Distinct Residues in the Carboxyl Tail Mediate Agonist-induced Desensitization and Internalization of the Human Dopamine D1 Receptor*

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We have shown in a previous study that desensitization and internalization of the human dopamine D1 receptor following short-term agonist exposure are mediated by temporally and biochemically distinct mechanisms. In the present study, we have used site-directed mutagenesis to remove potential phosphorylation sites in the third intracellular loop and carboxyl tail of the dopamine D1 receptor to study these processes. Mutant D1 receptors were stably transfected into Chinese hamster ovary cells, and kinetic parameters were measured. Mutations of Ser/Thr residues to alanine in the carboxyl tail demonstrated that the single substitution of Thr-360 abolished agonist-induced phosphorylation and desensitization of the receptor. Isolated mutation of the adjacent glutamic acid residue in the distal carboxyl tail (Thr-446, Thr-439, and Glu-359) also abolished agonist-induced phosphorylation, whereas receptors with Ser/Thr residues at these positions retained agonist-induced desensitization. These data suggest that Thr-360 in conjunction with Glu-359 may comprise a motif necessary for GRK2-mediated phosphorylation and desensitization. Agonist-induced internalization was not affected with mutation of either the Thr-360 or the Glu-359 residues. However, receptors with Ser/Thr residues mutated in the distal carboxyl tail (Thr-446, Thr-439, and Ser-431) failed to internalize in response to agonist activation, but were able to desensitize normally. These results indicate that agonist-induced desensitization and internalization are regulated by separate and distinct serine and threonine residues within the carboxyl tail of the human dopamine D1 receptor.

Dopamine D1 receptors belong to the family of G protein-coupled receptors (GPCRs) and mediate their physiological response by coupling with stimulatory G proteins, which in turn activate adenylyl cyclase and increase levels of intracellular cAMP (1). Desensitization of GPCRs is defined as a loss of responsiveness to agonist treatment following prolonged agonist exposure (2, 3). In the dopamine D1 receptor, we have shown that following short-term agonist exposure, desensitization and internalization were differentially regulated, both biochemically and temporally (4). Rapid desensitization results from receptor uncoupling from G protein following phosphorylation of intracellular domains of the receptor and subsequent internalization of receptor from the cell surface (5). The family of G protein-coupled receptor kinases (GRKs) and second messenger-dependent kinases, such as cAMP-dependent protein kinase (PKA), are serine/threonine kinases that function to uncouple the receptor from G protein (5–8). GRKs additionally promote the interaction of the receptor with arrestin proteins, which recruit the receptors to clathrin-coated pits for subsequent internalization (9).

The β2-adrenergic receptor is closely related structurally and functionally to the dopamine D1 receptor, and the definition of the precise sites mediating desensitization occurred in a stepwise manner. Initial studies identified a region of the β2-adrenergic receptor carboxyl tail that was involved in agonist-induced desensitization (10–13), and a recent study narrowed this region to the proximal portion of the carboxyl tail of the receptor and identified three serine residues essential for regulating desensitization (14).

Agonist activation of GPCRs may result in phosphorylation of many serines and threonines that are not necessarily directly involved in the process of desensitization. Therefore, functional desensitization studies in conjunction with the direct demonstration of phosphorylation are necessary to determine the specific residues essential for desensitization (12, 13). In the rat dopamine D1 receptor, a single consensus PKA site in the third intracellular loop (encompassing Thr-268) was shown to be responsible for agonist-induced homologous desensitization (15). Other studies have demonstrated that the human dopamine D1 receptor treated with specific PKA inhibitors desensitized normally, whereas co-expression of GRKs 2, 3, and 5 enhanced receptor desensitization (16, 17).

Although there are general consensus sequences that have been identified for kinase action to mediate GPCR desensitization (18, 19), there are no consistent consensus sequences defined for internalization (5–8) and a variety of motifs have been described. Stimulation of cAMP in opossum kidney cells that endogenously express the dopamine D1 receptor has been shown to be essential for internalization (20); however, a recent study with substitution mutations of all four PKA sites in the rat dopamine D1 receptor demonstrated that internalization was unaffected (15). Internalization has also been associated with several other motifs in GPCRs, such as a dileucine sequence (21) and the NPXYX motif (22). In many receptors, serine and threonine residues have been shown to have a role in internalization (23–26) as well. In some examples of GPCRs (27–29), mutations of particular Ser/Thr residues impaired...
both desensitization and internalization, whereas in others, the sites for desensitization and internalization were distinct (23, 30). In view of the variable regulation of GPCR internalization reported, we were interested in determining the residues mediating desensitization and internalization in the human dopamine D₁ receptor. In the present study, we have identified a single threonine and a neighboring acidic residue that are critical for rapid agonist-induced phosphorylation and desensitization of the human dopamine D₁ receptor. Furthermore, we have also identified a region containing one serine and two threonine residues in the distal carboxyl tail that is required for internalization of the human dopamine D₁ receptor.

EXPERIMENTAL PROCEDURES

Generation of Mutant and Stable Cell Lines Expressing Wild Type or Mutant Receptors—The full-length cDNA of the wild type human dopamine D₁ receptor was cloned into the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA). This construct became the template for site-directed mutagenesis using the Transformer site-directed mutagenesis kit (CLONTECH, Palo Alto, CA). The mutant dopamine D₁ constructs were made by substituting single or multiple serine and threonine residues. Briefly, mutagenic primers were used in PCRTM concert with selection primers designed to eliminate a unique restriction site (located at the 3′ region of the vector) or a unique NotI restriction site (located 5′ of the vector). The products from the DNA polymerase-T4 DNA ligase reaction were digested with the enzyme corresponding to the selection primer both before and after transformation into Escherichia coli. Clones lacking the unique restriction site were selected for dideoxy sequencing on both strands to confirm the incorporation of the desired nucleotide substitution. The mutated dopamine D₁ receptor cDNAs were then subcloned into the pIRESn eo expression vector (Invitrogen, Carlsbad, CA) at the EcoRV site. The sequence of the full-length-mutated cDNA and its orientation in pIRESn eo were confirmed by dideoxy sequencing. For stable expression, the cell line CHO-K₁ (CCRL6, American Type Culture Collection, Rockville, MD) was grown to ~60% confluency in 60-mm dishes and transfected with wild type or mutant dopamine D₁ receptor constructs using LipofectAMINE (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s recommendations. Stable transfectants were selected in 1 mg/ml Geneticin (Invitrogen), and clones with the appropriate expression level were screened by a radioligand saturation binding assay. Between 30 and 50 clones expressing varying receptor densities were screened to select those with comparable expression levels.

Cell Cultures and Membrane Preparation—CHO cells were maintained as a monolayer culture in Dulbecco’s modified essential medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were harvested and lysed in a buffer containing a protease and phosphatase inhibitors, 5 mM sodium pyrophosphate, 50 mM NaF) and mixed with 100 μM fluoridophosphate buffer and 1 M mixed with 100 μM sodium phosphate. Membranes were prepared as above.

Internalization Assays—Cell membranes were prepared as above and layered on top of a 35% sucrose cushion and centrifuged at 12,000 × g for 90 min at 4 °C to separate the light and heavy fractions of the membrane as described previously (32). The heavy fraction at the bottom of the sucrose cushion was resuspended in binding buffer and used for radioligand saturation binding assays to analyze the extent of receptor sequestration (33). Internalization is expressed as the percent decrease of cell surface receptors in the treated cells relative to the untreated cells.

Adenylyl Cyclase Assays—Adenylyl cyclase activity was determined essentially as described by Salomon et al. (34). Membranes were prepared as described above. The assay mix contained 20 μl of membrane suspension (20–25 μg of protein), 12 μM ATP, 100 μM GTP, 2.7 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, 1 unit of myokinase, 5 mM ascorbic acid, and 0.13 μCi of [3H]-ATP in a final volume of 50 μl. Enzyme activities were determined in duplicate in the absence of dopamine basal activity, or with various concentrations (1 nm to 1 μM) of dopamine for 20 min at 37 °C. Reactions were stopped by the addition of 1 ml of ice-cold solution containing 0.4 mM ATP, 0.3 mM GTP, 1 mM [3H]-CAMP, and [3H]-CAMP was isolated by sequential column chromatography using Dowex cation exchange resin and aluminum oxide columns. The amount of [3H]-CAMP was used to quantify individual column recovery. Desensitization is expressed as the percent decrease of the response of the treated cells relative to the untreated cells. All experiments were performed in duplicate, and each experiment was repeated at least three times.

Agnostic-Induced Phosphorylation—Phosphorylation assays, using a protocol modified slightly from that previously described (35), were conducted in COS-7 cells transiently expressing one of the HA-tagged wild type, mutant H (T9380A) or mutant J (E359A) dopamine D₁ receptor constructs, using LipofectAMINE 2000 (Invitrogen, Burlington, Ontario, Canada). Transfected cells were screened for expression of HA-tagged dopamine D₁ receptor by both radioligand binding and immunoblot using an anti-rat monoclonal antibody HA tag and a goat anti-rat polyclonal antibody conjugated to horseradish peroxidase (Roche Molecular Biochemicals).

For agonist-induced phosphorylation assays, transfected cells were grown overnight in serum-free medium that was then changed with PO₄-free DMEM. To this, H₂11005C with 155 mM NaCl and 50 mM NaF (Santa Cruz Biotechnology) was added. The supernatant was then dialyzed using Centriprep YM-30, Amicon using dialysis buffer (100 mM NaCl, 10 mM Tris, pH 7.4 and containing the inhibitors listed above), after which the volume was reduced to ~1 ml. The samples were incubated overnight in normal rat serum (0.5% v/v) with 0.5% BSA (Santa Cruz Biotechnology). Immunoprecipitation was performed with an anti-HA primary monoclonal antibody and a secondary antibody (anti-rat IgG with agarose conjugate). The samples were then centrifuged at 13,000 rpm for 20 min, and the pellet was washed four times with dialysis buffer and resuspended in 60 μl of buffer with β-mercaptoethanol and mixed well. 40 μl was loaded onto 12% Tris-glycine gels (Novex, La Jolla, CA). After electrophoresis, gels were transferred to PVDF membranes (Santa Cruz Biotechnology) and blocked for 1 h in TBS-T. Membranes were probed overnight in normal rat serum (0.5% v/v) with 0.5% BSA (Santa Cruz Biotechnology). Membranes were washed with 10 ml of ice-cold phosphate-buffered saline, scraped with a rubber policeman, and centrifuged at 100 × g for 10 min. Cells were thus lysed in hypotonic buffer (5 mM Tris-HCl, pH 7.8, 2 mM EDTA), containing a protease inhibitor mixture (10 mg/ml leupeptin, 5 mg/ml soybean trypsin inhibitor, and 5 mg/ml benzamidine) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for two 30-s bursts at the 5.5 setting. The lysate was centrifuged at 80 × g for 10 min to pellet unbroken cells and nuclei. The supernatant was then centrifuged at 30,000 × g for 20 min at 4 °C, and the resulting pellet was resuspended in buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, and 1 mM EGTA with the protease inhibitor mixture and used immediately for radioligand binding or adenylyl cyclase assays. Protein concentrations were determined by the method of Bradford (31), using bovine serum albumin as the standard.

For agonist-induced desensitization and internalization assays, CHO cells grown to 80–90% confluence were incubated overnight in serum-free DMEM. Serum-free DMEM was replaced prior to the experiment, and dopamine and ascorbic acid were added in final concentrations of 10 and 100 μM, respectively, for the indicated time. Control cells were treated with serum-free DMEM containing 100 μM ascorbic acid only.

Radioligand Binding Assays—For radioligand saturation binding assays, cells membranes (20–30 μg of protein/tube) were incubated with increasing concentrations of [³H]-SCH-23390 (specific activity 75.5 Ci/mmol, Mepharmaceuticals, Guelph, Ontario, Canada) in a total volume of 1 ml of binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1.5 mM CaCl₂, 5 mM MgCl₂, 5 mM KCl, and 120 mM NaCl). (+)-Butaclamol was added at a final concentration of 1 μM to define nonspecific binding. Tubes were incubated at room temperature for 90 min, and bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Montreal, Canada) onto Whatman GF/C filters. Filters were washed with 10 ml of ice-cold 50 mM Tris-HCl, pH 7.4, and incubated overnight in 5 ml of scintillation fluid (Cerenkov, Costa Mesa, CA). Tritium was counted using a Beckman LS 6500 scintillation counter at a counting efficiency of 40%. All experiments were performed in duplicate, and each experiment was repeated at least three times.

Data Analysis—The data obtained from radioligand saturation and adenylyl cyclase experiments were fitted by least-squares nonlinear regression using the computer program Prism (GraphPad Software, San Diego, CA). Data from multiple experiments were averaged and expressed as the means ± S.E. The results were considered significantly different when the probability of randomly obtaining a mean difference was <0.05 using the paired Student’s t test.
D1 receptor mutants were made as follows. Receptor A consisted of mutations of all serine and threonine residues in the third intracellular loop between amino acids 243 and 268. Receptors B, C, D, E, F, G, H, I, and J were located in the carboxyl tail and consisted of mutations of serine and threonine residues at the following positions. Receptor B consisted of a single Thr-446 substitution; receptor C consisted of substitutions between positions 372 and 446; receptor D consisted of substitutions between 428 and 439; receptor E consisted of substitutions between positions 372 and 446; receptor F consisted of substitutions between positions 342 and 446; receptors H, I, and J consisted of single substitutions of Thr-360 and Ser-362, and Glu-359, respectively. Each of the mutant and wild-type dopamine D1 receptors were stably expressed in CHO cells. For each construct, results are the mean ± S.E. n, number of independent experiments performed in duplicate.

**Table I**

| D1 construct | n | $B_{max}$ | $K_D$ |
|--------------|---|-----------|-------|
| Wild type    | 5 | 2.01 ± 0.05 | 0.43 ± 0.06 |
| Wild type    | 4 | 0.64 ± 0.03 | 0.11 ± 0.03 |
| Receptor A   | 3 | 2.05 ± 0.06 | 0.29 ± 0.04 |
| Receptor B   | 4 | 0.26 ± 0.03 | 0.37 ± 0.10 |
| Receptor C   | 3 | 0.49 ± 0.04 | 0.34 ± 0.11 |
| Receptor D   | 3 | 0.27 ± 0.03 | 0.21 ± 0.10 |
| Receptor E   | 4 | 1.89 ± 0.07 | 0.41 ± 0.07 |
| Receptor F   | 3 | 1.81 ± 0.11 | 0.23 ± 0.08 |
| Receptor G   | 3 | 2.27 ± 0.09 | 0.36 ± 0.06 |
| Receptor H   | 2 | 1.15 ± 0.08 | 0.31 ± 0.10 |
| Receptor I   | 2 | 0.50 ± 0.04 | 0.46 ± 0.10 |
| Receptor J   | 2 | 0.46 ± 0.03 | 0.36 ± 0.10 |

**Expression of Mutant Dopamine D1 Receptors**—We have shown in previous studies that treatment of the dopamine D1 receptor with 10 μM dopamine for 15 min caused rapid desensitization and internalization (4, 36). Therefore, to identify the specific residues involved, progressive substitution mutants were made in the carboxyl tail and third intracellular loop, replacing serines and threonines with alanine residues (Fig. 1). A receptor with all the serine and threonine residues substituted in the third intracellular loop between amino acids 243–268 was termed receptor A. Receptors B through J (Fig. 1) contained substitutions of Ser/Thr residues in the carboxyl tail as follows: B contained the single 446 substitution; C contained substitutions between positions 431 and 439; D contained substitutions between positions 431 and 439; E contained substitutions between positions 372 and 446; F contained substitutions between 342 and 354 and between residues 372 and 446; G contained substitutions at position 360 and between residues 372 and 446; receptors H, I, and J consisted of single substitutions of Thr-360, Ser-362, and Glu-359, respectively.

**Agonist-induced Desensitization**—Desensitization of wild type and D1 receptor mutants was measured as a loss of agonist potency and loss of cyclic AMP generation for a range of dopamine concentrations (10^{-10} to 10^{-4} M) following a 20-min pretreatment with 10 μM dopamine. The rate of dopamine-stimulated maximal adenylyl cyclase activity ($V_{max}$) was decreased in wild type receptor a by 28.6 ± 5.1% and the EC_{50} was shifted 1.5-fold to the right, consistent with agonist-induced desensitization (Fig. 2). For the lower density cell line, wild type receptor b, the desensitization results were similar (data not shown). Receptor A displayed a similar reduction in adenylyl cyclase activity as wild type (32.3 ± 4.2%) with a 1.5-fold shift to the right in the EC_{50} response. Receptor E also had a reduction in the $V_{max}$ similar to wild type at 36.5 ± 5.8% with no shift in the EC_{50} for dopamine. Likewise, receptor F also displayed a reduction in the $V_{max}$ of 22.5 ± 3.8%; however,
there was no shift in the EC_{50}. The desensitization response was completely abolished in receptor G with no change in V_{max} or in EC_{50} (Fig. 2).

Therefore, we examined the specific role of Thr-360 in desensitization. In mutant receptor H containing the single substitution of Thr-360, desensitization was abolished, suggesting an important role for Thr-360 in mediating the agonist-induced desensitization response (Fig. 2). Receptor I exhibited partial desensitization, because there was a 20.0/4.8% reduction in V_{max} but with a 1.2-fold shift to the right in the EC_{50} response (Fig. 2). Desensitization was also abolished in receptor J containing the Glu-359 mutation (Fig. 2). The changes in V_{max} of adenylyl cyclase stimulation following agonist exposure for all mutant receptors compared with wild type is summarized (Fig. 3).

**Fig. 2.** Effects of substitution of intracellular serine and threonine residues in the dopamine D_{1} receptor on rapid agonist-induced desensitization. CHO cells expressing wild type dopamine D_{1} receptor; receptor A (243–268), receptor E (372–446), receptor F (342–354, 372–446), receptor G (360, 372–446), receptor H (Thr-360), receptor I (Ser-362), and receptor J (Glu-359) were incubated in the presence (C) or absence (G) of 10 μM dopamine for 20 min, following which, the ability of increasing concentrations of dopamine (10^{−10} to 10^{−4} μM) to stimulate cAMP accumulation was tested. Data are presented as a percentage of maximal stimulated adenylyl cyclase activity of untreated cells and are the means ± S.E. of at least three independent experiments performed in duplicate. The numbers in parentheses indicate the amino acid sequences from which the Ser/Thr were mutated.

**Fig. 3.** Effect of mutations of the dopamine D_{1} receptor on the reduction in V_{max} of adenylyl cyclase activity. CHO cells expressing wild type or mutant dopamine D_{1} receptors were incubated in the absence and presence of 10 μM dopamine for 20 min, and, subsequently, the ability of increasing concentrations of dopamine to stimulate cAMP accumulation was tested. The percent reduction in V_{max} of wild type and mutant receptors is presented as the mean ± S.E. of at least three independent experiments. Significant differences from wild type are denoted by an asterisk (p < 0.05).
induced internalization of the dopamine D<sub>1</sub> receptor. For receptors D (Fig. 4) and E (data not shown), internalization was also abolished, which was expected because these receptors all included substitutions of the residues mutated in receptors B and C. Receptors H and J, previously shown not to desensitize, underwent agonist-induced internalization similar to wild type (18.7 ± 5.7%, 25.0 ± 3%) (Fig. 4). The extent of loss of cell surface receptors following agonist exposure for all mutant receptors compared with wild type is shown in Fig. 5.

**Agonist-induced Phosphorylation**—Because receptors H and J failed to desensitize in response to agonist, their ability to phosphorylate following agonist activation was evaluated. Following agonist treatment, the D<sub>1</sub> wild type receptor demonstrated a significant increase in agonist-induced phosphorylation above the basal level (Fig. 6, lanes 1 and 2). Exposure of receptor H and J to 10 μM dopamine for 20 min resulted in no increase in phosphorylation above basal (Fig. 6, lanes 3 and 4, lanes 7 and 8). It is also notable that receptors H and J both exhibited a modest reduction in the levels of basal phosphorylation (Fig. 6, lanes 5–7).

**DISCUSSION**

In this study, we show that rapid agonist-induced desensitization and phosphorylation of the human dopamine D<sub>1</sub> receptor is critically dependent on Thr-360 and the preceding Glu-359 in the proximal segment of the carboxyl tail. Mutation of

**FIG. 4.** Effects of substitution of intracellular serine and threonine residues in the dopamine D<sub>1</sub> receptor on rapid agonist-induced internalization. CHO cells expressing wild type, receptor A (243–268), receptor B (Thr-446), receptor C (431–439), receptor H (Thr-360), and receptor J (Glu-359) were incubated in the presence (○) or absence (■) of 10 μM dopamine at 37 °C for 20 min, and saturation binding of the heavy membrane fraction was estimated using increasing concentrations of [3H]SCH-23390. The percentages of dopamine-induced internalization are presented as the means ± S.E. of at least three independent experiments performed in duplicate.

**FIG. 5.** Effect of Ser/Thr mutations of the dopamine D<sub>1</sub> receptor on the loss of cell membrane receptors. CHO cells expressing wild type or mutant dopamine D<sub>1</sub> receptors were incubated in the absence and presence of 10 μM dopamine for 20 min, and receptor density was estimated using [3H]SCH-23390. The percentages of loss of cell surface receptors are presented as the means ± S.E. of at least three independent experiments. Significant differences from wild type are denoted by an asterisk (p < 0.05).
either Glu-359 or Thr-360 prevented agonist-induced phosphorylation, indicating that Thr-360 is the primary site or has a dominant role as the initiation site of agonist-induced phosphorylation. The motif we identified (consisting of Glu-359 and Thr-360), in which the threonine is flanked on its amino-terminal side by the acidic amino acid, defines a site of GRK2 recognition and phosphorylation (19). Because basal levels of phosphorylation of the D1 receptors mutated at Glu-359 or Thr-360 was also reduced, this indicates that these residues have a role in maintaining the basal level of agonist-independent phosphorylation. In addition, we have identified a cluster of residues consisting of two threonines and one serine in the distal portion of the carboxyl tail, Thr-446, Thr-439, and Ser-431, that is responsible for rapid agonist-induced internalization. Furthermore, we have shown that, in the human dopamine D1 receptor, desensitization and internalization are biochemically distinct mechanisms, because the residues that were involved in abolishing desensitization or internalization did not have a reciprocal effect on the other process.

The sequence in the dopamine D1 receptor that includes the putative GRK2 recognition motif, is shown in Fig. 7 in comparison with analogous sequences in the dopamine D5, the β2-adrenergic and the adenosine A3 receptors. Although no definitive consensus sequences have been identified for the various GRKs, both GRK1 and GRK2 are known as acidotropic kinases, because they most actively phosphorylate serines and threonines in close proximity to acidic amino acids (19). GRK2 has been shown to preferentially phosphorylate serines and threonines with acidic amino acids on the amino-terminal side. In contrast, GRK1 recognizes serines and threonines with acidic residues localized to the carboxyl-terminal side (19, 37, 38). GRK5 and GRK6, which preferentially act at serine residues with neighboring basic amino acids on the amino-terminal side (39, 40), are not likely to act at Thr-360, because there are no adjacent basic residues. Therefore, we propose that the effect of ablation of either Thr-360 or the adjacent Glu-359 resulting in loss of desensitization and agonist-induced phosphorylation indicates that GRK2 may be the critical regulator of rapid agonist-induced desensitization and phosphorylation in the human dopamine D1 receptor.

The β2-adrenergic and the dopamine D1 receptors share significant homology, including similarly sized carboxyl tails, and each receptor activates stimulatory G proteins and undergoes GRK-mediated homologous desensitization. Studies using antisense oligodeoxynucleotides to inhibit GRK2 synthesis (41) and specific antibodies to inhibit GRK2 availability (42) indicate that this kinase may be involved in the homologous desensitization of the β2-adrenergic receptor. The substantial increase in the extent of dopamine D1 receptor phosphorylation following overexpression of GRK2 has suggested a role for GRK2 in regulation of dopamine D1 receptor desensitization (17). Therefore, we anticipated that the Ser/Thr residues that are subject to GRK2 phosphorylation would be situated in similar regions of the β2-adrenergic and the dopamine D1 receptors because of the structural similarities of the two receptors. Recently, it has been shown in the β2-adrenergic receptor that, although Ser-364, together with Glu-362, might meet the criteria for a putative GRK2 recognition site, mutation of Ser-364 was shown to have no effect on desensitization (14). Mutation of the two serines, at positions 355 and 356, however, did eliminate desensitization of the β2-adrenergic receptor (14). Even though there is no acidic residue directly amino-terminal to these residues, the presence of a PKA site located on the amino-terminal side of Ser-355, when phosphorylated, may modify the charge distribution in the proximal carboxyl tail and, therefore, may have an additional effect to modulate GRK2-mediated desensitization of the β2-adrenergic receptor mediated through Ser-355 and Ser-366. As can be seen in Fig. 7, the putative GRK2 recognition motif present in the dopamine D1 receptor also differs from that identified in the rat adenosine A3 receptor, which contains two threonine residues at positions 318 and 319 in the carboxyl tail (43).

GPCRs may have GRK2 motifs located toward the distal or proximal regions of the carboxyl tails, or the third intracellular loop (8). Thus, there is considerable variability between both the location of GRK2 recognition motifs within GPCRs and the sequences that comprise GRK motifs in a given receptor. The sites of GRK phosphorylation of rhodopsin (37), the μ opioid (44), and the δ opioid (45) receptors are located in the distal carboxyl tails. In addition to being located more proximally, the putative GRK2 recognition site of the dopamine D1 receptor required only the presence of Glu-359 or Thr-360. The serine at position 362 does not appear to participate as a primary site of GRK action in the dopamine D1 receptor desensitization motif, because when Thr-360 was mutated, Ser-362 was not able to substitute and was found not to have a primary role in receptor desensitization.

The mechanism and putative motifs mediating internalization are variable, however, mutagenesis studies have suggested a role for carboxyl tail serine and threonine residues in internalization (23–26). In this study we determined that three residues in the distal portion of the carboxyl tail (Thr-446, Thr-439, and Ser-431) were involved in internalization of the rat dopamine D1 receptor mutants were incubated with 32P in the presence or absence of dopamine, 10 μM, for 20 min. Membranes were harvested, and the receptors were immunoprecipitated with the anti-HA antibody. The immunoprecipitate was electrophoresed and exposures were made to film. The position of the major protein band visualized by immunoblotting is indicated with an arrow. The Ser/Thr and Glu residues that are implicated in GRK-mediated desensitization are underlined. Arrowhead indicates the initiation site of agonist-induced phosphorylation is indicated with an arrow.
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Distinct Residues in the Carboxyl Tail Mediate Agonist-induced Desensitization and Internalization of the Human Dopamine D_1 Receptor
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