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Pharmacology

Single amino acid substitution affects desensitization of the 5-hydroxytryptamine type 3 receptor expressed in *Xenopus* oocytes

(site-directed mutagenesis/ligand-gated ion channel)

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ABSTRACT **5-Hydroxytryptamine type 3 receptors were expressed in *Xenopus* oocytes from a cloned cDNA. The peak inward current evoked by 5-hydroxytryptamine (30 μM) was linearly related to the holding potential (−100 to +20 mV) and reversed near 0 mV. The inward current (at −60 mV) declined during the continued presence of 5-hydroxytryptamine with a half-time of about 2 s; this desensitization was 20 times slower in calcium-free solution. Desensitization was markedly different in channels in which Leu^{286} was changed by site-directed mutagenesis; this residue is thought to lie near the middle of the M2 segment. Desensitization was faster with Phe, Tyr, or Ala in this position and slower with Thr. Phe and Thr substitutions in the equivalent position of the nicotinic acetylcholine receptor have similar effects on desensitization, suggesting that the underlying conformational change might be common to ligand-gated channels.**

One property common to ligand-gated ion channels is desensitization, whereby the current reaches a maximum amplitude and then declines during the continued presence of the agonist (see review by Huganir and Greengard, ref. 1). Desensitization of the 5-hydroxytryptamine type 3 (5-HT_3) receptor is well characterized in different preparations, including mouse hippocampal neurons (2), rat superior cervical ganglion neurons (3), and a variety of clonal cell lines, including N1E-115 cells (4, 5), N18 cells (6), and NG108-15 cells (7, 8). The 5-HT_3 receptor expressed in *Xenopus* oocytes also shows desensitization (9).

Even though virtually all ligand-gated ion channels have been shown to desensitize, the physiological relevance of desensitization is presently unclear. Desensitization could serve to terminate the postsynaptic response and/or as a mechanism for the short-term regulation of synaptic efficacy (1, 10). For example, Trussell and Fischbach (11) have reported that for the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate subtype of glutamate receptors, desensitization is rapid enough to account for the decay of synaptic currents. More recently it was reported that aniracetam, which reduces the desensitization of the AMPA/kainate receptors, also decreases the rate of decay of excitatory synaptic currents (12, 13), again suggesting a role for desensitization in the regulation of synaptic transmission.

The ionic selectivity of the 5-HT_3 receptor resembles that of the nicotinic receptor (6, 14) and there is considerable primary sequence homology (9). For the neuronal nicotinic acetylcholine receptor expressed in *Xenopus* oocytes (15), the identity of a single amino acid residue in the second hydrophobic domain dramatically affects desensitization; this domain is thought to form the pore of the channel. The purpose of the present experiments was to determine whether similar substitutions had a similar effect in the 5-HT_3 receptor, to test the hypothesis that the conformational change that underlies desensitization is similar in the two receptors.

METHODS

The cDNA encoding the 5-HT_3 receptor from NCB-20 clonal cells was kindly provided by D. Julius (9). Site-directed mutants were constructed using the phagemid pSelect(−), a derivative of pSelect (Promega) that allows rescue of the (+) strand (J.P.A., unpublished observation). Sequences encoding the 5-HT_3 receptor were subcloned; the pSelect(−) plasmid harboring 5-HT_3 sequences has been designated pS5-HT_3. Single-stranded DNA was rescued by superinfection with M13K07 helper phage. Mutagenic oligonucleotides were annealed to the single-stranded DNA in the presence of an oligonucleotide that corrects a frameshift mutation in the β-lactamase gene of the plasmid. cDNA was synthesized using T4 DNA polymerase and ligase (Bethesda Research Laboratories). The reaction products were transformed into BMH71-18 *mutS* and grown in 2 ml of liquid culture containing ampicillin. DNA was isolated and used to retransform JM101. Individual colonies were isolated and grown in liquid culture, and DNA was prepared for double-stranded DNA sequencing. Oligonucleotides were synthesized on an Applied Biosystems PCRmate, and DNA sequencing was performed by dideoxy chain termination, according to published methods. RNA was synthesized *in vitro* from the T7 promoter in pS5-HT_3 or in each one of the Leu^{286} mutants, according to established protocols. $m^7G(5')ppp(5')G$ (Pharmacia) was included in the reaction mixture to generate capped transcripts.

Harvesting, injection, and incubation of *Xenopus laevis* oocytes (stage V–VI) were as described (16). Oocytes were injected 3–7 days before electrical recordings were made. Two electrodes filled with 3 M KCl (resistances of ≤1 MΩ) were inserted into oocytes and used to measure membrane currents with a voltage-clamp amplifier (Dagan Instruments, Minneapolis). Unless otherwise mentioned, oocytes were held at −60 mV. The oocytes were continually bathed in a solution of the following composition (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and NaHepes 5, with the pH adjusted to 7.5, or a similar solution that lacked $CaCl_2$. 5-HT (Sigma) was dissolved in the bathing solution and applied by a perfusion system consisting of two glass capillary tubes (1.5 mm o.d.; 1.1 mm i.d.) mounted in parallel; one tube contained control bathing solution and the other contained 5-HT. The flow rate of the solution was ≈6 ml/min. The control tube was positioned directly in front of the oocyte (within 200 μm) with the solution flowing. Immediately before 5-HT application, the flow to the 5-HT-containing tube was opened, and

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Abbreviations: BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid; GR67330, (±)-1,2,3,9-tetrahydro-9-methyl-3-[(5-methyl-1*H*-imidazol-4-yl)-4*H*-carbazol-4-one; 5-HT, 5-hydroxytryptamine; *I–V*, current–voltage; AMPA, α-3-amino-3-hydroxy-5-methyl-4-isoxazolepriopionate.

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the tip of this pipette was manually positioned in front of the oocyte. Usually a delay of 5–8 min between 5-HT applications was used to avoid accumulation of desensitization. Bis(2-aminophenoxy)ethane-*N*,*N*,*N'N'*-tetraacetic acid (BAPTA; Research Biochemicals, Natick, MA) was injected into oocytes via a third microelectrode; 50 nl of a 50 mM stock solution prepared in water was injected.

Concentration–response curves were fitted to $X = 100\ [5\text{-HT}]^n/(K^n + [5\text{-HT}]^n)$, where X is the response amplitude normalized to the response to 5-HT (100 μM) in the same oocyte, K is the apparent dissociation constant for 5-HT, and n is the Hill coefficient. Current–voltage (*I–V*) curves were constructed by normalizing the peak current at any potential to its value at −100 mV. Results are given as mean ± SEM.

RESULTS

Desensitization. 5-HT (30 μM) elicited inward currents in oocytes that had been injected with RNA transcribed from the cloned cDNA; it had no effect in uninjected oocytes (n = 15). (±)-1,2,3,9-tetrahydro-9-methyl-3-[(5-methyl-1*H*-imidazol-4-yl)-4*H*-carbazol-4-one (GR67330; 50 nM), a highly selective antagonist of 5-HT_3 receptors (17), reversibly reduced the amplitude of the 5-HT response by >95% (Fig. 1*B*). The peak current was linearly dependent on membrane potential and reversed polarity at −3.5 ± 1.0 mV (n = 6). At −60 mV, the inward current reached its peak amplitude within 200 ms and then declined with a half-time ($t_{1/2}$) of about 2 s (Fig. 1*A*). Desensitization was voltage-dependent; at −100, −60, and −20 mV, $t_{1/2}$ was 0.5 ± 0.1 s, 2.2 ± 0.3 s, and 12.8 ± 2.9 s, respectively, in the same six oocytes.

Ca^{2+} has previously been shown to affect desensitization of the 5-HT_3 receptor (6, 18, 19). Initial reports indicated that the 5-HT_3 channel was impermeable to Ca^{2+}; however, other studies show significant Ca^{2+} permeability (3, 6, 14, 18, 20). During the continued application of 5-HT (30 μM) at a holding potential of −60 mV, $t_{1/2}$ was 2.3 ± 0.3 s (n = 13; Fig. 1*A*) when the perfusing solution contained Ca^{2+} (1.8 mM); however, in the absence of Ca^{2+} the rate of desensitization was much slower ($t_{1/2}$ at −60 mV was 47 ± 3 s, n = 16) (Fig. 1*A*). The peak amplitude of the inward current was not consistently altered in Ca^{2+}-free solution (ratio of peak amplitudes in Ca^{2+}-free and control solution was 0.96 ± 0.16; range, 0.51–1.87, n = 8). However, desensitization was not affected by potential in Ca^{2+}-free solution. The $t_{1/2}$ values were 58 ± 4 s at −100 mV, 52 ± 3 s at −60 mV, and 49 ± 2 s at −20 mV (n = 9).

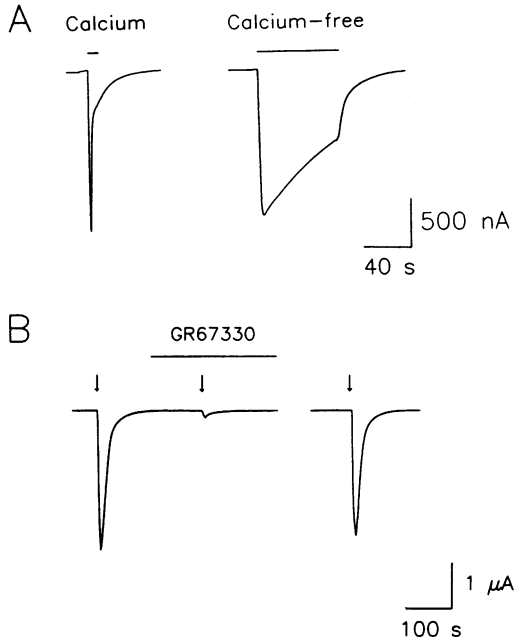

FIG. 1. (*A*) Calcium removal reduces desensitization. Inward currents were evoked by application of 5-HT (30 μM; period indicated by bar) in normal Ca^{2+} (1.8 mM) and in Ca^{2+}-free solution. Holding potential, −60 mV. (*B*) Inward current evoked by 5-HT (50 μM; 5-s application at times indicated by arrows) was blocked by GR67330 (50 nM; period of application indicated by bar). The righthand trace shows recovery of response to 5-HT after 22 min of washing. Traces are single (nonaveraged) records.

One interpretation of these results is that Ca^{2+} enters the cell through the opened 5-HT_3 channel and that this contributes to the rapid desensitization by acting at an intracellular site on the channel. Oocytes were therefore injected with the calcium chelator BAPTA (2.5 nmol; see *Methods*) to buffer intracellular Ca^{2+}. The desensitization $t_{1/2}$ (at −60 mV) increased from 3.7 ± 0.8 s to 11 ± 3 s (n = 8) after BAPTA injection. The peak amplitude of the current caused by 5-HT was not different after BAPTA injection (ratio of peak amplitude before injection to that after injection was 1.0 ± 0.12; range, 0.60–1.4).

Effects of Changing Leu^{286}. These experiments were carried out in Ca^{2+}-free solutions to avoid any effects that might result secondarily from changes in intracellular [Ca^{2+}] ($[Ca^{2+}]_i$), in view of possible differences in Ca^{2+} permeability among the mutated channels. Desensitization was significantly faster in channels having Phe, Tyr, or Ala in place of Leu^{286} [$t_{1/2}$ was 1.2 ± 0.1 s for L286F channel (n = 13), 4.7 ± 0.3 s for L286Y (n = 12), and 25 ± 0.8 for L286A (n = 10)] (Fig. 2). Desensitization was greatly reduced with the Thr substitution; indeed, the current induced by 5-HT in oocytes expressing 5-HT_3 (L286T) channels declined by only 19% ± 3% (n = 11) after 60 s. For the L286T mutant, a similar finding was made in normal solution (Ca^{2+} concentration, 1.8 mM); in wild-type channels the current declined to 9% ± 1% (n = 9) of its peak amplitude in 40 s and in L286T it declined only to 22% ± 2% (n = 9).

The apparent dissociation constant for 5-HT was not greatly different among the mutated channels examined; channels containing Phe, Tyr, Ala, Leu, and Thr at position 286 had values of 1.3, 0.3, 1.0, 1.4, and 0.6 μM, respectively (Fig. 3*A*). The Hill coefficient also showed no obvious correlation with the residue at this position: for Phe, Tyr, Ala, Leu, and Thr the values were 2.4, 1.6, 3.2, 2.7, and 3.4, respectively.

Steady-state *I–V* curves showed modest inward rectification in the wild-type channels (i.e., as observed in control Ca^{2+}-containing solution) as well as those with Phe or Tyr substitutions (Fig. 3*B*). However, the *I–V* relation was quite linear for the Ala and Thr mutants (Fig. 3*B*). The reversal potentials were not obviously different for any of the channels studied (Fig. 3*B*).

In the case of the α7 nicotinic receptor, a Leu → Thr mutation at the equivalent position results in certain antagonists behaving as agonists (21). GR67330 (50 nM) and (+)-tubocurarine (50 nM) had no agonist action on the L286T mutant 5-HT_3 channel (n = 4), although both reduced the amplitude of the current evoked by 5-HT through wild-type channels by >95%.

DISCUSSION

The basic properties of the responses to 5-HT were very similar to those reported by Maricq *et al.* (9). The only significant difference was our failure to observe the marked rectification in the *I–V* relation described by Maricq *et al.* (9) using solutions of normal magnesium and calcium. The reason for this discrepancy is not immediately obvious. The relatively linear *I–V* relation that we observed more closely

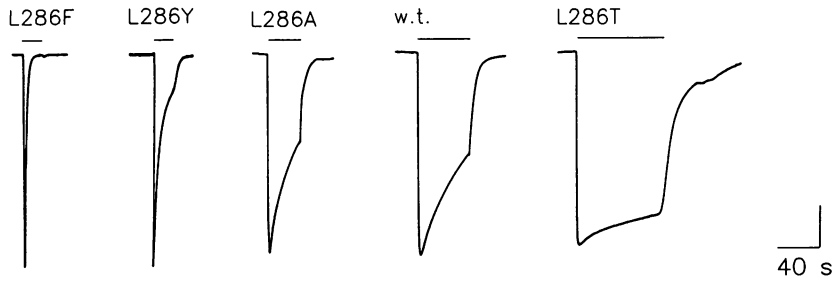

FIG. 2. Desensitization of the 5-HT current in 5-HT_3 receptors with different amino acid residues at the position of Leu^{286}. 5-HT (30 μM) was applied during the time indicated by the bars. w.t., Wild-type channel with Leu^{286}. Vertical calibration is 250 nA for the w.t., 500 nA for L286F and L286A, and 100 nA for L286Y and L286T. Ca^{2+}-free solution.

resembles that seen in NCB-20 cells, from which the cDNA clone was obtained.

The desensitization is also similar to that seen for 5-HT responses in NCB-20 cells, a mouse neuroblastoma × Chinese hamster brain cell hybrid cell line (22). These similarities to native 5-HT_3 receptors extend to the $t_{1/2}$ for decline of the response, the voltage-dependence of desensitization, and the acceleration of desensitization by increased extracellular concentration of divalent cations (6, 18, 19). It is likely that Ca^{2+} exerts at least a portion of its effect on desensitization at an intracellular site on the 5-HT_3 channel, because of the finding that intracellular BAPTA slowed the rate of desensitization.

Leu^{286} of the 5-HT_3 receptor is at a position near the middle of the second hydrophobic region, which, in the case of the nicotinic receptor, appears to contribute to the lining of the ionic pore. The corresponding amino acids in this region are ITLLL^{286}GYSVF (5-HT_3; ref. 9) and ITVLL^{247}SLTVF (α7 nicotinic; ref. 23). Desensitization of current induced by acetylcholine through expressed α7 nicotinic receptors is slightly slowed by hydrophobic substitutions [α7(L247V)] and greatly slowed by polar substitutions [α7(L247T) and α7(L247S)] (15). We also found that the substitution of Thr for the wild-type Leu [5-HT_3 (L286T)] caused a marked reduction of desensitization. Further evidence for the role of a polar side chain comes from our finding that desensitization was slower with Tyr than with Phe substituting for Leu^{286}. It is difficult to make direct comparisons with the results reported for the α7 nicotinic receptor (15) because of the different calcium concentrations used for most of our experiments. Desensitization of the wild-type 5-HT_3 channels ($t_{1/2}$ about 50 s) was much less than that reported for the wild-type α7 nicotinic channels ($t_{1/2}$ about 0.5 s), and this difference might be attributable to the lack of external Ca^{2+} in our experiments. This could also account for the observation that substitution of Phe for Leu had little effect in the nicotinic channels, but greatly *increased* the rate of desensitization in 5-HT_3 channels (Fig. 2). Taken together, the results imply that the underlying conformational change of this part of the molecule has similarities between nicotinic and 5-HT_3 receptors; in both cases, desensitization is greatly reduced by increasing the polarity of the side chain. It has recently been reported that amino acid substitutions at position 251 of the α7 nicotinic receptor, in addition to the previously described changes at position 247, have profound effects on the kinetics of desensitization (24).

The other changes in the properties of the 5-HT_3 receptor were relatively minor when compared with the effects on desensitization. Channels having residues with polar side chains (Thr and Tyr) had slightly higher affinity for 5-HT than channels with nonpolar residues; a similar observation was made in the case of the α7 nicotinic receptor, but there the changes were much more marked. Likewise, inward rectification in the whole-cell current was observed in wild-type channels and was unchanged by Leu → Phe substitution; however, the *I–V* relation was linear for the channel with Thr at this position. These substitutions had the same effects for the α7 nicotinic receptor, but size rather than side chain

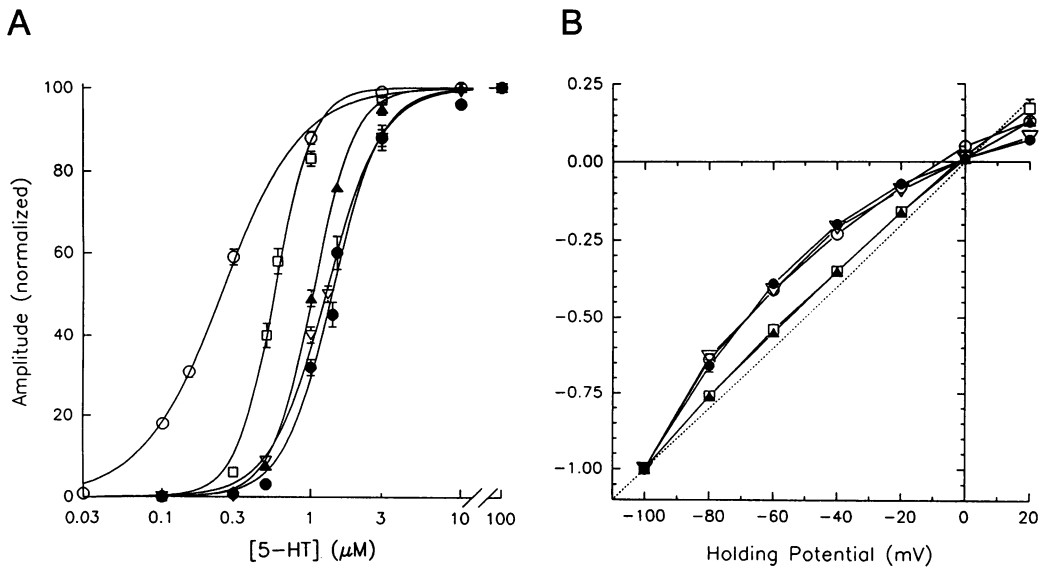

FIG. 3. Effects of residue at position of Leu^{286} on sensitivity to 5-HT and on *I–V*. (*A*) Concentration–response curves for the peak current evoked by 5-HT. (*B*) *I–V* relations. Filled circles, wild-type; open circles, L286Y; squares, L286T; filled triangles, L286A; open triangles, L286F. Each point shows the mean ± SEM for 3–11 cells.

polarity may be an important factor because the *I–V* relation was also linear in 5-HT_3 (L286A). We did not observe the conversion of antagonist to agonists as has been described for the Leu → Thr mutation in the $\alpha 7$ nicotinic receptor (21).

Most of the subunits of ligand-gated ion channels (nicotinic, γ-aminobutyric acid type A, glycine, 5-HT_3) have Leu at the equivalent position in M2 (9, 21, 25). AMPA/kainate subunits of glutamate channels have Leu or Ile or Phe, depending on the alignment (26–30); *N*-methyl-D-aspartate subunits have Ile, Leu, His, Thr, or Phe (29, 31, 32). These provide a number of "natural mutants" with which the hypothesis can be tested that differences in the residue at this position, or residues in the multimeric channel, correlate with the desensitization that occurs during the continued presence of the agonist.

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