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Molecular Basis of Partial Agonism: Orientation of Indoleamine Ligands in the Binding Pocket of the Human Serotonin 5-HT2A Receptor Determines Relative Efficacy

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ABSTRACT

Based on experiment and computational simulation, we present a structural explanation for the differing efficacies of indole agonists at the human serotonin 5-HT2A receptor (5HT2AR). We find that serotonin [5-hydroxytryptamine (5-HT)] forms hydrogen-bonds with Ser3.36 in helix 3 and Ser5.46 in helix 5. Disruption of these hydrogen bonds by methyl-substitution of the cationic primary amine or of the backbone N1-amine, respectively, leads to a reduction in agonist efficacy. Computational simulation predicts that mutation of Ser3.36 to Ala should allow a similar interaction with helix 3 both for agonists that have unmodified cationic amine side chains and for those with substituted amines. Experimentally, this mutation was found to largely eliminate the differences in efficacy caused by cationic amine substitution for a series of indole congeners. Similarly, substitution of the N1-amine, which interacts with Ser5.46, reduced efficacy more markedly at the wild-type (WT) than at the Ser5.46Ala mutant receptor. Computational modeling of binding pocket interactions of ligands with WT and mutant receptor constructs demonstrate how the Ser3.36 and Ser5.46 interactions serve to modify the agonist's favored position in the binding pocket. A striking correlation was found between differences in the position assumed by the indole ring and differences in agonist activity. These data support the hypothesis that the position of the agonist interacting with the receptor is influenced by specific interactions in helices 3 and 5 and determines the degree of receptor activation by agonist through a mechanism that is likely to be shared by other G-protein coupled receptors in this class.

Structure-function studies in many G protein-coupled receptors have identified ligand docking sites that are used differentially by specific agonists (for some reviews see (Ballesteros and Weinstein, 1995; Portoghese, 2001; Shi and Javitch, 2002; Visiers et al., 2002a). We showed that in binding to the 5-HT2AR, the charged amine side chain of 5-HT interacts with both Asp3.32 and Ser3.36, but that *N*,*N*-dimethyl 5-hydroxytryptamine (*N*,*N*-dMe-5-HT) and *d*-lysergic acid diethylamide (LSD) do not interact with Ser3.36 because the substituted amine produces a steric clash (Almaula et al., 1996b). Such differences in the mode of interaction of various ligands in the receptor binding pocket affect not only affinity (Almaula et al., 1996b) but can also change the manner in which the ligand is docked in the receptor. The 5HT2AR is ideally suited for probing the pharmacological consequences of such changes in the binding pattern of ligands and the specific molecular mechanisms by which ligand-receptor interactions can influence efficacy, because of the availability of a series of structural congeners that vary in maximal response they can elicit relative to that of 5-HT (E^r_{max}). We have previously reported that the partial agonists *N*,*N*-dMe-5-HT and LSD have reduced efficacy relative to 5-HT largely because only the amine group of 5-HT can interact favorably with Ser3.36 (Almaula et al., 1996b). These observations suggest that partial agonism can result from alterations in a specific mode of docking the ligand in the receptor. In the present study, we demonstrate that this is a general phenomenon for indoleamine agonists and explore the underlying molecular mechanism whereby altered positioning is translated into altered efficacy. The results provide the basis for proposing a structure-based mechanistic hypothesis for the molecular mechanism of partial agonism. This hypothesis is supported by data collected specifically with the 5-HT2AR, but it involves conserved structural motifs (Visiers et al., 2002a) that are likely to make the findings applicable to many neurotransmitter receptors.

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B.J.E. and I.V. contributed equally to this study

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; WT, wild-type; IP, inositol phosphate; 5HT2AR, 5-HT2A receptor; RMSD, root-mean-square difference value; E^r_{max}, maximal response relative to that of 5-HT; Me, methyl; dMe, dimethyl; MeO, methoxy; TRYP, tryptamine; LSD, *d*-lysergic acid diethylamide.

Materials and Methods

Residue Numbering Scheme. Receptor residues are numbered according to a consensus numbering scheme described previously (Ballesteros and Weinstein, 1995). For the 5HT2AR used in this study, Ser159 in helix 3 is Ser3.36 and Ser242 in helix 5 is Ser5.46.

Cell Lines and DNA Constructs. Constructs for the WT and mutant receptors were prepared as described previously (Sealfon et al., 1995). The inserts were subcloned from pAlter (Promega, Madison WI) by *Sma*I-*Xba*I digestion. For expression, the insert was subcloned into the *Eco*RV and *Xba*I sites of pcDNA3 (Invitrogen, Carlsbad, CA). The mutations were confirmed by sequencing the mutation site in the expression vector. For radioligand binding studies, COS-1 cells were transiently transfected with WT, Ser3.36Ala, and Ser5.46Ala mutant 5HT2AR constructs as described previously (Ebersole and Sealfon, 2002). For functional studies, stable cell lines were derived by transfection of human embryonic kidney 293 cells as described previously (Ebersole and Sealfon, 2002).

Radioligand Binding. [^{3}H]Ketanserin saturation and competition binding assays were carried out with membranes prepared from transfected COS-1 cells, and data were analyzed as described previously (Ebersole and Sealfon, 2002). Competition binding data were fit to a one-site model for reasons we have described previously (Almaula et al., 1996a). For those experiments in which the slope factors were less than unity, fit to a two-site model in general did not improve the fits. The addition of guanine nucleotides to the incubation mixtures did not change the slope factors of those competition curves with slope factors of less than 1, which suggests that the presence of interconverting affinity states is not the reason for shallow slope factors. Therefore, the K_i values reported are apparent K_i values to facilitate comparison of ligand affinities when different concentrations of radioligand were used.

Phosphatidylinositol Turnover. The accumulation of [^{3}H]inositol phosphates ([^{3}H]IP) was measured as described previously (Ebersole and Sealfon, 2002). The response to all ligands in all lines was linear for at least 20 min; therefore, assays were routinely carried out for 18 min. Because the response in WT cells was linear for at least 45 min, measurements of E_{max} for low-efficacy ligands (E^r_{max} <0.2) were determined at 30 min and were compared with results obtained for 5-HT at 30 min. E^r_{max} was calculated as (E_{max} drug)/(E_{max} 5-HT) in that experiment. The EC_{50} of 5-HT did not change as a result of the longer incubation. Baseline accumulation of [^{3}H]IP in the absence of added drug was subtracted and analysis performed as described previously (Ebersole and Sealfon, 2002).

Computational Methods. The protocol for constructing the three-dimensional model of the transmembrane bundle of the 5HT2AR based on the rhodopsin structure (Palczewski et al., 2000) template has been described previously (Ballesteros and Weinstein, 1995; Visiers et al., 2002a). The ends of all the transmembrane helices and their relative orientation were determined as described previously (Ballesteros and Weinstein, 1995; Ballesteros et al., 1998; Visiers et al., 2002a). Constraints include experimental data for ligand interaction at positions Asp3.32 (Wang et al., 1993) and Ser3.36 (Almaula et al., 1996b), the orientation of the conserved Arg-cage (Ballesteros et al., 1998; Shapiro et al., 2002; Visiers et al., 2002b) and the aromatic cluster in TM6 (Javitch et al., 1998; Visiers et al., 2002a), data from cysteine scanning mutagenesis (for review, see Ballesteros et al., 2001), fluorescence microscopy (Gether et al., 1995), spin labeling (Yang et al., 1996), and the rhodopsin crystal structure (Palczewski et al., 2000). Initial positions of the explicit internal waters included in the model were obtained from a cavity-biased grand canonical ensemble Monte Carlo simulation (Mezei, 1980, 1987, 1989) using the program MMC (http://inka.mssm.edu/~mezei/mmc). Bound waters were identified by chemical potential (Mezei and Guarnieri, 1998), which was tuned first to reproduce the experimental density in the "bulk" water beyond the 5-Å layer surrounding the receptor immersed in a large water reservoir. A final run of 106 MC step length was used to extract 10 equally spaced configurations and the external waters were stripped away, yielding an average of 50 waters per receptor.

5-HT was positioned such that the protonated nitrogen of the 5-HT side chain hydrogen bonds to both Asp3.32 (Wang et al., 1993) and Ser3.36 (Almaula et al., 1996b) in helix 3, the indole moiety of 5-HT interacts with the aromatic ring of Phe6.52 in helix 6 (Roth et al., 1997), as described recently (Visiers et al., 2002a), and the indole nitrogen interacts with Ser5.46 (Almaula et al., 1996a). The resulting initial orientation of the ligand in 5HT2AR binding pocket is the same for all ligands used in the computational simulations. The initial position of the ligands was relaxed by a short energy minimization followed by a molecular dynamics simulation at 300 K, initially constrained with a force constant (K) of 1 (in kcal/mol Å^2) that is released in three equilibration steps of 50 ps until K = 0 is reached. A new 50-ps equilibration preceded a production run of 300-ps during which no constraints were applied to the ligand and a K = 0.02 was applied to the backbone α carbons. The average of the last 50 ps was calculated after 300 and 600 ps of the production run, minimized, and used in further comparisons. Conformational differences are reported as Root Mean Square Difference value (RMSD). Simulations were done with CHARMM (Brooks et al., 1983) and CHARMM22 parameter sets (Mackerell Jr. et al., 1998).

Sources of Chemicals. 4-HT, 1-*N*-Me-5-HT, and 1-*N*-Me-TRYP were from Sigma/RBI (Natick, MA) as part of the NIMH Chemical Synthesis Program (contract N01MH30003). LSD, 2-bromo-LSD, *N*,*N*-dMe-4-HT were from the National Institute on Drug Abuse (Bethesda, MD). *N*-Me-5-MeOT was from the National Institute of Mental Health (Bethesda, MD). Other drugs were from Sigma/RBI.

Results

Effects of Ser3.36Ala and Ser5.46Ala Mutations on Ligand Affinity

The structures and abbreviations used for the ligands are shown in Fig. 1. The affinities of the ligands for the WT and mutant receptors were determined in membranes prepared from transiently transfected COS cells. Mutation of either Ser3.36 or Ser5.46 to Ala had little effect on the affinity of the antagonist [^{3}H]ketanserin [for WT, K_d = 0.84 nM; for Ser3.36Ala, K_d = 1.1 nM (Almaula et al., 1996b); for Ser5.46Ala, K_d = 0.49 nM (Almaula et al., 1996a], but the effects on agonists varied:

Ser3.36Ala. The effect of Ser3.36Ala mutation on binding affinity was greatest for ligands with an unsubstituted cationic nitrogen. Thus, 5-HT, 5-MeOT, 4-HT, and TRYP showed an 8- to 20-fold decrease in affinity for the mutant receptor (Table 1). In contrast, the affinities of ligands with di-substituted or ring-embedded cationic nitrogens for the mutant receptor changed only slightly. This was striking among the ergoline compounds, where affinities changed 2.2-fold or less. Within a congeneric series, changes in affinity of mono-substituted ligands (*N*-Me-5-HT, *N*-Me-5-MeOT, and *N*-Me-TRYP) were intermediate to those of the unsubstituted and disubstituted ligands, suggesting a reduced but significant ability to interact with Ser3.36 in the WT receptor.

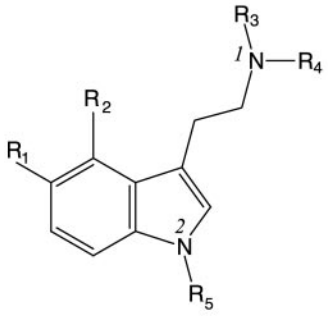
Ser5.46Ala. 5-HT and TRYP showed a 4-fold decrease in affinity for the Ser5.46Ala mutant receptor (Table 2), as would be predicted from loss of a favorable hydrogen-bonding interaction with Ser5.46. In contrast, 1-*N*-Me-5-HT and 1-*N*-Me-TRYP, whose N1-substitution would preclude hydrogen bonding with Ser5.46 in the WT receptor, showed an approximately 2-fold increase in affinity for the Ser5.46Ala mutant.

Effects of Ser3.36Ala and Ser5.46ala Mutations on Pharmacological Efficacy

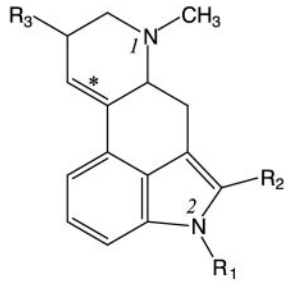
The elimination of the Ser3.36 or Ser5.46 side chains was predicted to lead to a differential repositioning of unsubstituted and substituted agonists in the binding pocket (see computational simulations below) so that their final orientation is more similar than in the WT receptor. If the positioning of a ligand influences the degree of receptor activation, then the Ser-to-Ala mutations should reduce the differences in relative efficacy of substituted and unsubstituted congeners. This predicted role of the interaction with Ser3.36 or Ser5.46 in determining the comparative ability of agonists to activate the receptor was evaluated by measuring the accumulation of [^{3}H]IP in cells stably expressing similar levels of WT or mutant receptors. B_{max} values derived from [^{3}H]ketanserin saturation binding studies for WT, Ser3.36, and Ser5.46 were 310 ± 53, 378 ± 73, and 249 ± 60 fmol/mg protein, respectively (mean ± S.E., n = 3–7 determinations).

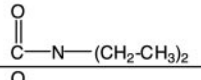
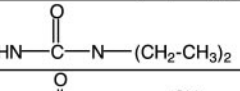
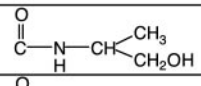
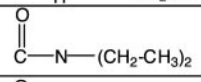
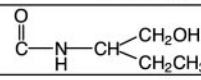
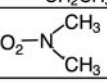
Ser3.36Ala. The E^r_{max} of all mono- and di-substituted tryptamine-related ligands tested increased as a result of mutation (Table 3). For example, in cells expressing WT receptor, *N*,*N*-dMe-5-HT was a partial agonist with E^r_{max} of 0.19 (Fig. 2A). However, in cells expressing the Ser3.36Ala receptor, the E^r_{max} of *N*,*N*-dMe-5-HT was greatly increased to 0.93 (Fig. 2B).

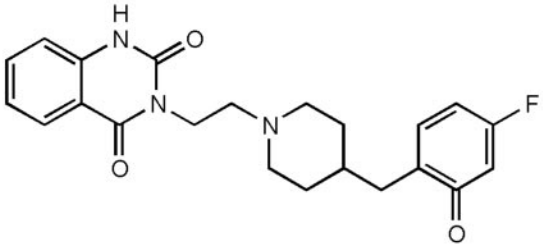
Some partial agonists at the WT 5HT2AR, such as 4-HT, 5-MeOT, and TRYP, have unsubstituted amines in their side chains (Fig. 1). The larger effect of mutation of Ser3.36 on the affinity of these ligands in comparison with their amine-substituted congeners (Table 1) is consistent with an interaction in the WT between Ser3.36 and the amine of these agonists. Therefore, the basis for their lower E^r_{max} at the WT receptor must be different from that of the congeners with substituted amine side chains, such as *N*,*N*-dMe-5-HT. Because mutation of Ser3.36 would be expected to have a similar effect on the positioning relative to helix 3 for all ligands with unsubstituted side chain amines, their E^r_{max} should remain comparable. This prediction is verified by the experimental results, showing that the E^r_{max} of the unsubstituted ligands TRYP, 5-MeOT, and 4-HT in the Ser3.36Ala mutant



	R1	R2	R3	R4	R5
Tryptamine (TRYP)	H	H	H	H	H
N-Me-TRYP	H	H	CH3	H	H
N,N-dMe-TRYP	H	H	CH3	CH3	H
1-N-Me-TRYP	H	H	H	H	CH3
5-OH-tryptamine (5-HT)	OH	H	H	H	H
N-Me-5-HT	OH	H	CH3	H	H
N,N-dMe-5-HT	OH	H	CH3	CH3	H
1-N-Me-5-HT	OH	H	H	H	CH3
5-methoxy-tryptamine (5-MeOT)	OCH3	H	H	H	H
N-Me-5-MeOT	OCH3	H	CH3	H	H
N,N-dMe-5-MeOT	OCH3	H	CH3	CH3	H
4-OH-tryptamine (4-HT)	H	OH	H	H	H
N,N-dMe-4-HT	H	OH	CH3	CH3	H



	R1	R2	R3
LSD	H	H	
Lisuride	H	H	
Ergonovine	H	H	
2-Br-LSD (BOL)	H	Br	
Methysergide	CH3	H	
Mesulergine * (*lacks double bond at this position)	CH3	H	



Ketanserin

Fig. 1. Structures of ketanserin, unsubstituted and substituted tryptamines and ergolines. *1*, amine nitrogen interacting with Ser3.36 and Asp3.32; *2*, N1 nitrogen interacting with Ser5.46

remained the same as in the WT, or slightly decreased relative to the activity of 5-HT (Fig. 2, A and B; Table 3).

Substitution of the amine of the partial agonists 4-HT, 5-MeOT, and TRYP leads to a decrease in E^r_{max} at the WT receptor (Table 3). If this decrease arises from repositioning caused by loss of interaction with Ser3.36, then the E^r_{max} of these compounds at the mutant receptor should be restored to values approaching those of the parent unsubstituted partial agonist. The results support these inferences (Table 3): *N*,*N*-dMe-TRYP, which has an E^r_{max} of 0.05 at the WT receptor, shows an increase to an E^r_{max} of 0.32 at the mutant receptor, which is comparable with the E^r_{max} values for TRYP (0.43 and 0.40 at the WT and mutant receptors, respectively). *N*,*N*-dMe-5-MeOT shows an E^r_{max} of 0.76 at the mutant receptor, comparable with 0.83 for 5-MeOT at the WT receptor. *N*,*N*-dMe-4-HT shows an E^r_{max} of 0.59 at the mutant receptor, similar to the value of 0.52 observed with 4-HT at the WT receptor.

The observed effects of the Ser3.36Ala mutation are similar for the ergoline-based ligands of the 5HT2AR. Notably, a striking increase in E^r_{max} relative to 5-HT was observed for compounds in this series in the mutant receptor. The two ergolines that showed slight agonist activity at the WT receptor, LSD and ergonovine, were found to approach the E_{max} of 5-HT in the Ser3.36Ala mutant receptor (Table 3). Lisuride and 2-bromo-LSD (a "classic" 5HT2AR antagonist), which were without detectable agonist activity at the WT receptor, were partial agonists at the mutant receptor. Of the ligands tested, only methysergide and mesulergine showed little or no activity for both WT and mutant receptors (Table 3). These results suggest that the positioning of the ergoline-based ligands relative to the helix 3 anchor is a major determinant of efficacy. This positioning in the binding pocket is influenced by the steric clash between the substituted, ring-embedded amine group of the ligands and the Ser3.36 side chain in helix 3.

Ser5.46Ala. At the WT receptor, the E^r_{max} values of 1-*N*-Me-5-HT and 1-*N*-Me-TRYP were 20 to 30% of those of the unsubstituted parent compounds (Table 3). Mutation of Ser5.46 to Ala, which was expected to remove the clash with this locus for N1-substituted ligands, resulted in an increase in E^r_{max} for both 1-*N*-Me-substitued compounds to about 60% of those of their parent compounds, 5-HT and TRYP. In contrast, the Ser3.36Ala mutation had little effect on the 1-*N*-Me compounds, as expected, because these have unsub-

TABLE 1

Ligand affinity parameters for WT and Ser3.36Ala 5-HT2A receptors

Values for K_i were determined from [^{3}H]ketanserin competition binding curves. Ratio = K_i for S3.36A/K_i for WT. Data are mean ± S.E. from 3–14 experiments.

	K_i		
	WT	WT S3.36A	Ratio
	nM		
5-HT	267 ± 86	7,460 ± 1,112	27.9
N-Me-5-HT	197 ± 19	1,589 ± 145	8.1
N,*N*-dMe-5-HT	162 ± 14	645 ± 88	4
5-MeOT	183 ± 16	3,631 ± 295	19.8
N-Me-5-MeOT	137 ± 35	748 ± 126	5.5
N,*N*-dMe-5-MeOT	121 ± 17	554 ± 90	4.6
4-HT	673 ± 106	6,085 ± 745	9
N,*N*-dMe-4-HT	95 ± 13	360 ± 76	3.8
TRYP	960 ± 133	11,250 ± 1,451	11.7
N-Me-TRYP	509 ± 56	2560 ± 452	5
N,*N*-dMe-TRYP	366 ± 41	982 ± 130	2.7
LSD	0.38 ± 0.05	0.54 ± 0.13	1.4
Lisuride	0.59 ± 0.01	0.58 ± 0.03	1
BOL	0.22 ± 0.01	0.38 ± 0.01	1.7
Ergonovine	0.76 ± 0.02	1.7 ± 0.2	2.2
Methysergide	6.7 ± 0.3	11.3 ± 0.5	1.7
Mesulergine	23 ± 2	38 ± 2	1.7

TABLE 2

Ligand affinity parameters for WT and 5.46Ala 5-HT2A receptors

Values for K_i were determined from [^{3}H]ketanserin competition binding curves. Ratio = K_i for S5.46A/K_i for WT. Data are mean ± S.E. from 3 to 14 experiments.

	K_i		
	WT	S5.46A	Ratio
	nM		
5-HT	267 ± 86	1150 ± 149	4.3
1-*N*-Me-5-HT	2213 ± 312	1305 ± 182	0.6
TRYP	960 ± 133	3493 ± 420	3.6
1-*N*-Me-TRYP	3211 ± 296	1677 ± 327	0.5

TABLE 3

Functional activity of WT, Ser3.36Ala, and Ser5.46Ala 5-HT2A receptors

Values for EC_{50} and E^r_{max} were determined from accumulation of [^{3}H]IP. Data are mean ± S.E. from at least three experiments.

	EC_{50}			E^r_{max}		
	WT	S3.36A	S5.46A	WT	S3.36A	S5.46A
5-HT	440 ± 15	5,085 ± 451	200 ± 14	1	1	1
N-Me-5-HT	233 ± 14	1,061 ± 140		0.21 ± 0.01	0.82 ± 0.01	
N,*N*-dMe-5-HT	217 ± 29	280 ± 98	110 ± 18	0.19 ± 0.03	0.93 ± 0.07	0.41 ± 0.04
1-*N*-Me-5-HT	4,206 ± 176	9,929 ± 2,052	293 ± 31	0.26 ± 0.02	0.11 ± 0.02	0.61 ± 0.03
5-MeOT	199 ± 21	3,277 ± 178	182 ± 28	0.83 ± 0.10	1.0 ± 0.04	0.86 ± 0.10
N-Me-5-MeOT	214 ± 33	1,037 ± 244		0.29 ± 0.01	0.86 ± 0.08	
N,*N*-dMe-5-MeOT	249 ± 49	504 ± 49	282 ± 64	0.26 ± 0.03	0.76 ± 0.06	0.43 ± 0.08
4-HT	1,040 ± 321	14,801 ± 1,737		0.52 ± 0.02	0.25 ± 0.03	
N,*N*-dMe-4-HT	366 ± 104	495 ± 89		0.07 ± 0.01	0.59 ± 0.04	
TRYP	2,647 ± 578	37,230 ± 3,302	2,995 ± 925	0.43 ± 0.03	0.40 ± 0.04	0.54 ± 0.08
N-Me-TRYP	1,736 ± 132	5,584 ± 400		0.12 ± 0.01	0.23 ± 0.04	
N,*N*-dMe-TRYP	888 ± 236	1,971 ± 370	N.D.	0.05 ± 0.02	0.32 ± 0.08	0
1-*N*-Me-TRYP	7,629 ± 1,662	51,453 ± 14,489	1142 ± 311	0.08 ± 0.03	0.07 ± 0.02	0.35 ± 0.03
LSD	1.4 ± 0.4	3.4 ± 0.6	4.1 ± 0.9	0.11 ± 0.01	0.87 ± 0.03	0.15 ± 0.01
Lisuride	N.D.	3.4 ± 1.0		0	0.32 ± 0.07	
BOL	N.D.	1.5 ± 0.2		0	0.37 ± 0.08	
Ergonovine	17 ± 6	4.4 ± 0.1	19 ± 9	0.08 ± 0.01	0.79 ± 0.05	0.16 ± 0.02
Methysergide	N.D.	23 ± 3	N.D.	0	0.08 ± 0.01	0
Mesulergine	N.D.	N.D.		0	0	

N.D., EC_{50} could not be determined because of low activity

stituted amine chains. Thus, the E^r_{max} of the 1-*N*-Me-substituted compounds at the Ser3.36Ala receptor were decreased (1-*N*-Me-5-HT) or unchanged (1-*N*-Me-TRYP). Clearly, the steric clash of the N1 methyl group with Ser5.46 contributes significantly to reduced efficacy by a similar mechanism of ligand repositioning.

The large increases in E^r_{max} for *N*,*N*-dMe-5-HT and *N*,*N*-dMe-5-MeOT and ergonovine that resulted from the Ser3.36Ala mutation were not seen in the Ser5.46Ala mutant, where only slight increases in E^r_{max} were seen (Table 3). In the Ser5.46Ala mutant, the E^r_{max} values for 5-MeOT, LSD, and *N*,*N*-dMe-Tryp were either unchanged or decreased. This pattern of activation is clearly different from that seen with the Ser3.36Ala mutation, further demonstrating the role of interaction of the amine and Ser3.36 in the WT.

Ligand Orientation in Molecular Models of Receptor Complexes

To explore the relationship of ligand positioning to E^r_{max}, we investigated the relative positioning of 5-HT, *N*,*N*-dMe-5-HT, and 1-*N*-Me-5-HT in the binding region of the transmembrane helix bundle using models of the WT, Ser3.36Ala mutant, and Ser5.46Ala 5HT2ARs (see *Materials and Methods*).

Orientation of 5-HT and *N*,*N*-dMe-5-HT in the WT Receptor. The initial placement of 5-HT in the WT was near optimal (Fig. 3A), yielding a small RMSD (0.4 Å) between the structures initially and after the optimization described under *Materials and Methods*. In contrast, when *N*,*N*-dMe-5-HT was placed in the same starting orientation as 5-HT in WT, a steric clash between the methyl group of *N*,*N*-dMe-5-HT and the hydroxyl group of Ser3.36 (distance, 1.8Å) caused a reorganization upon structural relaxation (RMSD, 2.1 Å compared with the starting structure). In the new orientation, the methyl-substituted side chain of *N*,*N*-dMe-5-HT avoids the steric clash at 3.6 Å from the Ser3.36 hydroxyl group. The RMSD between the final conformations adopted by 5-HT and *N*,*N*-dMe-5-HT in the WT receptor after 300 ps of simulation is 1.5 Å (Fig. 3B). At the end of 600 ps of production run, the two ligands are further separated (RMSD, 5.3 Å).

Orientation of 5-HT and *N*,*N*-dMe-5-HT in Ser3.36Ala Mutant Receptor. In this mutant, 5-HT loses the hydrogen bond with Ser3.36 and 5-HT moves away during the simulation (Fig. 3C). Because it is no longer restrained by a double hydrogen bond between its amine nitrogen and both Asp3.32

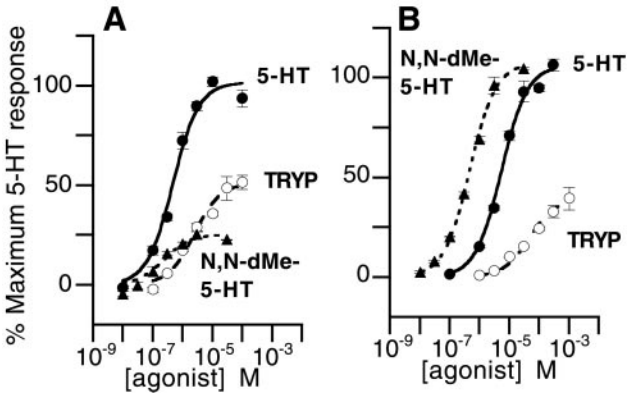


Fig. 2. Stimulation of accumulation of [^{3}H]IP in (A) WT and (B) Ser3.36Ala mutant cell lines normalized with fit E_{max} value for 5-HT equal to 100%, mean ± S.E. of triplicate determinations. In this representative experiment, the values for WT and mutant were, respectively, 539 and 301 dpm for basal and 4245 and 6195 dpm for 5-HT E_{max}.

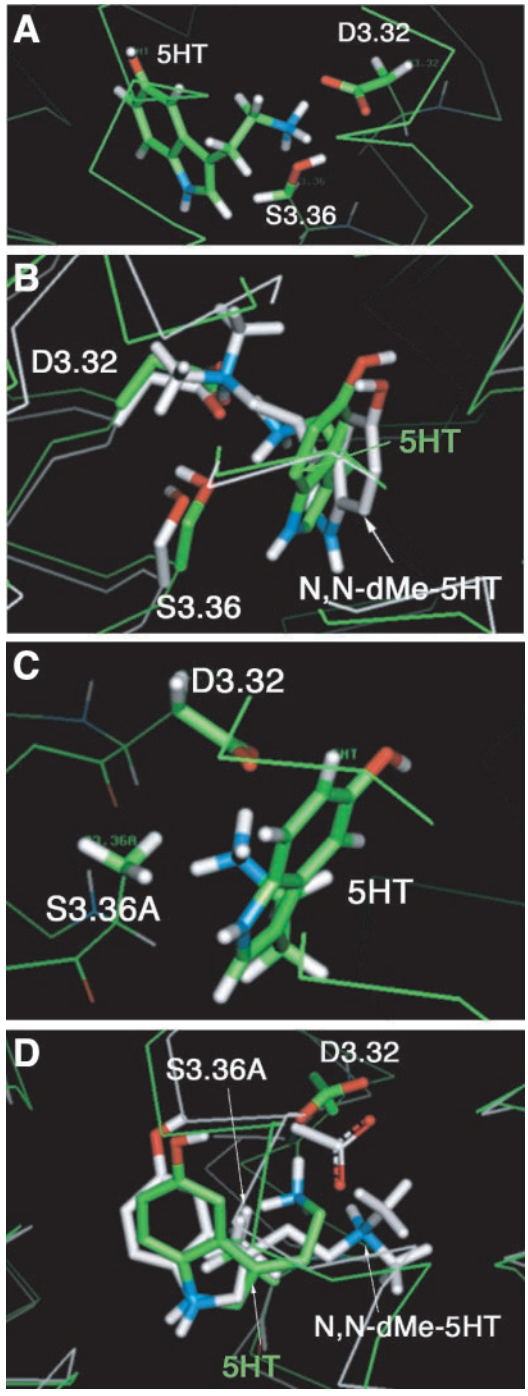


Fig. 3. A, orientation of 5-HT in the binding pocket of the WT receptor. The protonated nitrogen of the 5-HT side chain forms hydrogen bonds to both Asp3.32 (D3.32) and Ser3.36 (S3.36). B, comparison of the orientation of 5-HT (green) and *N*,*N*-dMe-5-HT (white) in the binding pocket of WT receptor. To avoid steric clash, the methyl-substituted side chain of *N*,*N*-dMe-5-HT has moved 3.6 Å from Ser3.36. C, orientation of 5-HT in the binding pocket of Ser3.36Ala mutant receptor. Because of substitution of Ala for Ser, 5-HT has lost the hydrogen bond with this side chain and has a position different from that in WT. D, comparison of orientation of 5-HT (green) and *N*,*N*-dMe-5-HT (white) in Ser3.36Ala mutant receptor. In the presence of the Ala mutation, the steric clash between the methyl group of *N*,*N*-dMe-5-HT is weaker than in WT receptor, and the final orientations of the indole moieties of the two ligands are similar.

and Ser3.36 (Fig. 3A), 5-HT is positioned differently from its orientation in the WT receptor. The final orientation of *N*,*N*-dMe-5-HT in the Ser3.36A mutant is also the result of the reorganization of the ligand and the protein, but in this case, the steric clash between the methyl group of *N*,*N*-dMe-5-HT and the Ala at position 3.36 is weaker than in the WT (the distance between the methyl group and Ala3.36 is 2.1 Å). Consequently, the indole moiety of *N*,*N*-dMe-5-HT adopts a final orientation that is near that of 5-HT in the same mutant receptor (RMSD, 1.0 Å for all nonhydrogen atoms) (Fig. 3D).

Orientation of 5-HT and 1-*N*-Me-5-HT in the WT and Ser5.46 Ala Mutant Receptor. In the starting orientation, the indole nitrogen of 5-HT is positioned at 2.8Å from the Ser5.46 hydroxyl group. In the structure of the complex resulting from the simulation, a water molecule acts as a bridge between the indole nitrogen of 5-HT and the Ser5.46 hydroxyl group (Fig. 4A). The methyl group on N1 in 1-*N*-Me-5-HT would clash against Ser5.46 in a 5-HT–like starting orientation, but it moves away during the simulation (Fig. 4B). The relatively large RMSD value (3.1 Å) for the two ligands, 5-HT and 1-*N*-Me-5-HT, in the WT indicates that they are positioned quite differently, as seen in Fig. 4, A and B.

Both the attractive interactions and the steric clashes change with the Ser5.46Ala mutation. In the absence of the Ser at position 5.46, the indole nitrogen of 5-HT is not anchored to this position, and moves during simulation to a distance of 4.8 Å (indole nitrogen to the Cβ of Ala5.46). When 1-*N*-Me-5-HT is docked in the binding site of the Ser5.46Ala mutant in the same starting orientation as 5-HT, the mutation decreases the steric clash of the N1-methyl group only modestly. Consequently, 1-*N*-Me-5-HT still moves away from residue 5.46 during the simulation. The displacement of 1-*N*-Me-5-HT induced by the steric clash avoidance is almost the same in magnitude and direction as the one that 5-HT undergoes in the same mutant receptor as a result of the lack of hydrogen bonding to 5.46. Consequently, after simulation, 5-HT and 1-*N*-Me-5-HT end up in nearly overlapping orientations (RMSD, 1.1Å) (Fig. 4C) (Video versions of Figs. 3, A–C, and 4C are available at http://transport.physbio.mssm.edu/5ht/index2.html).

Discussion

The combination of pharmacological studies of WT and mutant constructs of the 5HT2AR with modeling of the corresponding ligand-receptor complexes for the series of congeneric compounds explores the relationship between agonist activity and ligand position in the receptor. Although not specifically tested in this study, simulations and previous studies indicate that many side chains in helices 3, 5, 6, and 7 contribute to the stabilization of the complexes formed by the bulky ligands studied here. These residues include Asp3.32(155), Ser3.36(159), Ser5.38(234), Ser5.46(242), Phe6.51(339), Phe6.52(340), Asp6.55(343), and Ile6.56(344) (Almaula et al., 1996a,b; Roth et al., 1997; Kristiansen et al., 2000; Shapiro et al., 2000; Visiers et al., 2002a; I. Visiers and H. Weinstein, unpublished data). However, the present results and previous reports (Johnson et al., 1994; Almaula et al., 1996a,b) indicate that only ligands with free, unsubstituted primary amines interact with Ser3.36 and that ligands with substitutions on the indole N1 clash at the Ser5.46 position. The changes in the relative E^r_{max} of these ligands at the two mutant receptors documented here implicate these steric interactions as important determinants of ligand efficacy. Furthermore, the differences in ligand orientation predicted by the simulations with the mutants and substituted ligands suggest the hypothesis that these interactions are translated into changes in efficacy by influencing the preferred positioning of the agonist in the binding pocket. Notably, the affinity of ketanserin was unaffected by the mutations studied. The amine nitrogen of ketanserin is embedded in a six-membered ring, which, as for LSD, explains the lack of interaction with Ser3.36 in the WT receptor. However, ketanserin is longer than the ergoline ligands (see Fig. 1), and modeling of its position in the binding site suggests that it intercalates with the aromatic cluster in helix 6 (data not

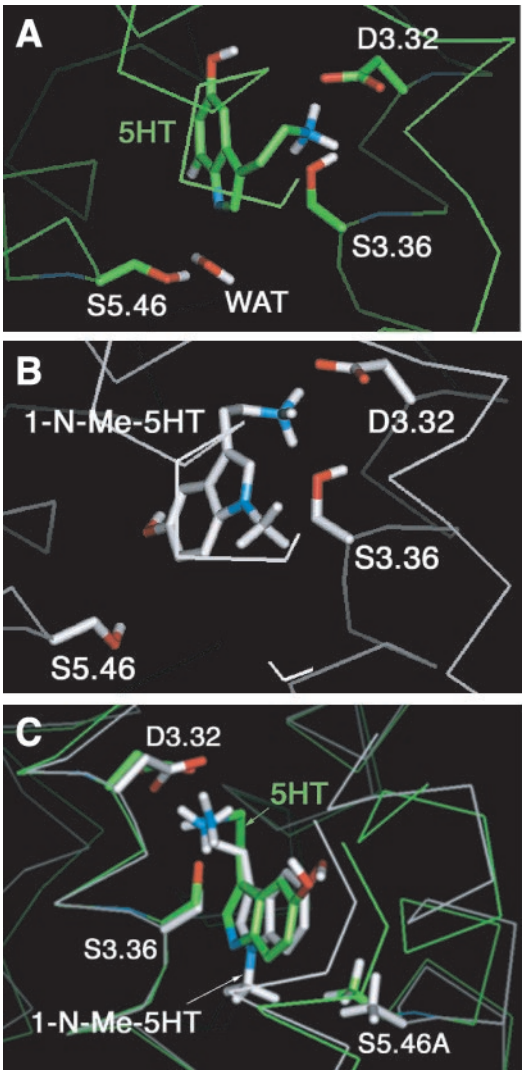


Fig. 4. A, orientation of 5-HT in the binding pocket of the WT receptor. A water molecule (WAT) bridges the indole nitrogen of 5-HT and the Ser5.46 hydroxyl group. B, orientation of 1-*N*-Me 5-HT in the binding pocket of the WT receptor. The clash of the *N*-1 methyl group with Ser5.46 causes the ligand to adopt an orientation different from that of 5-HT. C, orientation of 5-HT (green) and 1-*N*-Me-5-HT (white) in the binding pocket of Ser5.46Ala mutant receptor. Substitution of Ala for Ser at this position only modestly decreases the steric clash of the N1 methyl group with 5.46. For 5-HT, the lack of hydrogen bond with 5.46 results in an orientation that is nearly overlapping with that of 1-*N*-Me-5-HT.

shown). These additional interactions serve to minimize the role of Ser3.36 in the docking of ketanserin.

The largest changes in affinity caused by the Ser3.36Ala mutation are observed for ligands with unsubstituted amines (5-HT, 5-MeOT, 4-HT, and TRYP). Progressively smaller affinity changes are observed with methyl substitution, dimethyl substitution, and ring embedding, respectively. The larger decrease in the affinity of unsubstituted ligands for the Ser3.36Ala mutant receptor is consistent with the loss of one of the two sites of interaction of the ligand with helix 3. It is noteworthy how closely the relative changes in the EC_{50} values of the ligands for the [^{3}H]IP accumulation response curve parallel the relative changes in ligand binding affinity. Moreover, increases in EC_{50} values in the mutant receptor are largest for ligands with unsubstituted amines and smallest for ligands with di-substituted or ring-embedded amines (Table 1), thus strengthening the direct relationship between the substitution and the resulting pharmacological response.

The changes in E^r_{max} observed at the Ser3.36Ala mutant compared with WT indicate that this locus influences agonist efficacy for this series of ligands. Strikingly, for the tryptamine-based ligands studied, the E^r_{max} of substituted congeners (e.g., *N*,*N*-dMe-5-HT) at the WT receptor approximates that of the parent compound (5-HT) at the mutant receptor. The loss of the favorable interaction between Ser3.36 and the amine group of these ligands when they are substituted thus seems to be largely responsible for the decrease in E^r_{max}. In view of their marked difference in structure, it is noteworthy that the partial agonist ergolines LSD and ergonovine both show a marked increase in E^r_{max} at the mutant receptor. This suggests that the differences in efficacy between the ergolines and 5-HT in the WT 5-HT2AR is largely caused by the Ser3.36 interaction that is sterically allowed for the amine 5-HT but not for the ring-embedded amines of the ergolines.

The studies of the helix 5 interaction site identify a similar role for this locus in agonist positioning and agonist activation. The E^r_{max} of the two N1 methyl-substituted congeners is much greater with the Ser5.46Ala mutant than with the WT receptor. How does the presence or absence of an interaction with Ser3.36 and Ser5.46 influence agonist efficacy? The molecular models indicate clearly that the differences in steric clash with substituted and unsubstituted ligands lead to differences in their indole ring positioning in the binding pocket in the WT receptor. The effect of steric clash at the 5.46 locus, for example, is evident from the comparison of the position of 5-HT and 1-*N*-Me-5-HT in the WT receptor (Fig. 4, A and B). In the Ser5.46Ala mutant, the two ligands almost overlap (Fig. 4C), consonant with their similar pharmacological properties in this construct. The results of this study show that elimination of the steric clashes results in equalization of E^r_{max} and suggest that the positioning of a 5HT2AR ligand is influenced by anchoring sites in helix 3 and helix 5 and is a significant determinant of its capacity to activate the receptor. Structural considerations and computational studies indicate that the indole ring interacts with elements of a cluster of aromatic residues in helix 6 (Javitch et al., 1998; Visiers et al., 2002a). This aromatic cluster, anchored by the conserved Trp6.48, may serve as the main activation trigger used by this class of agonists (Visiers et al., 2000, 2002a), because it was shown that the orientation of Trp6.48 changes upon activation (Lin and Sakmar, 1996). It seems likely that the favored position and tilt of the indole ring of the ligands studied here determines its ability to interact with this aromatic cluster (Visiers et al., 2002a) and, thereby, its efficacy to activate the receptor. If eliciting a full agonist response requires a specific set of ligand-receptor interactions that are achieved in a particular position of the ligand in the receptor, then the energetically preferred position of a partial agonist must be different. The full agonist position and its dynamic consequences for receptor activation may be achievable by the partial agonist with lower probability than by the full agonist and hence apply only for a fraction of the ligand's residence time in the receptor.

Our data from mutagenesis and computational simulations provide insight into the molecular mechanisms underlying partial agonism and explain how such subtle alterations in ligand structure can lead to altered agonist efficacy. We propose that ligand positioning in the binding pocket that is determined by the orienting interactions of ligands with Ser3.36 and Ser5.46 are required for full agonist activity by virtue of their effects on ligand positioning in the binding pocket. These results thus implicate ligand positioning as a major determinant of drug efficacy. Notably, a cluster of aromatic residues in TM6 has been proposed as a sensor of such positioning of the agonist (Visiers et al., 2002a). This aromatic cluster belongs to the special class of conserved structural microdomain/functional microdomain motifs that include as well the Glu/Asp-Arg-Tyr motif in helix 3 and the Asn-Pro-Xxx-Xxx-Tyr motif in helix 7 and have been shown to conserve their role in mechanisms of G protein-coupled receptor function (for review, see Visiers et al., 2002a; Huang et al., 2001; Prioleau et al., 2002; Visiers et al., 2002b) for some recent examples). It is very likely, therefore, that the structurally specific mechanism described here for the first time to explain the manner in which the position of the ligand in the binding site affects its pharmacological efficacy, is generalizable to other families of G protein-coupled receptors in this class A of rhodopsin-like receptors.

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