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A Single Amino-Acid in the TM1 Domain Is an Important Determinant of the Desensitization Kinetics of Recombinant Human and Guinea Pig α-Homomeric 5-Hydroxytryptamine Type 3 Receptors

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ABSTRACT

Desensitization of ligand-gated ion channels shapes synaptic responses and provides critical neuroprotection at central synapses, yet the molecular mechanisms underlying the desensitization process are poorly understood. Using the whole-cell voltage-clamp technique, we investigated desensitization kinetics of recombinant human and guinea pig α-homomeric 5-hydroxytryptamine type 3 (5-HT_{3A}) receptors heterologously expressed in human embryonic kidney 293 cells. Human 5-HT_{3A} receptors desensitize 3.5 times faster than does the homologous receptor from guinea pigs. By constructing various chimeras and through site-directed mutagenesis, we have identified a single serine in the M1 region of the human 5-HT_{3A} receptor sequence (S248) that, when substituted with threonine found in the equivalent guinea pig sequence (T254), conferred guinea pig-like kinetics on the time course of desensitization of the human receptor. Correspondingly, the reverse mutation (guinea pig T254S) resulted in a fast, human-like time constant of desensitization. Thus, the primary structure of the M1 region is an important determinant of desensitization kinetics of recombinant 5-HT_{3A} receptors.

The 5-HT_3 receptor is a ligand-gated ion channel found in the central and peripheral nervous system, where it mediates fast synaptic transmission (Peters et al., 1992; Yakel et al., 1992; Roerig et al., 1997). 5-HT_3 receptors were thought to be homogeneous because for many years only a single class of 5-HT_3 receptor subunit, the 5-HT_{3A} subunit, had been cloned (Maricq et al., 1991; Hope et al., 1993; Werner et al., 1994; Miyake et al., 1995; Lankiewicz et al., 1998). Davies et al. (1999) succeeded in showing the existence of a β -subunit of the human 5-HT_3 (5-HT_{3B}) receptor, which is closely related to the known 5HT_{3A} (41% amino acid identity) and Hanna et al. (2000) reported the existence of mouse and rat 5-HT_{3B} receptor subunits. The existence of an additional subunit involved in the formation of heteromeric 5-HT_3 receptors was already proposed from the substantial heterogenity of the properties of native 5-HT_3 receptors (Derkach et al., 1989; Yang et al., 1992; Hussy et al., 1994; Jones and Surprenant, 1994; Fletcher and Barnes, 1998).

Agonist activation of the 5-HT_3 receptor depolarizes the cell, which desensitizes in the continuous presence of agonist. Based on work with ACh and glutamate receptors, desensitization is thought to shape the synaptic response. Altering desensitization of rapidly activated receptors has been proposed as a mechanism for synaptic plasticity (Huganir et al. 1986). Several factors are known to regulate the kinetics of desensitization of ion channel, including membrane voltage, amino acid sequence, both intracellular and extracellular calcium, phosphorylation, and the developmental state of the cells (Yakel, 1992; Yakel et al., 1993). The molecular mechanism of desensitization is still unknown and controversial (Lin and Stevens, 1994). Recently, we found that heterologous expression of homomeric human or guinea pig 5-HT_{3A} receptors in HEK 293 cells revealed great differences in time courses of desensitization in that the guinea pig receptor showed prolonged desensitization kinetics compared with the human 5-HT_{3A} receptor (Lankiewicz et al., 1998).

Knowing the molecular determinants for the species differences in the rate of desensitization (i.e., decline of amplitude in continuous presence of agonist) and inactivation (i.e., closing of channels after removal of agonist) properties will help us to understand this basic phenomenon of 5-HT_3 receptor function at the molecular level. Although nearly all ligand-gated channels have been shown to desensitize, there are only very limited data showing the physiological relevance of desensitization. Desensitization is mostly discussed in relation to termination of postsynaptic currents and as a mechanism for the modulation of synaptic efficacy. A better

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ABBREVIATIONS: 5-HT_3, 5-hydroxytryptamine type 3; HEK, human embryonic kidney; GP_s, guinea pig_{short}; H, human; ACh, acetylcholine.

understanding of the molecular mechanisms of desensitization may also trigger new insights into synaptic function and its regulation.

Materials and Methods

Construction of Chimeric Receptors. Random chimeric cDNA was constructed as described previously (Lankiewicz et al., 1998). For chimeras with guinea pig cDNA at the 5′ end, primers (50 pmol) were used that fit the 5′ region of the 5-HT_{3A} guinea pig_{short} (GP_s) (P6) and the 3′ region of the 5-HT_{3A} human cDNA (P10). For the reverse chimeras, we used primers P9 and P8 fitting the 3′ ending of the 5-HT_{3A} GP_s and the 5′ prime region of the 5-HT_{3A} human (H) cDNA, respectively. Chimeric cDNA was amplified in two cycles (45 s at 94°C, 1 s at 50°C) to generate incomplete polymerase chain reaction products, followed by 20 cycles (45 s at 94°C, 45 s at 60°C, 2 min at 72°C) using 0.125 U of *Pfu*-polymerase (Stratagene, La Jolla, CA), 2.5 U of *Taq*-polymerase, and a mixture of 1 ng of *Hin*dIII cut p5-HT_{3A} GP_s and p5-HT_{3A} H as the template. The reaction product was digested with *Hin*dIII and *Xba*I and subcloned into an eucaryotic expression vector (pRc/CMV; Invitrogen, Carlsbad, CA). The "switch-point" was mapped by restriction digestion with *Pst*I, and chimeric cDNAs of interest were sequenced on both strands. The switch-point was defined as the first detectable nucleotide of B in an A × B chimera. We produced the chimeric 5-HT_{3A} receptors C1, E1, E2, E4, and X23 (see under *Results*). The resulting pE1, pE2, pE4, and pX23 contained the 5′-end of the 5-HT_{3A} GP_s up to position 1367, 1026, 792, and 800, respectively, fused to the 3′-end of 5-HT_{3A} H beginning at position 1334, 1011, 867 and 785, respectively (Miyake et al., 1995). pC1 is a combination of the 5-HT_{3A} H 5′-end up to position 946 and 5-HT_{3A} GP_s 3′-end beginning at position 876: P6, CCCAAGCTTGCCACCATGGTGCTGTGGCTCCAGCTG; P8, TACCTT/CGACCAATCCTAT/CT/CCT/ATAGATCTTCGT; P9, ATTGGATCCAGACCATCTTCATTGTGCA/GGCTG; (5 ′) CCCAAGCTTGTCGCTATGCTGCTGTGGGTC; P10, (3′) CATCTAGACTTGGCTTGTGATTGCTGAGATG.

Site Directed Mutagenesis. Mutagenesis of the plasmids p5-HT_{3A} GP_s, p5-HT_{3A} H (Lankiewicz et al., 1998) was performed using the U.S.E. Mutagenesis Kit from Amersham Pharmacia Biotech (Piscataway, NJ) (GP_{I264V}) and using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (H_{S248T} and GP_{T254S}). In the case of the guinea pig 5-HT_{3A} receptor, threonine 254 was mutated to serine and isoleucine 264 was mutated to valine. For the human 5-HT_{3A} receptor, serine 248 was mutated to threonine. For the mutations, the following oligonucleotides were used (altered nucleotides are underlined): HS248T, 5′-CCT CTT CTA TGT GGT CAC GTT GCT ACT GCC CAG CAT-3′; 5′-GAT GCT GGG CAG TAG CAA CGT GAC CAC ATA GAA GAG-3′; GPT254S, 5′-CGG CGA CCT CTC TTC TAT GCA GTC AGC TTG CTG CTG- 3′; 5′-CAG CAG CAA G C T GAC TGC ATA GAA GAG AGG T CG CCG-3′; GPI264V: 5′-CAT CTT TCT CAT GGT CGT GGA CAT TGT GG-3′. A silent mutation, present in each oligonucleotide, introduced a new enzyme restriction site or deleted a restriction site to facilitate mutant screening. Mutations were confirmed by sequencing of both strands.

Functional Expression in HEK293 Cells. Culture and transfection of HEK293 cells was done as described previously (Gorman et al., 1990; Lankiewicz et al., 1998). Cells were grown in minimum essential medium supplemented with 10% fetal calf serum in 5% CO_2 at 37°C. Transfection was accomplished by mixing 15 μg of expression vector and 250 μl of 250 mM $CaCl_2$. The material was added dropwise to 250 μl of 2× HEPES buffered saline. The precipitate then was added to 20% confluent HEK293 cells and allowed to incubate for 5 h before washing the cells twice with phosphate-buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.2). Stable cell lines were established by selection with 500 μg/ml G418.

Electrophysiology and Solutions. Transfected HEK293 cells expressing the recombinant 5-HT_{3A} receptors (H_{S248T}, GP_{T254S}, GP_{I264V}, E4, C1, E1, E2, and X23) were recorded in the whole-cell voltage-clamp configuration (Hamill et al., 1981) under visual control using an inverted microscope (Zeiss, Jena, Germany). The cells were kept in an external solution containing: 145 mM NaCl, 10 mM glucose, 1 mM EGTA, and 10 mM HEPES. pH was adjusted to 7.3 with NaOH. Patch electrodes were pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, England) using a horizontal pipette puller (DMZ Universal Puller, Zeitz-Instruments, Munich, Germany) to yield pipettes with resistances of 3 to 6 MΩ. Pipettes were filled with a solution containing 145 mM CsCl, 10 mM glucose, 10 mM HEPES, and 1 mM EGTA. pH was adjusted to 7.2 with CsOH.

After establishing the whole-cell configuration, the series resistance was estimated and checked during the course of the experiment using the EPC-9 amplifier (List, Darmstadt, Germany). The series resistance was about 15 to 20 MΩ during a typical recording and was not compensated.

The cells were lifted from the substrate and serotonin (Sigma, Deisenhofen, Germany) was applied at the indicated concentrations using a fast superfusion device. A piezo-translator-driven, double-barreled application pipette was used to expose the raised cell to the serotonin containing solution (flow rate, 200 μl/min; solution exchange time, ~10 ms). Serotonin pulses (10 μM 5-HT) of varying lengths were delivered, and the kinetics of the activation of the 5-HT_{3A} receptors measured as the time from 10 to 90% of maximum current. Desensitization of the 5-HT_{3A} receptors in the presence of serotonin, or the time course of inactivation after fast removal of serotonin, was measured as the decay time from 90 to 10% of maximum current. The desensitization of the receptors was quantified using an experimental paradigm with prolonged application of 10 μM 5-HT for up to 240 s, depending on the type of receptor. Inactivation of the 5-HT_{3A} R channels was measured after application of a brief 5-HT pulse (500 ms duration).

Current signals were recorded at a holding potential of −50 mV using the Pulse software on a Macintosh Centris 650 computer. The data were analyzed using the Pulse Fit (HEKA, Lamprecht, Germany) and IgorPro (Wavemetrics, Lake Oswego, OR) software.

Results

HEK293 cells expressing human or guinea pig homomeric 5-HT_{3A} receptors were recorded in the whole-cell voltage-clamp configuration. Because it is widely accepted that calcium depresses the peak current and is intimately involved in the mechanism of desensitization (reviewed in Yakel, 1992), we first investigated the effect of calcium ions on the kinetics of 5-HT_{3A} receptor responses of wild-type human or guinea pig receptors, respectively. We measured the kinetics of current amplitudes of human and guinea pig receptor responses during prolonged application (240 s) of 10 μM 5-HT (see also Lankiewicz et al., 1998) in calcium-free solutions and in presence of 1 mM or 3 mM $CaCl_2$ and showed that $CaCl_2$ remarkably accelerated the desensitization kinetics of human or guinea pig 5-HT_{3A} receptors (Fig. 1, Table 1). In contrast, the activation kinetics of the 5-HT-induced currents were not significantly affected by calcium (data not shown).

The effect of calcium on the desensitization kinetics of guinea pig receptors is weaker than its effect on human 5-HT_{3A} receptors as measured by the decay time from 90 to 10% of current. To eliminate the accelerating effect of calcium ions on the desensitization kinetics, all further investigations were done in calcium-free solutions.

The rapid application of 10 μM 5-HT induced rapid currents ($activation_{10-90\%}$, 58.6 ± 2.6 ms for human, 124.5 ±

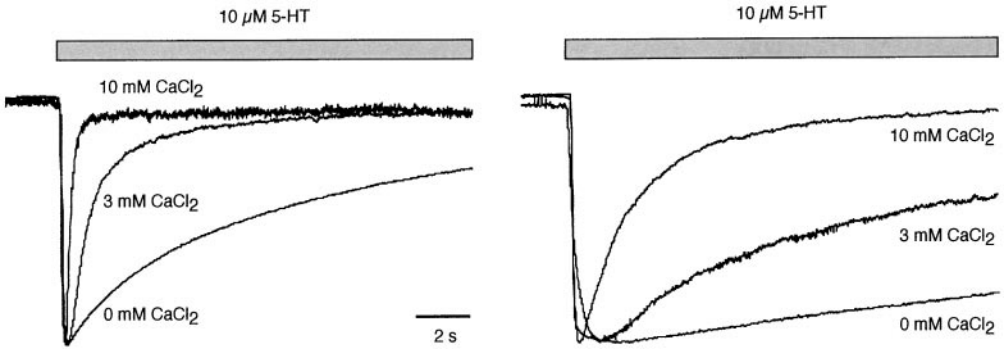

Fig. 1. Normalized whole-cell, voltage-clamp recordings of wild-type human (left) and guinea pig (right) 5-HT_{3A} receptors in absence of Ca^{2+} or in presence of 3 or 10 mM $[Ca^{2+}]_o$. The amplitudes were normalized, as the peak currents are depressed in a dose-dependent manner by extracellular calcium. The bar indicates the application of 10 μM 5-HT. The decay time of the current from 90 to 10% of amplitude of 5-HT-induced currents are indicated in the Table 1.

18.6 ms for guinea pig) that reached a maximum of several hundred picoamperes to a few nanoamperes, depending on the expression level of the receptor protein, and decreased with characteristic decay constants. Heterologous expression of homomeric human or guinea pig 5-HT_{3A} receptors revealed great differences in the time course of desensitization. The guinea pig receptor showed prolonged desensitization kinetics compared with the human 5-HT_{3A} receptor (Lankiewicz et al., 1998). Human 5-HT_{3A} receptors desensitized over 42.7 ± 3.4 s from 90 to 10% of maximum current (n = 19), whereas guinea pig 5-HT_{3A} receptors took 151.4 ± 9.2 s to desensitize (n = 15) (compare also Lankiewicz et al., 1998). Representative current traces are depicted in Fig. 1.

Figure 2 demonstrates the dependence of the desensitization kinetics of the human 5-HT_{3A} receptor on the 5-HT concentration. The time course of desensitization accelerates with increasing 5-HT concentration (73.6 ± 5.9 s with 1 μM 5-HT, 41.8 ± 4.5 s with 30 μM, and 12 ± 2.3 s with 1 mM 5-HT; n = 12, 10, and 11 cells, respectively). In contrast, the time course of inactivation (i.e., closing of the channels after removing of 5-HT) of this receptor is independent from the agonist concentration (8.4 ± 0.6 s with 1 μM 5-HT, 9.7 ± 0.7 s with 30 μM, and 8.5 ± 0.5 s with 1 mM 5-HT; n = 10, 10, and 8 cells, respectively). The same is true for the guinea pig 5-HT_{3A} receptor. Because of the slow desensitization of the guinea pig receptor in presence of 1 μM 5-HT the kinetics were not estimated for this concentration. With 30 μM 5-HT, the time course of desensitization was 120 ± 21.7 s (n = 5), and the desensitization time course from 90 to 10% of current was 51.2 ± 24.5 s with 1 mM 5-HT (n = 3).

To investigate recovery from desensitization, complete desensitization was induced and the agonist was subsequently washed off. The arrival of 5-HT_{3A} receptors in the resting, activatable state was measured from the amplitude of the inward current evoked by a near maximum effective concentration of agonist after a variable period of washing. Figure 3 shows the recovery time (resensitization) of the completely desensitized human or guinea pig 5-HT_{3A} receptor. Prolonged application of 10 μM 5-HT for up to 4 min completely desensitized the 5-HT_{3A} receptors. The 5-HT-induced currents regained 100% of the control amplitude after 30 s of wash (recovery) (n = 5–8 cells).

We investigated the molecular substrate (i.e., the primary structure) of the species difference in desensitization and inactivation kinetics by constructing chimeric receptors between the human and guinea pig 5-HT_{3A} receptor sequences (Lankiewicz et al., 1998). The chimeric receptors consisted of the guinea pig amino terminus, the human carboxy-terminal

TABLE 1

Effect of external Ca^{2+} on desensitization (time from 90 to 10% of amplitude) of human and guinea pig wild-type 5-HT_{3A} receptors (values are given as mean ± S.E.; n, number of cells).

Desensitization (90–10%) [s]	Ca^{2+}-free	3 mM $CaCl_2$	10 mM $CaCl_2$
H	42.7 ± 3.4 s (n= 19)	3.4 ± 1.0 s (n= 5)	1.9 ± 0.9 s (n= 6)
GP	151.4 ± 9.2 s (n= 15)	54.3 ± 14.1 s (n= 5)	21.5 ± 5.7 s (n= 4)

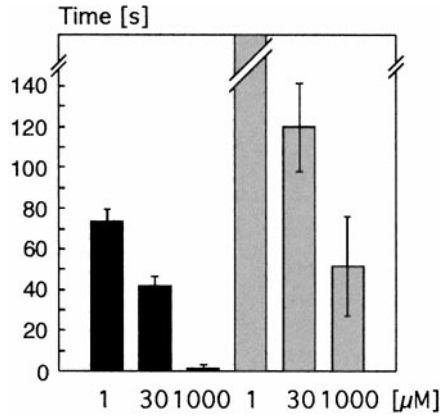

Fig. 2. Diagram showing the dependence of desensitization kinetics of the human (■) and guinea pig 5-HT_{3A} receptors (▨) on the 5-HT concentration. The ordinate indicates the decay time [s] from 90 to 10% of the current. Guinea pig 5-HT_{3A} receptors slowly desensitize in presence of 1 μM 5-HT. The breaks in the time-axis and the bar are set to indicate this fact.

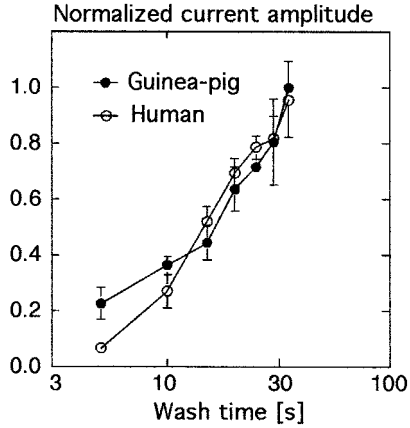

Fig. 3. Diagram showing the resensitization of human (○) or guinea pig (●) 5-HT_{3A} receptors. The receptors were completely desensitized by prolonged application of 10 μM 5-HT. The normalized amplitude of 5-HT (10 μM) induced currents is indicated as a function of recovery (wash) time [s].

domain (E1, E2, E4, and X23), and the chimeric receptor C1, which consisted of the human amino terminus and the guinea pig carboxy-terminal domain. The switch-points (see under *Materials and Methods*) are indicated in Fig. 6. Transient expression of the chimeric receptor plasmids in HEK 293 cells produced functional 5-HT$_{3A}$ receptor channels with apparent affinities (EC_{50}) for 5-HT (E1, 1.9 ± 0.01 μM, n_H = 2.1; E2, 1.2 ± 0.1 μM, n_H = 2.1 ± 0.2; E4, 1.3 ± 0.1 μM, n_H = 1.3; C1, 1.3 ± 0.1 μM, n_H = 2.1 ± 0.3 and X23, 1.6 ± 0.1 μM, n_H = 1.3) comparable with that of wild-type 5-HT$_{3A}$ receptors (human, 2.3 ± 0.2 μM, n_H = 2.3 ± 0.4 and guinea pig, 2.1 ± 0.9 μM, n_H = 2.8 ± 0.5) (Fig. 4). Application of 10 μM 5-HT revealed a correlation between the time constant of decay and the M1 domain. The chimeric receptors E1, E2, X23, and C1 that possessed the guinea pig M1 and N-terminal domains as common structural features showed GP-like desensitization kinetics (163.9 ± 16.3 s, 143.3 ± 17.3 s, 189 ± 22.7 s, and 172.8 ± 22.6 s, respectively; n = 8–18 cells) (Figs. 5, A and C, and 6 and Table 2). In contrast, the chimeric receptor E4 (with human M1 to M4) desensitized rapidly (48.8 ± 4 s; n = 18) (Figs. 5, A and C, and 6 and Table 2). The same held for the inactivation kinetics: The chimeric receptors E1, E2, X23, and C1 showed inactivation constants (90–10%) of 16.1 ± 1 s, 17.1 ± 0.9 s, 22.9 ± 2 s, and 20.8 ± 1.7 s, respectively (n = 13–18 cells), whereas the inactivation time constant of E4 was 7.2 ± 0.5 s (n = 17).

On the assumption that the M1 domain determines the desensitization and inactivation kinetics, we compared the amino-acid sequence of the M1 region in human and guinea pig 5-HT$_{3A}$ receptors and found four amino-acids in M1 of human 5-HT$_{3A}$ that differed from the GP sequence: position 252 was V in H and A in GP (i.e., V252A); the three remaining differences were S254T, V264I, and M265V.

The amino acids in the GP sequence at positions A252 and V265 are identical in GP, mouse, and rat. Because the human 5-HT$_{3A}$ receptor shows desensitization and inactivation kinetics in the same order of magnitude as mouse or rat 5-HT$_{3A}$ receptors (Lankiewicz et al., 1998), we concluded that these amino acid positions were not responsible for determining the desensitization and inactivation kinetics.

As the GP sequence T254 and I264 are different from the residues of the other species, we used site directed mutagenesis to replace the GP T254 with serine (GP_{T254S}) and I264 with valine (GP_{I264V}), according to the respective positions in the human 5-HT$_{3A}$ receptor sequence. The human S248 was replaced with threonine (H_{S248T}), corresponding to the respective position in the GP 5-HT$_{3A}$ receptor sequence. (Note that the position 254 of the GP sequence corresponds to position 248 of the human sequence).

Transiently transfected HEK293 cells expressing GP_{T254S} 5-HT$_{3A}$ receptors produced 5-HT-induced currents that desensitized and inactivated much more quickly than did currents of wild-type GP 5-HT$_{3A}$ receptors ($desensitization_{90-10\%}$, 47.5 ± 4 s, n = 17; $inactivation_{90-10\%}$, 8 ± 0.4 s, n = 23). The receptors showed human-like desensitization and inactivation kinetics (Fig. 5, B and D, and 7). In contrast to the GP_{T254S} receptors, the cells expressing the GP_{I264V} 5-HT$_{3A}$ receptors showed the same desensitization and inactivation

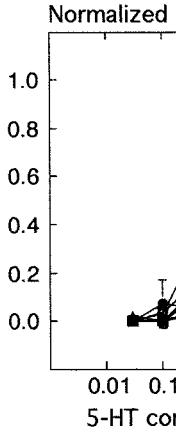

Fig. 4. Diagram showing the dose-response relationships of all 5-HT$_{3A}$ receptors investigated (wild-type human and wild-type guinea pig, C1, E1, E2, E4, X23, GP_{T254S}, H_{S248T}, and GP_{I264V}).

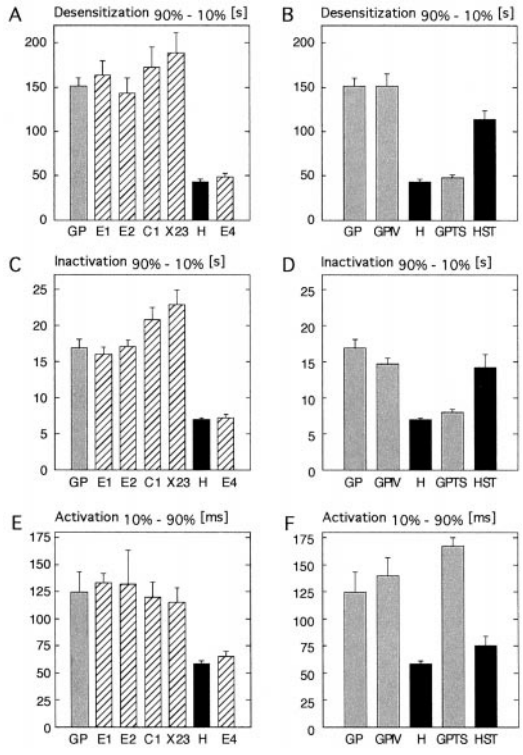

Fig. 5. A, diagram showing the desensitization kinetics of wild-type and chimeric 5-HT$_{3A}$ receptors. The decay time [s] (from 90 to 10% of maximum current) of 5-HT (10 μM) induced currents is indicated for guinea pig (GP, ▨), human (H, ■), and chimeric 5-HT$_{3A}$ receptors (▨, E1, E2, C1, X23, and E4). B, diagram showing the desensitization kinetics of wild-type and mutant 5-HT$_{3A}$ receptors. The decay time [s] (from 90 to 10% of maximum current) of 5-HT (10 μM) induced currents is indicated for guinea pig (GP, ▨), human (H, ■), and the mutant 5-HT$_{3A}$ receptors (GP_{I264V}, GP_{T254S}, H_{S248T}, ▨). C, diagram showing the inactivation kinetics of wild-type and chimeric 5-HT$_{3A}$ receptors. The decay time [s] (from 90 to 10% of maximum current) of 5-HT (10 μM, 500 ms) induced currents is indicated for guinea pig (GP, ▨), human (H, ■), and chimeric 5-HT$_{3A}$ receptors (▨, E1, E2, C1, X23 and E4). D, diagram showing the inactivation kinetics of wild-type and mutant 5-HT$_{3A}$ receptors. The decay time [s] (from 90 to 10% of maximum current) of 5-HT (10 μM, 500 ms) induced currents is indicated for guinea pig (GP, ▨), human (H, ■), and mutant 5-HT$_{3A}$ receptors (GP_{I264V}, GP_{T254S}, H_{S248T}, ▨). E, diagram showing the activation kinetics of wild-type and chimeric 5-HT$_{3A}$ receptors. The rise time [s] (from 10 to 90% of maximum current) of 5-HT (10 μM) induced currents is indicated for guinea pig (GP, ▨), human (H, ■) and the chimeric 5-HT$_{3A}$ receptors (▨, E1, E2, C1, X23, and E4). F, diagram showing the activation kinetics of wild-type and mutant 5-HT$_{3A}$ receptors. The rise time [s] (from 10 to 90% of maximum current) of 5-HT (10 μM, 500 ms) induced currents is indicated for guinea pig (GP, ▨), human (H, ■), and mutant 5-HT$_{3A}$ receptors (GP_{I264V}, GP_{T254S}, H_{S248T}, ▨).

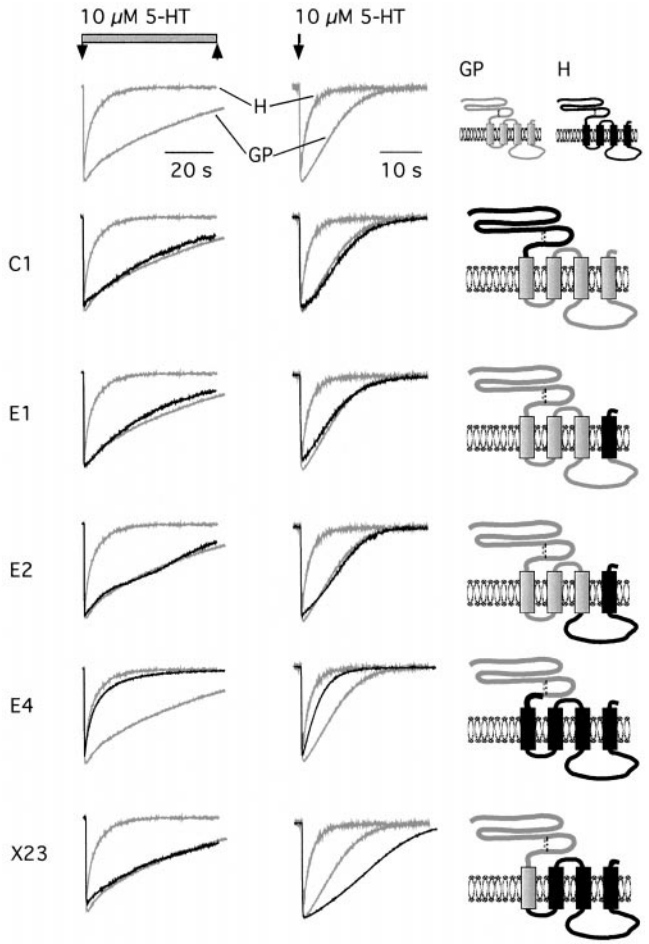

Fig. 6. Whole-cell voltage-clamp recordings of wild-type human (H) and guinea pig (GP) 5-HT_{3A} receptors (first row) and chimeric receptors (row 2 to 6; C1, E1, E2, E4, and X23), showing the different desensitization (left column), and inactivation kinetics (middle column). The application of 5-HT (10 μM) is indicated by the bar and the arrows. Representative current recordings of wild-type receptors are shown in gray (same signals in all rows). The amplitudes of the 5-HT-induced inward currents ranged from 2.1 nA to 4.6 nA ($V_{hold} = -50$ mV), depending on the level of protein expression. The right column depicts the structure of the chimeric receptors. The human sequence is shown in black, and the guinea pig sequence is shown in gray. The switch-point was defined as the first detectable nucleotide of B in an A × B chimera. The resulting pE1, pE2, pE4, and pX23 contained the 5′ end of the 5-HT_{3A} GP_s up to positions 1499, 1158, 792, and 932, respectively, fused to the 3′ end of 5-HT_3 H beginning at positions 1553, 1230, 865, and 1004, respectively. pC1 is a combination of the 5-HT_{3A} H 5′ end up to position 724 and 5-HT_{3A} GP_s 3′ end beginning at position 876.

kinetics as the wild-type 5-HT_{3A} (151.6 ± 13.4 s, $n = 12$ and 14.7 ± 0.8 s, $n = 19$, respectively); i.e., the I264V mutation did not change the kinetics (Fig. 5, B and D. and 7). On the other hand, the S248T mutation of the human sequence (H_{S248T}) produced currents with guinea pig-like desensitization and inactivation kinetics (113.9 ± 9.5 s, $n = 23$, and 14.2 ± 1.9 s, $n = 19$, respectively) (Fig. 5, B and D, and 7). The EC_{50} for 5-HT of all investigated receptors (chimeras and point mutations) were not changed compared with wild-type human or guinea pig 5-HT_{3A} receptors (Fig. 4, Table 3).

The wild-type guinea pig 5-HT_{3A} receptors showed no voltage-dependence of desensitization kinetics by application of 10 μM 5-HT: 151.4 ± 9 s at −50 mV and 126.8 ± 23 s at +50 mV ($n = 3$). In contrast, the human 5-HT_{3A} receptors did show a voltage dependence: the decay time ($Time_{90-10\%}$) was 42.7 ± 3.4 s at −50 mV and 12 ± 3 s at +50 mV ($n = 7$) (compare Lankiewicz et al., 1998).

In contrast to desensitization and inactivation kinetics, the time course of the activation of the 5-HT induced currents (rise time, 10–90% of current) was not changed by the mutations (Fig. 5, E and F). The activation kinetics of the wild-type guinea pig 5-HT_{3A} receptor was significantly slower than that of the wild-type human 5-HT_{3A} receptor (58.6 ± 2.6 ms for human and 124.5 ± 18.6 ms for guinea pig receptor, respectively; $n = 13$ and 10 cells). The point mutation of the guinea pig sequence resulting in GP_{T254S} and GP_{I264V} (167.5 ± 8 ms and 140 ± 16.5 ms, respectively) showed the same activation kinetics as did the wild-type. The mutation of the human sequence, resulting in H_{S248T}, also produced receptors with unaltered activation kinetics (75.4 ± 8.7 ms; $n = 13$). The chimeric receptors E1, E2, C1, and X23 showed slow (guinea pig-like) activation kinetics (133 ± 9 ms, 132 ± 31 ms, 120 ± 13.6 ms, and 115 ± 13.5 ms, respectively; $n =$ 8 to 10 cells), whereas the E4 chimeric receptor showed fast (human-like) activation kinetics (65 ± 5 ms; $n = 10$). Taking these data together, we conclude that the M1 region determines the activation kinetics.

Discussion

We demonstrate that the time course of desensitization and inactivation of 5-HT-induced currents in recombinant human or guinea pig homomeric 5-HT_{3A} receptors is strongly influenced by a single amino acid in the M1 region of the receptor protein.

The effect of extracellular Ca^{2+} on 5-HT_3 receptor currents has been studied in different preparations, revealing sometime opposing effects on desensitization kinetics (reviewed in Yakel, 1992). Our data demonstrated calcium-mediated effects on the peak amplitudes and desensitization kinetics of the currents. To avoid these effects, we investigated the

TABLE 2

Desensitization and inactivation times of human and guinea pig wild-type, chimeric, and mutant 5-HT_{3A} receptors (n, number of cells). Times represent the duration from 90 to 10% of amplitude.

Receptor	Desensitization (90–10%) [s]	SE	n Cells
H	42.7	3.4	19
GP	151.4	9.2	15
C1	172.8	22.6	9
E1	163.9	16.3	13
E2	143.3	17.3	11
E4	48.8	4	18
X23	189	22.7	8
GP_{T254S}	47.5	4	17
H_{S248T}	113.9	9.5	23
GP_{I264V}	151.6	13.4	12
Receptor	**Inactivation (90–10%) [s]**	**SE**	**n Cells**
H	7	0.2	34
GP	16.9	1.1	39
C1	20.8	1.7	15
E1	16.1	1	18
E2	17.1	0.9	13
E4	7.2	0.5	17
X23	22.9	2	18
GP_{T254S}	8	0.4	23
H_{S248T}	14.2	1.9	19
GP_{I264V}	14.7	0.8	19

desensitization kinetics of 5-HT-induced currents under calcium-free conditions.

Desensitization Kinetics of 5-HT$_{3A}$ Receptors. The decline of amplitude in continuous presence of agonist is the phenotype of receptor desensitization. The process of 5-HT$_3$ receptor desensitization is thought to be coupled to binding of the agonist to a specific site on the receptor protein. However, little is known about the molecular structures and mechanisms underlying the gating process. By following the changes in desensitization behavior of various chimeras between the human and guinea pig sequences, we could delimit the molecular substrate for determining this difference in desensitization kinetics to the first transmembrane region (M1) of the receptor protein. Using site directed mutagenesis, a single serine residue in the human 5-HT$_{3A}$ receptor sequence (S248) was identified that, when substituted with the threonine residue found in the equivalent guinea pig sequence (T254), conferred guinea pig-like slow kinetics on the time course of desensitization of the human receptor. Correspondingly, the reverse mutation (guinea pig T254S) resulted in a fast, human-like time course of desensitization.

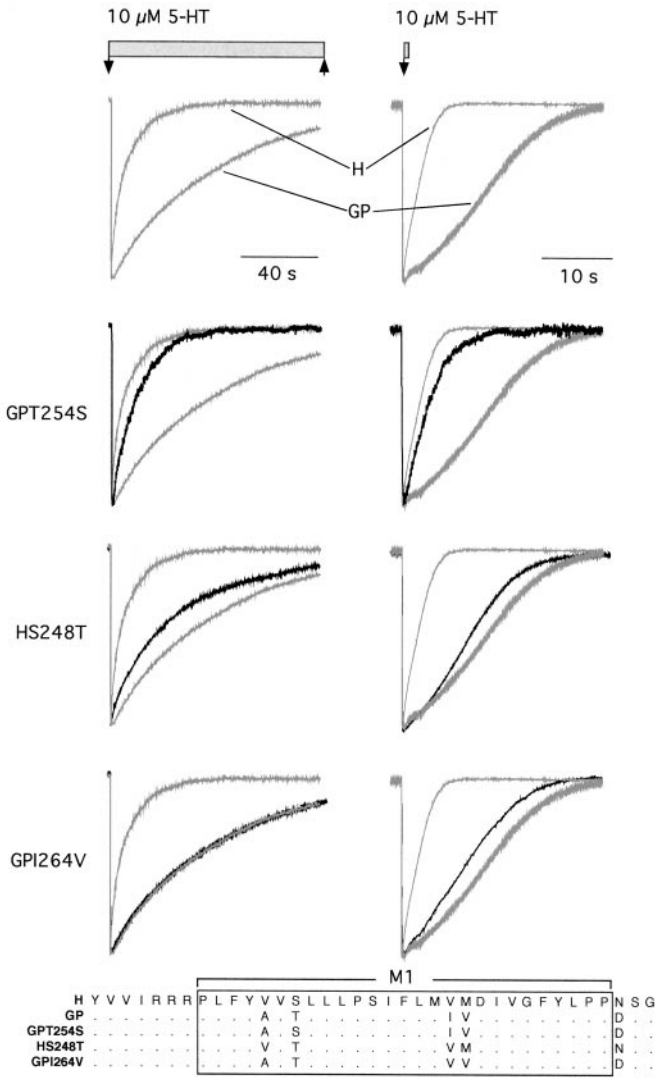

Fig. 7. Whole-cell, voltage-clamp recordings of wild-type human (H) and guinea pig (GP) 5-HT$_{3A}$ receptors (first row) and mutant receptors (row 2–4; GP$_{T254S}$, HS$_{248T}$, and GP$_{I264V}$), showing the different desensitization (left column) and inactivation kinetics (right column). The bar and the arrows indicate application of 10 μM 5-HT. Representative current recordings of wild-type receptors are shown in gray (same signals in all rows). The amplitudes of the 5-HT-induced inward currents ranged from 400 pA (GP$_{T254S}$) to 4.4 nA (V_{hold} = −50 mV). The lower panel shows the comparison of the amino acid sequence of the M1 (putative transmembrane) region (box) of H, GP, GP$_{T254S}$, HS$_{248T}$, and GP$_{I264V}$ 5-HT$_{3A}$ receptors. The consensus sequence is shown in the first row of the alignment.

Currently, most elements found to affect desensitization are contained within the M2 region (i.e., the pore region) or the agonist binding domain. For instance, the lysine residue at the 4′ position in the M2 domain of the murine 5-HT$_{3A}$ receptor has an important role in receptor desensitization (Gunthorpe et al., 2000). Mutation of leucine 286 in the M2 domain of the cloned 5-HT$_{3A}$ receptor protein profoundly altered desensitization kinetics (Yakel et al., 1993). Replacement of this latter residue by threonine slowed the time course of desensitization dramatically, whereas replacement by alanine, phenylalanine, or tyrosine accelerated desensitization. Striking parallels have been found in the M2 (pore) region of two other ligand-gated channels: in nicotinic ACh receptors, the mutation of a highly conserved leucine 247 or valine 251 also induces profound alterations in the desensitization kinetics (Revah et al., 1991; Galzi et al., 1992). Similar results were obtained in a mutant γ-aminobutyric acid$_A$ receptor after replacement of threonine by alanine or serine in the M2 region of the protein (Im et al., 1995). These results show a pronounced conservation of function within the M2 domain of the protein and may reflect a common structural feature involved in conformation changes of ligand-gated channels. Recently, England et al. (1999) reported a contribution of the M1 region in addition to the crucial role of the M2 domain in the mechanism of gating of nicotinic acetylcholine receptors heterologously expressed in *Xenopus laevis* oocytes. Replacing specific backbone peptide bonds with an ester, mutations were identified that produced measurable changes in EC$_{50}$ throughout the α-M2 domains, especially in the region near the extracellular surface of the M2 domains. The role of a conserved proline that is found in the M1 region of every subunit in the ligand-gated ion channel superfamily, for gating of the channel, has been demonstrated recently by Dang et al. (2000). Our data support the important role of those conserved amino acids in the M1 domain for the process of gating in ligand-gated ion channels.

The geometry of quaternary structure of the 5-HT$_3$ receptor protein and its ability to undergo conformational transitions might be important parameters for determining the time courses of desensitization. Eisele et al. (1993) found that the tertiary and quaternary structures of the whole receptor

TABLE 3

Apparent affinities (EC$_{50}$) and Hill coefficients (n_H) of human and guinea pig wild-type, chimeric, and mutant 5-HT$_{3A}$ receptors (n = number of cells).

Receptor	EC$_{50}$	SE	n_H	n Cells
	μM			
H	2.3	0.2	2.3 ± 0.4	4–11
GP	2.9	0.1	2.8 ± 0.5	4–11
C1	1.3	0.1	2.1 ± 0.3	3
E1	1.9	0.02	2.1	5–17
E2	1.2	0.1	2.1 ± 0.2	3–13
E4	1.3	0.1	1.3	3–7
X23	1.6	0.1	1.3	4–8
GP$_{T254S}$	1.4	0.1	1.8 ± 0.3	5–16
H$_{S248T}$	0.7	0.2	1.4 ± 0.3	4–14
GP$_{I264V}$	1.1	0.1	1.8 ± 0.3	3–15

protein are responsible for the desensitization kinetics. Here we report for the first time that the substitution of the serine residue 254 with threonine in the first transmembrane region of the protein lead to a dramatically change in desensitization kinetics. The difference in size of the residue (methyl group instead of —H) might play a role in the conformational changes during desensitization. We identified a serine (the S254 residue) that seems to be an essential element that undergoes significant structural changes during desensitization and may form important physical contacts in the stabilization of different receptor conformations.

Concentration and Voltage Dependence of the Desensitization Rate. The desensitization rate of the 5-HT_{3A} receptor wild-type and mutant varies with agonist concentration, higher concentrations of agonist desensitize the receptor more rapidly. The time course of desensitization of human 5-HT_{3A} receptors was about six times longer at 1 μM than at 1000 μM 5-HT. For the guinea pig receptors, we could only compare the decay times for 30 μM and 1000 μM 5-HT, and found a similar relation of kinetics.

The voltage dependence of the desensitization rate was different in the human and guinea pig 5-HT_{3A} receptors. Applying 10 μM 5-HT, no voltage dependence was evident in guinea pig receptors, whereas the human 5-HT_{3A} receptors showed accelerated desensitization at positive potentials (compare Lankiewicz et al., 1998).

Recovery from Desensitization of 5-HT_{3A} Receptors. The rate of recovery from desensitization can determine the ability of the synapse to respond to repetitive firing and thus represents a major factor contributing to the plasticity of synapses. As we show, recovery of the slowly desensitizing guinea pig type of receptor is in the same magnitude as the fast human receptors. The nature of this phenomenon is unknown. The data on sigmoid recovery from desensitization (see Fig. 3) excludes the possibility that the kinetics is determined by a single rate limiting dissociation step or conformational transition. Therefore, it has been assumed that recovery from desensitization involves multiple steps that occur at similar rates. Similar multistep mechanisms might also apply to the recovery from desensitization of nicotinic ACh receptors (Franke et al., 1991; Dilger and Liu, 1992).

Activation and Inactivation Kinetics of 5-HT_{3A} receptors. The response of the human 5-HT_{3A} receptor reached 90% of its peak within approximately 59 ms after application of 10 μM 5-HT, and the guinea pig receptor within 125 ms. Similarly, Gunthorpe et al. (2000) reported a 10 to 90% rise time of 103 $\pm$ 9 ms for 5-HT-induced currents in HEK293 cells heterologously expressing murine 5-HT_{3A} receptors. However, similarly low forward rate constants are described for 5-HT_3 receptors in neuroblastoma cells (Mienville, 1991) and γ-aminobutyric $acid_A$ receptors (Adelsberger et al., 1996).

If our measurements indeed reflect a true activation process, then the rate of binding of this receptor is slower than that of many other ligand-gated channels (ACh, glutamate), in which the rate of activation approaches the diffusion limit (Dudel et al., 1992). These measurements are important in establishing the intimate association between receptor and channel characteristics of ligand-gated channels and in defining the possibilities for function in rapid forms of synaptic transmission.

The activation was not influenced by the mutations, reflecting the unchanged binding affinities resulting from the 5-HT dose-response curves (apparent K_d and EC_{50} values). We found the inactivation and desensitization kinetics of human and guinea pig 5-HT_{3A} receptors affected by the mutations in the same direction.

Interestingly, a single amino acid (S248 or T254) is an important determinant for two independent processes—desensitization and inactivation—of a ligand-gated ion channel. This indicates that the amino acid at this respective position is part of a structure that is possibly involved in gating of the receptor-associated channel. Substitution of these amino acids may have consequences on protein conformation, modulating the open probability.

Conclusion

Taken together, our results imply that the underlying conformational change of this part of the molecule during desensitization and inactivation has a high degree of similarity. 5-HT_3 receptors mediate fast excitatory synaptic transmission in the central and pheripheral nervous system and play roles in synaptic plasticity and development as well as in several chronic and acute neurological disorders. Receptor desensitization is a way to regulate synaptic responses. The identification of specific structural elements of the 5-HT_3 receptor involved in desensitization may facilitate new insights into synaptic function and its regulation. 5-HT_3 receptors stripped of their normal desensitization provide a powerful tool for investigating receptor channel properties otherwise masked by the fast onset of receptor desensitization. The 5-HT_3 receptor makes it possible for 5-HT to function in rapid excitatory synaptic transmission. Until recently, 5-HT was thought to act primarily as a modulator of neuronal excitability. Characterization of the kinetic and pharmacological properties of the 5-HT_3 receptor, its presence in the mammalian central nervous system (Bloom and Morales, 1998), and the clinical and behavioral actions of 5-HT_3 receptor-specific ligands (see also Apud, 1993) suggest a new physiological function for 5-HT in the brain (Yakel et al., 1990).

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