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Hydrophobic Residues of the D_2 Dopamine Receptor Are Important for Binding and Signal Transduction

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Abstract: Dopamine receptors belong to the seven transmembrane helix-containing, G protein-coupled receptor superfamily. Mutagenesis studies suggest that dopamine and its analogues interact with aspartate-114 in helix 3 and two helix 5 serines (194 and 197) of the D_2 receptor. In addition to these amino acids, hydrophobic residues within the receptor core may be important not only for binding but also for receptor activation. Described is a site-directed mutagenesis investigation into the roles of these hydrophobic residues in the long isoform of the human D_2 receptor. Replacement of helix 6 phenylalanines (389 or 390) with alanines resulted in disrupted binding to several agonists and antagonists and impaired inhibition of adenylyl cyclase activity. Replacement of the helix 5 phenylalanine-198 with an alanine selectively disrupted [^{3}H]N-0437 binding, whereas the affinities for other agonists and antagonists remained unchanged. This mutant remained functionally intact when stimulated with dopamine or bromocriptine. Replacement of the helix 7 phenylalanine-411 or the helix 6 leucine-387 with alanines produced receptors that bound agonists well but were unable to inhibit adenylyl cyclase. Based on these data, two conserved helix 6 phenylalanines (389 and 390) appear to be crucial for ligand binding, and phenylalanine-411 in helix 7 and leucine-387 in helix 6 may be important for propagating conformational changes from the agonist binding site(s) to G protein coupling domain(s) of the D_2 receptor. **Key Words:** Site-directed mutagenesis—Phenylalanine—Coupling—Seven transmembrane helix-containing receptors.
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Molecular biological techniques have led to the identification of five distinct dopamine receptor subtypes (D_{1-5}), each encoded by a different gene (for review, see Sibley and Monsma, 1992). Hydrophobicity plots and analyses of their deduced amino acid sequences suggest the receptor proteins are members of the seven transmembrane (TM), guanine nucleotide binding (G) protein-coupled superfamily of membrane receptors. Although the nature of ligand varies widely, the members of this superfamily display high amino acid homology, and this similarity in primary structure presumably leads to similar secondary and tertiary structures.

The proteins of this superfamily perform at least two functions: (a) ligand binding and (b) G protein coupling or signal transduction. It follows that G protein-coupled receptors can be conceptually and perhaps spatially divided into a ligand binding domain and a G protein-coupling domain. Interactions with G proteins appear to involve the intracellular loops and the carboxy-terminus, as evidenced by various mutational strategies (Strader et al., 1987*a*; Dixon et al., 1988; Kobilka et al., 1988; O'Dowd et al., 1988; Cotecchia et al., 1990). Depending on the type of ligand to which the receptor binds, the ligand binding domain may involve extracellular (Fong et al., 1992) and/or TM (Dixon et al., 1987; Strader et al., 1988, 1989) regions. Receptors of small cationic ligands, such as adrenergic and dopaminergic receptors, appear to bind ligands at the TM helices.

Mutagenesis efforts have greatly detailed the binding "pocket" within the lumen of the transmembranous protein core of the adrenergic receptors. These studies demonstrate the importance of several conserved amino acids for binding to the endogenous catecholamine ligands epinephrine and norepinephrine. Key amino acids include a negatively charged aspartate in the third TM helix (TM3) and two serine residues in TM5 (Strader et al., 1988, 1989; Wang et al., 1991). Dopamine receptors, although they bind a different endogenous catecholamine, also possess these cognate amino acids; mutagenesis of these residues in the D_2 subtype has produced similar but not identical results. Mansour et al. (1992) have corroborated the importance of the TM3 aspartate and revealed potential agonist interactions with two TM5 serines (194 and 197); however, the two serine residues of the D_2 dopamine receptor were not equally important, with serine-197

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Abbreviations used: cAMP, cyclic AMP; CMV, cytomegalovirus; G protein, guanine nucleotide binding protein; NPA, *R*(−)-propylnorapomorphine; PBS, phosphate-buffered saline; TM, transmembrane.

being more important for agonist binding. In addition, ligand binding may also be influenced by serine-193 in TM5 and a TM7 serine (Cox et al., 1992). It is interesting that D_1 dopamine receptors have four closely packed serines in TM5 (197, 198, 199, and 202), three of which may be involved in binding with dopamine (Pollock et al., 1992).

Many explorations into seven-TM receptor topography have emphasized ligand binding regions, but the mechanism(s) by which receptor occupancy leads to receptor activation remains unclear. Recombinant DNA mutagenesis data, such as those outlined above, have contributed greatly to the refinement of seven-TM receptor models built on the framework of the bacteriorhodopsin three-dimensional structure (Henderson et al., 1990). Refinement of these models has led to detailed speculation about residues that may not only stabilize binding at the aforementioned key anchor points, but that may also play a role in receptor activation and signal transduction. One such model for receptors of small cationic molecules has recently been proposed by Trumpp-Kallmeyer et al. (1992) in which catecholamine agonists interact with the binding pocket at three conserved residues, consistent with existing mutagenesis data: an aspartate in TM3 and two serines in TM5. A similar model has been independently developed by our laboratory, specifically for the D_2 dopamine receptor subtype. In both these models, several hydrophobic, largely aromatic, residues are located within the lumen of the receptor and project into the vicinity of the putative catecholamine binding site. Some of these residues are predicted to move on agonist binding, potentially initiating conformational shifts of the protein.

As an alternative to the models developed by Trumpp-Kallmeyer et al. (1992) and by our laboratory, a model proposed by Dahl et al. (1991) presents a different perspective on D_2 receptor topography and function. The Dahl model is based on binding at aspartate-80 in TM2 and asparagine-390 in TM7 of the rat D_2 short isoform, equivalent to asparagine-418 of the human D_2 long isoform, placing the dopamine molecule deeper within the receptor core than is predicted by binding at aspartate-114. A few studies (Strader et al., 1987*b*, 1988; Horstman et al., 1990; Neve et al., 1991) indicate that these residues, or their cognates in other seven-TM receptor subtypes, may indeed play some role either in ligand binding or in allosteric regulation of receptor conformation. Dahl et al. (1991) hypothesized that signal transduction is due to disruption of electrostatic fields initiated by binding of a protonated agonist near aspartate-80; no mention of hydrophobic residues is made in this model.

The present study therefore was designed to test the strength of such computer-generated models by selectively mutating several hydrophobic residues that are highly conserved among receptors of small cationic ligands (for sequence comparison, see Trumpp-Kallmeyer et al., 1992) and are predicted to participate in both stabilization of binding and the initiation of signal transduction in several seven-TM subtypes. For this particular study, the pharmacologically and functionally well-characterized D_2 receptor subtype was used. The specific point mutations (Fig. 1) include three phenylalanine residues hypothesized to stabilize the binding of dopamine at aspartate-114, serine-194, and serine-197. Specifically, phenylalanine-389 in TM6 may electronically shield the electrostatic interaction between the positively charged amine group of dopamine and the negatively charged carboxyl group of aspartate-114, and phenylalanine-198 in TM5 and phenylalanine-390 in TM6 may stabilize the binding of dopamine by interacting with the aromatic nucleus of the catecholamine. According to the Trumpp-Kallmeyer model, these phenylalanines must move to accommodate the binding of dopamine and may therefore participate in a cascade of conformational changes leading to receptor activation. Two additional residues not mentioned in the model of Trumpp-Kallmeyer et al. (1992) were also mutated: phenylalanine-411 in TM7 and leucine-387 in TM6. According to our model, phenylalanine-411 and leucine-387 may also be involved in signal transduction. In attempts to preserve the tertiary conformation of the receptor, all replacement mutations were made to alanine.

MATERIALS AND METHODS

Mutagenesis

A cDNA containing the entire coding region and ~1,000 bp of the 3′ untranslated region of the long isoform of the human D_2 receptor was ligated into the eukaryotic expression vector pCMVneo, which contains the SV40 origin of replication (pCMVD2L). Transcription of the D_2 receptor was driven by the cytomegalovirus (CMV) promoter. A full-length cDNA for the long isoform of the human D_2 receptor was simultaneously subcloned into m13mp18, which was used to derive the single-stranded template for mutagenesis reactions. Site-directed mutants were created using the Amersham mutagenesis kit V2.1. Complementary, mutant oligonucleotides of 24 bases were synthesized and purified twice by HPLC. Mutants were verified by dideoxy chain termination DNA sequencing (Sequenase, V3.0). Mutated fragments were then subcloned into pCMVD2L. A β-galactosidase cDNA construct was also created in pCMVneo for transfection efficiency corrections (pCMVβ-gal).

Cell culture and transfection

COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2/95% O_2 and subcultured every 3–5 days. Cells were seeded at a density of $1-1.5 \times 10^6$ cells per 10-cm-diameter dish for transfections. For receptor binding assays, transfections were performed according to the calcium phosphate precipitation protocol of Chen and Okayama (1987), using 20 μg of pCMVD2L and 5 μg of pCMVβ-gal for transfection efficiency corrections. Transfected cells were incubated at 37°C in 3% CO_2 for 18–24 h, following which the cells were washed twice with Versene and once with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfected cells were then returned to

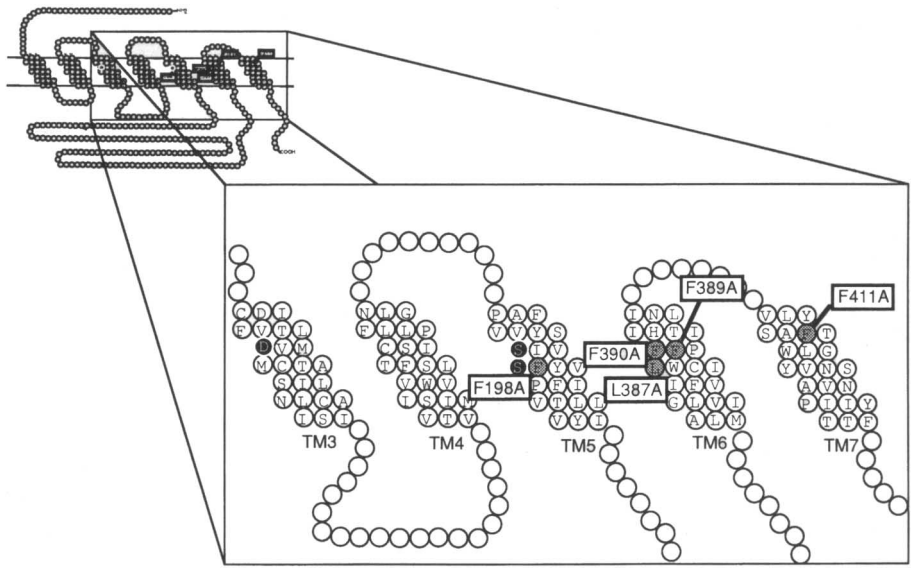

FIG. 1. Schematized topography of the long isoform of the human D_2 dopamine receptor. Solid circles represent putative ligand binding sites. Stippled circles represent residues mutated to alanine. The specific mutants are labeled with boxes.

incubation at 37°C in 5% CO_2 for 24–48 h before receptor binding and β-galactosidase activity assays were performed. For radioimmunoassay assessment of intracellular cyclic AMP (cAMP) accumulation, COS-1 cells were transfected by electroporation. One 10-cm-diameter dish of subconfluent cells was washed in phosphate-buffered saline (PBS) and then trypsinized (0.5% trypsin in 5.3 m*M* EDTA) for 2 min at 37°C. Cells were then harvested and washed twice in PBS at 0°C to remove the trypsin. The cellular pellet was resuspended in 1 ml of PBS and electroporated with 5 μg of pCMVβ-gal and 20 μg of either wild-type or mutated pCMVD2L at 250 V and 330 μF at 0°C in disposable 1-ml electroporation chambers using a BRL Cell-Porator system. As a control, mock-transfected COS-1 cells were electroporated with 5 μg of pCMVβ-gal. Electroporated cells were allowed to recover at 0°C for 10 min before being diluted in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The diluted cells were then aliquoted into 24-well culture dishes and incubated at 37°C in 5% CO_2 for 48–72 h before quantification of cAMP.

Radioligand binding assays

Cells transfected by the calcium phosphate method were washed twice and harvested in either antagonist binding buffer (50 m*M* Tris, 120 m*M* NaCl, 0.1% ascorbic acid, 5 m*M* KCl, and 5 m*M* $MgCl_2$, pH 7.1 at 22°C) or agonist binding buffer (50 m*M* Tris, 5 m*M* KCl, 5 m*M* $MgCl_2$, and 1 m*M* EDTA, pH 7.4 at 22°C). Harvested cells were pelleted at 500 *g* for 5 min, resuspended in binding buffer, and homogenized (Virtishear homogenizer; 80% maximum rpm, 30 s). For Scatchard analysis, cellular homogenates were incubated with the D_2 antagonist [^{3}H]raclopride (specific activity, 75.1 Ci/mmol; Du Pont/NEN) or [^{3}H]spiperone (specific activity, 17.7 Ci/mmol; Du Pont/NEN) or the D_2 agonist [^{3}H]N-0437 (specific activity, 105 Ci/mmol; Amersham) at six to eight concentrations. Nonspecific binding was determined by displacement with the D_2 antagonist (+)-butaclamol (1 μ*M*). The total volume in each incubation reaction was 250 μl per tube. Incubations were performed in duplicate for 60 min at 22°C. For the generation of competition isotherms, cellular homogenates were incubated in the presence of saturating concentrations of [^{3}H]spiperone (1–5 n*M*) displaced by increasing concentrations of unlabeled agonist [*R*(−)-propylnorapomorphine (NPA), apomorphine, 2-bromo-α-ergocryptine (bromocriptine), or dopamine]. The total volume in each incubation reaction was 240 μl per tube. Incubation reactions were performed in duplicate at 22°C for 60–90 min. K_D values from Scatchard plots and K_i values from competition curves were determined by using LIGAND (Munson and Rodbard, 1980) (version 4.0 for the Macintosh) and averaged from a minimum of two isotherms.

Quantification of cAMP

Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was aspirated from 24-well culture dishes containing COS-1 cells electroporated with either wild-type or mutated pCMVD2L and replaced with Dulbecco's modified Eagle's medium. Cells were then preincubated with 0.5 m*M* 3-isobutyl-1-methylxanthine, 10 μ*M* SCH-23390 (to prevent any D_1-mediated effects), and 10 μ*M* (−)-alprenolol (to prevent any adrenergic effects) for 30 min at 37°C before incubation with 50 μ*M* forskolin, at which time varying concentrations of either dopamine or bromocriptine were applied for 15 min at 37°C. The drug-containing medium was then quickly removed by aspiration. Each well was washed twice with ice-cold PBS and then lysed in 65% ethanol for 8–12 h at 4°C. The lysate was collected, centrifuged at 2,000 *g* for 15 min, and lyophilized. The lyophilized cAMP extract was resuspended and diluted in 0.05 *M* sodium acetate (pH 6.2), and the amount of cAMP was assessed by equilibrium radioimmunoassay. Triplicate tubes of resuspended cAMP were incubated with anti-cAMP antiserum (Sigma Immunochemical) for 4 h at 4°C. ^{125}I-cAMP tracer was then added to each tube. Tubes were quickly vortex-mixed and incubated for an additional 18–24 h. Antigen/antibody complexes were coprecipitated with bovine serum albumin (fraction V; Sigma) in ethanol, by centrifugation at 2,000 *g* for 15 min at 4°C. The supernatant was aspirated, and the remaining pellets were assayed for undisplaced ^{125}I-cAMP using a GammaTrac 1290/TM analytical γ-radiation counting system. The amount of cAMP in each tube was

TABLE 1. *Antagonist binding*

Receptor	[^{3}H]Spiperone (n*M*)	[^{3}H]Raclopride (n*M*)
D_2 wild-type	0.083 ± 0.009	0.98 ± 0.14
F198A	0.091 ± 0.010	1.4 ± 0.28
F389A	2.8 ± 0.84	NSB
F390A	NSB	NSB
F411A	0.061 ± 0.002	0.40 ± 0.05
L387A	0.085 ± 0.008	0.40 ± 0.06

Data are mean ± SEM K_D values (n = 2–3) from two or three Scatchard plots for wild-type and mutant (F198A, phenylalanine-198 → alanine; F389A, phenylalanine-389 → alanine; F390A, phenylalanine-390 → alanine; F411A, phenylalanine-411 → alanine; and L387A, leucine-387 → alanine) D_2 receptors. NSB indicates that no specific binding was measurable.

determined by comparison with a standard curve of known concentrations of unlabeled cAMP. All cAMP level measurements were determined from six to nine independent experiments.

Western immunoblot assay

COS-1 cells transfected with 25 μg of either the wild-type or mutant pCMVD2L by calcium phosphate precipitation were scraped off and stored as loose pellets until electrophoresis. Cells were resuspended in deionized water, and an aliquot containing 30 μg of total protein was diluted in a 2× loading buffer [24.8 m*M* Tris base, 192 m*M* glycine, and 0.1% (wt/vol) sodium dodecyl sulfate] and boiled for 5 min. The proteins were electrophoresed through a sodium dodecyl sulfate/10% polyacrylamide resolving gel using a BRL Mini-V 8.10 Vertical Gel Electrophoresis System. Proteins were then transferred to a nitrocellulose membrane in transfer buffer (24.8 m*M* Tris base, 192 m*M* glycine, and 10% methanol) for ~2 h at 0°C and 150 V. The membrane was incubated in a blocking buffer of 1% nonfat powdered milk in PBS for 1 h with gentle agitation at 22°C, washed three times in PBS, and incubated overnight at 22°C with a 1:5,000 dilution of rabbit anti-D_2 antiserum raised against the third cytosolic region (amino acids 246–316) of the long isoform of the human D_2 receptor. The membrane was then washed three times in PBS and incubated with goat IgG directed against rabbit IgG for 1 h at 22°C. The membrane was again washed three times in PBS and incubated with a rabbit anti-horseradish peroxidase antibody for 1 h at 22°C. After washing in PBS, the membrane was then incubated in horseradish peroxidase (4 μg/ml) for 1 h at 22°C. Following the final washes in PBS, the membrane was added to a diaminobenzidine substrate solution [20% (wt/vol) 3,3′-diaminobenzidine, 63 m*M* nickel ammonium persulfate, and 0.0005% H_2O_2 in PBS]. The color reaction was terminated after incubation for 10 min at 22°C by gently rinsing the membrane in water.

RESULTS

Radioligand binding

The long isoform of the wild-type human D_2 receptor and five individual substitution mutations (F198A, F389A, F390A, F411A, and L387A) were characterized by their abilities to bind two D_2-specific antagonists in saturation binding experiments and five agonists in saturation and competition assays (Tables 1 and 2). The mutant and wild-type D_2 constructs, when transiently transfected into COS-1 cells by calcium phosphate precipitation, produced high levels of receptor binding as detected by the antagonist [^{3}H]-spiperone (ranging from 0.3 to 1.5 pmol/mg of protein) except for mutant F390A, which was unable to bind any labeled ligand used in this study ([^{3}H]-spiperone, [^{3}H]raclopride, or [^{3}H]N-0437). The presence of D_2-like immunoreactive protein in transfected COS-1 cells was verified by western blot analysis. A predominant band at ~42 kDa was observed in cells transfected with the wild-type D_2 and all mutants, including F390A, but not in untransfected COS-1 (data not shown). In addition, transfection efficiencies were estimated by expression of the cotransfected β-galactosidase reporter construct, which demonstrated similar efficiencies across mutants (data not shown). Scatchard analysis using the labeled agonist [^{3}H]N-0437 produced K_D values in the nanomolar range; K_D values for the antagonists [^{3}H]spiperone (Fig. 2) and [^{3}H]-raclopride were in the picomolar and nanomolar ranges, respectively. Agonist competition of [^{3}H]-spiperone produced the following rank order of potency for the wild-type D_2: bromocriptine > NPA

TABLE 2. *Agonist binding*

Receptor	[^{3}H]N-0437 (n*M*)	Bromocriptine (n*M*)	NPA (n*M*)	Apomorphine (n*M*)	Dopamine (μ*M*)
D_2 wild-type	1.7 ± 0.58	8.2 ± 2.0	22 ± 12	200 ± 162	1.0 ± 0.40
F198A	NSB	6.3 ± 2.7	23 ± 15	39 ± 13	1.6 ± 1.1
F389A	NSB	850 ± 642	7,200 ± 6,800	16,000 ± 740	59 ± 55
F390A	NSB	—	—	—	—
F411A	1.2 ± 0.045	6.6 ± 0.02	25 ± 4.9	250 ± 134	0.89 ± 0.40
L387A	1.2 ± 0.065	5.9 ± 2.2	34 ± 9.4	220 ± 177	10 ± 0.87

Data are mean ± SEM K_D and K_i values (n = 2–4) from two to four isotherms for wild-type and mutant (F198A, phenylalanine-198 → alanine; F389A, phenylalanine-389 → alanine; F411A, phenylalanine-411 → alanine; and L387A, leucine-387 → alanine) D_2 receptors. In competition experiments, [^{3}H]spiperone was used as the labeling ligand. NSB indicates that no specific binding was detectable. K_i values for mutant F390A (phenylalanine-390 → alanine) could not be measured because this mutant did not display any specific binding for the labeled ligands.

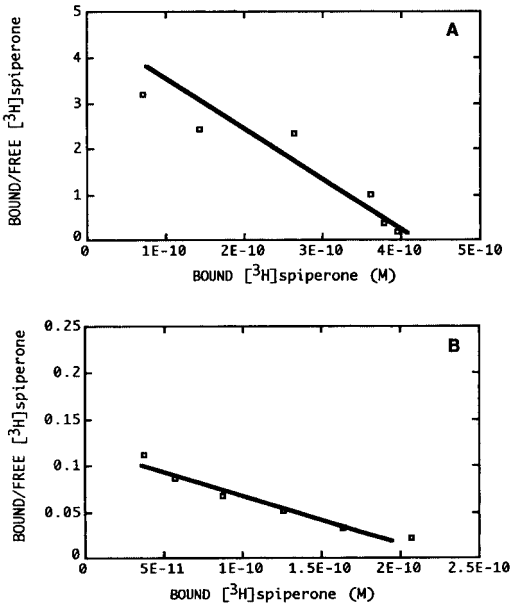

FIG. 2. Representative Scatchard plots of [^{3}H]spiperone binding for (**A**) the wild-type D_2 receptor and (**B**) replacement of phenylalanine at position 389 with an alanine (mutant F389A). Nonspecific binding was determined with 1 μM (+)-butaclamol.

> apomorphine > dopamine (Table 2 and Fig. 3). Specific results of binding experiments with the various mutants are detailed below and in Tables 1 and 2 and Figs. 2 and 3.

F198A. Mutation of phenylalanine-198 to alanine in TM5 (F198A) exhibited antagonist binding characteristics essentially unchanged from those of the wild-type D_2 (Table 1). Affinities for the D_2 agonists bromocriptine, NPA, apomorphine, and dopamine were also relatively unaffected (Table 2 and Fig. 3); however, no specific binding for the agonist [^{3}H]N-0437 could be measured.

F389A and F390A. Individual mutations of phenylalanine-389 to alanine (F389A) and phenylalanine-390 to alanine (F390A), both in TM6, produced dramatic effects on binding to both antagonists and agonists (Tables 1 and 2). Specific binding was detectable for F390A neither to the agonist [^{3}H]N-0437 nor to the antagonists [^{3}H]spiperone and [^{3}H]raclopride. F390A's failure to bind any labeled ligand used in this study precluded further pharmacological characterization of this mutant. Saturation binding experiments with F389A produced similarly dramatic K_D changes; specific binding was not detectable to the agonist [^{3}H]N-0437 or to the antagonist [^{3}H]raclopride. However, F389A was able to bind [^{3}H]spiperone, but with a 34-fold decreased affinity, as compared with the wild-type D_2 (Fig. 2). Competition isotherms for F389A, using [^{3}H]spiperone as the labeled ligand, demonstrated dramatic shifts in K_i values (59–327-fold) for binding with the agonists bromocriptine, apomorphine, NPA, and dopamine (Fig. 3).

L387A and F411A. In saturation studies, the individual mutations of phenylalanine-411 to alanine (F411A) in TM7 and leucine-387 to alanine (L387A) in TM6 demonstrated no appreciable changes in binding affinities for the antagonists [^{3}H]spiperone and [^{3}H]raclopride or to the agonist [^{3}H]N-0437 (Tables 1 and 2). For the most part, competition isotherms with agonists produced similarly undramatic shifts in K_i values (Table 2 and Fig. 3) with one exception: Mutant L387A produced a 10-fold decrease in affinity for dopamine.

Effect of mutations on intracellular cAMP accumulation

The wild-type D_2 receptor and the D_2 mutants (F198A, F389A, F390A, F411A, and L387A) were tested for their ability to inhibit forskolin-stimulated accumulation of intracellular cAMP by incubation with the D_2 agonist dopamine or bromocriptine in electroporated COS-1 cells. Electroporation, in contrast to transfection by calcium phosphate precipitation, yielded much higher transfection efficiencies, based on β-galactosidase transfection efficiency estimates (70–90% of all cells by electroporation in comparison with 5–15% by calcium phosphate). As a result, electroporation was the transfection method of choice for performing functional assays. Dose–response curves derived from either the wild-type D_2 receptor or the mutant F198A were biphasic for the agonists dopamine and bromocriptine (Fig. 4). The wild-type D_2 produced statistically significant inhibition of forskolin-stimulated cAMP accumulation between 20 nM and 2 μM dopamine (Fig. 4). This inhibition was maximal (49%) at 20 nM dopamine (Table 3). The wild-type D_2 also demonstrated significant inhibition at 3 and 10 nM bromocriptine, maximally by 62% at 10 nM bromocriptine. Inhibition by F198A reached 25% for dopamine at 200 nM dopamine and 55% for 10 nM bromocriptine (Table 3 and Fig. 4). However, at higher dopamine or bromocriptine concentrations, the inhibition was attenuated in a dose-dependent manner. The mutants F389A, F411A, and L387A did not display discernible dose–response curves for dopamine ($n = 7$) or for bromocriptine ($n = 6$). This impaired inhibition could not be explained simply by lack of expression due to electroporation. Receptor levels for F198A, F389A, F411A, and L387A were similar to those obtained with the *wild-type* D_2 (200–500 fmol/mg of protein); electroporated F390A was not detectable by [^{3}H]spiperone binding. F390A did not display statistically significant inhibition by bromocriptine but did demonstrate 33% inhibition at 2 μM dopamine (Table 3 and Fig. 4).

DISCUSSION

The results from mutations of hydrophobic residues within the putative TM regions of the D_2 dopamine receptor demonstrate that single amino acid changes

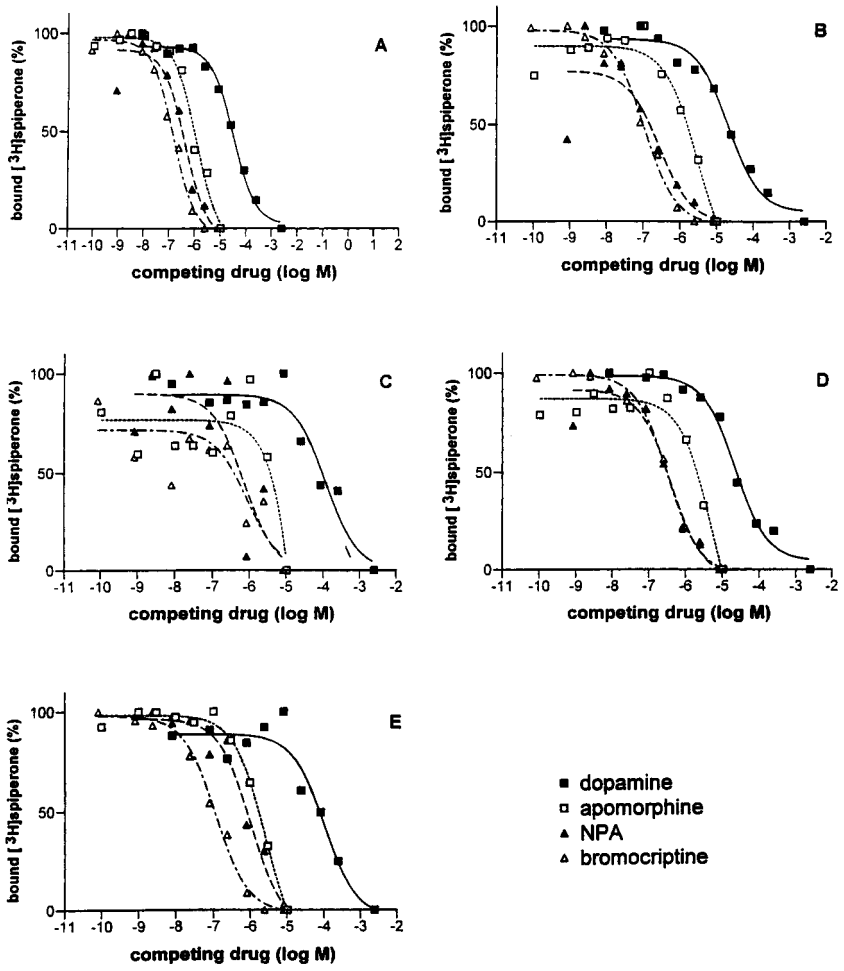

FIG. 3. Representative competition binding assays for (**A**) wild-type D_2, (**B**) mutant F198A (phenylalanine-198 → alanine), (**C**) mutant F389A (phenylalanine-389 → alanine), (**D**) mutant F411A (phenylalanine-411 → alanine), and (**E**) mutant L387A (leucine-387 → alanine) receptors. Saturating concentrations of [^{3}H]spiperone were displaced by bromocriptine, NPA, apomorphine, and dopamine. Data are not presented for mutant F390A (phenylalanine-390 → alanine) because this mutant was unable to bind any labeled ligand used in this study.

can markedly affect binding and signal transduction properties of this seven-TM receptor. Two TM6 phenylalanine mutants, F389A and F390A, bound ligands very poorly. In contrast, mutants in the TM7 phenylalanine (F411A) and the TM6 leucine (L387A) displayed pharmacological profiles similar to that of the wild-type D_2. The TM5 mutant F198A exhibited relatively high affinity for most of the ligands tested but displayed no specific binding for the agonist [^{3}H]N-0437. When analyzed for functional characteristics, i.e., abilities to inhibit cAMP accumulation when stimulated with agonists, it was clear for all the mutants except for F198A that signal transduction was impaired to some degree. For all mutants, however, western analysis suggests that a receptor of the correct molecular weight was expressed. Because F389A and F390A displayed poor binding as well as poor signal transduction, it is difficult to assess whether the diminished functional capacities of these particular mutants were secondary to binding affinity changes or to entirely different mechanisms. Although the functional deficits of F389A and F390A may have been predicted from their poor binding affinities to agonists, it is interesting that it proved *not* to be the case that the mutants that successfully bound agonists were necessarily the same mutants that inhibited adenylyl cyclase; that is, a non-identity between binding and signal transduction was observed in mutants F411A and L387A. F411A and L387A retained high affinities to agonists but were unable to inhibit adenylyl cyclase. These mutants are most interesting as minor structural changes have highlighted separately the binding and signal transduction functions of the D_2 receptor.

The wild-type D_2 receptor, when analyzed for binding characteristics, produced a typical D_2-like pharmacological profile. When analyzed for functional characteristics, the wild-type receptor produced an unexpected biphasic response to agonist dose. It has been amply demonstrated that the D_2 receptor is able to inhibit adenylyl cyclase activity (De Camilli et al., 1979; Albert et al., 1990), and measurement of intracellular cAMP level changes demonstrates that in this experimental paradigm, the agonists dopamine and bromocriptine were able to inhibit elevated intracellular cAMP levels in COS-1 cells transiently transfected with the wild-type D_2 receptor. Both agonists were able to inhibit cAMP accumulation maximally by ~50–60%. It is interesting that the inhibition curves

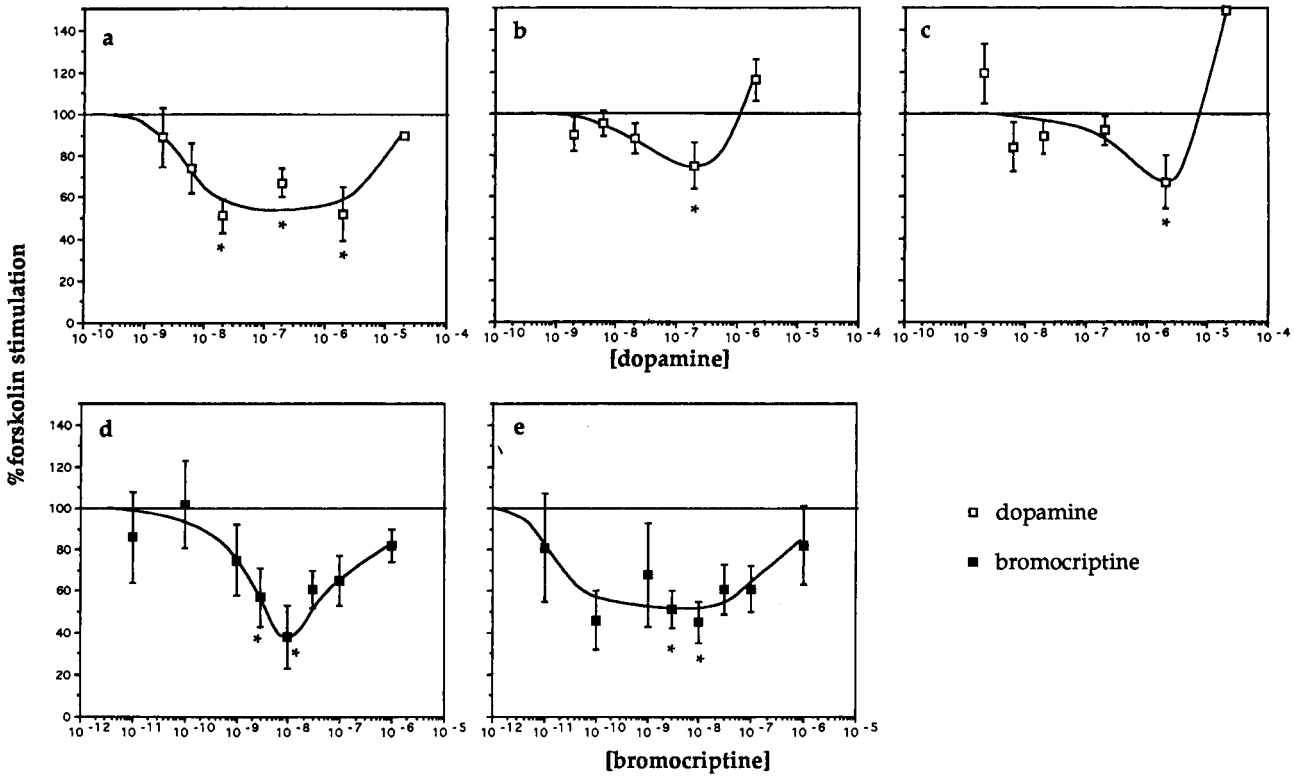

FIG. 4. Dose–effect curves for stimulation with either dopamine (n = 7) or bromocriptine (n = 6): (**a**) D_2 wild-type, (**b**) F198A (phenylalanine-198 → alanine), (**c**) F390A (phenylalanine-390 → alanine) with dopamine, (**d**) D_2 wild-type, and (**e**) F198A with bromocriptine. Data are mean ± SEM (bars) values at each agonist dose as percentages of the stimulated state without agonist (50 μM forskolin + 0.5 mM 3-isobutyl-1-methylxanthine). Other mutants did not produce statistically significant dose effects. *Significance by Fisher PLSD post hoc analysis of one-factor ANOVAs at 95%.

were not sigmoidal but rather U-shaped. This biphasic pattern was very reproducible. Similar responses in adenylyl cyclase activity have been reported in Chinese hamster ovary cells transfected with variants of the α_2-adrenergic receptor (Eason et al., 1992) and by m4-muscarinic acetylcholine receptors, also transfected into Chinese hamster ovary cells (Jones et al., 1991). Both the α_2-adrenergic receptor and the m4-muscarinic acetylcholine receptors, like the D_2 dopamine receptor, have been traditionally categorized as G_i-coupled. Federman et al. (1992) have reported that stimulation of D_2 receptors expressed in COS-7 cells increases adenylyl cyclase activity when these cells are cotransfected with a mutationally active α subunit of G_s; the "conditional" stimulation is thought to be due to activation of type II adenylyl cyclase by $\beta\gamma$ subunits released from G_i. The study by Eason et al. (1992) suggests that the stimulatory arm of the biphasic curve is due to direct coupling with G_s, as a result of overexpression of the recombinant receptors. In the present study, attenuation of adenylyl cyclase inhibition at high agonist doses may similarly be due to overexpression of the D_2 receptor, either causing a "spillover" coupling with G_s, which acts to increase adenylyl cyclase activity, or allowing G_i-released $\beta\gamma$ subunits to stimulate conditionally adenylyl cyclase. The mechanism and physiological relevance of this phenomenon have yet to be determined. Using the binding profile and functional characteristics of the wild-type D_2 receptor as reference standards, the properties of the mutated receptors were assessed and are described in detail below.

F198A

Phenylalanine-198, located in TM5, is hypothesized to stabilize binding through $\pi-\pi$ interactions with the aromatic nucleus of the endogenous catecholamine ligand dopamine. Yet replacement of this aromatic group with the methyl group of alanine only disrupted binding of the agonist [^{3}H]N-0437, whereas the binding of other ligands remained essentially unaffected. These data suggest that if phenylalanine-198 does offer a stabilizing influence, its absence is not detected by the binding of most dopaminergic ligands. In other words, all aromatic ligands may not depend on this particular influence to the same degree. The differential effects are not surprising given that the molecular structures of the ligands tested vary widely. Alternatively, phenylalanine-198 may be highly conserved owing to a generalized structural role that does not affect the nature of the binding pocket.

TABLE 3. *Maximal inhibition of cAMP accumulation by dopamine and bromocriptine*

Receptor	Maximal inhibition (%)	
	Dopamine	Bromocriptine (10 n*M*)
Wild-type D_2	49 ± 8% (20 n*M*)	62 ± 15%
F198A	25 ± 11% (200 n*M*)	55 ± 10%
F389A	—	—
F390A	33 ± 14% (2 μM)	—
F411A	—	—
L387A	—	—

Intracellular levels of cAMP were elevated with 50 μM forskolin and 0.5 m*M* 3-isobutyl-1-methylxanthine in COS-1 cells transfected with wild-type and mutant (F198A, phenylalanine-198 → alanine; F389A, phenylalanine-389 → alanine; F390A, phenylalanine-390 → alanine; F411A, phenylalanine-411 → alanine; and L387A, leucine-387 → alanine) D_2 receptors. Mean ± SEM inhibition of this accumulation (n = 6–9) is reported at 10 n*M* bromocriptine, the dose that produced maximal inhibition in the wild-type D_2 and mutant F198A, and at indicated doses of dopamine. Dashes indicate no significant inhibition when analyzed by one-factor ANOVA at 95%.

Mutant F198A was able to bind dopamine and bromocriptine with wild-type D_2-like affinities and accordingly demonstrated near wild-type functional characteristics when stimulated with these agonists. Both the models generated by Trumpp-Kallmeyer et al. (1992) and by our laboratory predict the importance of the TM5 phenylalanine-198 for both binding stabilization and receptor activation. However, the pharmacological profile of F198A was largely unchanged, and inhibition of adenylyl cyclase activity did not appear to be dramatically affected, although maximal inhibition by dopamine may have been reduced somewhat. It is interesting to note that the dose–response profiles of F198A were also biphasic. Thus, at least with respect to the agonists dopamine and bromocriptine, it appears that the bulk and aromatic properties of phenylalanine-198 are not overtly important for agonist-induced signal transduction or for stabilization of binding as originally predicted. However, the functionally intact appearance of F198A does not rule out the potential role of phenylalanine-198 in signal transduction. The models tested in this study implicate several other hydrophobic side chains in receptor activation, as mentioned in the introductory section. It is conceivable that a ligand as large as the ergot derivative, bromocriptine, is able to trigger critical conformational changes by interacting with multiple, parallel triggering mechanisms that remain intact in F198A. In addition, the slightly reduced maximal inhibition by dopamine (25% vs. 49% for the wild-type D_2) and the narrower range of doses that produced significant inhibition may indicate some reduction in signal transducing capabilities induced by the endogenous ligand. To characterize this mutant completely, functional assays using N-0437 would be desirable because the altered binding affinity for [^{3}H]N-0437 in the absence of similar changes for other agonists suggests the existence of some specific interaction between this particular molecule and phenylalanine-198. Unfortunately, this agonist was not available in the unlabeled form.

F389A and F390A

Of the hydrophobic residues mutated in this study, it appears that the conserved phenylalanines at positions 389 and 390 in TM6 are most critical for binding to both agonists and antagonists. The importance of the phenylalanine residue at position 389 is demonstrated by the severely altered binding profile of the D_2 receptor when this amino acid was mutated to an alanine. Computer-generated models of the D_2 receptor set forth by Trumpp-Kallmeyer et al. (1992) and by our laboratory predict that this conserved residue forms part of a hydrophobic pocket that stabilizes the electrostatic interactions between the negatively charged TM3 aspartate-114 and the positively charged head group of dopamine. Based on these models, one would expect that alteration of this stabilizing pocket would alter binding of any ligand that utilizes this binding site. The presently reported binding profile is constructed of five agonists and two antagonists; the binding affinities to all of these ligands were reduced, suggesting the "universal" use of the binding site at aspartate-114 stabilized by phenylalanine-389. Similar to the dramatic binding changes observed on mutation of phenylalanine-389, mutation of phenylalanine-390 to an alanine disrupted binding of the D_2 receptor to the agonist [^{3}H]N-0437 and the antagonists [^{3}H]raclopride and [^{3}H]spiperone to such a degree that no specific binding could be observed for either of these ligands. The importance of this phenylalanine for binding is supported by mutation of the equivalent residue in β-adrenergic (Dixon et al., 1988) and the serotonin 5-HT_2 (Choudhary et al., 1993) receptors. Unlike phenylalanine-389, which may shield the interaction between aspartate-114 and positively charged groups of the ligands, phenylalanine-390 may interact with the aromatic nucleus of catecholamines. Although the predictions governing binding stability were originally modeled on catecholamine binding, these predictions are applicable to the agonist [^{3}H]N-0437 and the antagonist [^{3}H]raclopride, because both ligands contain an aromatic core, and both appear to utilize binding sites at the D_2 receptor similar to those used by dopamine (Mansour et al., 1992). Although the alterations in binding may be due to loss of stabilizing influences, the phenylalanines at positions 389 and 390 are located very close to a proline residue at position 388, and binding shifts may be due to local alterations in protein conformation about this potential "hinge" site (Brandl and Deber, 1986).

The replacement of the large aromatic TM6 phenylalanines not only disrupted binding but also signal transduction. Mutants F389A and F390A were unable to demonstrate significant inhibition of cAMP accumulation when stimulated with bromocriptine. These results may have been predicted from the relatively poor

binding affinity of F389A for bromocriptine (K_i = 850 n*M*, compared with 8.2 n*M* for the wild-type D_2) and the presumably poor affinity of F390A for this ligand. The impaired receptor function observed on bromocriptine stimulation is most likely due to impaired binding to these mutants. Similarly, F389A was unresponsive to dopamine. It is surprising that F390A, which was presumed unable to bind any agonists, was capable of inhibiting cAMP accumulation when stimulated with the endogenous ligand dopamine, although only at a much higher concentration. Dopamine binding with F390A could not be directly assessed owing to this mutation's inability to bind any radioligand tested; however, in light of these functional data, it must be assumed that this mutant is capable of some binding with dopamine.

The results obtained from F389A and F390A are in some disagreement with the results of a recent mutagenesis study in the 5-HT_2 receptor subtype in which mutation of phenylalanine-340 (equivalent to phenylalanine-390 in the human D_2) to a leucine diminished binding of several antagonists and agonists and also diminished functional coupling to phosphoinositide hydrolysis, whereas mutation of phenylalanine-339 (equivalent to the D_2 phenylalanine-389) to leucine had less dramatic effects on binding and virtually no effect on 5-HT_2 receptor coupling (Choudhary et al., 1993). The present study indicates that mutation of either one of these highly conserved phenylalanines significantly disrupts D_2 binding and function, more so for phenylalanine-389 than phenylalanine-390. The 5-HT_2 receptor mutations, however, were made to leucine, thus preserving the bulk of the substituted residues while eliminating their aromaticity. By examining the results of the 5-HT_2 receptor mutations together with those of the present study, it seems that the size and hydrophobicity, rather than the aromaticity, of phenylalanine-389 (or phenylalanine-339 in the 5-HT_2) are important for proper ligand binding and signal transduction, whereas the aromaticity of phenylalanine-390 (phenylalanine-340 in the 5-HT_2 receptor) appears critical for stabilization of ligand binding and for signal transduction. Alternatively, the stabilizing role of phenylalanine-389 may not be conserved between dopaminergic and serotonergic receptors, because these receptor families bind different classes of small molecules, i.e., catecholamines and indoleamines, respectively. Both classes of ligands possess a positively charged amine group that presumably binds to the TM3 aspartate-114 of D_2 or its serotonergic equivalent. But whereas catecholamines appear to utilize the two hydroxyl groups of the catechol moiety for binding to two TM5 serines (Mansour et al., 1992), indoleamines have only the hydroxyl group, which potentially binds to only one TM5 serine (Trumpp-Kallmeyer et al., 1992). Thus, the slight variation in ligand anchor points may produce variation in the importance these TM6 phenylalanines. In both receptor families, however, it is clear that replacement of either of these conserved phenylalanine residues in TM6 disrupts ideal binding and function of the receptor, perhaps by eliminating stabilizing stacking interactions with the ligand or by altering the native conformation of the receptor.

F411A and L387A

The TM7 phenylalanine at position 411 and the TM6 leucine at position 387 were *not* residues predicted to be important for ligand binding stabilization. Accordingly, replacement of phenylalanine-411 with an alanine (F411A) failed to produce dramatic changes in the wild-type binding affinities to various antagonists and agonists. Similarly, uninteresting changes were observed for the replacement of leucine-387 with alanine (L387A) except for a modest (10-fold) increase in the K_i value for dopamine. Binding with dopamine may have been less than optimal owing to subtle changes in receptor conformation caused by the replacement of the large branched chain of leucine-387 with the relatively small methyl group of alanine. If the immediately adjacent proline-388 represents a critical point of flexure for maintaining optimal receptor conformation, a change in the adjacent conformational space may have produced slight changes in the tertiary structure of the protein to which only the endogenous ligand, dopamine, is sensitive.

Although replacement of the bulky side chains of phenylalanine-411 and leucine-387 did not dramatically affect ligand binding, these mutated residues proved to be critical for signal transduction through the G_i/adenylyl cyclase pathway. Neither dopamine nor bromocriptine was able to inhibit adenylyl cyclase through either of these mutants despite their high-affinity binding to these agonists. Thus, single amino acid substitutions within either the sixth or seventh transmembrane helices apparently uncoupled ligand binding from signal transduction. Although both the TM7 phenylalanine-411 and the TM6 leucine-387 are suspected to be involved in a common conformational shift of the entire protein, the "micromechanisms" by which these mutations disrupt receptor activation are probably different. Computer modeling performed in our laboratory suggests that, on agonist binding, phenylalanine-411 may move owing to steric interactions. It is conceivable that removal of this particular TM7 phenylalanine could eliminate sterically induced shifts in the position of the seventh TM helix and, as a consequence, the position of the carboxyl-terminal tail, a region shown to be involved in G protein coupling (Dixon et al., 1988; Cotecchia et al., 1990). In contrast, the leucine at position 387 of TM6 probably does not move in response to steric influences. Instead, this residue may be an important influence on the "hinge" action of the adjacent proline at position 388. Prolines are exceptional not only for placing kinks in α-helices, but possibly also for providing membrane proteins with natural hinges (Brandl and Deber, 1986) owing to the relatively high probability of *trans*/*cis* isomerization

about the X–proline peptide bond, where X is any amino acid to the N-terminal side (Brandts et al., 1975). There is some evidence that the residue immediately preceding proline may affect this isomerization because the bulky pyrrolidine ring restricts the conformational space of the preceding amino acid (Brandts et al., 1975). Replacement of the large branched leucine residue with the small, hydrophobic methyl group of alanine changes the steric environment near the adjacent proline and may affect not only its isomerization but also the motion of the entire sixth TM barrel. It is not difficult to believe that a critical hinge motion of TM6 is involved in receptor activation and G protein coupling because this helix is directly linked to the third cytosolic loop of the receptor protein.

It is interesting that replacement of any single one of these residues (phenylalanine-411 and leucine-387) with an alanine was sufficient to impair coupling. The simplest explanation for these observations is a linear or chain-like arrangement of steps involving phenylalanine-411 and leucine-387 (and also possibly phenylalanines at positions 389 and 390) in which removal of any one of the "links" would block the propagation of conformational changes. However, one cannot rule out the possibility that these residues may individually be involved in disparate pathways leading to a common activated receptor conformation.

Conclusions

To summarize the present study, mutations of several hydrophobic amino acids in the TM domains of the human D_2 receptor, long isoform, were constructed and characterized. The mutated residues were chosen based on computer-generated models devised by our laboratory and that of Trumpp-Kallmeyer et al. (1992) that predicted their importance in binding and signal transduction. Such computerized models serve as excellent tools for visualizing the receptor protein and providing starting points for mutagenesis studies. However, the present study produced some interesting details of the D_2 receptor that could not have been completely predicted from the tested models. Perhaps the most mundane interpretations would have arisen from mutations that simultaneously disrupted agonist binding and inhibition of adenylyl cyclase activity (such as F389A) or mutations that significantly altered neither of these receptor-mediated events (such as F198A). From the results presented, however, one observes that single amino acid mutations were able to separate ligand binding events from the ultimate signal transduction event. As cases in point, the mutants F411A and L387A were capable of binding dopamine and bromocriptine with near wild-type affinities, yet they were unable to demonstrate functional coupling. Previous site-directed mutagenesis studies have successfully elaborated regions of the receptor critical for ligand binding and regions important for coupling with G proteins. However, before a complete understanding of seven-TM receptor function may be achieved, further mutagenesis efforts with the aid of computer modeling, such as the study reported here, must be aimed at disclosing mechanisms linking these events.

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REFERENCES

Albert P. R., Neve K. A., Bunzow J. R., and Civelli O. (1990) Coupling of a cloned rat dopamine-D_2 receptor to inhibition of adenylyl cyclase and prolactin secretion. *J. Biol. Chem.* **265,** 2098–2104.

Brandl C. J. and Deber C. M. (1986) Hypothesis about the function of membrane-buried proline residues in transport proteins. *Proc. Natl. Acad. Sci. USA* **83,** 917–921.

Brandts J. F., Halvorson H. R., and Brennan M. (1975) Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis–trans* isomerism of proline residues. *Biochemistry* **14,** 4953–4963.

Chen C. and Okayama H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7,** 2745–2752.

Choudhary M. S., Craigo S., and Roth B. L. (1993) A single point mutation ($Phe^{340} \rightarrow Leu^{340}$) of a conserved phenylalanine abolishes 4-[^{125}I]iodo-(2,5-dimethoxy)phenylisopropylamine and [^{3}H]mesulergine, but not [^{3}H]ketanserin binding to 5-hydroxytryptamine$_2$ receptors. *Mol. Pharmacol.* **43,** 755–761.

Cotecchia S., Exum S., Caron M., and Lefkowitz R. (1990) Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc. Natl. Acad. Sci. USA* **87,** 2896–2990.

Cox B. A., Henningsen R. A., Spanoyannis A., Neve R. L., and Neve K. A. (1992) Contributions of conserved serine residues to the interactions of ligands with dopamine D_2 receptors. *J. Neurochem.* **59,** 627–635.

Dahl S. G., Edvardsen Ø., and Sylte I. (1991) Molecular dynamics of dopamine at the D_2 receptor. *Proc. Natl. Acad. Sci. USA* **88,** 8111–8115.

De Camilli P., Macconi D., and Spada A. (1979) Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. *Nature* **278,** 252–254.

Dixon R. A. F., Sigal I. S., Rands E., Register R. B., Candelore M. R., Blake A. D., and Strader C. D. (1987) Ligand binding to the β-adrenergic receptor involves its rhodopsin-like core. *Nature* **326,** 73–77.

Dixon R. A. F., Sigal I. S., and Strader C. D. (1988) Structure–function analysis of the β-adrenergic receptor. *Cold Spring Harbor Symp. Quant. Biol.* **53,** 487–497.

Eason M. G., Kurose H., Holt B. D., Raymond J. R., and Liggett S. B. (1992) Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. *J. Biol. Chem.* **267,** 15795–15801.

Federman A. D., Conklin B. R., Schrader K. A., Reed R. R., and Bourne H. R. (1992) Hormonal stimulation of adenylyl cyclase through G_i-protein $\beta\gamma$ subunits. *Nature* **356,** 159–161.

Fong T. M., Huang R.-R. C., and Strader C. D. (1992) Localization of agonist and antagonist binding domains of the human neurokinin-1 receptor. *J. Biol. Chem.* **267,** 25664–25667.

Henderson R., Baldwin J., Ceska T. H., Zemlin F., Beckmann E., and Downing K. (1990) Model of the structure of bacteriorhodopsin based on high resolution electron cryo-microscopy. *J. Mol. Biol.* **213,** 899–929.

Horstman D. A., Brandon S., Wilson A. L., Guyer C. A., Cragoe E. J., and Limbird L. E. (1990) An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* **265,** 21590–21595.

Jones S. V. P., Heilman C. J., and Brann M. R. (1991) Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.* **40,** 242–247.

Kobilka B. K., Kobilka T. S., Daniel K., Regan J. W., Caron M. G., and Lefkowitz R. J. (1988) Chimeric α_2-,β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* **240,** 1310–1316.

Mansour A., Meng F., Meador-Woodruff J. H., Taylor L. P., Civelli O., and Akil H. (1992) Site-directed mutagenesis of the human dopamine D_2 receptor. *Eur. J. Pharmacol.* **227,** 205–214.

Munson P. J. and Rodbard D. (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107,** 220–230.

Neve K. A., Cox B. A., Henningsen R. A., Spanoyannis A., and Neve R. L. (1991) Pivotal role for aspartate-80 in the regulation of dopamine D_2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.* **39,** 733–739.

O'Dowd B. F., Hnatowich M., Regan J. W., Leader W. M., Caron M. G., and Lefkowitz R. J. (1988) Site-directed mutagenesis of the cytoplasmic domains of the human β_2-adrenergic receptor. Localization of regions involved in G protein–receptor coupling. *J. Biol. Chem.* **263,** 15985–15992.

Pollock N. J., Manelli A. M., Hutchins C. W., Steffey M. E., MacKenzie R. G., and Frail D. E. (1992) Serine mutations in transmembrane V of the dopamine D_1 receptor affect ligand interactions and receptor activation. *J. Biol. Chem.* **267,** 17780–17786.

Sibley D. R. and Monsma F. J. Jr. (1992) Molecular biology of dopamine receptors. *Trends Pharmacol. Sci.* **13,** 61–69.

Strader C. D., Dixon R. A., Cheung A. H., Candelore M. R., Blake A. D., and Sigal I. S. (1987*a*) Mutations that uncouple the β-adrenergic receptor from Gs and increase agonist affinity. *J. Biol. Chem.* **262,** 16439–16443.

Strader C. D., Sigal I. S., Register R. B., Candelore M. R., Rands E., and Dixon R. A. F. (1987*b*) Identification of residues required for ligand binding to the β-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84,** 4384–4388.

Strader C. D., Sigal I. S., Candelore M. R., Rands E., Hill W. S., and Dixon R. A. F. (1988) Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **263,** 10267–10271.

Strader C. D., Candelore M. R., Hill W. S., Sigal I. S., and Dixon R. A. F. (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J. Biol. Chem.* **264,** 13572–13578.

Trumpp-Kallmeyer S., Hoflack J., Bruinvels A., and Hibert M. (1992) Modeling of G-protein-coupled receptors: applications to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. *J. Med. Chem.* **35,** 3448–3462.

Wang C. D., Buck M. A., and Fraser C. M. (1991) Site-directed mutagenesis of α_{2A}-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.* **40,** 168–179.