{3}H]SCH-23390, with similar affinities. Moreover, we were able to select cell lines expressing similar levels of receptor expression, indicating that the quadruple mutant receptor can be expressed

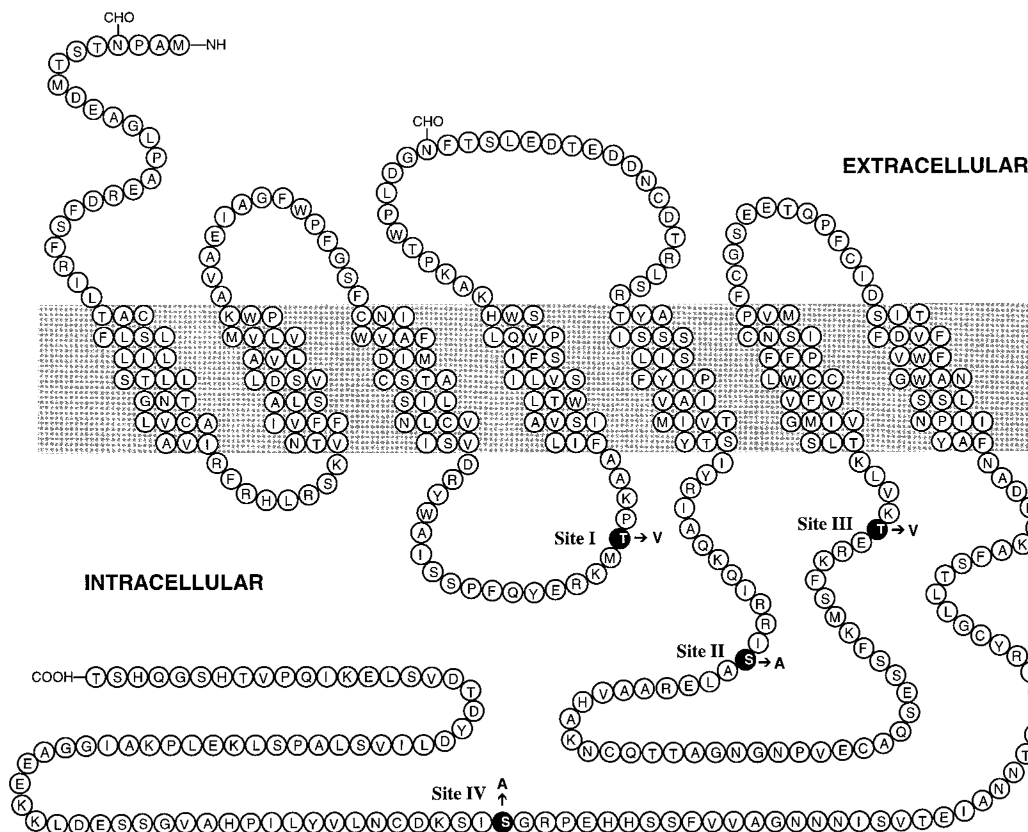

Fig. 1. Sites of potential PKA-mediated phosphorylation on the rat D_1 dopamine receptor protein. The membrane topography of the D_1 receptor was predicted from hydropathy analysis and the four solid circles indicate potential sites for PKA phosphorylation in the protein (see text). In one mutant construct (the quadruple mutant), all four potential PKA phosphorylation sites were simultaneously mutated to the indicated amino acids (T→V and S→A). Four additional mutant constructs were made in which only one the indicated residues was mutated: Thr135 (site I), Ser229 (site II), Thr268 (site III), or Ser380 (site IV).

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to the same degree as the wild-type construct. Table 1 summarizes the radioligand binding data for all of the mutant and wild-type D_1 receptors stably expressed in C6 glioma cells. There do not seem to be any noticeable differences in the ligand binding properties of these receptor constructs and we were able to select cell lines with similar levels of receptor expression for further characterization.

Figure 3 shows dose-response curves for dopamine-stimulation of cAMP accumulation in intact C6 glioma cells expressing either the wild-type or quadruple mutant receptors. As can be seen, there are no noticeable differences in the two receptor constructs for promoting this response—dopamine exhibits a similar EC_{50} value for stimulation in addition to a similar maximum cAMP response (V_{max}). Table 1 summarizes the cAMP response data for all of the mutant and wild-type D_1 receptors stably expressed in C6 glioma cells. As with the radioligand binding data, there are no noticeable differences between the various D_1 receptor constructs for this response. All of the receptors stimulated cAMP production to a similar extent and exhibited a similar potency for dopamine stimulation. Taken together, the data in Figs. 2 and 3 and Table 1 indicate that the mutations introduced into the D_1 receptor have no effect on the receptor's ability to be expressed, bind antagonist (SCH-23390) or agonist (dopamine) ligands, or couple efficiently to generate cAMP within the cell.

Characterization of Agonist-Induced Desensitization and Down-Regulation of the Wild-Type and Quadruple Mutant Receptors. To maximize the opportunity to see an effect of the mutagenesis, we decided to initially characterize the regulatory properties of the quadruple mutated D_1 receptor. Figure 4 shows an experiment in which we have examined the agonist-induced loss of receptor-ligand binding activity after dopamine pretreatment of the cells. In Fig. 4 (top), it can be seen that dopamine pretreatment of cells expressing the wild-type receptor results in a loss of subsequently examined radioligand-binding activity. This loss of ligand binding activity is primarily manifested as a reduction in the maximum binding capacity (B_{max}) with little or no change in the affinity (K_d) of the radioligand. Furthermore, the loss of radioligand binding seems to be time-dependent; a greater reduction is observed at 3 h of dopamine pretreatment than at 1 h. Most importantly, there does not seem to be any difference in the down-regulation (defined as the loss of binding activity) response of the quadruple mutant receptor (Fig. 4, bottom) compared with the wild-type receptor.

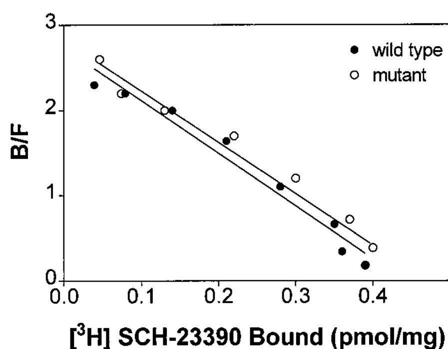

Fig. 2. Saturation analysis of [^{3}H]SCH-23390 binding to wild-type and quadruple mutant D_1 receptors. Membranes of the stably transfected C6 cells expressing either the wild-type or quadruply mutated D_1 receptors were prepared and assayed for [^{3}H]SCH23390 binding as described in *Experimental Procedures*. The saturation binding isotherms are presented in Scatchard coordinates of bound/free versus bound radioligand. Computer analysis of the data reveals the following ligand binding parameters: wild-type receptor, K_D = 0.16 nM and B_{max} = 0.44 pmol/mg membrane protein; mutant receptor, K_D = 0.17 nM and B_{max} = 0.47 pmol/mg membrane protein. A single experiment is shown that is representative of three such experiments. Average ligand binding parameters from three experiments are shown in Table 1.

To compare the agonist-induced down-regulation of the wild-type and quadruple mutant receptor constructs in more detail, we performed a time course experiment for the dopamine-induced loss of radioligand binding activity. Figure 5 definitively shows that there is not a difference in the two receptor constructs for this response—both receptors exhibit a $T_{1/2}$ of about 2 h for receptor loss and both receptors are maximally down-regulated (by about 75%) after 7 h. The results in Figs. 4 and 5 thus suggest that the site mutations have no effect on the receptor's ability to undergo agonist-induced down-regulation on agonist exposure and receptor activation.

We next tested the wild-type and quadruple mutant D_1 receptors for their ability to undergo functional agonist-induced desensitization. Figure 6 (top) shows the effects of pretreating cells expressing the wild-type D_1 receptor on the subsequent ability of dopamine to stimulate cAMP production. As can be seen, there is a time-dependent loss of dopamine stimulation of cAMP production such that after about 2 h of dopamine pretreatment, there is a nearly complete loss of this response. Also, it can be observed that the desensitization of the response involves a reduction in potency (increase in EC_{50} value) for dopamine stimulation of cAMP accumulation in addition to a reduction in the maximum response (V_{max}). Figure 6 (bottom) shows a similar experiment, in which cells expressing the quadruple mutant receptor were pretreated with dopamine for increasing amounts of time. It can be readily seen that this receptor also exhibits an agonist-induced functional desensitization response; however, the onset of the desensitization seems to occur much more slowly compared with that of the wild-type receptor (compare Fig. 6, top and bottom).

Because the mutant receptor also seems to demonstrate an agonist-induced increase in EC_{50} value and a reduction in V_{max} for dopamine-stimulated cAMP production, we thought it would be informative to compare these two parameters as a function of dopamine pretreatment time for both the wild-type and mutant receptors. Figure 7 shows the agonist-induced increase in EC_{50} values as determined from dose-response curves that were generated in experiments similar to those shown in Fig. 6. As can be seen, there is a progressive time-dependent decrease (increase in EC_{50} values) in the potency for dopamine to stimulate cAMP production. Importantly, there does not seem to be any noticeable difference between the wild-type and mutant receptors for this agonist-induced shift in the dopamine dose-response curve. Because the EC_{50} value of the dopamine dose-response curve could not be reliably calculated after 1 h of dopamine pretreatment using the wild-type receptor (see Fig. 6), we did not extend the analysis beyond this time point. Consequently, we could not determine a maximum effect for the shift in EC_{50}, although it does seem to be leveling off between 40 and 60 min (Fig. 7).

Figure 8 shows the relationship between the maximum response (V_{max}) for dopamine-stimulated cAMP production

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and time of dopamine pretreatment of the cells. The onset of desensitization for this functional parameter occurs relatively rapidly for the wild-type receptor exhibiting a $T_{1/2}$ of about 20 min with a maximum desensitization occurring at about 1 h. In striking contrast, the desensitization of the mutant receptor is slower in onset, with a $T_{1/2}$ of about 75 min and does not exhibit a maximum response until >150 min. The data in Figs. 6 to 8 thus indicate that although the site mutations have no effect on the dopamine-induced shift in agonist potency for cAMP production, these mutations dramatically reduce the time of onset for desensitization of the maximum response. Because reduction of the maximum response seems to be a greater determinant of desensitization (see Fig. 6), the mutant D_1 receptor is significantly impaired in its agonist-induced desensitization response.

Characterization of Agonist-Induced Desensitization of the Single Point Mutant Receptors. Because the quadruple mutant receptor was significantly delayed in its onset of agonist-induced desensitization, it was important to determine which of the putative phosphorylation sites was responsible for this effect. We thus examined each of the single point-mutated receptors for their ability to undergo agonist-induced desensitization. We chose a single time point for dopamine pretreatment (1 h), which exhibited the largest difference in the wild-type and mutant receptors (compare Fig. 8). Figure 9 shows dopamine dose-response curves for cAMP generation in cells expressing the wild-type and all mutant receptors with and without dopamine pretreatment for 1 h. Figure 9, a and f, replicates the results for the wild-type and quadruple (Q) mutant receptors, respectively, as was shown in Fig. 6. Figure 9, b through e, show the results for the single point mutant receptors (sites I to IV; Fig. 1). It is readily apparent that site mutants I, II, and IV show results similar to those observed with the wild-type receptor. In contrast, site mutant III seems to show results that are more similar to those of the quadruple (Q) mutant receptor (Fig. 9, d and f). Based on these results, we are concluding that it is the mutation of site III, Thr268 in the carboxyl end of the third cytoplasmic loop of the receptor (Fig. 1) that results in an attenuation of the rate of agonist-induced desensitization of the D_1 receptor.

Forskolin-Induced Desensitization of D_1 Receptor-Mediated cAMP Accumulation. As previously noted, prior studies have shown that intracellular activation of PKA can partially mimic agonist-induced desensitization of D_1 receptors (Bates et al., 1991; Zhou et al., 1991; Black et al., 1994). Consequently, we thought it necessary to examine the effects of raising intracellular levels of cAMP in the absence of D_1 receptor activation using forskolin, which potently elevates cAMP levels in intact C6 cells (data not shown). Figure 10 shows experiments using C6 cells transfected with either the wild-type (Fig. 10, top) or the quadruple mutant (Fig. 10, bottom) D_1 receptors. As can be seen, treatment of the cells with forskolin results in a 25 to 30% reduction in the maximum cAMP response to dopamine, an effect that is maximal between 2 and 3 h of pretreatment. Forskolin treatment did not seem to alter the potency of dopamine for elevating cAMP levels. Interestingly, there did not seem to be any differences between the wild-type and mutant receptors with respect to the forskolin-induced desensitization of the D_1 receptor-mediated response (compare Fig. 10, top and bottom).

Discussion

Previous studies have indicated that agonist-induced regulation of catecholamine receptors is complex process that involves multiple mechanisms. Using β-adrenergic receptors as model systems, evidence has been provided for at least two regulatory pathways. One involves agonist-stimulated phosphorylation of the receptor protein by a member(s) of the GRK family of protein kinases, which leads to functional uncoupling of the receptor and subsequent binding of a member of the arrestin family (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). Arrestin binding to the phosphorylated receptor results in further uncoupling and may also target the receptor for internalization (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). A second regulatory pathway involves the phosphorylation of the receptor by PKA (Hausdorff et al., 1989). This results in functional uncoupling of the receptor, although the exact mechanisms by which this occurs is unclear. Originally thought not to be important in agonist-specific forms of catecholamine receptor desensitization, recent findings (including our current data) have suggested that this may not be entirely correct (Chuang et al., 1996; Post et al., 1996).

Similar to the β-adrenergic receptors, evidence has accumulated that suggests a role for both PKA- and GRK-mediated phosphorylation events in D_1 receptor desensitization, although the mechanism and relative importance of each remains to be determined. In this study, we have investigated the possibility that some of the PKA-mediated effects may involve phosphorylation of the D_1 receptor. Site-directed mutagenesis techniques were used to alter each of the putative PKA phosphorylation sites in the D_1 receptor protein followed by heterologous expression in C6 glioma cells. The C6 cells seemed to represent good transfection hosts to study

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TABLE 1
Characterization of D_1 wild-type and mutant receptors
The wild-type and mutant D_1 receptor constructs, including the quadruple mutant (Mut Q) and the single point mutants, were stably expressed in C6 glioma cells and examined for their radioligand binding and functional properties as described in Figs. 2 and 3. The data represent mean ± S.E.M. values from three independent experiments.

D_1 Construct	[^{3}H]SCH-23390 Binding		cAMP Production	
	B_{max}	K_d	V_{max}	EC_{50}
	pmol/mg of protein	*nM*	*pmol/60,000 cells*	*μM*
Wild-type	0.43 ± 0.03	0.17 ± 0.01	22.2 ± 2.2	0.09 ± 0.014
Mut I	0.47 ± 0.03	0.21 ± 0.03	24.7 ± 3.0	0.05 ± 0.001
Mut II	0.42 ± 0.01	0.25 ± 0.03	27.4 ± 2.3	0.10 ± 0.007
Mut III	0.27 ± 0.04	0.26 ± 0.04	22.9 ± 2.5	0.19 ± 0.015
Mut IV	0.42 ± 0.07	0.23 ± 0.05	28.3 ± 2.9	0.09 ± 0.009
Mut Q	0.40 ± 0.07	0.18 ± 0.01	23.6 ± 3.2	0.12 ± 0.015

agonist-induced regulation of the D_1 receptor; the time courses for agonist-induced desensitization and down-regulation of receptor binding seemed to be almost identical with those observed previously in NS20Y cells, which endogenously express the D_1 receptor (Barton and Sibley, 1990). Strikingly, we found that mutagenesis of the putative phosphorylation sites on the D_1 receptor significantly attenuated its rate of agonist-induced desensitization. This effect was caused by an attenuation of the desensitization of the maximum cAMP response to dopamine, whereas there was no effect on the reduction of agonist potency after dopamine pretreatment. The impaired desensitization could not have been caused by reduced agonist activation or coupling of the mutated receptors because there were no differences in their

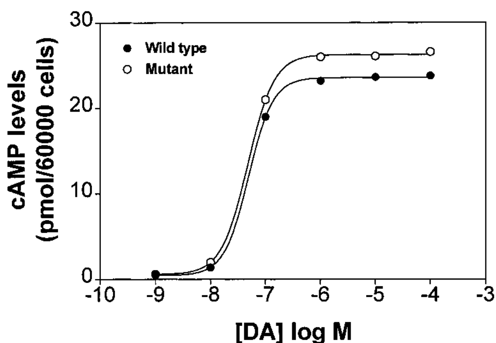

Fig. 3. Dopamine dose-response curves for cAMP production in wild-type and quadruple mutant D_1 receptors. C6 glioma cells stably transfected with either wild-type or quadruple mutant rat D_1 receptors were stimulated with 10^{-9} to 10^{-4} M of dopamine (DA) for 15 min at 37°C. Accumulation of cAMP was measured as described in *Experimental Procedures*. The calculated EC_{50} value for the wild-type D_1 receptor was 59 nM, whereas the EC_{50} value for the mutant receptor was 57 nM. The data shown are representative of three independent experiments.

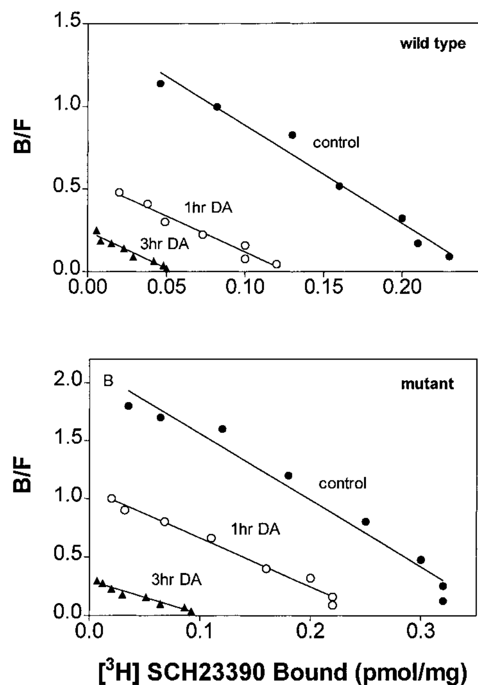

Fig. 4. Dopamine-induced loss of D_1 receptor binding activity. C6 cells expressing either the wild-type (top) or quadruple mutant (bottom) D_1 receptors were incubated in the absence (control) or presence of 10 μM dopamine (DA) for 1 or 3 h at 37°C. The cells were then washed and used to prepare membranes for [^{3}H]SCH-23390 binding assays as described in *Experimental Procedures*. The saturation binding isotherms are presented in Scatchard coordinates of bound/free versus bound radioligand. A representative experiment is shown and was performed three times with similar results.

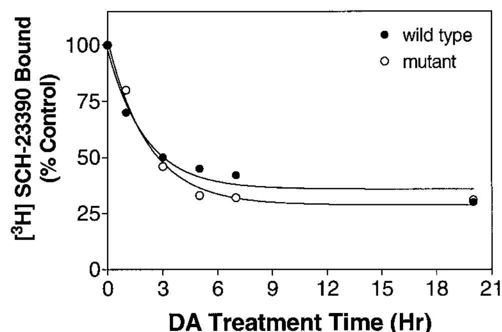

Fig. 5. Time course of dopamine-induced loss of D_1 receptor binding activity. Stably transfected C6 glioma cells expressing either wild-type or quadruple mutant D_1 receptors were pretreated with media alone (control) or with 10 μM dopamine at 37°C for the indicated time periods. Cells were subsequently washed and membranes were prepared for radioligand binding assessments using 1 nM [^{3}H]SCH-23390 as described in *Experimental Procedures*. The data are expressed as a percentage of the control [^{3}H]SCH-23390 binding for each treatment group. The results shown are representative of three independent experiments.

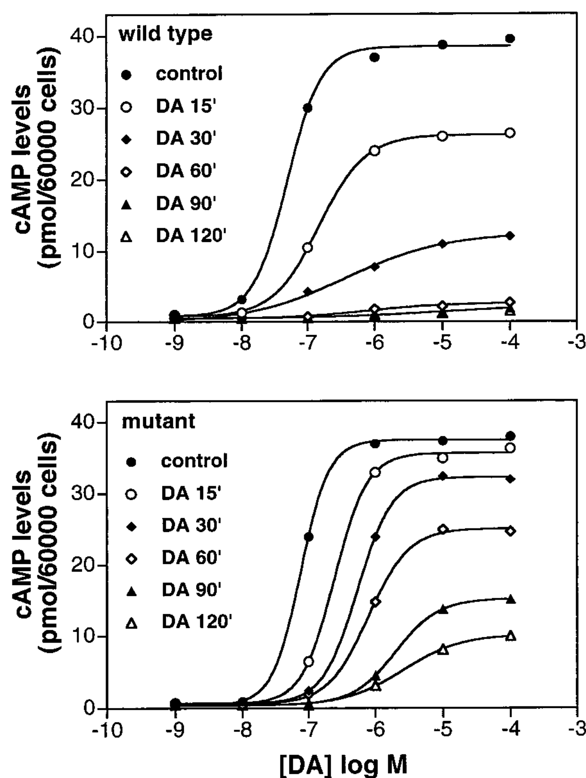

Fig. 6. Dopamine-induced desensitization of D_1 receptor-mediated cAMP generation. Stably transfected C6 glioma cells expressing either wild-type (top) or quadruple mutant (bottom) D_1 receptors were pretreated with media alone (control) or with 10 μM dopamine at 37°C for the indicated time periods. Cells were subsequently washed and then stimulated with 10^{-9} to 10^{-4} M dopamine (DA) for 15 min at 37°C. Accumulation of cAMP was measured as described in *Experimental Procedures*. The calculated EC_{50} values for the generated curves are as follows. Wild-type receptor: control, 0.05 μM; 15 min dopamine, 0.15 μM; 30 min dopamine, 0.37 μM; 60 min dopamine, 0.81 μM; 90 and 120 min dopamine, not determinable. Mutant receptor: control, 0.07 μM; 15 min dopamine, 0.25 μM; 30 min dopamine, 0.54 μM; 60 min dopamine, 0.8 μM; 90 min dopamine, 2 μM; and 120 min dopamine, 2.6 μM. The experiment shown is representative of three independent experiments.

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ability to stimulate cAMP accumulation compared with the wild-type receptor.

Further analyses of single mutated receptors, in which only one of the four putative phosphorylation sites is modified, revealed that Thr268 in the carboxyl end of third cytoplasmic loop of the receptor protein is primarily responsible for regulating the desensitization kinetics. Although it is conceivable that other cryptic PKA phosphorylation sites might exist in the D_1 receptor, Thr268 is located in an ideal location to influence functional G protein coupling of the D_1 receptor. Previous studies (Neve and Neve, 1997; Robinson and Caron, 1997) using site-directed mutagenesis and receptor chimeras have suggested that the G_s coupling regions reside within the third cytoplasmic loop of the D_1 receptor protein. Moreover, mutagenesis work in other G protein-coupled receptor systems has suggested that G protein coupling regions are frequently located in areas of the intracellular loops near the plasma membrane (Wess, 1997), which is the location of Thr268 in the D_1 receptor.

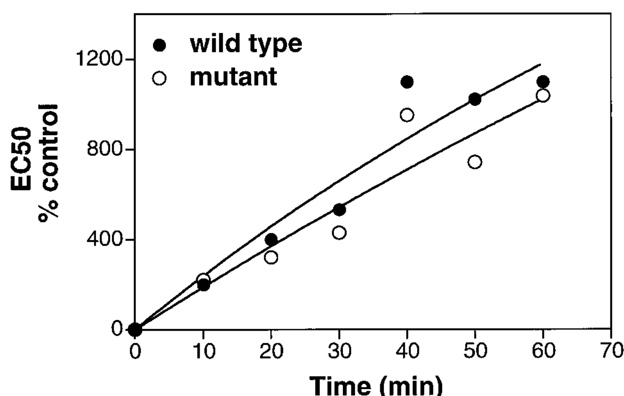

Fig. 7. Time course for the effect of dopamine pretreatment on the EC_{50} value for D_1 receptor-mediated cAMP generation. Stably transfected C6 glioma cells expressing either wild-type or quadruple mutant D_1 receptors were pretreated with media alone (control) or with 10 μM dopamine at 37°C for the indicated time periods. Cells were subsequently washed and then stimulated with 10^{-9} to 10^{-4} M dopamine for 15 min at 37°C. Accumulation of cAMP was measured as described in *Experimental Procedures*. The EC_{50} values were derived from the resulting dopamine dose-response curves as described in Fig. 6 and Table 1. The EC_{50} values from the control groups of either the wild-type or mutant receptors were designated as 100% and all other EC_{50} values (dopamine pretreated) were expressed a percentage of these control values and plotted as a function of dopamine pretreatment time. The data presented are representative of two independent experiments.

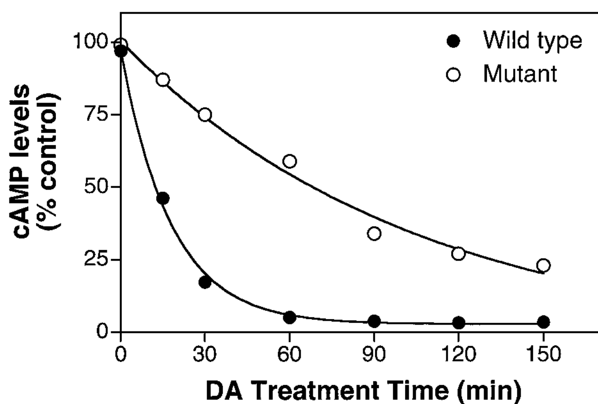

Fig. 8. Time course for the effect of dopamine-pretreatment on the V_{max} for D_1 receptor-mediated cAMP generation. Stably transfected C6 glioma cells expressing either wild-type or quadruple mutant D_1 receptors were pretreated with media alone (control) or with 10 μM dopamine at 37°C for the indicated time periods. Cells were subsequently washed and then stimulated with 10^{-9} to 10^{-4} M dopamine for 15 min at 37°C. Accumulation of cAMP was measured as described in *Experimental Procedures*. The V_{max} values were derived from the resulting dopamine dose-response curves as described in Fig. 6 and Table 1. The V_{max} values from the control groups of either the wild-type or mutant receptors were designated as 100% and all other V_{max} values (dopamine pretreated) were expressed a percentage of these control values and plotted as a function of dopamine pretreatment time. The data presented are representative of three independent experiments.

Although mutation of Thr268 attenuated the *rate* of agonist-induced desensitization, the receptor was still able to undergo complete functional desensitization. This observation, as well as the finding that the site mutations had no effect on the dopamine-induced shift in agonist potency, argues strongly for the existence of another regulatory pathway, presumably mediated by GRK phosphorylation and arrestin binding. Given this observation, it is interesting to speculate on the presumed mechanism by which phosphorylation of Thr268 may accelerate desensitization of the D_1 receptor. One possibility is that, as discussed above, phosphorylation of Thr268 impairs its ability to activate G_s and that this functional uncoupling occurs simultaneously with and is additive to that produced via a GRK/arrestin pathway. Previous evidence has suggested that PKA activation can indeed result in impaired G_s-D_1 receptor coupling (Bates et al., 1991; Zhou et al., 1991; Black et al., 1994). A second, perhaps more intriguing, explanation for the accelerated desensitization is that PKA-mediated phosphorylation works in a synergistic fashion with that of the GRK/arrestin pathway. In this scenario, PKA phosphorylation of the D_1 receptor could enhance the rate of GRK phosphorylation and/or arrestin binding to the GRK-phosphorylated receptor. Further experiments will obviously be required to investigate these possible explanations, however, neither is mutually exclusive. In this regard, it is interesting to note that recent investigations by Bouvier and colleagues (M. Bouvier, personal communication) have shown that, of the two predicted PKA sites in the human β_2-adrenergic receptor, one is phosphorylated by PKA in an agonist-specific fashion and that phosphorylation of this site enhances subsequent receptor phosphorylation by GRK2.

It was interesting to find that forskolin-induced elevation of intracellular cAMP levels resulted in a partial desensitization of the D_1 receptor cAMP response. Moreover, there was no difference between the wild-type and mutant receptors with respect to the forskolin-induced desensitization. There are several possible interpretations of these data. First, the forskolin-induced desensitization may be occurring through a pathway or mechanism that does not involve direct phosphorylation of the D_1 receptor on the mutated sites. Second, the mutated sites (specifically Thr268) may not be directly phosphorylated by PKA as a result of D_1 receptor activation. Although Thr268 is present within the context of a PKA recognition motif (Kennelly and Krebs, 1991), it is possible that other protein kinases may phosphorylate this site. Finally, PKA phosphorylation of the D_1 receptor on Thr268 may occur in a strictly agonist-dependent fashion, as Bouvier and colleagues have found for the β_2-adrenergic receptor (see above). Obviously, definitive proof that PKA directly phosphorylates the D_1 receptor on Thr268, or elsewhere, must await the production of sufficient quantities of

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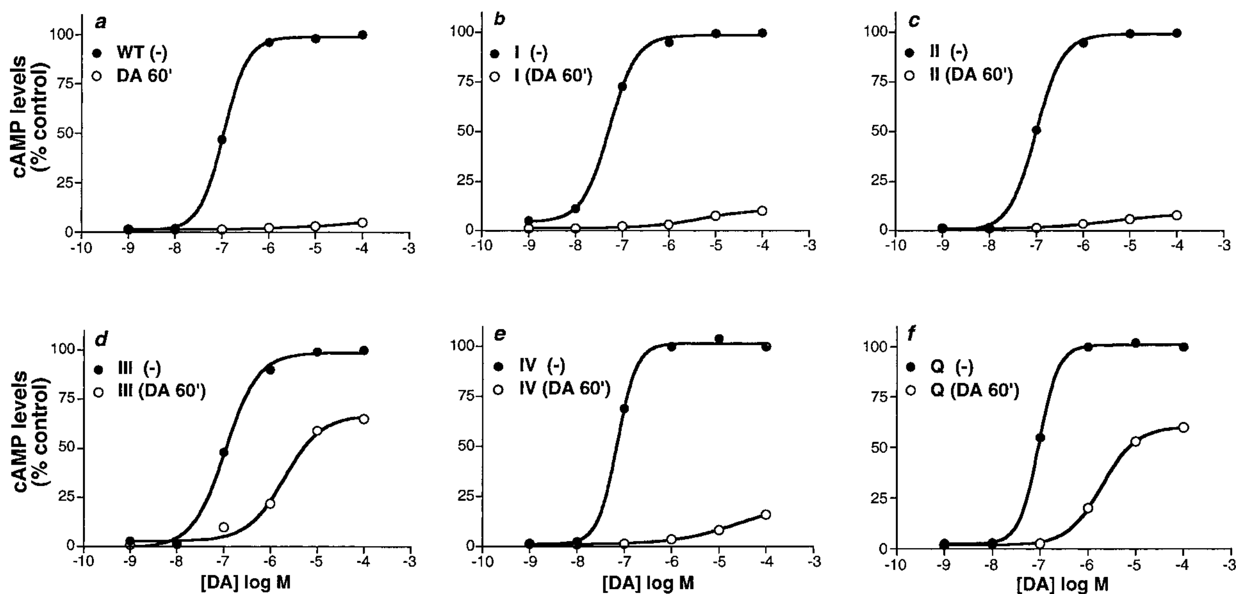

Fig. 9. Dopamine-induced desensitization of D_1 receptors with single amino acid substitutions. The effect of dopamine pretreatment on the cAMP production by the wild-type (WT) receptor (a), single mutant (I-IV) receptors (b, c, d, and e), or quadruple (Q) mutant receptor (f) was compared. Mutants I to IV refer to the sites designated in Fig. 1, Mut Q refers to the quadruple mutant. Stably transfected C6 glioma cells were pretreated with media alone (−) or with 10 μM dopamine at 37°C for 1 h (dopamine 60′). Cells were subsequently washed and then stimulated with 10^{-9} to 10^{-4} M dopamine for 15 min at 37°C. Accumulation of cAMP was measured as described in *Experimental Procedures*. The maximal cAMP production in the control group of each cell line was designated 100% and cAMP data for the dopamine-pretreated cells were normalized to these values. The data shown are representative of three independent experiments.

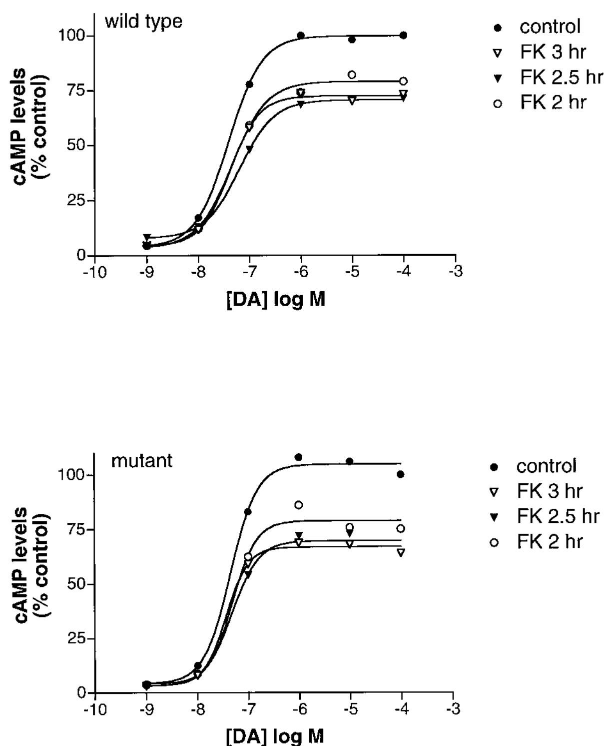

Fig. 10. Forskolin-induced desensitization of D_1 receptor-mediated cAMP accumulation. C6 glioma cells stably transfected with either the wild-type (top) or quadruple mutant (bottom) receptors were treated with media alone (control) or 10 μM forskolin for the indicated times at 37°C. The cells were subsequently washed and then stimulated with 10^{-9} to 10^{-4} M dopamine for 15 min at 37°C. Accumulation of cAMP was measured as described in *Experimental Procedures*. The maximal cAMP production in the control group of each cell line was designated 100% and cAMP data for the forskolin-pretreated cells were normalized to these values. The data shown are representative of three independent experiments.

purified receptor for use in in vitro phosphorylation assays with purified PKA.

It was notable that the putative PKA site mutants were not impaired in their ability to undergo agonist-induced down-regulation of ligand binding activity. This implies that the loss of ligand binding activity, which could be caused by either receptor sequestration and/or degradation, is not mediated by PKA phosphorylation of the D_1 receptor protein. Because previous data have suggested a role for PKA in agonist-induced D_1 receptor down-regulation (Bates et al., 1991, 1993; Zhou et al., 1991; Black et al., 1994), this suggests that PKA phosphorylation must regulate the activity of some other protein involved in the expression and/or degradation of the D_1 receptor. Because the expression of the D_1 receptor in the transfected C6 cells is under the control of a strong viral promoter, this cAMP/PKA-mediated regulation of receptor expression is probably not occurring at the transcriptional level. Further experiments, using morphological techniques, will be directed at examining the role of receptor phosphorylation in the internalization and intracellular trafficking of the D_1 receptor.

Recently, Zamanillo et al. (1995) have shown that PKA could phosphorylate Ser380 in the carboxyl tail of the D_1 receptor. This study was conducted in vitro and used a fusion protein of the receptor's carboxyl terminus and purified PKA. Mutagenesis of Ser380 in the fusion protein precluded its phosphorylation by PKA (Zamanillo et al., 1995). There are at least two possible explanations that would reconcile our current data with that of Zamanillo et al. (1995). First, it is conceivable that the tertiary conformation of the native D_1 receptor protein in vivo could preclude phosphorylation of Ser380 in its carboxyl terminus. These conformational constraints would not be present in the carboxyl terminus fusion protein. Second, it is possible that Ser380 is indeed phosphor-

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ylated by PKA in vivo, however, this phosphorylation is functionally silent, at least with respect to regulating the functional activity of the receptor as examined in this manuscript. In this regard, it is interesting to note that functionally silent GRK phosphorylation sites have recently been proposed to exist within the β_2-adrenergic receptor (Seibold et al., 1998).

In summary, our results support the hypothesis that phosphorylation of the D_1 receptor on Thr268 is important for rapid agonist-induced homologous desensitization. Future experiments will be directed toward confirmation of this hypothesis using purified protein kinase and receptor components and addressing the molecular mechanism(s) by which the accelerated desensitization occurs.

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