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Differential Ergoline and Ergopeptine Binding to 5-Hydroxytryptamine$_{2A}$ Receptors: Ergolines Require an Aromatic Residue at Position 340 for High Affinity Binding

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SUMMARY

In this paper we show that a highly conserved aromatic residue, phenylalanine at the 340-position, is essential for ergoline binding to 5-hydroxytryptamine$_{2A}$ receptors. We hypothesized that F340 was essential for a specific aromatic-aromatic interaction (e.g., π-π or hydrophobic) between the phenyl moiety of F340 and the aromatic ring of the ergoline nucleus. To test this hypothesis, eight point mutations of adjacent (F340 and F339) and nonadjacent (F125) phenyalanines were made, using conservative (phenylalanine to tyrosine) and nonconservative (phenylalanine to leucine, alanine, or serine) substitutions. The binding affinities of all of the tested simple ergolines were greatly reduced by specific mutations of F340 in which aromatic-aromatic interactions (e.g., F340A and F340L) were abolished, but they were unaffected when the replacement residue was aromatic (e.g., F340Y). In contrast, the binding affinities of four ergopeptines (bromocryptine, ergocryptine, ergocornine, and ergotamine) were relatively unaffected by the F340L substitution. Neither ergoline nor ergopeptine affinities were consistently altered by F339 mutations. These results support the notion that aromatic-aromatic interactions (either π-π or hydrophobic) with F340 are essential for the binding of simple ergolines but not ergopeptines to 5-hydroxytryptamine$_{2A}$ receptors. Our findings support models of ergoline and ergopeptine binding to serotonin receptors that suggest that the nature of the substituent at the 8-position of the ergoline nucleus may give rise to different modes of binding for the two classes of agents, particularly with respect to the phenyl ring of F340.

Serotonin (5-HT) is an indolamine neurotransmitter that has important actions affecting most major organ systems. These include the regulation of gastrointestinal motility, central nervous system function, and cardiovascular tone (1). To mediate this large array of physiological effects of 5-HT, no fewer than 14 different receptors have evolved, all of which have distinct pharmacological properties, regional distributions, and physiological functions (2). Historically, ergots (e.g., ergoline and ergopeptine alkaloids) have been used as probes to study various 5-HT receptor subtypes. Some ergots, for instance LSD, bind to most 5-HT receptors, whereas mesulergine binds with high affinity only to receptors in the 5-HT$_2$ family (2). Additionally, some ergolines (e.g., amersergide and methysergide) and ergopeptines (e.g., bromocryptine and ergotamine) have real or potential utility in the treatment of a number of psychiatric, neurological, and medical diseases, despite their relative nonselectivity for 5-HT receptors. A detailed understanding of ergoline-5-HT receptor interactions at the molecular level could enhance our ability to design new ergoline and nonergoline derivatives with greater selectivity and efficacy.

In the absence of detailed structural information regarding the molecular details of ligand-receptor interactions, a combination of techniques such as site-directed mutagenesis, study of structure-activity relationships, and molecular modeling may be profitably used to arrive at testable models for drug-receptor binding. Previous models of 5-HT and ergoline binding to 5-HT$_{2A}$ receptors have emphasized potential face-to-face ring stacking between the aromatic ring of 5-HT (or its homologue in the ergoline nucleus) and various highly conserved phenylalanine residues. Thus, studies by ourselves (3–5) and others (6, 7) have suggested that "stacking" or "edge-to-face" π-π interactions could be essential for anchoring the aromatic groups of many serotonin receptor ligands. Based on the models proposed by Westkaemper and

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamide; DMEM, Dulbecco's modified Eagle medium; PCR, polymerase chain reaction.

Glennon (3–5) and Hibert and co-workers (6, 7), two highly conserved phenylalanine residues, F340 and F339, were implicated and mutated. Our prior work (8, 9) showed that F340L greatly diminished the 5-HT$_{2A}$ receptor affinities of 5-HT agonists and selected antagonists; in contrast, the F339L mutation significantly diminished only the affinity of ketanserin. The demonstration of the differential effects of the mutations on drug binding helped to more clearly establish a sequence alignment between helix 6 of bacteriorhodopsin and the putative helix 6 of the 5-HT$_{2A}$ receptor, allowing the generation of a more refined three-dimensional receptor model (4).

Others have studied the role of the F340 cognate amino acid for drug binding to β-adrenergic, dopaminergic, and muscarinic receptors (10–12). For all three receptors, mutation of the F340 cognate amino acid greatly affected agonist and/or antagonist binding. Although all of these studies strongly suggest that F340, or its cognate amino acid, is essential for the binding of many compounds to 5-HT$_{2A}$ and other receptors, the mechanism by which drugs utilize this residue for high affinity binding is unclear.

To further clarify the role of F340 and F339 in ligand binding, a series of eight point mutations of conserved and nonconserved phenylalanine residues in the 5-HT$_{2A}$ receptor were used to determine the affinities of eight simple ergolines and four ergopeptines. Our results are consistent with molecular models that predict that aromatic-aromatic interactions between F340 and ergolines are essential for high affinity binding to 5-HT$_{2A}$ receptors.

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO/BRL (Gaithersburg, MD). [^{3}H]Ketanserin (67 Ci/mmol), [^{3}H]inositol (15 Ci/mmol), and α-^{35}S-dATP (1500 Ci/mmol) were from New England Nuclear (Boston, MA), whereas [^{3}H]mesulergine (81 Ci/mmol) was from Amersham Corporation. Restriction enzymes were from New England Biolabs (Boston, MA), and most other molecular biology reagents were purchased from Stratagene (Torry Pines, CA) or United States Biochemicals (Cleveland, OH). *Thermus aquaticus* polymerase was from Cetus Corporation. COS-7 cells were a gift from B. Kobilka (Stanford University), whereas the 5-HT$_2$ cDNA was from D. Julius (University of California, San Francisco).

Site-directed mutagenesis. Site-directed mutagenesis was performed using a PCR technique detailed previously, using the following mutant oligonucleotide primers (bold type = mutant bases): F340L, CCATTCTTGATCACCAA; F340Y, GCCCATTCTACATCACCAA; F340A, TGCCCATTCGCCATC-ACCAA; F339L, GTGCCCATTGTTCATCACC; F339Y, GGTGCCCA-TACTTCATCAC; F339A, TGGTGCCCAGCCTTCATCACC; F125L, GCTGGGTTTGCTTGTCATG. Additional primers were upstream of the *Xho*I site and downstream of the *Bcl*I site. Asymmetric PCR was performed precisely as detailed previously (13). The F125S mutation was made via a random error in the PCR process; all sequences were verified by double-stranded DNA sequencing using overlapping primers (14) to verify that only the desired single-point mutations were produced.

Transient transfection. Transient transfection of COS-7 cells was performed exactly as detailed previously, using the DEAE-dextran technique (15, 16). Cells were harvested into DMEM (using cell scrapers) 72 hr after transfection, centrifuged at 10,000 × *g* for 10 min, and then lysed in binding buffer (50 mM Tris·HCl, pH 7.40). Lysed cells were then harvested by centrifugation at 20,000 × *g* for 20 min at 4° and were stored frozen as tight pellets at −80° until use.

Binding assays. Binding assays were performed for 90 min at 25°, in total volumes of 0.5 ml, with 5–20 μg of membrane protein in 50 mM Tris·HCl buffer, pH 7.40, as described previously (8). Membranes were harvested with a Brandel SM-24 cell harvester, followed by three ice-cold washes on polyethyleneimine (0.1%)-pretreated glass fiber filters. Filters were soaked for 18 hr in scintillation fluid before counting, with efficiency determined by the external standard method. Specific binding (determined with 10 μM mianserin) represented 90–97% of total binding in the experiments reported here; no more than 10% of total counts/assay tube were bound.

Phosphoinositide hydrolysis. COS-7 cells were transiently transfected as detailed previously, using the DEAE-dextran technique (8). At 24 hr after transfection, cells were harvested by trypsinization and split into 24-well plates with complete medium. Twenty-four hours later, cells were washed with inositol-free DMEM and then incubated for an additional 18 hr with inositol-free DMEM containing 1 μCi/ml [^{3}H]inositol and 10% dialyzed fetal calf serum. Cells were then rinsed three times with Krebs-bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 11 mM glucose). Before use the buffer was equilibrated with 95% O_2/5% CO_2. Cells were then incubated for 30 min with test agents together with 10 mM LiCl in Krebs-bicarbonate buffer. The reaction was then terminated by aspiration and the addition of 1.2 ml of methanol/water/HCl (25:25:0.1). Cells were then harvested into glass tubes and 0.6 ml of chloroform was added, followed by vigorous vortex-mixing. After phase separation, the upper aqueous phase was removed and [^{3}H]inositol monophosphate was isolated and quantified as detailed previously (17, 18).

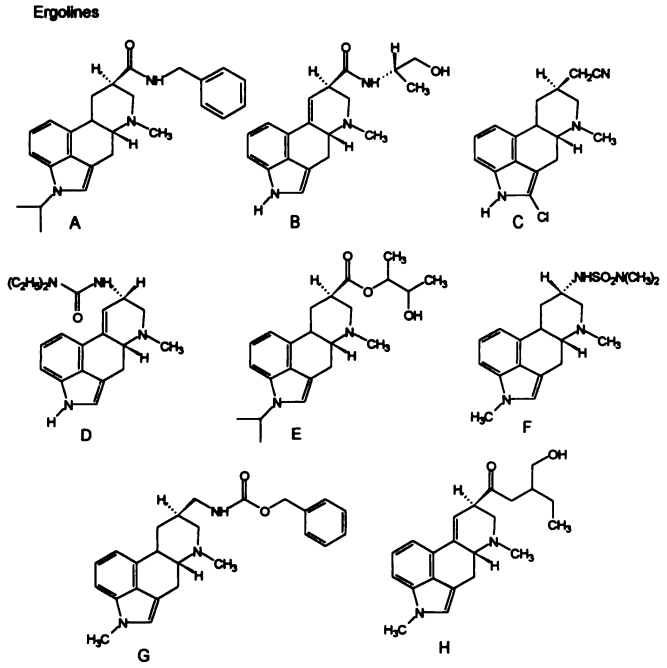
Data analysis. Protein was determined using a kit from Bio-Rad (Richmond, CA). Binding data were analyzed using the weighted, nonlinear, least-squares LIGAND program of Munson and Rodbard (19), which determines binding to multiple sites according to the law of mass action, as detailed previously (8, 19); nonspecific binding (N1 and N2 being shared parameters), B_{max} (R1 in the program), K_d (of the labeled ligand; K11 in the program), and K_i (of the unlabeled ligand; K21 in the program) were fitted parameters. For all experiments, data were sequentially analyzed by one- and two-site models. In all cases a two-site model did not significantly improve the fit (*F* test; $p > 0.05$). The goodness of fit was determined by a runs test

Molecular modeling. The 5-HT$_{2A}$ receptor model used here is identical to that described previously (4). Modeling procedures described were performed using version 6.04 of the SYBYL molecular modeling package from Tripos Associates (St. Louis, MO). Molecular mechanics minimizations were carried out using the Tripos force field with explicit consideration of all of the atoms without constraints. Conjugate gradient minimizations were carried out to a root mean squares gradient of <0.5 kcal/mol-Å^2, with a dielectric constant of 4.

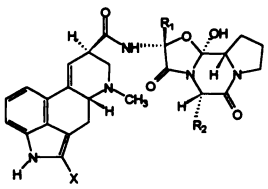
Results

Effect of the F340L mutation on ergoline and ergopeptine binding. In preliminary studies, we discovered that three simple ergolines (lisuride, mesulergine, and methysergide) (See Fig. 1 for structures) showed greatly diminished binding to the F340L mutant. Subsequent studies (Fig. 2; Table 1) with a total of eight simple ergolines demonstrated that all had greatly diminished affinities (mean, 175-fold decrease) for 5-HT$_{2A}$ receptors with the F340L mutation. In contrast, four ergopeptines (Fig. 3; Table 1) were relatively unaffected by the F340L mutation (mean, 2.9-fold decrease in affinity). [^{3}H]Ketanserin binding was unaffected by the F340L mutation, in agreement with our previous study (Table 2).

Effects of conservative and nonconservative substitutions at F340, F339, and F125 on the binding of ergolines and ergopeptines to the 5-HT$_{2A}$ receptor. Our results led us to hypothesize that an aromatic-aromatic interaction is essential for ergoline binding to 5-HT$_{2A}$ recep-



Ergopeptines



	R_1	R_2	X
Ergotamine	CH_3	$CH_2C_6H_5$	H
Ergocryptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	H
Ergocornine	$CH(CH_3)_2$	$CH(CH_3)_2$	H
Bromocriptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	Br

Fig. 1. Structures of the ergolines and ergopeptines. The following simple ergolines were used: A, amersergide; B, ergonovine; C, lergotrile; D, lisuride; E, LY53857; F, mesulergine; G, metergoline; H, methysergide.

tors via F340. To test this hypothesis, we prepared a series of point mutations at F340, F339, and, as a control, F125, to help determine the nature of the bond disrupted by the F340L mutation. Table 2 shows the results of these experiments. Table 3 shows relative numbers of receptors for each mutant receptor type; all mutants tested expressed large numbers of receptors in the transient expression system (Table 3). We also found that all receptors were coupled to phosphoinositide hydrolysis (data not shown).

As can be clearly seen, of the simple ergolines tested only LY53857 was affected by the F340Y mutation, whereas F340L and F340A mutations produced markedly diminished affinities for ergolines (Fig. 4; Table 2). The F340Y mutation altered the affinity of LY53857 to a minor extent (6-fold). If an aromatic group at the F340 position is essential, then our hypothesis would predict that F340Y should behave similarly to F340, at least with respect to ergoline binding. Because the

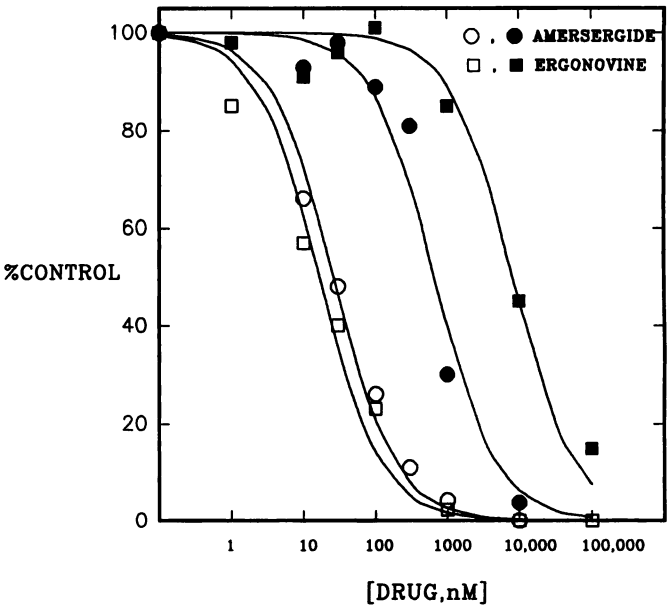


Fig. 2. Effect of the F340L mutation on amersergide and ergonovine binding to 5-HT_{2A} receptors. Shown are typical competition binding isotherms for amersergide (○, ●) and ergonovine (□, ■) inhibition of [^{3}H]ketanserin binding to 5-HT_{2A} (○, □) and F340L (●, ■) receptors transiently expressed in COS-7 cells.

TABLE 1

Effect of the F340L mutation on ergoline binding to 5-HT_{2A} receptors

Data represent mean ± standard error of computer-derived estimates for three or more separate experiments.

Drug	Binding affinity		ΔΔG
	5-HT_{2A}	F340L	
	nM		*kcal/mol*
Mesulergine	2.9 ± 0.5	1,924 ± 779[a]	3.9
Metergoline	0.4 ± 0.08	6.6 ± .96[a]	1.7
Methysergide	2.4 ± 1.7	385 ± 117[a]	3.0
Lisuride	6.2 ± 4.4	505 ± 83[a]	2.6
LY53857	1.0 ± 0.8	35 ± 12	2.1
Ergonovine	11.6 ± 5	2,287 ± 456[a]	3.1
Lergotrile	111 ± 45	>10,000[a]	ND[b]
Amersergide	6.6[c]	489[c]	2.6
Ergocryptine	43 ± 11	95 ± 43	0.5
Bromocryptine	83 ± 13	97 ± 76	0.09
Ergocornine	22 ± 5	125 ± 27[a]	1.0
Ergotamine	4.2 ± 1.2	10.6 ± 5	0.6

[a] $p < 0.01$ versus 5-HT_{2A} (*F* test).
[b] ND, not determined.
[c] Two experiments.

F340Y mutation had minimal effects on ergoline binding, our results are in accord with predictions of a binding model that proposes that an aromatic-aromatic interaction is essential for anchoring the ergoline nucleus to F340. The two ergopeptines studied (ergotamine and ergocryptine) were relatively unaffected by any tested F340 mutation.

We also examined the effects of conservative and nonconservative substitutions at F339 and F125. Ergoline binding was relatively unaffected by the F339L and F339A mutations, whereas the F339Y mutation had modest effects on both ergoline and ergopeptine binding (Table 2). The results are consistent with the notion that F339 plays no major role in ergoline or ergopeptine binding but that introduction of a new functional group (e.g., an hydroxyl group with F339Y)

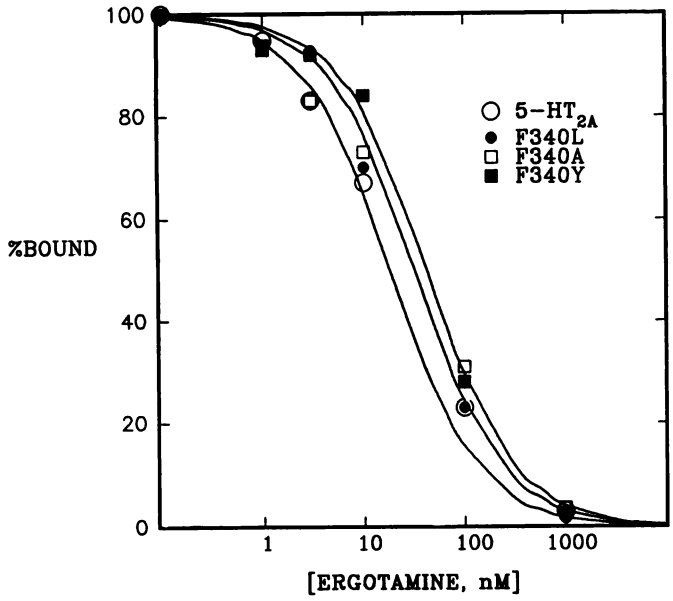


Fig. 3. Lack of effect of F340L, F340Y, and F340A mutations on ergotamine binding to 5-HT$_{2A}$ receptors. Shown are typical competition binding isotherms for ergotamine binding to 5-HT$_{2A}$, F340L, F340Y, and F340A receptors transiently expressed in COS-7 cells.

can have nonspecific effects on ligand binding (Table 2). A similar, possibly nonspecific, effect was seen with the F125S mutation, in which an aromatic residue is replaced with a polar moiety (i.e., phenylalanine to serine). With the F125S mutation, mesulergine binding affinity, but not that of ketanserin, was diminished (Table 2).

Effects of point mutations on ketanserin binding to the 5-HT$_{2A}$ receptor. Kristiansen *et al.* (20) have recently proposed a model of ketanserin binding to the 5-HT$_{2A}$ receptor. Their model predicts that F339 but not F340 is essential for ketanserin binding. Table 2 lists the affinity of ketanserin for the various 5-HT$_{2A}$ receptor mutants. Mutations of F339 had substantial effects on the affinity of ketanserin for the 5-HT$_{2A}$ receptor (7–24.5-fold loss in affinity). F340L and F340A substitutions had no effect on ketanserin binding affinity, whereas F340Y caused a substantial decrease in ketanserin affinity (72.5-fold decrease). The unexpected effects of the F340Y mutation might be explained by the introduction of a new functional group into a generally hydrophobic area. These results are, then, generally consistent with the model of Kristiansen *et al.* (20), which proposes that ketanserin is mainly anchored by F339 but that F340 is close to ketanserin and may be altered by mutations that introduce new functional groups at the 340-position.

Results of molecular modeling studies. Given the overwhelming structural similarities between the simple ergolines and the ergopeptines, it was initially difficult to reconcile the differential effects of this F340L mutation on the affinities of these two related classes of ligands. Several possible molecular interpretations of the mutagenesis data were next considered. An obvious possibility is that ergopeptines are not affected by the F340L mutation because they do not interact significantly with F340. The lack of interaction with F340 could be due to either of two possibilities, as follows: 1) the conformation of F340 is different in the ergopeptine-receptor and ergoline-receptor complexes (e.g., inaccessible to ergopeptines but accessible to ergolines) or, 2) although ergolines and ergopeptines share a common heterocyclic nucleus, they bind at different but overlapping sites (i.e., they bind in slightly different orientations). An explanation for the second possibility is that the bulky peptide portion of the ergopeptines prevents them from binding far enough down into the receptor aggregate to interact favorably with F340, whereas compounds with a smaller 8-substituent can. This second possibility was explored using the 5-HT$_{2A}$ receptor model that has recently been described (4).

Simple ergolines are conformationally rigid, with the exception of a few degrees of rotational freedom in the relatively small 8-position substituents. On the other hand, the peptide sustituent of the ergopeptines is bulky and can exist in numerous conformations, particularly with respect to its orientation relative to the ergoline ring. Crystal structures (21), NMR spectroscopy studies performed in solution (21), and computational conformation analysis studies (22) of ergopeptines show that there are a multitude of accessible conformations falling into two broad classes, i.e., those in which the peptide portion is folded over the ergoline ring (most like the X-ray crystal structures) and those in which the peptide structure is extended away from the indole ring. Conceivably, then, ergopeptines (e.g., ergotamine) can bind in either a "folded" or "extended" form (Figs. 5 and 6). Either way, ergopeptines can present a greater steric challenge to accommodation within the helical bundle of the receptor and at the same time offer greater opportunities for favorable binding interactions, compared with the ergolines.

Representatives of both the folded and extended conformational families of ergotamine were next evaluated computationally by manual docking, and the results were compared with those generated in a similar fashion for ergonovine, a representative simple ergoline. The docking process was performed with the intention of evaluating whether ergotamine and ergonovine could bind differently but in a manner consistent with a theoretical receptor model. Using the DOCK facility within SYBYL to provide real-time approximation of complex energies, multiple complexes containing ergotamine and ergonovine were generated and subjected to extensive molecular mechanics minimization. Model complexes that showed favorable calculated binding energies were evaluated with respect to potential differences in the proximity to F340. A major outcome was the realization that, as suspected, ergonovine can be accommodated "deeper" into, and more to the center of, the helical aggregate than ergotamine, due to the less sterically demanding 8-position substituent of ergonovine.

Both complexes maintained an ionic bond between the aspartate (D155) of helix 3 and the ligand ammonium ion. Fig. 7 is a superimposed stereo diagram of the ergonovine and ergotamine complexes with the 5-HT$_{2A}$ receptor. Only F340 and D155 amino acid side chains are shown. It can be seen that the phenyl ring of F340 is positioned over and nearer the indole ring of ergonovine, compared with that of ergotamine (Fig. 7).

Discussion

The major finding of this study is that a highly conserved phenylalanine, F340, is essential for optimal ergoline binding to 5-HT$_{2A}$ receptors. Because the F340Y mutant behaved essentially identically to the native receptor (i.e., F340), at

TABLE 2

Binding of ergots to various 5-HT$_{2A}$ receptor mutants

Means ± standard errors of computer-derived estimates (from the LIGAND program) are given for three or more separate experiments; otherwise results from two experiments are shown, with individual values in parentheses.

Drug	Binding affinity								
	F340A	F340L	F340Y	F339A	F339L	F339Y	F125L	F125S	5-HT$_{2A}$
					nM				
Ketanserin	0.9 ± 0.6[a]	0.3 ± 0.1[a]	29 ± 12	4.6 ± 0.4[a]	9.8 ± 4.7	2.8 ± 0.7[a]	0.38 ± 0.2[a]	0.8 (0.6, 1.0)[a]	0.4 ± 0.2[a]
Mesulergine	191 ± 30[b]	1924 ± 779[b]	2.8 ± 0.8[c]	3.1 ± 0.2[c]	5.6 ± 1.8[c]	21 ± 15[b]	1.45 ± 0.38[c]	48 ± 27[b]	2.9 ± 0.5[c]
LY53857	142 ± 16[b]	35 ± 12[b]	5.7 ± 3	6.0 ± 2	1.1 ± 0.8	10 ± 4	4.6 ± 0.6	19.6 (23, 16.2)[b]	1.0 ± 0.8
Ergotamine	6.4 ± 1.2	10.6 ± 5	9 (11, 7)	10.7 (12, 9.4)	3.9 (6, 1.8)	32 ± 4	8.95 (11, 6.9)	24.5 ± 3.5[b]	4.2 ± 1.2
Ergocryptine	15 ± 6[b]	95 ± 43	ND[d]	ND	29 (35, 23)	244 ± 130	ND	10.9 (13, 8.8)	43 ± 11
Lisuride	230 ± 91[b]	546 ± 127[b]	2.6 ± 1.8	5.2 ± 3	9 ± 3.6	18 ± 8	3.6 ± 0.7	8.0 ± 1.9	6.2 ± 4.5

[a] Ketanserin K_d determined by saturation binding studies.
[b] $p < 0.01$ versus 5-HT$_{2A}$.
[c] Mesulergine K_d determined by saturation binding studies.
[d] ND, not determined.

TABLE 3

Receptor expression for 5-HT$_{2A}$ receptor mutants

Data represent mean ± standard deviation of computer-derived estimates of the maximum number of binding sites for various 5-HT$_{2A}$ receptor mutants (three or more separate experiments). The following radioligands were used to determine the B_{max} values for each receptor: [^{3}H]ketanserin, 5-HT$_{2A}$, F340L, F340A, F339A, F339Y, F125L, and F125S; [^{3}H]mesulergine, F339L and F340Y.

Receptor	B_{max}
	pmol/mg
5-HT$_{2A}$	1.9 ± 0.25
F340L	1.1 ± 0.137[a]
F340A	1.7 ± 0.2
F340Y	1.8 ± 0.17
F339L	0.9 ± 0.15[a]
F339A	1.2 ± 0.3[a]
F339Y	1.46 ± 0.16[a]
F125L	2.15 ± 0.25
F125S	1.7 ± 0.3

[a] $p < 0.05$ versus 5-HT$_{2A}$ (F test).

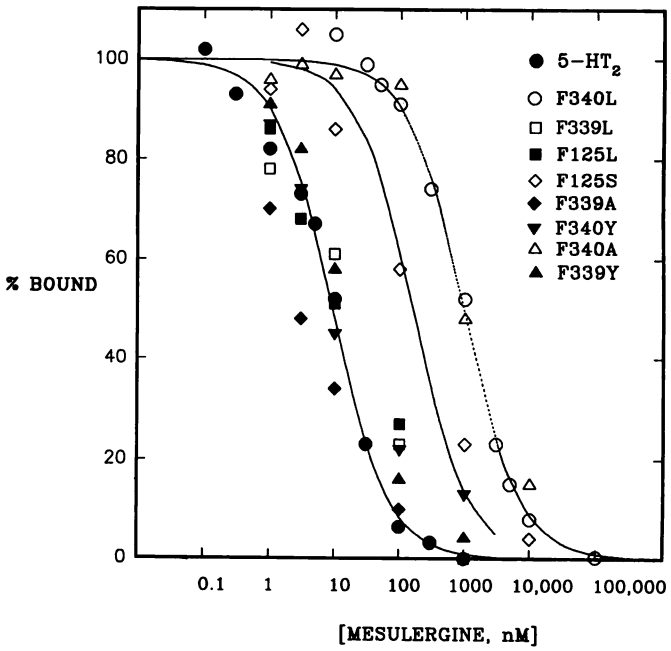


Fig. 4. Effects of multiple point mutations of conserved and nonconserved phenylalanine residues on mesulergine binding to 5-HT$_{2A}$ receptors. Shown are typical competition binding isotherms for mesulergine binding to 5-HT$_{2A}$, F340L, F340Y, F340A, F339L, F339Y, F339A, F125L, and F125S receptors transiently expressed in COS-7 cells.

least with respect to ergolines, the data are consistent with the hypothesis that an aromatic residue at position 340 is essential for high affinity ergoline binding. These results support previous molecular modeling studies that implicated a role for F340 in the binding of serotonergic ligands. The present findings also support models of ergoline binding that predict that the aromatic rings of the ergoline nucleus are stabilized by face-to-face ring stacking (π-π bonds) or edge-to-face interactions. Because F340 is conserved among all G protein-coupled 5-HT receptors and because LSD and other ergolines bind nonselectively to many 5-HT receptors, our results predict that the F340 cognate amino acid should be important for ergoline binding to other G protein-coupled 5-HT receptors.

Our results also imply that the phenylalanine residue adjacent to F340 (F339) is relatively unimportant for ergoline and ergopeptine binding, because the F339L and F339A mutations had little effect on ergoline or ergopeptine binding. Interestingly, all mutations of F339 markedly diminished the affinity of ketanserin for the 5-HT$_{2A}$ receptor, whereas the only mutation of F340 that altered ketanserin binding was F340Y. In general, the results of the F339 mutations are consistent with the notion that a phenyl ring at position 339 stabilizes ketanserin in the binding pocket. Apparently F340 is close to the binding site, because F340Y showed greatly diminished affinity for ketanserin (35-fold loss of affinity). The unexpected effect of the F340Y mutation on ketanserin binding might be explained on the basis of the introduction of a new functional group into a hydrophobic binding pocket (see below). Taken together, these results support a model for ketanserin binding to the 5-HT$_{2A}$ receptor that was recently published by Dahl and co-workers (20).

One possibility that was not addressed by these studies is that the mutations we constructed induced their effects by changes in overall protein conformation and not by selective effects near the binding site. To completely rule out this possibility will require structural information of a type that is currently unavailable with current technology (e.g., crystallography). Fersht and colleagues (23–25), in their studies of mutations of enzymes for which detailed structural information is available, provided a number of tests that could be applied to mutated proteins to determine whether gross structural changes are induced by the mutations.

Fersht *et al.* (23) proposed, first, that the replacement

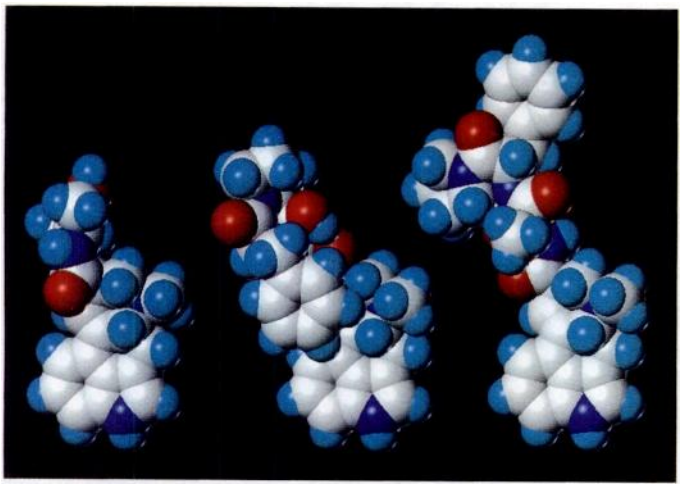


Fig. 5. Space-filling models viewed perpendicularly to the plane of the indole ring. Shown are space-filling models perpendicular to the plane of the indole rings of ergonovine (*left*), the folded conformation of ergotamine (*middle*), and the extended conformation of ergotamine (*right*).

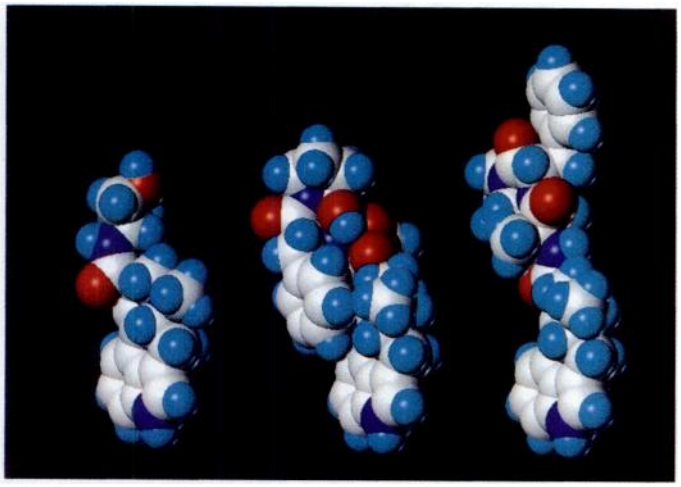


Fig. 6. Space-filling models viewed from the edge of the indole ring. Shown are space-filling models viewed from the edge of the indole rings for ergonovine (*left*), the folded conformation of ergotamine (*middle*), and the extended conformation of ergotamine (*right*).

residue be smaller than the native residue and that additional functional groups not be added. With the exception of the F340Y, F339Y, and F125S mutations, all of our mutants fulfill these criteria. The inconsistent results with the F340Y, F339Y, and F125S mutations can be explained by the fact that potentially polar residues have been inserted into a generally hydrophobic binding pocket.

Fersht (25) also proposed that point mutations that affected the binding of certain substrates (but not others) were unlikely to grossly affect the conformation of the protein. Because all of the mutations we produced had relatively selective effects on ligand binding, it is unlikely, by this criterion, that large conformational changes were induced by the mutations we constructed. We also found experimentally that the $\Delta\Delta G$ values for the F340L mutation were all <4 kcal/mol, as predicted by Fersht and co-workers (23–25) for mutations that do not affect the overall conformation of the protein. Finally, all mutant receptors were positively coupled to second messenger production,[1] indicating that the functional properties of the receptor were not abolished by the various mutations we constructed.

Another major finding of this study is that F340 is not essential for ergopeptine binding. Given the structural similarities between ergolines and ergopeptines, this was not anticipated. A typical assumption made when contemplating the ways in which two structurally related compounds may bind to a receptor is that a correspondence exists between the shared structural features and that neither the ligand conformation nor the receptor binding site conformation changes upon complex formation. That is, ligands are thought to bind at the receptor in a fashion suggested by superimposition of the ligands themselves. Although there are many cases for which this assumption proves accurate, there are some recent experimentally verified exceptions (see Ref. 3 for a recent review). The ergopeptines and ergolines share a common polycyclic nucleus, with the only major difference between the two classes of compounds being the nature of the 8-position substituent. It was, therefore, tempting to assume that the common structural elements of both occupy the same space within the receptor. The mutagenesis data presented here strongly suggest otherwise.

The differences in the sensitivity of ergopeptines and ergolines to the F340 mutations must be attributed to their difference in structure, which, in turn, results in different orientations of the ligands in the receptor-ligand complex, at least with respect to F340. In either the extended or folded conformation, the peptide portion of ergopeptines could provide a steric encumbrance and may limit the access of the aromatic portion of the ligand to F340, which is deep within the helical aggregate (see Results). The presence of the peptide substituent may also provide alternative ligand-receptor interactions not available to simple ergolines that lack the peptide moiety.

The computer simulation studies showed that ergotamine can be accommodated in the cavity formed by helices 3, 4, 5, and 6, but in a fashion somewhat different from that for simple ergolines. Examination of the affinities of ligands for the F340L mutant separates compounds into two groups, 1) those compounds whose affinities are negligibly affected (<5-fold) (group 1) and 2) those compounds whose affinities are greatly affected (>35-fold) (group 2). Group 1 compounds are all ergopeptines and group 2 compounds are all ergolines, with a relatively smaller substituent in the 8-position. We hypothesize that ergolines bind to the transmembrane domain 3 aspartate (which is near the extracellular side of the receptor) with the indole nitrogen pointed toward the intracellular side and the 8-position pointed toward the extracellular surface (i.e., "up"). F340 is at the level of the indole of the ergolines, in a position such that it could interact favorably (in either a ring-stacking or edge-to-face configuration). The peptide portion of the ergopeptines would then be pointed "up" and might be near the helical terminus; this may be the only way such a bulky substituent can be sterically accommodated.

The single exception to this categorization is metergoline, for which affinity is decreased approximately 17-fold by the F340L mutation. Although modeling studies were explicitly performed only for ergotamine and ergonovine, it is possible

[1] M. S. Choudhary and B. L. Roth, manuscript in preparation.

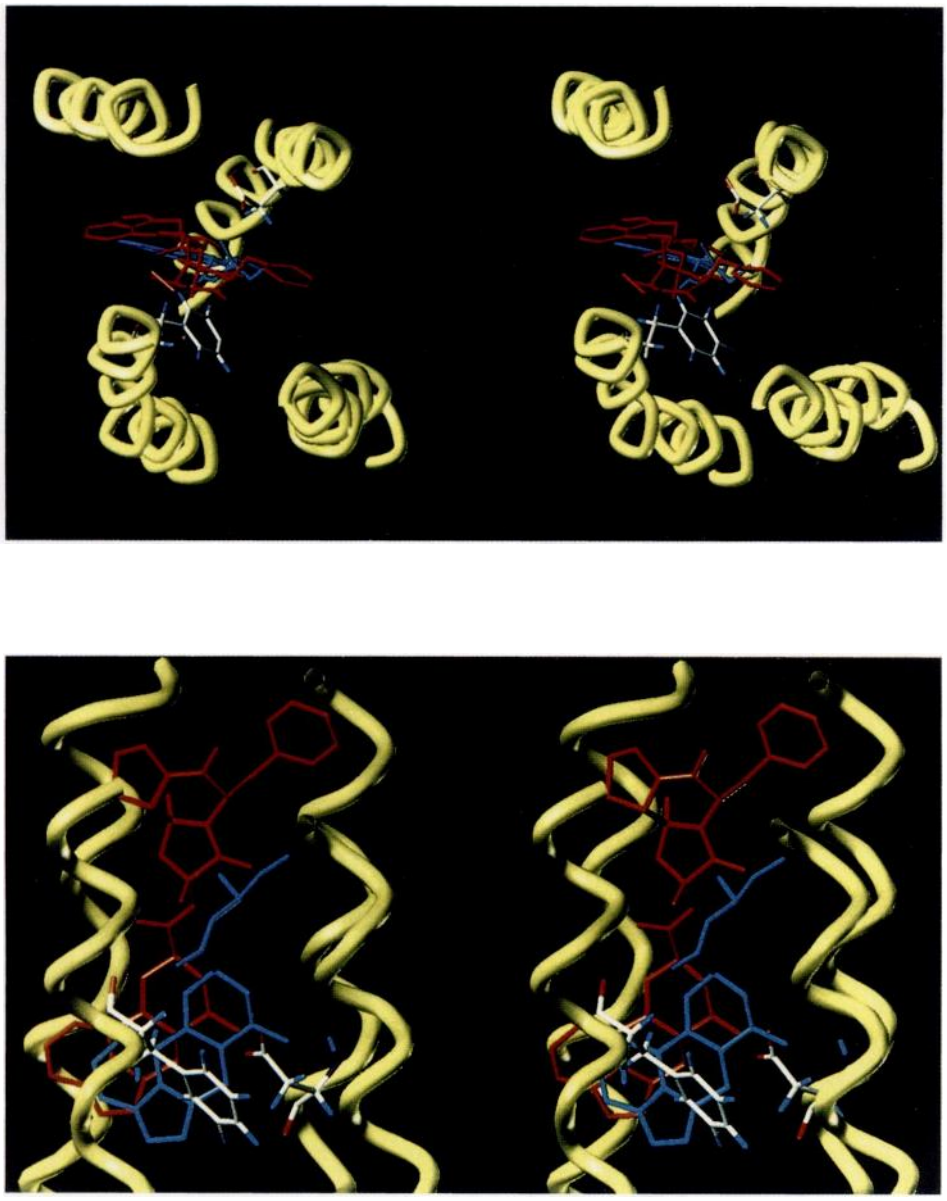


Fig. 7. Stereo views of the 5-HT_{2A} receptor complexes with ergonovine (*cyan*) and ergotamine (*red*). The helical backbones for transmembrane domains 3, 4, 6, and 7 are shown in *yellow*, with the side chain atoms for D155 and F340 colored according to atom type. Other helices and residues are not shown, for clarity. *Top*, view from extracellular side perpendicular to the membrane surface; *Bottom*, view in the plane of the membrane.

that metergoline binds to the receptor in a fashion that is intermediate between the two limiting cases represented by ergopeptines and ergolines. Interestingly, the 8-position substituent of metergoline is the largest of all of the ergolines and may result in a more ergopeptine-like ligand-receptor complex.

In fact, the introduction of the peptide substituent on the ergot nucleus does not significantly change affinity, as can be clearly seen with the native receptor. Although the substituent contributes many functional groups that could provide favorable interactions with many receptor features, any such favorable interactions must be accompanied by a loss of favorable interactions attributable to the ergot nucleus, perhaps because the ergot component binds in a different manner than if the peptide were absent (i.e., with the indole nuclei of ergopeptines and ergolines binding at different sites).

There is another possibility that is not addressed by these studies. Previous studies of peptide ligand binding to G protein-coupled receptors have suggested that many peptides interact, in part, with the extracellular domains (see Ref. 26 for review). It is conceivable that the peptide portion of ergopeptines also binds near the extracellular surface of helical aggregates of the 5-HT_{2A} receptor, thus preventing the indole nucleus of ergopeptines from reaching the distant F340.

Our results also allow us to tentatively dock the ergoline nucleus at three loci within the 5-HT_{2A} receptor, i.e., D155, F340, and S242. Prior studies by Wang *et al.* (27) demonstrated that D155 was essential for LSD binding, whereas Johnson *et al.* (28) found that an A242S mutation inhibited the binding of *N*-substituted ergolines. Our results highlight the importance of an aromatic group at position 340. Further studies, which are in progress, will allow us to assign additional residues essential for ergoline-5-HT_{2A} receptor binding.

Clearly the presence of a phenylalanine at the cognate 340-position, as may be seen in α- and β-adrenergic receptors, is not sufficient for ergot binding, because these two receptor types differ markedly in their affinities for ergolines. Whether ergot binding is affected by mutations of the F340

cognate in α- and β-adrenergic receptors is unknown. Obviously, there must be additional residues that are found in α- and not β-adrenergic receptors that are essential for high affinity ergot binding.

In summary, our results clearly demonstrate that a highly conserved aromatic residue, F340, is essential for the binding of simple ergolines, but not ergopeptines, to the 5-HT$_{2A}$ receptor. Our results with the ergolines are in agreement with models that postulate that ergolines utilize aromatic residues for aromatic-aromatic interactions in binding to G protein-coupled receptors. The observed lack of effect of the F340L and F340A mutations on ergopeptine binding is unexpected and would not have been predicted based on existing 5-HT$_{2A}$ receptor models. Additional modeling studies were conducted in an attempt to account for the different findings, and several possible explanations, including one that envisions ergopeptines and ergolines binding at "shallower" and "deeper" positions, respectively, in the receptor cavity, are provided. These results highlight the power of a combined mutagenesis/molecular modeling approach to understanding the features of receptor-ligand interactions. Our results further suggest that the processes that govern ligand-receptor binding are likely to be more complicated than previously contemplated.

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