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# Site-Directed Mutagenesis of the Serotonin 5-Hydroxytrypamine$_2$ Receptor: Identification of Amino Acids Necessary for Ligand Binding and Receptor Activation

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**SUMMARY**

Serotonin 5-hydroxytryptamine (5-HT)$_2$ receptors are implicated in the etiology of mental disease and depression. Drugs that interact with the 5-HT$_2$ receptor are used therapeutically to treat such illnesses, and their mechanisms of action are of great interest. In this study 5-HT$_2$ receptor-ligand interactions were examined by site-directed mutagenesis in which three aspartic acid to asparagine mutants (Asn-120, Asn-155, and Asn-172) were created and expressed in NIH3T3 cells. The Asp-120 to asparagine mutant exhibited the same affinity for $^{125}$I-lysergic acid diethylamide ($^{125}$I-LSD) as did the wild-type receptor and showed a decreased and GTP-insensitive binding affinity for the agonists 5-HT and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (~10-fold) and the antagonists ketanserin and mianserin (~10-fold) but not spiperone. The mutation also abolished agonist-stimulated formation of [$^3$H]polyphosphoinositides (PI). The Asn-155 mutant showed reduced binding affinity for $^{125}$I-LSD ($K_d$, 2.8 nM versus 0.6 nM for the wild-type receptor) and had reduced affinity for agonists (~30-fold) and for antagonists (14–75-fold). However, the Asn-155 receptor retained GTP sensitivity and the ability to stimulate PI formation. The Asn-172 mutant retained the wild-type $K_d$ value for $^{125}$I-LSD, exhibited only ~5-fold reduced affinity for 5-HT and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane while retaining GTP-sensitive agonist binding, showed no change in affinity for ketanserin, and had a small decrease in mianserin and spiperone binding (~6-fold). The Asn-172 receptor also retained the ability to form PI. These results indicate that Asp-120 is necessary for allosteric activation of the guanine nucleotide-binding protein. Asp-155 is necessary for high affinity binding, probably by acting as a counterion for the amine group of the ligand. The different effects of the three mutations on ketanserin, mianserin, and spiperone binding affinity suggest that these antagonists may share overlapping but different binding domains. The information provided by this study may facilitate the design of therapeutic site-selective compounds based on the structure of the 5-HT$_2$ receptor.

The structure and function of 5-HT$_2$ receptors are of great interest because drugs that interact with the receptors exert profound effects on mental states and are used therapeutically. The 5-HT$_2$ receptor is implicated in depression and the 5-HT$_2$ receptor antagonist mianserin is used as an antidepressant agent (1). Hallucinogenic drugs interact specifically with 5-HT$_2$ receptors (2, 3), and this action is believed to account for the hallucinogenic state that resembles psychosis. Consistent with the actions of hallucinogenic drugs as agonists of 5-HT$_2$ receptors is the therapeutic use of 5-HT$_2$ receptor blockers as antipsychotic agents (4). Chlorpromazine and spiperone are potent antipsychotic agents that block 5-HT$_2$ receptor function, but the actions of these two compounds are not specific for only the 5-HT$_2$ receptor. So far no 5-HT$_2$-specific ligands are available. A greater knowledge of the binding sites for 5-HT$_2$ ligands would be of great benefit for understanding the molecular mechanisms of serotonergic signal transduction and for understanding the mechanisms of these drugs. Molecular information concerning the 5-HT$_2$ receptor binding site will allow a structurally based approach for drug design to create highly specific serotonergic agents.

Since 5-HT$_2$ receptors were first defined in 1979 (5) a detailed pharmacological profile has been established for the receptor (6). The primary structure of 5-HT$_2$ receptors has been deduced by the molecular cloning of the cDNA coding for the receptor in rat (7, 8), mouse (9), and human (10, 11). The primary structure places the 5-HT$_2$ receptor in the class of membrane protein receptors that traverse the lipid bilayer seven times and work in tandem with a G protein to elicit the cellular second messenger response (12, 13). Transfection of the cDNA into mammalian cell lines has indicated that one gene codes for the pharmacologically defined receptor, to account for the ligand-

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**ABBREVIATIONS:** 5-HT, 5-hydroxytryptamine; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; G protein, guanine nucleotide-binding protein; PI, phosphoinositide(s); LSD, lysergic acid diethylamide.

binding properties of the 5-$HT_2$ receptor (14). Thus, the 471-amino acid polypeptide encoded by a multiple-exon gene (9) confers the specific pharmacological profile for 5-$HT_2$ receptors. This profile includes a relatively low affinity for the agonist 5-HT, a higher affinity for the agonist DOI, and high affinity for the antagonists ketanserin, spiperone, and mianserin. The binding sites for these compounds are unknown but, because agonist inhibition of antagonist binding (and vice versa) is competitive, are believed to overlap or share binding site residues. However, agonists are GTP sensitive, whereas antagonists are not, indicating that all ligands may not bind by the same mechanism. Knowledge of the 5-$HT_2$ receptor primary structure makes it possible to examine the interactions of agonist and antagonist with the receptor using site-directed mutagenesis in combination with stably transfected mammalian cells and to determine the receptor binding site residues.

Rhodopsin and the $\beta$-adrenergic receptor have served as model proteins for the study of the structure and function of the G protein-coupled receptor family. It is established that the agonist binding site for the $\beta$-adrenergic receptor is in the inner membrane regions of the receptor, analogously to the structure of the retinol chromophore in rhodopsin (15). Subsequent studies of the $\beta$-adrenergic, $\alpha$-adrenergic, and muscarinic receptors have demonstrated that the inner membrane binding site is a common feature of all these receptors, and each receptor contains an aspartic acid in the third transmembrane domain that is necessary for high affinity agonist binding (15). The agonists for these receptors, epinephrine, norepinephrine, and acetylcholine, respectively, all contain an amine group that is believed to form a complex with the negatively charged aspartate residue in the third transmembrane domain as one of the epitopes necessary for high affinity binding. The primary structures of the members of the G protein-coupled receptor family indicate that this binding mechanism is conserved in all the receptors that have a charged amine group as a feature of their activating ligand. All cloned 5-HT receptors also contain the aspartic acid residue in the third transmembrane domain at a comparable position in the bilayer (7–11, 16–21), whereas other receptors that have agonists without amine groups do not have this conserved feature. Mutagenesis of the 5-$HT_2$ receptor at this aspartic acid should reduce high affinity agonist binding if this structural-functional mechanism is conserved.

Another aspartic acid that has been indicated to play a functional role in G protein receptors is an aspartic acid in the second transmembrane domain. In the 5-$HT_2$ receptor this residue is Asp-120. Cognate residues in $\beta$-adrenergic (22), $\alpha_{2A}$-adrenergic (23), and muscarinic receptors (24), when mutated to asparagine, cause the loss of the ability to stimulate second messenger formation. This cognate residue in the $\alpha_2$-adrenergic receptor has also been shown to be necessary for the allosteric regulation of receptor affinity by sodium (25). Interestingly, this same mutation in the an $\alpha_2$-adrenergic receptor results in the inability to increase potassium currents but does not cause an inability to stimulate inhibition of cAMP or inhibition of calcium currents (26). The question follows as to whether mutation of Asp-120 of the 5-$HT_2$ receptor will render it insensitive to agonist-induced second messenger formation.

One other aspartic acid residue that is conserved in virtually all types of G protein-coupled receptors is found at the interface of the third transmembrane domain and the second cytoplasmic loop (Asp-172 in the 5-$HT_2$ receptor). The almost complete conservation of this residue within a three-amino acid sequence of Asp-Arg-Tyr in all G protein-coupled receptors suggests its functional importance. Mutagenesis studies of this aspartic acid residue in $\alpha$-adrenergic receptors, $\beta$-adrenergic receptors, and muscarinic receptors indicate that different functional consequences occur in different receptors upon the mutation of this aspartic acid. Its role cannot yet be precisely identified.

We have created three aspartic acid to asparagine mutant receptors (Asn-120, Asn-155, and Asn-172) and stably expressed the receptors in NIH3T3 cells. The effects of the mutations were assessed by $^{125}$I-LSD radioligand binding analysis and competition analysis of this binding with the 5-$HT_2$ receptor agonists 5-HT and DOI and antagonists ketanserin, mianserin, and spiperone. The mutants were also assessed for 5-HT- or DOI-stimulated PI formation. The three 5-$HT_2$ receptor mutations we created are at sites in the primary structure that are conserved in all the cloned 5-HT receptors (7–11, 16–21) and are cognates to the aspartic acid residues examined in the receptors discussed earlier. Our examination indicates the roles of these aspartic acid residues in the 5-$HT_2$ receptor. We discuss our results in terms of the emerging model of receptors for amine-containing agonists.

## Experimental Procedures

**Materials.** Tissue culture reagents and Geneticin (G418 sulfate) were from GIBCO Laboratories. NIH3T3 cells were from the American Type Culture Collection. $^{125}$I-LSD (2200 Ci/mmol) and *myo*-[$^3$H]inositol were from New England Nuclear. Mianserin, ketanserin, spiperone, and serotonin were from Research Biochemicals. Restriction endonucleases were from Boehringer Mannheim. Polypropylene Econo-column chromatography columns and AG1–8X anion exchange resin were from Bio-Rad. The oligonucleotide-directed *in vitro* mutagenesis system kit was from Amersham. The pcDNA1neo vector was from Invitrogen. All other reagents were from Sigma. Mutant oligonucleotides were from Keystone.

**Site-directed mutagenesis and stable expression of receptors in NIH3T3 cells.** The entire coding region of the rat 5-$HT_2$ receptor contained within a 2-kilobase fragment of cDNA was cloned into M13 at *Eco*RI and *Xba*I sites, for mutagenesis. The single-base mutation that converts aspartic acid (GAT or GAC) to asparagine (AAT or AAC) was introduced into the rat 5-$HT_2$ receptor cDNA at codon 120, 155, or 172 by oligonucleotide-directed mutagenesis, according to the method of Kunkel (27). The authenticity of each mutation was confirmed by single-stranded dideoxy sequencing using the DNA sequencing kit from Sequenase. For gene expression, the wild-type or mutant 2-kilobase cDNA was excised from M13 mp18 at the *Hind*III and *Eco*RI sites and inserted into pcDNA1neo expression vector at the *Hind*III site and at the blunt end *Eco*RV site after creating a blunt end at the *Eco*RI end of the excised cDNA.

NIH3T3 cells, which do not express 5-$HT_2$ receptors, were transfected by $CaPO_4$ precipitation (28) with the pcDNA1neo/5-$HT_2$ receptor expression vector, which contains the entire receptor coding region. pcDNA1neo contains the aminoglycosidase phosphotransferase enzyme, which acts as a dominant selectable marker that confers resistance to Geneticin (G418) in cells expressing the enzyme. After 2 days the transfected cells were grown continuously in selective medium containing 600 mg/ml G418. Surviving colonies were isolated, expanded, and assayed for [$^3$H]ketanserin binding activity.

**Membrane preparation and radioligand binding analysis.** Transfected cells were harvested at full confluence by scraping the cells from the culture dish after removal of the medium and addition of 6 ml of ice-cold phosphate-buffered saline. The scraped cells were collected in a 50-ml tube and pelleted at ~500 × *g* for 5 min at 4°. The

cells were gently resuspended in ice-cold phosphate-buffered saline and pelleted as before, to remove any residual medium. This pellet was homogenized in ice-cold binding buffer (50 mM Tris·HCl, pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$) at approximately 1:3 (w/v) pellet to buffer ratio. This homogenate was used immediately for radioligand binding analysis. Protein concentrations were determined by the method of Lowry *et al.* (29). Radioligand binding assays were performed in duplicate with 0.1 mg of protein in 0.25-ml total volume. 5-HT$_2$ receptors were labeled with increasing concentrations of $^{125}$I-LSD for saturation isotherms or with a fixed concentration of 0.6 nM for competition assays. Nonspecific binding was defined by the presence of 1 μM mianserin. Assay mixtures were incubated in the dark at 37° for 40 min and then rapidly filtered through glass fiber filters (no. 32; Schleicher and Schuell). The filters were immediately washed three times with ice-cold buffer. Scatchard analysis of saturation isotherms was done to determine $K_d$ and $B_{max}$ values by linear regression of the binding data. $K_i$ values were determined from the Hill slope by using linear regression of the data and the Cheng-Prussof equation, $K_i$ = $IC_{50}$/[1 + (radioligand concentration/$K_d$ of the radioligand)].

**Measurement of PI hydrolysis.** The measurement of PI hydrolysis in the transfected cells was carried out essentially as described (30). Cells were seeded into 12-well dishes at a density of 2 × $10^5$ cells/well and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 1 day of growth, the cells were grown for another 24 hr in the presence of *myo*-[$^3$H]inositol (2 μCi/1 ml of medium in each well). Before the assay, cells were treated for 20 min with 10 mM LiCl and 10 μM pargyline in Dulbecco's modified Eagle's medium. For the assay the appropriate amount of 5-HT or antagonist was added in a 20-μl volume, and the samples were incubated for 60 min at 37°. The reaction was terminated by the addition of 1 ml of ice-cold 75% methanol. The cells were scraped and the suspensions were added to ice-cold tubes containing 1 ml of chloroform and 0.5 ml of double-distilled water. The contents were mixed thoroughly and centrifuged at 2000 rpm at 4° for 10 min. One milliliter of the upper aqueous phase was added to 2 ml of ice-cold double-distilled water; the solution was mixed and loaded onto a 10-ml column of AG1-X8 anion exchange resin. The column was washed with 3 × 5 ml of ice-cold double-distilled water followed by 2 × 5 ml of 5 mM sodium borate/60 mM sodium formate. The total [$^3$H]inositol phosphates were then eluted into scintillation vials with 2 × 2 ml of 1 M ammonium formate and were mixed with 14 ml of scintillation cocktail (Budget-Solve; Research Products International). Radioactivity was determined by liquid scintillation counting.

## Results

The effect of three individual aspartic acid to asparagine point mutations on 5-HT$_2$ receptor function was examined by comparing the radioligand-binding properties and agonist-stimulated PI production of the wild-type receptors with those of the mutant receptors, using stably transfected NIH3T3 cells.

### Radioligand Binding

**Scatchard analysis.** $^{125}$I-LSD was used to determine the dissociation constant ($K_d$) and maxiumum bound ($B_{max}$) values for each of the mutant receptors and the wild-type receptor. The wild-type receptor and the Asn-120 mutant bound the ligand with equal affinity ($K_d$ = 0.6 ± 0.1 nM for both) (Table 1), whereas the Asn-172 mutant binding affinity was slightly increased ($K_d$ = 0.4 ± 0.1 nM) (Table 1). The Asn-155 mutant exhibited the lowest affinity binding for $^{125}$I-LSD, with a 5-fold reduction in binding affinity ($K_d$ = 2.8 ± 0.6 nM) (Table 1). The $B_{max}$ values of the wild-type and mutant receptors determined by Scatchard analysis indicated that, despite variation in maximum bound values ($B_{max}$ = 180 ± 20, 110 ± 20, 220 ± 50, and 70 ± 10 fmol/mg of protein for the wild-type, Asn-120, Asn-155, and Asn-172 receptors, respectively) (Table 1), the three mutant receptors were processed and expressed by the cells. Thus, the mutations did not affect the expression of the receptors to result in lack of binding or signal-generating capabilities (see PI Turnover). Data on the site mutations therefore reflect the effect of the amino acid substitutions on the function of the receptors, rather than indicating a complete loss of activity due to improper processing of the mutant receptors.

**Agonist competition and GTP effects.** Displacement of $^{125}$I-LSD by two 5-HT$_2$ receptor agonists, 5-HT and DOI, was analyzed and the wild-type values were compared with the properties of the mutant receptors. The Asn-120 mutant showed a significant difference in binding of 5-HT, compared with the wild-type receptor ($K_i$ = 2.1 ± 0.02 μM versus 300 ± 10 nM) (Fig. 1, A and B; Table 1). Mutation of Asn-155 resulted in a mutant receptor with an even more decreased affinity for 5-HT ($K_i$ = 11 ± 0.5 μM) (Fig. 1C; Table 1). The Asn-172 mutant showed a greater change in binding affinity, compared with wild-type, than did the Asn-120 mutant but a smaller change than did the Asn-155 mutant for $^{125}$I-LSD sites (Asn-172 $K_i$ = 1.4 ± 0.2 μM) (Fig. 1D; Table 1). Of interest is the finding that the wild-type rank order of potency of DOI > 5-HT was retained in all the mutants despite the reduction of agonist afinity conferred by each mutation.

The effects of 100 μM GTP on the inhibition of $^{125}$I-LSD binding by the two agonists were examined. The wild-type receptor was highly sensitive to GTP, which caused a decrease in 5-HT and DOI binding affinity of 14-fold ($K_i$ = 4.1 ± 0.1 μM) and 10-fold ($K_i$ = 300 ± 10 nM), respectively (Fig. 1A; Table 1). Such a GTP-induced decrease is well established for 5-HT$_2$ receptors (14, 32) and reflects the low affinity binding state of the receptor in the G protein-uncoupled state. The Asn-120 mutant was virtually insensitive to the effects of GTP. Both 5-HT and DOI exhibited $K_i$ values nearly identical to values determined in the absence of GTP (Fig. 1B; Table 1). Asn-155 and Asn-172 mutants were both sensitive to the presence of GTP but displayed less sensitivity than did the wild-type receptor. The $K_i$ for 5-HT binding to the Asn-155 mutant decreased ~2-fold to 20.6 ± 1.7 μM and the affinity of DOI for the Asn-155 mutant decreased 1.5-fold to 1.2 ± 0.07 μM due to the presence of GTP (Fig. 1C; Table 1). The affinity of the Asn-172 receptor for 5-HT decreased 3.5-fold to 5.1 ± 0.2 μM in the presence of GTP, and the DOI affinity of the Asn-172 receptor decreased 2-fold to 360 ± 10 nM in the presence of the guanine nucleotide (Fig. 1D; Table 1).

**Competiton by antagonists.** The $K_i$ values for three 5-HT$_2$ receptor antagonists, ketanserin, mianserin, and spiperone, were also examined in the wild-type and mutant receptors to determine the effect of the aspartic acid to asparagine mutation on mutant receptor binding affinity determined by competition for bound $^{125}$I-LSD. All the antagonists showed a pattern of decreased binding affinity for the $^{125}$I-LSD site similar but not identical to that seen for 5-HT and DOI, with the Asn-155 receptor exhibiting the largest loss in binding affinity.

Ketanserin binding was least affected by the Asn-172 mutation. The $K_i$ of 0.7 ± 0.1 nM for the Asn-172 receptor was virtually identical to the value for the wild-type receptor ($K_i$ = 0.8 ± 0.1 nM) (Fig. 2A; Table 1). The Asn-120 mutation affected ketanserin binding less than did the Asn-155 mutation ($K_i$ = 8 ± 1 nM and 60 ± 1 nM, respectively) (Fig. 2A; Table 1), with

TABLE 1

**Ligand binding**

Results of binding experiments using $^{125}$I-LSD to label wild-type and mutant receptors are shown. The values presented are the results of experiments illustrated in Figs. 1 and 2.

| Receptor | $B_{max}$ | $K_d$, $^{125}$I-LSD | $K_i$ | | | | |
|---|---|---|---|---|---|---|---|
| | | | Serotonin | DOI | Ketanserin | Mianserin | Spiperone |
| | fmol/mg | nM | nM | | | | |
| Wild-type | | | | | | | |
| −GTP | 180 ± 20 | 0.6 ± 0.1 | 300 ± 10 | 29 ± 3 | 0.8 ± 0.1 | 1.4 ± 0.1 | 8.5 ± 0.2 |
| +100 μM GTP | ND[a] | ND | 4,100 ± 100 | 300 ± 10 | ND | ND | ND |
| Asn-120 | | | | | | | |
| −GTP | 110 ± 20 | 0.6 ± 0.1 | 2,100 ± 20 | 370 ± 40 | 8 ± 1 | 24 ± 1 | 12 ± 1 |
| +100 μM GTP | ND | ND | 2,000 ± 10 | 250 ± 10 | ND | ND | ND |
| Asn-155 | | | | | | | |
| −GTP | 220 ± 50 | 2.8 ± 0.6 | 11,000 ± 500 | 810 ± 10 | 60 ± 1 | 76 ± 1 | 120 ± 10 |
| +100 μM GTP | ND | ND | 20,600 ± 1,700 | 1,200 ± 70 | ND | ND | ND |
| Asn-172 | | | | | | | |
| −GTP | 70 ± 10 | 0.4 ± 0.1 | 1,400 ± 20 | 180 ± 10 | 0.7 ± 0.1 | 7.8 ± 0.1 | 48 ± 1 |
| +100 μM GTP | ND | ND | 5,100 ± 200 | 360 ± 10 | ND | ND | ND |

[a] ND, not determined.

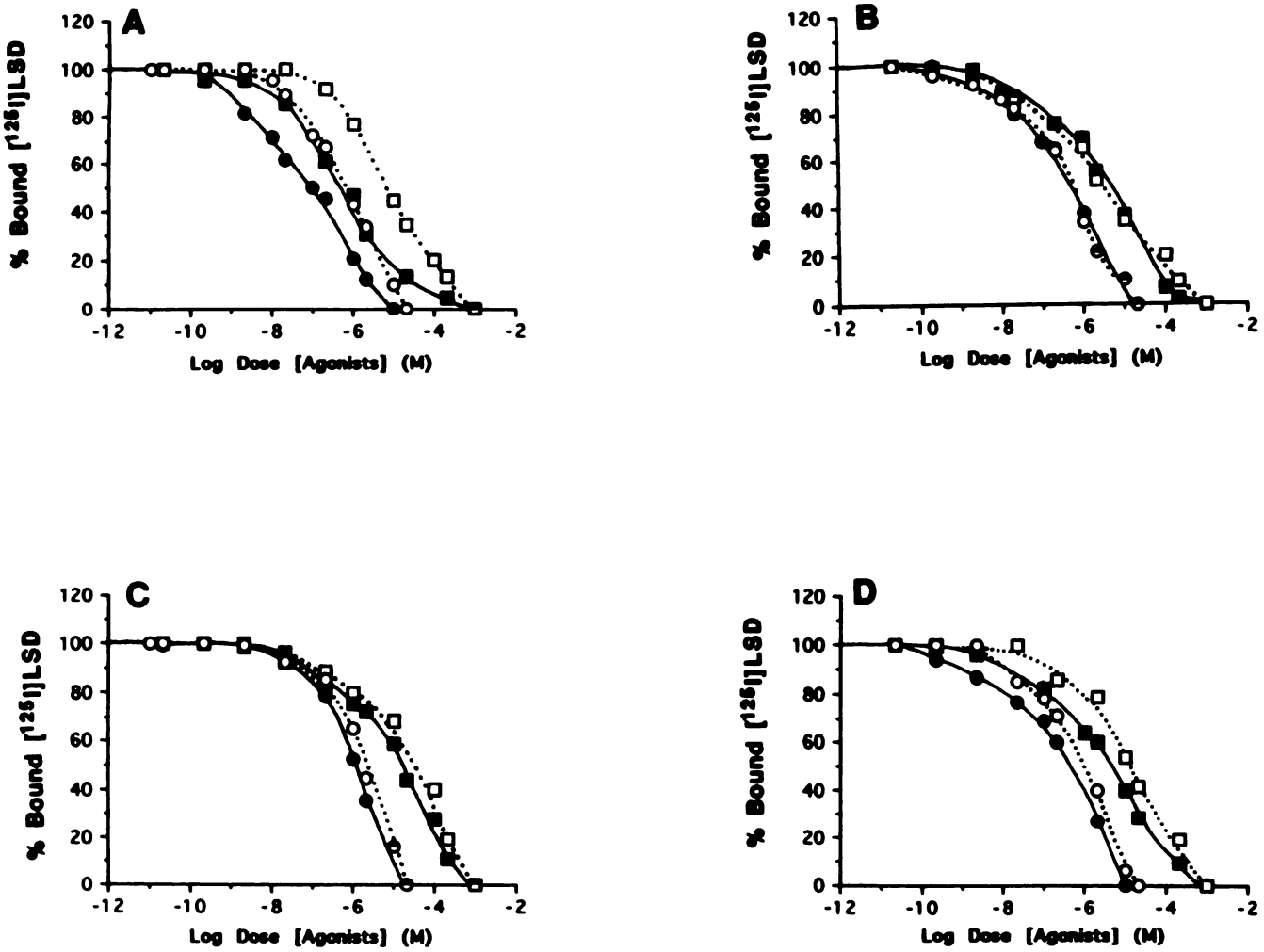


Fig. 1. Inhibition of $^{125}$I-LSD binding by 5-HT and DOI in the presence or absence of 100 mM GTP. A, Wild-type receptor; B, Asn-120; C, Asn-155; D, Asn-172. 5-HT: ■, no GTP; □, with GTP. DOI: ●, no GTP; ○, with GTP. Table 1 presents the $K_i$ values for the graphs. The experiments were carried out as described in Experimental Procedures.

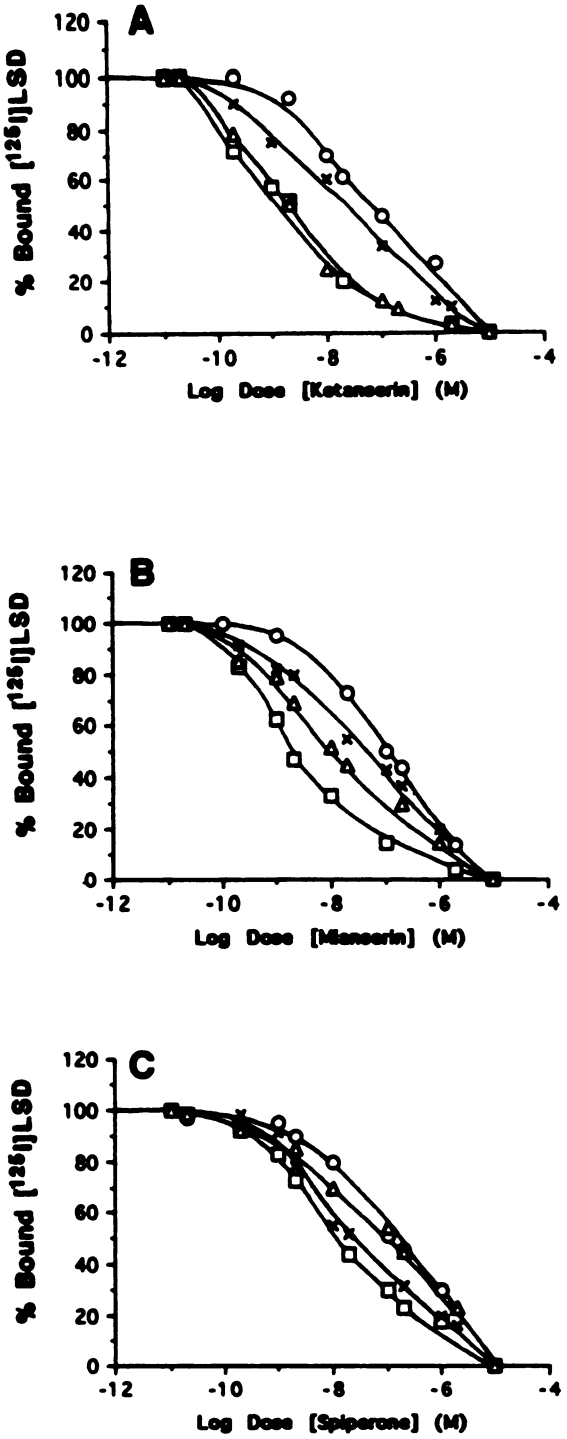


Fig. 2. Inhibition of $^{125}$I-LSD binding by ketanserin (A), mianserin (B), and spiperone (C). □, Wild-type; ×, Asn-120; ○, Asn-155; △, Asn-172. The experiments were carried out as described in Experimental Procedures. $K_i$ values are presented in Table 1.

ketanserin binding to the Asn-120 receptor being 10-fold less than that to the wild-type receptor and binding to the Asn-155 receptor being 75-fold less.

Mianserin affinity was closest to the wild-type value in the Asn-172 receptor, with a $K_i$ value of 7.8 ± 0.1 nM, a 6-fold decrease compared with the wild-type $K_i$ of 1.4 ± 0.1 nM (Fig. 2B; Table 1). The Asn-120 mutant showed a greater loss of affinity for mianserin (17-fold decrease; $K_i$ = 24 ± 1 nM) (Fig. 2B; Table 1) than did the Asn-172 mutant, and the Asn-155 receptor exhibited the largest decrease in affinity for mianserin (54-fold), compared with the wild-type receptor, with a $K_i$ of 76 ± 1 nM (Fig. 2B; Table 1).

Spiperone exhibited different sensitivities to the effects of the three mutations. The Asn-120 receptor showed the highest spiperone binding affinity, with a 1.4-fold loss in affinity, compared with the wild-type receptor ($K_i$ = 12 ± 1 nM, compared with 8.5 ± 0.2 nM for the wild-type receptor) (Fig. 2C; Table 1). The Asn-172 receptor had the next highest affinity, with a 6-fold affinity decrease ($K_i$ = 48 ± 1 nM) (Fig. 2C; Table 1). The Asn-155 receptor was the least sensitive to spiperone, exhibiting a 14-fold loss of affinity ($K_i$ = 120 ± 10 nM) (Fig. 2C; Table 1).

The most consistent finding in both the agonist and antagonist binding studies was that the Asn-155 receptor exhibited the lowest affinity binding of agonists and antagonists alike (Table 1). Of note are the differential binding patterns of ketanserin and spiperone. Ketanserin binding to the Asn-172 mutant was near wild-type affinity, whereas spiperone had almost 6-fold lower binding affinity (Table 1). On the other hand, spiperone binding to the Asn-120 mutant was only slightly affected, with a 1.4-fold loss in affinity, but ketanserin binding affinity was reduced 10-fold (Table 1).

### PI Turnover

The consequences of the three mutations on 5-HT-stimulated PI formation were examined by growing the cells for 24 hr in medium containing *myo*-[$^3$H]inositol and then measuring the amount of tritium in the inositol phosphates isolated from cellular extracts, using anion exchange chromatography to separate the negatively charged PI.

**Agonist-stimulated PI turnover.** 5-HT at 10 mM generated 1.6–1.8 fold increases in the level of [$^3$H]PI in wild-type ($EC_{50}$ = 128 ± 2 nM), Asn-155 ($EC_{50}$ = 7800 ± 500 nM), and Asn-172 ($EC_{50}$ = 290 ± 20 nM) receptors (Fig. 3A; Table 2), compared with [$^3$H]PI levels in cells not exposed to 5-HT. These levels of PI formation above basal are consistent with those seen for 5-HT-stimulated increases in PI produced by 5-HT$_2$ receptors in brain and smooth muscle cells (33, 34). In contrast to the three other receptors, 5-HT did not stimulate PI formation with the Asn-120 receptor (Fig. 3A; Table 2).

DOI also stimulated PI formation in wild-type, Asn-155, and Asn-172 receptors. $EC_{50}$ values for DOI stimulation were 4.3 ± 0.1 nM for wild-type, 6800 ± 200 nM for Asn-155, and 110 ± 10 nM for Asn-172 receptors (Fig. 3B; Table 2). For both agonists the Asn-155 mutation caused the greatest decrease in sensitivity for agonist-stimulated PI turnover (61-fold decrease for 5-HT and 1581-fold decrease for DOI), a result consistent with the decrease in binding affinity of agonists seen with the Asn-155 mutant.

**Antagonist inhibiton of PI turnover.** Inhibition of 5-HT-stimulated PI formation by the three 5-HT$_2$ receptor antagonists resulted in results qualitatively similar to the binding assay results. Ketanserin inhibited wild-type receptor 5-HT-stimulated PI formation with an $IC_{50}$ value of 6.5 ± 0.3 nM. The Asn-172 mutant showed decreased sensitivity to ketanserin with an $IC_{50}$ of 15 ± 1 nM, corresponding to a ~2-fold decrease in potency, and the Asn-155 mutant exhibited an $IC_{50}$ value of 150 ± 10 nM, which corresponded to a ~20-fold loss of potency for ketanserin in inhibiting 5-HT-stimulated PI formation with the Asn-155 mutant (Fig. 4A; Table 2).

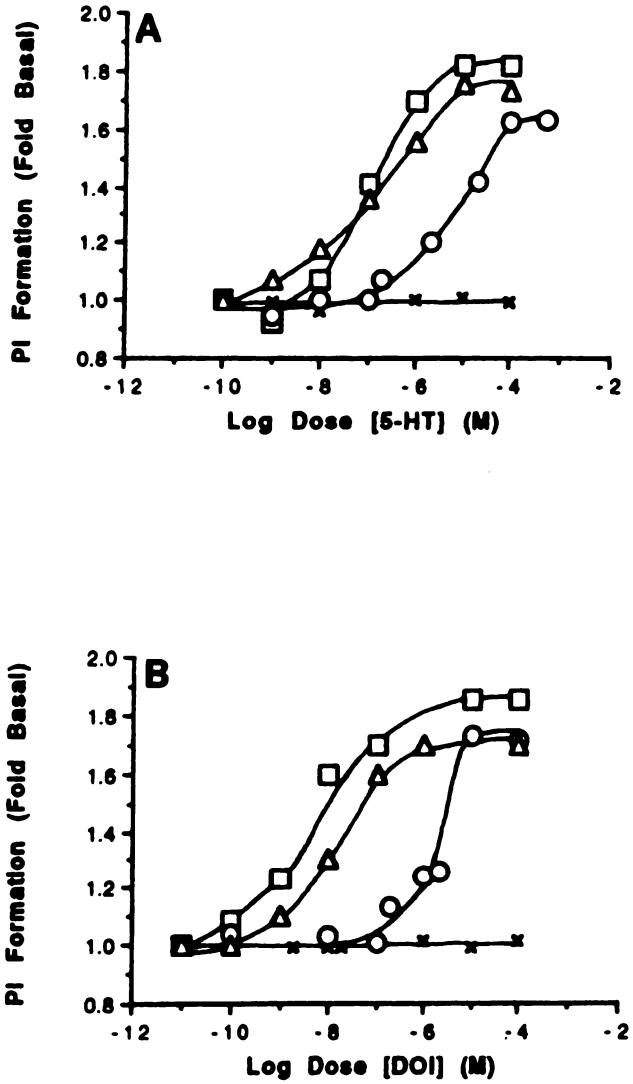


Fig. 3. Agonist-stimulated PI turnover. PI turnover stimulated by 5-HT (A) or DOI (B) was determined as described in Experimental Procedures and is presented in terms of fold increase over basal level, where basal level is defined as the amount of PI present in the cell without agonist stimulation. □, Wild-type; ×, Asn-120; ○, Asn-155; △, Asn-172. $EC_{50}$ values are presented in Table 2.

Mianserin inhibited 5-HT-stimulated PI formation with an $IC_{50}$ value of 4.5 ± 0.1 nM at the wild-type receptor but was less effective with the Asn-172 ($IC_{50}$ = 9.1 ± 0.2 nM) and Asn-155 ($IC_{50}$ = 32 ± 1 nM) receptors (Fig. 4B; Table 2). These values correspond to 2- and 7-fold losses, respectively, in the effectiveness of mianserin to inhibit second messenger formation, compared with wild-type formation.

Spiperone inhibition of wild-type receptors reached half-maximal at 4.9 -± 0.2 nM, compared with 12 ± 1 nM with Asn-172 and 42 ± 1 nM with Asn-155 receptors. The decreases in affinity relative to the wild-type value were 2.5-fold and 8.5-fold, respectively (Fig. 4C; Table 2). The antagonist inhibition of PI formation was most affected by the Asn-155 mutation for each compound (Table 2), a result consistently observed for all agonists and antagonists (Tables 1 and 2).

## Discussion

The point mutations we have created produced three mutant proteins with an asparigine substituted for aspartic acid. The result of this mutation is to produce a receptor lacking a negatively charged amino acid at the mutated position. The ligand binding studies and PI turnover studies presented here, combined with evolutionary and theoretical considerations concerning the folding of intregal membrane proteins, i.e., the "inside-out" nature of the proteins (35, 36), allow us to speculate about the functional structure of the 5-$HT_2$ receptor. The results indicate that the 5-$HT_2$ receptor shares structurally based functional properties with other G protein-coupled receptors but also indicate that individual G protein-coupled receptors have unique functional properties for shared stuctures.

**Asn-120.** Asp-120 is necessary for agonist-stimulated PI turnover. The Asn-120 mutant did not respond to 5-HT or DOI, at up to 10 μM, to stimulate PI turnover (Fig. 3; Table 2). This loss of second message formation in Asn-120 cannot be attributed to a loss of agonist affinity, because Asn-155 and Asn-172 receptors were able to stimulate PI formation but bound agonists with lower affinity (Asn-155) or near-comparable affinity (Asn-172), compared with the Asn-120 receptor (Table 1). Nor is this loss likely to be due to low levels of mutant receptor expression in the cells, because the Asn-172 receptor was expressed at lower levels in the cells (Table 1) but retained the ability to instigate PI formation. The loss of ability to stimulate PI formation of the Asn-120 mutant, despite the retention of agonist affinity, indicates that the mutation of Asp-120 to asparagine impairs the ability of the receptor to generate the second messenger, either because the receptor and G protein become functionally uncoupled or because the receptor becomes insensitive to agonist-induced activation.

**Asn-155.** Asp-155 serves as a binding site residue for the amine-containing agonists and antagonists. Evidence for this includes the following. 1) The loss of affinity for agonists and antagonists is greater than that for Asn-120 or Asn-172 receptors (Tables 1 and 2), which indicates a more direct role in

TABLE 2

**PI formation**

Results of PI turnover assays using [$^3$H]inositol to label cellular inositol phosphate-containing lipids. The values presented are the results of experiments illustrated in Figs. 3 and 4.

| Receptor | $EC_{50}$ | | Maximum response to 5-HT (% wild-type) | $IC_{50}$ | | |
|---|---|---|---|---|---|---|
| | Serotonin | DOI | | Ketanserin | Mianserin | Spiperone |
| | nM | | Fold over basal | | nM | |
| Wild-type | 128 ± 2 | 4.3 ± 0.1 | 1.82 (100) | 6.5 ± 0.3 | 4.5 ± 0.1 | 4.9 ± 0.2 |
| Asn-120 | ND* | ND | 1.01 (1.2) | ND | ND | ND |
| Asn-155 | 7800 ± 500 | 6800 ± 200 | 1.64 (78.0) | 150 ± 10 | 32 ± 1 | 42 ± 1 |
| Asn-172 | 290 ± 20 | 110 ± 10 | 1.76 (88.4) | 15 ± 1 | 9.1 ± 0.2 | 12 ± 1 |

* ND, not determined.

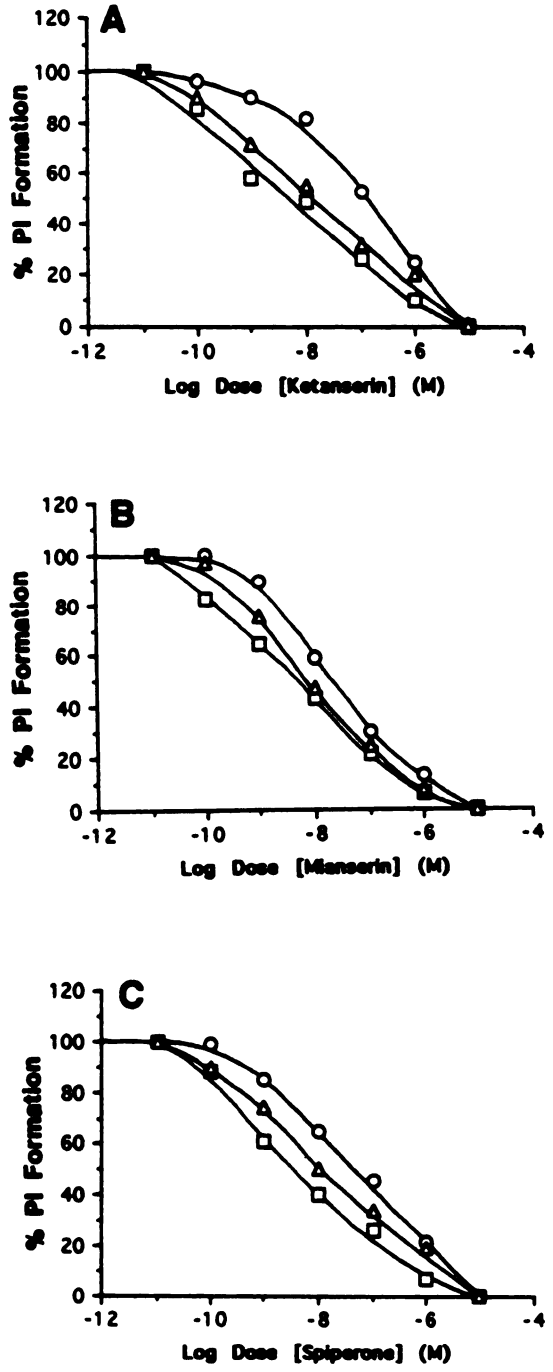


Fig. 4. Inhibition of 5-HT-stimulated PI formation by ketanserin (A), mianserin (B), and spiperone (C). □, Wild-type; ○, Asn-155; △, Asn-172. $IC_{50}$ values are presented in Table 2. The experiments were carried out as described in Experimental Procedures.

binding for Asp-155 than an allosteric conformational change due to the mutation. 2) The ability to stimulate PI turnover is not abolished in the Asn-155 mutant. Only a small decrease in the maximum response to 5-HT and DOI for PI turnover results, but the $EC_{50}$ is increased, which reflects the loss of agonist affinity (Table 2). 3) GTP decreased affinity for agonists (Fig. 1, A and B; Table 1), indicating that allosteric coupling of the receptor-G protein complex is still present.

**Asn-172.** Asp-172 is necessary for wild-type properties but does not play a major role in G protein coupling or agonist binding for the 5-HT$_2$ receptor. 5-HT and DOI both exhibited lower affinity for the Asn-172 mutant, compared with the wild-type receptor, but retained GTP-sensitive binding, albeit less sensitive than that with the wild-type receptor (Table 1). Mianserin and spiperone were affected by this mutation and exhibited lower affinity binding to the Asn-172 receptor than to the wild-type receptor, but ketanserin competition for $^{125}$I-LSD was unaffected by the mutation (Table 1). Asp-172 is predicted to be found not in the hydrophobic interior of the protein but at the cytoplasmic surface in the second cytoplasmic loop (37). This residue is found in a three-amino motif of Asp-Arg-Tyr found in most G protein-coupled receptors (38) at the analogous site of the second intracellular loop. The three-amino acid structure, and consequently Asp-172, is predicted to be of structural-functional importance because of its ubiquity, but the exact role is unknown. One possibility is that the Asp-Arg-Tyr sequence acts as a determinant for topological positioning of the third transmembrane domain and the second cytosolic domain in the membrane. The fact that charged residues in membrane proteins can serve this purpose has been demonstrated (39). Our results indicate that the Asp-172 mutant contributes to the binding affinity of agonists and some antagonists (mianserin and spiperone) possibly because of changes in the three-dimensional structure of the receptor, rather than having a direct ligand interaction.

**Theoretical considerations concerning the structural-functional roles of Asp-120 and Asp-155.** The functional roles we have ascribed to 5-HT$_2$ receptor aspartic acid residues 120 and 155 are consistent with evolutionary and membrane protein structure theory. The evidence indicating functional roles for these aspartic acid residues can be summarized as follows. 1) Seven hydrophobic regions determined by hydrophobicity analysis are postulated to form seven transmembrane domains as the main determinants of the topological structure of the membrane receptor. Physical studies of bacteriorhodopsin (40) provide strong evidence for the seven-transmembrane domain model. 2) Thermodynamically unstable charged residues in hydrophobic domains 2 and 3 (Asp-120 and Asp-155, respectively) are found in 5-HT$_2$ receptors. 3) The thermodynamic unfavorability and large electrical fields of the Asp-120 and Asp-155 residues in the hydrophobic portion of the lipid bilayer suggest their structural and functional importance (41). 4) An Asp-120 cognate is found in all G protein-coupled receptors. This 100% conserved structure suggests the importance of this residue for the function of each member of this family of proteins in signal transduction, as opposed to ligand binding. Our results clearly indicate that the major effect of mutagenizing this residue to asparagine is to abolish signal transduction, a result consistent with evolutionary analysis of 5-HT$_2$ receptor and G protein-coupled receptor primary structures. Also, the lack of GTP-sensitive agonist binding seen with the Asn-120 receptor (Fig. 1B; Table 1) indicates that allosteric modulation of the receptor does not occur for this mutant. The $K_i$ values for 5-HT and DOI competition for $^{125}$I-LSD binding are nearly identical in the presence and absence of GTP, and the $K_i$ values are most similar to wild-type receptor affinity in the presence of GTP, i.e., the uncoupled state of the receptor-G protein complex. The loss of GTP-sensitive agonist binding alone does not indicate the G protein-coupling role of Asp-120, because Asn-155 also shows reduced GTP-sensitve agonist binding (Table 1). These data support the argument, based upon the

evolutionary conservation of Asp-120 cognates in all members of the G protein-coupled receptor family, that G protein coupling is mediated by Asp-120 cognates. We feel the evolutionary analysis of G protein-coupled receptor primary structures is the strongest evidence to implicate Asp-120 as being necessary for the high affinity coupled state of the receptor and for allosteric activation of the G protein upon ligand binding. 5) An Asp-155 cognate is seen in amine ligand receptors (5-HT, dopamine, adrenergic, muscarinic, tyramine, and octopamine receptors). The conserved nature of the aspartic acid residue in the third transmembrane domain (corresponding to Asp-155 in the 5-$HT_2$ receptor) found in each of these receptors, but not found in receptors for ligands that do not contain amine groups [e.g., the cAMP receptor (42) and adenosine receptor (43)], strongly suggests that this residue is used for binding the amine group of agonists. Indeed, our results indicate that Asp-155 is necessary for agonists and antagonists to bind the 5-$HT_2$ receptor. The large decreases in binding affinities corresponding to $\Delta\Delta G$ values of 2.3, 2.0, and 2.6 kcal/mol for 5-HT, DOI, and ketanserin, respectively, are consistent with a direct interaction between ligand and receptor taking place at this site. The ability of the Asn-155 receptor to stimulate PI turnover in the presence of 5-HT or DOI indicates that this reduction of GTP-sensitive agonist binding is not reflected in the activation of the G protein, as is the case for the Asn-120 receptor. Evolutionary considerations indicate that this residue is not essential for G protein coupling, and our results confirm this.

**Receptor-ligand interactions.** Asp-155 acts as a counterion for the amine group of the ligands examined here but is clearly not the only determinant for ligand binding. The ligands varied in the decreases in binding affinity, from 75-fold for ketanserin to ~5-fold for $^{125}$I-LSD. The binding of $^{125}$I-LSD and spiperone were least affected by mutating Asp-155 (spiperone affinity was decreased by ~14-fold), compared with larger decreases seen for 5-HT (~37-fold), DOI (~28-fold), ketanserin, and mianserin (~54-fold). The binding of these compunds is therefore not solely dependent on their interactions with Asp-155. Spiperone and $^{125}$I-LSD are the most hydrophobic of these compunds, and it is possible that a great deal of their binding energy ($\Delta$G) is contributed by hydrophobic "forces," compared with the more water-soluble ligands. Consequently, $^{125}$I-LSD and spiperone are less affected by abolishing the counterionic interaction provided by Asp-155 in the binding energy than are the other compounds, which have smaller contributions to their binding energy by hydrophobic forces.

This study examined only the mutation of the three aspartic acid residues. It is safe to assume there is more than one binding site residue that forms an interaction with one of the chemical groups of the ligand. The amine group is shared by all the ligands examined here but the three-dimensional structures of the ligands vary and the geometries of the various functional groups are not shared among ligands. The binding sites for each ligand most likely consist of different binding domains that overlap at Asp-155 (and possibly other residues), thus accounting for competitive binding without sharing all of the same epitopes. The results described here for antagonists support this idea, in that antagonists are differentially affected by the Asn-120 and Asn-172 mutations (Table 1). Also indicative of a multiepitopic binding site is the retention of the rank order of potencies of the agonists 5-HT and DOI, with DOI > 5-HT in the wild-type receptor and in each mutant (Table 1). The postulated direct interaction between Asp-155 and the amine group of 5-HT or DOI is lost in the Asn-155 receptor, resulting in lower affinities for both agonists, but whatever other binding site interactions or binding mechanisms exist that confer rank order of potency on the two agonists are retained in all the mutants we have created and examined here.

**Comparison with adrenergic and muscarinic receptor mutagenesis studies.** All members of the G protein-coupled family of membrane-bound signal-transducing proteins have the hallmark seven-transmembrane domain structure and are coupled to G proteins to elicit their cellular responses. 5-HT is similar to the adrenergic, dopaminergic, and muscarinic receptor agonists, in that these ligands contain an amine group. Qualitatively similar results have been reported concerning the roles of the Asp-120 and Asp-155 cognates in the $\alpha$-adrenergic (23), $\beta$-adrenergic (22, 44), and muscarinic receptors (24). Those results and the findings reported here confirm the idea that Asp-155 of the 5-$HT_2$ receptor and the cognate residues in adrenergic and muscarinic receptors interact with the amine moiety of their agonists. Asp-120 is also seen to couple the 5-$HT_2$ receptor to second messenger signal transduction via the G protein. Asp-120 mutation and mutation of cognate amino acids in adrenergic and muscarinic receptors generally abolish the G protein-mediated second messenger response. One exception was reported for the $\alpha_2$-adreneric receptor when this mutant was expressed in mouse pituitary tumor cells (26). Only one of three second messenger responses was abolished due to this mutation of the $\alpha_2$-adrenergic receptor. The ability to inhibit cAMP formation was one of the two responses retained by this adrenergic mutant. It is difficult to reconcile this result with the results of a previous study of the $\alpha_{2A}$-adrenergic receptor, where this same mutation abolished the ability of the receptor to inhibit cAMP formation in Chinese hamster ovary cells (23), which makes the results of these two studies quite interesting. Here we clearly see an inability to stimulate PI formation with the Asn-120 5-$HT_2$ receptor mutant expressed in NIH3T3 cells. Evidence has mounted that activation of the G protein by adrenergic receptors requires multiple residues of the receptor (15). Asn-120 and its cognates seem to play an integral role in producing the second messenger response but, as demonstrated for the $\alpha_2$-adrenergic receptor (26), are not always necessary for this response. As G protein-coupled receptors of all classes are examined by site-directed mutagenesis, it will be of great interest to see how many aspartic acid to asparagine mutants at the second transmembrane domain cognate site will lose the ability to produce a second messenger response.

The significance of these results and observations is to allow predictions to be made concerning G protein-coupled receptor family receptors that have not yet been subjected to mutagenesis. Other 5-HT receptors (5-$HT_{1a}$, 5-$HT_{1b}$, 5-$HT_{1c}$, and 5-$HT_{1d}$), dopamine D1 and D2 receptors (46, 47), tyramine receptors (48), and octopamine receptors (49) are predicted to lose agonist affinity upon mutation of their Asp-155 cognate aspartic acids. Mutation of the Asp-120 cognate aspartic acid is predicted to abolish G protein-mediated second messenger formation in most members of this family, regardless of the ligand specificity of the receptor. Exceptions to these predictions, as the one cited above (26), would be of great interest.

The role of Asp-172 is more enigmatic for 5-$HT_2$ receptors

and other members of the G protein-coupled family. Here, the 5-HT$_2$ receptor Asp-172 mutated to asparagine did not cause a loss of second messenger formation. Similar results were observed for the $\alpha$-adrenergic receptor mutated at this cognate position (23), but the muscarinic receptor (24) and the human $\beta$-adrenergic receptor (50) mutated at this position exhibited profound losses in second messenger formation. If the Asp-Arg-Tyr sequence is a topological determinant for the membrane protein, the discrepancies in second messenger formation due to the aspartic acid to asparagine mutation among G protein-coupled receptors may reflect specific receptor interactions with individual G protein subtypes.

Due to the difficulties of purifying and crystallizing membrane receptors such as the 5-HT$_2$ receptor, computer modeling is the strongest tool available for determining receptor structure and receptor-ligand binding sites. The results of this study concerning the roles of aspartic acids 120, 155, and 172 provide empirical information for molecular modeling of the 5-HT$_2$ receptor, which is necessary to produce a model that accurately reflects the three-dimensional structure of the protein. We feel that this study and further mutagenesis studies of the 5-HT$_2$ receptor will continue to yield structural and functional information concerning the signal transduction mechanisms and ligand binding sites for this important membrane protein.

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