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Nonconserved Residues in the Second Transmembrane-Spanning Domain of the D_4 Dopamine Receptor Are Molecular Determinants of D_4-Selective Pharmacology

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ABSTRACT

The molecular determinants that govern selective ligand binding to the rat D_4 dopamine receptor were investigated by substituting D_2 dopamine receptor sequences into a D_4 dopamine receptor background. The resulting mutant D_4 dopamine receptors were then screened with a panel of 10 selective and nonselective ligands, which included two allosteric modulators as sensitive measures of protein conformational changes. Mutation of a phenylalanine at position 88 in the second transmembrane-spanning domain (TMS2) of the D_4 receptor to the corresponding valine in the D_2 receptor D_4-F88V resulted in an ~100-fold decrease in the affinity of the highly D_4-selective drug 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine (L-750,667) without substantially affecting the binding of the other ligands. Mutations at the extracellular side of D_4-TMS3 produced moderate decreases in L-750,667 binding affinities with concomitant increases in binding affinity for the D_2/D_3-selective antagonist (−)-raclopride. However, the binding affinities of these same D_4-TMS3 mutants for the allosteric modulator isomethylbutylamiloride also were an anomalous 6- to 20-fold higher than either wild-type receptor. In the combined D_4-F88V/TMS3 mutants, L-750,667 binding affinity was further decreased, but this decrease was not additive. More importantly, the combined D_4-F88V/TMS3 mutants had (−)-raclopride and isomethylbutylamiloride binding properties that reverted back to those of the wild-type D_4-receptor. In contrast to the D_4-F88V mutant, the adjacent D_4-L87W mutant had an increased affinity for ligands with extended structures, but had essentially no effect on ligands with compact structures. These findings demonstrate that two residues near the extracellular side of D_4-TMS2 are critical molecular determinants for the selective binding of L-750,667 and ligands with extended structures.

Dopamine receptors belong to a superfamily of protein receptors that transmit chemical signals across plasma membranes by coupling to intracellular GTP-binding proteins. The prototypical G protein-coupled receptor (GPCR) is thought to have seven helical transmembrane-spanning (TMS) domains that assume a three-dimensional conformation similar to rhodopsin (Unger et al., 1997) and bacteriorhodopsin (Kimura et al., 1997). The ligand-binding crevice is, also by analogy with bacteriorhodopsin and rhodopsin, thought to reside within the helical TMS domains approximately two helical turns from the extracellular surface. In addition, studies with the scanning cysteine accessibility method (SCAM) have demonstrated that residues at the center and extracellular side of TMS2, TMS3, TMS5, TMS6, and TMS7 are readily solvent accessible within the D_2 dopamine receptor and appear to form a part of the "binding-site crevice" (Javitch et al., 1994, 1995, 1996, 1998, 1999; Fu et al., 1996). In other GPCR receptor systems, residues at the extracellular mouth of the binding-site crevice have been identified as molecular determinants for the binding of subtype-selective ligands (Fukuda et al., 1995; Hjorth et al., 1995; Hwa et al., 1995; Minami et al., 1995, 1996; Valiquette et al., 1996; Zhao et al., 1996; Cotte et al., 1998).

On the basis of the crystallographic structures of bacteriorhodopsin (Kimura et al., 1997) and rhodopsin (Unger et al., 1997), and homology modeling and mutational analysis of adrenergic receptors (Kobilka, 1992; Strader et al., 1994; Baldwin et al., 1997; Elling et al., 1997), TMS3 of GPCRs is thought to form a long, tilted α-helical structure that contains a critical ligand-docking site located approximately two helical turns from the extracellular membrane surface. For

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ABBREVIATIONS: GPCR, G protein-coupled receptor; TMS, transmembrane spanning; SCAM, scanning cysteine accessibility method; L-750,667, 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine; A-69024, 1-(2-bromo-4,5-dimethyoxybenzyl)-7-hydroxy-6-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline; OPC14597, 7-(−4(4-(2,3-dichlorophenyl)-1-piperazinyl)butyloxy)-3,4-dihydro-2(1H)-quinolinone; MIA, methylisobutylamiloride.

D_2 dopamine receptors, the docking of dopamine has been largely attributed to the charge interaction of the (protonatable) amine moiety of dopamine with the carboxyl group of a conserved aspartate residue (Mansour et al., 1992; Javitch et al., 1994, Miller et al., 1988). More recently, amino acids predicted to lie on the same helical face as the critical aspartate at position 114 in TMS3 of the D_2 dopamine receptor also were found to be part of the water-accessible binding-site crevice (Javitch et al., 1994). Specifically, chemical modification of TMS3 residues that occupy these highly solvent accessible positions interferes with the binding of substituted benzamide antagonists (Javitch et al., 1994). In addition, D_4/D_2 dopamine receptor chimera studies have provided corroborating evidence that TMS3 harbors residues involved in the binding of D_2- and D_4-selective antagonists (Burgess and Sibley, 1994). An amino acid sequence comparison of the TMS3 region in all cloned rat (and human) dopamine receptors is consistent with this notion as there are three TMS3 residues unique to the D_4 dopamine receptor but conserved in the other four dopamine receptor subtypes (D_1, D_2, D_3, and D_5). Collectively, these findings provided the impetus for investigating TMS3 residues as possible determinants of the selective pharmacology of the D_4 dopamine receptor subtype. Accordingly, nonconserved D_4 residues near the extracellular side of TMS3, as well as some of those unique to D_4 and predicted to face the binding-site crevice, were mutated to their corresponding residues in the D_2 receptor, and then screened for their nonselective, D_4-selective, and D_2-selective ligand-binding properties. Neighboring residues in the adjacent TMS2 were likewise investigated, either alone or in combination with TMS3 mutations, because models of GPCR structure suggest that some residues in TMS2 and TMS3 may be in proximity. Allosteric modulators also were used as sensitive measures of conformational rearrangements to distinguish between mutations that anomalously alter the general conformation of the binding-site crevice and those that play a more specific role in protein-protein interactions or ligand docking. We find that two amino acids located two helical turns from the extracellular side of TMS2 are critical molecular determinants for the selective binding of 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine (L-750,667) and ligands with extended structures. In addition, two amino acids in the first helical turn of TMS3 also contribute to selective drug binding but the effects are less pronounced, and most of them seem to rely on the orientation of the extracellular surface of TMS2 relative to TMS3.

Materials and Methods

Reagents. All drugs were either purchased from Research Biochemicals Inc. (Natick, MA) or received as generous gifts from the various sources listed in the acknowledgments. Analytical grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and cell culture supplies were purchased from Life Technologies Biomedical Research Laboratories (Gaithersburg, MD). Zinc chloride was purchased from Aldrich Chemical (catalog no. 22,999–7; Milwaukee, WI) and [^{3}H]methylspiperone (NET856; 84 Ci/mmol) was purchased from DuPont NEN (Boston, MA).

Site-Directed Mutagenesis. Regions within TMS3 of the rat D_4 dopamine receptor were replaced with the corresponding residues from the rat D_2 receptor with a DpnI-based site-directed mutagenesis kit called QuikChange (Stratagene, La Jolla, CA). In other experiments, reciprocal D_4 mutations were constructed into a D_{2L} wild-type receptor background. The full-length sequences of all mutant receptors were subsequently confirmed by [^{33}P]dideoxy-nucleotide sequencing with Thermo Sequenase (Amersham, Cleveland, OH). The naming convention we adopted begins with the wild-type background receptor name followed by the single-lettered code for the amino acid(s) to be mutated and their position(s), then ending with the corresponding amino acid substitution(s). For example, the D_4-F88V mutant has a D_4-background that has been mutated from a phenylalanine at position 88 to a valine present at this corresponding position in D_2 dopamine receptors.

Transfection. pcDNA3 plasmid constructs containing either the wild-type or a mutant dopamine receptor were transiently transfected into COS7 cells with $CaPO_4$ precipitation (Invitrogen, San Diego, CA). Specifically, 20 μg of plasmid DNA was mixed with a final volume of 1 ml of $CaPO_4$/HEPES solution and the resulting precipitate was added dropwise to 20 to 30% confluent COS cells attached to a 150-cm^2 plate in a total media volume of 20 ml. The following day, the media was removed by aspiration and replaced with fresh media. Cells were grown to confluence before harvesting.

Preparation of Membranes for Binding Assays. COS cells expressing the desired receptor were dislodged by a 5-min incubation in Earle's balanced saline solution lacking Ca^{2+} and Mg^{2+} and supplemented with 5 mM EDTA. After centrifugation, the cell pellet was lysed in lysis buffer (5 mM Tris, 5 mM $MgCl_2$, pH 7.4). The lysate was glass-glass homogenized (13 strokes) and the membranes were centrifuged at 35,000*g* for 30 min. The pellet was resuspended in 50 mM Tris, pH 7.4, at 25°C and centrifuged again. The washed membrane pellet was resuspended by light homogenization (2–3 strokes) in binding buffer (see below) immediately before use.

Radioligand-Binding Assays. Membranes containing wild-type or mutant dopamine receptors were assayed for specific [^{3}H]methylspiperone (84 Ci/mmol) (DuPont NEN) binding activity. The binding buffer consisted of 50 mM Tris, pH 7.4, at 25°C. The binding buffer for agonists was supplemented with 1 mM sodium ascorbate, whereas for the substituted benzamide antagonists the buffer was supplemented with 120 mM NaCl. Nonspecific binding was defined by 5 μM (+)-butaclamol. The reaction was allowed to proceed at 25°C for 1.5 h before rapid filtration through GF/C filters pretreated with 0.3% polyethyleneimine. The wash buffer consisted of ice-cold binding buffer (pH 7.4; 0°C). Radioactivity bound to the filters was quantified by scintillation spectroscopy at a counting efficiency of 47%. Membrane protein concentrations were determined with the bicinchoninic acid protein reagent (Pierce, Rockford, IL) and a BSA standard curve. Drug-binding affinity values were determined by either saturation isotherms or inhibition curves. Only some mutants were screened with 1-(2-bromo-4,5-dimethyoxybenzyl)-7-hydroxy-6-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline (A-69024), nafadotride, and 7-(−4(4-(2,3-dichlorophenyl)-1-piperazinyl)butyloxy)-3,4-dihydro-2(1H)-quinolinone (OPC-4392) due to a limited supply of these drugs.

Calculations and Data Analysis. All points were sampled in triplicate for each experiment. All experiments were repeated from two to five times, and averaged values were reported with their associated standard deviation or standard error. The equilibrium dissociation constant or K_D of the primary radioligand was measured by saturation isotherm analysis. The inhibition constant (K_i) values for all drugs, except for zinc binding to D_4 receptors, were calculated from IC_{50} values with the Cheng-Prusoff equation $K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$ (Cheng and Prusoff, 1973). This form of the equation assumes a competitive interaction and a pseudo Hill slope = 1. In cases where the best-fit curve did not have a pseudo Hill slope approximating unity, the apparent K_i affinity values are better described as $K_{0.5}$ values. Even though methylisobutylamiloride (MIA) is an allosteric modulator of D_4 dopamine receptors, the competitive form of the Cheng-Prusoff equation was used as an appropriate estimate of MIA K_i values because MIA/methylspiperone-binding interactions are highly cooperative (Philip G. Strange and Sam R. J.

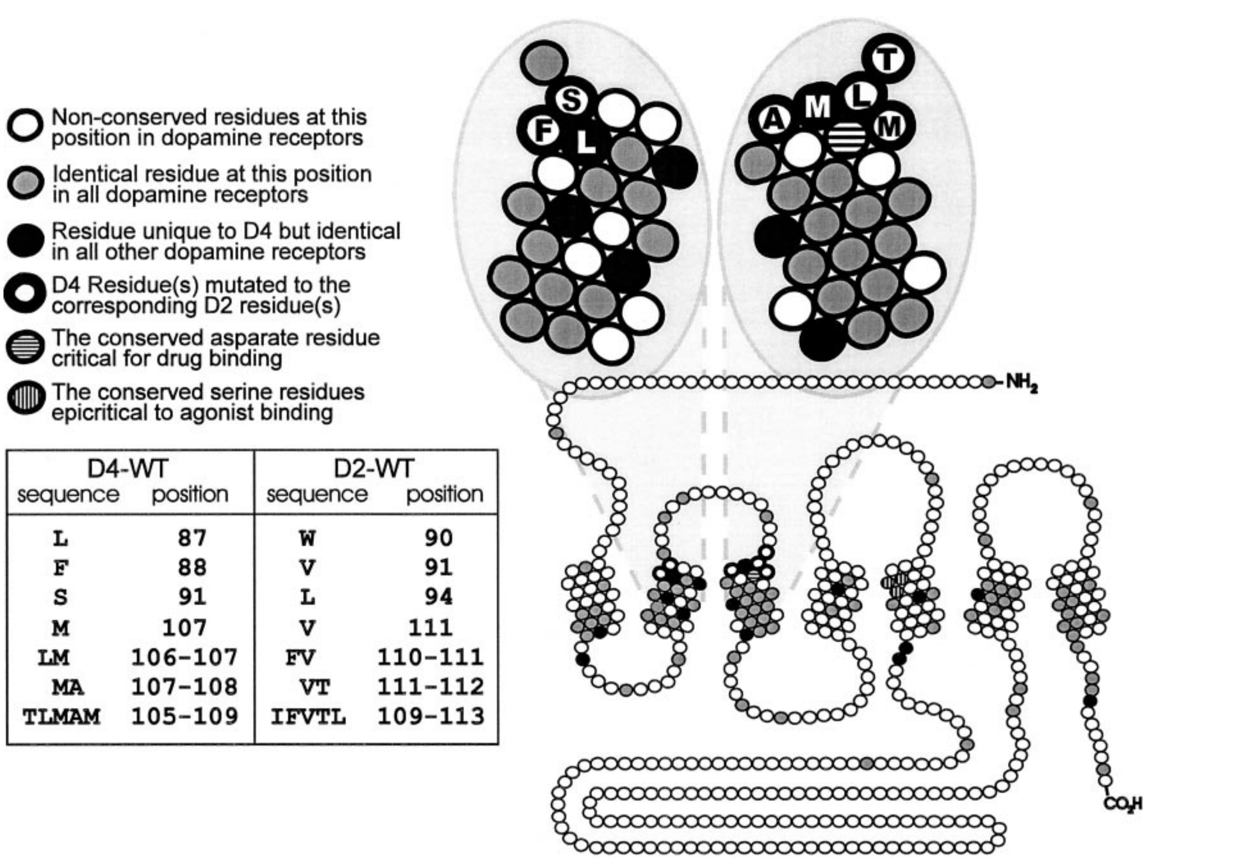
Hoare, personal communication, concerning the mechanism of allosteric modulation of the wild-type D_4 dopamine receptor subtype by MIA) and a relatively low concentration of [^{3}H]methylspiperone was used in these assays (the concentration of [^{3}H]methylspiperone is approximately equal its K_D). Because zinc is a noncompetitive allosteric modulator of methylspiperone binding to D_4 receptors, its K_i-binding affinity values are taken to be directly equivalent to its IC_{50} values, i.e., the noncompetitve form of the Cheng-Prusoff equation (Cheng and Prusoff, 1973). A 95% CI was used for all curve-fitting procedures and for comparing different curve-fitting models. The statistical measures of fit were the *F* test, the run test, and a correlation coefficient. By convention, any change in drug-binding affinity that is ≤2.5-fold different from the wild-type background is considered to be the same as the wild type when analyzing pharmacological differences.

Results

Amino acids critical for the binding of subtype-selective drugs to D_2 and D_4 dopamine receptors were identified by measuring the binding properties of mutant dopamine receptors that were constructed by substituting D_2 dopamine receptor amino acids into a D_4 dopamine receptor background. To narrow the pool of potential mutation sites, D_4 dopamine receptors were mutated at those positions that are 1) unique to D_4 but conserved in all other dopamine receptors, i.e., D_1, D_2, D_3 and D_5; 2) in proximity to the known docking sites for nonselective drug binding, e.g., Asp114 of the D_2 receptor; and 3) postulated to be facing the binding-site crevice. Nonconserved amino acids surrounding these uniquely D_4 sites were likewise mutated by replacing them with their corresponding D_2 amino acids. On the basis of these selection criteria, several residues within TMS2 and TMS3 of the wild-type rat D_4 receptor were mutated to the corresponding residues in the wild-type rat D_2 dopamine receptor, either singly or in combination (Fig. 1). The binding affinity and pseudo Hill slope values for these mutant D_4 receptors were then determined for a panel of 10 ligands with varying subtype selectivities (Table 1). Mutation of two amino acids in D_4-TMS2 had the most selective and dramatic effects on ligand binding. In contrast, neighboring amino acids in D_4-TMS3 played a relatively nonselective role in drug binding, which appears to be mediated by more global changes in receptor conformation.

All the mutant and both wild-type dopamine receptors were first screened by saturation isotherm analysis for high-affinity binding of the primary radioligand [^{3}H]methylspiperone (Table 2). Determining the K_D values for each of the mutants and both the wild-type receptors was necessary to calculate the K_i values for the other, nonradiolabeled drugs with the Cheng-Prusoff equation. With the exception of the D_4-L87W mutant, which had a significantly more D_2-like [^{3}H]methylspiperone-binding profile (7-fold), all other D_4 mutants bound [^{3}H]methylspiperone with affinities similar to the wild-type D_4 receptor background. In contrast, the reciprocal TMS3 cassette mutant constructed into a D_2-background (D_2-IFVTL109–113TLMAM) bound [^{3}H]methylspiperone with 4-fold lower affinity than the wild-type D_2 receptor.

Starting with the D_4 subtype-selective ligands, we next

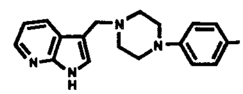
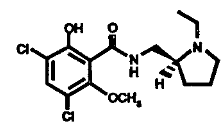
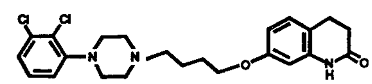
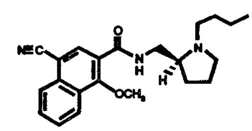
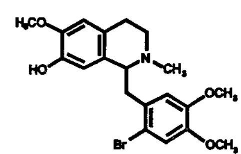
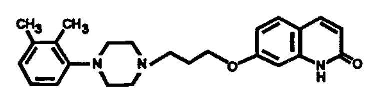
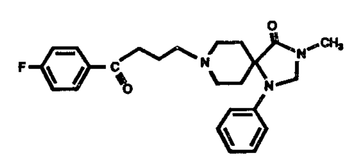
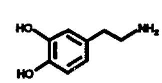
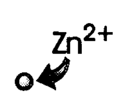
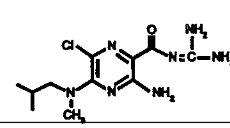


D4-WT sequence	D4-WT position	D2-WT sequence	D2-WT position
L	87	W	90
F	88	V	91
S	91	L	94
M	107	V	111
LM	106-107	FV	110-111
MA	107-108	VT	111-112
TLMAM	105-109	IFVTL	109-113

Fig. 1. A schematic of a generalized dopamine receptor protein. The specific residues in TMS2 and TMS3 that were analyzed by mutagenesis are highlighted with a bold border and a letter corresponding to the one-lettered amino acid code for the sequence in the wild-type D_4 dopamine receptor. The table compares corresponding residues determined from the alignment of the amino acids in the rat D_4 sequence relative to the rat D_{2L} sequence.

measured the binding affinities for nonradiolabeled ligands via [^{3}H]methylspiperone competition-binding assays. The D_4-M107V mutant, as well as all of the D_4-TMS3 cassette mutations centered around methionine 107, bound the highly D_4-selective antagonist L-750,667 (Patel et al., 1996), with lower affinity than did the wild-type D_4 receptor (Table 3). Although the D_4-M107V mutation alone produced only a 3-fold decrease in L-750,667 binding affinity (Table 3), additionally substituting a phenylalanine for the adjacent leucine at position 106 resulted in a more pronounced affinity change (20-fold) (Fig. 2; Table 3). Much larger decreases in L-750,667 binding affinity (96-fold) were observed for the TMS2 mutant D_4-F88V, but not for mutations made adjacent to or one helical turn above phenylalanine at position 88 (Table 3). Moreover, double mutations involving phenylalanine 88 in TMS2 in combination with phenylalanine 106 and methionine 107 in TMS3 bound L-750,667 with even lower affinity (145–242-fold) than either TMS domain mutation alone, but

TABLE 1
Ligand nomenclature, structures, and pharmacological selectivities

Drug Name	Dopamine Receptor Selectivities	Pharmacophore Chemical Class	Drug Structure
L-750,667	D4-selective D4:D2 ratio >13,500	Piperazine	
(−)-Raclopride	D2/D3-selective D2:D4 ratio = 4000	Substituted benzamide	
OPC-14597	D2/D3-selective D2:D4 ratio = 250	Piperazine	
(−)-Nafadotride	D3/D2-selective D2:D4 ratio = 235	Substituted benzamide	
A-69024	D1/D4-selective D4:D2 ratio = 70	Isoquinoline	
OPC-4392	D2/D3-selective D2:D4 ratio = 24	Piperazine	
Methylspiperone	D2/D3/D4-selective D2:D4 ratio = 15	Butyrophenone	
Dopamine	Not selective D4:D2 ratio = 12	Catechol	
Zinc	Not selective	Pseudo-noble-gas metal	
Methylisobutylamiloride	Not selective	Pyrazine	

the magnitude was less than expected if the effects of the "single" TMS2 and TMS3 mutations were independent, i.e., additive (Table 3). Although the effects of mutations in D_4-TMS3 are relatively modest, amino acids at the extracellular surface of TMS3 are clearly contributing to L-750,667 binding because substituting the corresponding D_4-TMS3 residues into a D_2-background (i.e., the mutant D_2-IFVTL109–113TLMAM) produces a mutant D_2 receptor with significantly increased affinity (>20-fold) for L-750,667 compared with the wild-type D_2 receptor (Fig. 2; Table 3). Like L-750,667, the less selective, mixed D_1/D_4-selective antagonist A-69024 (Kerkman et al., 1989; Burgess and Sibley, 1994) bound the five amino acid cassette D_4 mutant centered around and including M107V with moderately lower affinity than the wild-type D_4 receptor (10-fold) (Table 3). In contrast to L-750,667, A-69024 bound the D_4-M107V mutants with an anomalous 4-fold higher affinity than the wild-type D_4 receptor (Table 3).

Next, the mutant dopamine receptors were tested for their ability to bind the relatively D_2-selective ligands of the substituted benzamide class, (−)-raclopride and (−)-nafadotride (Seeman, 1992; Sautel et al., 1995). Both (−)-raclopride and (−)-nafadotride bound D_4-M107V and associated D_4 cassette mutants, which included substitution of leucine 106 for phenylalanine, with moderately higher affinity than the D_4 wild-type receptor (7–29-fold) (Table 3). Furthermore, the reciprocal TMS3 cassette mutant in a D_2-background produced a D_2 mutant receptor with modestly decreased affinity for (−)-raclopride (6-fold). In contrast to the opposing effects observed for reciprocal TMS3 mutants, none of the D_4 mutations in TMS2 disrupted (−)-raclopride binding. However, double D_4-TMS2/3 mutations that include the F88V substitution had the surprising effect of reverting the (−)-raclo-

TABLE 2

Binding affinities for [^{3}H]methylspiperone at mutant and wild-type D_2 and D_4 dopamine receptors

Mutant and wild-type dopamine receptors were transiently expressed in COS7 cells. Membranes prepared from these cells were equilibrated with increasing concentrations of [^{3}H]methylspiperone (~10–2000 pM). Nonspecific binding was defined by 5 μM (+)-butaclamol. The amount of specifically bound [^{3}H]methylspiperone after rapid filtration was plotted as bound/free versus bound, and the best-fit linear regression lines for these saturation isotherms were used to calculate the corresponding [^{3}H]methylspiperone equilibrium dissociation constants (K_D) for each receptor (n = 2–3).

Receptor	K_D ± S.D.	Fold change in K_i (away from wild-type background)
	pM	
D_4-WT	294 ± 30	1
D_4-M107V	138 ± 22	−2
D_4-TLMAM 105-109IFVTL	269 ± 49	1
D_4-LM106-107FV	515 ± 57	2
D_4-MA107-108VT	300 ± 48	1
D_4-L87W	44 ± 7	−7
D_4-F88V	474 ± 94	1.5
D_4-S91L	169 ± 26	−2
D_4-LM106-107FV+F88V	196 ± 75	1.5
D_4-TLMAM105-109IFVTL+F88V	105 ± 4	−3
D_2-WT[a]	20 ± 4	−15
D_2-IFVTL109-113TLMAM[b]	75 ± 11	4

[a] The fold changes for the D_2 wild-type dopamine receptor are relative to the D_4 wild-type dopamine receptor.
[b] The fold changes for this D_2 mutant are relative to the D_2 wild-type background from which it was derived.

TABLE 3

Drug-binding affinities for mutant and wild-type D_2 and D_4 dopamine receptors

Mutant and wild-type dopamine receptors were transiently expressed in COS7 cells. Membranes prepared from these cells were equilibrated with a fixed concentration of [^{3}H]methylspiperone (~500 pM) and increasing concentrations of the competing drug. The amount of specifically bound [^{3}H]methylspiperone after rapid filtration is plotted as a function of the logarithm of the concentration of drug. Nonspecific binding was defined by 5 μM (+)-butaclamol. The averaged IC_{50} values (n = 3–4) derived from best-fit curves to the data were then used to calculate the corresponding drug inhibition constants (K_i) for each receptor with the competitive form of the Cheng-Prusoff equation and [^{3}H]methylspiperone K_D value listed in Table 1. All affinity values are listed in the table as K_i ± S.D. (in nanomoles). The numbers in parentheses below each K_i value represent the fold change in affinity and the sign indicates the direction of the change relative to the wild-type receptor background.

Receptor or Mutant	L-750,667	A-69024	Raclopride	Nafadotride	OPC-14597	Dopamine[a]	OPC-4392
D_4-WT	0.11 ± 0.02 (1)	7.9 ± 0.7 (1)	2205 ± 300 (1)	399 ± 49 (1)	46.5 ± 6.8 (1)	37 ± 8 (1)	62 ± 12 (1)
D_4-M107V	0.33 ± 0.05 (3)	2.0 ± 0.15 (−4)	266 ± 28 (−8)	57 ± 7.1 (−7)	88.3 ± 14.4 (2)	139 ± 32 (4)	132 ± 28 (2)
D_4-TLMAM 105-109IFVTL	1.6 ± 0.04 (15)	79 ± 12 (10)	76 ± 10 (−29)	38 ± 10 (−11)	103.3 ± 23.3 (2)	32 ± 12 (1)	149 ± 33 (2)
D_4-LM106-107FV	2.2 ± 0.38 (20)	ND	187 ± 30 (−12)	ND	ND	41 ± 8 (1)	ND
D_4-MA107-108VT	0.42 ± 0.13 (4)[b]	ND	533 ± 131 (−4)	ND	ND	82 ± 29 (2)	ND
D_4-L87W	0.066 ± 0.018 (−2)[b]	ND	645 ± 110 (−3)	ND	2.3 ± 0.76 (−20)[b]	19 ± 4 (−2)	3.8 ± 0.71 (−16)
D_4-F88V	10.6 ± 2.2 (96)	ND	3218 ± 840 (1.5)	ND	66.4 ± 16.6 (1)	53 ± 14 (1)	ND
D_4-S91L	0.38 ± 0.14 (3)[b]	ND	1036 ± 15 (−2)	ND	63.2 ± 18.3 (1)	14 ± 4 (−3)	ND
D_4-LM106-107FV+F88V	15.9 ± 3.3 (145)	ND	1337 ± 321 (−1.5)	ND	ND	178 ± 64 (5)	ND
D_4-TLMAM105-109IFVTL+F88V	26.7 ± 4.4 (242)	ND	682 ± 158 (−3)	ND	ND	111 ± 38 (3)	ND
D_2-WT[c]	>1500[d] (>13,500)	543 ± 71 (69)	0.56 ± 0.03 (−3938)[b]	1.7 ± 0.4 (−235)	0.19 ± 0.04 (−247)[b]	442 ± 126 (12)	2.6 ± 0.5 (−24)
D_2-IFVTL109-113TLMAM[e]	80.4 ± 21.9 (−20)	ND	3.5 ± 0.7 (6)[b]	ND	3.8 ± 1.8 (−20)[b]	1196 ± 310 (3)	ND

ND, not determined.
[a] A significantly better fit was achieved at 95% CI with a slope <0.8, thus values reflect an apparent $K_{0.5}$ rather than a K_i value.
[b] A significantly better fit was achieved at 95% CI with a slope <0.9, thus values reflect an apparent $K_{0.5}$ rather than a K_i value.
[c] The fold changes in parentheses for the D_2 wild-type dopamine receptor are relative to the D_4 wild-type dopamine receptor.
[d] Only partial inhibition (~20%) was achieved at the highest concentration.
[e] The fold changes in parentheses for the D_2 mutants are relative to the D_2 wild-type background from which they were derived.

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pride binding properties back to those of the wild-type D_4-background (Table 3). Like the substituted benzamide antagonists, the antagonist OPC-14597 has selectivity for the D_2 subtype over the D_4 subtype (Lawler et al., 1999) (OPC-14597 also has weak partial agonist activity in some systems). However, only OPC-14597 has an extended ligand structure and its binding was not affected by any of the D_4-TMS3 mutants (Table 3). Given this lack of effect of TMS3 mutations in a D_4-background, it was indeed surprising that the reciprocal TMS3 cassette mutant in a D_2-background had moderately decreased affinity (20-fold) for OPC-14597 (Table 3). Despite this incongruence for OPC-14597 binding between the reciprocal TMS3 mutants constructed in D_2- and D_4-backgrounds, OPC-14597 binding to the D_4-L87W TMS2 mutant was significantly increase, thereby making it more D_2-like (20-fold) (Table 3).

In addition to drugs with relatively high selectivity for the D_2- versus the D_4 subtype, we tested the much less selective endogenous agonist dopamine and the agonist OPC-4392 (Lawler et al., 1999). The largest change in the binding affinity among the relatively nonselective agonists was the 16-fold increase in OPC-4392-binding affinity to the D_4-L87W mutant, which makes it considerably more D_2-like (Table 3). This same mutant had dopamine-binding properties similar to the wild-type D_4 receptor (Table 3). Spuriously, the D_4-M107V and one of the double D_4-TMS2/3 mutants became modestly more D_2-like with respect to dopamine receptor binding (4–5-fold decreases in affinity), even though no such change was observed for several other mutations in this region, which encompassed the M107V mutation (Table 3).

Typically, a range of ligands is tested on a given mutation to ensure that the observed effects are specific for a given drug rather than being due to gross conformational changes that would affect the binding properties of a variety of ligands. In an effort to detect the more subtle conformational effects induced by the mutations, all mutant dopamine receptors were additionally screened with the allosteric modulators zinc and MIA (Hoare and Strange, 1996; Schetz et al., 1999). Because the affinities for a range of drugs were only modestly altered in the D_4-M107V mutant, with most of the changes being intermediate to those of the wild-type D_2 and D_4 receptors, it was somewhat of a surprise that the D_4-M107V mutant also had an estimated 20-fold higher affinity for MIA than either of the wild-type receptors. Furthermore, this "anomalous" decrease in K_i was accompanied by a significantly shallower pseudo Hill slope ($n_H = 0.72$) than either of the wild-type receptors whose slopes were approximately equal to one (Fig. 4). Likewise, the D_4-TMS3 cassette mutant, centered around and including the M107V substitution, had the same unusually shallow and anomalously higher affinity MIA-binding profile (14-fold) (Table 4). Mutation of M107V in combination with one or the other adjacent amino acid also increased binding affinity for MIA beyond the wild-type receptors (6–14-fold), but without a change in pseudo Hill slopes. Despite this, the reciprocal TMS3 cassette mutation

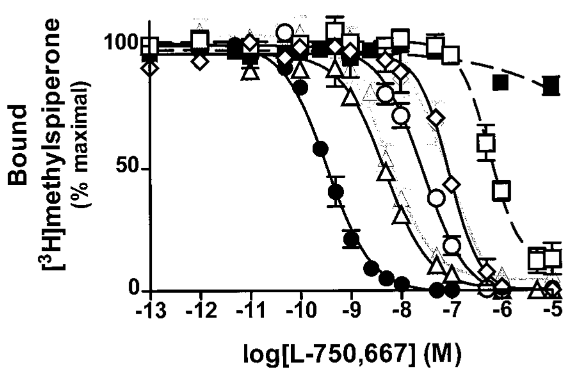

Fig. 2. L-750,667 inhibition of [^{3}H]methylspiperone binding to wild-type and mutant D_2 and D_4 dopamine receptors. Mutant and wild-type dopamine receptors were transiently expressed in COS7 cells. Membranes prepared from these cells were equilibrated with a fixed concentration of [^{3}H]methylspiperone (~500 pM) and increasing concentrations of the competing drug L-750,667. The amount of specifically bound [^{3}H]methylspiperone following rapid filtration is plotted as a function of the logarithm of the concentration of L-750,667. Nonspecific binding was defined by 5 μM (+)-butaclamol. The averaged IC_{50} values (n = 3–4) derived from best fit curves to the data were then used to calculate the corresponding L-750,667 inhibition constants (K_i) for each receptor with the competitive form of the Cheng-Prusoff equation and [^{3}H]methylspiperone K_D values listed in Table 2. These corresponding K_i affinity values are listed in Table 3. The key to the data symbols is as follows: ■, D_2-WT; □, D_2-IFVTL109–113TLMAM; ●, D_4-WT; △, D_4-LM106–107FV; ◬, D_4-TLMAM105–109IFVTL; ○, D_4-F88V; ◇, D_4-LM106–107FV+F88V; and ◈, D_4-TLMAM105–109IFVTL+F88V.

TABLE 4

Comparison of the binding affinities of wild-type and TMS2 and TMS3 mutant D_4 and D_2 dopamine receptors

Receptor or Mutant	Drug Equilibrium Affinity Constants K_i (Fold change in K_i away from the wild-type background)[a]	
	Methylisobutylamiloride *nM*[b]	*Zinc* *μM*
D_4-WT	199 ± 32 (1)	39 ± 5.8 (1)
D_4-M107V	10 ± 2.4 (−20)[c]	18 ± 2.5 (−2)
D_4-TLMAM105-109IFVTL	14 ± 4 (−14)[c]	17 ± 3.9 (−2)
D_4-LM106-107FV	14 ± 2.1 (−14)	ND
D_4-MA107-108VT	31 ± 8.7 (−6)	ND
D_4-L87W	290 ± 31 (1.5)	20 ± 0.52 (−2)
D_4-F88V	253 ± 52 (1)	11 ± 4.7 (−4)[c]
D_4-S91L	221 ± 27 (1)	23 ± 3.8 (−2)
D_4-LM106-107FV+F88V	133 ± 36 (−1.5)	8 ± 3.4 (−5)[c]
D_4-TLMAM105-109IFVTL+F88V	105 ± 22 (−2)	4.6 ± 1.1 (−8)[c]
D_2-WT[d]	270 ± 81 (8421 ± 2526) (1 {40})	3.2 ± 0.75 {184 ± 43} (−12 {5})
D_2-IFVTL109-113TLMAM[d]	>1000[e] (>5)	17.1 ± 1.9 {131 ± 14.4} (5 {1})

ND, not determined.

[a] The fold changes in parentheses for the D_2 wild-type dopamine receptor are relative to the D4 wild-type dopamine receptor, whereas all fold changes for mutant receptors are relative to the wild-type background from which they were derived.

[b] K_i values for MIA binding to the wild-type and mutant D_4 receptor were calculated with the competitive form of the Cheng-Prusoff equation because MIA inhibition of [^{3}H]methylspiperone binding to the wild-type D_4 dopamine receptor is highly cooperative.

[c] A significantly better fit at 95% CI with a shallow slope ($n_H < 0.8$). In these cases, the values reflect an apparent $K_{0.5}$ rather than a K_i value.

[d] Because both MIA and zinc binding to the D_2 subtype are characterized by negative heterotropic cooperativity, their actual affinity values lie between their IC_{50} values and K_i values derived from the competitive form of the Cheng-Prusoff equation. The values in brachets are IC_{50} values. Note that the K_i value for zinc at the "unoccupied" D_2 receptor expressed in Chinese hamster ovary cells was previously determined to be ~40 μM (Schetz and Sibley, 1997).

[e] Only partial inhibition (~30%) was achieved at the highest concentration.

in a D_2-background did not produce the opposite effect, rather it had an estimated >6-fold decrease in MIA affinity beyond both wild-type dopamine receptors. In contrast to the TMS3 mutants, none of the D_4-TMS2 mutants affected MIA-binding affinity. However, the relatively pronounced and anomalous MIA binding observed for the D_4-TMS3 mutants was completely reversed in both of the D_4-TMS2/3 double mutants, which included the F88V substitution (Table 4). This remarkable undoing of the effects of D_4-TMS3 mutants on (−)-raclopride and MIA, but not L-750,667 binding, suggests that the binding of certain drugs is sensitive to the relative conformation of TMS2 and TMS3. Unlike MIA binding, the binding of zinc to the mutant D_4-TMS3 dopamine receptors was mostly unaffected. However, the D_4-TMS2 mutation D_4-F88V had an apparent 4-fold increase in zinc affinity that persisted in the D_4-TMS2/3 double mutants involving F88V.

Overall, the most selective and drastic individual effects were for the interaction between L-750,667 binding and the D_4-F88V mutant as well as D_4-TMS2/3 double mutations involving F88V. Specifically, the binding the D_4-selective antagonist L-750,667 to the D_4-F88V mutant was reduced ~100-fold, even though the binding of nine other ligands was essentially unaffected. Furthermore, double D_4-TMS2/3 mutations involving the amino acid substitutions F88V in TMS2 and LM106–107VT in TMS3 had a synergistic effect, rather than a reversal effect as was seen for (−)-raclopride and MIA binding to the D_4-TMS2/3 double mutants. Mutation of the adjacent leucine at position 87 to the corresponding tryptophan in the D_2 receptor categorically altered the binding of drugs with extended structures, but not drugs with compact structures. Relative to mutations in a D_4-background, the effects of the D_2-TMS3 cassette mutation as well as other mutations in a D_2-background (unpublished data) produced, in general, more nonspecific effects.

Discussion

Previous mutational studies of D_1 and D_2 dopamine receptors have identified a conserved aspartic acid residue located two helical turns from the extracellular surface of TMS3 as a critical docking site for the protonatable amine moiety of agonists and antagonists (Miller et al., 1988; Mansour et al., 1992; Javitch et al., 1994; Mailman et al., 1997). Other mutational studies of D_1 and D_2 dopamine receptors have additionally identified three conserved serine residues located three helical turns from the extracellular surface of TMS5 as critical-docking sites primarily for the catechol moiety of agonists (Cox et al., 1992; Mansour et al., 1992; Pollock et al., 1992; Coley et al., 1995). Furthermore, a series of studies on the D_2 receptor with SCAM suggests that neighboring residues in TMS2–5 and TMS6–7 are readily solvent accessible and are oriented toward the same helical face as the critical-docking sites for substituted benzamide antagonists, and therefore, seem to form a part of the binding-site crevice (Javitch et al., 1994, 1995, 1996, 1998, 1999; Fu et al., 1996). Although no dopamine receptor studies have specifically identified residues that are responsible for the binding of highly subtype-selective ligands, residues at the extracellular mouth of the binding-site crevice are molecular determinants for the binding of subtype-selective ligands in other GPCR systems (Fukuda et al., 1995; Hjorth et al., 1995; Hwa et al., 1995; Minami et al., 1995, 1996; Valiquette et al., 1996; Zhao et al., 1996; Cotte et al., 1998). Consequently, our initial strategy for locating subtype-selective drug-binding sites was to first compare dopamine sequences and identify unique amino acids in the D_4 subtype that are either near or somehow associated with previously determined ligand-docking sites for nonselective drugs. If only the amino acid sequences for the seven TMS domains of the rat D_1–D_5 dopamine receptors are aligned and compared, then a total of 11 amino acids are unique to the D_4 subtype but conserved in all other dopamine receptor subtypes. Of these 11 positions, seven of them reside in the second and third TMS domains: four in TMS2 and three in TMS3. Of these seven, only three (L80, L87, and M107) are predicted by homology modeling (Baldwin et al., 1997; Unger et al., 1997) and SCAM (Javitch et al., 1994, 1995, 1996, 1998; Fu et al., 1996) to face toward the solvent-accessible binding-site crevice. Amino acids at two of these sites were mutated to the corresponding amino acids found in all the other dopamine receptor subtypes, i.e., L87→W and M107→V, because the physicochemical properties of these corresponding residues are very different. In addition, the surrounding nonconserved residues in the D_4 dopamine receptor were mutated to their corresponding residues in the D_2 dopamine receptor, either alone or in combination. The pharmacological characteristics of the resulting mutant D_4 dopamine receptors were then measured by screening a variety of D_2-selective, D_4-selective, and nonselective dopamine receptor ligands.

Substitution of the methionine at position 107 of the D_4 dopamine receptor to the corresponding valine in the D_2 sequence (D_4-M107V) modestly alters the binding of all dopamine receptor ligands with compact structures. With the exception of A-69024 and MIA, the changes in drug-binding affinities resulted in a mutant D_4 receptor with increased D_2-like pharmacology. Incidentally, all three ligands whose binding is unaffected by the D_4-M107V mutation have a long spacer arm (≥4 atoms) extending from their central pharmacophore, i.e., [^{3}H]methylspiperone, OPC-14597, and OPC-4392. The same distinction among ligand binding also is observed for the cassette-style mutations centered around M107, including the D_4-TLMAM105–109IFVTL, D_4-LM106–107FV, and D_4-MA107–108VT mutants. Although intriguing, this distinction based on spacer arm length does not completely generalize to the reciprocal mutant in a D_2-background, D_2-IFVTL109–113TLMAM, because the binding of OPC-14597 as well as the binding of L-750,667 and (−)-raclopride are more D_4-like. Furthermore, all mutations at the extracellular side of D_4-TMS3, i.e., D_4-M107V, D_4-TLMAM105–109IFVTL, D_4-LM106–107FV, and D_4-MA107–108VT anomalously increased the affinity for MIA beyond both the wild-type D_2 and D_4 receptors, and for D_4-M107V and D_4-TLMAM105–109IFVTL mutations, the competition curves slopes were distinctly shallow. The discordant effects of the D_4-M107V and D_4-TLMAM105–109IFVTL mutants on dopamine and A-69024-binding affinity, the imperfect distinction between extended and compact ligand structures for reciprocal substitutions in both a D_4- and a D_2-background, and the unusual D_4-TMS3 MIA binding profile suggest that residues at the extracellular side of TMS3 are located in a conformationally delicate region. Consequently, some, if not all, of the observed effects of the D_4-TMS3 mutants on drug binding may be due to a more general, rather than a specific, rearrangement of the conformation of the binding-site crevice. This finding is consistent with the location of these residues in the region directly proceeding the critical aspartate docking site and extending another 1.5 helical turns to the extracellular surface.

Because differences within TMS3 of D_2 and D_4 could not fully account for the binding of selective drugs and models of GPCR structure suggest that TMS2 is in proximity to TMS3, nonconserved or uniquely D_4 residues at the extracellular side of TMS2 that are thought to lie on the same face as the binding-site crevice were subjected to mutational analysis. Remarkably, the D_4-F88V mutant drastically decreased (~100-fold) the binding affinity of L-750,667 without substantially altering the pharmacological profile of the allosteric modulators or any other drug tested. The adjacent D_4-L87W mutation and D_4-S91L mutation one helical turn above it had no apparent effect on L-750,667 binding affinity, but both of their L-750,667 competition curves were significantly shallow, perhaps indicating that they are in proximity to a critical site. In contrast to the pronounced and specific effects of the D_4-F88V mutant on the binding affinity of the compact D_4-selective ligand L-750,667, the D_4-L87W mutant specifically increased the binding affinity only for ligands with extended structures, i.e., methylspiperone, OPC-14597, and OPC-4392, thereby making them significantly more D_2-like. This correlation between ligand structure and interaction with residue 87 in D_4-TMS2 appears to be predominantly an effect of ligand spacer arm length, rather than the functional activity or D_2 versus D_4 selectivity of the ligand per se. Specifically, OPC-14597 is a selective antagonist, OPC-4392 is a comparatively nonselective agonist, and methylspiperone is a comparatively nonselective antagonist, yet the binding affinity of each is increased by substituting a tryptophan for a leucine at positions 87 in D_4-TMS3. In contrast, both L-750,667 and OPC-14597 are selective antagonists with a piperazine pharmacophore, but L-750,667 has a compact structure and its binding is unaffected by the D_4-L87W mutation.

The two D_4-TMS3 mutants with the largest effects on L-750,667 and (−)-raclopride binding were chosen for double mutation studies with D_4-F88V. The resulting D_4-TLMAM105–109IFVTL+F88V and D_4-LM106–107FV+F88V double mutants (D_4-TMS2/3) had lower affinity for L-750,667 than the single D_4-F88V mutant, but the decrease in affinity was not as large as would be expected if the effects of the "single" mutations were additive. However, the D_4-F88V mutation dominated the effect observed for the double mutations that included D_4-F88V and the extracellular side of D_4-TMS3. Much more intriguing was that the double mutants no longer had aberrant MIA-binding profiles as were observed for the single TMS domain mutations at the extracellular side of D_4-TMS3. Remarkably, the binding affinities of the D_4-TMS2/3 double mutants for (−)-raclopride also were restored to the level of the wild-type D_4 receptor, and with the exception of L-750,667, the remaining ligands were either unaffected or bound the double mutants with somewhat higher affinities. These results strongly imply that the dependence of L-750,667 binding affinity on phenylalanine 88 of the D_4 receptor is contingent on a delicate interaction between TMS2 and TMS3. However, the dependence of this TMS2–3 interaction on phenylalanine 88 is somewhat surprising given that the proposed three-dimensional models of GPCRs have phenylalanine 88 oriented toward TMS1 (at least when the receptor is in the nonligated conformational state) and leucine 87 oriented toward TMS2 (Baldwin et al., 1997; Javitch et al., 1999). A more detailed analysis that considers the effects of several different amino acid substitutions at position 88 of the D_4 dopamine receptor may explain the nature of these interhelical conformational effects on the shape of the binding-site crevice.

In summary, a single phenylalanine residue located at position 88 in the second helical turn from the extracellular side of TMS2 is a primary molecular determinant responsible for the subtype-selective binding of the D_4-selective antagonist L-750,667. Furthermore, it seems that L-750,667 may interfer with the interaction phenylalanine 88 imposes on TMS3 because mutation of phenylalanine 88 to valine overrides, and in some cases reverses, the moderate effects observed for mutations in the first helical turn of TMS3. Specifically, the magnitude of the influence of the leucine106→phenylalanine mutation in TMS3 of the D_4 dopamine receptor on the binding of D_2- and D_4-subtype-selective drugs with compact structures depends on whether a phenylalanine or a valine occupies position 88 in TMS2. In contrast, the binding of drugs with extended structures was selectively increased when the unique leucine at position 87 of the D_4 receptor is mutated to the conserved tryptophan present at the corresponding position in all other dopamine receptors. A notable overall outcome is that the largest effects on selective drug binding are due to nonconserved amino acids directly adjacent to two uniquely D_4 amino acids in TMS2 and TMS3 that are proposed to face the binding-site crevice. A significant aside to this study is the finding that the allosteric modulators functioned as useful sensors of the more subtle mutations that anomalously influence the conformation of the receptor-binding pocket.

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