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Differential Modes of Agonist Binding to 5-Hydroxytryptamine$_{2A}$ Serotonin Receptors Revealed by Mutation and Molecular Modeling of Conserved Residues in Transmembrane Region 5

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ABSTRACT

Site-directed mutagenesis and molecular modeling were used to investigate the molecular interactions involved in ligand binding to, and activation of, the rat 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) serotonin (5-HT) receptor. Based on previous modeling studies utilizing molecular mechanics energy calculations and molecular dynamics simulations, four sites (S239[5.43], F240[5.44], F243[5.47], and F244[5.48]) in transmembrane region V were selected, each predicted to contribute to agonist and/or antagonist binding. The F243A mutation increased the affinity of (+/−)4-iodo-2,5-dimethoxyphenylisopropylamine, decreased the binding of α-methyl-5HT, *N*-ω-methyl-5HT, ketanserin, ritanserin, and spiperone and had no effect on the binding of 5-HT and 5-methyl-*N,N*-dimethyltryptamine. The F240A mutant had no effect on the binding of any of the ligands tested, whereas F244A caused an agonist-specific decrease in binding affinity (3- to 10-fold). S239A caused a 6- to 13-fold decrease in tryptamine-binding affinity and a 5-fold increase in affinity of 4-iodo-2,5-dimethoxyphenylisopropylamine. A subset of the agonists used in binding studies were used to determine the efficacies and potencies of these mutants to activate phosphoinositide hydrolysis. The F243A and F244A mutations reduced agonist stimulated phosphoinositide hydrolysis, whereas the S239A and F240A mutations had no effect. There was little correlation between agonist binding and second messenger production. Furthermore, molecular dynamics simulations, considering these data, produced ligand-bound structures utilizing substantially different bonding interactions even among structurally similar ligands (differing by as little as one methyl group). Taken together, these results suggest that relatively minor changes in either receptor or ligand structure can produce drastic and unpredictable changes in both binding interactions and 5-HT$_{2A}$ receptor activation. Thus, our finding may have major implications for the future and feasibility of receptor structure-based drug design.

5-Hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptors are the major site of action for at least three classes of hallucinogens, including the ergolines (e.g., lysergic acid diethylamine), phenylisopropylamines (e.g., 4-iodo-2,5-dimethylphenylisopropylamine, DOI), and substituted tryptamines (e.g., *N,N*-dimethyltryptamine, DMT). Our limited, but expanding, understanding of 5-HT$_{2A}$ receptor function has led to the development of novel clinical therapies, including the atypical antipsychotics (e.g., clozapine, risperidone, olanzepine, and quietiapine), and the atypical antidepressants (e.g., nefazadone, mirtazepine, and mianserin; for review, see Roth et al., 1998). Efforts to improve clinical therapies by developing more receptor-specific, novel therapeutic agents have been hampered in part by a lack of knowledge concerning the precise three-dimensional structure of ligand-receptor interactions. In the absence of a high-resolution crystal structure for 5-HT$_{2A}$ (or any other G-protein-coupled receptor; GPCR), site-directed mutagenesis accompanied by molecular modeling has been used to uncover the details of ligand binding.

Based on analogies with rhodopsin and bacteriorhodopsin, a number of models of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have been constructed and, occasionally, subjected to testing by fitting of site-directed mutagenesis data (Choudhary et al., 1993; Westkaemper and Glennon, 1993; Holtje and Jendretzki, 1995; Almaula et al. 1996a,b; Kristiansen and Dahl, 1996; for example, see Roth et al., 1997a). Most models of

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ABBREVIATIONS: 5-HT$_{2A}$, 5-hydroxytryptamine$_{2A}$; DOI, 4-iodo-2,5-dimethylphenylisopropylamine; DMT, *N,N*-dimethyltryptamine; GPCR, G-protein-coupled receptor; PI, phosphoinositide; Me, methyl; DOM, 4-methoxy-2,5-dimethylphenylisopropylamine; NMSP, *N*-methylspiperone; vdW, van der Waals; TM, transmembrane.

agonist-5-HT_{2A} receptor complexes share the following features: a key stabilizing ionic interaction between the positively charged amine moiety of the agonist with a negatively charged aspartic acid residue (D155[3.32 using the numbering system of Ballesteros and Weinstein (1995), see below] in helix 3) and the OH group of S159[3.36] (helix 3), one or more serine residues (S207[4.57], S239[5.43] in helix 5, S372[7.45], S373[7.46] in helix 7), which serve to stabilize NH or OH moieties via hydrogen bonds, and a central core of aromatic residues, which stabilize aromatic and hydrophobic moieties of the ergoline nucleus (previously hypothesized to include F240[5.44], F340[6.52], W336[6.48], and W367[7.40]). Of these key residues, a great deal of attention has been focused on the ligand interactions involving D155[3.32] (Wang et al., 1993; Kristiansen et al., 2000) and F340[6.52] (Choudhary et al., 1993, 1995; Roth et al., 1995, 1997b).

In the present study, we have combined site-directed mutagenesis and molecular modeling techniques to identify additional residues in the rat $5HT_{2A}$ receptor that are important for agonist binding, antagonist binding, and agonist-stimulated phosphoinositide (PI) hydrolysis. The effects of four single-point mutations in transmembrane helix 5 (S239A, F240A, F243A, and F244A) of the rat $5HT_{2A}$ receptor on ligand binding and agonist-stimulated PI hydrolysis were examined. Our results suggest a key role for F243[5.47] in binding and activation of the $5HT_{2A}$ receptor. Depending on the nature of the bound ligand, F243[5.47] and F244[5.48] likely participate in a cluster of stabilizing, aromatic amino acids lining the binding pocket, exerting their influence through π-π stabilization, favorable van der Waals (vdW) interactions and, indirectly, by influencing the position of neighboring side chains. Our findings imply that changes in agonist binding not only affect binding pocket interactions but may also induce helical perturbations, which variously affect the receptor's ability to interact with G-proteins. Agonist binding was particularly sensitive to disruption by S239A, most likely interacting via formation of an OH-N1 hydrogen bond with the ring nitrogen of the indolealkylamines. Finally, the receptor binding and activation data from this and previous studies were utilized in molecular dynamics simulations to construct detailed molecular models for complexes between the 5-HT_{2A} receptor and structurally diverse agonists.

Materials and Methods

Receptor-Numbering Schemes. Where appropriate, amino acid residues have been labeled both by standard amino terminus-based numbering and by a numbering scheme introduced by Ballesteros and Weinstein (1995) in which relative amino acid positions are highlighted. This scheme facilitates the efficient comparison of residues within different GPCRs. With this method, every amino acid identifier starts with the transmembrane helix number and is followed by the position relative to a reference residue (arbitrarily assigned the number 50) among the most conserved amino acids in that transmembrane helix. In the seven transmembrane (TM) GPCRs, this generalized numbering scheme utilizes N1.50, D2.50, R3.50, W4.50, P5.50, P6.50, and P7.50 as seven reference positions, corresponding in rat 5-HT_{2A} receptors to N92, D120, R173, W200, P246, P338, and P377, respectively. F340, for example, lies two positions positive relative to P338, yielding the identifier [6.52].

Site-Directed Mutagenesis and Plasmid Construction. The mammalian expression vector pSVK3-SR2 was used as previously detailed (Roth et al., 1992; Choudhary et al., 1993, 1995). Mutants were developed using a polymerase chain reaction-based technique (Quikchange kit; Stratagene, La Jolla, CA) following the recommendations of the manufacturer, with mutagenesis and sequencing primers from Life Technologies/BRL (Gaithersburg, MD). For each mutant made, the full length of the receptor insert was sequenced using the dideoxy method (T7 Sequenase Quick Denature kit; Amersham, Piscataway, NJ).

Cell Culture. COS-7 cells were grown as previously detailed (Choudhary et al., 1993, 1995; Roth et al., 1997b) and transiently transfected with various receptor mutants using Fugene6 (Roche, Indianapolis, IN) in 100-mm dishes by scaling up the recommended procedure supplied by the manufacturer.

Binding Assays. Transiently transfected cells were switched from medium containing dialyzed serum to serum-free medium for 24 h before harvest to remove residual 5-HT and then harvested using a cell scraper as previously described (Roth et al., 1995, 1997b). Binding assays were performed using membrane preparations in a total volume of 1.0 ml using [^{3}H]ketanserin or [^{3}H]*N*-methylspiperone as the labeled ligand. For saturation-binding assays, six concentrations of labeled ligand spanning a range of 100-fold (typically 0.1–10 nM) were used. For competition-binding assays, six to 10 concentrations of unlabeled ligand spanning a range of 10,000-fold (typically 1–10,000 nM) were used. Agonist and antagonist competition-binding assays and saturation-binding assays were performed in a buffer of the following composition: 50 mM Tris-Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.1% ascorbic acid, pH 7.4. (Roth et al., 1995, 1997b) Typically, specific binding represented 90% of total binding with no more than 10% of the total counts bound. Data were analyzed using the LIGAND program (Munson and Rodbard, 1980) as previously detailed (Roth et al., 1997a,b) with differences in binding parameters analyzed using the *F* test.

Phosphoinositide Hydrolysis Assays. For measurements of [^{3}H]PI release, cells were loaded for 18–24 h with 1 Ci/ml [^{3}H]inositol in serum-free and inositol-free medium as previously described (Roth et al., 1997a,b). Measurements of PI hydrolysis were performed as previously detailed (Roth et al., 1984, 1997a,b). K_{act} and V_{max} values were determined using a nonlinear curve-fitting routine as previously described (Roth et al., 1997a,b).

Molecular Modeling of the Rat 5-HT_{2A} Receptor. A model of the transmembrane domain in the rat 5-HT_{2A} receptor was constructed using computer graphics, molecular mechanics, and molecular dynamics simulations. The MIDASPLUS and SYBYL (6.4 beta) programs (University of California, San Francisco, CA) were used for computer graphics. Molecular mechanics and molecular dynamics simulations were performed with the AMBER 4.1/5.0 programs (Oxford Molecular, Oxford Science Park, UK) using the all atomic force field (Cornell et al., 1995). All calculations were performed with a distance-dependent dielectric function ($\epsilon = r_{ij}$) and a 10-Å cutoff radius for nonbonded interactions. Energy minimizations of ligand-receptor complexes were performed by 500 steps of steepest descent minimization followed by conjugate gradient minimization until convergence with a 0.02-kcal mol^{-1} $Å^{-1}$ root mean square energy gradient difference between successive minimization steps.

Models of TM I to VII with standard α-helical geometries (PHI = $-65°$ and PSI = $-40°$) were constructed. Each helix was capped by acetamide at its N terminus and *N*-methyl-amide at its C terminus. Each of the seven helices were then energy minimized. The resulting energy-minimized structures were assembled into a seven TM bundle according to the projection map of the frog rhodopsin structure (Baldwin et al., 1997). Information about interhelical interactions proposed according to data obtained in site-directed mutagenesis experiments with various G-protein-coupled receptors was also utilized in the model building. According to the present mutation data, the longitudinal and rotational orientation of helix 5 was modified such that the F240[5.44] side chain pointed away from the binding site, the F243[5.47] and F244[5.48] side chains pointed into the

binding site, and the S239[5.43] side chain was placed at the interface to helix 4.

Molecular Modeling of Ligand-5HT$_{2A}$ Receptor Interactions. Energy minimizations of ligands excluding electrostatic interactions were performed by 500 steps of steepest descent minimization followed by conjugate gradient minimization until convergence with a 0.0001-kcal mol^{-1} Å^{-1} root mean square energy gradient difference between successive minimization steps. Electrostatic potentials around 5-HT, α-methyl (Me)-5-HT, 5-methyl-DMT, *N*-ω-methyl-5-HT, and (R)DOM were calculated at the HF/6–31G* level by using the GAMESS (October 31, 1996 version; Gordon Research Group, Ames, IA) and MOLDEN programs (Molden 3.6; Schaftenaar and Noordik, 2000) or by using the GAUSSIAN programs (Gaussian94, revision D.4; Gaussian, Inc., Carnegie, PA). Atomic point charges were projected from these potentials by using the RESP program of the AMBER program package.

Ligand-receptor complexes were constructed by using interactive computer graphics. 5-HT, α-Me-5-HT, 5-methyl-DMT, and *N*-ω-methyl-5-HT were placed with the nitrogen atom of the fused ring system close to S239[5.43] in helix 5 and the protonated amine moiety close to D155[3.32]. The tryptamines were placed such that the 5-OH substituent of the 5-HT analogs was positioned close to S159[3.36]. However, an alternative model for the α-Me-5-HT-receptor complex was also constructed in which the 5-OH substituent was close to the hydroxyl group of S239[5.43] and the α-methyl substituent was close to the F243[5.47] side chain (simulation 2). (R)DOM was placed into the binding pocket with its 2-methoxy substituent close to S159[3.36], its 5-methoxy substituent close to N343[6.55] (helix 6), and its protonated amine side chain close to D155[3.32].

All ligand-receptor complexes were refined by energy minimization, followed by 100 ps of restrained molecular dynamics simulation (temperature: 310 K) and energy minimization of the 100-ps structure. During the molecular dynamics simulations, positional restraints were placed on Cα atoms (1 kcal mol^{-1}Å^{-1}). Distance restraints on backbone hydrogen bonds between the NH moiety of residue i and the oxygen atom of residue i-4 (50 kcal mol^{-1}Å^{-1}, excluding the backbone hydrogen bond between S372 and N376 in helix 7), and distance restraints (50 kcal mol^{-1}Å^{-1}) on specific ligand-receptor hydrogen bonds were applied. In the case of α-methyl-5HT, the oxygen atom of the S239[5.43] side chain was restrained to the centroide of the 5-ring in the agonist during simulation 1, whereas a restraint between the 5-OH substituent of α-methyl-5HT and S239[5.43] was applied during simulation 2. Y370[7.43] has been demonstrated to be important for binding of serotonin and DOM, but not for the binding of α-methyl-5HT or bufotenine to the rat 5-HT$_{2A}$ receptor (Roth et al., 1997b). Therefore, we performed two additional simulations with serotonin and DOM in which a restraint was used to move the OH group of Y370[7.43] toward the protonated amine group of the agonist (serotonin, simulation 2: 5OH-S159 OG (*O*-O distance; r3 = 3.5 Å), indole NH-S239 OG (N-O; r3 = 3.5 Å), NH3+−D155 OD1/OD2 (N-O; r3 = 3.5 Å), NH3+−Y370 OH (N-O; r3 = 3.5 Å); DOM, simulation 2: 2-methoxy-S159 OG (*O*-O; r3 = 3.5 Å), 5-methoxy-N343 ND2 (*O*-N; r3 = 3.5Å), 5-methoxy-S159 OG (*O*-O; r3 = 4.5 Å), NH3+−D155 OD1/OD2 (N-O; r3 = 3.5 Å), NH3+−Y370 OH (N-O; r3 = 3.5 Å).

Results

Characterization of Mutant Receptor Expression. Saturation-binding isotherms were established for [^{3}H]ketanserin and [^{3}H]*N*-methylspiperone (NMSP) binding to wild-type and mutant receptors. [^{3}H]NMSP was used for the F243A mutant because ketanserin did not bind with high affinity to this mutant. A summary of K_d and B_{max} values for each mutant is shown in Table 1. Receptor expression among the transfectants was variable, ranging from a low of 574 ± 111 fmol/mg (F243A) to a high of 2367 ± 583 fmol/mg (F244A). Native receptors bound [^{3}H]ketanserin and [^{3}H]*N*-methylspiperone with affinities of 1.7 ± 0.29 and 0.78 ± 0.17 nM, respectively. S239A and F240A had slightly increased affinities for ketanserin (2.7- and 2.9-fold, respectively), whereas the F244A mutant showed a 3.1-fold decrease in binding affinity. The F243A mutant had a 7.7-fold lower affinity than the native receptor for [^{3}H]*N*-methylspiperone.

Binding Affinities for the Wild-Type and Mutant Receptors. We next measured the K_i values for eight structurally diverse agonists and antagonists at native and mutant receptors. Structures of the various compounds used in this study are presented in Fig. 1A. The relative position of each residue along a theoretical α-helix is presented in the helical net diagram in Fig. 1B. Binding affinities for the drugs tested at the various helix V mutants as well as the native receptor are summarized in Table 2. The F240A mutation had no significant effect on binding except for ritanserin, which displayed a 2-fold increase in binding affinity. The S239A mutant showed 6- to 13-fold decreases in binding affinity for 5-HT, α-Me-5HT, 5-methyl-DMT, and *N*-ω-methyl-5HT and 2- and 5-fold increases in binding affinity to ritanserin and DOI, respectively. The F244A mutant exhibited similar changes in ligand binding, ranging between 2.9- and 10.1-fold decreases in binding affinities for 5-HT, α-Me-5HT, 5-methyl-DMT, *N*-ω-methyl-5HT, and ketanserin but showing a 3.0-fold increase in binding affinity for ritanserin. There was no measurable difference in spiperone binding for the F244A mutant. The most striking alterations in ligand-binding affinities were seen with the F243A mutation, ranging from no effect (5HT and *N*-ω-methyl-5HT) to 48.7- and 216.6-fold reductions in binding affinities for α-Me-5HT and ritanserin, respectively.

Effects of S239A, F240A, F243A, and F244A Mutations on Agonist-Mediated PI Hydrolysis. We next examined the ability of the various 5-HT$_{2A}$ mutants to activate PI hydrolysis. None of the mutants altered the basal levels of PI hydrolysis, indicating that they did not alter the constitutive activity of the 5-HT$_{2A}$ receptor (not shown). The abilities of four different 5-HT$_{2A}$ agonists to stimulate PI hydrolysis in

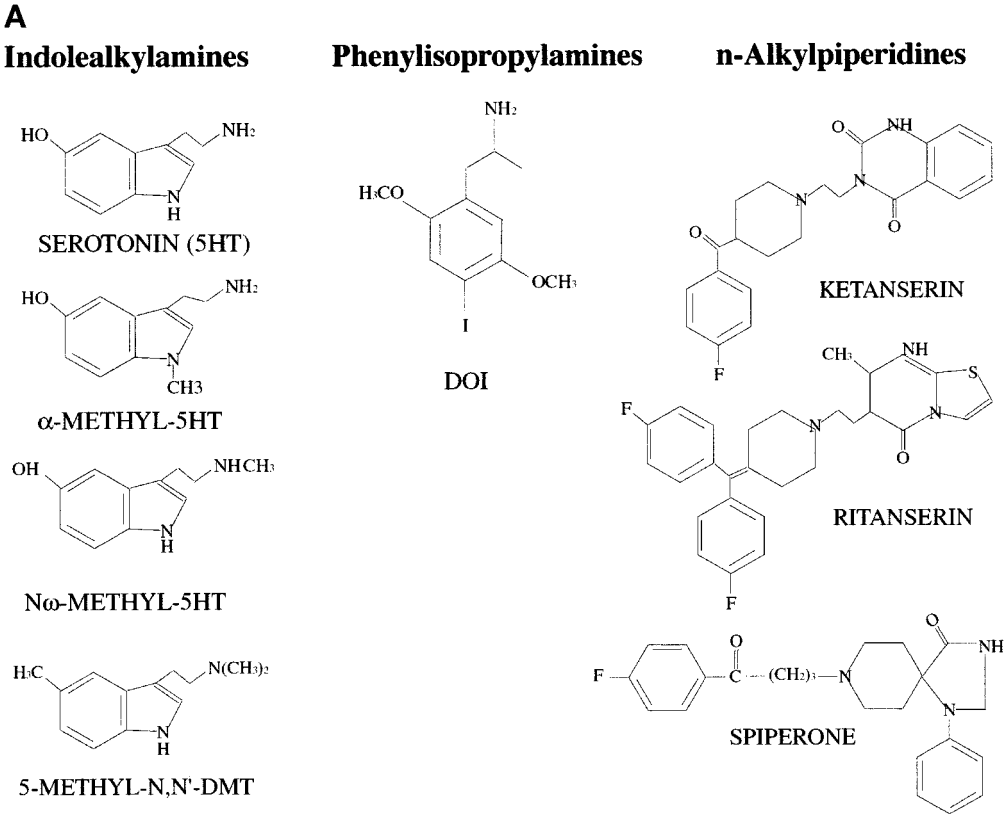
TABLE 1

[^{3}H]Ketanserin and [^{3}H]-*N*-methylspiperone binding by native and mutant 5-HT$_{2A}$ receptors

Data represent means ± S.E. for three separate experiments of mean K_d (nM) and B_{max} (fmol/mg protein). For the F243A mutant, [^{3}H]NMSP was used as the radioligand because of the low affinity of ketanserin for F243A. For all other mutants, [^{3}H]ketanserin was used. In a typical experiment, 90% of total binding was specific, and ≤10% of the total radioligand was bound.

	Drug	5-HT$_{2A}$	S239A	F240A	F243A	F244A
K_d (nM)	Ketanserin	1.7 ± 0.29	0.62 ± 0.21	0.58 ± 0.09		5.3 ± 2.0
B_{max} (fmol/mg protein)		1803 ± 143	1089 ± 125	1658 ± 85		2367 ± 583
K_d (nM)	NMSP	0.78 ± 0.17			6.0 ± 1.7	
B_{max} (fmol/mg protein)		713 ± 57			574 ± 111	

A



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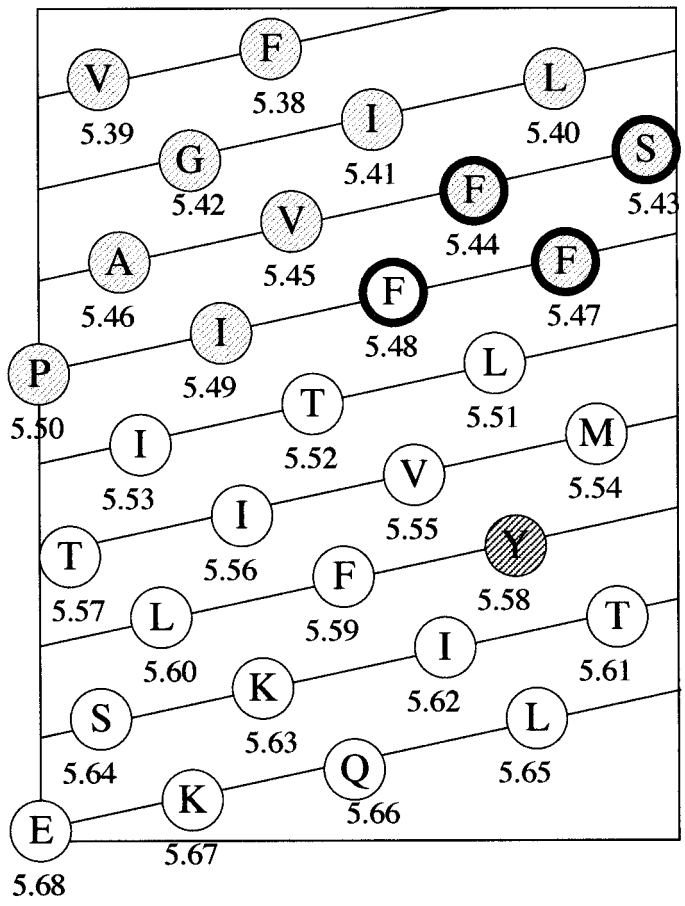

Fig. 1. Ligand structures and helical net diagram of TM V. Shown are the structure of ligands used in this study (A), and a helical net diagram of the relative orientations of residues within transmembrane region V in a theoretical α-helix (B). Residues evaluated in this study are highlighted in bold (S239, F240, F243, F244, corresponding to 5.43, 5.44, 5.47, 5.48, respectively). Homologous residues in the human D2 receptor shown to be accessible to MTSEA (Javitch et al., 1995) are cross-hatched.

cells with each receptor mutation are presented in Table 3, and a representative activation isotherm is shown in Fig. 2.

The K_{act} values for mutants S239A, F243A, and F244A ranged between 3- and 316-fold higher in these mutants than in wild-type cells. 5-HT and the other tryptamines appeared to be partial agonists for F243A and F244A, giving less than 70% of the maximum wild-type response (values ranged between 31 and 65%). α-Me5HT was similar to 5HT in its ability to promote PI hydrolysis. 5-methyl-DMT appeared to be a partial agonist of the F244A mutant but failed to significantly stimulate PI hydrolysis in the F243A mutant. PI hydrolysis in response to DOI was attenuated 216-fold by the

TABLE 2

Ligand affinity for the rat 5-HT_{2A} receptor and receptor mutants

Data represent means ± S.E. of K_i (nM) for three or more separate experiments. Eight different concentrations spanning 5 to 6 log U of test drug were used to displace 1 nM [^{3}H]ketanserin (WT, S239A, F240A, F244A) or [^{3}H]NMSP (F243A).

	K_i				
	5-HT_{2A}	S239A	F240A	F243A	F244A
			nM		
5HT	1,124 ± 239	8,500 ± 3,287[a]	773 ± 104	2,667 ± 817	11,348 ± 4,464[a]
α-Me-5HT	1,300 ± 207	8,950 ± 2,724[a]	947 ± 430	63,300 ± 15,616[a]	4,100 ± 992[a]
N-ω-Me-5HT	616 ± 75.0	3,800 ± 673[a]	667 ± 272	2,167 ± 481	2,000 ± 408[a]
5-Me-DMT	2,357 ± 219	31,575 ± 17,971[a]	2,450 ± 1,021	4,033 ± 1,934[a]	8,033 ± 3,888[a]
DOI	78.5 ± 25.6	15.6 ± 1.6[a]	44.4 ± 6.1	2.0 ± 0.58[a]	123.9 ± 36.8
Ketanserin	2.3 ± 0.42	1.5 ± 0.65	0.93 ± 0.32	10.4 ± 4.5[a]	6.7 ± 3.2[a]
Ritanserin	0.67 ± 0.24	0.21 ± 0.06[a]	0.32 ± 0.01[a]	145.1 ± 51.0[a]	0.22 ± 0.06[a]
Spiperone	0.71 ± 0.09	0.44 ± 0.09	0.52 ± 0.05	3.5 ± 1.1[a]	0.68 ± 0.12

[a] Significantly different from the wild type (5-HT_{2A}) control ($P < .05$, ANOVA).

TABLE 3

Ability of agonists to activate PI hydrolysis at native and mutant 5-HT_{2A} receptors

Data represent means ± S.E. of computer-derived estimates for K_{act} (nM) and V_{max} values that have been replicated at least three times. V_{max} values are expressed relative to 5-HT expression of native 5-HT_{2A} receptors transfected at the same time. For a typical experiment, basal activity ranged from 200 to 500 cpm, whereas the maximum stimulation elicited by 5-HT was 3000 to 5000 cpm. No significant effect of any of the mutations on basal PI hydrolysis was measured.

	5-HT_{2A}		S239A		F240A		F243A		F244A	
	K_{act}	V_{max}	K_{act}	V_{max}	K_{act}	V_{max}	K_{act}	V_{max}	K_{act}	V_{max}
	nM	%	*nM*	%	*nM*	%	*nM*	%	*nM*	%
5-HT	15.0 ± 1.3	95 ± 1.3	275 ± 41.2[a]	111 ± 2.8	7.9 ± 2.2	97 ± 4.0	3070 ± 1327[a]	44 ± 3.9[a]	110 ± 30[a]	65 ± 3.2[a]
α-Me-5HT	26.5 ± 5.1	85 ± 3.0	861 ± 97.3[a]	99 ± 2.1	20.2 ± 5.2	79 ± 3.6	8364 ± 2766[a]	31 ± 2.6[a]	178 ± 24[a]	61 ± 1.4[a]
5-Me-DMT	882 ± 219	62 ± 2.9	4566 ± 920[a]	87 ± 3.9	395 ± 81	77 ± 2.7	UD	UD	1311 ± 218	45 ± 2.0[a]
DOI	7.5 ± 2.4	95 ± 4.7	27.4 ± 9.1	80 ± 5.1	5.9 ± 2.6	95 ± 6.1	1623 ± 849[a]	49 ± 5.1[a]	23.3 ± 5.9	90 ± 4.5
DOI S(+)	8.3 ± 2.1	103 ± 4.4	32.7 ± 14.5	61 ± 5.9	9.2 ± 4.2	86 ± 13	ND	ND	24.6 ± 15.4[b]	104 ± 14
DOI R(−)	1.7 ± 1.5	87 ± 9.5	5.5 ± 7.2[b]	99 ± 19	1.6 ± 1.2	82 ± 7.5	ND	ND	9.3 ± 2.8[b]	111 ± 6

UD, undetectable (<10% activation); ND, not determined.
[a] Significantly different from the wild type (5-HT_{2A}) control ($P < .05$, ANOVA).

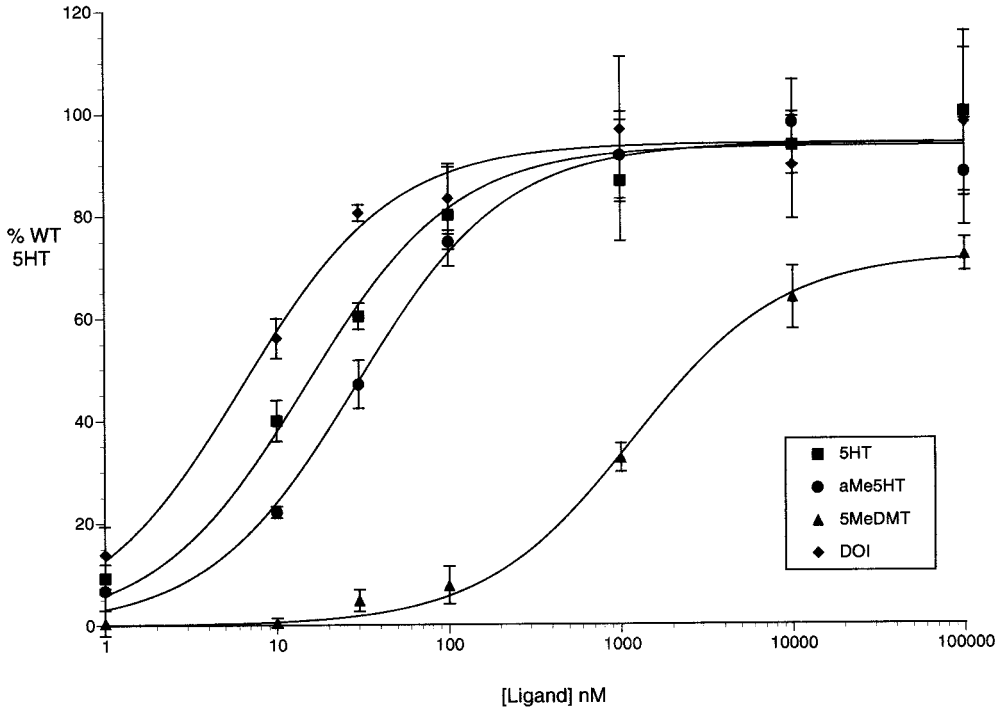

Fig. 2. Agonist-mediated PI hydrolysis in cells transfected with wild-type 5-HT_{2A} receptors. Data represent means ± S.E.M. (of triplicate determinations) for the percentages of stimulation of PI hydrolysis compared with the native rat 5-HT_{2A} receptors. Six log-serial dilutions of 5-HT were incubated in 24-well tissue culture plate wells containing transiently transfected COS-7 cells, and the accumulation of [^{3}H]inositol monophosphate was determined as described in *Materials and Methods*. Data are expressed as percentages of native receptor stimulation.

F243A mutation. The F240A and F244A mutations had no effect on PI formation with any of the agonists tested.

DOM Interacts with the Rat 5HT$_{2A}$ Receptor in a Manner Different from Serotonin. Figures 3 and 4 show the binding pockets of agonist-5HT$_{2A}$ receptor complexes obtained after energy minimizations and 100-ps molecular dynamics simulations. In all of these models, the aromatic ring of the agonist molecule was surrounded by V156[3.33], F243[5.47], F244[5.48], and F340[6.52]. However, subtle differences (described below) in the detailed receptor-ligand interactions for the structurally diverse agonists were also apparent.

The Y370[7.43] side chain was located in the proximity of the highly conserved D120[2.50] after simulation 1 and to D155[3.32] after simulation 2 with serotonin and DOM (Fig. 3). After simulation 1 with serotonin, the indole NH substituent was hydrogen bonded to the main chain carbonyl group of S239[5.43], and the protonated amine side chain interacted with the carboxylate of D155[3.32] and the side chain CO group of N343[6.55]. The 5-OH group interacted with both D155[3.32] and S159[3.36]. After simulation 2, the protonated amine side chain of serotonin interacted with the carboxylate group of D155[3.32] and the main chain carbonyl group of F365[7.38], and the 5-OH substituent interacted with S159[3.36], whereas the indole NH substituent interacted with the OH group of S239[5.43] after the second simulation.

After both simulations 1 and 2 with DOM, the primary amine side chain interacted with D155[3.32] and S159[3.36], whereas the 5-methoxy substituent was hydrogen bonded to the NH_2 group of N343[6.55]. The 2-methoxy substituent was close to the hydroxyl group of S159[3.36] (heteroatomic distance: 3.9 and 4.10 Å) but in unfavorable positions for formation of a hydrogen bond. After simulation 1, the 5-methoxy substituent was close to the S239[5.43] side chain (*O*-O distance, simulations 1 and 2: 3.8 and 6.1 Å).

N1-Unsubstituted Tryptamines and α-Methyl-5HT Differentially Interact with the Rat 5-HT$_{2A}$ Receptor. Figure 4 shows the binding pocket of agonist-receptor complexes with N1-unsubstituted tryptamine analogs and α-Me-

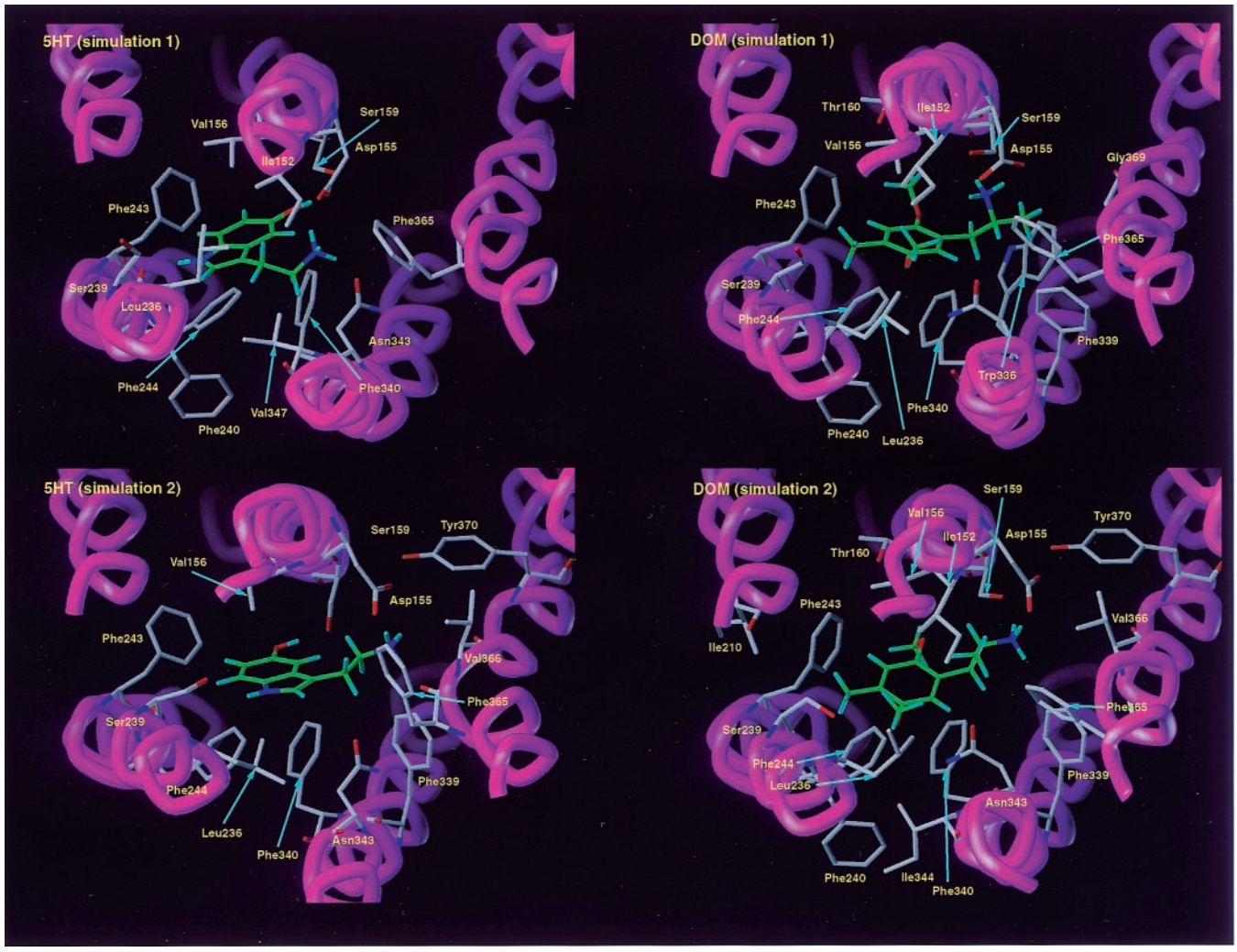

Fig. 3. Serotonin and DOM-binding pockets in the rat 5HT$_{2A}$ receptor. Views of the transmembrane helices (represented as tubes) from the extracellular side are shown after various molecular dynamics simulations. These views show the agonists 5-HT or DOM and all residues within a 3.5-Å sphere radius of the agonist molecule in energy minimized complexes after 100-ps molecular dynamics simulations. The left panels represent simulations obtained with 5-HT, and the right panels represent simulations obtained with DOM. The top section represents simulation 1, and the bottom panel represents simulation 2. Color coding of ligand and receptor atoms: red, O; green, N; cyan, H; white, C in the receptor; green, C in the ligand.

5HT obtained after energy minimizations and 100-ps of molecular dynamics simulations. The NH group of the unsubstituted tryptamines interacted as a hydrogen bond donor with the OH group of S239[5.43]. As seen with the 5HT-receptor model, the secondary amine side chain of *N*-ω-methyl-5HT interacted with both the carboxylate group of D155[3.32] and the side chain CO group of N343[6.55]. In contrast, the tertiary amine side chain of 5-methyl-DMT interacted with the carboxylate group of D155[3.32] only. The 5-OH substituent of *N*-ω-methyl-5HT was hydrogen bonded between the hydroxyl groups of S159[3.36] and T160[3.37].

The second set of panels in Fig. 4 shows the binding pocket of two α-methyl-5HT-receptor complexes featuring different agonist orientations. After simulation 1, the N1-methyl substituent pointed in the direction of helix 5, whereas the 5-OH substituent was hydrogen bonded between S159[3.36] and T160[3.37]. The OH group of S239[5.43] was located above the α-nitrogen atom (*O*-N distance: 3.8 Å). After simulation 2, the α-methyl substituent and the tryptamine ring were close to the F243[5.47], whereas the OH group of S239[5.43] was hydrogen bonded to the 5-OH substituent of the agonist. The protonated amine group was hydrogen bonded to the carboxylate of D155[3.32] and the side chain CO group of N343[6.55] after both simulations.

Discussion

Very little is known about the complex ligand-receptor interactions that regulate 5-HT receptor actions, primarily because no direct structural information is currently available regarding any details of the receptor or the ligand-receptor complexes. The best characterized receptor belonging to family A of G-protein-coupled receptors is rhodopsin for which electron density maps and models to approximately 6 Å in the plane of the membrane have been published (Baldwin et al., 1997). This level of resolution has been insufficient to provide a detailed model for binding pocket interactions. Furthermore, these electron density maps represent the structure of bovine and frog rhodopsin in the inactive state. Thus, although these models provide the only available scaffolds for the development of rat 5-HT_{2A} receptor models, there will no doubt be significant structural and

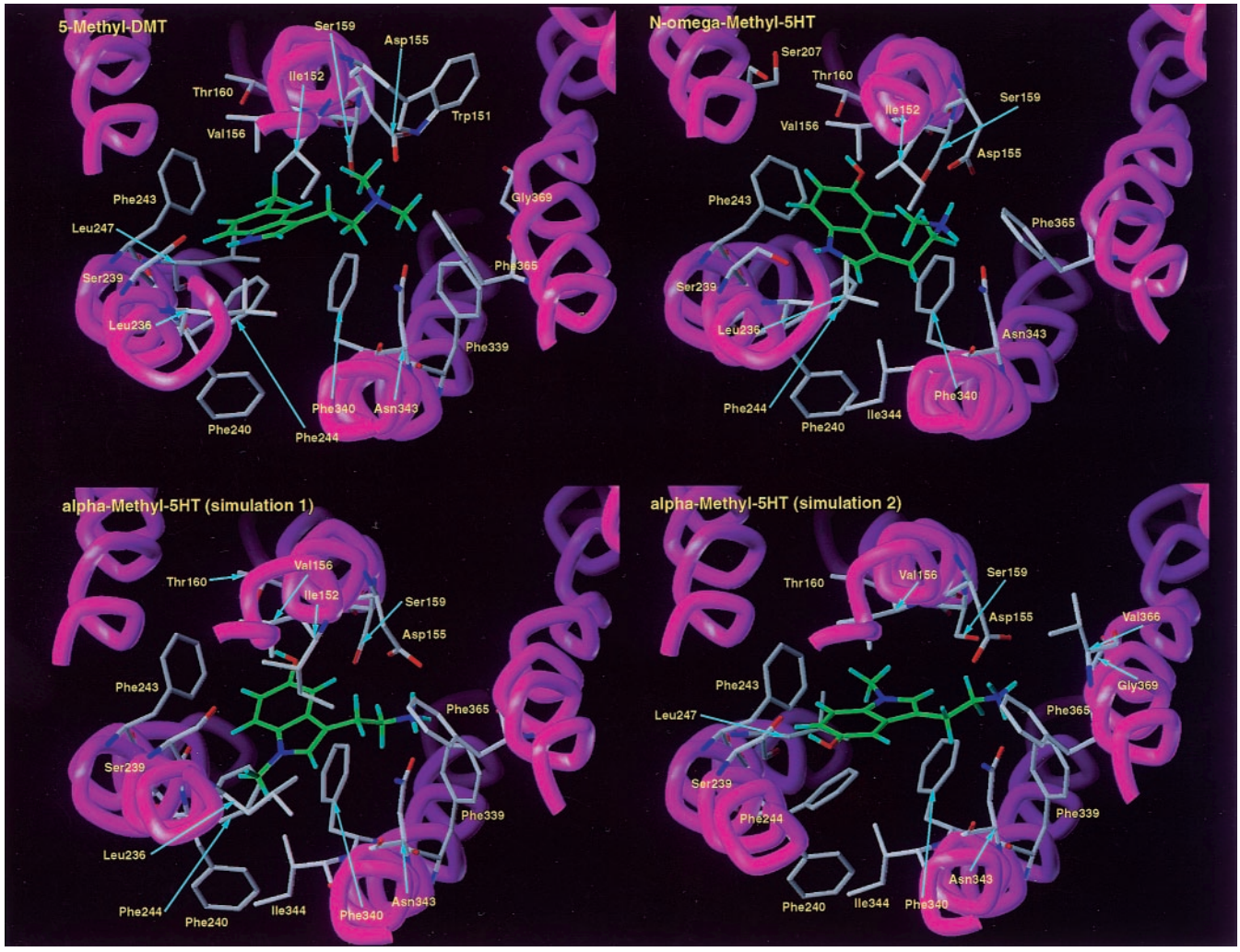

Fig. 4. Molecular modeling and site-directed mutagenesis reveal differential binding of α−methyl 5HT to the 5-HT_{2A} receptor. Views (from the extracellular side of the receptor) of the transmembrane helices (represented as tubes), showing various agonists (green) and all residues within a 3.5-Å sphere radius of the agonist molecule in energy-minimized complexes after 100-ps molecular dynamics simulations. The top two panels show the simulated binding of 5-methyl-DMT and *N*-ω-methyl-5HT. The bottom two panels show two views of the simulated binding of α-Me-5HT to the 5-HT2A receptor after two different molecular dynamics simulations (see *Results* and *Discussion* for details). Color coding of ligand and receptor atoms: red, O; green, N; cyan, H; white, C in the receptor; green: C in the ligand.

functional species differences in both the inactive and activated states.

As described below, our accumulated data suggest that minor changes in ligand structure have variable effects on the apparent orientations of bound ligands. These results could not have been predicted based on the assumptions made with previous models using similar drug structures. The resulting apparent differences in ligand-receptor interactions and the changes in ligand binding and receptor activation caused by substituting drugs differing by as little as one methyl group are likely to be of tremendous importance for structure-based drug design.

Structure of Transmembrane Region 5. Because our prior rat 5-HT_{2A} receptor model (Kristiansen et al., 2000) could not account for all effects of the present mutation data in helix 5, an updated model was constructed. In this new model, F243[5.47] and F244[5.48] are pointing into the binding site, F240[5.44] is pointing toward the membrane, and S239[5.43] and A242[5.46] are pointing more in the direction of helix 4. In a regular α-helix, there is a 200° arc between the F244[5.48] and A242[5.46] side chains, making it impossible to place both simultaneously into the binding pocket. Theoretically, the presence of a highly conserved proline in transmembrane helix 5 (P246[5.50]) might lead to a partial unwinding of the helix in the region containing S239[5.43] and A242[5.46]. A very extreme unwinding for the corresponding region of helix 5 (F189–F198) in the human dopamine D_2 receptor has been proposed from experiments in which the substituted cysteine accessibility method has been used (Javitch et al., 1995). Alternatively, binding of ligands may influence the rotational orientation of helix 5 by specifically interacting with residues on different faces of the helix as supported by data from the human α_{2A} adrenergic receptor (Marjamäki et al., 1999). The finding that A242[5.46] was inaccessible for ligand binding in the present models was supported by the lack of effect of the A242S and A242T mutations in the rat 5-HT_{2A} receptor on binding of ketanserin, DOI, and serotonin (Johnson et al., 1994). However, the Ser/Ala difference between human 5-HT_{2A} and 5-HT_{2C} receptor (Almaula et al., 1996a) and between rat and human 5-HT_{2A} receptors (Kao et al., 1992; Johnson et al., 1994) have been identified as a major determinant for the subtype specificity of N-1-substituted ergolines, such as mesulergine.

Role of Aromatic Residues in Receptor Folding, Ligand Binding, and Signal Transduction. In the absence of a crystal structure for the receptor, structure activity relationships, coupled with molecular modeling, have been used to define the three-dimensional binding interactions between receptor and ligand (Kristiansen et al., 1993; Westkaemper and Glennon, 1993; Choudhary et al., 1995; Holtje and Jendretzki, 1995; Kristiansen and Dahl, 1996). Many of these models suggest the presence of key aromatic residues, including W200[4.50], F340[6.52], W336[6.48], W367[7.40], and Y370[7.43], which account for much of the binding affinity and specificity of the receptor. Mutation of these positions caused a markedly reduced agonist affinity and efficacy at 5-HT_{2A} receptors (Choudhary et al., 1995; Roth et al., 1997b), whereas mutation of other aromatic residues (e.g., F365[7.38]), predicted to be near the binding pocket, had little or no effect on agonist affinity (but did diminish agonist efficacy; Roth et al., 1997b). Mutation of other, nonconserved residues (F125[2.55], M132[2.62], and T134[2.64]) had no significant effect on agonist- or antagonist-binding affinity.

In the present analysis, F243[5.47], F244[5.48], F332[6.44], W336[6.48], F339[6.51], and F340[6.52] are predicted to form one large cluster of aromatic residues between the helices. Among these residues, only F332[6.44] and W336[6.48] are highly conserved within family A of the GPCRs. All biogenic amine receptors have aromatic side chains corresponding to F243[5.47], F244[5.48], F332[6.44], W336[6.48], and F339[6.51] and N (all muscarinic receptors) or F (all other biogenic amine receptors) corresponding to F340[6.52]. Although the nonconserved F240[5.44] side chain was located in the cluster at the extracellular edge, its side chain pointed away from the binding site, toward the membrane. Previous reports have demonstrated the importance of several of these aromatic residues surrounding the serotonin-binding pocket, including W200[4.50], W336[6.48], F339[6.51], F340[6.52], W367[7.40], and Y370[7.43] (Choudhary et al. 1995; Roth et al., 1997b).

Our results build on the growing body of research that suggests that these aromatic residues stabilize bound ligand by providing π-π stacking interactions, structural stabilization, or both. Figures 3 and 4 contain a composite of the energy-minimized, agonist-bound receptors, based on 100-ps molecular dynamics simulations. The stabilizing influence of the aromatic core of the binding pocket is most clearly evidenced in the 5-HT, 5-methyl-DMT, *N*-ω-Me-5HT, and the DOM/DOI panels. In the first three, residues F243[5.47], F244[5.48], F340[6.52], and F365[7.38] surround the indole ring of the ligand, whereas W336[6.48] may also participate in core stabilization with bound DOM. Notably, F240[5.44] is oriented out of the binding pocket in all models. The aromatic residues interact not only with ligand but with other core residues as well. For example, because of their relative orientations, it is likely that F244[5.48] and W336[6.48] are important for keeping F340[6.52] in the proper orientation and position for stabilizing interactions with the indole ring of the indolealkylamines.

The present mutational data suggest that both F243[5.47] and F244[5.48] interact specifically with agonists and antagonists. The detailed interactions with F243[5.47] and F244[5.48] varied among the structurally diverse compounds investigated. Site-directed mutagenesis experiments with one other biogenic amine receptor showed that the F corresponding to F243[5.47] specifically interacts with agonists (Cho et al., 1995; Javitch et al., 1995). However, the direct involvement of F244[5.48] in ligand binding and signal transduction has not yet been reported for any other biogenic amine receptor (Wetzel et al., 1996).

The Rat 5HT_{2A} Receptor Interacts Differently with Phenylisopropylamines than with Tryptamines. According to the present modeling, (R)DOM was located closer than the tryptamines to helix 6, and the hydrophobic part of the protonated side chain had vdW contacts with F339[6.51] and W336[6.48] after simulation 1. The F243[5.47] residue was located close to the 2-methoxy group of DOM and may, therefore, sterically interfere with formation of a hydrogen bond between 2-methoxy in DOI/DOM and the hydroxyl group of S159[3.36]. This hypothesis is in agreement with the 40-fold increase in DOI binding observed for the F243A mutation. The 5-fold increase in affinity of DOI observed for the S239A mutation could be due to a direct vdW interaction

between the A239 side chain and the 5-methoxy substituent (simulation 2) or indirect structural effects on the binding pocket.

Distinct Binding Modes for α-Methyl-5HT and the N1-Unsubstituted Tryptamines. Based on prior molecular dynamics simulations, the agonist-binding site was suggested to occupy the same general location inside the helix bundle for structurally diverse agonists. However, results from the most recent molecular dynamics simulations and mutagenesis experiments suggest the existence of different binding modes for N1-unsubstituted tryptamines and α-methyl-5HT.

In general, the indole NH group in N1-unsubstituted tryptamines was predicted to interact with the S239[5.43] side chain as a hydrogen bond donor. This observation was supported by the fact that the S239A mutation decreases the binding of 5-methyl-DMT more than the binding of tryptamines having a 5-OH substituent. The lack of a 5-OH group, which might form additional compensating hydrogen bonds in the receptor, might lead to particularly high sensitivity for the S239A mutation for this compound. Similar Trp-Ser interactions are observed in many protein structures where the side chain OH group of Ser prefers to act as a hydrogen bond acceptor in interactions with the NE1 atom of Trp (Samanta et al., 2000).

According to the present modeling and mutagenesis data, α-methyl-5HT might interact in a different manner with the binding site than N1-unsubstitued tryptamines. The lack of a hydrogen bond donor at the N1 position caused the α-methyl-5HT molecule to bind in a different orientation compared with N1-unsubstituted tryptamines. In the present models, it is also possible that the OH group of S239[5.43] is hydrogen bonded to the 5-OH group of α-methyl-5HT and that the F243[5.47] side chain is located in close proximity to both the α-methyl substituent and the tryptamine ring (simulation 2).

The presence of a methyl group in the 5 position appears to shift the agonist molecule upward (toward the extracellular side) and away from the F243[5.47] residue. This hypothesis is also supported by the fact that the mutation F243A causes only a minor 1.7-fold reduction in the binding affinity of 5-methyl-DMT.

An alternative model suggests that the OH group of S239[5.43] forms an OH-aromatic interaction with the indole ring of α-methyl-5HT (simulation 1), although this interaction seems to be difficult to fulfill in the context of the present model. The substantial decrease in affinity of α-methyl-5HT by the F243A and S239A mutations suggests that the interaction between the indole ring of the agonist and the F243[5.47] side chain places the agonist in a position where an interaction between the S239[5.43] OH group and a hydrogen-bonding group in the agonist could take place.

According to the present simulations, the protonated amine of tertiary amine agonists may interact only with the side chain carboxyl group of D155[3.32]. In contrast, the protonated amine group of agonists that are primary or secondary amines may, in addition, interact with other polar residues, such as the OH of S159[3.36], the side chain CO of N343[6.55], and the OH of Y370[7.43]. In the case of agonists that are tertiary amines, the steric accessibility of the protonated amine side chain is restricted such that only one hydrogen-bonding partner is allowed. Mutation data for the human 5-HT_{2A} receptor have previously demonstrated that the binding of primary amines is more reduced than the binding of tertiary amines by the S159A and S159C mutations (Almaula et al., 1996b). The present simulations suggest that the 5-OH group of tertiary amines may, instead, interact with the OH groups of S159[3.36] and/or T160[3.37], which is in accordance with data from Almaula et al. (1996b). The importance of hydrogen bonding to ligand stabilization is evidenced by the numerous proposed interactions with S159[3.36], T160[3.37], D155[3.32], S239[5.43], and N343[6.55]. Similar results for the S239A mutant were obtained in a previous report (Johnson et al., 1997) in which the rat 5-HT_{2A} receptor was shown to have a 10-fold decrease in binding affinity to serotonin (but not DOI) on Ala substitution at S239[5.43]. Our results support this finding, where 5-HT showed an 8-fold decrease in binding affinity at the same position. It is unknown why the DOI data are dissimilar.

It is particularly interesting to note that the mutations at each residue did not always lead to positive correlations between ligand-binding and receptor activation data. In particular, DOI at the F243A mutation experienced a 40-fold increase in binding affinity with an accompanying 216-fold decrease in the ability to stimulate PI hydrolysis. At this position, 5-HT binding was virtually unaffected, whereas receptor activation experienced a dramatic 200-fold decrease in efficiency. 5-Methyl-DMT binding decreased only slightly at the F243A mutation while completely uncoupling receptor activation, yielding no detectable PI hydrolysis. In contrast, α-Me-5HT-binding and G-protein-coupling results were in strong agreement at F243A. These data suggest that changes in ligand binding within the central core can have significant changes in structural features located at a considerable distance from the binding interactions (in the case of G-protein coupling to the 5-HT_{2A} receptor, these changes take place at residues within the third intracellular loop). Additional perturbations in the loop structure could arise from the availability of "cavities" because of the loss of a bulky aromatic side chain.

One possible explanation for the differential effects of various mutations on ligand binding and efficacy is that the isomerization states of the receptor that lead to G-protein activation are selectively altered. To address this possibility we examined the basal PI hydrolysis activity of the various mutant and native receptor constructs. No change in basal activity was measured, suggesting that none of the mutants resulted in constitutive activation or inactivation of a spontaneously isomerizing, constitutively active state of the receptor. Importantly, our recent studies of another series of mutations at the D155 locus (Kristiansen et al., 2000) demonstrated that mutations that directly impair signal transduction lead to lower levels of basal activity.

The results of this study demonstrate the importance of highly conserved residues in transmembrane region 5 for agonist binding and efficacy at the rat 5-HT_{2A} receptor. In the absence of crystallographic structural data, these models remain our best guides to the development of novel clinical therapies for the treatment of psychiatric disorders involving the use and regulation of serotonin receptors. These results also demonstrate that, although the interactions of a set of diverse ligands with a single receptor are complex, they are amenable to experimental analysis. Furthermore, our results demonstrate that molecular predictions can be made regarding differential modes of ligand binding and receptor activation.

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