

Identification of Conserved Aromatic Residues Essential for Agonist Binding and Second Messenger Production at 5-Hydroxytryptamine_{2A} Receptors

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SUMMARY

Several models of agonist binding to G protein-coupled 5-hydroxytryptamine [5-HT] (serotonin) receptors have highlighted the potential importance of highly conserved aromatic residues for ligand binding and agonist efficacy. In this study, we tested these models by constructing and characterizing a number of point mutations of conserved and nonconserved aromatic residues using the 5-HT_{2A} receptor as a model system. Mutations of three highly conserved tryptophans (W200A, W336A, and W367A) proposed to reside near the binding pocket markedly reduced agonist affinity and efficacy at 5-HT_{2A} receptors. Mutations of two other highly conserved aromatic residues postulated to be near the agonist binding site (F340L and Y370A) also had dramatic effects on agonist binding and efficacy. Point mutations of neighboring conserved phenylalanines (F339L and

F365L) had minimal effects on agonist binding, although the F365L mutation diminished agonist efficacy. Finally, mutations of two nonconserved aromatic residues (F125L and F383A) not predicted to be near the binding pocket had no effects on agonist binding, potency, or efficacy. Our results are best explained by models that suggest that helices III, V, VI, and VII can form a unit of interacting helices in which highly conserved aromatic residues are oriented toward the center of the helical aggregate to form an aromatic pocket. In addition, our novel results identify a series of aromatic residues essential for agonist-induced second messenger production. These results demonstrate that highly conserved aromatic residues residing in neighboring helices provide the optimum environment for both agonist binding and activation of 5-HT_{2A} receptors.

The molecular mechanisms by which drugs bind to and activate GPCRs remain a central unresolved problem for biochemists and pharmacologists. In the absence of direct structural data, most investigators have relied on a combination of site-directed mutagenesis studies and molecular modeling approaches. For the past few years, we have used the 5-HT_{2A} receptor as a convenient model for investigating the molecular requirements for binding structurally diverse ligands to a single GPCR (1–3) because a large number of clinically important but structurally diverse ligands (e.g., indoles, ergolines, ergopeptines, piperazines, tricyclic and heterocyclic compounds, substituted amphetamines, butyrophenones, and others) have high affinity for 5-HT_{2A} receptors (4). Mutagenesis and structure-activity studies have demonstrated that many of these compounds probably bind to unique regions of the receptor (3–6). Determination of how diverse agents bind to a single receptor will clarify the structural features essential for ligand recognition.

Current models of GPCR structure are mainly derived from analogies with high-resolution structures of bacteriorhodopsin (7) and relatively low-resolution structures of rhodopsin (8). Like other GPCRs, the 5-HT_{2A} receptor is believed to have a topology of seven-transmembrane α -helices (3, 5, 9–12), although the actual helical connectivity, arrangement, and three-dimensional structure are unknown.

Most explicit models of 5-HT_{2A} receptor structure (3, 5, 9–12) have a proposed negatively charged carboxylate anion in the third transmembrane domain (D155) that anchors the positively charged amine moiety of 5-HT, in analogy with β -adrenergic and other biogenic amine receptors. A highly conserved aromatic residue in helix VI (F340) apparently stabilizes the aromatic ring of 5-HT and related compounds (2, 3), whereas nonconserved serines in helix V (6) are essential for binding N1-substituted tryptamines and ergolines. An additional serine in helix III (5) may assist in anchoring the amine portion of 5-HT and related ligands.

High-resolution studies of bacteriorhodopsin (7) have determined that a number of aromatic residues located in adjacent helices stabilize the chromophore; mutagenesis studies on rhodopsin (13) have also highlighted the importance of

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TABLE 1
Expression of [³H]ketanserine binding by native and mutant 5-HT_{2A} receptors

Data represent mean \pm standard deviation of computer-derived estimates for three separate experiments, except where noted. For the W336A, F339L, and Y370A mutants, [³H]mesulergine was used as the radioligand. For all other mutants, [³H]ketanserine was used. In a typical experiment, 90% of total binding was specific, and $\leq 10\%$ of the total radioligand was bound. B_{\max} data for the F125L, F339L, and F340L mutations are from Ref. 3.

Drug	5-HT _{2A}	W76A	F125L	W200A	W336A	F339L	F340L	F365L	W367A	Y370A	F383A
K_d (nM)	0.98 \pm 0.3	9.8 \pm 0.7	1.5 \pm 1.3	0.65 (n = 2)	899 \pm 133	8 \pm 3.8	2.2 \pm 0.8	3.9 \pm 1.4	N.D.	18.4 (n = 2)	3.5 (n = 2)
B_{\max} (fmol/mg of protein)	1900 \pm 250	1763 \pm 152	2150 \pm 248	400 (n = 2)	296 \pm 130	912 \pm 150	1100 \pm 137	1862 \pm 428	N.D.	1382 \pm 414	2820 \pm 225

N.D., no binding detected.

bulky aromatic groups for chromophore stabilization and photoisomerization. Recent molecular models of GPCRs have predicted that highly conserved aromatic residues are also directly involved in ligand binding (9–12). These aromatic residues include tryptophans (W200, W336, and W367), phenylalanines (F340, F365), and a moderately conserved tyrosine (Y370) (9–12). It has been proposed that these residues may participate in direct hydrophobic-type interactions with the ligands, although tryptophans can also act to stabilize the positively charged amine substituents. Despite the postulated critical role of these aromatic residues for ligand binding, to our knowledge no systematic evaluation have been reported of the relevance of these residues for ligand binding and activation of GPCRs.

We constructed and characterized a number of nonconservative mutations of the aromatic residues most frequently identified by molecular modeling studies of GPCRs. To our knowledge, this is the first study in which all of these residues have been systematically studied by nonconservative mutagenesis. Our findings implicate most of these residues for the processes of agonist binding and receptor activation. Our findings are consistent with models (3, 9–12) that predict that some of these highly conserved aromatic residues could face the interior of the helical aggregate and act to form a hydrophobic pocket that allows for optimal ligand binding.

Experimental Procedures

Construction and expression of mutant 5-HT_{2A} receptors.

Ten mutant 5-HT_{2A} receptors were constructed using a modified polymerase chain reaction-based mutagenesis method as previously described (1–3, 14). Highly conserved tryptophan and phenylalanine residues in several adjacent and nonadjacent helices were targeted for site-directed mutagenesis, including W200A (helix IV), W336A (helix VI), W367A (helix VII), F339L (helix VI), F340L (helix VI), F365L (helix VII), and Y370A (helix VII). In addition, nonconserved, aromatic residues were targeted to control for nonspecific effects resulting from the loss of an aromatic moiety in the transmembrane helix: W76A (helix I), F125L (helix II), and F383A (helix VII). Mutations were confirmed by double-strand DNA sequencing using Sequenase (United States Biochemical Corp., Cleveland, OH) and the dideoxynucleotide technique (15). Mutant 5-HT_{2A} receptors were transiently expressed using the DEAE-dextran technique, as previously detailed (1–3), into COS-7 cells. Cells were harvested 72 hr later for binding assays.

Analytical procedures. PI hydrolysis and binding assays were performed as previously detailed (1–3). K_{act} and V_{max} values for agonist-stimulated [³H]IP release were calculated using a modified Michaelis-Menton equation and a nonlinear least-squares curve-fitting program available on SigmaPlot (1–3). For binding assays, specific binding represented 80–95% of total binding; no more than 10% of the total counts added/tube were specifically bound. Binding data were analyzed using a computerized, nonlinear least-squares curve-fitting routine [LIGAND program (16)] that calculates K_i , K_d , and B_{max} values according to the law of mass action. For competition binding studies, 6–12 concentrations of unlabeled ligand were used, whereas for saturation binding studies, 11 concentrations were used. A runs test was used to ensure that the predicted parameter estimates were appropriate. An F test was used at the $p < 0.05$ level of significance to determine the differences between K_i values and K_{act} values. Protein determinations were performed using the BioRad kit.

Results

Mutations of highly conserved aromatic residues diminish agonist binding at 5-HT_{2A} receptors. Our prior

studies (2, 3) showed that a point mutation of a highly conserved phenylalanine residue (F340L) greatly diminished the binding of 5-HT and several ergolines to the 5-HT_{2A} receptor. Other studies involving β -adrenergic (17), opiate (18), neuropeptide (19), and muscarinic (20) receptors have highlighted the importance of this cognate residue for ligand binding to GPCRs.

Within the general vicinity of F340, several additional aromatic residues are potentially located that could play a role in ligand binding and/or efficacy. Several of the aromatic residues within the predicted vicinity of F340 are highly conserved among various GPCRs, including W200 (helix IV), W336 (helix VI), and W367 (helix VII), whereas Y370 (helix VII) is less widely conserved. To determine the importance of these residues for agonist binding to 5-HT_{2A} receptors, we made point mutations of these conserved aromatic residues as well as of several other nonconserved aromatic residues (W76A, F339L, F365L, and F383A) to control for nonspecific mutagenesis effects.

All together, 10 mutant 5-HT_{2A} receptors (W76A, F125L, W200A, W336A, F339L, F340L, F365L, W367A, Y370A, and F383A) were prepared and analyzed by radioligand binding studies. With the exception of the W336A, W367A, and

Y370A mutants, all of the mutant 5-HT_{2A} receptors were able to bind [³H]ketanserin with high affinity (Table 1). The W336A and Y370A mutations bound [³H]mesulergine with high affinity ($K_d = 1.5$ nM for W336A and 2.2 nM for the Y370A mutations); thus, ketanserin affinity for these two mutants was determined by competition binding assays. The W367A mutation did not bind any tested radioligand, including [³H]spiperone, [³H]mianserin, and [³H]ketanserin. Immunofluorescence studies demonstrated expression of the W367A mutation on the cell surface of transiently expressed COS-7 cells (not shown) and PI hydrolysis studies (see below) demonstrated the expression of functional receptors.

Fig. 1 shows that mutations involving three of these residues (W336A, F340L, and Y370A) had dramatic effects on agonist binding affinities, whereas the W200A mutation affected only the two agonists tested. In confirmation of our prior studies, the F340L mutation significantly diminished the binding affinities of four agonists: 5-HT (Fig. 1A), DOM (Fig. 1B), α -methyl-serotonin (Fig. 1C), and bufotenine (Fig. 1D). Mutation of a nearby, highly conserved tryptophan, W336A, also significantly diminished the binding affinities of the four tested agonists (Fig. 1, A–D). The Y370A mutation affected two of the agonists (5-HT and DOM) but had no

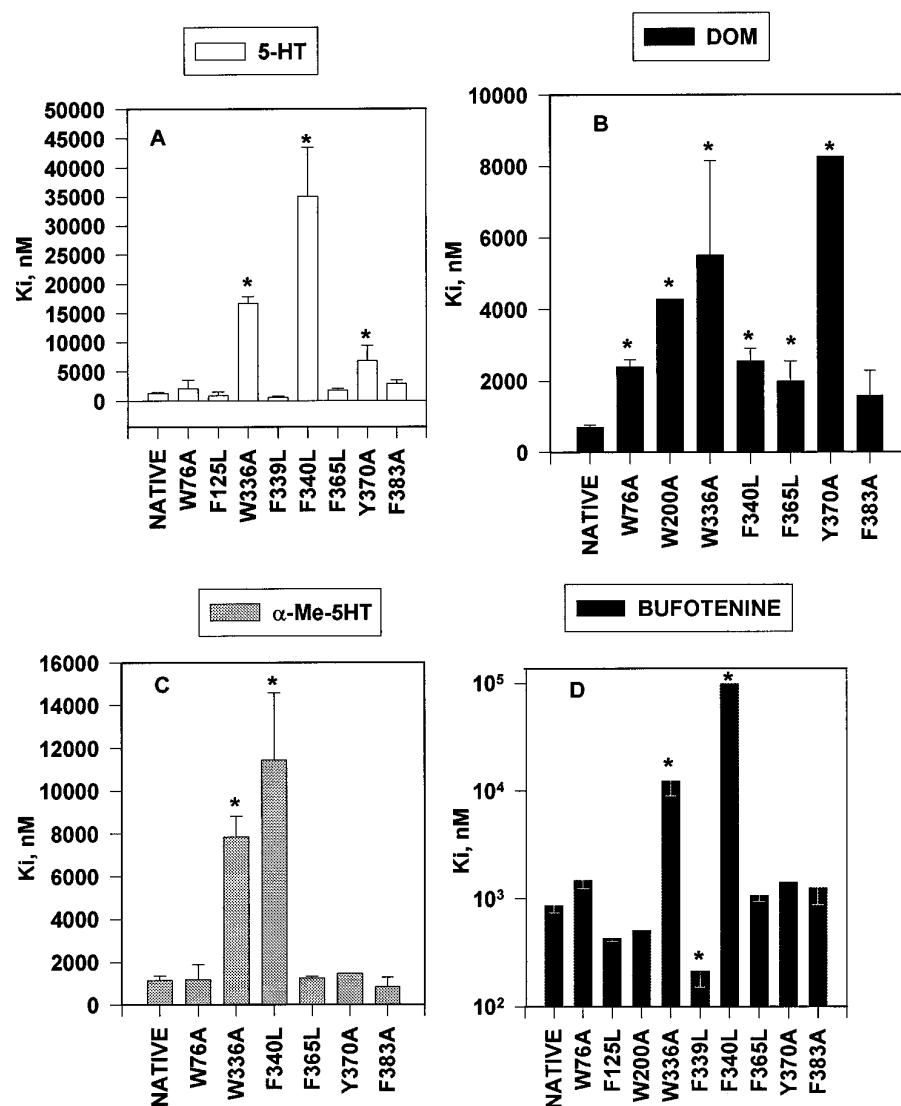


Fig. 1. Mutations of highly conserved aromatic residues diminish agonist binding. Native and mutant 5-HT_{2A} receptors were transiently expressed in COS-7 cells, and the ability was determined of 5-HT to compete with [³H]ketanserin-labeled (native, W76A, F125L, F340L, F365L, and F383A) or [³H]mesulergine-labeled (W336A, F339A, and Y370A) receptors. A, 5-HT binding affinities. B, DOM binding affinities. C, α -Methyl-5-HT binding affinities. D, Bufotenine binding affinities. Data represent mean \pm standard deviation of computer-derived estimates for three separate experiments in which 5–12 doses of 10–100,000 nM agonist were used with duplicate determinations for each point. *, $p < 0.05$ versus native (F test).

effect on the binding affinities of either α -methyl-serotonin or bufotenine (Fig. 1, A–D).

The other tested mutations (W76A, F125L, F339L, F365L, and F383A) had few consistent effects (<10 -fold change in K_i) on the binding of any of the agonists tested (Fig. 1). These results show that merely altering the aromatic environment of the proposed binding pocket does not necessarily alter agonist binding affinity.

Mutations of highly conserved aromatic residues affect agonist efficacies and potencies for activating PI hydrolysis. We next investigated if these same mutations affected agonist efficacy or potency. As shown in Fig. 2 and Table 2, several of the tested mutations involving highly conserved aromatic residues (e.g., W200A, W336A, F340L, and Y370A) substantially impaired the ability of DOM to augment PI hydrolysis. Fig. 3 shows that several of these same point mutations also diminished the ability of 5-HT to activate PI hydrolysis. Similar results were obtained when two other agonists (bufotenine and α -Me-5-HT) were also examined for their ability to augment PI hydrolysis at native and mutant 5-HT_{2A} receptors (Table 2).

Table 2 shows the K_{act} and percent V_{max} (relative to the efficacy of 5-HT at the native receptor) values we obtained for all four agonists at the various 5-HT_{2A} receptor mutants. As is clear from Table 2, the following aromatic residues are essential for optimal agonist efficacy at 5-HT_{2A} receptors: W200, W336, F340, F365, W367, and Y370. Interestingly, even though the W367A mutation was unable to bind any tested radioligand, the W367A mutant was able to activate PI hydrolysis, albeit at a greatly reduced level. It is also clear that the W367A mutant greatly reduced the potency of all four agonists tested.

Interestingly, the W76A mutation did not substantially

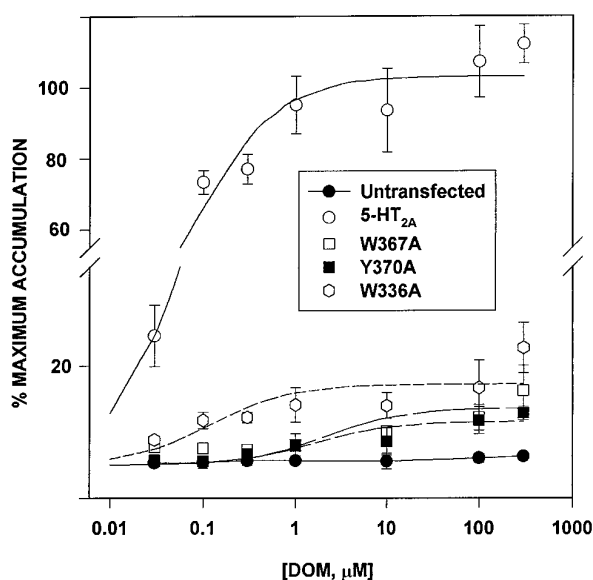


Fig. 2. Mutations of conserved aromatic residues diminish the ability of DOM to activate PI hydrolysis. Native and mutant 5-HT_{2A} receptors were transiently expressed in COS-7 cells, and the ability of graded doses of DOM to augment [³H]-IP release was determined as described in the text. Data represent mean \pm standard error of triplicate determinations of [³H]IP release from a typical experiment and are expressed as percent maximum accumulation. In a typical experiment, basal activity ranged from 200 to 400 cpm, whereas the maximum stimulation elicited by agonist was 6,000 to 10,000 cpm.

alter agonist binding affinity or the K_{act} value, whereas the percent V_{max} value for PI hydrolysis was diminished for several tested agonists, even though receptor expression was similar to the native receptor (see Table 1). These results suggest that even though W76A does not directly participate in ligand binding, W76 may be important for signal transduction for the 5-HT_{2A} receptor.

In addition, the F340L mutation greatly diminished agonist efficacy for all tested agonists, which is in support of our previous findings (3). Interestingly, however, the K_{act} and percent V_{max} values for DOM were greatly attenuated, while the K_i value for DOM was only minimally altered (compare Fig. 2 with Table 2). These results imply that the molecular processes governing agonist binding and signal transduction potency are likely to be related but not identical.

It is also clear in a comparison of Tables 1 and 2 that several of the mutations that affected agonist efficacy also decreased receptor expression, although this was not a uniform observation. Thus, the F339L mutations diminished receptor expression by 58% but did not alter agonist efficacy, whereas the F365L mutation did not alter receptor expression but diminished agonist efficacy. Thus, it is unlikely that the low agonist efficacies seen for several of the mutations were due solely to low receptor expression.

We directly tested this possibility by transfecting COS-7 cells with increasing amounts of native 5-HT_{2A} receptor cDNA (0.3–10 μ g/100-mm plate) and determined the effects of various levels of receptor 5-HT_{2A} receptor expression on K_{act} and percent V_{max} values for 5-HT. As is seen in Fig. 4, levels of expression as low as 390 fmol/mg of protein had little effect on the dose-response curve for 5-HT; lower levels of expression decreased the percent V_{max} value but had little apparent effect on the K_{act} value. These results imply that the apparently decreased efficacy noted with certain 5-HT_{2A} receptor mutants is not likely due *solely* to decreased receptor expression because all of the mutant receptors, with the exception of the W336A mutant, had levels of expression of >390 fmol/mg.

Discussion

Our major finding is that multiple, highly conserved aromatic residues are essential for both agonist binding and efficacy at 5-HT_{2A} receptors. A unique feature of this study is the large number of highly conserved aromatic residues were targeted using nonconservative substitutions. An additional novel feature of the present studies is that both agonist binding and second messenger production (i.e., PI hydrolysis) were simultaneously determined. Prior studies used nonconservative substitutions (21), targeted fewer numbers of residues (13, 22, 24–29), and did not uniformly assess second messenger production (13, 22, 24–29). Given the large number of mutants simultaneously studied, our results allow us to conclude that aromatic residues have three independent functions for GPCRs: (i) coupling to second messenger production (W76), (ii) direct ligand binding (W200, W336, F340, and Y370), and (iii) a combination of these two functions (W336, F340, W367, and Y370).

Interestingly, the W76A mutation modestly (4–5-fold) diminished the K_{act} values for two of the agonists tested (5-HT and DOM). Our results suggest that W76 participates directly in signal transduction, perhaps by facilitating helical/

TABLE 2

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

Data represent mean \pm standard deviation of computer-derived estimates for K_{act} and V_{max} values for experiments that have been replicated at least three times (except where noted). V_{max} values are expressed relative to 5-HT expression of native 5-HT_{2A} receptors. In a typical experiment, basal activity ranged from 200 to 400 cpm, whereas the maximum stimulation elicited by 5-HT was 6,000–10,000 cpm.

Drug	5-HT _{2A}	W76A	F125L	W200A	W336A	F339L	F340L	F365L	W367A	Y370A	F383A
Bufotenine											
K_{ACT} (nM)	84 \pm 2	65 \pm 26	109 \pm 128	881 \pm 83 ^a	1838 \pm 179 ^a	158 \pm 96	1,900 \pm 190 ^a	585 \pm 271 ^a	N.D.	1346 \pm 796 ^a	84 \pm 2
V_{MAX} (% 5-HT)	72 \pm 1	27 \pm 2	67 \pm 20	11 \pm 2 ^a	16 \pm 2 ^a	75 \pm 14	10.5 \pm 1.4 ^a	30 \pm 14 ^a		21 \pm 2	72 \pm 2
α -Methyl-serotonin											
K_{ACT} (nM)	57 \pm 14	241 \pm 63 ^a		N.D.	3090 \pm 836 ^a		N.D.	311 \pm 133 ^a	14,140 \pm 6810 ^a	3258 \pm 900 ^a	117 \pm 18
V_{MAX} (% 5-HT)	103 \pm 13	31 \pm 12 ^a		N.D.	23 \pm 5 ^a		N.D.	100 \pm 12	22 \pm 18 ^a	59 \pm 3 ^a	100 \pm 3
5-HT											
K_{ACT} (nM)	47 \pm 13	91 \pm 15	249 \pm 110	2637 \pm 439 ^a	716 \pm 403 ^a	249 \pm 110	7,200 \pm 2000 ^a	118 \pm 41	7100 \pm 3100 ^a	14280 \pm 960 ^a	43 \pm 6
V_{MAX} (% 5-HT)	100	31 \pm 10 ^a	100 \pm 7	3 \pm 2 ^a	27 \pm 2 ^a	100 (N = 2)	18 \pm 3 ^a	32 \pm 2 ^a	7 \pm 1 ^a	9 \pm 2 ^a	100 \pm 2
DOM											
K_{ACT} (nM)	42 \pm 16	166 \pm 33 ^a		N.D.	180 \pm 123		28,1000 \pm 14,000	98 \pm 26	2360 \pm 1075 ^a	11,410 \pm 599 ^a	67 \pm 6
V_{MAX} (% 5-HT)	87 \pm 4	42 \pm 3 ^a		N.D.	21 \pm 2 ^a		50 \pm 3 ^a	56 (N = 2)	8 \pm 1 ^a	9 \pm 1 ^a	81 \pm 2

N.D., no activation of PI hydrolysis detected (>30% elevation over background).

^a $p < 0.05$ versus native receptor (F test).

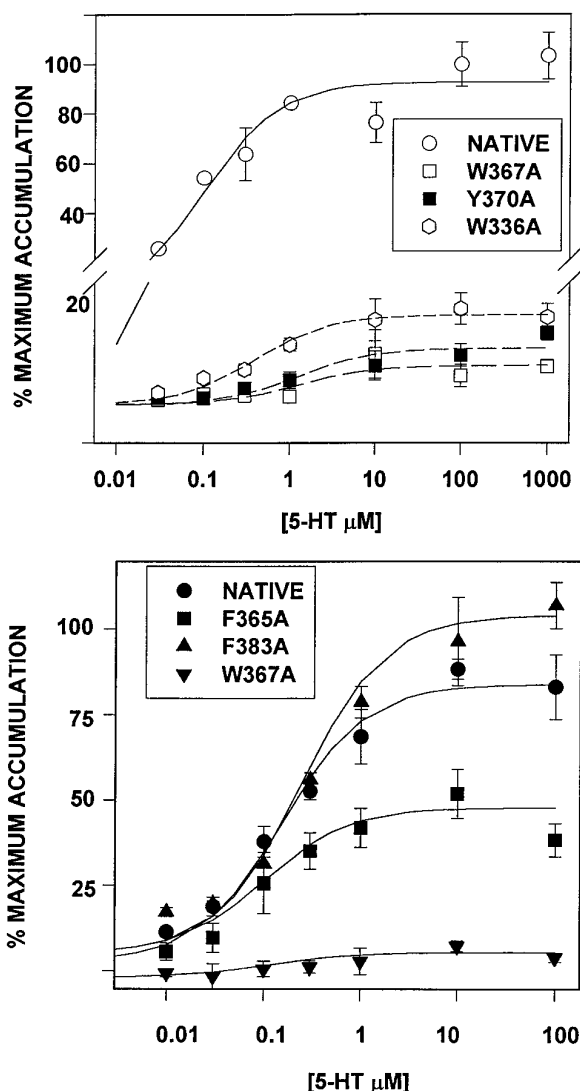


Fig. 3. Mutations of conserved aromatic residues diminish the ability of 5-HT to activate PI hydrolysis. Native and mutant 5-HT_{2A} receptors were transiently expressed in COS-7 cells, and the ability of graded doses of 5-HT to augment [³H]IP release determined as described in the text. Data represent mean \pm standard error of triplicate determinations of percent maximal accumulation of [³H]IP accumulation from three separate experiments. In a typical experiment, basal activity ranged from 200 to 400 cpm, whereas the maximum stimulation elicited by agonist was 6,000 to 10,000 cpm.

helical interactions. W76 is predicted to be located near the top of helix I and could be involved in altering the general helical orientation of helix I, although structural studies will be necessary to test this hypothesis. Our results demonstrate a previously unsuspected role in signal transduction for this nonconserved residue. It is conceivable, of course, that the results obtained with the W76A mutation are due to an allosteric effect, and further studies are necessary to investigate this possibility.

Several other mutations (W200A, W336A, F340L, W367A, and Y370A) dramatically altered agonist affinity and/or efficacy. With few exceptions, the tested agonists had substantially reduced binding affinities and efficacies for these mutant receptors. These results imply that these aromatic residues (W200, W336, F340, W367, and Y370) are involved in signal transduction and ligand binding. Because most of

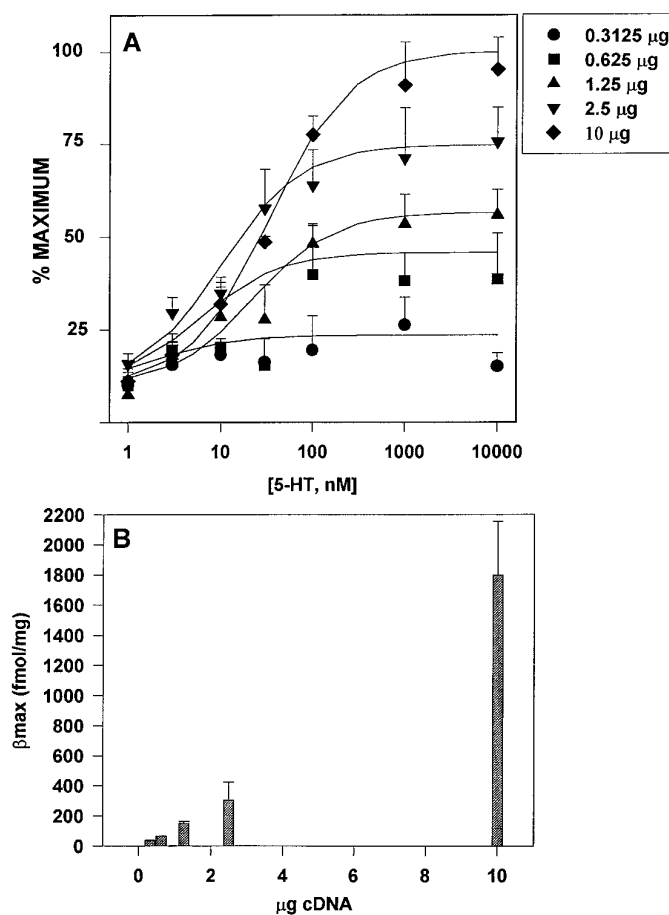


Fig. 4. Effect of variable levels of receptor expression on agonist-induced [³H] IP accumulation in Cos-7 cells. COS-7 cells were transfected with 0.3–10 μ g of 5-HT_{2A} receptor cDNA in pSVK3 as described in the text, and the ability of graded doses of 5-HT to augment [³H]IP accumulation was determined (A). Data represent mean \pm standard error of triplicate determinations from a typical experiment that was replicated twice. B, Effect of transfecting COS-7 cells with 0.3–10 μ g of 5-HT_{2A} cDNA on [³H]ketanserin binding. Data represent mean \pm standard deviation of maximum binding/ μ g of protein.

the critical residues are located in helices VI and VII, it is likely that these helices interact, perhaps via hydrophobic or aromatic/aromatic interactions.

An alternative hypothesis is that the W200A, W336A, F340L, W367A, and Y370A mutations all induce gross structural changes in the 5-HT_{2A} receptor, which results in a change in signal transduction because of allosteric effects. Our recent results (30) imply that the F340L mutation, for instance, has distinct and separate effects on agonist affinity and efficacy. To test this notion, we assessed the abilities of the native and F340L mutant 5-HT_{2A} receptor to bind to G_{αq}, which can couple 5-HT_{2A} receptor to PI hydrolysis. These new findings¹ demonstrate that the F340L mutation significantly impairs the ability of the receptor to bind to G_{αq} in the absence of radioligand, suggesting that the F340L mutation has allosteric effects as well as direct effects on ligand binding. Obviously, it will be of great interest to determine

¹E. A. Hyde, W. Kroeze, and B. L. Roth. A helix-six mutation that diminishes basal activity of the 5HT_{2A} receptor directly interferes with G_{αq} binding, submitted for publication.

whether these other mutations have similar effects on $G_{\alpha q}$ binding.

Although none of these residues have been targeted in previous studies of serotonergic receptors, in prior studies of various other GPCRs, some of the amino acid cognates of the aromatic residues targeted in our study have been examined. It should be noted, however, that in some of these studies, conservative mutations were constructed (W-to-F; see, for example, Ref. 22), so the aromatic nature of the residue was maintained. In the present study, only nonconservative mutations (W-to-A) were constructed. Thus, the present studies could reflect the true contribution of the aromatic moiety to agonist binding and/or efficacy. It will be important, of course, in future studies to systematically investigate the effects of conservative and nonconservative mutations at these various locations to verify this notion.

For the W200 cognate, Wess *et al.* (21) found that a W292F mutation slightly decreased agonist potency and affinity, whereas a W161A mutation in bovine opsin was without effect (13). For the W336 cognate, diminished agonist efficacy was seen at the muscarinic m3 receptor (21), the angiotensin II receptor (22), and bovine opsin (13). A W279A mutation (analogous to W336A) in the thyrotropin-releasing hormone receptor was without effect (31). Taken together with our findings of greatly decreased agonist binding affinity, potency, and efficacy, it seems that the highly conserved tryptophan found in helix VII is of general importance for signal transduction and ligand binding for GPCRs.

For the F339 and F340 cognates, studies of β -adrenergic (see review in Ref. 17), bradykinin₂ (24) and neurokinin₂ (25) receptors have demonstrated diminished agonist binding and/or efficacy. One prior study targeted the F365 position (27) and concluded that its orientation was toward helices VI and III. For the W367 cognate, Wess *et al.* (21) found slightly decreased agonist efficacy at the m3 muscarinic receptor. For the Y370 cognate, agonist and/or antagonist binding affinities were decreased at the m3 muscarinic (21) and β_2 -adrenergic (17) receptors, although agonist efficacy was not determined.

Recent models of GPCR structure based on rhodopsin templates (3, 5, 9–12) have suggested that some of the residues implicated by our mutagenesis studies are predicted to participate in agonist binding to 5-HT and other receptors. To examine this hypothesis, a model was constructed derived from a 6-Å projection map of frog rhodopsin (8) using the residue assignments of Baldwin (32) and Schwartz (33) (Fig. 5). This model, using the residue assignments of Schwartz (32), correctly predicts that some of the amino acids identified by our site-directed mutagenesis findings (W336 and F340) reside near the central portion of the helical aggregate (Fig. 5) but does not account for the critical nature of W200, W367, and Y370 for both agonist binding and efficacy.

Kristiansen and Dahl (12) recently proposed, based on dynamic simulations of a rhodopsin-based serotonin receptor model, that nearly all of the residues we identified by mutagenesis (W200, W336, F340, F365, and Y370) are involved in 5-HT binding. The single exception was W367, which was previously identified as being involved in β -adrenergic receptor antagonist binding based on photoaffinity labeling studies (see Ref. 32 for discussion). We discovered a substantial contribution from W367 for both ligand binding and second messenger production. In this regard, a recent study by Fu *et*

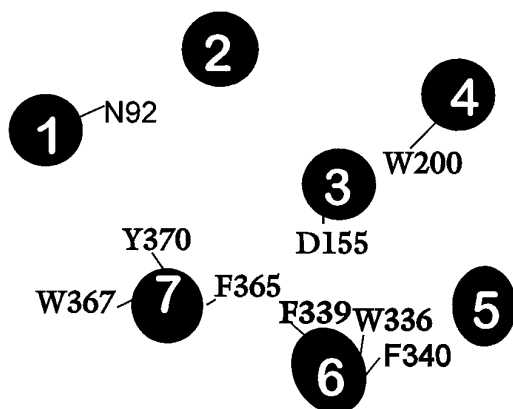


Fig. 5. Summary of various models that account for the orientation of aromatic residues in helix VII. Shown are the theoretical locations of the mutations constructed in this study. A template derived from the projection map recently published for frog rhodopsin at 6 Å resolution (7) was used. Assignment of residues are based on models published by Baldwin (8) and Schwartz (9), with the view being from the cytoplasmic surface. This assignment suggests that W367 and Y370 are oriented away from the center of the helical aggregate and that F365 points toward the center.

al. (29) indicates that the W367 cognate found in the D₂ dopamine receptor forms part of the binding crevice.

These findings of Fu *et al.* (29) included substantial contributions by the Y370 cognate for D₂ antagonist binding, although agonist binding and efficacy were not assessed in these studies. Our findings agree with this notion and prompted us to evaluate a preliminary model to determine whether it more readily accounts for our findings. This preliminary model² orients Y370 toward the center of the helix and F365 toward the M6/M7 helical interface, as implied by Fu *et al.* (29). The model also introduces a substantial proline kink to account for accessibility data obtained for the D₂ receptor F383 cognate, as suggested by Fu *et al.* (29) for the D₂ dopamine receptor. Although our studies do not directly address the question of accessibility of these residues or the precise orientation of these residues with regard to the helical aggregate, future studies using the techniques of Fu *et al.* (29) could clarify this issue.

An additional new finding of the current study was the dissociation we discovered between ligand binding and agonist potency when aromatic residues were targeted. To our knowledge, this relationship has not previously been reported. Several mutations had relatively modest effects on ligand binding affinities (<10-fold) and substantial (100–1000-fold) effects on agonist potencies (compare Figs. 1 and 4). These results suggest that these mutations disrupt the ability of agonists to activate second messenger production in a manner that is greater than their effects on binding affinity. The precise biochemical mechanism responsible for these findings is unclear but could be due to disruption of agonist-induced conformational changes that are essential for agonist efficacy. In agreement with this hypothesis, we recently discovered that the F340L mutation disrupts the ability of purified 5-HT_{2A} receptors to bind to $G_{\alpha q}$.³

We discovered that several highly conserved aromatic residues are essential for agonist binding and efficacy at 5-HT_{2A}

² M. Shoham and B. L. Roth, unpublished observations.

³ Hyde *et al.*, manuscript submitted.

receptors. Our results predict that models based strictly on prior rhodopsin-based helical assignments may not faithfully account for the mutagenesis data. Our data are in general agreement with three prior studies that highlight the importance of certain conserved aromatic residues for ligand binding to 5-HT_{2A} (3), opiate (28), and D₂ dopamine (29) receptors, although none of these studies examined the effects of these mutations on agonist efficacy. It is conceivable that these aromatic residues form a hydrophobic pocket that allows for optimum agonist binding.

In conclusion, our results show in a dramatic and novel fashion that several highly conserved aromatic residues are important for agonist binding and efficacy. One can speculate that these results imply that aromatic residues in general play a previously unsuspected role in agonist binding and signal transduction.

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