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Modeling and Mutational Analysis of a Putative Sodium-Binding Pocket on the Dopamine D_2 Receptor

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ABSTRACT

A homology model of the dopamine D_2 receptor was constructed based on the crystal structure of rhodopsin. A putative sodium-binding pocket identified in an earlier model (PDB 1I15) was revised. It is now defined by Asn-419 backbone oxygen at the apex of a pyramid and Asp-80, Ser-121, Asn-419, and Ser-420 at each vertex of the planar base. Asn-423 stabilizes this pocket through hydrogen bonds to two of these residues. Highly conserved Asn-52 is positioned near the sodium pocket, where it hydrogen-bonds with Asp-80 and the backbone carbonyl of Ser-420. Mutation of three of these residues, Asn-52 in helix 1, Ser-121 in helix 3, and Ser-420 in helix 7, profoundly altered the properties of the receptor. Mutants in which Asn-52 was replaced with Ala or Leu or Ser-121 was replaced with Leu exhibited no detectable binding of radioligands, although receptor immunoreactivity in the membrane was similar to that in cells expressing the wild-type D_{2L} receptor. A mutant in which Asn-52 was replaced with Gln, preserving hydrogen-bonding capability, was similar to D_{2L} in affinity for ligands and ability to inhibit cAMP accumulation. Mutants in which either Ser-121 or Ser-420 was replaced with Ala or Asn had decreased affinity for agonists (Ser-121), but increased affinity for the antagonists haloperidol and clozapine. Interestingly, the affinity of these Ser-121 and Ser-420 mutants for substituted benzamide antagonists showed little or no dependence on sodium, consistent with our hypothesis that Ser-121 and Ser-420 contribute to the formation of a sodium-binding pocket.

The dopamine D_2 receptor belongs to a subfamily of 7-transmembrane domain (TM) G protein-coupled receptors that interact with the G proteins $G\alpha_i$ and $G\alpha_o$ to modulate several effectors, including adenylate cyclase and potassium channels (Limbird, 1988). A characteristic of $G\alpha_i$-coupled receptors is that they are regulated by sodium, which decreases the affinity of the receptors for agonists (Pert and Snyder, 1974; Limbird et al., 1982). Sodium inhibits the binding of agonists by acting directly on the receptor (Limbird et al., 1982; Urwyler, 1989) at a site that is accessible from the intracellular surface of the cell membrane (Motulsky and Insel, 1983; Nunnari et al., 1987). Although the binding of antagonist ligands to some $G\alpha_i$-coupled receptors is modestly enhanced (<10-fold) by sodium (Limbird et al., 1982), the D_2 receptor is unusual in that the affinity of certain antagonists, most of them substituted benzamide derivatives, is enhanced 10- to 40-fold by sodium (Stefanini et al., 1980; Neve, 1991).

Homology modeling of the dopamine D_2 receptor (Teeter et al., 1994) has suggested the presence of a pyramidal sodium-binding pocket defined by residues Asp-$80^{2.50}$, Ser-$121^{3.39}$, Asn-$124^{3.42}$, and Ser-$420^{7.46}$ at each vertex of the base, and Asn-$423^{7.49}$ at the apex. Asn-$52^{1.50}$ is positioned near the sodium pocket, where it could interact with Asp-80. This putative sodium regulatory site is contiguous with a proposed nonpolar binding pocket; an attractive hypothesis is that drugs that occupy the nonpolar binding pocket may be particularly sensitive to the binding of sodium to the regulatory site (Teeter et al., 1994; Teeter and DuRand, 1996).

Mutation of Asp-80 of the D_2 receptor and the corresponding residue in the α_{2A}-adrenergic receptor, one of the putative sodium pocket residues, abolishes regulation of these receptors by sodium (Horstman et al., 1990; Neve et al., 1991). This highly conserved TM2 residue has been a frequent target of mutagenesis studies, having been mutated in at least 31 different receptor subtypes (Beukers et al., 1999).

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ABBREVIATIONS: TM, transmembrane domain; 7-OH-DPAT, hydroxy-2-dipropylaminotetralin; HEK, human embryonic kidney.

In addition to eliminating regulation of receptors by sodium (Horstman et al., 1990; Neve et al., 1991; Barbhaiya et al., 1996; Martin et al., 1999), mutation of this residue eliminates or greatly reduces functional coupling of many receptors to G proteins and second messengers (Chung et al., 1988; Fraser et al., 1989; Neve et al., 1991; Perlman et al., 1997a; Donnelly et al., 1999; Martin et al., 1999). Thus, binding of sodium to this residue may be important for stabilizing an active receptor conformation.

We now describe a revised D_2 receptor model, derived from the newly determined X-ray structure of rhodopsin, in which Asn-419$^{7.45}$ replaces Asn-124 at one vertex of the pyramidal sodium-binding pocket and the carbonyl of Asn-419 replaces Asn-423 at the apex. Mutation of three additional residues within this region, Asn-52 in TM1, Ser-121 in TM3, and Ser-420 in TM7, profoundly altered the properties of the receptor. We constructed mutant receptors in which Asn-52 of the rat D_{2L} was replaced with alanine (N52A), leucine (N52L), or glutamine (N52Q), and Ser-121 was replaced with alanine (S121A), asparagine (S121N), or leucine (S121L). The mutants N52A, N52L, and S121L exhibited no detectable binding of radioligands, although receptor targeting to the plasma membrane did not appear grossly impaired. The mutant N52Q, which retains hydrogen-bonding capability at this residue, was similar to the wild-type receptor in affinity for ligands and ability to inhibit adenylate cyclase. S121A and S121N had decreased affinity for agonists but increased affinity for the antagonists haloperidol and clozapine. Interestingly, the binding of substituted benzamide antagonists to S121A and S121N showed a loss of sodium sensitivity, with decreased binding compared with wild-type D_{2L} only in the presence of sodium. A similar loss of sodium sensitivity was observed for Ser-420 mutants (S420A, S420N, S420L, and S420V) but not for mutants of a residue now predicted to be oriented away from the putative sodium-binding pocket, Asn-124 (N124A, N124L, N124Q).

Experimental Procedures

Materials. [^{3}H]Spiperone was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). [^{3}H]cAMP and [^{3}H]YM09151–2 were from PerkinElmer Life Science Products (Boston, MA). (+)-Butaclamol, clozapine, haloperidol, spiperone, sulpiride, quinpirole, 7-OH-DPAT, pergolide, bromocriptine, and lisuride were purchased from Sigma/RBI (Natick, MA). Tropapride and YM09151–2 were obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program. Epidepride was a generous gift from Dr. T. de Paulis (Vanderbilt University, Nashville, TN). Serum was from HyClone (Logan, UT). Most other reagents, including culture media and dopamine, were purchased from Sigma Chemical Co. (St. Louis, MO).

D_2 Receptor Homology Modeling. An initial model of the D_2 receptor was built from the two-dimensional electron microscopy structure of bacteriorhodopsin, as described previously (Teeter et al., 1994). In brief, based on the three-dimensional bacteriorhodopsin structure obtained experimentally and related adrenergic receptor ligand binding mutagenesis, rhodopsin was aligned with bacteriorhodopsin. Alignment of low homology sequences was aided by establishing polar and nonpolar helix faces via helical wheels. From the alignment, D_2 receptor residues were substituted into the coordinates of bacteriorhodopsin (Henderson et al., 1990) to build an initial data-based D_2 receptor model. Local geometry optimization, Pro template replacements, and side chain rotation consistent with protein structure knowledge refined this model (1I15 in the Protein Data Bank). Global energy minimization was not used for the final model for reasons discussed previously (see footnote 1 in Teeter et al., 1994).

An improved model was based on the rhodopsin X-ray structure at 2.8-Å resolution (Palczewski et al., 2000). The sequence alignment was straightforward and identical to that of the online "Comprehensive alignment of G protein-coupled receptor sequences" (http://mgddk1.niddk.nih.gov:8000/comprehensive.html). Residues in rhodopsin were substituted with rat D_{2L} residues. Unless otherwise specified, residues are numbered according to their position in the receptor being discussed. In some cases, a superscript is added to indicate the position of the residue relative to the most conserved residue in that TM (Ballesteros and Weinstein, 1995)

Modeling the Pro residues required consideration of the structural location and geometry of the Pro. Of the changed residues, Pro-53$^{1.48}$ is not significantly bent in rhodopsin (Palczewski et al., 2000). The backbone of this residue, changed to Phe in the D2 receptor, was not remodeled. Residues that were different from rhodopsin in the termini or loops (one in the nonhelical N terminus region, one in cytoplasmic loop 1 between TM1 and TM2, two in extracellular loop 2 between TM4 and TM5, 11 in cytoplasmic loop 3, and one in extracellular loop 3) were not modeled, because loops are not included at this time. Five of the seven remaining Pro changes were at the N terminus of helices 1, 2, 3, 5, and 7 (1.29, 2.38, 3.22, 5.36, 7.31), were considered to be involved in the turns, and were not modeled. The same reasoning was applied to the Pro$^{4.60}$ at the C-terminus of TM4.

Pro-89$^{2.59}$ replaced a Thr that, in the rhodopsin crystal structure, is near the kink and helix unwinding at Gly$^{2.56}$–Gly$^{2.57}$ (Palczewski et al., 2000). This kink on the N-terminal side of Pro-89$^{2.59}$ in D2 eliminates most bad contacts in the Pro side chain direction. Only small backbone torsion adjustments were required. However, this substitution resulted in the new D_2 model having overlapped side chains in TM2 and TM3 near the extracellular surface. The rhodopsin unwinding at GG is not favorable for the β-carbon containing residues in D_2 in this region. Thus, one might expect some steric hindrance. The backbone torsions (Φ,Ψ angles) of residues around the new Pro, particularly the four residues N-terminal to it (residues i to i-4), were adjusted to relieve the close contacts with TM3. These changes made the helix more regular (near $-60°$,$-40°$ in Φ,Ψ) and accommodated the Pro side chain

No energy minimization was performed nor were molecular dynamics run. This is in concert with our earlier modeling (Teeter et al., 1994), based on calculations that show molecular mechanics techniques can distort a model that has been determined from a 1-Å resolution crystal structure (Whitlow and Teeter, 1986).

Production of Cell Lines. Mutants of the rat D_{2L} receptor were constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Wild-type and mutant receptors in pcDNA3.1 were stably expressed in HEK293 cells by calcium phosphate precipitation, and clonal cell lines were isolated after selection with G418 (800 μg/ml). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 5% iron-supplemented calf bovine serum, 5% fetal bovine serum, 600 μg/ml G418, 0.05 U/ml penicillin, and 50 μg/ml streptomycin at 37°C and 10% CO_2.

Immunocytochemical Detection of the D_2 Receptor. Cells grown on glass coverslips were fixed in 4% paraformaldehyde/phosphate-buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl, pH 7.4) for 15 min, permeabilized in 0.5% Triton X-100 for 15 min, then blocked with 5% goat serum for 1 h at room temperature. Cells were incubated with rabbit anti-D2 receptor antiserum (AB5084P, 1/500 dilution; Chemicon International, Temecula, CA), washed, and incubated for 1 h with Oregon Green-tagged goat anti-rabbit IgG (1/100 dilution; Molecular Probes, Eugene, OR). The cells were washed, mounted onto a slide with Slowfade (Molecular Probes), and imaged with a Leica SP laser scanning confocal microscope (Leica, Deerfield, IL).

[^{3}H]Spiperone Binding Assay. Cells were lysed in ice-cold hypotonic buffer (1 mM Na^+HEPES, pH 7.4, 2 mM EDTA) for 10 min, scraped from the plate, and centrifuged at 18,000*g* for 20 min. The

resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 6 to 10 s in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 0.9% NaCl). Membrane proteins (5–20 μg) were incubated in duplicate for 45 min at 37°C in a total reaction volume of 1 ml with [^{3}H]spiperone at concentrations ranging from 0.006–0.2 nM for saturation binding or ~0.1 nM with the appropriate concentration of the competing drug for competition binding. (+)-Butaclamol (2 μM) was used to define nonspecific binding. In some experiments, binding in sodium-free buffer was compared with binding in the presence of 50 mM NaCl. Data for saturation and displacement binding were analyzed by nonlinear regression using the computer program Prism (GraphPad, San Diego, CA) to determine K_D and IC_{50} values. Apparent affinity (K_I) values were calculated from the IC_{50} value by the method of Cheng and Prusoff (1973). The free concentration of radioligand was calculated as the concentration added minus the concentration specifically bound.

cAMP Accumulation Assay. The ability of D_2 receptor agonists to inhibit 30 μM forskolin-stimulated cAMP accumulation was measured in intact cells. Cells were plated at a density of 18,000 cells/cm^2 in 48-well tissue culture plates and used in experiments 2 to 3 days later. Before the assay, cells were preincubated with Earle's balanced salt solution with 0.2% ascorbic acid and 2% calf bovine serum, pH 7.4, for 10 min at 37°C. The assay was terminated after 10 min by decanting the medium, and the cells were placed on ice and lysed with 3% trichloroacetic acid. Lysates were incubated on ice at least 30 min before cAMP accumulation was measured using a competitive protein binding assay as described previously (Watts and Neve, 1996). Dose-response data were analyzed as described above for radioligand binding.

Results

Based on the crystal structure of rhodopsin (1F88 in the Protein Data Bank; Palczewski et al., 2000) a new model of the dopamine receptor was constructed (Fig. 1A; Teeter and DuRand, unpublished observations). In this model, the sodium-binding pocket is similar to that in the previous model (Teeter et al., 1994), except that Asn-124$^{3.42}$ is pointed away from the sodium site and hydrogen-bonds to Ser-75$^{2.45}$ at the back side of tilted TM3. Conserved residue Asn-419$^{7.45}$ is now in the sodium-binding site, which has a pyramidal shape defined by side chain atoms of Asp-80$^{2.50}$, Ser-121$^{3.39}$, Asn-419, and Ser-420$^{7.46}$ at each vertex of the planar base, and the carbonyl oxygen of Asn-419 at the apex (Fig. 1B). Asn-423$^{7.49}$ stabilizes the pyramid by hydrogen-bonding to Asp-80 and to the carbonyl oxygen of Asn-419. In addition, the Asn-52$^{1.50}$ side chain hydrogen-bonds to Asp-80 (Fig. 2A) without changing rotamers, although a rotamer change had been proposed previously (Teeter et al., 1994), and it also binds to the backbone carbonyl of Ser-420 on TM7. The sodium pocket region thus involves residues from TM1 (Asn-52), TM2 (Asp-80), TM3 (Ser-121), and TM7 (Asn-419, Ser-420) (Fig. 3).

Three substitution mutants were constructed for both Asn-52 (N52A, N52Q, and N52L) and Ser-121 (S121A, S121N, S121L). Wild-type and mutant receptors were stably expressed in HEK293 cells and characterized by saturation analysis of the binding of the D_2 receptor antagonist [^{3}H]spiperone. Three of the mutants retained high affinity for the radioligand, and clonal cell lines were selected that expressed approximately equal densities of receptors (Table 1). Membranes from cell lines expressing the other three mutants (N52A, N52L, and S121L) showed no detectable specific binding of [^{3}H]spiperone or a second D_2 receptor antagonist, [^{3}H]YM09151–2 (data not shown). Expression of N52A, N52L, and S121L in the cell membrane was confirmed by immunocytochemistry (Fig. 4). D_2 receptor immunoreactivity was present in the membrane of cells expressing each of the nonfunctional mutants, although the cell membrane was labeled less sharply and uniformly than in cells expressing wild-type D_{2L} receptor.

The affinity of the mutant receptors for agonists and antagonists was determined by inhibition of the binding of [^{3}H]spiperone. Whereas N52Q was indistinguishable from wild-type D_{2L} with regard to the affinity of all the agonists

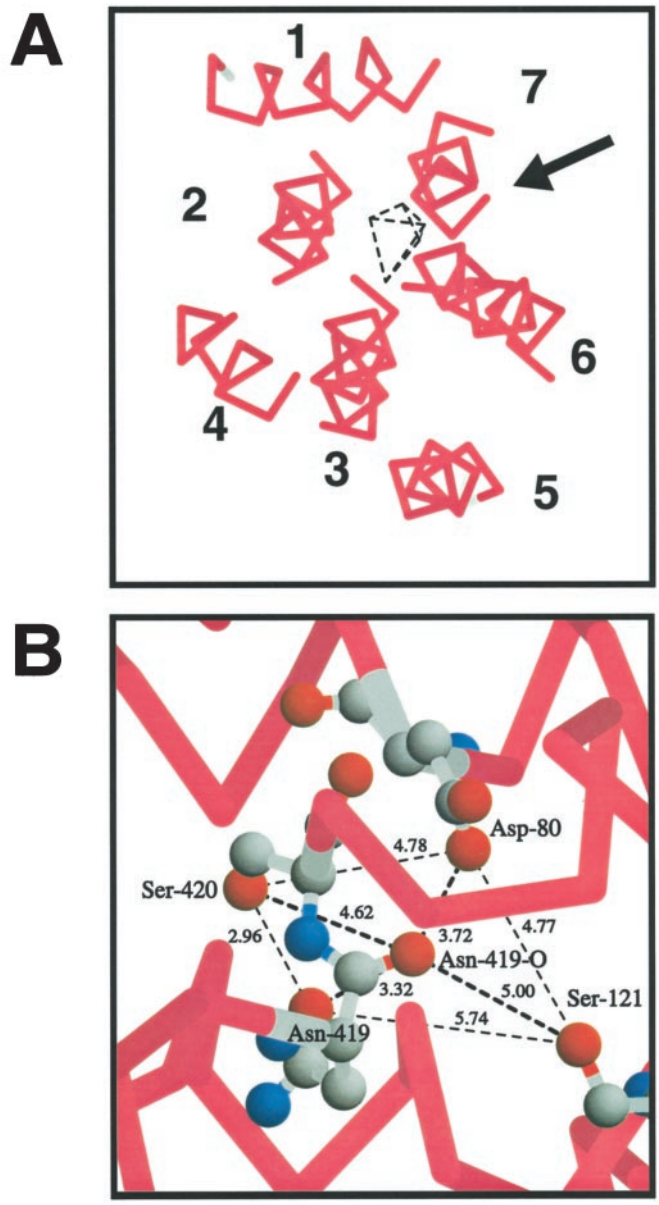

Fig. 1. Model of a putative sodium-binding pocket on the D_2 receptor. A, overview of helices in new D_2 dopamine receptor model, as viewed from the extracellular side of the membrane. TM1 is at the top and TM2 to TM7 are counterclockwise from this helix. A square pyramid occupies the region between TM2, TM3, and TM7, near the cytoplasmic side of the receptor. The vertices of the pyramid represent side chains and backbone carbonyl of four residues in the sodium-binding pocket. The arrow indicates the viewpoint of Fig. 1B. B, square pyramidal sodium site consisting of polar groups ~3.5 to 6 Å apart. Asn-419 carbonyl oxygen is at the apex of this pyramid. The four-sided base is viewed from the apex and consists of side chains of Asp-80 from TM2, Ser-121 from TM3, and Asn-419 and Ser-420 from TM7.

and antagonists that were tested, S121A had 4- to 5-fold enhanced affinity for haloperidol and clozapine, and a significantly decreased affinity for the agonists 7-OH-DPAT and dopamine (Table 2). Substitution of Asn for Ser-121 caused a 2-fold increase in the affinity of the receptor for clozapine and also caused a greater disruption of agonist binding compared with S121A, so that the affinity of S121N for dopamine, quinpirole, and 7-OH-DPAT was decreased 10-, 4-, and 25-fold, respectively.

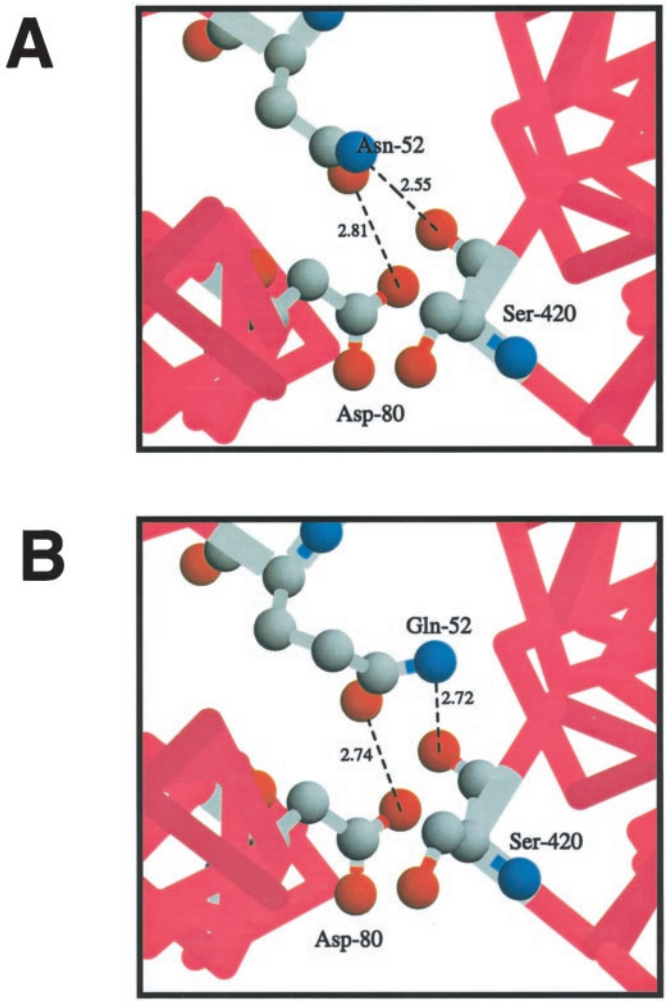

Fig. 2. Conserved Asn-52 in TM1 and its interactions. A, Asn-52 (TM1) hydrogen-bonds to Asp-80 (TM2) and to the carbonyl of Ser-420 (TM7). B, the N52Q mutation maintains the same hydrogen bonds (to Asp-80 and Ser-420 carbonyl) that are made by Asn-52.

The affinity and sodium-sensitivity of the binding of four antagonists, piquindone (Teeter and DuRand, 1996) and three substituted benzamide derivatives, was determined by inhibition of the binding of [^{3}H]spiperone in the presence and absence of 50 mM NaCl (Table 3; Fig. 5). The binding of the drugs to wild-type D_{2L} depended on the presence of sodium; the affinity of D_{2L} for the compounds was 13- to 48-fold greater in the presence than in the absence of sodium. The sodium sensitivity of binding to N52Q was similar to that of D_{2L}, so that the affinity of N52Q for the drugs was 13- to 106-fold greater in the presence of sodium. On the other hand, the sodium sensitivity of the binding of these antagonists to S121A and S121N was greatly reduced, so that affinities for the drugs were enhanced only 2- to 5-fold by the presence of sodium and, except for sulpiride at S121A, were more similar to K_I values of the wild-type receptor obtained in the absence than in the presence of sodium.

The ability of agonists to activate the mutant receptors was determined by measuring inhibition of forskolin-stimulated cAMP accumulation (Table 4). All three of the mutant receptors that were capable of binding radioligands with high affinity also inhibited forskolin-stimulated cAMP accumulation; maximal inhibition was similar for the mutant and wild-type receptors (~60% of total accumulation). Consistent with the decreased affinity of S121N for agonists, dopamine and quinpirole were significantly less potent at inhibition of cyclic AMP accumulation via S121N than the wild-type receptor.

To compare the sodium-binding pockets defined in the earlier homology model (Teeter et al., 1994) and the revised model, we constructed substitution mutants of Ser-420, which is part of the sodium site in both models, and Asn-124, which was suggested to be part of the sodium site in the earlier model but is pointed away from the sodium site in the revised model. All of the mutants bound [^{3}H]spiperone, in

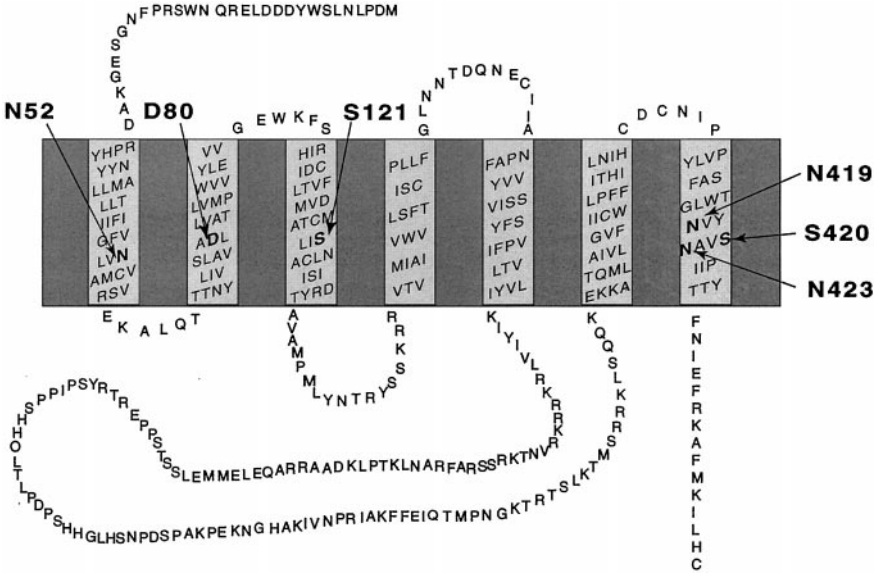

Fig. 3. Schematic of the rat D_{2L} receptor. Amino acid residues that form the sodium-binding pocket or that interact with its residues are indicated.

some cases with modestly decreased affinity compared with wild-type D_{2L} (Table 1). The affinity of N124A and N124L for most antagonists was decreased by 2- to 5-fold, whereas the mutation of N124Q was more deleterious, resulting in decreases in affinity of up to 30-fold (Table 5). Nevertheless, mutation of Asn-124 tended to decrease the potency of sodium-sensitive drugs whether or not sodium was present, so that the magnitude of the sodium-dependent increase in affinity for substituted benzamide antagonists was similar for wild-type D_{2L} and all three Asn-124 mutants. In contrast, all four mutants of Ser-420 (S420A, S420L, S420N, and S420V) displayed a loss of affinity for substituted benzamide antagonists that was greater in the presence of sodium than in its absence, so that the effect of sodium on the binding of these antagonists was abolished (Table 6). As observed for Ala and Asn mutants of Ser-121, the affinity of S420A and S420N for clozapine and haloperidol was increased 2- to 9-fold compared with wild-type D_{2L}.

TABLE 1

Saturation analysis of [^{3}H]spiperone binding to wild-type and mutant receptors Wildtype (D_{2L}) and mutant D_2 receptors were stably expressed in HEK293 cells and clonal cell lines were selected. The density of receptors on membranes prepared from the cells was determined by saturation analysis of the binding of [^{3}H]spiperone. The results from three to four independent experiments are shown as the mean ± S.E. for the density of binding sites (B_{max}), expressed as femtomoles per milligram of protein, or the geometric mean followed by the range of the S.E. for the affinity of each receptor subtype for [^{3}H]spiperone, expressed as picomolar.

Receptor	[^{3}H]Spiperone Binding	
	K_d	B_{max}
	pM	*fmol/mg*
D_{2L}	40 (33–48)	828 ± 168
N52Q	35 (28–43)	330 ± 87
S121N	32 (28–36)	444 ± 127
S121A	34 (24–48)	471 ± 63
N124A	84 (75–95)[a]	455 ± 26
N124L	96 (78–117)[a]	791 ± 122
N124Q	524 (419–656)[a]	1610 ± 247
S420A	27 (24–39)	886 ± 187
S420L	105 (91–122)[a]	522 ± 53
S420N	44 (41–48)	1789 ± 190
S420V	157 (139–177)[a]	1421 ± 233

[a] $P < 0.05$ compared with the affinity of D_{2L} for that drug.

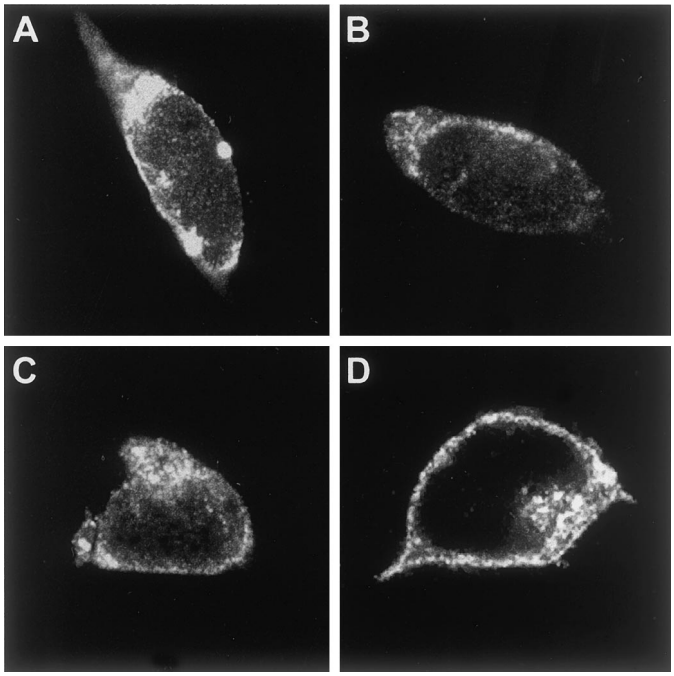

Fig. 4. Localization of D_2 receptor immunoreactivity in human embryonic kidney cells stably expressing wild-type or mutant receptors. A, S121L; B, N52A; C, N52L; D, D_{2L} wild-type.

Discussion

A D_2 receptor model was built based on the crystal structure of rhodopsin (Palczewski et al., 2000). When this model is viewed from the perspective of the arrow in Fig. 1A, a cluster of highly conserved polar residues that form a roughly pyramidal region is apparent. This pyramid is defined by Asp-80$^{2.50}$, Ser-121$^{3.39}$, Asn-419$^{7.45}$, and Ser-420$^{7.46}$ at each vertex of the base, and Asn-419 backbone oxygen at the apex (Fig. 1B). A sodium ion at the center of this site could form nearly ideal interactions, neutralizing the negative charge in this region. Four of these interactions appear to be closer (~3 Å) than the others (~4 Å). This differs from the previous model (Teeter et al., 1994) in several ways. First, conserved Asn-423$^{7.49}$, the former apex, appears to be in sodium's second coordination shell (~4 Å away). Asn-423 stabilizes the pocket by hydrogen-bonding to the new apex, the carbonyl oxygen of Asn-419, as well as to the side chain of Asp-80 at one vertex. Asn-419 is found one turn away from the highly conserved NP sequence (Asn-423, Pro-424$^{7.50}$ in D_{2L}), at a potentially conserved bend in TM7 (Fu et al., 1996). This bend frees the Asn-419 carbonyl oxygen to hydrogen-bond to the sodium ion. Second, Asn-124$^{3.42}$ has been replaced by Asn-419 at one of the vertices. Asn-124 is pointed away from the sodium site and hydrogen bonds to Ser-75$^{2.45}$. In rhodopsin, the corresponding hydrogen bonding pair is Asn-78 on TM2 and Ser-127 on TM3 (Palczewski et al., 2000), suggesting that this is a conserved interaction. Third, the side chains of Asn-419 and Ser-420 are hydrogen-bonded, further strengthening the sodium-binding pocket. Finally, the Asn-52$^{1.50}$ side chain hydrogen-bonds to Asp-80 (Fig. 2A), without changing rotamers as previously proposed (Teeter et al., 1994), and also binds to the backbone carbonyl of Ser-420 on TM7. Both of these interactions as well as the exposed carbonyl of Asn-419 are conserved in the rhodopsin crystal structure (Palczewski et al., 2000) and are likely to be conserved in all G protein-coupled receptors.

We constructed mutants in which conservative or nonconservative substitutions were made for each of two polar amino acid residues postulated to contribute to the formation of the sodium-binding pocket (Ser-121 and Ser-420) for one residue thought to hydrogen-bond with the highly conserved aspartate residue Asp-80 (Asn-52) and for one residue that was predicted to be part of the sodium-binding pocket in our earlier model, but not in the revised model (Asn-124). Three of the six mutants, N52L, N52A, and S121L, were not detectable by radioligand binding, suggesting that the structure of the D_2 receptor is very sensitive to changes in the properties of the residues at these positions. Residues with nonpolar side chains, in particular, were not well tolerated. Immunocytochemical localization of the nonfunctional mutant receptors suggested that each was expressed and targeted to the cell membrane, although the mutant receptors may have been distributed less homogenously around the perimeter of the cell than was the wild-type D_2 receptor. Although conclu-

sive demonstration of appropriate cell membrane targeting would require labeling of nonpermeabilized cells with an antibody directed against an extracellular epitope, the immunocytochemical data demonstrate the synthesis of D2 receptor protein from the transfected plasmid.

The D_{2L} receptor mutant N52Q was indistinguishable from wild-type D_{2L} with regard to the potency of agonists and antagonists, sensitivity to sodium, and inhibition of forskolin-stimulated cyclic AMP accumulation. In our model, this is explained by the ability of Gln to preserve the postulated hydrogen-bonding interaction of the native Asn-52 with Asp-80 (Fig. 2B). Interaction of $Asn^{1.50}$ with $Asp^{2.50}$ is also predicted by models of the TRH and α_{1B}-adrenergic receptors, with mutagenesis data that support the interaction (Scheer et al., 1996; Perlman et al., 1997a). Interestingly, substitution of Ala or Asp for $Asn^{1.50}$ in the TRH receptor (N43A and N43D) or the α_{1B}-adrenergic receptor (N63A and N63D) has less severe consequences than substitution with Ala or Leu in the D_2 receptor in the present study. In the case of the TRH receptor, the mutants are detectable by ligand binding and are functional, albeit with reduced affinity for ligands, decreased density of expression, and decreased abil-

TABLE 2

Apparent affinity of Asn-52 and Ser-121 mutant receptors for agonists and antagonists

Apparent affinity (K_I) values for the indicated drugs were determined by inhibition of the binding of [^{3}H]spiperone, as described under *Experimental Procedures*. Each value is the geometric mean of three to five independent experiments, followed in parentheses by the limits defined by the asymmetrical S.E.

Drug	K_I values			
	D2	N52Q	S121A	S121N
			nM	
(+)-Butaclamol	0.70 (0.53–0.91)	0.83 (0.67–1.04)	0.71 (0.55–0.92)	0.51 (0.44–0.59)
Haloperidol	1.42 (1.20–1.54)	1.27 (1.14–1.4)	0.36 (0.31–0.44)[a]	0.61 (0.52–0.71)[a]
Clozapine	155 (142–170)	151 (138–164)	29 (24–35)[a]	164 (137–196)
Lisuride	0.57 (0.47–0.68)	0.37 (0.27–0.50)	0.55 (0.48–0.65)	0.98 (0.75–1.28)
Bromocriptine	1.29 (1.04–1.60)	1.00 (0.75–1.32)	1.62 (1.43–1.85)	1.86 (1.55–2.23)
Pergolide	59 (40–89)	41 (37–45)	37 (26–53)	86 (72–103)
7-OH-DPAT	396 (366–428)	344 (307–385)	1,500 (916–2,460)[a]	10,100 (6,960–14,700)[a]
Dopamine	14,800 (11,400–19,100)	8,200 (4,400–15,100)	41,900 (36,900–47,400)[a]	148,400 (121,300–181,600)[a]
Quinpirole	13,300 (11,000–16,000)	12,800 (8,600–19,100)	12,100 (7,700–19,100)	47,800 (37,100–61,600)[a]

[a] $P < 0.05$ compared with the affinity of D_{2L} for that drug.

TABLE 3

Apparent affinity of Asn-52 and Ser-121 mutant receptors for sodium-sensitive antagonists

Apparent affinity (K_I) values for the indicated drugs were determined by inhibition of the binding of [^{3}H]spiperone, as described under *Experimental Procedures*. Each value is the geometric mean of three to five independent experiments, followed in parentheses by the limits defined by the asymmetrical S.E. The fold change in affinity resulting from the presence or absence of sodium (Δsodium) is also given.

Drug		K_I values			
		D2	N52Q	S121A	S121N
				nM	
Epidepride	+ sodium	0.068 (0.059–0.079)	0.039 (0.031–0.050)	0.33 (0.23–0.48)[a]	0.57 (0.45–0.72)[a]
	− sodium	1.85 (1.07–3.18)	1.45 (1.18–1.79)	1.34 (0.92–1.96)	1.80 (1.37–2.36)
	Δsodium	27	37	4	3
Tropapride	+ sodium	0.096 (0.060–0.153)	0.15 (0.10–0.21)	1.38 (0.99–1.93)[a]	1.70 (1.21–2.39)[a]
	− sodium	4.65 (3.79–5.72)	4.17 (2.97–5.85)	4.31 (3.71–5.0)	6.86 (5.12–9.20)
	Δsodium	48	28	3	4
Piquindone	+ sodium	10.7 (8.3–13.8)	5.5 (4.6–6.5)	21.1 (13.7–32.5)	46.7 (35.7–61.2)[a]
	− sodium	137 (103–183)	71 (56–91)	89 (68–117)	181 (143–229)
	Δsodium	13	13	4	4
Sulpiride	+ sodium	33 (26–44)	17 (13–21)	126 (89–178)[a]	300 (229–394)[a]
	− sodium	1440 (1030–2030)	1810 (1310–2510)	898 (740–1090)	1260 (990–1610)
	Δsodium	44	106	7	4

[a] $P < 0.05$ compared with the affinity of D_{2L} for that drug.

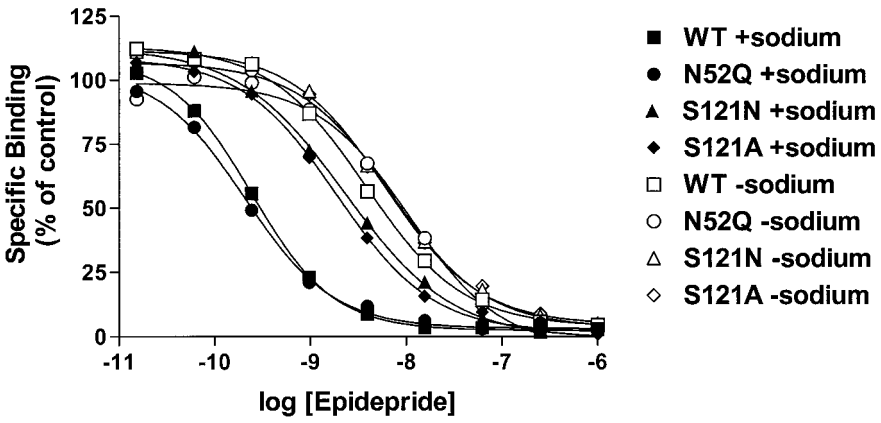

Fig. 5. Loss of sodium-sensitive binding of epidepride to S121A and S121N. Membranes were prepared from HEK293 cells stably expressing wild-type (WT) and mutant D_{2L} receptors. The apparent affinity of the receptors for epidepride was determined by inhibition of the binding of [^{3}H]spiperone in the presence (+ sodium) or absence (− sodium) of 50 mM NaCl, as described under *Experimental Procedures*. The results from one experiment representative of four independent experiments are shown.

ity to stimulate inositol phosphate formation (Perlman et al., 1997a). N63A and N63D mutants (but not N63L or N63F mutants) of the α_{1B}-adrenergic receptor also bind agonist and antagonist ligands with high affinity and stimulate inositol phosphate formation (Scheer et al., 1996). Studies of the tachykinin NK_2 receptor suggest that the primary effect of mutation of $Asn^{1.50}$ to Asp or His (N51D and N51H) is to greatly reduce the expression of the receptors, without markedly altering the binding of ligands or functional coupling (Donnelly et al., 1999). Similarly, an $N33A^{1.50}$ mutant of the

TABLE 4

Inhibition of cAMP accumulation by wild-type and mutant D_{2L} receptors

HEK293 cells stably expressing the indicated wild-type or mutant D_{2L} receptor were incubated with 30 μM forskolin and increasing concentrations of the indicated drugs, and cAMP formation was determined as described under *Experimental Procedures*. EC_{50} values, expressed in nanomolar, were calculated from the dose-response curves and are the geometric means of three to five independent experiments, followed in parentheses by the limits defined by the asymmetrical S.E. The mean ± S.E. is shown for maximal inhibition of forskolin-stimulated activity (Max), expressed as a percentage of total cAMP accumulation.

Drug		D2	N52Q	S121A	S121N
Dopamine	EC_{50} (nM)	27 (17–42)	21 (14–32)	55 (31–99)	198 (114–340)[a]
	Max (%)	64 ± 10	78 ± 4	49 ± 8	54 ± 7
Quinpirole	EC_{50} (nM)	10 (8–14)	36 (20–67)	19 (18–20)	105 (97–114)[a]
	Max (%)	63 ± 12	69 ± 7	59 ± 5	57 ± 1
7-OH-DPAT	EC_{50} (nM)	41 (20–84)	20 (15–27)	30 (16–53)	102 (98–107)
	Max (%)	57 ± 8	74 ± 3	58 ± 12	64 ± 5

[a] $P < 0.05$ compared with the potency of D_{2L} for that drug.

TABLE 5

Apparent affinity of Asn-124 mutant receptors for antagonists

Apparent affinity (K_I) values for the indicated drugs were determined by inhibition of the binding of [^{3}H]spiperone, as described under *Experimental Procedures*. Each value is the geometric mean of four to six independent experiments, followed in parentheses by the limits defined by the asymmetrical S.E. For the substituted benzamide derivates, the fold change in affinity resulting from the presence or absence of sodium (Δsodium) is also given.

Drug		K_I			
		D2	N124A	N124L	N124Q
		nM			
Epidepride	+ sodium	0.08 (0.07–0.09)	0.35 (0.29–0.42)[a]	0.26 (0.2–0.33)[a]	1.6 (1.2–2.0)[a]
	− sodium	1.6 (1.0–2.5)	3.8 (2.6–5.5)	3.0 (2.0–4.7)	17 (12–25)[a]
	Δsodium	19	11	12	11
YM09151-2	+ sodium	0.03 (0.02–0.03)	0.12 (0.10–0.14)[a]	0.11 (0.09–0.12)[a]	0.48 (0.37–0.62)[a]
	− sodium	0.22 (0.19–0.26)	0.67 (0.59–0.76)[a]	0.63 (0.49–0.81)[a]	5.7 (5.0–6.4)[a]
	Δsodium	8	6	6	12
Tropapride	+ sodium	0.07 (0.06–0.08)	0.28 (0.21–0.38)[a]	0.29 (0.23–0.36)[a]	2.2 (1.4–3.3)[a]
	− sodium	0.74 (0.34–1.6)	1.4 (1.2–1.8)	2.0 (1.6–2.5)	19 (16–23)[a]
	Δsodium	11	5	7	9
Sulpiride	+ sodium	48 (47–50)	139 (114–169)[a]	132 (116–150)[a]	454 (403–512)[a]
	− sodium	410 (360–480)	1,310 (1,110–1,550)[a]	1,206 (962–1,510)[a]	8,525 (6,062–11,990)[a]
	Δsodium	9	5	9	18
(+)-Butaclamol	+ sodium	0.2 (0.1–0.2)	0.8 (0.7–0.9)[a]	0.9 (0.7–1.0)[a]	2.8 (2.1–3.8)[a]
Haloperidol	+ sodium	1.0 (0.9–1.2)	0.6 (0.5–0.8)	0.4 (0.3–0.5)[a]	5.0 (4.3–5.7)[a]
Clozapine	+ sodium	72 (63–82)	147 (128–170)[a]	84 (76–93)	336 (321–351)[a]

[a] $P < 0.05$ compared with the affinity of D_{2L} for that drug.

TABLE 6

Apparent affinity of Ser-420 mutant receptors for antagonists

Apparent affinity (K_I) values for the indicated drugs were determined by inhibition of the binding of [^{3}H]spiperone, as described under *Experimental Procedures*. Each value is the geometric mean of four to six independent experiments, followed in parentheses by the limits defined by the asymmetrical S.E. For the substituted benzamide derivates, the fold change in affinity resulting from the presence or absence of sodium (Δsodium) is also given.

Drug		K_I				
		D2	S420A	S420L	S420N	S420V
		nM				
Epidepride	+ sodium	0.08 (0.07–0.09)	0.28 (0.24–0.32)[a]	9.4 (7.4–11.9)[a]	1.3 (1.0–1.7)[a]	5.5 (4.0–7.6)[a]
	− sodium	1.6 (1.0–2.5)	0.42 (0.32–0.54)[a]	14 (13–16)[a]	2.4 (1.7–3.5)	13 (9–18)[a]
	Δsodium	19	2	2	2	2
YM09141-2	+ sodium	0.03 (0.02–0.03)	0.13 (0.11–0.15)[a]	1.0 (0.8–1.4)[a]	0.28 (0.23–0.34)[a]	1.3 (1.0–1.7)[a]
	− sodium	0.22 (0.19–0.26)	0.16 (0.13–0.19)	0.98 (0.73–1.3)[a]	0.41 (0.30–0.57)	2.1 (1.6–2.6)[a]
	Δsodium	8	1	1	1	2
Tropapride	+ sodium	0.07 (0.06–0.08)	0.25 (0.19–0.34)[a]	15 (5–50)[a]	0.67 (0.57–0.8)[a]	3.5 (2.9–4.3)[a]
	− sodium	0.74 (0.34–1.6)	0.15 (0.10–0.23)	8.7 (6.1–12.5)[a]	0.73 (0.52–1.03)	6.5 (5.1–8.1)[a]
	Δsodium	11	1	1	1	2
Sulpiride	+ sodium	48 (47–50)	140 (110–170)	3280 (2,580–7,770)[a]	680 (600–780)[a]	2,580 (2200–3020)
	− sodium	410 (360–480)	180 (140–250)	3100 (2,710–3,540)	1230 (1100–1370)	3,040 (2450–3780)
	Δsodium	9	1	1	2	1
(+)-Butaclamol	+ sodium	0.2 (0.1–0.2)	0.23 (0.17–0.3)	0.26 (0.22–0.3)	0.18 (0.15–0.22)	0.59 (0.45–0.77)
Haloperidol	+ sodium	1.0 (0.9–1.2)	0.2 (0.1–0.2)[a]	1.0 (0.8–1.2)	0.3 (0.2–0.4)[a]	2.0 (1.5–2.5)
Clozapine	+ sodium	72 (63–82)	8.4 (7.6–9.2)[a]	160 (120–220)[a]	38 (34–42)[a]	130 (120–140)

[a] $P < 0.05$ compared with the affinity of D_{2L} for that drug.

platelet activating factor receptor binds agonist and antagonist radioligands (Ishii et al., 1997). In the case of the peptide receptors, the less severe consequences of substituting Ala at this position, compared with the consequences for the D_{2L} receptor, could be caused by a different mode of ligand binding, which is primarily to the extracellular loops rather than to the transmembrane helices, but it is not clear why the Ala substitution at this position is better tolerated by the α_{1b}-adrenergic receptor (Scheer et al., 1996) than by the D_{2L} receptor (present results).

The deleterious consequences of the N52A substitution compared with the sparing of function in S121A is interesting. Ser-121 is part of the sodium site, which is an open cavity with considerable water around. Indeed, a water molecule is at the center of a site in the rhodopsin structure that corresponds to the presumed sodium-binding site in the D_2 receptor (Palczewski et al., 2000). Thus, the sparing of function after replacement of Ser-121 with a nonpolar alanine residue in S121A is probably caused by a polar water molecule filling the space vacated by the lost hydroxyl of the serine side chain (Alber et al., 1987). On the other hand, in our model, Asn-52's site has no cavity for water and is quite "tight" but hydrophilic, and the Ala substitution would therefore be more deleterious.

In addition to binding sodium, the polar pocket formed by $Asn^{1.50}$, $Asp^{2.50}$, and $Asn^{7.49}$ (Asn-423 in the D_{2L} receptor) has been postulated to be involved in regulating the equilibrium between active and inactive receptor conformations (Scheer et al., 1996). According to this model, $Arg^{3.50}$ of the highly conserved Asp-Arg-Tyr (DRY) sequence at the base of TM3 is stabilized within the polar pocket by hydrogen-bonding and electrostatic interactions with $Asn^{1.50}$ and $Asp^{2.50}$, and mutation of $Asn^{1.50}$ shifts $Arg^{3.50}$ out of the polar pocket, mimicking the active conformation of the receptor. However, in the rhodopsin crystal structure (Palczewski et al., 2000) and in our new D_2 receptor model, the distance between $Arg^{3.50}$ in the DRY (or ERY) sequence and either $Asn^{1.50}$ or $Asp^{2.50}$ is about 20 Å. Instead of interacting with TM1 and TM2, $Arg^{3.50}$ in the ERY in rhodopsin has a salt link with Glu-$369^{6.30}$ (D_{2L} numbering) in the TM6 helical extension.

The S121A and S121N mutants of the D_{2L} receptor differed from the wild-type receptor in several respects. The affinity of the receptors for some antagonists (clozapine and haloperidol) was enhanced modestly, whereas there was a moderate decrease in affinity for several agonists. Both mutant receptors mediated agonist inhibition of adenylate cyclase, with decreases in agonist potency that roughly paralleled the decreased agonist affinity observed in ligand binding studies. The most robust effect of these mutations was to decrease the sodium sensitivity of the binding of those antagonists that have markedly higher affinity for the wild-type D_2 receptor in the presence of sodium. As was observed previously for the D80A mutant (Neve et al., 1991), regardless of the presence or absence of sodium, the affinity of the mutants for substituted benzamide derivatives was similar to that of the wild-type receptor in the absence of sodium. Similar results were observed for Ala and Asn substitutions of Ser-420, whereas Val or Leu substitution for Ser-420 abolished the sodium sensitivity of the receptor without increasing the affinity of the mutants for haloperidol and clozapine. In contrast, mutation of Asn-124 had little effect on the sodium sensitivity of the D_{2L} receptor, supporting the revised receptor model in which this residue is not predicted to be part of the sodium-binding pocket.

The S121N mutation caused decreased binding of benzamides despite the potential of the Asn to hydrogen-bond to sodium. This could be explained by direct interference of the larger Asn with the binding of benzamides. In our model, distances between Asn-121 and benzamide ligands are approximately 2 Å. Furthermore, the side chains of Asn-121 (TM3) and Asn-423 (TM7) could hydrogen-bond (distance 3.4 Å). In rhodopsin, TM3 and TM6 are shown to move apart at the interior in the activated receptor (Farrens et al., 1996). TM7 also changes its position later in the rhodopsin photocycle (Kim et al., 1997). A hydrogen bond between TM3 and TM7 would constrain this movement. Ser-$121^{3.39}$ has been a target in mutagenesis studies of many G protein-coupled receptors, and a cysteine-scanning study indicates that the residue is within the water-accessible ligand binding pocket of the D_2 receptor (Javitch et al., 1995). Ala mutants of this residue have been reported to not be expressed (β_2-adrenergic receptor; Strader et al., 1989), to be expressed but unable to bind ligands (adenosine A1 receptor; Barbhaiya et al., 1996), or to be expressed and able to bind ligands but unable to stimulate inositol phosphate accumulation (AT_1 receptor; Monnot et al., 1996). On the other hand, mutation of this residue to Ala has little or no effect on ligand binding or receptor signaling for the M_1 acetylcholine receptor (Lu and Hulme, 1999) and the platelet-activating factor receptor (Ishii et al., 1997) and little or no effect on ligand binding for the angiotensin II AT_1 receptor (Monnot et al., 1996; Perlman et al., 1997b), the bradykinin B_2 receptor (Jarnagin et al., 1996), and the adenosine A_{2a} receptor (Jiang et al., 1996). The S123A mutant of the C5a chemotactic peptide receptor is also functional (Baranski et al., 1999), as is the S115G mutant of the AT_1 receptor (Noda et al., 1996). These studies are all consistent with our observation that mutation of Ser-121 to Ala or Asn had little effect on the binding of ligands to the D_{2L} receptor or the ability of the receptor to inhibit adenylate cyclase, but selectively altered the regulation of the receptor by sodium.

The question arises whether the sodium pocket, documented to be accessible from the cytoplasmic side only, is permeable to sodium ions in the new D_2 receptor model. This might be expected because of the proximity of the nonpolar ligand binding pocket to the water-accessible Ser-121 (see above). However, TM3 is tilted in rhodopsin and in our model, and Met-$116^{3.35}$ (first M in DVMM) lies to the extracellular side of the sodium pocket, partially blocking the path of sodium to the extracellular side but not occluding Ser-121 from that side. In addition, the nonpolar atoms of the pocket are incompatible with the polar sodium ion but well suited for ligand binding or disulfide bond formation by MTSEA. Although MTSEA has a polar end that demonstrates proximity to water, in cysteine-scanning studies, the alkyl sulfide end could access the Ser-121 site that has been mutated to the less polar Cys through the pocket. Thus, our model is consistent with these residues forming a sodium-binding pocket, as opposed to a pore or channel.

Mutation of the residue corresponding to Ser-$420^{7.46}$ to Ala had little effect on ligand binding to the β_2-adrenergic receptor (Strader et al., 1989) or the $P2Y_1$ purinoceptor (Jiang et al., 1997), whereas the same mutation decreased the affinity of the $5HT_{1A}$ receptor (S393A) for agonists (Chanda et al., 1993) and the affinity of the adenosine A_{2A} receptor (S281A) for agonist and antagonist ligands. Interestingly, the conservative substitution $S281N^{7.46}$ in the A_{2A} receptor increased

receptor affinity for agonists but not antagonists (Jiang et al., 1996), whereas in the present study, the potency of several antagonist ligands for either S420A or S420N mutants of D_{2L} was enhanced. Consistent with the present results, the S391A$^{7.46}$ mutation of the short form of the D_2 receptor, D_{2S}, has little effect on the binding of most ligands but decreases the potency of substituted benzamide antagonists in the presence of sodium (Cox et al., 1992). Because this residue is not exposed to the water-accessible ligand binding crevice (Fu et al., 1996), effects of mutation of the residue on ligand affinity are probably indirect.

Asn-124$^{3.42}$ is not within the water-accessible ligand binding pocket of the D_2 receptor (Javitch et al., 1995), and probably does not participate directly in the binding of ligands. The effects of mutation of this residue on ligand binding are probably secondary to disruption of helix packing. In this regard, it is interesting that replacing the polar Asn side chain with a polar but bulkier Gln side chain was more deleterious than substitution with nonpolar residues that are smaller than or similar in size to Asn. Mutation of the corresponding residue in the M_1 muscarinic receptor (N115A) has little effect on the function of that receptor (Lu and Hulme, 1999).

In summary, these results support our hypothesis that Asn-52 and Ser-121 are crucial for maintaining the conformation of the ligand-binding pocket of the D_{2L} dopamine receptor, because certain nonconservative substitutions result in a profound loss of receptor function. Furthermore, the loss of sensitivity to sodium of the S121A and S121N mutants and all of the Ser-420 substitution mutants is in accord with our prediction that Ser-121 and Ser-420 participate in the formation of a sodium-binding pocket on the receptor.

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