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A Single Point Mutation (Phe^{340}→Leu^{340}) of a Conserved Phenylalanine Abolishes 4-[^{125}I]Iodo-(2,5-dimethoxy)phenylisopropylamine and [^{3}H]Mesulergine But Not [^{3}H]Ketanserin Binding to 5-Hydroxytryptamine$_2$ Receptors

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SUMMARY

The molecular processes by which agonists and antagonists bind to serotonin$_2$ [5-hydroxytryptamine (5-HT$_2$)] receptors are currently unknown. Three molecular models have proposed that conserved aromatic residues help to anchor the phenyl ring of 5-HT via stacking or π-π-type interactions with the 5-HT$_2$ receptor. To test these models we made single point mutations (Phe^{339}→Leu^{339} and Phe^{340}→Leu^{340}) of two aromatic residues that are conserved among all guanine nucleotide-binding protein-coupled 5-HT receptors and a single point mutation (Phe^{125}→Leu^{125}) that exchanges a 5-HT$_2$ for a 5-HT$_{1c}$ sequence. [^{3}H]Mesulergine binding was abolished by Phe^{340}→Leu^{340} and unchanged with the Phe^{339}→Leu^{339} and Phe^{125}→Leu^{125} mutations, whereas [^{3}H]ketanserin binding affinity was diminished by the Phe^{339}→Leu^{339} mutation and unchanged by Phe^{340}→Leu^{340} and Phe^{125}→Leu^{125}. We also found that the affinities of three ergot derivatives (mesulergine, methysergide, and lisuride) were decreased by 88–1079-fold with only the Phe^{340}→Leu^{340} mutation. We also discovered that 4-[^{125}I]iodo-2,5-(dimethoxy)phenylisopropylamine (DOI) binding was abolished in COS-7 cells expressing 5-HT$_2$ (Phe^{340}→Leu^{340}) receptors but maintained in cells expressing the Phe^{339}→Leu^{339} and Phe^{125}→Leu^{125} mutations. Additionally, the K_i values for several agonists and partial agonists (5-HT, DOI, *m*-chlorophenylpiperazine, trifluoromethylphenylpiperazine, bufotenine, and MK-212) were greatly diminished (26–14,000-fold decrease) only with the Phe^{340}→Leu^{340} receptor mutation. Finally, the Phe^{340}→Leu^{340} mutant receptors displayed an attenuated or abolished ability to augment phosphoinositide hydrolysis in COS-7 cells with four separate agonists (5-HT, MK-212, bufotenine, and quipazine). Taken together, these results are consistent with the idea that agonists and certain ergot derivatives anchor to 5-HT$_2$ receptors, in part, via specific interactions with aromatic residue Phe^{340} located in transmembrane region VI.

5-HT$_2$ serotonin receptors appear to mediate several important effects of 5-HT (serotonin), including vascular and uterine smooth muscle contraction (1), platelet aggregation (2), and, possibly, certain central nervous system effects of 5-HT (3). Additionally, many hallucinogens, including lysergic acid diethylamide, DOI, 4-bromo-(2,5-dimethoxy)phenylisopropylamine, and others (4, 5), bind to 5-HT$_2$ receptors with high affinity (6, 7). Finally, a large number of psychotherapeutic agents, including certain antidepressants and antipsychotic agents (8–13), display high affinities for 5-HT$_2$ receptors. Interestingly, it appears that a larger variety of compounds can bind to the 5-HT$_2$ receptor than to the closely related 5-HT$_{1C}$ receptor (14, 15). The molecular mechanisms by which agonists and antagonists bind to 5-HT$_2$ receptors are currently unknown.

We recently began to investigate the molecular details of the binding of various drugs to 5-HT$_2$ and 5-HT$_{1C}$ receptors, and we discovered that, for instance, typical and atypical antipsychotic agents apparently prefer the 5-HT$_2$ receptor over the 5-HT$_{1C}$ receptor (15). We also determined, using chimeric 5-HT$_2$/5-HT$_{1C}$ receptors, that 5-HT$_2$-selective antagonists of various structural classes utilize, in part, distinct regions of the 5-HT$_2$ receptor for high affinity binding (16, 17).

Several recently described molecular models predict certain types of interactions between 5-HT and specific substituents of the 5-HT$_2$ receptor molecule. These models (18–19b) differ markedly with respect to which aspartic acid residues are

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; DOI, 4-iodo-(2,5-dimethoxy)phenylisopropylamine; G protein, guanine nucleotide-binding protein; DMEM, Dulbecco's modified Eagle medium; TM, transmembrane region; PCR, polymerase chain reaction.

proposed to anchor the charged amine moiety of 5-HT and the specific aromatic residues necessary for stabilizing the phenyl ring of 5-HT. These models are interesting because they tend to agree with previous models of 5-HT and hallucinogen binding to 5-HT_2 receptors that emphasized a planar type interaction with unknown moieties of the receptor molecule (20, 21).

Based upon these models and our previous chimeric receptor studies, we made three single-point mutations, i.e., two of conserved phenylalanine residues located in TMVI (Phe^{339}→Leu^{339} and Phe^{340}→Leu^{340}) and one of a phenylalanine that is found in the 5-HT_2 receptor but not the 5-HT_{1c} receptor (Phe^{125}→Leu^{125}). Phe^{340} but not Phe^{339} was explicitly proposed by Hibert *et al.* (18) to be essential for 5-HT binding; Edvardssen *et al.* (19a) proposed that Phe^{340} is not involved in 5-HT binding but is involved in ritanserin binding to 5-HT_2 receptors. We discovered that mutating Phe^{340} to Leu^{340} abolished both [^{125}I]DOI and [^{3}H]mesulergine binding to 5-HT_2 receptors. The Phe^{340}→Leu^{340} mutation also diminished the affinity of 5-HT for the 5-HT_2 receptor. Our results are in accord with previous models that suggested that Phe^{340} is important for 5-HT binding.

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO/BRL (Gaithersburg, MD). [^{3}H]Ketanserin (67 Ci/mmol), [^{125}I]DOI (2200 Ci/mmol), [^{3}H]inositol (15 Ci/mmol), ^{125}I-lysergic acid diethylamide (2200 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), with most other molecular biology reagents being purchased from Stratagene (Torry Pines, CA) or United States Biochemicals (Cleveland, OH). Taq polymerase was from Cetus Corporation. COS-7 cells were a gift of B. Kobilka (Stanford University), whereas the 5-HT_2 cDNA was from D. Julius (University of California, San Francisco).

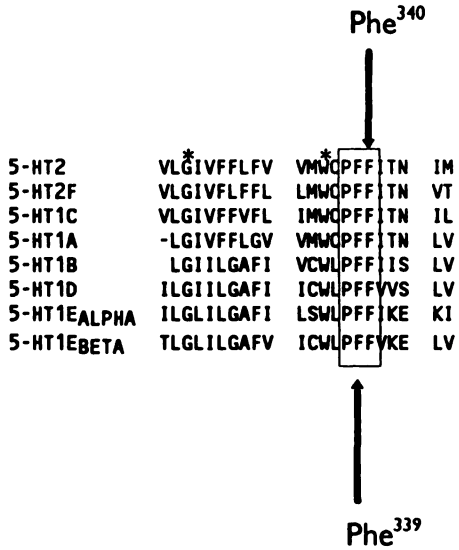

Fig. 1. Phe^{340} and Phe^{339} are conserved among G protein-coupled serotonin receptors. Shown are the aligned sequences for the 5-HT_2 (28), 5-HT_{1C} (29), 5-HT_{2F} (30a, 30b), 5-HT_{1A} (31), 5-HT_{1B} (32), 5-HT_{1D} (33), 5-$HT_{1E\alpha}$ (34, 35), and 5-$HT_{1E\beta}$ (36) receptors for putative TMVI. Sequences were aligned using the Hitachi software command ALIGN and the published sequences of the individual receptors. *, Conserved residue. Phe^{340} and Phe^{339} are found in analogous locations in all G protein-coupled catecholamine and histamine receptors thus far cloned (see Refs. 18 and 19a).

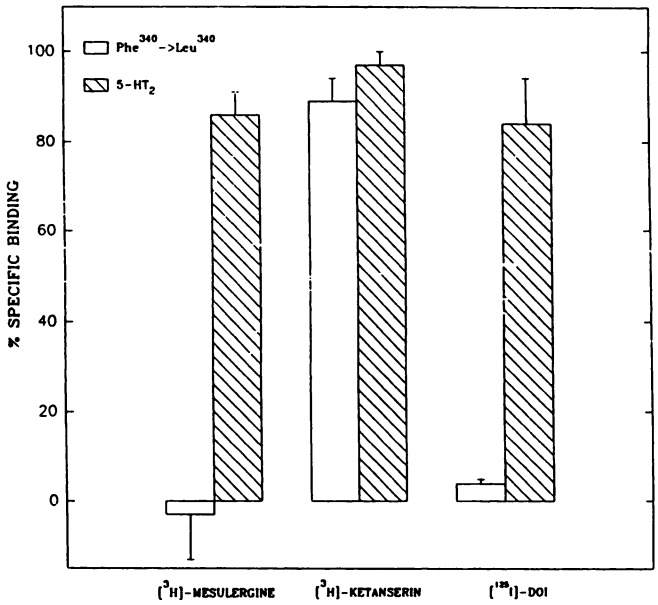

Fig. 2. A single point mutation abolishes [^{3}H]mesulergine and [^{125}I]DOI but not [^{3}H]ketanserin binding to 5-HT_2 receptors. Shown are typical results obtained by measuring [^{3}H]mesulergine (1 nM), [^{3}H]ketanserin (1 nM), or [^{125}I]DOI (0.5 nM) binding to 5-HT_2 (■) and 5-HT_2(Phe^{340}→Leu^{340}) (□) receptors transiently expressed in COS-7 cells. Data are expressed in terms of percentage of specific binding.

TABLE 1

Expression of [^{3}H]ketanserin binding by various mutant 5-HT_2 receptors

Shown are means ± standard deviations of computer-derived estimates for K_d and B_{max} values for 5-HT_2 and mutant 5-HT_2 receptors transiently expressed in COS-7 cells.

Receptor	K_d	B_{max}
	nM	fmol/mg
5-HT_2	0.4 ± 0.2	1900 ± 250
Phe^{340}→Leu^{340}	0.23 ± 0.03	1100 ± 137[a]
Phe^{339}→Leu^{339}	9.9 ± 5[b]	912 ± 150[a]
Phe^{125}→Leu^{125}	0.37 ± 0.3	2150 ± 248

[a] $p < 0.02$ versus 5-HT_2 B_{max}.
[b] $p < 0.01$ versus 5-HT_2 K_d.

Site-directed mutagenesis. Site-directed mutagenesis was performed using a PCR technique (22), with the mutant oligonucleotides CCATTCTTGATCACCAA (Phe^{340}→Leu^{340}), GTGCCCATTGTTCATCACC (Phe^{339}→Leu^{339}), and GCTGGGTTTGCTTGTCATG (Phe^{125}→Leu^{125}) (boldtype, point mutation), as follows for Phe^{339}→Leu^{339} and Phe^{340}→Leu^{340}. A 1000-base pair *Xho*I fragment of the 5-HT_2 cDNA was subcloned into pKS−. For Phe^{125}→Leu^{125} a *Kpn*I fragment was subcloned into KS−. Asymmetric PCR was performed using 100 pmol of T7 primer, 1 pmol of mutant oligonucleotide, 0.1 pmol of template, and standard PCR buffer conditions for 30 cycles (16), with annealing at 22° for 1 min, extension at 72° for 1.5 min, and denaturation at 94° for 0.5 min. The single-stranded product was gel purified and used to prime double-stranded PCR as follows: single-stranded product (5 pmol), T3 primer (5 pmol), template (0.1 mol), and standard PCR buffer conditions, with 30 cycles of 55° annealing (1 min), 72° extension (1.5 min), and 94° denaturation (0.5 min). The product was gel purified, digested with *Xho*I, and subcloned into *Xho*I-digested pSVK3-SR2 (16). The inserted fragment was completely sequenced using the dideoxynucleotide technique (23), with overlapping primers, to verify that only single-point mutations occurred.

Transient transfection. Transient transfection of COS-7 cells was

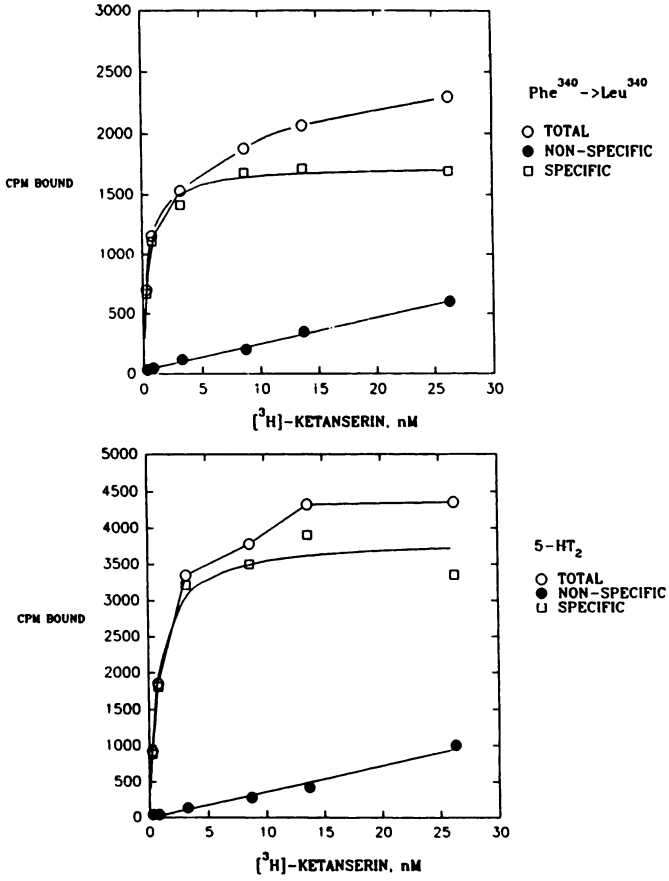

Fig. 3. Saturation binding isotherms for [^{3}H]ketanserin for control and mutant receptors. Shown are typical results obtained with 5-HT$_2$ and 5-HT$_2$(Phe^{340}→Leu^{340}) receptors for total binding (○), nonspecific binding determined with 10 μM mianserin (●), and specific binding (□). The B_{max} for the 5-HT$_2$ receptors was 1900 ± 250 fmol/mg, whereas the 5-HT$_2$(Phe^{340}→Leu^{340}) receptors had a B_{max} of 1100 ± 137 fmol/mg ($p < 0.01$ versus 5-HT$_2$). The experiment has been replicated three times.

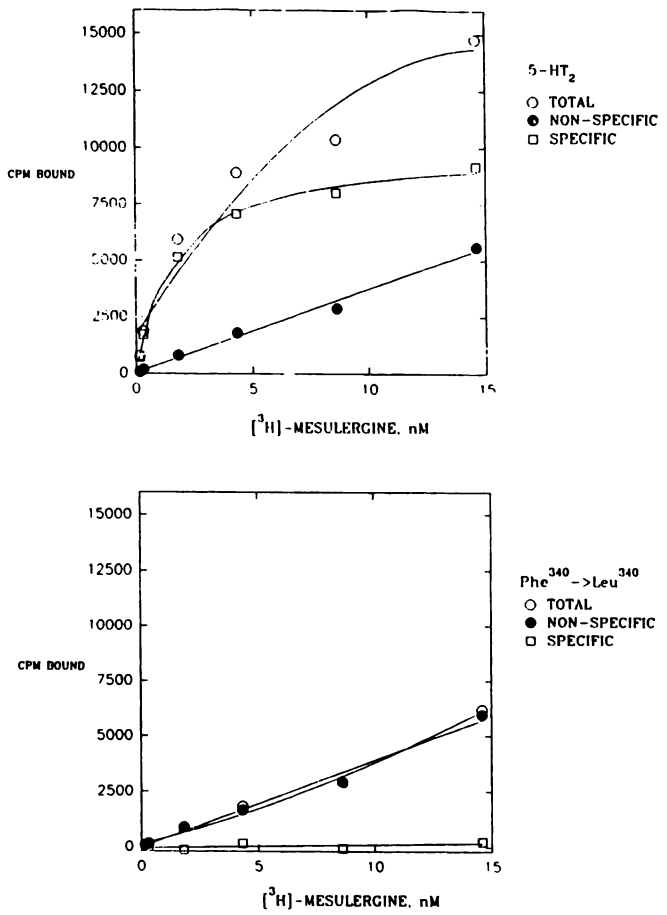

Fig. 4. Saturation binding isotherms for [^{3}H]mesulergine for control and mutant receptors. Shown are typical results obtained with 5-HT$_2$ and 5-HT$_2$(Phe^{340}→Leu^{340}) receptors for total binding (○), nonspecific binding determined with 10 μM mianserin (●), and specific binding (□). The experiment was performed three times.

performed exactly as detailed previously (15, 16). Cells were harvested into DMEM using cell scrapers, 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 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 in total volumes of 0.5 ml (for ^{3}H-radioligands) or 0.2 ml (for ^{125}I-radioligands) at 25° for 90 min, with 5–20 μg of membrane protein in 50 mM Tris·HCl buffer (pH 7.40), as described previously (9, 15, 16). For agonist binding a different binding buffer was used (50 mM Tris·HCl, 0.5 mM EDTA, 10 mM $MgCl_2$, 0.05% ascorbic acid). Membranes were harvested with a Brandel SM-24 cell harvester followed by three ice-cold washes onto polyethyleneimine-pretreated (0.1%) glass fiber filters. Filters were allowed to soak 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 using the DEAE-dextran technique, as detailed previously (15, 16). At 24 hr after transfection, cells were harvested by trypsinization and split into 24-well plates with complete medium. Cells were washed with inositol-free DMEM 24 hr later 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 a Krebs bicarbonate buffer of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 25; and glucose, 11. 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 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 vortexing. After phase separation, the upper aqueous phase was removed and [^{3}H]inositol monophosphate was isolated and quantified as detailed previously (24, 25).

Data analysis. Binding data were analyzed using a weighted, nonlinear, least-squares program that determines binding to multiple sites using the law of mass action (LIGAND program), as detailed previously (26). Protein was determined using a Bio-Rad kit (Richmond, CA).

Results

Expression of [^{3}H]ketanserin but not [^{3}H]mesulergine binding in cells expressing 5-HT$_2$(Phe^{340}→Leu^{340}). Fig. 1 shows the location of the specific point mutations for the present study. Binding studies performed with [^{3}H]ketanserin, [^{3}H]mesulergine, and [^{125}I]DOI (Fig. 2) demonstrated that only specific [^{3}H]ketanserin binding was measurable in COS-7 cells expressing 5-HT$_2$(Phe^{340}→Leu^{340}) receptors. Experiments performed at the same time with COS-7 cells transfected with the 5-HT$_2$ cDNA demonstrated that the 5-HT$_2$ receptor expressed appropriate amounts of specific [^{3}H]ketanserin, [^{3}H]mesulergine, and [^{125}I]DOI binding (see Fig. 2). Additionally, cells expressing Phe^{339}→Leu^{339} and Phe^{125}→Leu^{125} receptors ex-

TABLE 2

A single point mutation profoundly alters agonist and ergot binding to 5-HT$_2$ receptors

Shown are means ± standard deviations of K_i (or K_d) values for computer-derived estimates for three or more separate experiments. Typically [^{3}H]ketanserin (0.3–1.0 nM) was used for competition binding studies, in which 90% specific binding was routinely found.

Drug	K_i			
	5-HT$_2$	Phe^{340}→Leu^{340}	Phe^{339}→Leu^{339}	Phe^{125}→Leu^{125}
	nM			
Ketanserin	0.4 ± 0.2[a]	0.23 ± 0.03[a]	9.9 ± 5[a,b]	0.37 ± 0.3[a]
Ritanserin	0.1 ± 0.05	0.17 ± 0.08	ND[c]	ND[c]
Spiperone	0.29 ± 0.05	4.8 ± 1.5[b]	0.83 ± 0.3	0.63 ± 0.17
Haloperidol	30 ± 2.4	202 ± 72[a]	101 ± 64	43 ± 13
Mesulergine	3.28 ± 0.83[a]	3,540 ± 1,784[b]	5.6 ± 1.8[a]	1.45 ± 0.38[a]
Methysergide	2.95 ± 1.8	785 ± 117	13 ± 5[b]	1.6 ± 0.6
Lisuride	6.2 ± 4.5	546 ± 127	9 ± 3.6	3.6 ± 0.7
5-HT	1,320 ± 226	35,065 ± 10,873[b]	557 ± 266	929 ± 600
m-CPP[d]	231 ± 99	16,362 ± 934[b]	ND	576 ± 74
TFMPP	256 ± 37	30,000 ± 9,900[b]	ND	602 ± 143
DOI	0.92 ± 0.4[a]	13,667 ± 10,877[b]	3.5 ± 2.1[a]	3.2 ± 1.6[a]
Quipazine	886 ± 63	448 ± 246	2,667 ± 1,626	949 ± 294
MK-212	8,532 ± 2,955	≫100,000[b]	7,555 ± 4,165	5,597 ± 1,194
Bufotenine	649 ± 52	>100,000[b]	217 ± 65	438 ± 35

[a] K_d value.
[b] $p < 0.001$ versus 5-HT$_2$ K_i, using F test.
[c] ND, not determined.
[d] *m*-CPP, *m*-chlorophenylpiperazine; TFMPP, trifluoromethylphenylpiperazine.

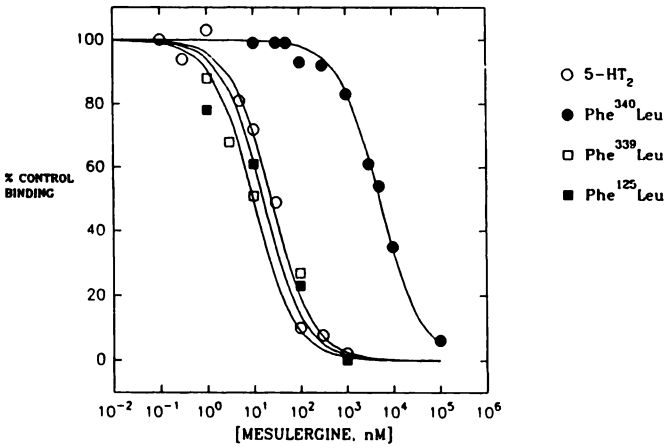

Fig. 5. Effect of a single point mutation on mesulergine binding to the 5-HT$_2$ receptor. Shown are typical competition binding isotherms for mesulergine inhibition of [^{3}H]ketanserin binding to 5-HT$_2$ (○), Phe^{125}→Leu^{125} (■), Phe^{339}→Leu^{339} (□), and Phe^{340}→Leu^{340} (●) receptors transiently expressed in COS-7 cells.

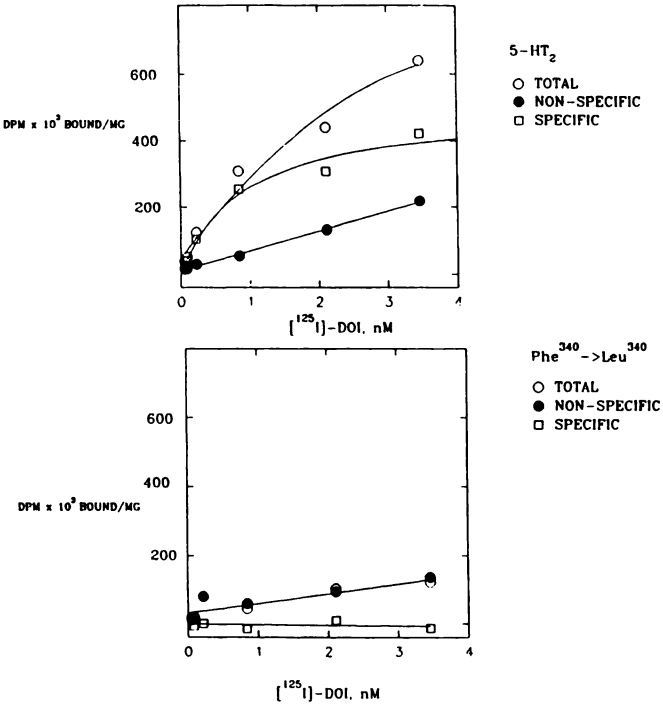

Fig. 6. Effect of a single point mutation on [^{125}I]DOI binding. Shown are typical saturation binding isotherms for [^{125}I]DOI binding to 5-HT$_2$ and 5-HT$_2$(Phe^{340}→Leu^{340}) receptors transiently expressed in COS-7 cells, for total binding (○), nonspecific binding determined with 10 μM mianserin (●), and specific binding (□). The experiment was replicated three times.

pressed specific [^{3}H]ketanserin, [^{3}H]mesulergine, and [^{125}I]DOI binding (data not shown). Table 1 gives the K_d and B_{max} values for the mutant and parent 5-HT$_2$ receptors. As is seen, the Phe^{339}→Leu^{339} mutation significantly attenuated [^{3}H]ketanserin affinity for the 5-HT$_2$ receptor. All four receptor cDNAs expressed large numbers of receptors, although both the Phe^{339}→Leu^{339} and Phe^{340}→Leu^{340} mutant receptors expressed approximately 50% fewer [^{3}H]ketanserin binding sites. We next performed saturation binding experiments to determine whether the loss of [^{3}H]mesulergine binding with the Phe^{340}→Leu^{340} receptors was due to altered affinity or the number of binding sites.

Figs. 3 and 4 show saturation binding isotherms for [^{3}H]ketanserin and [^{3}H]mesulergine binding to 5-HT$_2$ and 5-HT$_2$(Phe^{340}→Leu^{340}) receptors transiently expressed in COS-7 cells. As is clear, both receptors bound [^{3}H]ketanserin, whereas only the native receptor apparently bound [^{3}H]mesulergine with high affinity. The calculated affinity constant for [^{3}H]mesulergine is found in Table 2.

Characterization of antagonist binding affinities for mutant 5-HT$_2$ receptors. We next studied the pharmacology of the mutant receptors. For these experiments, the mutant receptors and the native receptor were studied simultaneously. Additionally, the same stock of unlabeled competing drug was used for all studies.

Fig. 5 shows competition binding isotherms for [^{3}H]mesuler-

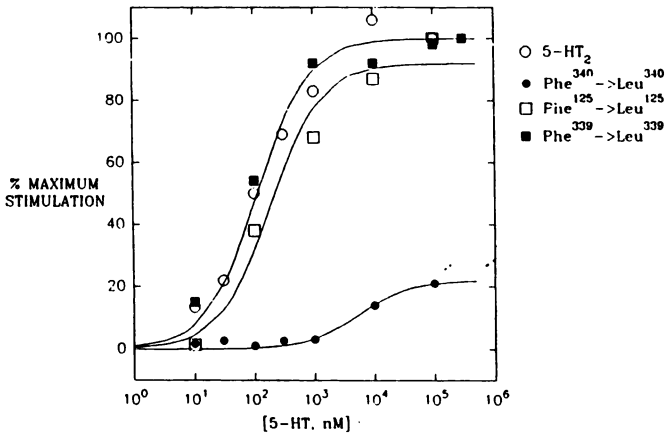

Fig. 7. Effect of a single point mutation on the ability of 5-HT to augment [^{3}H]inositol monophosphate accumulation in COS-7 cells. Dose-response studies for 5-HT-induced accumulation of [^{3}H]inositol monophosphate for a typical experiment are shown for 5-HT$_2$ (○), Phe^{340}→Leu^{340} (●), Phe^{339}→Leu^{339} (■), and Phe^{125}→Leu^{125} (□) receptors. For comparison, the maximum amount of [^{3}H]inositol monophosphate accumulation induced by 5-HT$_2$ receptors, in a typical experiment, was 12,000 cpm, with a basal accumulation of 920 cpm.

gine binding to Phe^{340}→Leu^{340}, Phe^{339}→Leu^{339}, Phe^{125}→Leu^{125}, and 5-HT$_2$ receptors. As is clearly seen, 5-HT$_2$(Phe^{340}→Leu^{340}) receptors showed diminished affinities for unlabeled mesulergine (Fig. 5; Table 2). Table 2 shows the calculated K_i values for several additional 5-HT$_2$ antagonists. Spiperone and haloperidol affinities were decreased only with the Phe^{340}→Leu^{340} mutation, whereas ketanserin affinity was significantly altered with the Phe^{339}→Leu^{339} mutation. As is clear from Table 2, three ergot derivatives (lisuride, methysergide, and mesulergine) had 88–1079-fold lower affinities for the Phe^{340}→Leu^{340} mutant receptors. In contrast, the Phe^{339}→Leu^{339} mutation significantly diminished only methysergide binding (4.4-fold decrease). These results imply that Phe^{340} is essential for mesulergine, methysergide, and lisuride binding to 5-HT$_2$ receptors.

Characterization of agonist binding affinities for 5-HT$_2$(Phe^{340}→Leu^{340}) receptors. We next examined agonist binding profiles. As is shown in Fig. 6, [^{125}I]DOI had high affinity for the parent receptors, whereas no specific [^{125}I]DOI binding was detected in COS-7 cells that expressed 5-HT$_2$(Phe^{340}→Leu^{340}) receptors. Table 1 shows the data obtained in competition binding assays for a variety of agonists and partial agonists for the 5-HT$_2$ receptor. As is clear, the Phe^{340}→Leu^{340} mutant receptors displayed greatly attenuated affinities for 5-HT and the partial agonists *m*-chlorophenylpiperazine, trifluoromethylphenylpiperazine, and DOI. The other mutations (Phe^{339}→Leu^{339} and Phe^{125}→Leu^{125}) did not significantly affect agonist binding (Table 1).

Fig. 7 shows the ability of the transiently expressed receptors to augment phosphoinositide hydrolysis in COS-7 cells. The 5-HT$_2$, Phe^{340}→Leu^{340}, Phe^{339}→Leu^{339}, and Phe^{125}→Leu^{125} receptors were all positively coupled to phosphoinositide hydrolysis. 5-HT had a significantly diminished affinity (K_{act}) for activating phosphoinositide hydrolysis in the 5-HT$_2$(Phe^{340}→Leu^{340}) receptors (Table 2). Table 3 lists the K_{act} values of MK-212, quipazine, 5-HT, and DOI for the various mutant 5-HT$_2$ receptors. As can be seen, the incapacity of agonists to augment phosphoinositide hydrolysis was matched by a similar inability to inhibit [^{3}H]ketanserin binding to Phe^{340}→Leu^{340} receptors. Interestingly, although both the Phe^{339}→Leu^{339} and Phe^{340}→Leu^{340} mutants expressed lower levels of [^{3}H]ketanserin binding (see Table 1), the Phe^{339}→Leu^{339} receptors were able to augment phosphoinositide hydrolysis to an extent equivalent to that of the parent receptors, indicating that there may be considerable receptor reserve in the transient assay system.

Discussion

The major finding of this study is that a single point mutation of a conserved phenylalanine residue (Phe^{340}; see Fig. 1) greatly attenuates the abilities of agonists, three ergots, and selected antagonists to bind to 5-HT$_2$ receptors (see Fig. 8 for structures of relevant compounds). To our knowledge, this is the first published report detailing the importance of a conserved aromatic residue for agonist and selected antagonist binding to G protein-coupled receptors. A report by Strader and co-workers (37) suggested that an equivalent phenylalanine found in the β-adrenergic receptor (Phe^{290}) was essential for high affinity isoproterol binding. Taken together, these results suggest that a specific interaction with a conserved aromatic residue (found in all G protein-coupled serotonin, catecholamine, and histamine receptors thus far cloned) is essential for the binding of agonists and three ergot derivatives to 5-HT$_2$ receptors. These results are in accord with recently published models of serotonin-receptor interactions (18–19b) that predict that serotonin, via stacking-type interactions, utilizes aromatic residues in binding to 5-HT$_2$ receptors. These results also support our findings with chimeric serotonin receptors that showed that TMVI-VII are important for 5-HT binding (16).

Several models have been presented that attempt to predict how serotonin and, in certain cases, other compounds bind to

TABLE 3

Ability of mutant 5-HT$_2$ receptors to activate phosphoinositide hydrolysis in COS-7 cells

Shown are computer-derived mean ± standard deviation values for K_{act} and percent of V_{max} (relative to 5-HT$_2$ receptors) for [^{3}H]inositol phosphate accumulation in COS-7 cells. For a typical experiment, 5-HT induced a maximum accumulation of [^{3}H]inositol phosphate of 12,000 dpm/well, which represents an approximately 13-fold activation over basal accumulation.

Drug	K_{act} (% of V_{max})			
	5-HT$_2$	Phe^{340}→Leu^{340}	Phe^{339}→Leu^{339}	Phe^{125}→Leu^{125}
	nM			
5-HT	92 ± 18 (100)	24,000 ± 10,000[a] (29[a])	188 ± 49 (100)	249 ± 110 (100)
Quipazine	395 ± 60 (100)	UD[b]	568 ± 71 (85 ± 16)	565 ± 243 (99 ± 6)
MK-212	5,248 ± 289 (100)	UD	1,726 ± 774[a] (93 ± 12)	4,905 ± 1,224 (94 ± 5.3)
Bufotenine	182 ± 77 (80 ± 14)	UD	158 ± 96 (107)	109 ± 28 (67 ± 20)

[a] $p < 0.001$ versus 5-HT$_2$ K_{act} or V_{max}.
[b] UD, undetectable activation (<30% above basal).

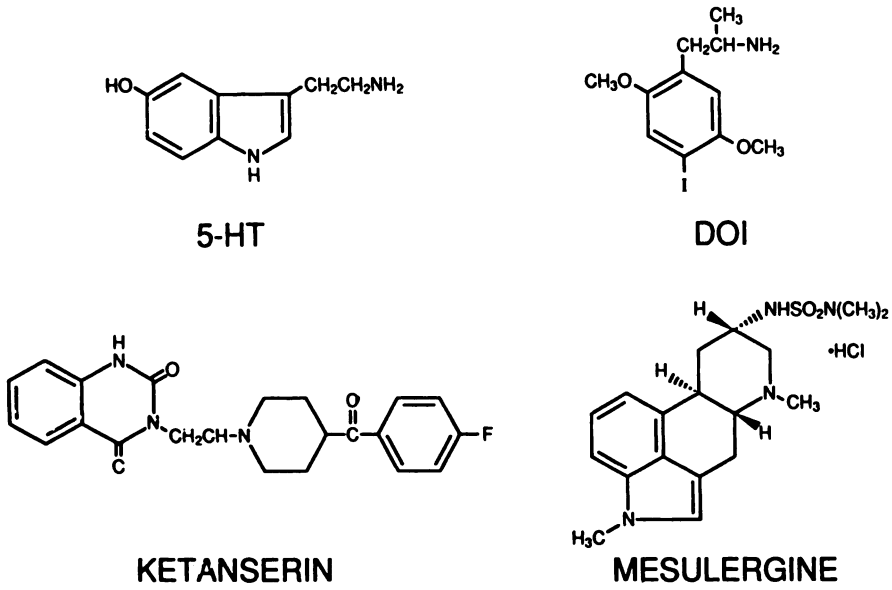

Fig. 8. Structures of 5-HT, DOI, ketanserin, and mesulergine. Shown are the structures of the relevant compounds used in these studies.

5-HT_2 receptors. Three models have explicitly identified particular amino acids as being important for anchoring serotonin to 5-HT_2 receptors. The model of Hibert *et al.* (18) suggests that Phe^{340} but not Phe^{339} anchors the aromatic ring of serotonin via a π-π (or "stacking") interaction. Edvardssen *et al.* (19a) proposed that Phe^{243} and Phe^{244} (in TMV) are essential for 5-HT binding but that Phe^{340} is involved in anchoring ritanserin. Based upon these considerations, we reasoned that a single point mutation of Phe^{340} should allow us to distinguish between these molecular models. Our results appear to support the proposal of Hibert *et al.* (18) that Phe^{340} is involved in anchoring serotonin and do not appear to provide evidence in favor of the model of Edvardssen *et al.* (19a).

Additionally, the model of Edvardssen *et al.* (19a) predicts that there should be an approximately 5.1 kcal/mol shift in the affinity of ritanserin for the 5-HT_2 receptor with a mutation that removes potential interactions with Phe^{340}. We did not discover a statistically significant shift in the affinity of ritanserin for the mutant receptor. It should be realized that our findings do not necessarily imply that any one of these models is correct. It is more likely that they represent useful approximations and will be further refined when additional biochemical and structural findings are available.

The decrease of mesulergine, lisuride, and methysergide binding affinities is interesting in light of hypotheses that have been suggested for many years, speculating that serotonin and certain ergot derivatives have similar conformations (27). Indeed, our findings appear to support the idea that serotonin and some ergots might utilize the same amino acids, at least in part, in binding to 5-HT_2 receptors.

Our discovery that [^{125}I]DOI binding was abolished was quite interesting and deserves comment. Previous workers have reported that compounds similar in structure to DOI (e.g., mescaline and lysergic acid diethylamide) could interact with a hydrophobic portion of the receptor in a 'planar' orientation (20, 21). Future studies investigating the binding of additional hallucinogens should help us determine whether Phe^{340} is essential for binding tryptamine and indolamine hallucinogens. Indeed, recent findings[1] suggest that many DOI derivatives also fail to bind with high affinity to the Phe^{340}→Leu^{340} mutant receptor.

In these experiments, we also evaluated two other point mutations, one of a highly conserved phenylalanine (Phe^{339}) and the other, of a nonconserved phenylalanine (Phe^{125}). We reasoned that, if the interactions we observed with the Phe^{340} mutation were specific, then mutations of other conserved and nonconserved phenylalanines would be relatively inconsequential. On the other hand, if π-π type interactions can occur with any phenylalanine, then mutations of Phe^{339} and Phe^{125} should yield alterations in the binding properties of the 5-HT_2 receptor. As predicted by the Hibert model, only mutation of Phe^{340} had a dramatic effect on agonist and ergot binding. Thus, even though Phe^{339} is adjacent to Phe^{340} it apparently, because of the turn of the helix, is out of the active binding pocket for 5-HT and other agonists. Phe^{339} does, however, appear to be important for ketanserin binding, because a 24-fold decrease in affinity for [^{3}H]ketanserin was noted with the Phe^{339}→Leu^{339} mutation. The role of Phe^{339} in ketanserin binding is consistent with a model proposed by Dahl.[2] These results imply that relatively specific interactions occur between the 5-HT_2 receptor and selected agonists and antagonists.

We also discovered that the transiently expressed receptor showed attenuated coupling to phosphoinositide hydrolysis. These results represent the first published findings for any G protein-coupled receptor that imply that an aromatic amino acid is important for second messenger production. The most obvious explanation for the decreased coupling to phosphoinositide hydrolysis is that the high affinity agonist state of the 5-HT_2 receptor (represented by [^{125}I]DOI binding) is abolished. It is equally conceivable, however, that Phe^{340} serves an additional function in G protein coupling. According to this idea, the loss of DOI binding and serotonin-stimulated phosphoinositide hydrolysis could be due to faulty G protein coupling and not due to alterations in a specific binding site for serotonin and other drugs.

In conclusion, we discovered a single point mutation (Phe^{340}→Leu^{340}) that abolishes high affinity agonist binding,

[1] B. L. Roth and R. Glennon, unpublished observations.

[2] S. G. Dahl, personal communication.

drastically alters the affinity for three ergots, and diminishes the ability of 5-HT to activate phosphoinositide hydrolysis. Because this residue is conserved among all of the G protein-coupled serotonin receptors thus far cloned, it will be interesting to determine the effect of the same mutation on other receptors within the serotonin receptor family. Finally, because the affinities of several antagonists such as ketanserin, ritanserin, and cinanserin are unaffected by the Phe^{340}→Leu^{340} mutation, our results buttress our previous findings that suggested that antagonists of different chemical classes utilize distinct residues for high affinity binding to 5-HT$_2$ receptors (see Refs. 16 and 17). The results also imply that the process of agonist binding is extremely specific, because mutation of an adjacent residue (Phe^{339}) did not affect agonist affinity. These findings should prove to be of fundamental importance for modeling the interactions of agonists and antagonists with serotonin receptors.

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