Structural Features of the Ligand-binding Domain of the Serotonin 5HT₃ Receptor*

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The serotonin type 3 receptor (5HT₃R) is a member of a superfamily of ligand-gated ion channels, which includes the muscle and neuronal nicotinic acetylcholine receptor (AChR), the glycine receptor, and the γ-aminobutyric acid type A receptor (1–3). Like the other members of the gene superfamily, the 5HT₃R exhibits a large degree of sequence similarity, and thus presumably structural homology, with the AChR (4). Chimeras containing the amino-terminal domain of the α7 neuronal AChR and the carboxyl-terminal domain of the 5HT₃R form functional ligand-gated channels with ligand specificities characteristic of AChR and permeability properties of 5HT₃Rs (5). These experiments suggest that the AChR and 5HT₃R have quite similar structures and signal transduction mechanisms.

A large number of studies have been conducted to elucidate the structure of the ligand-binding site for AChRs (6, 7); however, very little is known about the interactions between ligands and the 5HT₃R. A common competitive antagonist of the AChR and 5HT₃R is d-tubocurarine (curare), which inhibits both receptors at nanomolar concentrations (8, 9). If both receptors are similar in structure, it seems reasonable to assume that similar regions of the receptors may be involved in curare binding. Affinity labeling studies using the Torpedo electroplax AChR have identified two tryptophan residues, one on the γ(γTrp-55) and the other on the δ(δTrp-57) subunit, as being involved in curare-AChR interactions (10). Mutagenesis data are consistent with γTrp-55 contributing to the high affinity curare-binding site (11), which is known to exist at the α-γ subunit interface (12, 13).

Comparison of the deduced amino acid sequences of this region of a number of members of the gene superfamily shows that tryptophan is found in this position in the AChR γ(γTrp-55) and δ(δTrp-57) subunits and the 5HT₃R (position Trp-89) but not most other receptors (Fig. 1). In support of the notion that Trp-89 may contribute to the curare-binding site of 5HT₃Rs, chemical modification studies have suggested that one or more tryptophan residues contribute to the binding of a number of 5HT₃R ligands (14). To determine the role of Trp-89 in ligand-5HT₃R interactions, we have carried out an alanine scan mutational analysis (15) of the region around Trp-89. We have identified several residues in this region that play a role in ligand-receptor interactions and have further identified residues that may make differential contributions to agonist and antagonist binding. Furthermore, the periodicity of the effects of mutations on ligand affinity suggests that this particular region of the ligand-binding domain is in a β-strand conformation.

EXPERIMENTAL PROCEDURES

Isolation of 5HT₃R cDNA and Mutagenesis—A pair of oligonucleotides (GCCCTACTAGTCTCCATTTGA and CTCCCACTCGCCCTGATTTAT) derived from the published sequence of the original 5HT₃R cDNA (4) was used to amplify a 460-base pair fragment from RNA isolated from the murine neuroblastoma line NIE-115 using reverse transcription-polymerase chain reaction (RNA polymerase chain reaction kit, P/E/Express). The fragment was then used to screen a plasmid cDNA library made from N1E-115 mRNA. A full-length cDNA clone corresponding to the 5HT₃Rα, form (16) was isolated and subcloned into vector pCI (Promega, Madison, WI). Site-directed mutagenesis was performed using the QuickChange system (Stratagene, La Jolla, CA). All mutants were verified through sequence analysis. Because the amino terminus of the mature protein is unknown, the amino acid numbering system used here includes the signal sequence and has the initial methionine as position 1.

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1 The abbreviations used are: 5HT₃R, serotonin type 3 receptor; AChR, nicotinic acetylcholine receptor.
Secondary Structure Analysis—Protein secondary structure analysis was carried out using the Chou-Fasman (17) and Garnier-Robson (18) algorithms contained in a commercially available software package (Lasergene, DNASTar, Inc., Madison, WI).

Transfection— Cultures of the tsA201 cell line, a derivative of the widely used HEK 293 cell line, were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cultures at 30–40% confluence were transfected with 20 μg of 5HT3R cDNA/100-mm dish using the calcium phosphate technique. After 12-h exposure to the DNA/calcium phosphate solution, the medium was replaced with fresh medium, and the cells were allowed to grow for another 24–36 h prior to use. Maximal expression was obtained 36–72 h after transfection.

Membranes—[3H]granisetron binding was defined as that binding not displaced by 100 nM of the competing ligand and radioligand ([3H]granisetron; NEN Life Science Products, 85 Ci/mmol). Binding was terminated by rapid vacuum filtration onto GF/B filters that had been pre-treated in 0.2% polyethyleneimine in 50 mM Tris-HCl, pH 7.4, and the filters were washed with cold 50 mM Tris-HCl, pH 7.4. Nonspecific binding was defined as that binding not displaced by 100 μM m-chlorophenyl biguanide. Kd values for [3H]granisetron were determined by fitting the saturation binding data to Equation 1 using a Levenberg-Marquardt algorithm in a commercially available software package (Igor Pro, WaveMetrics, Oswego, OR),

\[
B = \frac{B_{\text{max}}}{1 + \left(\frac{[I]}{Kd}\right)}
\]

where \(B\) is the amount of [3H]granisetron specifically bound at concentration \([L]\), \(B_{\text{max}}\) is the maximal binding at saturation, and \(K_d\) is the dissociation constant. IC50 values for various inhibitors were determined by fitting the data to Equation 2,

\[
\theta = \left(1 + \frac{\left(I\right)}{IC50}\right)^{-1}
\]

where \(\theta\) is the fractional amount of [3H]granisetron bound in the presence of the inhibitor at concentration \([I]\) compared with that in the absence of inhibitor and IC50 is the concentration of inhibitor at which \(\theta = 0.5, K_d\) values were calculated from the IC50 values using the Cheng-Prusoff relation (19) (Equation 3),

\[
K_d = \frac{IC50}{1 + \left(I\right) / IC50}
\]

where \([L]\) is the concentration of [3H]granisetron used to determine the IC50 value in the experiment and \(K_d\) is the dissociation constant for [3H]granisetron.

Electrophysiology—Transfected cells were transferred to 35-mm dishes containing 140 mM NaCl, 1.7 mM MgCl2, 5 mM KCl, 1.8 mM CaCl2, 25 mM HEPES, pH 7.4, and currents elicited by bath application of agonists at a holding potential of ~70 mV were measured in the whole-cell configuration (20) with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) under computer control using custom programs written in AxoBasic. Patch electrodes contained 145 mM KCl, 2 mM MgCl2, 1 mM EGTA, 25 mM HEPES, pH 7.4, and had resistances of 3–5 MΩ. Agonist and antagonists were dissolved in extracellular solution and delivered to the cell by means of a fast perfusion system (Warner Instruments, Hamden, CT). For experiments involving inhibition of 5-HT-elicited currents by curare, cells were perfused with the desired concentration of curare for 2 min before switching to a solution containing 5-HT and curare.

Dose-response curves from individual cells were normalized to the maximum current and were fit to Equation 4 as described previously (21),

\[
\theta = \left(1 + \frac{EC50}{[A]}\right)^{-n}
\]

where \(\theta\) is the normalized current at 5-HT concentration \([A]\), EC50 is the concentration of 5-HT required to obtain half-maximal current, and \(n\) is the apparent Hill coefficient.

RESULTS

Our initial focus was on Trp-89, which is in the homologous position to the two tryptophan residues in the Torpedo AChR.

**Fig. 2.** Amino acid substitution at position Trp-89 markedly reduces curare sensitivity. Cells expressing either wild type (left) or W89F 5HT3R were voltage-clamped, and currents elicited by bath application of 1 μM 5-HT in the presence or absence of 10 nM curare (dTC) were recorded. Note that the mutant receptors are relatively insensitive to curare.
that are photolabeled by curare, γ-Trp-55 and δ-Trp-57 (10). Initially, Trp-89 was replaced by alanine, which should have little effect on α-helical or β-sheet structure and thus should produce little structural perturbation. However, we were unable to elicit whole cell currents with 5-HT concentrations up to 10 mM from cells transfected with W89A receptors nor were we able to detect specific binding of the antagonist [3H]granisetron in membranes prepared from transfected cells (data not shown). Obviously, substitution of Trp-89 with alanine either interfered with the proper assembly or the functioning of the receptor. We then replaced tryptophan with another aromatic amino acid, phenylalanine, which should represent a less severe substitution. Fig. 2 shows 5-HT-elicited currents recorded from cells transfected with either wild type (Fig. 2, left) or W89F (Fig. 2, right). Although the activation properties of the two receptors are essentially identical (Table I), the sensitivity to the competitive antagonist curare was markedly different. Wild type receptors were inhibited 85% by 10 nM curare, whereas W89F receptors were inhibited less than 15% under the same conditions.

This reduction in curare sensitivity is also seen in ligand binding studies. Saturation binding isotherms for the competitive antagonist [3H]granisetron were determined for both wild type and W89F receptors (Fig. 3A). The \( B_{\text{max}} \) value for wild type receptors was on the order of 2–4 pmol/mg of protein, whereas that of the W89F receptors was 1–2 pmol/mg of protein (data not shown). The expression levels of wild type receptors are 5–10 times that reported for N1E-115 neuroblastoma cells (22) or transiently transfected COS-1 (4) cells. The affinity of the W89F receptors for [3H]granisetron was markedly reduced compared with wild type (\( K_d = 10.9 \pm 2.9 \text{nM} \) (W89F) versus \( 1.3 \pm 0.1 \text{nM} \) (wild type)).

We examined the effects of the W89F mutation on the affinity of other agonists and antagonists through the use of analysis of the reduction in [3H]granisetron binding in the presence of increasing concentrations of agonists and antagonists. Inhibition of [3H]granisetron binding by curare to wild type and W89F receptors showed a similar reduction in ligand affinity in the mutant (Fig. 3B). The \( K_i \) for curare inhibition in wild type receptors was \( 138 \pm 22 \text{nM} \), whereas the value for W89F receptors was \( 1063 \pm 179 \text{nM} \), a reduction in affinity comparable with that seen in the [3H]granisetron saturation binding isotherms. A similar reduction in affinity was also observed for another 5HT3R antagonist, MDL-72222 (data not shown). In all three cases, the W89F mutation results in a reduction of ligand affinity. However, when agonists were used in the competition studies, a different picture emerges. No significant difference in affinity between mutant and wild type receptors was observed for the full agonist serotonin (Fig. 3C) or the partial agonists phenylbiguanide or m-chlorophenylbiguanide (data not shown). The serotonin data are consistent with the electrophysiological assays in which no difference in the activation parameters was observed between wild type and W89F receptors.

The above findings suggest that Trp-89 interacts specifically with antagonists but not with agonists. To explore this in more detail, alanine-scanning mutagenesis (15) of this region of the
receptor was carried out. In this approach, functionally important residues are identified by sequential replacement of residues within a specific region with alanine. Alanine is used as the replacement because it eliminates the side chain beyond the $\beta$-carbon yet, unlike glycine, generally does not alter main chain conformation. We carried out the analysis on nine residues flanking Trp-89 (Thr-85 to Trp-94) using $[^3]$H]granisetron, curare, and serotonin as the ligands to examine the properties of the binding site. All nine alanine-substituted receptors were expressed on the cell surface and formed $5HT_3Rs$. The receptor density (determined as the $B_{max}$ values for $[^3]$H]granisetron binding) of the mutant receptors ranged from 0.1 pmol/mg (Q92A) to 2.2 pmol/mg (T86A).

The results of the alanine scan in terms of ligand affinities are presented in Fig. 4. Each of the three ligands shows a distinct profile. For example, the only substitution that significantly affects the interaction of curare with the receptor is W89F; all other replacements in this region have little effect on affinity. In the case of the agonist serotonin, the only substitution that alters the affinity of the receptor is R91A. Finally, the affinity of $[^3]$H]granisetron is reduced by substitutions at both Trp-89 and Arg-91, as well as by a third, Y93A. These results suggest that whereas this region of the receptor is clearly involved in ligand binding, each of these three structurally different ligands interacts in a slightly different fashion with the ligand-binding domain of the receptor. These differences, in turn, can provide some information on some of the structural features of the receptor.

**DISCUSSION**

Previous studies have suggested the involvement of tryptophan residues in ligand binding in both the AChR (10, 11) and the $5HT_3R$ (14). The data presented in this study demonstrate that Trp-89, which is in the same homologous position as the two tryptophan residues in the AChR that interact with curare (Trp-55, Trp-57), plays an important role in curare-$5HT_3R$ interactions. The fact that this is a region in the $5HT_3R$ homologous to a domain known to be involved in ligand binding in the AChR lends support to the notion that similar regions in the two receptors are involved in ligand binding.

The data presented in this study suggest that although the region around Trp-89 in the $5HT_3R$ is clearly involved in ligand binding, the specific interactions of various compounds with the binding site may be different. In particular, the fact that in the region Thr-85 to Trp-94 substitution at Trp-89 affects curare, but not serotonin, affinity indicates that there are differential interactions between the binding site and the ligands. This is presumably because of differences in ligand structure and/or positioning within the binding site.

In addition to the notion that the “fine structure” of ligand-receptor interactions may differ for different ligands, our data also provide some insight into the structure of the binding site itself. Unlike curare and serotonin, whose binding is sensitive to mutations at only one residue, the affinity of the antagonist granisetron is affected by mutations at three different residues in this region (Trp-89, Arg-91, Tyr-93). The fact that the relevant positions are in an every-other-residue pattern suggests very strongly that this region of the receptor is in a $\beta$-strand conformation. In support of this hypothesis, secondary structure predictions using the Chou-Fasman (17) and Garnier-Robson (18) algorithms predict that this region of the protein should exist in a $\beta$-sheet structure. This aspect of the study demonstrates the power of the alanine scan mutagenesis ap-
Secondary Structure of 5HT₃R Ligand-binding Domain

The data presented here are consistent with the notion that there is a significant amount of structural and functional homology between the AChR and 5HT₃R (and by inference, the other members of the ligand-gated ion channel family). Changeux and co-workers (6) have proposed that the ligand-binding domain of the AChR is formed by several distinct regions of the α and non-α subunits. We envision that the ligand-binding domain of the 5HT₃R is also formed by several different parts of the extracellular domain that are homologous to those regions of the AChR known to be involved in ligand binding. The present study has focused on one of these regions, and the data indicate that this notion that homologous regions are involved in ligand binding is indeed correct. The expansion of our studies to the other regions of 5HT₃R that are homologous to the ligand-binding domains of the AChR will allow us to test the notion that the functional homology of the members of the ligand-gated ion channel family extends to a fairly resolved level. Given the success of our initial foray, as well as our findings concerning the secondary structure of a portion of the binding site, we envision this as a somewhat reciprocating process, in which information obtained from one member of the gene family can be used to guide the studies on another, and vice versa.

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