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Site-directed mutagenesis of the human dopamine D_2 receptor

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Based on amino acid sequence and computer modeling, two conflicting three-dimensional models of the dopamine D_2 receptor have been proposed. One model (Dahl et al., 1991, Proc. Natl. Acad. Sci. USA 88, 8111) suggests that dopamine interacts with aspartate 80 of transmembrane (TM) 2 and asparagine 390 of TM6 with the transmembranes arranged in a clockwise manner, while a second model (Hibert et al., 1991, Mol. Pharmacol. 40, 8) suggests that dopamine interacts with aspartate 114 of TM3 and the serines of TM5 (194 and 197) with the transmembranes arranged in a counterclockwise manner when viewed from the extracellular space. The present study tests the latter model by selectively mutating aspartate 114 and serines 194 and 197 of the human dopamine D_2 receptor by site-directed mutagenesis. In addition, two methionines (116 and 117) were mutated to evaluate whether residues near aspartate (114) of the dopamine D_2 receptor are critical in differentiating dopamine receptor agonists from adrenoceptor agonists. Removal of the negative charge with the mutation of aspartate (114) to either asparagine or glycine led to a total loss of both agonist and antagonist binding. Individual or dual methionine mutations in positions 116 and 117, to make the dopamine D_2 binding pocket more closely resemble the β_2-adrenoceptor, did not result in a change in selectivity toward noradrenergic agonists or antagonists. The serine mutations revealed interesting differences between the dopamine D_2 receptor and the adrenoceptors. In particular, serine 197 appeared more important than serine 194 for agonist binding. In addition, the binding of one agonist (N-0437) was unaffected by individual serine mutations, while the binding of some antagonists, such as raclopride and spiperone, was significantly altered. These findings are discussed in relation to ligand structure and their interactions with the putative binding pocket.

Dopamine D_2 receptors; Mutagenesis; Receptor binding; Catecholamine receptors

1. Introduction

The recent cloning (Bunzow et al., 1988; Dearry et al., 1990; Mahan et al., 1990; Monsma et al., 1990; Sokoloff et al., 1990; Sunahara et al., 1990, 1991; Zhou et al., 1990; Van Tol et al., 1991) of the dopamine receptors (D_1–D_5) suggests that they are members of a larger family of G-protein coupled, seven transmembrane receptors (Gilman, 1987) whose other members include the adrenoceptor (Dixon et al., 1986; Frielle et al., 1987; Kobilka et al., 1987a,b; Cotecchia et al., 1988; Regan et al., 1988), muscarinic acetylcholine receptor (Bonner et al., 1987; Peralta et al., 1987; Ramachandran et al., 1989), and peptidergic receptors (Masu et al., 1987; Hershey and Krause, 1990; Tanaka et al., 1990). These receptors share a common structural motif and are similar to the better characterized rhodopsin protein (Henderson et al., 1990) which spans the plasma membrane seven times, with the transmembrane (TM) domains forming a binding pocket. The N-terminal region is thought to be extracellular and the COOH-terminus intracellular. Three intracellular and three extracellular loops are formed with this organization and the second and third cytoplasmic loops are thought to be involved in G-protein coupling (Kobilka et al., 1988; Strader et al., 1987a).

Site-directed mutagenesis studies of the β_2-adrenoceptor suggest that specific amino acids deep in the binding pocket formed by the TM domains are critical for the recognition of catecholamines (Fraser, 1989; Strader et al., 1987, 1989a,b; Neve et al., 1991). In particular, aspartate 79 in TM2 and aspartate 113 in TM3 are thought to interact with the amino group of epinephrine and norepinephrine (Strader et al., 1987b), while two serines (204 and 207) in TM5 form hydrogen bonds with the hydroxyl groups of the catechol moiety (Strader et al., 1989a). Studies by Strader et al. (1989a)

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further suggest that the serines in TM5 are important only for agonist binding and their mutation to alanine does not alter antagonist binding.

Site-directed mutagenesis of corresponding residues in the α_2-adrenoceptor (Wang et al., 1991) suggests that there may be differences in the way catecholamines bind in the α_2- and β_2-receptors. Unlike the β_2-receptor, serine mutations in TM5 of the α_2-receptor can still exhibit a maximal cAMP inhibition with higher agonist concentrations despite a reduced receptor affinity. In the case of the β_2-receptor, similar mutations result in a complete loss of cAMP effects. Further, the two series (204 and 207) in TM5 of the β_2-receptor appear equally necessary for agonist affinity, while in the α_2-receptor the serines (200 and 204) are asymmetrically important, with serine 204 being primary in forming hydrogen bonds with the parahydroxyl group of the catecholamines. In both the α_2- and β_2-receptors, aspartate 113 of TM3 appears critical for agonist and antagonist binding.

Amino acid analysis of the adrenoceptor (α_1, α_2, β_1, β_2) and the dopamine receptors (D_1–D_5) demonstrate that the aspartates in TM2 and TM3 and the serines in TM5 are conserved across these catecholamine receptors, consistent with a common catecholamine binding pocket (table 1). Yet despite this common structure, other amino acids likely extend into the binding pocket to confer receptor affinity and selectivity. Identifying these residues is particularly important for better understanding of the catecholamine binding pocket and developing more efficacious and selective therapeutic drugs. In addition, as the majority of the information concerning catecholamine receptor structure has been derived from the α_2- and β_2-adrenoceptor, it is important to extend these studies to other receptors to determine whether substantial differences may exist. In the case of the dopamine receptors, only one site-directed mutagenesis study is available in which the effects of aspartate 80 mutations on D_2 receptor binding were evaluated (Neve et al., 1991). No information concerning the aspartate in TM3 or the serines in TM5 of the dopamine receptors is available.

Based on the amino acid sequence of the dopamine D_2 receptor and computer modeling, two conflicting three-dimensional models of the D_2 receptor have been proposed. One model (Dahl et al., 1991) suggests that dopamine interacts with aspartate 80 of TM2 and asparagine 390 of TM6 with the transmembranes being arranged in clockwise manner when viewed from the extracellular space. A second model (Hibert et al., 1991) suggests that dopamine may interact with aspartate 114 of TM3 and the serines of TM5 (194 and 197) with the transmembranes arranged in a counterclockwise manner. To further evaluate these models and their relevance to other catecholamine receptors, the present study examines the importance of the asparate 114 by site-directed mutagenesis, altering it to either asparagine or glycine, two neutral amino acids. The roles of serines 194 and 197 in TM5 of the D_2 receptor in agonist and antagonist binding were also examined to determine whether dopaminergic compounds interact with these amino acid residues. Pharmacological data with dopaminergic ligands (Seeman, 1981) indicate that the meta-hydroxyl group of dopaminergic agonists is primarily important in stabilizing agonist binding, suggesting that the serine residues (194 and 197) of the D_2 receptor may not be equally important for binding affinity as appears to be the case for the β_2-receptor. D_2 receptors with single or dual serine (194 and 197) mutations were, therefore, evaluated in the present study.

As the chemical structures of dopamine and norepinephrine are nearly identical, differing only in a beta-hydroxyl group present in norepinephrine, we hypothesized that there may be specific amino acids near the critical aspartate (114) in TM3 that might aid in conferring receptor selectivity To test this possibility, two adjacent methionines in TM3 of the D_2 receptor in positions 116 and 117 (table 1) were mutated individually or in combination to leucine (116) and cysteine (117) to more closely approximate the amino acid sequence of the β_2-adrenoceptor. Methionine 116 is conserved across all the dopamine receptors (D_1–D_5), but is replaced by leucine in the adrenoceptors (α_1, α_2, β_1, β_2). Methionine in position 117 is conserved in D_2 and D_3, but is replaced by either cysteine (D_1 and D_5) or leucine (D_4) in other dopamine receptors. It was hypothesized that one or both these methionines may provide steric interference with the side chain OH group of epinephrine and norepinephrine, but would allow dopamine, without this moiety, to bind to the D_2 receptor.

2. Materials and methods

2.1. Mutagenesis and expression

Mutant receptors of the human D_2 were prepared with the oligonucleotide-directed mutagenesis system provided by Amersham. Oligonucleotides (20–45 bases) were synthesized, purified on polyacrylamide gels (20%), and annealed to a M13 single-stranded bacteriophage that contained the entire protein coding region of the human D_2 receptor (1–1627 bp) including the 87 bp addition in the third cytosolic loop ($D_2\beta$). Briefly, the method involves extending the oligonucleotide with Klenow polymerase in the presence of T_4 DNA ligase to generate a mutant heteroduplex. The non-mutated strand is then selectively removed with exonuclease digestion and filtration, leaving the mutant

TABLE 1

Amino acid alignments of transmembrane three and five of the adrenoceptors and dopamine receptors.

Arrows indicate the aspartate, methionine and serine residues that have been mutated for the D_2 receptor. Both human (H) and rat (R) sequences are provided.

		III		V
A1	(H)	..CDIWAAVDVLCCTASILSLCAISLDRY	....	EPFYALFSSLGSFYIPLAVILVMYCR
A2A	(H)	..CEIYLALDVLFCTSSIVHLCAISLDRY	....	QKWYVISSCIGSFFAPCLIMILYYVR
A2B	(H)	..CGVYLALDVLFCTSSIVHLCAISLDRY	....	ETWYILSSCIGSFFAPCLIMGLVYAR
B1	(H)	..CEIWTSVDVLGVTASIETLCVIALDRY	....	NRAYAIASSVVSFYVPLVIMAFVYLR
B2	(H)	..CEFWTSIDVLCVTASIETLCVIAVDRY	....	NQAYAIASSIVSFYVPLVIMVFVYSR
B2	(R)	..CEFWTSIDVLCVTASVETLCVIAVDRY	....	NQAYAIASSIVSFYVPLVVMVFVYSR
D1	(H)	..CNIWVAFDIMCSTASILNLCVISVDRY	....	SRTYAISSSVISFYIPVAIMIVTYTR
D1	(R)	..CNIWVAFDIMCSTASILNLCVISVDRY	....	SRTYAISSSLISFYIPVAIMIVTYTS
D2	(H)	..CDIFVTLDVMMCTASILNLCAISIDRY	....	NPAFVVYSSIVSFYVPFIVTLLVYIK
D2	(R)	..CDIFVTLDVMMCTASILNLCAISIDRY	....	NPAFVVYSSIVSFYVPFIVTLLVYIK
D3	(R)	..CDVFVTLDVMMCTASILNLCAISIDRY	....	NPDFVIYSSVVSFYVPFGVTVLVYAR
D4	(H)	..CDALMAMDVMLCTASIFNLCAISVDRF	....	DRDYVVYSSVCSFFLPCPLMLLLYWA
D5	(H)	..CDVWVAFDIMCSTASILNLCVISVDRY	....	NRTYAISSSLISFYIPVAIMIVTYTR
		↑ ↑↑		↑ ↑

strand to regenerate the replicative DNA form that is then subcloned in a pCMV expression vector for transfection into eukaryotic cells. A total of seven single amino acid mutations were made: Asp (114) to Asn or Gly, Met (116) to Leu, Met (117) to Cys or Gly, Ser (194) to Ala, Ser (197) to Ala. Two dual mutations were also designed: Met (116) and Met (117) to Leu (116) and Cys (117) and Ser (194) and Ser (197) to Ala (194) and Ala (197). All pCMV mutant constructs were sequenced to verify that the mutations were correct.

2.2. Transfection

COS-1 cells were grown in Dulbeco's modified Eagle's medium with 10% fetal calf serum and subcultured into 90 mm tissue culture plates ($1-1.5 \times 10^6$ cells) 24 h prior to transient transfection using a calcium phosphate precipitation procedure (Chen and Okayama, 1987). Each 90 mm plate of cells was transfected with 20 μg of pCMV-D_2 wild type or pCMV-D_2 mutant DNAs. Plasmid DNAs were added to 0.5 ml 0.25 M $CaCl_2$ to which 0.5 ml of $2 \times$ BBS (50 mM N,N-bis(2-hydroxy-ethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM $NaHPO_4$, pH = 6.95) was added. This mixture was allowed to remain at 22°C for 10–20 min, then slowly dripped onto one 90 mm plate of cells. The cells were then grown overnight at 37°C and 3% CO_2, washed twice in Versine and once in medium and allowed to grow for an additional 24 h (37°C, 5% CO_2) prior to harvesting. To assess the efficiency of transfection across experiments, all studies involved the co-transfection of β-galactosidase that was subcloned into pCMV (10 μg of plasmid). Based on β-galactosidase assays, transfection efficiencies using this procedure in COS cells ranged from 10 to 40%.

2.3. Radioligand binding assays

At time of cell harvest, the culture medium was removed and each plate of cells was then washed with 10 ml of ligand incubation buffer. For [^{3}H]raclopride (New England Nuclear, 62.7–83.4 Ci/mmol) this buffer consisted of 50 mM Tris (pH = 7.4, 25°C), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 0.1% ascorbic acid. This buffer was then replaced by fresh ligand incubation buffer (7 ml) in which the transfected cells were harvested by scraping. For cells harvested for [^{3}H]N-0437 (Amersham, 54.0–98.0 Ci/mmol) binding studies, the same incubation buffer less the 120 mM NaCl and 0.1% ascorbic acid was used. Prior to homogenization (kinematic polytron), an aliquot of the cell suspension was saved for cell counting. The remaining sample was homogenized, with 200 μl of the homogenate added to each incubation tube bringing the total membrane homogenate and ligand volume to 250 μl per tube. Nonspecific binding for [^{3}H]raclopride and [^{3}H]N-0437 was defined by 1 μM (+)-butaclamol. After a 90 min incubation (22°C), membranes were filtered under vacuum through glass filters (Schleicher and Schuell, No. 32) using a Brandel cell harvester. The filters were then rinsed twice with 3.5 ml of washing buffer (50 mM Tris (pH = 7.5), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 4°C) and counted by liquid scintillation spectrometry. The wash buffer for [^{3}H]N-0437 was the same as above except that it did not contain 120 mM NaCl and 0.1% ascorbic acid. For saturation studies a minimum of eight concentrations

of radioligand (10–0.075 nM) with duplicates were used to determine Scatchard plots and all studies were replicated at least once. Similarly, competition studies used a minimum of eight concentrations of unlabelled compounds, performed in duplicate and repeated at least once. The following drugs were used to evaluate the affinity of dopaminergic agonists and antagonists: apomorphine, bromocriptine, (+)-butaclamol, chlorpromazine, clozapine, dopamine, LY171555, NPA (N-propylnorapomorphine), and spiperone. Apomorphine, bromocriptine, and dopamine were purchased from Sigma (St. Louis, MO, USA), and NPA was purchased from Research Biochemical (Natick, MA, USA). All other compounds were a kind gift from James Woods, University of Michigan. All binding data was analyzed with the LIGAND program developed by Munson and Rodbard (1980).

To assess whether the mutations altered the selectivity of the dopamine receptor, [^{125}I]CYP (cyanopindolol, New England Nuclear, 2000 Ci/mmol) binding was performed on transfected cells. [^{125}I]CYP binding studies were performed as above except that the incubation and wash buffers consisted of 50 mM Tris (pH = 7.4–7.6), 12.5 mM $MgCl_2$, and 1.5 mM EDTA. In addition, competition studies were performed with epinephrine (Sigma, St. Louis, MO, USA) and [^{3}H]N-0437.

2.4. β-Galatosidase (β-gal) assays

Aliquots (500 μl) of the transfected cell homogenates described above were centrifuged at 15,600 × *g* for 1 min and stored on ice. 200 μl of the supernatant was then added to an equal volume of β-gal assay solution (1.34 mg/ml O-nitrophenyl-β-D-galactoside, 166 mM 2-mercapteothanol, 500 mM Na_2HPO_4, 1 M KCl, 500 mM $MgCl_2$) and transferred to a cuvette. The reaction was stopped by adding 500 μl of 1 M Na_2CO_3. Spectrophotometric absorbance of this yellow reaction product was measured at 410 nm. Absorbance values of the homogenized samples were compared to a standard curve constructed with commercially available β-galactosidase (Sigma, St. Louis, MO, USA) and units of activity were determined. To calculate the percentage of cells expressing β-gal, 90 mm cell culture plates were rinsed twice in PBS, fixed in a formaldehyde (2.2%)/glutaraldehyde (0.2%) solution for 5 min at 22°C and treated with β-gal (1 mg/ml) for 1–12 h at 37°C. Cells positive for β-galactosidase yielded a blue color.

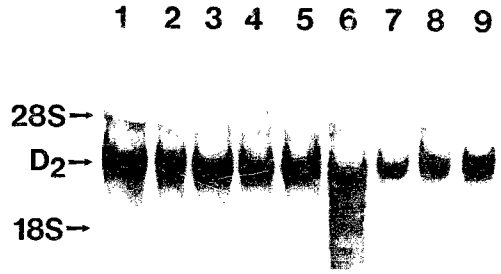

Fig. 1. Northern analysis comparing COS-1 cells transfected with the D_2 receptor mutants. The lanes are as follows: (1) Asp114-Asn, (2) Asp114-Gly, (3) Met116-Leu, (4) Met117-Cys, (5) Met117-Gly, (6) Ser194-Ala, (7) Ser197-Ala, (8) Ser194 and Ser197-Ala, (9) Met116-Leu and Met117-Cys.

2.5. Northern analysis

To aid in determining whether the receptor mutants were expressed, mRNA was extracted from cells transfected with the D_2 wild type and mutant D_2 receptors and Northern analysis was performed. RNA samples from transfected COS-1 cells were extracted with 4 M guanidium isothiocyanate and resuspended in 50% formamide, 20 mM morpholinepropanesulfonic acid (MOPS, pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. The RNA was denatured at 65°C for 10 min and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. The RNA samples were passively transferred to Nytran membranes (Schleicher and Schuell) with 10 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.2) and baked for 2 h at 80°C. The membranes were then prehybridized in 50% formamide, 5 × SSC, 5 × Denhardt's, 50 mM sodium phosphate (pH 6.5), and 0.5% SDS for a minimum of 2 h at 42°C. The hybridization buffer was the same as the prehybridization buffer except that it contained 1 × Denhardt's, 20 mM sodium phosphate, and 10% dextran sulfate. A random-primed ^{32}P-labelled fragment (BstEII/KpnI) of the human D_2 receptor was used in the hybridization

TABLE 2

K_d values (nM ± S.D.) of [^{3}H]raclopride.

Asp114-Asn	Asp114-Gly	Met116-Leu	Met117-Cys	Met117-Gly	Ser194-Ala	Ser197-Ala	Wild type
NSB	NSB	0.180 (±0.07)	0.124 (±0.005)	0.211 (±0.024)	0.235 (±0.020)	0.714 [a] (±0.044)	0.166 (±0.015)

Aspartate mutations in position 114 failed to show specific binding (NSB). Analysis of variance of the remaining mutants indicated that there was a difference between mutants and wild type D_2 ($F = 106.95$, $P < 0.0001$). Post-hoc Scheffe comparisons suggested that the serine 197 mutation significantly reduced ([a] $P < 0.05$) the affinity for raclopride.

TABLE 3

K_d Values (nM ± S.D.) of [^{3}H]N-0437.

Asp114-Asn	Asp114-Gly	Met116-Leu	Met117-Cys	Met117-Gly	Ser194-Ala	Ser197-Ala	Ser194-Ala Ser197-Ala	Wild type
NSB	NSB	0.771 (±0.244)	0.906 (±0.060)	1.14 (±0.39)	0.926 (±0.264)	0.906 (±0.118)	NSB	0.880 (±0.096)

Aspartate 114 mutations and dual serine 194 and 197 mutations demonstrated no specific binding (NSB). Other mutations failed to produce any differences in binding compared to the wild type D_2.

buffer to probe the filter overnight at 42°C. The blot was washed once in 2 × SSC and 0.5% SDS at 22°C for 15 min, transferred to 0.1 × SSC and 0.5% SDS at 65°C for 30 min and apposed to X-ray film at 22°C for 10 min.

In all of the above procedures, the D_2 mutants and the wild type human D_2 receptor were assessed in parallel. The data were analyzed by either one- or two-way ANOVAs and Schiffe post-hoc comparisons were performed.

3. Results

3.1. Ligand binding

The affinity of [^{3}H]raclopride for the wild type D_2 varied (0.17–0.55 nM) with tritiated ligand shipments and in all the following experiments the wild type D_2 was evaluated with the D_2 mutants using the same shipment of tritiated ligand. The agonist [^{3}H]N-0437 also failed to consistently demonstrate two binding sites with transiently transfected COS cells. In most cases, the LIGAND program fit the data best to one site and these are the affinities presented in table 3.

3.2. Transfection controls

All mutant receptors, when transiently transfected into COS cells, expressed high levels of D_2 receptor mRNA (fig. 1). While mRNA levels are not necessary reflections of protein levels, the qualitative differences observed between mutations are not likely due to a lack of receptor transcription. The mRNA levels in lanes 6 and 7 are somewhat lower compared to the other lanes of the Northern blot; however, these results are not consistently observed across experiments. Similarly, co-transfection results with β-gal that is measured in every experiment suggest that transfection efficiency was equivalent across mutant and wild type D_2 receptors (data not shown).

3.3. Aspartate (114) mutations

Mutation of the negatively charged aspartate (114) residue in TM3 to either asparagine or glycine produced a dramatic loss of binding affinity for both agonists and antagonists. Saturation studies with both [^{3}H]raclopride (table 2) and [^{3}H]N-0437 (table 3) failed to demonstrate any consistent dopaminergic receptor binding with aspartate mutations to either asparagine or glycine. These effects have been replicated over a series of studies and suggest that the negative charge of this aspartate is critical for D_2 receptor binding.

3.4. Serine 194 and 197 mutations

Individual mutation of serines 194 and 197 in TM5 to alanine produced asymmetrical effects on dopamine receptor binding. Saturation studies with antagonist [^{3}H]raclopride (table 2) and competition studies with [^{3}H]N-0437 (table 4) suggest that serine 197 may be differentially important for dopaminergic binding. As can be seen from table 2, elimination of the potential hydrogen bonds with the serine 197 mutation produced a 4-fold reduction in [^{3}H] raclopride binding affinity. In contrast, similar mutations of serine 194 to alanine had no effect on raclopride affinity.

Surprisingly, individual serine mutations in position 194 and 197 had no effect on the binding affinity of agonist [^{3}H]N-0437 as determined by saturations studies (table 3). Interestingly, however, competition studies using this ligand suggest that this is not the case for other dopaminergic agonists (table 4). While the loss of dopaminergic binding affinity varied with agonist, all

TABLE 4

K_i values (nM ± S.D.) of dopamine receptor agonists competing with [^{3}H]N-0437.

	Ser194-Ala	Ser197-Ala	Wild type
Apomorphine	5.01 ± 1.48	89.0 ± 17.0 [a]	2.78 ± 0.43
Bromocriptine	1.60 ± 0.19	3.45 ± 0.6 [a]	1.82 ± 0.31
Dopamine	488.0 ± 134.0	1,270.0 ± 270.0 [a]	152.0 ± 36.0
LY171555	114.0 ± 52.0	1,230.0 ± 440.0 [a]	217.0 ± 14.0
NPA	2.18 ± 0.57	39.6 ± 3.6 [a]	0.82 ± 0.10

Two-way ANOVA indicated there were significant differences for drug ($F = 60.2$, $P < 0.0001$), serine mutation ($F = 63.1$, $P < 0.0001$) and drug × mutation interaction ($F = 19.6$, $P < 0.0001$). The affinity for all agonists tested was significantly reduced with the serine 197 mutation as compared to the wild type ([a] $P < 0.05$). The affinity of all the agonists tested was not altered significantly with serine 194 mutations as compared to the wild type.

TABLE 5

K_i values (nM ± S.D.) of dopamine receptor antagonists competing with [^{3}H]raclopride.

	Ser194-Ala	Ser197-Ala	Wild type
(+)-Butaclamol	0.243 ± 0.066	0.265 ± 0.119	0.2301 ± 0.025
Clozapine	75.6 ± 6.8 [a]	27.7 ± 2.1	41.2 ± 5.8
Chlorpromazine	0.788 ± 0.143 [a]	0.227 ± 0.040 [a]	0.472 ± 0.028
Spiperone	0.079 ± 0.008	0.298 ± 0.01 [a]	0.096 ± 0.046

Two-way ANOVA indicated there were significant differences for drug ($F = 925.5$, $P < 0.0001$), serine mutation ($F = 101.9$, $P < 0.0001$) and drug × mutation interaction ($F = 81.8$, $P < 0.0001$). [a] Mutations that significantly differed from the wild type ($P < 0.05$). With the exception of (+)-butaclamol, the affinity of the other antagonists tested was significantly different ($P < 0.05$) for the serine 194 mutation as compared to serine 197.

compounds tested showed a substantially greater loss of binding affinity with serine mutations in position 197 as compared to 194 (table 4). Apomorphine and NPA showed the largest effect with a 30–48-fold shift in K_i with serine 197 mutations. Mutation of serine 194 produced only modest 2- to 3-fold shifts in affinity to dopamine, apomorphine and NPA that were not significantly different from the wild type. In addition to both drug and mutant effects, statistical analysis further suggested a drug-mutation interaction, indicating that the serine 197 mutation may have produced an altered receptor binding profile. The rank order of agonists tested in competition with [^{3}H]N-0437 varies when comparing the D_2 wild type and the serine 197 mutation.

The importance of serines 194 and 197 for antagonist binding is less clear. Competition studies (table 5) with a number of antagonists suggest that serine 197 may not be important for most antagonists tested, as the affinity for (+)-butaclamol, clozapine and chlorpromazine is not reduced by this mutation. However, as seen with [^{3}H]raclopride, there was a 3-fold reduction in affinity for spiperone with a serine 197 mutation. Small differences in affinity (less than 2-fold) for clozapine and chlorpromazine were seen with the serine 194 mutation; however, the significance of this slight shift is unclear. Again, two-way analysis of variance demonstrated a drug-mutation interaction, suggesting that these serine mutations may alter the binding profile and relative affinities of antagonists for the D_2 receptor.

Dual mutations of serine 194 and 197 to alanine produced a nearly complete loss of specific [^{3}H]N-0437 binding (table 3). While dual serine 194 and 197 mutations also produced a somewhat greater loss in binding affinity for [^{3}H]raclopride than mutations of serine 197 alone, the effects were comparatively small and not statistically significant.

3.5. Methionine 116 and methionine 117 mutations

Individual methionine mutations in positions 116 and 117 to leucine and cysteine, as well as dual mutations in these positions, failed to produce a change in the selectivity of the dopamine receptor. There was no enhanced adrenergic binding as measured by saturation studies with the adrenergic antagonist cyanopindolol or by competition studies with epinephrine and [^{3}H]N-0437. No differences in [^{125}I]CYP binding were observed between untransfected COS cells or COS cells transfected with the wild type D_2 or individual and dual methionine mutations (data not shown). Similarly, epinephrine did not display an enhanced affinity in displacing [^{3}H]N-0437 binding in cells transfected with the methionine mutants compared to the wild type control. Met (116) and Met (117) mutations also failed to show any change in affinity to [^{3}H]raclopride or [^{3}H]N-0437 as compared to the wild type D_2.

4. Discussion

Despite the structural similarities of the adrenoceptors and dopamine receptors, these results suggest that there are substantial differences in the way catecholamines interact with their respective binding pockets. The serine mutations in position 194 and 197 failed to discriminate agonists from antagonists, as was the case for the β_2-adrenoceptor, and, as will be explained more fully below, dopamine likely binds in a different orientation in the D_2 receptor pocket as compared to epinephrine and norepinephrine in the adrenoceptor pocket. Statistical analysis of the serine mutation binding results demonstrates a drug-mutation interaction, suggesting that the binding profile or the rank order of agonists and antagonists may be altered by these mutations, further emphasizing the importance of examining a wide series of compounds in evaluating the effects of receptor mutations.

While clearly a concern, it is unlikely that the effects that have been observed with the aspartate, serine or methionine mutations are due to nonspecific effects, such as the lack of receptor expression and variable transfection efficiency. Northern analysis indicates that all mutant receptor mRNAs were expressed and measurement of β-gal activity suggests that efficiency of transfection is an unlikely factor in interpreting these results. While these controls do not guarantee that D_2 mutant proteins were expressed correctly, the unaltered affinities for N-0437 for the single serine and dual methionine mutations argue that the proteins formed by these mutant receptors are essentially intact. Similarly, mutants with dual serine mutations which showed no binding with [^{3}H]N-0437, exhibited substantial binding with [^{3}H]raclopride. With regard to the

aspartate mutation, similar mutations in the β_2-receptor did not produce an aberrantly expressed protein (Strader et al., 1987b).

Consistent with the D_2 receptor model proposed by Hibert et al. (1991), the results clearly demonstrate the importance of the negatively charged aspartate (114) for both dopaminergic agonist and antagonist binding. Elimination of the negative charge by mutating this critical amino acid to either asparagine or glycine markedly reduced the affinity of both agonists and antagonists to the D_2 receptor. As is the case for the adrenoceptor, the negative charge of Asp (114) of the D_2 receptor likely forms a salt bridge with the amino group of dopaminergic ligands, stabilizing the binding to the receptor. The aspartate residue (114) in TM3 serves as a possible anchoring point, then, to which all catecholamines likely bind. Aspartate 80 of the D_2 receptor, on the other hand, is primarily important only for agonist binding and may be involved in inducing the conformation changes necessary for G-protein coupling (Neve et al., 1991).

Unlike the β_2-adrenoceptors in which both serines in TM5 are equally important for agonist binding, corresponding serines in the D_2 receptor (194 and 197) appear to be asymmetrically involved. Mutation of serine 197 to alanine produced far greater decreases in agonist affinities compared to mutation of serine 194. Given that the amino group of dopamine is likely anchored at aspartate 114, the meta- and para-hydroxyls of the catechol moiety are free to form hydrogen bonds with either serine 194 or serine 197. Structure-activity studies, however, have demonstrated that the meta-hydroxyl group is of primary importance for dopaminergic activity, with the para-hydroxyl group having only an accessory function (Seeman, 1981, for review). The meta-hydroxyl group of N-n-propylnorapomorphine, for example, is sufficient for dopamine receptor binding and the addition of the para-hydroxyl group enhances binding affinity only 9–10-fold The mutagenesis results pointing to the primary importance of serine 197 and these pharmacological data would suggest, then, that dopaminergic binding is stabilized by hydrogen bonds formed between the meta-hydroxyl group and serine 197, while the para-hydroxyl group forms hydrogen bonds with serine 194. This orientation for dopamine in the D_2 receptor pocket differs markedly from norepinephrine in the β_2-receptor, where the meta- and the para-hydroxyl groups form hydrogen bonds with serines 204 and 207, respectively (Strader et al., 1989a). It also appears to be different from the α_2-receptor where the para-hydroxyl group epinephrine is more important for ligand affinity compared to the meta-hydroxyl group (Wang et al., 1991).

Also, unlike the adrenoceptors, mutations of serine 194 and 197 of the D_2 receptor did not produce selective changes in agonists. Serine 197 mutations produced a 4- to 5-fold decrease in affinity for raclopride and a 3-foid change in affinity for spiperone, two D_2 antagonists. Examination of the structures of raclopride and spiperone and computer modeling of the D_2 receptor (Hibert et al., 1991; and this laboratory), suggest that unlike the other antagonists, both of these compounds could form hydrogen bonds with serine 197 (fig. 2). In the case of raclopride it is the carbonyl, while in spiperone it is the cyclic amide proton that likely forms hydrogen bonds with serine 197. Mutation of serine 197 to alanine would eliminate these possible hydrogen bonds and reduce raclopride's and spiperone's binding affinities. These suggestions are speculative, but they provide a possible framework for interpreting the receptor binding results. Other antagonists, such as (+)-butaclamol, showed no effect with the serine mutations, while clozapine and chlorpromazine did not show a reduced affinity by the serine 197 mutation, but were slightly affected by serine 194 mutations.

In general, agonist affinities were more greatly affected than antagonists by serine mutations 194 and 197. This is clearly evident from the dual serine mutations which resulted in a complete loss of agonist binding and only a 4-fold change in raclopride binding. Single serine 197 mutations similarly produced comparatively large (30- to 50-fold) decreases in apomorphine and NPA affinities. The precise loss of binding affinity with the serine 194 and 197 mutations is largely dependent on the agonist tested, suggesting that various dopaminergic drugs may bind in the pocket in subtly different ways. In the case of apomorphine and dopamine, for example, there are two hydroxyl groups in the meta and para positions, respectively, that can form hydrogen bonds with serine 194 and 197, while LY171555 has only one site capable of forming a hydrogen bond (fig. 2). If the quaternary nitrogen of LY171555 is aligned near the negative center of aspartate 114, then the only hydrogen within the ligand capable of forming a hydrogen bond would be positioned adjacent to serine 197. This observation, while speculative, is consistent with the binding results which demonstrate that a mutation of serine 197 produces a 5–6-fold decrease in LY171555 binding affinity, while a similar mutation of serine 194 produces no change in LY171555 affinity.

Of the agonists tested, N-0437 is particularly interesting as single serine 194 or 197 mutations produced no effect on its binding affinity, while dual serine mutations produced a complete loss of specific binding. Structurally, N-0437 is a very flexible compound whose phenol group may be capable of free rotation (fig. 2) and interacting with, and being stabilized by either serine 194 or 197. It is only with dual serine mutations that the binding of N-0437 is affected. It will be inter-

esting in future studies to examine whether receptors with single serine 194 or 197 mutations will be able to couple to G-proteins and whether N-0437 can induce an inhibition of cAMP.

Neither individual nor dual methionine mutations designed to more closely approximate the β_2-adrenoceptor produced a change in dopamine receptor selectivity. Direct binding studies with [^{125}I]CYP failed to demonstrate a change in binding affinity or capacity with each of the methionine mutants tested. Similarly, competition studies suggested that epinephrine's affinity was not enhanced by methionine mutations in positions 116 and 117. Perhaps single or dual amino acid mutations are not sufficient to alter receptor selectivity and domain swapping of longer amino acid stretches are necessary. For example, the transfer of TM7 of the α_2-receptor was necessary to confer α_2 binding characteristics to the β_2-receptor (Kobilka et al., 1988). Similarly here, larger domain switching between the adrenoceptors and dopamine receptors in the form of chimeras may be necessary to alter the selectivity of the dopamine receptor.

In summary, while aspartate (114) is absolutely critical for binding, the role of the two serines (194 and 197) in TM5 appears to differ as a function of the nature of the ligand. The serine mutations 194 and 197, while producing more dramatic effects on agonists, did not result in a sharp distinction between agonists and antagonists as has been the case for the adrenoceptors. Beyond the critical anchoring at aspartate 114 there may be multiple ways for a ligand to occupy the binding pocket and trigger the cascade of events which either result in or block the activation of G-protein events. The differential effects observed could be explained by different planar orientations or by more dramatic differences in the way that different ligands fit within the putative D_2 binding pocket, such that some ligands may be stabilized by interacting with residues other than the two serines in TM5. Alternatively, the differential sensitivity of agonists to the mutations could be due to differences in flexibility or rigidity of the ligands, and the possibility that interactions with a single serine may be sufficient. Future studies aim to further define the binding pocket of the

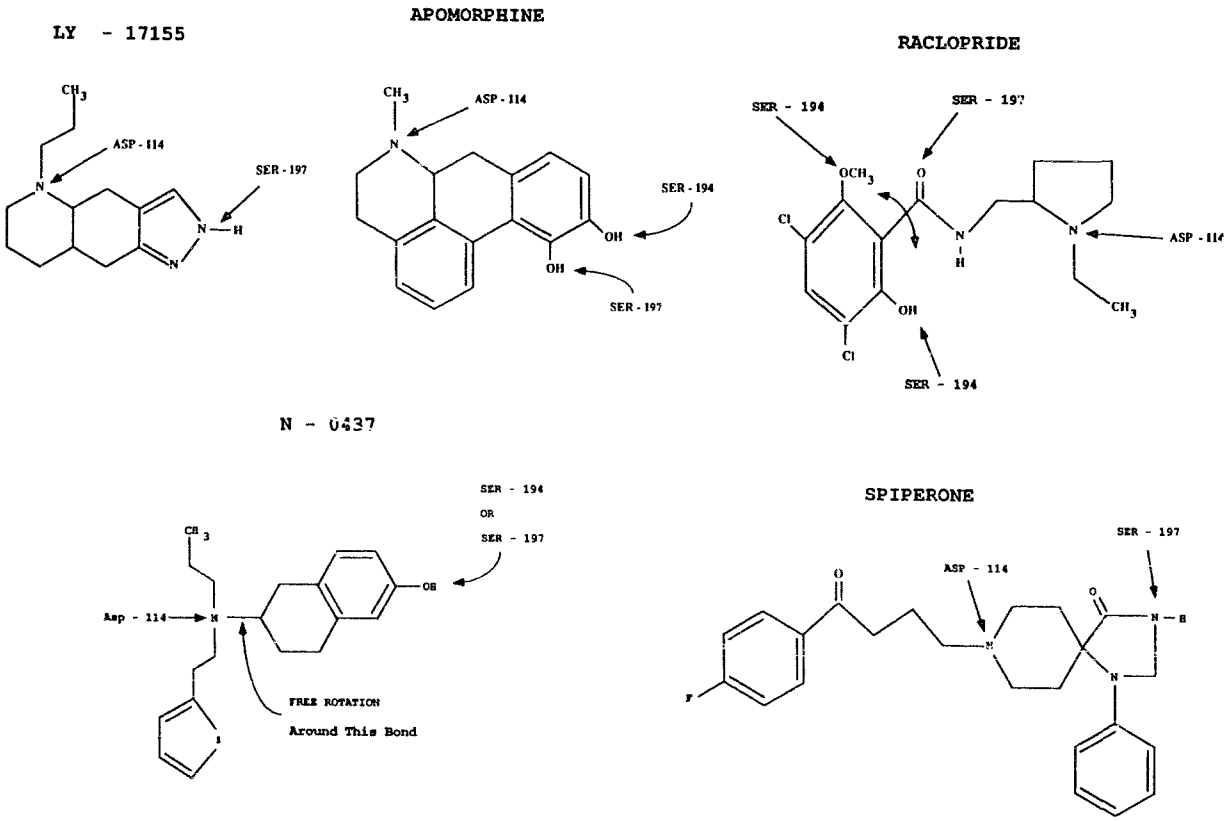

Fig. 2. Selected agonist and antagonist structures as they may interac. in the D_2 receptor pocket. Sites of interaction with aspartate 114 and serine 194 and 197 are based on binding results presented here and computer modeling of the D_2 receptor.

dopamine receptors and the residues involved in comformationally changing the receptor to induce changes in G-protein coupling and signal transduction.

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