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Importance of Phenylalanine 107 in Agonist Recognition by the 5-Hydroxytryptamine$_{3A}$ Receptor

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ABSTRACT

The 5-hydroxytryptamine (5-HT)$_3$ receptor is a member of the ligand-gated ion channel receptor family with significant homology to the nicotinic acetylcholine, γ-aminobutyric acid$_A$, and glycine receptors. In this receptor class, the agonist binding site is formed by parts of the extracellular amino-terminal region. This study examines the effects of altering phenylalanine 107 (F107) of the 5-HT$_{3AL}$ subunit, obtained from NG108-15 cells, using site-directed mutagenesis. The wild-type (WT) and mutant receptors were expressed in HEK 293 cells and characterized using both whole-cell patch-clamp and radioligand binding. The tyrosine mutant F107Y exhibits a significantly lower affinity for the agonist 5-HT (K_i = 203 versus 15.6 nM) and an increase of similar magnitude in the EC$_{50}$ value (10.6 versus 1.2 μM) compared with WT. The activation kinetics of the maximal currents generated by 5-HT with this mutant were markedly slower than those of the WT receptor, but application of supramaximal concentrations of the agonist markedly decreased the time to half-peak. The asparagine mutant F107N displayed a significantly higher affinity for 5-HT than the WT receptor (1.62 versus 15.6 nM), which was mirrored in direction and magnitude by changes in the EC$_{50}$ value for this agonist (0.2 versus 1.2 μM). In contrast to the WT receptor, the mutant F107N was activated by acetylcholine (EC$_{50}$ = 260 μM). The response to acetylcholine was blocked by the 5-HT$_3$ receptor antagonist renzapride with a similar IC$_{50}$ value as that determined against currents generated by 5-HT in the WT receptor. These data suggest that F107 is an important determinant of agonist recognition at the 5-HT$_3$ receptor.

The 5-hydroxytryptamine (5-HT)$_3$ receptor is a member of the family of ligand-gated ion channels. It consists of five subunits arranged pseudosymmetrically around an integral cation channel (Boess et al., 1995; Green et al., 1995). Initially, a single subunit was cloned (5-HT$_{3A}$; Maricq et al., 1991), and two splice variants have been identified in various species, designated 5-HT$_{3AL}$, with an additional five or six amino acids between the third and fourth hydrophobic segments, and 5-HT$_{3AS}$, without the insertion (Hope et al., 1993; Isenberg et al., 1993; Uetz et al., 1994; Werner et al., 1994; Belelli et al., 1995; Miquel et al., 1995). The 5-HT$_{3A}$ cDNA forms functional receptors that, when expressed in various cell lines, exhibit positive cooperativity to 5-HT and show a similar pharmacological profile as native 5-HT$_3$ receptors (Jackson and Yakel, 1995). Recently, a second subunit has been cloned, designated 5-HT$_{3B}$, that forms functional 5-HT$_3$ receptors when expressed together with 5-HT$_{3A}$ but not when expressed alone. The hetero-oligomer exhibits distinctive pharmacological properties compared with those found in the homo-oligomer and a markedly increased single-channel conductance (Davies et al., 1999).

The deduced amino acid sequence of the 5-HT$_{3A}$ receptor protein shows greatest similarity within the ligand-gated ion channel family with the nicotinic acetylcholine (ACh) receptor (nAChR) α7 subunit, with a sequence identity of 31% (55% including conservative substitutions). The construction of a chimera with the amino terminus of nAChR α7 subunit together with the carboxyl terminus of 5-HT$_{3AL}$ subunit exhibited agonist activation by ACh but not 5-HT (Eisele et al., 1993). This provided definitive evidence that agonist recognition is encoded by the amino terminus, thought to be extracellularly located. Biochemical and site-directed mutagenesis studies of the nAChR have identified three hydrophilic loops within the α-subunit amino-terminal domain that are involved in ligand recognition (Changeux et al., 1992; Karlin and Akabas, 1995). In particular, photoaffinity labeling of the tyrosine residue (Y93 in the *Torpedo* α-subunit) in the most amino terminal of these hydrophilic loops, loop 1, with [^{3}H]ACh mustard (Cohen et al., 1991), *p*-dimethylamino-ben-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; WT, wild type; HBSS, Hanks' buffered salt solution; mCPBG, *m*-chlorophenylbiguanide.

zenediazonium fluoroborate (Galzi et al., 1990), and subsequent mutagenic studies (Aylwin and White, 1994a,b; Nowak et al., 1995) have implicated this residue in the recognition of ACh (Fig. 1).

Within the 5-HT_{3A} receptor sequence, the amino acid triplet NEF aligns with YNN in the *Torpedo* α-subunit and YNS in the α7 sequence (Fig. 1). The markedly different character of this amino acid triplet suggests that this may play an important role in the differential ligand recognition of these two sequences. In an extension of our previous studies in which we carried out mutations of glutamate (E106) of the 5-HT_{3AL} sequence (Boess et al., 1997), we have now mutated the aromatic amino acid phenylalanine, F107, conservatively to tyrosine (F107Y) or to asparagine (F107N). We characterized the mutants with both radioligand binding and whole-cell patch-clamp studies.

Experimental Procedures

Materials. *BSu*36I was obtained from Promega (Madison, WI). *Sal*I was obtained from Stratagene (La Jolla, CA). The Altered Sites Mutagenesis Kit was purchased from Promega and included the vector pAlter. The expression vector pRC/CMV was supplied by InVitrogen (San Diego, CA). HEK 293 cells were obtained from American Type Culture Collection (Rockville, MD). TSA201 cells were obtained from COR Therapeutics (San Francisco, CA). Dulbecco's modified Eagle's medium, Hanks' balanced salt solution (HBSS), and penicillin/streptomycin were purchased from BioWhittaker (Walkersville, MD). Fetal bovine serum was purchased from Life Technologies (Burlington, Ontario, Canada). 5-HT creatinine sulfate, N^{ω}-methyl-5-HT oxalate, and ACh chloride were obtained from Sigma-Aldrich Canada Ltd. (Mississauga, Ontario, Canada). *m*-Chlorophenylbiguanide (mCPBG), 2-methyl-5-HT, and metoclopramide were obtained from Research Biochemicals International (Natick, MA). [^{3}H]GR65630 (61.4–64.4 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). [^{3}H](*S*)-Zacopride (84.0 Ci/mmol) was purchased from Amersham Life Science Inc. (Arlington Heights, IL). Ondansetron was donated by Glaxo (Ware, UK). Renzapride and granisetron were donated by SmithKline Beecham Pharmaceuticals (Harlow, UK). All drugs were prepared in HEPES (10 mM, pH 7.5). Other chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd., BDH (Toronto, Ontario, Canada), and FischerBiotech (Nepean, Ontario, Canada).

Site-Directed Mutagenesis of 5-HT_{3AL} cDNA. The 5-HT_{3AL} receptor cDNA from NG108-15 cells (generously provided by Dr. Eric Kawashima, Glaxo Molecular Biology Institute, Geneva, Switzerland) was subcloned into the mutagenesis vector pAlter and the eukaryotic expression vector pRC/CMV. The Altered Sites Mutagenesis Kit (Promega) was used to introduce mutations of F107 to tyrosine (F107Y) and asparagine (F107N) using the following mutagenic oligonucleotides: WT 5′-AGA-CTT-CCC-CAC-GTC-**C**AC-AAA-CTC-ATT-GAT-GAG-AAT-3′, F107Y 5′-AGA-CTT-CCC-CAC-GTC-**G**AC-**ATA**-CTC-ATT-GAT-GAG-AAT-3′, and F107N 5′-AGA-CTT-CCC-CAC-GTC-**G**AC-A**TT**-CTC-ATT-GAT-GAG-AAT-3′.

A silent mutation, present in each oligonucleotide, introduced a new *Sal*I restriction site to facilitate mutant screening. The sequence between two *Bsu*36I restriction sites in the 5-HT_{3AL} sequence encodes the amino terminus and the first two hydrophobic segments of the protein. Mutant pAlter-5-HT_{3AL} vectors were digested with *Bsu*36I, and the resulting 891-bp fragments were ligated into cut pRC/CMV-5-HT_{3AL} vector to replace the WT *Bsu*36I fragment. Mutations were confirmed using an automated fluorescent sequencing system (Applied Biosystems, Foster City, CA) for the coding region between, and including, the two *Bsu*36I restriction sites.

Torpedo nAChR α	DVWLPDLVL**YNN**AD	(97)
α7 nAChR	QIWKPDILL**YNS**AD	
5-HT_{3AL}R	SIWMPDILI**NEF**VD	(109)

Fig. 1. Alignment of deduced amino acid sequences of the 5-HT_{3A} receptor subunit and the α-subunits of nAChRs in recognition loop 1. Conserved residues are shown boxed, and the residues referred to in the text are in bold. The C-terminal residues are numbered.

Transient Expression of cDNAs, Cell Culture, and Membrane Preparation. WT and mutant cDNAs in the eukaryotic expression vector pRC/CMV were transiently expressed in either HEK 293 or the HEK 293-derived cell line TSA201 (Heinzel et al., 1988). TSA201 cells produced increased expression levels compared with HEK 293 cells. The results obtained with WT and mutant receptors expressed in the two cell types were similar, and the data were pooled. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The same medium was used to culture the TSA201 cells with the exception of low glucose (1 g/l). Cells were incubated at 37°C in a humidified 7% CO_2 atmosphere on either 150-or 35-mm-diameter plates.

Cells were transiently transfected with WT or mutant cDNAs using a modification of the calcium phosphate coprecipitation method (Chen and Okayama, 1988). For radioligand binding studies, cells were grown on 150-mm-diameter plates, transfected with 35 to 45 μg of DNA, and grown in a 3% CO_2 incubator. At 20 h after transfection, the cells were washed with HBSS, fresh medium was added, and the plates were returned to the incubator (3% CO_2). Then, 24 h later, the cells were harvested using ice-cold protease inhibitor buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 50 mg/l bacitracin, 50 mg/l soybean trypsin inhibitor, and 100 μM phenylmethylsulfonyl fluoride). The cells were homogenized with an Ultra-Turax (20,000 rpm, 10 s), and the homogenate was centrifuged (27,000*g* for 20 min at 4°C). The resultant membrane pellet was gently resuspended in ice-cold HEPES buffer (10 mM, pH 7.5) and frozen at −20°C until further use. For electrophysiological studies, cDNAs were transiently expressed in cells grown on 35-mm-diameter plates using 1 to 2 μg of DNA. Plates were incubated in 3% CO_2 for 18 h and then washed with HBSS. Fresh medium was added, and the cells were placed in the 7% CO_2 incubator until their use within 24 to 48 h.

Radioligand Binding. Membranes were thawed and centrifuged (27,000*g*, 4°C for 20 min), and the pellets resuspended in ice-cold HEPES (10 mM, pH 7.5) for both [^{3}H]GR65630 and [^{3}H](*S*)-zacopride binding. Assay tubes contained 800 μl of the HEPES buffer, with or without the competing drug, and 100 μl of radioligand [^{3}H]GR65630 (for competition studies, 0.6 nM; for saturation studies, 0.02–26.7 nM) or [^{3}H](*S*)-zacopride (for competition studies, 0.5 nM; for saturation studies, 0.02–10 nM). Metoclopramide (300 μM) was used to define specific binding. The assay tubes were preincubated on ice for 2 min before the addition of 100 μl of the membrane suspension, equivalent to approximately 100 μg of protein. After further incubation either on ice for 2 h with [^{3}H]GR65630 or at 37°C for 2 h with [^{3}H](*S*)-zacopride, the assay was terminated by rapid filtration through Whatman GF/B filters that had been pretreated with 0.3% polyethylenimine in the above HEPES buffer. The filters were then washed with 2 × 5 ml of ice-cold HEPES buffer, and the resultant radioactivity was determined by conventional liquid scintillation counting (Ecoscint; National Diagnostics, Atlanta, GA) at an efficiency of about 60%.

Electrophysiology. Membrane currents were recorded under voltage-clamp from single cells with the whole-cell configuration of the patch-clamp technique using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA). Series resistance compensation was used, and recordings were performed at a holding potential of −60 mV. Data acquisition, storage, and analysis were performed using pClamp 6 (Axon Instruments). Pipettes were pulled from borosilicate

glass and had resistances of 2 MΩ when filled with pipette solution consisting of 135 mM CsCl, 0.5 mM $MgCl_2$, and 10 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrapotassium salt, pH 7.2, with CsOH. Ligands were applied to the cells using a gravity-feed rapid perfusion system (half-time of solution change was 100 ms) based on the design of Carbone and Lux (1987). The cell was continually perfused with solution containing 130 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4 with NaOH. The solutions were changed by means of a manually operated valve that housed a manifold connected to solution reservoirs. Recordings were performed at room temperature (19–21°C). Cells with phase-bright "inclusion bodies" that were visible with a phase contrast microscope were generally found to express receptors. To allow recovery from desensitization, 3 min was allowed to elapse between agonist applications. Because the current was subject to run-down during the course of recording, data for concentration-effect curves were collected using the following sequence: maximal concentration, test concentration, maximal concentration. Data were accepted for analysis only if the current for the second maximal concentrations did not decline by more than 10%. For concentration-effect curves, data were pooled from different cells.

Data Analysis. Radioligand binding data were analyzed by computer-assisted iterative curve fitting (Kaleidagraph; Synergy Software, Reading, PA) according to the equation: $B = B_{max}[L]^n/([L]^n + K^n)$, where B is the bound ligand, B_{max} is the maximum binding at equilibrium, K is the molar equilibrium dissociation constant (K_d) for saturation studies or molar concentration of competing compound to reduce the specific binding to 50% for competition studies (IC_{50}), L is the free molar concentration of radioligand for saturation studies or molar concentration of competing compound for competition studies, and n is the Hill coefficient. The Cheng-Prusoff equation was used to calculate the K_i values of competing drugs, with $K_i = IC_{50}/(1 + ([L]/K_d)$.

Concentration-effect curves were fitted to the equation: $I = I_{max}/(1 + EC_{50}/[A]^n)$ using a Levenberg-Marquardt algorithm in PSI-PLOT (Polysoft). I and I_{max} are the currents at a given agonist concentration [A] and the maximal value, respectively. The EC_{50} value is the concentration of agonist required to obtain half-maximal current, and n is the apparent Hill coefficient.

Statistical significance of radioligand binding values were tested using pK_i and pK_d values with the Student's t test.

The pK_a values in Fig. 7 were calculated with the PALLAS system software package obtained from StandBy Software Inc. (Burlingame, CA).

Results

Radioligand binding studies were performed with both [^{3}H]GR65630 and [^{3}H](*S*)-zacopride on either HEK 293 or TSA201 cell membranes expressing WT, F107Y, or F107N mutant receptors. The densities of mutant and WT receptors determined with either radioligand were similar, suggesting that the mutations had not compromised expression. Saturation studies with either [^{3}H]GR65630 (Fig. 2) or [^{3}H](*S*)-zacopride appeared to label a homogeneous population of receptors, and there were no significant differences in the K_d values for either ligand between the WT and mutant receptors (Table 1 and 2).

Mutant F107Y. Competition studies in the presence of [^{3}H]GR65630 indicated that the affinity for the natural agonist 5-HT was decreased by a factor of 13 in F107Y compared with the WT (K_i = 203 and 15.6 nM, respectively; Fig. 3, Table 1). The direction of the change for the close structural analog 2-methyl-5-HT was the same, although the magnitude of the change was only 3.5-fold (K_i = 610 and 173 nM for the mutant and WT, respectively; Table 1). The agonist N^{ω}-methyl-5-HT had a 35-fold decrease in affinity compared with WT (K_i = 6695 and 187 nM, respectively; Table 1). This mutation had no significant effects on the affinity for the agonist mCPBG or the antagonists ondansetron, granisetron, or renzapride (Table 1). Competition studies, in which [^{3}H](*S*)-zacopride replaced [^{3}H]GR65630, gave similar results for this mutant, although the magnitude of the observed changes was reduced (Table 2).

Whole-cell patch-clamp studies carried out with the WT or mutant F107Y receptors expressed in either HEK 293 or TSA201 cells indicated that the whole-cell currents were comparable in magnitude. This was consistent with the radioligand binding data, suggesting that the mutation did not significantly affect receptor expression. No responses to 5-HT application were recorded in mock-transfected cells. Concentration-effect curves for mCPBG were similar in both the WT and F107Y mutant-expressing cells, although the Hill coefficient obtained with F107Y was reduced compared with WT (Table 3). The EC_{50} value of 5-HT was increased 9-fold by the mutation F107Y (10.6 μM) compared with WT (1.2 μM; Table 3, Fig. 4). The Hill coefficient for WT was 2.0, whereas for F107Y it was 1.6, which is indicative of positive cooperativity (Table 3). The EC_{50} value for N^{ω}-methyl-5-HT was also increased by this mutation compared with WT (7.1 and 0.96 μM, respectively; Table 3).

The activation kinetics of the currents generated by mutant F107Y receptors were markedly slower than those exhibited by WT receptors (Fig. 5A). Concentrations of 5-HT that elicited currents of maximum amplitude (approximately 10 × EC_{50}) produced a time to half-peak amplitude of 108 ± 25 ms (mean ± S.E.; n = 16) for WT (10 μM) that was limited, in our experimental system, by the rapidity with which the agonist could be applied. In contrast, the F107Y mutant receptors maximally activated by 5-HT at a concentration of 100 μM (approximately 10 × EC_{50}) exhibited a time to half-peak amplitude that was approximately 16 times longer ($t_{1/2}$ = 1795 ± 783 ms; n = 23; Fig. 5A). The application of a supramaximal concentration of 5-HT (10 mM, 1000 × EC_{50}) to these mutant receptors decreased the time to half-peak amplitude to 494 ± 180 ms (n = 18, Fig. 5A). This result is consistent with the notion that the slower activation kinetics of the mutant receptor were responsible, at least in part, for

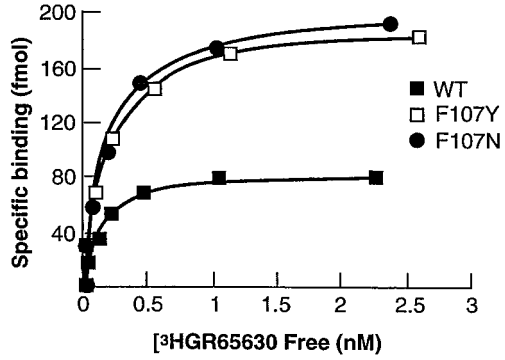

Fig. 2. Equilibrium saturation binding isotherms determined with [^{3}H]GR65630. Studies were carried out on cell membranes obtained from HEK 293 cells expressing WT (F107), F107Y, or F107N mutant receptors. Specific binding was defined with 300 μM metoclopramide. Results are shown from a single experiment that was repeated 3 to 10 times. Scatchard transformation of the data in all cases was consistent with the expression of a single homogeneous receptor population.

its lower apparent affinity and increased EC_{50} value compared with the WT receptor (Fig. 4). In contrast, the activation kinetics of mCPBG-elicited currents were unaltered (Fig. 5B). The IC_{50} value of the 5-HT_3 receptor antagonist renzapride to block 5-HT-induced currents was 10 nM when the agonist was applied at its EC_{50} value for both WT (1 μM) and F107Y (10 μM), suggesting no change in the affinity of renzapride for the mutant receptor (data not shown).

Mutant F107N. Competition studies with [^{3}H]GR65630 showed that the affinity for the agonist 5-HT was increased 10-fold for mutant receptor F107N compared with the WT (K_i = 1.62 and 15.6 nM, respectively; Fig. 3 and Table 1), whereas the increase in affinity for 2-methyl-5-HT was only 4-fold compared with WT (41.7 and 173 nM, respectively; Table 1). The affinity for N^{ω}-methyl-5-HT increased 35-fold in mutant F107N compared with WT (K_i = 5.2 and 187 nM, respectively; Table 1). mCPBG, ondansetron, and renzapride exhibited no change in affinity for the mutant F107N compared with WT, although the affinity of granisetron was decreased 10-fold (Table 1). In competition studies with [^{3}H](*S*)-zacopride, granisetron showed no difference in affinity to the WT receptor, whereas renzapride exhibited a significant increase in affinity compared with WT (Table 2).

Both the direction and magnitude of the changes in affinity for the agonists 5-HT and N^{ω}-methyl-5-HT, found in the binding studies with mutant F107N, were also reflected in the changes of the EC_{50} (Fig. 4, Table 3). However, the Hill coefficients for these agonists were decreased, suggesting a loss of cooperativity. The EC_{50} value for mCPBG for mutant F107N was not significantly different than that determined for the WT receptor (Table 3).

Interestingly, F107N mutant receptors could be activated by ACh (Fig. 6A) with an EC_{50} value of 256 μM (95% confidence limits, 197 and 359 μM; n = 5). The agonist response to ACh displayed positive cooperativity (Hill coefficient = 1.9) and was blocked in an apparently competitive fashion by renzapride (Fig. 6B), which displayed an IC_{50} value of 22 nM (95% confidence limits, 14 and 34 μM), very similar to that required to inhibit 5-HT responses in the WT receptor. The K_i values for ACh in both F107N and WT receptors were greater than 400 μM as determined in competition with [^{3}H]GR65630.

Discussion

The mutation of the aromatic residue F107 in the 5-HT_{3AL} receptor subunit sequence produced parallel shifts in radioligand binding affinity (K_i) and EC_{50} value determined electrophysiologically for the natural agonist 5-HT. The apparent affinity for the mutant F107Y decreased by about 10-fold compared with the WT receptor, whereas for the mutant F107N, the apparent affinity for 5-HT increased by a similar magnitude. The mutations exhibited similar characteristics when N^{ω}-methyl-5-HT replaced 5-HT in these experiments. The change in binding affinity of 5-HT for the mutant receptors, determined by radioligand binding, was mimicked qualitatively by its structural analog 2-methyl-5-HT, although the magnitude of the change for this agonist was smaller. Accurate determination of the EC_{50} values for 2-methyl-5-HT was compromised by its low efficacy in this preparation, approximately 20% of that found for the natural agonist. The mutations did not affect the affinity of the agonist mCPBG, and the currents elicited by it were indistinguishable from those found in the WT receptor. The mutations did not significantly compromise the affinity of the majority of the antagonist ligands studied, although in competition studies with [^{3}H]GR65630, the affinity of granisetron for mutant F107N was decreased about 10-fold compared with WT. It is interesting in this regard that Yan et al. (1999) located, by alanine-scanning mutagenesis, a region of the 5-HT_3 receptor sequence, N-terminal to that studied here, that exhibits distinct amino acids that interact with the agonist 5-HT and the antagonist granisetron. Although our observation in this regard merits further exploration, the main focus of this work concerns the agonist interaction with these mutants.

The mutation of F107 to tyrosine (F107Y) produced a decrease in radioligand binding affinity of 5-HT and a concomitant increase in EC_{50}. In addition, the rate of activation of the 5-HT current was markedly slowed compared with WT. Maximum 5-HT-induced currents were obtained in both WT and F107Y mutant receptors by agonist activation at 10 × EC_{50}. In the WT receptor, the time to half-peak current amplitude was 108 ± 25 ms (10 μM 5-HT), whereas in the mutant F107Y receptors, this was increased to 1795 ± 783 ms (100 μM 5-HT). However, on application of a supramaximal 5-HT concentration (10 mM, approximately 1000 × EC_{50}) to mutant F107Y, the activation rate of the macroscopic whole-cell current was markedly increased, showing a time to half-peak current of 494 ± 180 ms. These observations are consistent with decreased association rate of the agonist 5-HT for the F107Y mutant receptor. Currents induced by the agonist mCPBG were indistinguishable, in both magnitude and rate of activation, from those found in the WT receptor.

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AR + A \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A2R^* \quad (1)$$

TABLE 1

Comparison of radioligand binding affinities in WT and mutant receptors determined with [^{3}H]GR65630

K_d and K_i values (nanomolar) were determined as described in the text at a temperature of 0°C. Each value is the mean ± S.E., with the number of determinations given in parentheses.

Ligand	WT (F107)	F107Y	F107N
[^{3}H]GR65630	0.27 ± 0.03 (10)	0.23 ± 0.04 (3)	0.35 ± 0.08 (3)
5-HT	15.6 ± 3.2 (8)	203 ± 59 (5)*	1.62 ± 0.41 (4)*
N^{ω}-Methyl-5-HT	187 ± 73 (5)	6695 ± 2721 (8)*	5.2 ± 2.1 (5)*
2-Methyl-5-HT	173 ± 18 (9)	610 ± 81 (5)*	41.7 ± 8.4 (5)*
mCPBG	5.01 ± 1.09 (6)	11.0 ± 1.2 (3)	2.36 ± 0.79 (4)
Granisetron	0.28 ± 0.03 (6)	0.35 ± 0.07 (4)	2.43 ± 0.69 (5)*
Ondansetron	0.58 ± 0.10 (5)	0.74 ± 0.14 (5)	0.74 ± 0.08 (5)
Renzapride	1.29 ± 0.17 (4)	0.85 ± 0.14 (3)	1.68 ± 0.17 (3)

Significance was determined using the Student's t test on pK_d and pK_i values (*$P < .001$).

There is no state model currently available that reproduces all of the properties of 5-HT$_3$ receptor activation. However, it can be seen in this simplified agonist binding eq. 1 that the rates of both agonist association/dissociation (k_{+1}/k_{-1}, k_{+2}/k_{-2}) and channel opening/closing (gating, β/α) of the receptor determine its apparent affinity (EC_{50}). However, the observation that 5-HT activation in F107Y mutant receptors is markedly increased by supramaximal 5-HT concentrations suggests that it is the decreased agonist association rate that is responsible for the decrease in 5-HT apparent affinity for the F107Y receptor. Once maximum current amplitude is reached, the agonist association rate alone affects activation rate, as channel opening and closing rates are independent of concentration. The observation that both the K_i and EC_{50} values obtained for mCPBG are unaffected by this mutation is consistent with the suggestion that this mutant does not compromise channel gating. mCPBG recognition appears to be primarily associated with an amino acid segment immediately before the first hydrophobic domain of the sequence (Lankiewicz et al., 1998). Interestingly, mutation of the neighboring tyrosine residue to phenylalanine in the nAChR α7 subunit results in decreased agonist affinity for the natural agonist (ACh; Galzi et al., 1991). Aylwin and White (1994b), using mouse muscle cDNA-derived nAChRs, suggested that this was most likely due to a decrease in agonist association rate because, using single-channel analysis, they determined that the channel opening and closing rates were similar to those of WT. The low conductance of 5-HT$_3$ receptor homo-oligomer used in this study does not permit single-channel analysis, and we have thus been unable to further explore this.

The most parsimonious explanation of this observation is that the phenolic hydroxyl substituent in this mutant sterically compromises the access of 5-HT to its recognition site, an effect that can be overcome by driving the interaction with increased ligand concentrations. The increased bulk introduced with an additional aliphatic methyl group in N^{ω}-methyl-5-HT reduced the radioligand binding affinity by a factor of 35, although the magnitude of this decreased affinity was not entirely reflected in the EC_{50} determinations. A detailed electrophysiological characterization was not carried out with this compound.

The mutation of F107 to asparagine (F107N) caused an increase in affinity for 5-HT. The parallel changes in K_i and EC_{50} values are again consistent with a direct effect on binding of the agonist at the recognition site rather than with an indirect effect on channel gating. Asparagine is able to both accept and donate hydrogen bonds. However, phenylalanine acts only as a weak hydrogen bond acceptor by virtue of its delocalized π-electrons (Levitt and Perutz, 1988). The relatively small effects of the mutation on affinity of 2-methyl-5-HT, in which the methyl substituent will increase the partial charge on the pyrrole nitrogen (Fig. 7), seem to preclude an interaction with this site. In comparison with 5-HT, the analog N^{ω}-methyl-5-HT exhibits more pronounced changes in both K_i and EC_{50} for this mutant. The additional methyl substituent in this compound will increase the electron density on this aliphatic amine compared with 5-HT, thus making N^{ω}-methyl-5-HT a stronger hydrogen bond acceptor than 5-HT but a weaker hydrogen bond donor (Fig. 7). This suggests not only that it is the aliphatic amine of 5-HT that interacts directly with the amino acid in this position but also that in F107N, asparagine donates a hydrogen bond to the aliphatic amine of the indoleamines.

The mutant F107N also exhibited a significant affinity for ACh (EC_{50} = 256 μM), producing similar maximal currents to those found with 5-HT. Both the 5-HT and ACh responses were blocked, in an apparantly competitive manner, by ren-

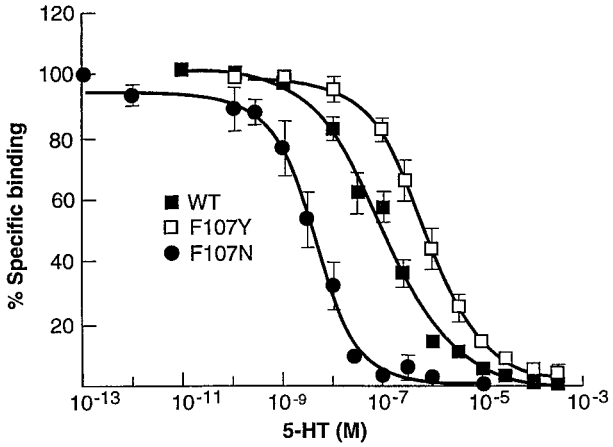

Fig. 3. Competition of 5-HT for [^{3}H]GR65630 binding to WT (F107), F107Y, and F107N mutant receptors on membranes obtained from expressing HEK 293 cells. Data are expressed as the mean ± S.E. (n = 4–8).

TABLE 2

Comparison of radioligand binding affinities in WT and mutant receptors determined with [^{3}H](S)zacopride

K_d and K_i values (nM) were determined as described in the text at a temperature of 37°C. Each value is the mean ± S.E., with the number of determinations given in parentheses.

Ligand	WT (F107)	F107Y	F107N
[^{3}H](S)-Zacopride	0.12 ± 0.04 (3)	0.11 ± 0.02 (3)	0.05 ± 0.02 (4)
5-HT	153 ± 8 (3)	434 ± 109 (3)*	51.1 ± 13.0 (3)*
2-Methyl-5-HT	395 ± 28 (3)	1677 ± 250 (3)*	246 ± 40 (3)
Granisetron	3.90 ± 0.73 (4)	1.87 ± 0.32 (3)	4.46 ± 0.71 (4)
Renzapride	8.81 ± 1.26 (4)	6.04 ± 1.63 (3)	4.52 ± 0.70 (5)*

Significance was determined using the Student's t test on pK_d and pK_i values (*$P < .05$).

TABLE 3

Comparison of macroscopic agonist dose-response data for WT and mutant receptors

The concentrations of agonist required for half-maximal (EC_{50}) and apparent Hill coefficients (n) were estimated from fits of dose-response data as described in the text. Each value is the mean, with 95% confidence limits given in parentheses; each value is the mean of 5 to 11 determinations.

	5-HT		N^{ω}-Methyl-5-HT		mCPBG	
	EC_{50}	Hill Coefficient	EC_{50}	Hill Coefficient	EC_{50}	Hill Coefficient
	μM		μM		μM	
WT	1.2 (1.1–1.3)	2.0 (1.6–2.3)	0.96 (0.82–1.12)	1.6 (1.2–2.0)	0.3 (0.3–0.4)	1.6 (1.3–1.9)
F107Y	10.6 (9.1–12.3)	1.6 (1.1–2.0)	7.1 (5.7–8.7)	1.6 (1.1–2.0)	0.34 (0.24–0.48)	1.0 (0.6–1.2)
F107N	0.2 (0.15–0.25)	1.0 (0.7–1.2)	0.05 (0.03–0.08)	0.4 (0.3–0.6)	0.20 (0.14–0.29)	0.6 (0.5–0.7)

zapride, the specific 5-HT_3 receptor antagonist. This mutation is thus perhaps simply permissive of the ACh interaction in that the majority of nAChR α-subunits have a conserved asparagine at this position (Fig. 1), although in the α7 subunit, the equivalent position is occupied by serine, which is also a hydrogen bond donor and acceptor.

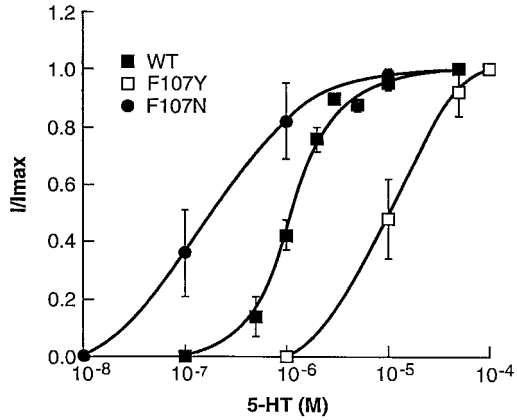

Fig. 4. Concentration-effect curves for 5-HT from WT (F107), F107Y, and F107N mutant receptors transiently expressed in HEK 293 cells. I and I_{max} are the currents at a given agonist concentration and the maximal current, respectively. Data are expressed as the mean ± S.E. (5–11 points were used for each determination).

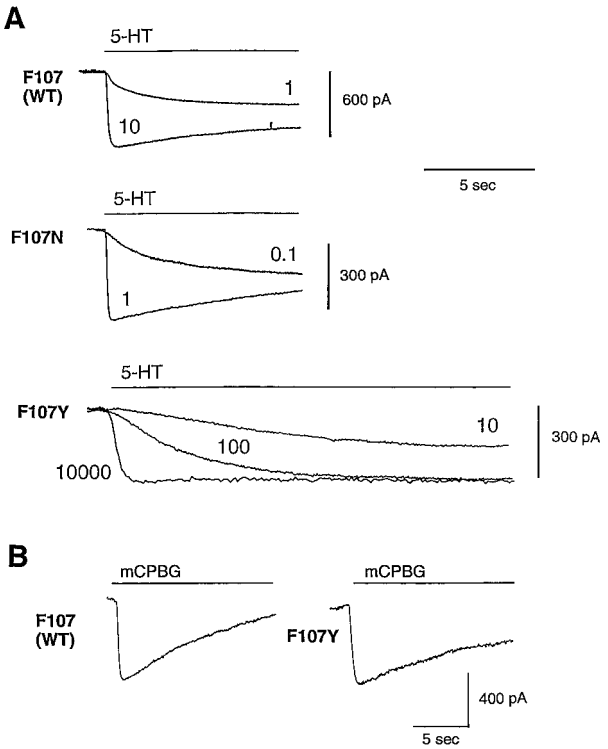

Fig. 5. Comparison of the agonist-induced inward currents in HEK 293 cells transiently transfected with WT (F107), F107N, and F107Y receptors. Currents were recorded from cells that were voltage-clamped at a holding potential of −60 mV. A, 5-HT application at micromolar concentrations indicated. In each case, current traces are shown at approximate EC_{50} and 10 × EC_{50} agonist concentrations. In the case of the mutant F107Y, an additional trace is shown at 1000 × EC_{50}. B, mCPBG application at a concentration of 10 μM. The horizontal bar indicates the duration of agonist application. Note that the time to maximum current amplitude is markedly longer for the current elicited by 100 μM 5-HT (10 × EC_{50}) in mutant F107Y receptors compared with the current elicited by 10 μM 5-HT (10 × EC_{50}) in WT receptors. Note also that the time to maximum current amplitude is reduced by 10 mM 5-HT (1000 × EC_{50}) for mutant F107Y receptors.

Previous studies with the nAChR have clearly shown the requirement for an aromatic residue at position 93 in the nAChR α-subunits to support a high affinity for the natural agonist. The studies of Nowak et al. (1995), in which a variety of unnatural amino acids were substituted at this position, suggested that this particular tyrosine was involved in hydrogen bond donation. In this 5-HT_3 receptor mutant, the equivalent alignment position is occupied by asparagine (105; Fig. 1), which will also act as a hydrogen bond donor. Our observation that the 5-HT_3 receptor mutant F107N but not the WT receptor is activated by ACh indicates that the asparagine at position 107 may allow hydrogen bond formation, further stabilizing the interaction with the acetyl moiety of ACh. This would increase the energy of the interaction between ACh and the receptor, allowing receptor activation. Although it is reasonable to suppose that this is a direct binding interaction, our attempts to identify high-affinity ACh displacement of [^{3}H]GR65630 in mutant F107N were unsuccessful, and it is thus possible that the mutation simply provided a means by which the receptor conformation could proceed to the channel open state. The apparent affinity of this 5-HT_3 receptor mutant for ACh is reduced by only a factor of about 2 over that found for the chick α7 homo-oligomer.

The results of mutational analysis of phenylalanine 107 in the $5\text{-}HT_{3AL}$ sequence suggest that this position provides important recognition properties for the natural agonist 5-HT. The mutant F107Y results in a marked decrease in the association rate of the agonist 5-HT. Our studies with both 5-HT and N^{ω}-methyl-5-HT in mutant F107N have been rationalized with the suggestion that the amino acid in this position acts as a hydrogen bond donor to the terminal aliphatic amine of 5-HT. In this mutant, ACh functioned as a

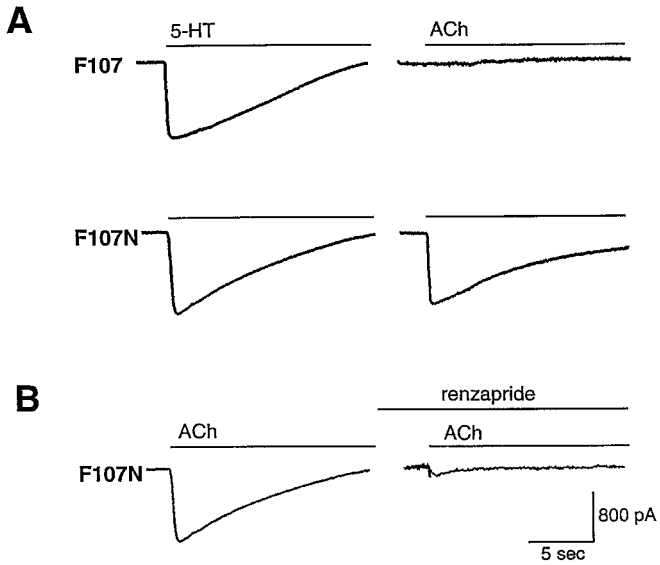

Fig. 6. Comparison of the whole-cell currents recorded in WT (F107) and mutant F107N receptors as a consequence of 5-HT or ACh application. A, agonist application is indicated by the horizontal bars. The concentration of 5-HT was 10 μM, whereas the concentration of ACh was 10 mM for F107 and 3 mM for F107N. B, renzapride application, at a concentration of 100 nM, preceded that of ACh by 30 s and was followed by coapplication with ACh.

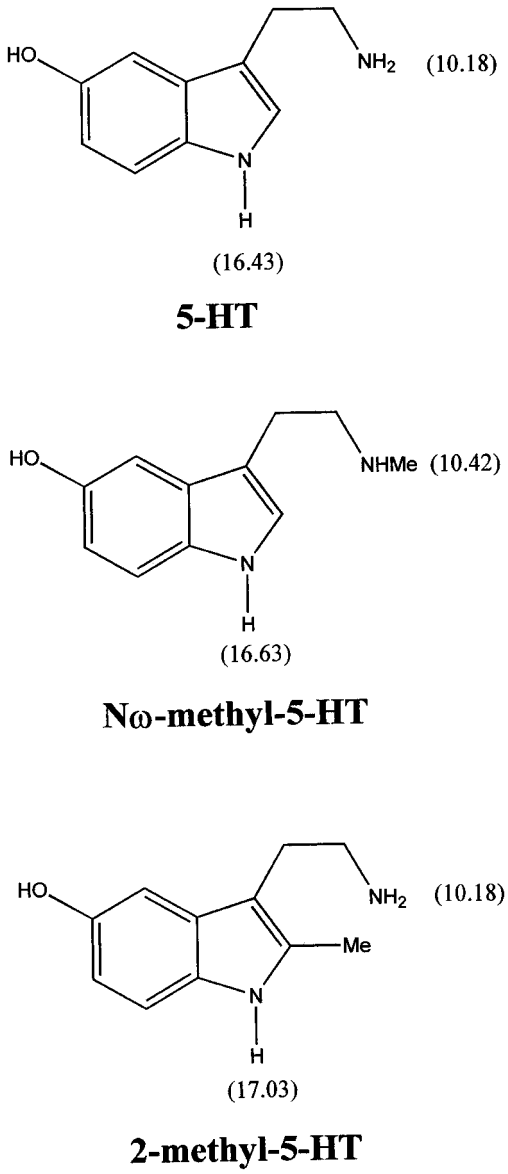

Fig. 7. The structures of 5-HT, N^{ω}-methyl-5-HT, and 2-methyl-5-HT. The numbers in parentheses adjacent to the pyrrole nitrogen and the aliphatic amine, in each structure, are pK_a values calculated with the PALLAS software system.

full agonist, and this response was inhibited by the 5-HT$_3$ receptor antagonist renzapride.

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