0026-895X/93/040516-05$03.00/0

MOLECULAR PHARMACOLOGY, 43:516-520

Identification of Residues Important for Ligand Binding to the Human 5-Hydroxytryptamine$_{1A}$ Serotonin Receptor

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Received June 24, 1992; Accepted February 2, 1993

SUMMARY

The functional significance of the conserved amino acids within transmembrane regions II and VII of the human 5-hydroxytryptamine (5-HT)$_{1A}$ receptor was analyzed by oligonucleotide-directed mutagenesis followed by transient expression of the mutated receptor genes in COS-1 cells. The substitution of a conserved asparagine at position 396 (transmembrane region VII) with either alanine, phenylalanine, or valine resulted in a receptor that did not bind the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-*n*-[^{3}H]propylamino)tetralin. In contrast, replacement of Asn^{396} with glutamine did not affect agonist binding. In addition, serine residues at positions 391 and 393 (transmembrane domain VII) were changed to alanine. Changing the less conserved Ser^{391} to alanine had no effect on ligand binding. However, replacement of the conserved Ser^{393} with alanine reduced ligand binding by 86%. Replacement of a conserved aspartate at position 82 (transmembrane region II) with alanine also produced a receptor without detectable agonist binding. Protein immunoblotting detected receptor protein of approximately 51 kDa in both wild-type and mutant receptor-expressing cells, indicating that these mutations probably did not affect expression or processing of the protein. Importantly, the sequence of the human 5-HT$_{1A}$ receptor described in this paper differs from the published sequence [*Nature (Lond.)* **329**:75–79 (1987)] in transmembrane region IV. The present sequence encodes a protein of 422 amino acids, instead of the 421-amino acid protein that has been described previously [*Nature (Lond.)* **329**:75–79 (1987)], and has a change in the sequence in transmembrane region IV from . . .RPRAL. . . to . . .RRAAA.., which corresponds to the published sequence [*J. Biol. Chem.* **265**:5825–5832 (1990)] of the rat 5-HT$_{1A}$ receptor. Moreover, conversion of the transmembrane region IV sequence of the present clone to that of the published sequence by site-directed mutagenesis abolished ligand binding to the receptor.

Serotonin receptors fall into four major classes, 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$, based on interactions of various agonists and antagonists (1). Of these, the 5-HT$_1$ receptors fall into five subtypes, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$, and 5-HT$_{1E}$, based on radioligand binding and functional analysis. The nucleic acid sequences of six of the serotonin receptors, the 5-HT$_{1A}$ (2, 3), 5-HT$_{1B}$ (4), 5-HT$_{1C}$ (5), 5-HT$_{1D}$ (6), 5-HT$_{1E}$ (7), and 5-HT$_2$ (8) receptors, reveal that all of these receptors are coupled to guanine nucleotide-binding proteins and have putative seven-transmembrane region topology. However, little is known regarding which residues in each polypeptide are responsible for ligand binding.

Recently, however, Guan *et al.* (9) reported a detailed study of the role of Asn^{385} in the seventh transmembrane domain of the human 5-HT$_{1A}$ receptor. They found that mutation of Asn^{385} to valine caused a highly selective decrease (about 100-fold) in the affinity of the β-adrenergic antagonist pindolol and other aryloxyalkylamines for the mutant receptor, while producing only minor changes in the binding of other classes of ligands. These results provide evidence that Asn^{385} is responsible for a high affinity binding interaction between the 5-HT$_{1A}$ receptor and aryloxyalkylamines but not other 5-HT$_{1A}$ ligands.

The present study was undertaken to determine whether other residues in the human 5-HT$_{1A}$ receptor are responsible for agonist ligand binding. The choice of residues for these site-directed mutagenesis studies was determined by the observation that in the seventh transmembrane domain of several cloned monoamine receptors (α- and β-adrenergic, 5-HT$_{1A}$, 5-HT$_{1D}$, and 5-HT$_2$) Asn^{396} and Ser^{393} are highly conserved. We now report that these residues are important for binding of the selective 5-HT$_{1A}$ agonist [^{3}H]8-OH-DPAT. Furthermore, a residue in the second transmembrane domain, Asp^{82}, is also involved in this binding.

We further show that the sequence of our 5-HT$_{1A}$ receptor clone differs from that previously published (2), in transmembrane domain IV (. . .RRAAA. . ., compared with . . .RPRAL. . .

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; IC$_{50}$, concentration of drug displacing 50% of bound ligand; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; PCR, polymerase chain reaction.

in the previously described clone). Conversion of the present sequence to the published sequence abolished [^{3}H]8-OH-DPAT binding to the transfected COS-1 cells.

Materials and Methods

Cloning and sequence determination. The PCR, using AmpliTaq (Perkin Elmer Cetus, Norwalk, CT), was used to amplify the coding region of the human 5-HT$_{1A}$ serotonin receptor from a human genomic library in EMBL3 (Clontech, Palo Alto, CA). The 5′ and 3′ primers were designed from the published human 5-HT$_{1A}$ sequence (2). The primer 5′ to the coding sequence contained the optimal Kozak sequence for translation initiation and suitable restriction sites to facilitate subcloning. The amplified product was verified by dideoxy DNA sequence analysis (10).

Mutagenesis. Oligonucleotide-directed *in vitro* mutagenesis was performed in the different regions of the receptor gene using the Mutagene phagemid *in vitro* mutagenesis kit (Bio-Rad, Richmond, CA). The DNA sequences of all the mutant receptors were confirmed by sequencing.

Expression of the cloned genes in COS-1 cells. A 1.27-kilobase *Hin*dIII-*Xba*I fragment containing the coding sequence of the 5-HT$_{1A}$ receptor was ligated between the *Hin*dIII and *Xba*I sites of the eukaryotic expression vector pcDNA-1 (In Vitrogen, San Diego, CA). Wild-type and mutant receptor DNA was transiently transfected into COS-1 cells using the procedure described by Chen and Okayama (11). Cells were harvested for binding assay 45–50 hr after transfection.

Membrane preparation. Transfected cells were washed in cold binding buffer (50 mM Tris·HCl, pH 7.4, 4 mM $CaCl_2$) and homogenized for 20–30 sec in a Tekmar Tissumizer (setting 50). Membranes were pelleted at 23,000 × *g* and rehomogenized, and the membrane protein content was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Radioligand binding assay. All membranes, drugs, and radioligand were made up in binding buffer (50 mM Tris·HCl, pH 7.4, 4 mM $CaCl_2$). Binding experiments were performed in a 1-ml volume of binding buffer for 30–60 min at 37°, using 90 μg of membrane protein, unless otherwise stated. The bound radioactivity was separated from free by filtration through Whatman GF/B filters with a Brandel cell harvester. Saturation isotherms were performed by incubating the membrane with varying concentrations of [^{3}H]8-OH-DPAT (Amersham Corp., Arlington Heights, IL). Nonspecific binding was determined by adding 10 μM methysergide to the binding assay. Binding assays were always performed in triplicate. Equilibrium dissociation constants were determined from saturation isotherms analyzed by the nonlinear curve-fitting program LIGAND.

Generation of 5-HT$_{1A}$ receptor antiserum. The entire cytoplasmic domain (between transmembrane regions V and VI) from amino acid isoleucine at position 218 to amino acid lysine at position 345 (total length, 128 amino acids) was amplified by PCR and cloned in the *Escherichia coli* expression vector pGEX-2T (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) behind the glutathione *S*-transferase gene from *Schistosoma japonicum*. This construct produced a fusion protein of approximately 26 kDa as the fusion partner at the amino terminus of the 5-HT$_{1A}$ gene fragment. The fusion protein was obtained after induction with isopropyl β-D-thiogalactopyranoside and was subsequently purified (>95% purity). New Zealand white female rabbits were injected biweekly with 300–400 μg of protein in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) for the first injection and incomplete Freund's adjuvant (Sigma) for subsequent injections. Hyperimmune serum was collected and tested for 5-HT$_{1A}$ antibody.

Protein immunoblotting. Membrane samples isolated from cells expressing the wild-type or mutant receptor were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose paper by electroblotting using a Novex Western blot apparatus. Electroblotting was performed according to the manufacturer's instructions. The nitrocellulose paper was incubated with rabbit anti-5-HT$_{1A}$ receptor antiserum at a dilution of 1/150. The nitrocellulose paper was then washed three times and incubated for 1 hr at room temperature with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit antibody at a dilution of 1/1000. After three washes, the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium/phosphatase (Kirkegaard & Perry Laboratories, Inc., MD) was added and the color was allowed to develop.

Materials. Buspirone was purchased from Research Biochemicals Inc. (MA), and both BMY-7378 and WAY-100,135 were synthesized at Wyeth Research (UK) Ltd. (Taplow, UK). Methysergide was a gift from Sandoz (Basel, Switzerland).

Results and Discussion

A fragment from genomic DNA containing the entire human 5-HT$_{1A}$ region was amplified using PCR and appropriate end-specific primers. The DNA sequence of the amplified fragment revealed a stretch of 1266 base pairs coding for a protein of 422 amino acids, with two changes, compared with the previously published 5-HT$_{1A}$ sequence (2).

One change (Asn^{396} to Tyr) was found in transmembrane domain VII and the other change in transmembrane domain IV. After position 100, amino acid numbers described here are increased by one because the 5-HT$_{1A}$ receptor described in this paper is one amino acid longer than the published sequence (2). Comparison of transmembrane domain IV of the present clone with that of the published sequence (Fig. 1) reveals that the present clone shares exact homology in transmembrane domain IV with the rat 5-HT$_{1A}$ sequence (3); thus, this human 5-HT$_{1A}$ clone is identical in size (422 amino acids) to the rat clone.

To test whether the present clone encodes the human 5-HT$_{1A}$ receptor, a 1.27-kilobase *Bam*HI/*Xba*I fragment containing the 5-HT$_{1A}$ receptor was placed in the expression vector pcDNA-1 containing the human cytomegalovirus promoter and the bovine growth hormone polyadenylation signal and was transfected into COS-1 cells. COS-1 cells were used for transfection because these cells appear to be devoid of a serotonin response (12). The study showed no specific [^{3}H]8-OH-DPAT binding in the transfected cells. In contrast, when the mutation in transmembrane domain VII was corrected from Tyr^{396} to asparagine, the resulting clone gave very high specific [^{3}H]8-OH-DPAT binding to the transiently transfected cell membranes (Table 1).

Membranes prepared from COS-1 cells transfected with the expression vector pcDNA-1 containing this corrected 5-HT$_{1A}$

Human 5-HT$_{1A}$	ACG	CCC	CGG	CCG	CGT	GCG	CTC	ATC
(Clone G21)	T	P	R	P	R	A	L	I
Our clone	ACG	CCC	CGG	CGC	GCC	GCT	GCG	CTC	ATC
	T	P	R	R	A	A	A	L	I
Rat 5-HT$_{1A}$	ACG	CCC	CGG	CGC ↓	GCC	GCT	GCG	CTC	ATC
(Clone D)	T	P	R	R	A	A	A	L	I

Fig. 1. Comparison of the sequence around transmembrane domain IV between the published (2) human 5-HT$_{1A}$ receptor, the human 5-HT$_{1A}$ receptor described in this paper, and the rat 5-HT$_{1A}$ receptor (3). *Arrow at the top of the rat 5-HT$_{1A}$* clone, boundary of the extracellular domain and hydrophobic transmembrane domain IV.

TABLE 1

Effect of mutations of 5-HT$_{1A}$ receptors on [^{3}H]8-OH-DPAT binding to membranes isolated from transfected COS-1 cells

The eukaryotic expression vector pcDNA-1 containing the 5-HT$_{1A}$ mutant receptor clones was used to transfect COS-1 cells. Membranes prepared from these cells as described in Materials and Methods were assayed for their ability to bind 1 nM [^{3}H]8-OH-DPAT. This concentration was chosen because it is close to the K_d for [^{3}H]8-OH-DPAT and binding, therefore, should be sensitive to small changes in the binding isotherm. Nonspecific binding varied between 0.5 and 2.5 fmol/mg of protein. The results are the average of three independent binding assays, and each binding assay was performed in triplicate.

Mutation	Transmembrane domain	Specific binding
		fmol/mg of protein
None (wild-type)	VII	277.5 ± 35.0
Asn^{396} → Phe	VII	1.2 ± 0.3
Asn^{396} → Ala	VII	0.5 ± 0.1
Asn^{396} → Val	VII	1.0 ± 0.1
Asn^{396} → Gln	VII	274.1 ± 34.8
Ser^{391} → Ala	VII	274.4 ± 34.4
Ser^{393} → Ala	VII	38.2 ± 2.1
Tyr^{396} → Asn	VII	273.9 ± 34.4
Asp^{82} → Ala	II	2.4 ± 0.3

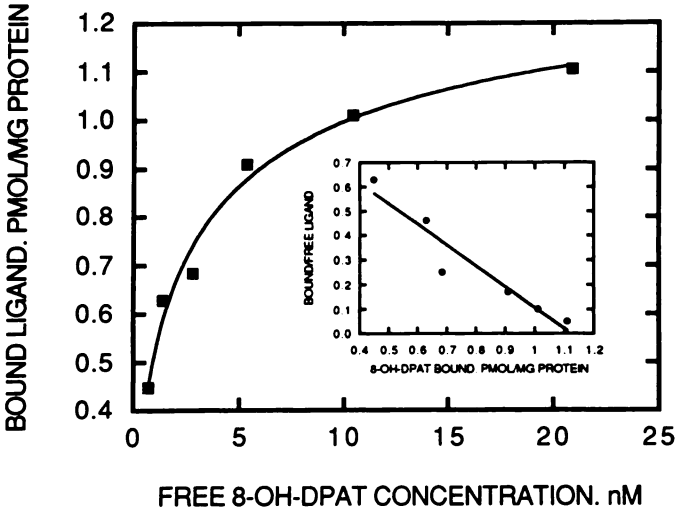

Fig. 2. Saturation analysis of [^{3}H]8-OH-DPAT binding to membranes prepared from COS-1 cells. *Abscissa*, free concentration of [^{3}H]8-OH-DPAT; *ordinate*, concentration of membrane-bound [^{3}H]8-OH-DPAT. *Inset*, Scatchard transformation of the data, which gave K_d = 1.2 nM and B_{max} = 1.25 pmol/mg of protein.

coding sequence were subjected to saturation binding analysis using [^{3}H]8-OH-DPAT (Fig. 2). Parallel incubations in the presence of 10 μM methysergide were conducted to determine nonspecific binding as a function of [^{3}H]8-OH-DPAT concentration; nonspecific binding was subtracted from total binding to yield specific binding. Membranes prepared from either COS-1 cells or cells transfected with the expression vector alone produced no detectable [^{3}H]8-OH-DPAT binding (data not shown).

Scatchard analysis of the binding data from a representative experiment gave K_d and B_{max} values of 0.98 ± 0.19 nM and 977 ± 69 fmol/mg of protein, respectively. Similar values have been reported previously for the human 5-HT$_{1A}$ receptor (12–14). This experiment clearly indicates that this clone indeed encodes the human 5-HT$_{1A}$ receptor.

Displacement of specific [^{3}H]8-OH-DPAT binding from the 5-HT$_{1A}$ receptor was also studied using buspirone, BMY-7378, and WAY-100,135 (Fig. 3). The first two are partial agonists and the last is a novel pure antagonist at the 5-HT$_{1A}$ receptor (15). Each of these compounds displaced specific [^{3}H]8-OH-DPAT binding from the 5-HT$_{1A}$ receptor with appropriate affinities (13, 16) (Fig. 3). When further analyzed by DNA sequencing, two clones, obtained from several independent PCR reactions, showed only asparagine at position 396 in transmembrane region VII. This rules out the possibility of polymorphism at this residue.

Because the rat and human 5-HT$_{1A}$ receptors have identical transmembrane region VII sequences (containing Asn^{396}), we introduced an Asn^{396} to Tyr mutation into the rat 5-HT$_{1A}$ receptor sequence. This mutation resulted in the elimination

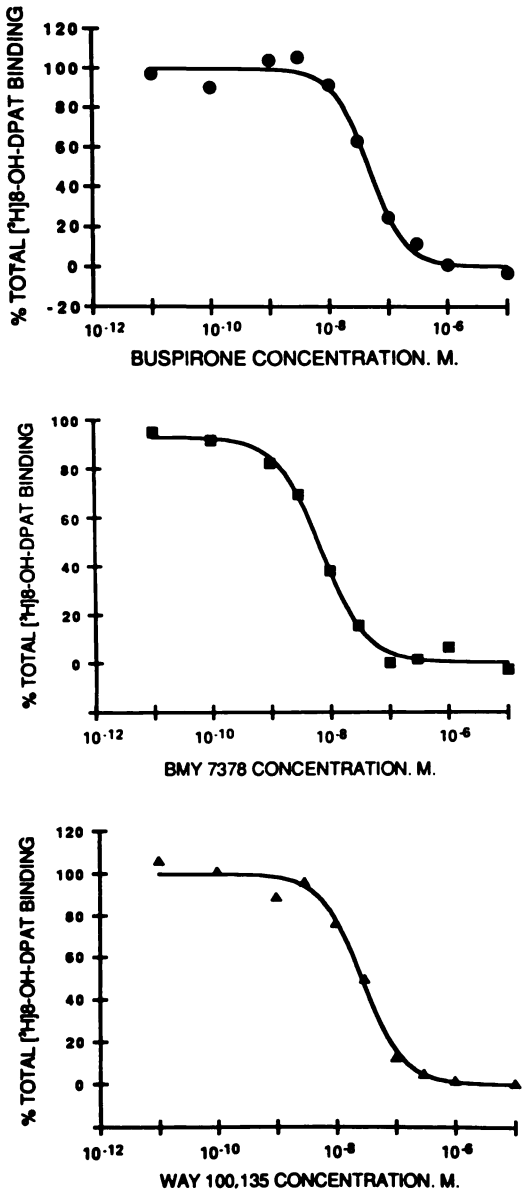

Fig. 3. Competition by 5-HT ligands for [^{3}H]8-OH-DPAT binding to COS-1 membranes. *Ordinate*, percentage of total [^{3}H]8-OH-DPAT binding at a radioligand concentration of 1 nM, in the absence (100%) or presence of increasing concentrations of displacing compounds (*abscissa*). *Top*, displacement by buspirone; *middle*, displacement by BMY-7378; *bottom*, displacement by WAY-100,135. Total counts in the absence of displacing compounds were typically 3000 cpm. IC$_{50}$ values were calculated as follows: buspirone, 48 nM; BMY-7378, 7.2 nM; WAY-100,135, 27 nM.

of [^{3}H]8-OH-DPAT binding to the rat 5-HT$_{1A}$ receptor (data not shown), as seen in the original human clone containing Tyr^{396}.

When additional mutations were introduced at Asn^{396} in transmembrane region VII (asparagine to alanine, asparagine to valine, asparagine to phenylalanine, and asparagine to glutamine), none of the mutant receptors demonstrated detectable agonist binding, with the exception of the asparagine to glutamine mutation. Because glutamine substitution at this position did not affect agonist binding, this finding suggests that the substitution of Asn^{396} with alanine, valine, or phenylalanine may prevent the receptor from assuming a conformation that is essential for agonist binding by preventing the seventh hydrophobic domain from forming a close interaction with one of the other hydrophobic domains, possibly by eliminating hydrogen-bond donor/acceptor interactions of this residue.

Alternatively this asparagine reside might also be involved directly in ligand binding. At present, we have no direct evidence to either prove or disprove this hypothesis. Because glutamine has an additional side-chain methylene group, compared with asparagine, and because glutamine substitution does not affect agonist binding, there apparently is some side-chain tolerance at this residue that should be explored further.

Because the radioligand binding assays were performed at a radioligand concentration equal to the K_d (~1 nM) of the 5-HT$_{1A}$ receptor, low affinity receptors formed with the apparent inactivating mutations may not have been detectable. When we increased the radioligand concentration to ~15 times the K_d, no specific binding of the radioligand was observed for any of the mutant receptors that failed to bind at the lower [^{3}H]8-OH-DPAT concentration. It is therefore likely that the mutant receptor genes did not produce any functional receptors of either high affinity or low affinity. However, it is important to note that both high and low affinity 5-HT$_{1A}$ receptors exist (17).

In the β-adrenoreceptor, the hydroxyl groups in the catechol ring appear to bind via two serine residues (positions 207 and 204) in transmembrane domain V; a third residue, Ser^{319}, is also important for agonist binding (18). We wondered, therefore, whether the ring hydroxyl in [^{3}H]8-OH-DPAT might also interact with a serine residue at the 5-HT$_{1A}$ receptor site.

Ser^{319} in the β-adrenoreceptor of several species is in a position in transmembrane region VII analogous to that of Ser^{393} in the 5-HT$_{1A}$ receptor (19). Accordingly, we mutated two serine residues, at positions 391 and 393, in transmembrane domain VII. Whereas Ser^{393} is conserved throughout a number of monoamine receptors (8, 20), Ser^{391} is not. Replacement of the latter (Ser^{391}) with alanine had no effect on [^{3}H]8-OH-DPAT binding, whereas mutation of the conserved Ser^{393} to alanine reduced binding considerably.

Scatchard analysis of this mutation yielded K_d and B_{max} values from three experiments of 35.5 ± 1.3 nM and 63.9 ± 7.8 fmol/mg of protein, respectively (Fig. 4). The Ser^{393} to alanine mutation therefore reduced the affinity for [^{3}H]8-OH-DPAT 36-fold, compared with the value for the wild-type receptor given above. Because the alanine residue in the mutant receptor cannot form hydrogen bonds, these data suggest that hydrogen bonding between Ser^{393} and [^{3}H]8-OH-DPAT (probably the ring hydroxyl) is important for binding of this ligand.

Previous mutagenesis studies (20) have argued for a role for negatively charged residues within the hydrophobic core of the

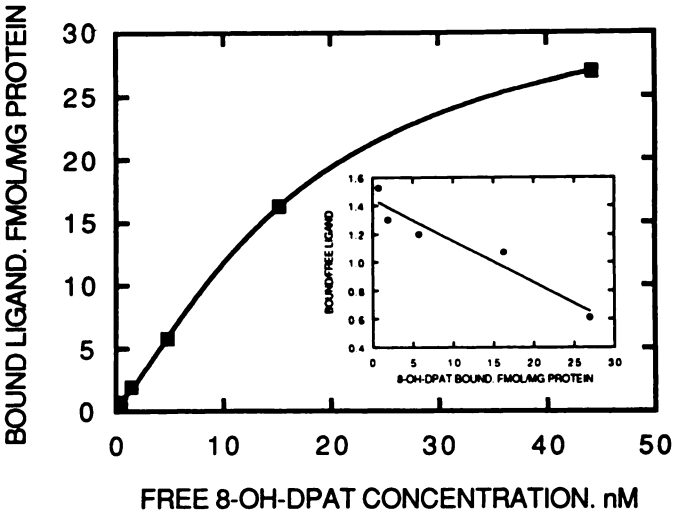

Fig. 4. Saturation analysis of [^{3}H]8-OH-DPAT binding to membranes prepared from COS-1 cells transfected with the gene containing the Ser^{393} to Ala mutation. *Abscissa*, free concentration of [^{3}H]8-OH-DPAT; *ordinate*, concentration of membrane-bound [^{3}H]8-OH-DPAT. *Inset*, Scatchard transformation of the data, which gave K_d = 33.4 nM and B_{max} = 48.4 fmol/mg of protein.

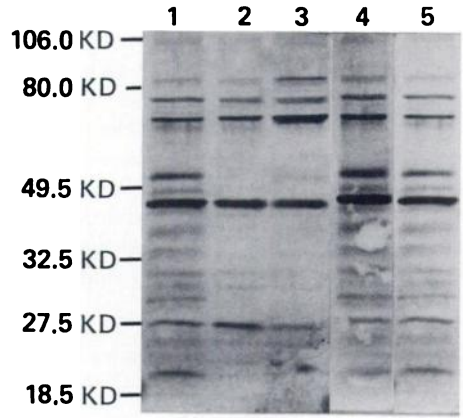

Fig. 5. Protein immunoblotting of the mutant and wild-type human 5-HT$_{1A}$ receptors. COS-1 cells were transfected with the wild-type or mutant human 5-HT$_{1A}$ receptor coding sequence, as described in Materials and Methods. Membranes were isolated and boiled in Laemmli buffer, and then proteins were separated on 10% polyacrylamide gels (22). After electrophoresis, proteins were transferred to nitrocellulose membrane and the immunoreactive receptor band was detected by protein immunoblotting. The antibody used in the immunoblot was raised, in a rabbit, to the entire cytoplasmic region located between transmembrane regions V and VI, using a fusion protein expressed in *E. coli* (to be described elsewhere). *Lane 1*, human 5-HT$_{1A}$ (Tyr^{396} → Asn); *lane 2*, mock-transfected COS-1 cells; *lane 3*, COS-1 cells transfected with the expression vector pcDNA-1; *lane 4*, 5-HT$_{1A}$ (Asp^{82} → Ala); *lane 5*, 5-HT$_{1A}$ (Asn^{396} → Tyr).

β-adrenergic receptor in mediating ligand binding. Single-amino acid substitution for the conserved Asp^{79} and Asp^{113} in transmembrane regions II and III of this receptor had profound effects on its ability to bind ligand. Sequences surrounding the conserved aspartic acid residues of hydrophobic domains II and III of 5-HT receptors cloned to date are very similar (21). To probe further the functional nature of these conserved regions, we replaced Asp^{82} of domain II with alanine, resulting in a receptor without detectable agonist binding (Table 1). This strongly argues for a role for this residue in either 5-HT$_{1A}$

ligand binding or maintenance of conformation as a result of its charge or hydrogen-bond acceptor properties.

Because the 5-HT_{1A} human receptor sequence described in this work differs from the published sequence (2), we investigated the effect of this sequence difference. Conversion of our sequence to the previously published sequence (2) by site-directed mutagenesis produced receptors without detectable binding. The original 5-HT_{1A} clone (2) has been resequenced and found to be identical in sequence to the present clone.[1]

To address the issue of whether the point mutations described in this work caused improper biosynthesis incorrect folding, and/or incorrect insertion of the receptor into the membrane (thereby eliminating ligand binding), we performed protein immunoblotting of COS-1 membranes prepared from transfected cells carrying the mutant and wild-type receptor genes. These data, shown in Fig. 5, indicate expression of an immunoreactive polypeptide with an apparent molecular weight of 51,000 in both the wild-type and mutant receptor-expressing cells suggesting that the point mutations did not cause any abnormality in the receptor proteins. It is also possible that the mutant proteins are misfolded and, hence, inactive but are still expressed and detectable by antiserum.

There are also additional bands that can be seen only in the lanes containing receptor constructs. Although we do not know the nature of these bands, we suspect that they are degradation products of the receptor that might be produced during purification. A band located just below the 49-kDa marker was detected in all the Western blots that were carried out. This might well be the unglycosylated form of the receptor protein.

Further studies based on the observations presented in this paper and the availability of antagonists such as WAY-100,135 should provide additional insight into the differences between agonist and antagonist binding, as well as the structure-function relationships among 5-HT_{1A} receptor subtypes.

Acknowledgments

We gratefully acknowledge Dennis Austin for synthesizing oligonucleotides and Phyllis Totaro for typing the manuscript.

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