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Molecular Cloning, Functional Expression, and Pharmacological Characterization of 5-Hydroxytryptamine$_3$ Receptor cDNA and Its Splice Variants from Guinea Pig

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ABSTRACT

Polymerase chain reaction and rapid amplification of cDNA ends were used to isolate cDNAs encoding a 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor subunit and its splice variants from guinea pig intestine. The amino acid sequence predicted from this cDNA is 81% homologous to the murine 5-HT$_3$ receptor subunits cloned from NCB20 and N1E-115 cells. The splice variants code for two proteins differing by a deletion of six amino acids located in the large intracellular loop between transmembrane domains M3 and M4. For characterization, the cloned 5-HT$_3$ cDNA was expressed in HEK 293 cells, and the electrophysiological and pharmacological properties of the recombinant ion/channel/receptor complex were investigated by patch clamping. Our data reveal that the cloned cDNAs code for guinea pig 5-HT$_3$ receptors, which functionally assemble as homo-oligomers. The kinetic behavior of the ion channel and its sensitivity to several agonists and antagonists were markedly different from those of the cloned 5-HT$_3$ receptors from mouse and human under similar experimental conditions. The agonists used were 5-hydroxytryptamine, 2-methyl-5-hydroxytryptamine, 1-phenylbiguanide (PBG), *m*-chlorophenylbiguanide, and the antagonists tropisetron and metoclopramide. In addition, 5-HT, PBG, and tropisetron were investigated through radioligand binding to isolated membranes. Compared with the human and murine 5-HT$_3$ receptors, the guinea pig receptor showed prolonged desensitization kinetics. In addition, the guinea pig 5-HT$_3$ receptor did not respond to the selective 5-HT$_3$ receptor agonist PBG. Construction of chimeric receptors between guinea pig and human 5-HT$_3$ receptor sequences localized the differences in desensitization kinetics to the carboxyl-terminal domain and the ligand binding site to the amino-terminal domain of the receptor protein. Molecular determinants of the PBG binding site of the human 5-HT$_3$ receptor were localized to a 28-amino-acid spanning region adjacent to the M1 region.

5-HT$_3$Rs belong to the superfamily of ligand-gated ion channels that mediate fast synaptic transmission in the peripheral and central nervous systems (Peters *et al.*, 1992; Yakel, 1992). These channels are composed of five identical or homologous subunits and their functional diversity generally is attributed to the presence of several different subunits that can coassemble to yield receptors with specific pharmacological and physiological properties (Betz, 1990). No such diversity has emerged for 5-HT$_3$Rs. A single 5-HT$_3$ inotropic receptor subunit (5-HT$_3$R-A) was cloned 6 years ago from the NCB20 neuroblastoma cell line (Maricq *et al.*, 1991), but despite evidence for both pharmacological and biophysical variations between tissues and species, no further 5-HT$_3$R subunits, like different α or β subunits, have been identified. 5-HT$_3$R-A cDNA and a splice variant have been cloned from additional neuroblastoma cell lines and from mouse, rat, and human tissues. These subunits form functional homo-oligomeric 5-HT$_3$Rs when expressed in oocytes or HEK 293 cells (Maricq *et al.*, 1991; Hope *et al.*, 1993; Werner *et al.*, 1994; Miyake *et al.*, 1995).

Electrophysiological recordings from neurons and neuroblastoma cell lines have established that the 5-HT$_3$R is a cation-selective channel with similar permeability to Na^+ and K^+, although its conductance differs among preparations (Yakel, 1992). Although alternative splicing in mouse and rat generates two receptor isoforms, there is no evidence that this contributes to functional diversity (Hope *et al.*, 1993; Werner *et al.*, 1994; Miquel *et al.*, 1995). The electrophysiological evidence in favor of 5-HT$_3$R heterogeneity is sup-

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ABBREVIATIONS: 5-HT$_3$R, 5-hydroxytryptamine$_3$ receptor; 5-HT, 5-hydroxytryptamine; PBG, 1-phenylbiguanide; mCPBG, *m*-chlorophenylbiguanide; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; RT, reverse transcriptase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; ORF, open reading frame; RACE, rapid amplification of cDNA ends; HEK, human embryonic kidney.

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ported by pharmacological studies, which suggest the existence of receptor subtypes in different species such as rat, rabbit, and guinea pig that differ in their affinities for antagonists (Peters *et al.*, 1992).

In guinea pig, 5-HT$_3$Rs in various tissues have been subject to extensive pharmacological characterization. The receptor from colon and vagus nerve exhibits considerably lower sensitivity to all 11 antagonists tested compared with the respective tissues in rat (Butler *et al.*, 1990). In contrast to receptors from mouse, rat, and human, PBG does not act as an agonist in guinea pig.

Within the central nervous system, the 5-HT$_3$R is expressed predominantly in neurons in the area postrema and mesolimbic system (Kilpatrick *et al.*, 1987, 1988; Tecott *et al.*, 1993). Thus, 5-HT$_3$Rs seem to be a potential target for the development of drugs for the treatment of nausea and behavioral disorders (Aput, 1993). 5-HT$_3$R antagonists prevent emesis induced by cytostatic drugs that are commonly used in cancer therapy (Gralla *et al.*, 1991). Moreover, based on animal models and preliminary clinical studies, it has been suggested that 5-HT$_3$R antagonists display anxiolytic (Rodgers *et al.*, 1995) and atypical antipsychotic (Costall *et al.*, 1993; Zoldan *et al.*, 1993; Warburton *et al.*, 1994) properties.

In the present study, we isolated cDNA for two splice variants of the 5-HT$_3$R from guinea pig intestine. The electrophysiological and pharmacological properties of the recombinant receptor from guinea pig were compared with those from mouse and human. Functional expression and electrophysiological and pharmacological characterizations of the recombinant and chimeric 5-HT$_3$R subunits were obtained in HEK 293 cells by patch-clamp measurements. In addition, pharmacological data were obtained in radioligand binding studies.

Materials and Methods

mRNA isolation and cDNA synthesis. mRNA was isolated from adult guinea pig small intestine with the Pharmacia (Vienna, Austria) QuickPrep Micro mRNA Purification Kit. cDNA was constructed by using an oligo(dT) primer with a T7-promotor sequence at its 5′-end (P7 TTCGAAATTAATACGACTCACTATAGGGAGAT$_{20}$) or a gene-specific primer (P4 CAGGAGCTCCCAC/TTCICCC/TTGA/GTT) and 200 units of Moloney murine leukemia virus RT (GIBCO BRL, Paisley, Scotland). (The nucleotide sequences of guinea pig 5-HT$_3$R cDNAs have been submitted to GenBank with accession numbers AF006461 and AF006462, respectively.)

PCR. Nucleotide sequences derived from the previously reported mouse 5-HT$_3$ subunit (Maricq *et al.*, 1991) were used to design PCR primers (P1 ATCCTCGAGGTGGATGAGAAGAACCAA/GGT and P2 TTCATCGATGGCTGCAGTGGTTA/G/C/TCCCAT). P7 primed cDNA and 50 pmol of primers P1 and P2 were incubated in *Taq* buffer (20 mM Tris·HCl, pH 8.4, 50 mM KCl, GIBCO BRL) containing 0.2 mM concentrations of dNTPs and 2.5 units of *Taq* DNA polymerase (GIBCO BRL). Thirty-five cycles (94° for 1 min, 55° for 1 min, 72° for 1 min) were performed with a programmable thermocycler. One fifth of the reaction products were analyzed by gel electrophoresis. The amplified fragment was digested with *Xho*I and *Cla*I, subcloned into pBluescript II KS$^+$ (Stratagene, Heidelberg, Germany), and sequenced with PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and an ABI 373 Version 2.0.1 DNA Sequencer (Applied Biosystems, Foster City, CA).

5′- and 3′-RACE. Isolation of 5′- and 3′-termini of guinea pig 5-HT$_3$R cDNA was done according to the method described for human 5-HT$_3$R by Lankiewicz *et al.* (1997). Briefly, primers were designed on the basis of sequence information obtained with sequenced guinea pig PCR products. For the amplification of 3′-ends, 0.5 μg of P7 primed cDNA and 50 pmol of primer P3 (CCACCAAGACTGATGAC) and T7-primer (AATTAATACGACTCACTATAGG) were cycled 30 times (94° for 1 min, 50° for 1 min, 72° for 1.5 min, 2.5 units of *Taq*-polymerase). The specific reaction product was purified with Streptavidin-Paramagnetic Particles (Promega, Madison, WI) and biotinylated internal primer P2, reamplified (30 times: 94° for 1 min, 50° for 1 min, 72° for 1 min, 2.5 units of *Taq*-polymerase, 50 pmol of P3 and T7), cloned blunt end into pBluescript II KS$^+$ (Stratagene), and sequenced. For 5′-RACE, cDNA was constructed with the gene-specific primer P4, and a poly(dA) tail was added with terminal transferase. The next step was one cycle of PCR with 10 pmol of P7 at 94° for 1 min, 40° for 1.5 min, and 72° for 2.5 min and 2.5 units of *Taq*-polymerase followed by 30 cycles with 50 pmol of primer P5 (ACAGAATTCTGIACA/GTCA/GAAIGGA/GAA) and T7 at (94° for 1 min, 50° for 1 min, 72° for 1 min). The specific reaction product was purified with Streptavidin-Paramagnetic Particles (Promega) and biotinylated internal primer P1, reamplified 30 times (94° for 1 min, 50° for 1 min, 72° for 1 min, 2.5 units of *Taq*-polymerase, 50 of pmol P5, 50 pmol of T7), cloned, and sequenced as described.

PCR detection of alternative splicing. The specific oligonucleotides P9 (ATTGGATCCAGACCATCTTCATTGTGCA/GGCTG) and P2 were designed to anneal to cDNA sequence corresponding to the long cytosolic loop between M3 and M4. PCR was done with cDNA from guinea pig cortex, intestine, spleen, liver, and muscle and NG108–15 cells. Amplification was done for 30 cycles (94° for 1 min, 60° for 1 min, 72° for 30 sec, 2.5 units of *Taq*-polymerase). The PCR products were resolved on a 2.5% agarose gel (Boehringer-Mannheim Biochemica, Mannheim, Germany).

Construction of recombinant plasmids p5-HT$_3$GP$_s$, p5-HT$_3$GP$_l$, and p5-HT$_3$H. The recombinant plasmids p5-HT$_3$GP carrying the entire protein coding sequence of guinea pig 5-HT$_3$R splice variants were constructed as follows. PCR was done with intestine P7 primed cDNA as template and the primers P6 (CCCAAGCTTGCCACCATGGTGCTGTGGCTCCAGCTG) containing a *Hin*dIII restriction side followed by a consensus sequence for the initiation of translation in vertebrates (Kozak, 1989) and the first 21 nucleotides from the coding region of guinea pig 5-HT$_3$R and P8 (TACCTT/CGACCAATCCTAT/CT/CCT/ATAGATCTTCGT) containing an *Xba*I restriction side. The cDNA was amplified by 40 cycles (1 min for 94°, 1 min for 65°, 2 min for 72°) using 2.5 units of *Pfu*-Polymerase (Stratagene). The reaction product was digested with *Hin*dIII and *Xba*I, subcloned into an eucaryotic expression vector (pRc/CMV; InVitrogen, San Diego, CA), and sequenced on both strands. The expression plasmid p5-HT$_3$H for the human 5-HT$_3$R was constructed in the same manner as above using oligo(dT)-primed cDNA from human colon and primers P9 (5′) CCCAAGCTTGTCGCTATGCTGCTGTGGGTC and P10 (3′) CATCTAGACTTGGCTTGTGATTGCTGAGATG (Miyake *et al.*, 1995; Lankiewicz *et al.*, 1997).

Construction of chimeric receptors. Random chimeric cDNA was constructed essentially as described previously (Klug *et al.*, 1991). For chimeras with guinea pig cDNA at the 5′-end, primers (50 pmol) were used that fit the 5′ region of 5-HT$_3$GP$_s$ (P6) and the 3′ region of 5-HT$_3$H cDNA (P10). For the reverse chimeras, we used primers P9 and P8 fitting the 3′ region of 5-HT$_3$GP$_s$ and the 5′ region of 5-HT$_3$H cDNA, respectively. Chimeric cDNA was amplified in 2 cycles (45 sec at 94°, 1 sec at 50°) to generate incomplete PCR products, followed by 20 cycles (45 sec at 94°, 45 sec at 60°, 2 min at 72°) using 0.125 unit of *Pfu*-polymerase (Stratagene) and 2.5 units of *Taq*-polymerase and a mixture of 1 ng of *Hin*dIII cut p5-HT$_3$GP$_s$ and p5-HT$_3$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). The "switch-point" was mapped by restriction digestion with *Pst*I, and chimeric cDNAs of interest were sequenced on both strands. The resulting pE4 contained the 5′-end of 5-HT$_3$GP$_s$ up to position 792 (Fig. 1) fused to the 3′ end of 5-HT$_3$H

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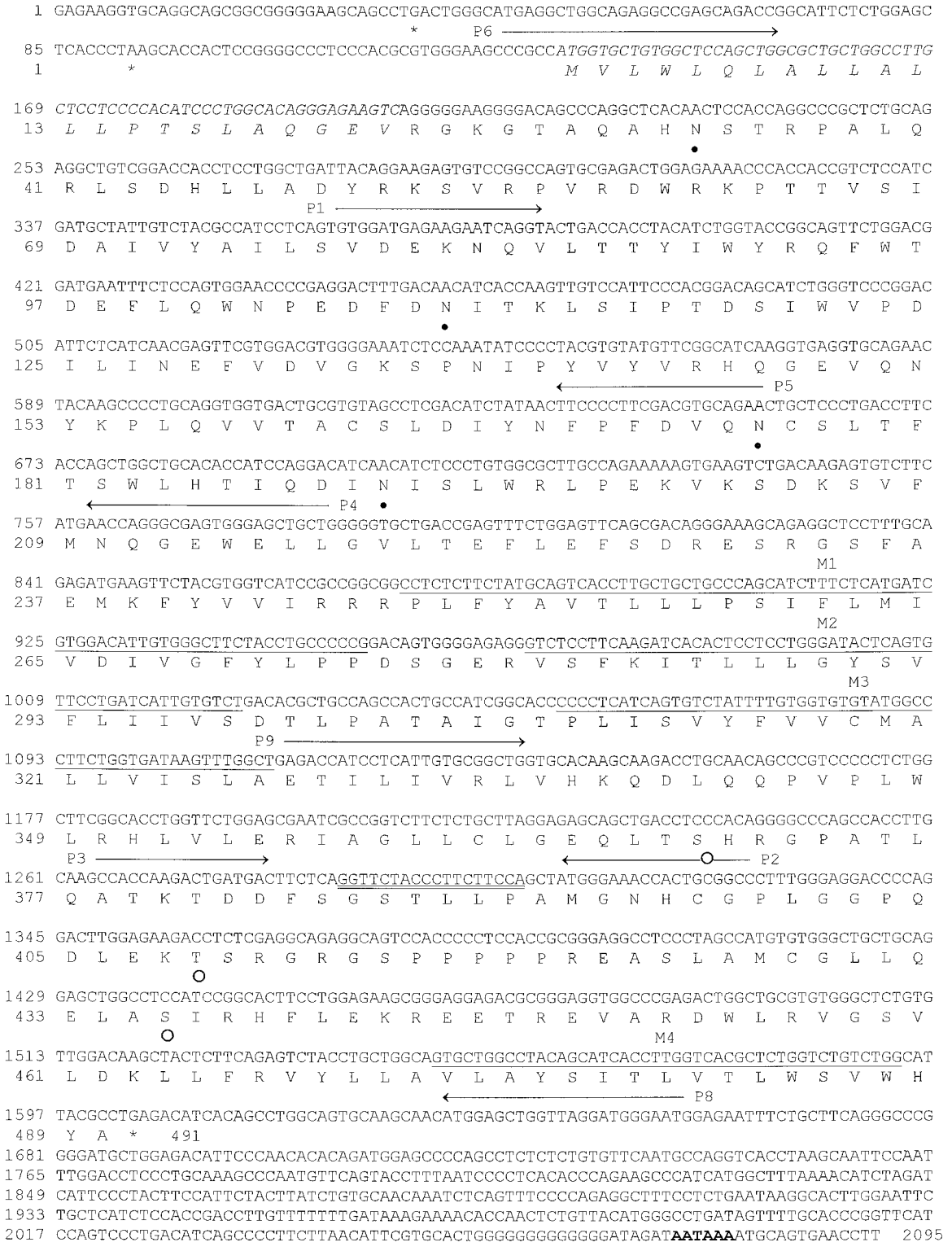

Fig. 1. Nucleotide and amino acid sequence of cloned cDNA encoding the 5-HT_3R channel subunit from guinea pig small intestine. *Numbers*, position of the residue (*left side of each line*). Nucleotide 2095 is followed by a poly(A)$^+$ tract. *, Stop triplet of the coding sequence and the two in-frame stop triplets upstream of the first ATG. *Bold*, poly(A)$^+$ signal sequence. *Italics*, signal sequence. *Double-underlined*, insertion of the long form. *Underlined*, putative transmembrane regions (M1–M4). ●, Consensus sites for glycosylation. ○, Consensus sites for protein kinase C.

beginning at position 865 (Miyake *et al.*, 1995). pC1 is a combination of the 5-HT_3H 5′-end up to position 724 and 5-HT_3GP_s 3′-end beginning at position 876. The switch-point was defined as the first detectable nucleotide of B in an A×B chimera.

Functional expression in HEK 293 cells. Culture and transfection of HEK 293 cells was done as described previously (Gorman *et al.*, 1990). Cells were grown in minimum essential medium supplemented with 10% fetal calf serum in 5% CO_2. Transfection was

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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 HEK 293 cells and allowed to incubate for 5 hr before washing the cells twice with phosphate-buffered saline (0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8.0 g/liter NaCl, 1.15 g/liter Na_2HPO_4). Stable cell lines were established by selection with 500 μg/ml G418.

Electrophysiology and solutions. Transfected HEK 293 cells stably expressing the recombinant 5-HT$_3$Rs [human (H), mouse (M_1), guinea pig (GP_1 and GP_s)] 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 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, Augsburg, Germany) to yield pipettes with resistances of 3–6 MΩ. Pipettes were filled with a solution containing 145 mM CsCl, 10 mM glucose, 10 mM HEPES, and 1 mM EGTA, pH adjusted to 7.2 with CsOH.

Substance application. After establishment of the whole-cell configuration, the cells were lifted from the substrate, and 5-HT or 5-HT$_3$R agonists or antagonists were applied at the indicated concentrations using a fast superfusion device. A piezotranslator-driven double-barreled application pipette was used to expose the lifted cell to 5-HT (agonist)-free or 5-HT (agonist)-containing external solution (flow rate, 200 μl/min). A 2-sec agonist pulse was delivered every 60 sec unless otherwise stated. To study the inhibitory properties of the antagonists, they were presented at the indicated concentration in both 5-HT-free and 5-HT (10 μM)-containing solutions. 5-HT; the agonists 2-Me-5-HT, PBG, and mCPBG; and the antagonists metoclopramide and tropisetron (Sigma, Deisenhofen, Germany; RBI, Köln, Germany) were dissolved in an external solution.

Data acquisition and analysis. Current signals were recorded at a holding potential of −50 mV with an EPC-9 amplifier using the Pulse software on a Macintosh Centris 650 computer. The data were analyzed using PulseFit (Heka, Lamprecht, Germany) and IgorPro (Wavemetrics, Lake Oswego, OR) software.

Binding of the radioligand [^{3}H]GR65630 to membrane fractions of cells expressing the 5-HT$_3$R. HEK 293 cells stably expressing the human or guinea pig 5-HT$_3$R were grown as described above. The cells were harvested, washed with phosphate-buffered saline, and homogenized in 5 volumes of 0.32 M sucrose, 50 mM Tris·HCl, and 1 mM EDTA, pH 7.5, containing the protease inhibitors aprotinin (10 μg/ml), pepstatin (0.75 μg/ml), benzamidine (0.1 mM), phenylmethylsulfonyl fluoride (0.5 mM), and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (1 μM) as described previously (Maricq *et al.*, 1991). After centrifugation at 750 × *g* for 10 min, the supernatant fraction was recentrifuged at 100,000 × *g* for 45 min. The resulting pellet was resuspended in 50 mM Tris·HCl, and 1 mM EDTA, pH 7.5, containing the same protease inhibitors described above. For ligand binding experiments, ≈200 μg of protein was incubated in microtiter plates in a total volume of 250 μl at 37° for 30 min with the indicated concentrations of [^{3}H]GR65630 (64 Ci/mM; New England Nuclear Research Products, Boston, MA). Bound ligands were separated from free ligands by washing with ice-cold assay buffer (50 mM Tris·HCl, 1 mM EDTA, pH 7.5) and rapid filtration through Whatman GF/B filters with a Titertek cell harvester (Nunc, Wiesbaden, Germany). Radioactivity was determined by liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 10 μM MDL 72222. Specific binding represented 65–80% of the total binding. Binding data were analyzed with the EBDA and LIGAND programs, which provide a nonlinear, least-squares regression analysis (Munson and Rodbard, 1980). This weighted curve-fitting program assumes binding according to the law of mass action to independent classes of binding sites.

Results

Structure of guinea pig 5-HT$_3$R-A. Using primers P1 and P2 (Fig. 1) deduced from conserved regions of known 5-HT$_3$Rs, we were able to isolate a 959-bp cDNA fragment from guinea pig small intestine mRNA through RT-PCR that was 63% homologous to the partial cDNA sequence coding for the murine 5-HT$_3$R (Maricq *et al.*, 1991). The RACE technique was used to obtain the full-length cDNA sequence (Lankiewicz *et al.*, 1997). Missing 3′- and 5′-termini of the 5-HT$_3$ cDNA were amplified from small intestine cDNA as described in Materials and Methods. Four independent cDNA clones of the 3′-end of 813 bp were isolated. The clones were equal in sequence except for some inhomogeneity at position 2067 in the 3′-untranslated region. The number of guanosines varied from 10 to 13, and the subsequent adenosine occurred in only one clone. Four independent cDNA clones of the 5′-end were isolated. Sequencing showed that the isolated fragments varied in length by four nucleotides; this may be caused by incomplete cDNA synthesis.

Fig. 1 shows the resulting cDNA sequence of 2095 nucleotides of the guinea pig 5-HT$_3$R-A$_{s/l}$ subunits assembled from sequences of the initial PCR fragment and the longest 5′- and 3′-RACE products. The sequence contains a 5′- and 3′-nontranslated region and a complete ORF of 1473 nucleotides. The translation initiation site was assigned to the ATG at position 133, which is surrounded by an almost perfect ribosome-binding consensus sequence (Kozak, 1989), which is proposed to be used as the starting point for protein translation. A second ORF can be located at position 43–93 at the very 5′-end encoding a short polypeptide of 16 amino acids. Similar ORFs can be found in the cDNA coding for human 5-HT$_3$R (Miyake *et al.*, 1995) and proto-oncoproteins, growth factors, or cell surface receptors (Geballe and Morris, 1994) and are postulated to have regulatory function. The 3′-untranslated region has a poly(A)$^+$ termination signal at position 2076 followed by a poly(A)$^+$ tract 14 nucleotides downstream.

The large ORF of the guinea pig 5-HT$_3$R encodes for a protein of 484 amino acids (5-HT$_3$R$_s$) or 490 amino acids (5-HT$_3$R$_l$), respectively. Both encode the same mature polypeptide except for a deletion of 6 amino acids in 5-HT$_3$R$_s$. Amino acid sequence comparison reveals 81% homology to the mouse and rat 5-HT$_3$R splice variants and 86% homology to the human 5-HT$_3$R (Fig. 2). The structural features correspond to other ligand-gated ion channels (Stroud *et al.*, 1990). The guinea pig 5-HT$_3$R has a putative signal sequence of 23 amino acids, four transmembrane spanning regions (M1–M4) containing a large cytosolic loop between M3 and M4, and a cystine bridge spanning 13 amino acids, which are typical sequence features of nicotinic acetylcholine, glycine, and γ-aminobutyric acid$_A$ receptor channels. Four potential sites for *N*-glycosylation (Marshall, 1972) and three potential sites for protein kinase C (Woodgett *et al.*, 1986) and casein kinase II (Pinna, 1990) were located at the extracellular amino terminus and cytoplasmatic loop between M3 and M4, respectively (Figs. 1 and 2).

Alternative Splicing

To analyze alternative splicing, we performed RT-PCR experiments with primers P2 and P9 (Fig. 1) flanking the position of the deletion. mRNA extracted from different

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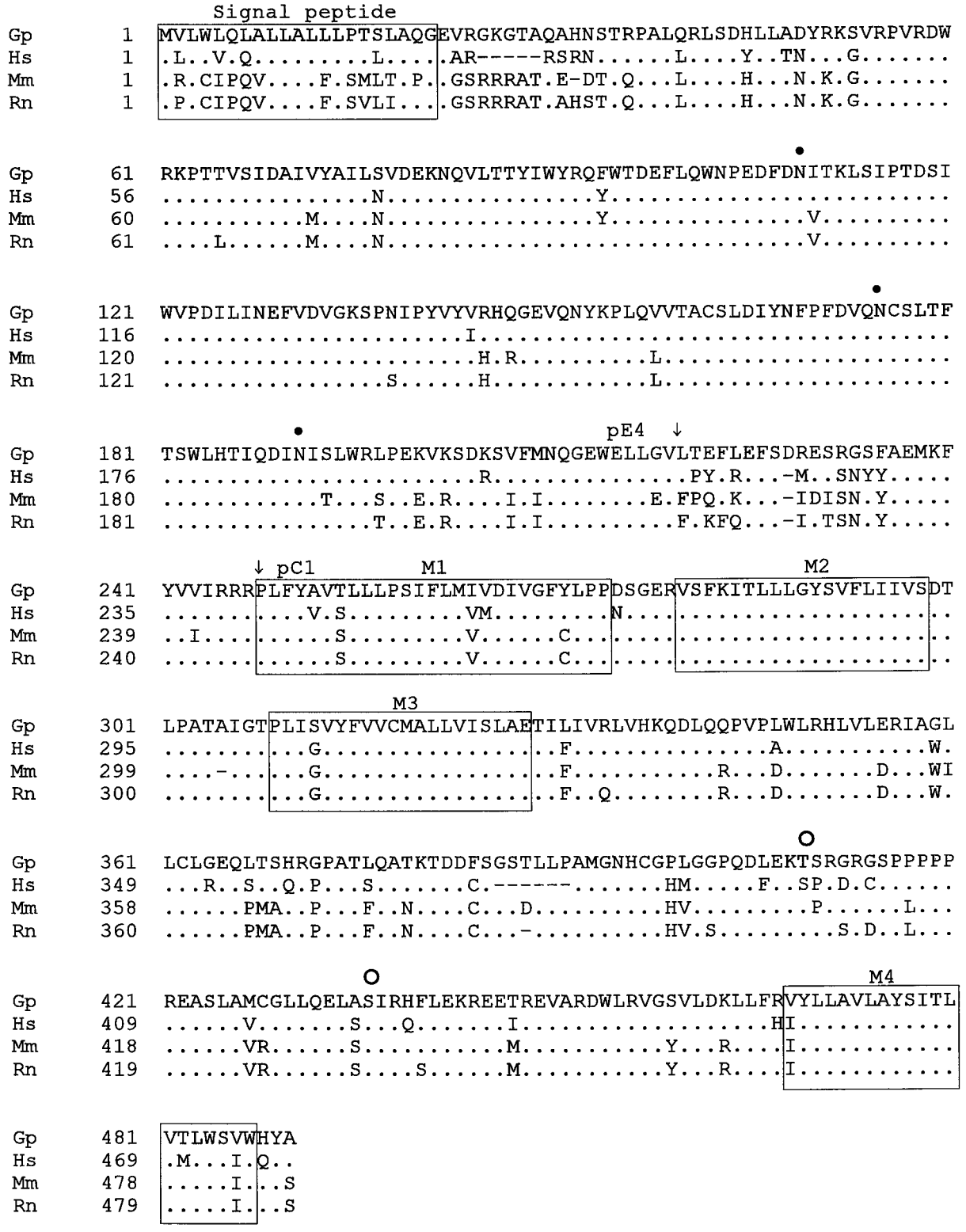

Fig. 2. Comparison of the amino acid sequence of guinea pig (*Gp*), human colon (*Hs*), mouse NCB20 cells (*Mm*), and rat brain (*Rn*) 5-HT_{3R}s. *Boxed*, M1–M4 segments and the putative signal peptide. ○, Potential conserved protein kinase C sites. ●, Potential conserved *N*-glycosylation sites. ↓, Switch point of the chimeric receptors.

guinea pig tissues was analyzed and compared with the murine cell line NG108–15, which is known to express both forms (Emerit *et al.*, 1995). PCR resulted in fragments of 207 and 225 bp, respectively, as predicted from the nucleotide sequences of the splice variants. Gel electrophoresis of these PCR products revealed that both forms of the 5-HT_3R occur together in murine NG108–15 cells and guinea pig cortex, intestine, and liver but not in guinea pig spleen and muscle. Fig. 3 shows that 5-HT_3R_l has a lower level of expression compared with 5-HT_3R_s. This finding is supported by inves-

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tigation of subcloned PCR fragments (p5-HT$_3$GP plasmids). Restriction analysis revealed that only 4 of 40 clones contain cDNA for the 5-HT$_3$R$_l$.

Electrophysiological Recordings

HEK 293 cells expressing 5-HT$_3$Rs from mouse (M$_l$), human (H), or guinea pig (GP$_l$ or GP$_s$), respectively, were recorded in the whole-cell voltage-clamp configuration. 5-HT (10 μM) induced currents that developed fast, reached a maximum, and decreased with characteristic decay constants (Fig. 4, *column 1*).

To characterize the 5-HT$_3$Rs of mouse, human, and guinea pig in more detail, we investigated the activation and desensitization kinetics as well as the reversal potential of the 5-HT-induced currents.

Activation kinetics of 5-HT-induced currents. The activation kinetics of murine, human, and guinea pig 5-HT$_3$Rs were dose dependent (*i.e.*, the currents developed faster with increasing 5-HT concentrations). We compared the rise time [time to reach the maximum of the current (*i.e.*, time to peak)] of 10 μM 5-HT-induced currents. Currents induced by 10 μM 5-HT developed quickly in cells transfected with murine and human 5-HT$_3$Rs but slower in cells expressing guinea pig 5-HT$_3$Rs (Table 1).

Current/voltage relationship. The 5-HT-induced currents of murine, human, and guinea pig 5-HT$_3$Rs reversed polarity at holding potentials close to 0 mV (Table 1). Given our ionic conditions for the pipette and bath solutions (see Materials and Methods), the resulting reversal potential predicts 5-HT-induced currents through nonselective cation channels. The current/voltage relationship of all investigated 5-HT$_3$Rs showed no pronounced rectification (Fig. 5), indicating equal permeability for Na^+ and Cs^+ ions.

Desensitization kinetics of 5-HT-induced currents. In the continuous presence of the agonist 5-HT, the induced currents declined with time (*i.e.*, desensitized). Murine and human 5-HT$_3$Rs showed a rather fast desensitization kinetics that were best fit by single- and double-exponential functions, respectively. The application of 10 μM 5-HT to cells expressing murine 5-HT$_3$Rs induced currents that declined with time constants of τ_{fast} = 155 ± 60 msec and τ_{slow} = 1226 ± 169 msec in cells showing double-exponential time courses and with τ = 1047 ± 79 msec in cells showing monoexponential time courses of desensitization. Currents through human 5-HT$_3$Rs declined with time constants of τ_{fast} = 280 ± 49 msec and τ_{slow} = 2313 ± 659 msec in cells showing double-exponential time courses and with τ = 639 ± 68 msec in cells showing monoexponential time courses of desensitization. The desensitization kinetics of guinea pig 5-HT$_3$Rs showed a more linear decrease (Fig. 4) and could not be fit with an exponential function. For this reason, we calculated the decrease in amplitude after 2 sec of 5-HT-application. The data presented in Table 1 show rather fast desensitization of 5-HT-induced currents in HEK 293 cells expressing murine and human 5-HT$_3$Rs, in contrast to only slight desensitization of both types of the guinea pig 5-HT$_3$Rs. In addition, we investigated the presensitization characteristics of the human and GP$_s$ 5-HT$_3$Rs. We evaluated the amplitude of the response to application of 300 μM 5-HT in various background concentrations of 5-HT. The presensitization EC$_{50}$ value (IC$_{50}$) was 0.2 ± 0.002 μM for human and 0.5 ± 0.008 μM for GP$_s$ 5-HT$_3$Rs (n = 5).

Desensitization kinetics of guinea pig 5-HT$_3$Rs (GP$_l$ and GP$_s$) showed no consistent voltage dependence, whereas about half of the cells expressing human 5-HT$_3$Rs (53%) showed an acceleration of desensitization kinetics at positive holding potentials. In these cells, the normalized amplitudes of the induced currents after 2 sec of 5-HT (10 μM) application at positive holding potentials (+50 mV) were about half (56% ± 7%) of the respective amplitudes at negative holding potentials (−50 mV).

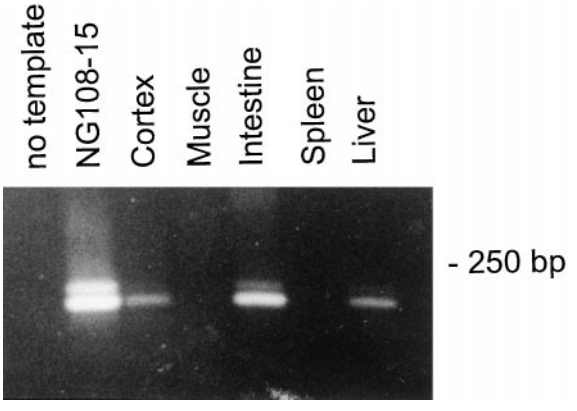

Fig. 3. RT-PCR analysis of 5-HT$_3$ transcripts in different guinea pig tissues. PCR was performed with P2 and P8 primers on 100 ng of oligo(dT)-primed cDNA. PCR products were separated on a 2.5% agarose gel. The fragments of 207 and 225 bp correspond to 5-HT$_3$R$_s$ and 5-HT$_3$R$_l$, respectively. NG108–15 cDNA served as control.

Pharmacology of Human, Murine, and Guinea Pig 5-HT$_3$Rs

Agonists at 5-HT$_3$Rs. We investigated the potencies of the 5-HT$_3$R agonists 5-HT, 2-Me-5-HT, PBG, and mCPBG. The expressed 5-HT$_3$Rs of all species responded to 5-HT and the 5-HT$_3$R agonist 2-Me-5-HT in a dose-dependent way and with very similar apparent affinities (Fig. 6; for EC$_{50}$ values, see Table 2). The agonists PBG and mCPBG discriminated between the 5-HT$_3$Rs of the various species. In murine 5-HT$_3$Rs, mCPBG in nanomolar concentration induced marked responses, whereas in human 5-HT$_3$Rs, micromolar concentration of mCPBG is required. At least, the guinea pig 5-HT$_3$Rs GP$_l$ and GP$_s$ showed a 10-fold lower apparent affinity for mCPBG.

Higher concentrations of agonist (especially mCPBG) inhibited the induced current. Increasing concentrations of agonist first accelerated the time constant of decay and finally reduced the maximum amplitude of the response (Figs. 4 and 6). This block was not voltage dependent between −90 and +50 mV (data not shown). Reducing the agonist concentration at the end of the application removed channel block and led to a pronounced "off response": ions were able to permeate through the still opened but no longer blocked channel. The subsequent decline in the current indicates channel closing due to receptor inactivation.

The murine 5-HT$_3$R had a four times higher apparent affinity for PBG than did the human 5-HT$_3$R. The guinea pig 5-HT$_3$Rs (GP$_l$ and GP$_s$) did not respond to PBG, even in millimolar concentrations. We also found that PBG did not antagonize guinea pig 5-HT$_3$Rs (data not shown).

Antagonists at 5-HT$_3$Rs. We also tested the effectiveness of the competitive 5-HT$_3$R antagonists metoclopramide and tropisetron (Fig. 7). The murine and human 5-HT$_3$Rs were

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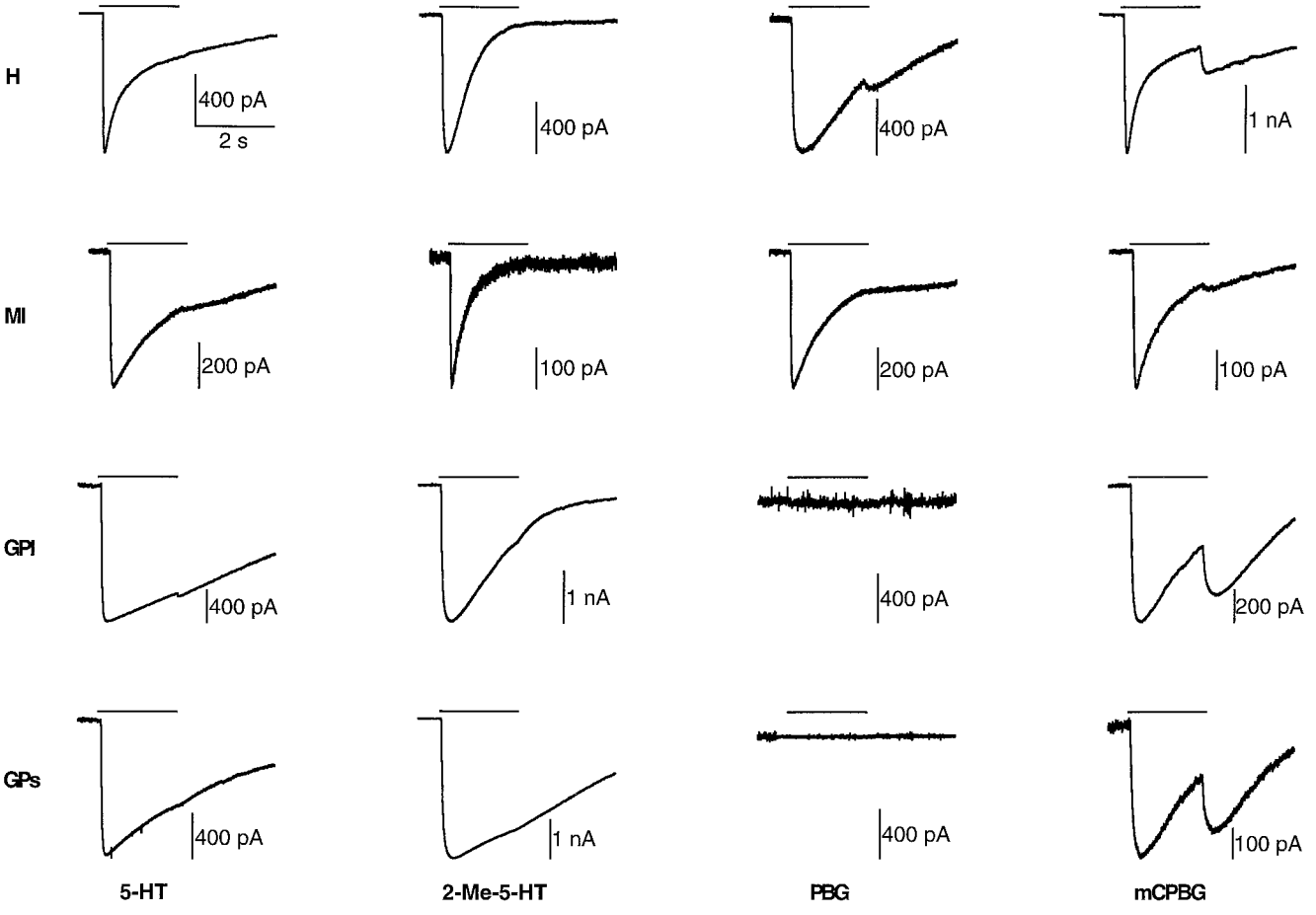

Fig. 4. Whole-cell currents of HEK 293 cells expressing the human (*first row*), murine (*second row*) or guinea pig (GP_l, *third row*; GP_s, *fourth row*) 5-HT_3Rs. The currents were induced by application of 10 μM 5-HT (*first column*), 10 μM 2-Me-5-HT (*second column*), 100 μM PBG (*third column*), or mCPBG (*fourth column*: H, 10 μM; M_l, 100 μM), respectively. The guinea pig 5-HT_3Rs did not respond to PBG. Higher concentrations of mCPBG produced a voltage-independent (data not shown) channel block (note the fast time constant of decay and the pronounced "off response" at decreasing mCPBG concentrations after agonist application).

TABLE 1

Time to peak, reversal potential of 10 μM 5-HT-induced currents, and the normalized amplitude after 2 sec of 10 μM 5-HT application of recombinant and chimeric 5-HT_3 receptors

The peak amplitude of the induced current was set at 1. Transfected HEK 293 cells were recorded at a holding potential of −50 mV. The data were obtained from 4 to 11 independent experiments. Values are presented as mean ± standard error.

5-HT_3 receptor	Time to peak	E_{rev}	Normal amplitude after 2 sec of 5-HT application
	msec	*mV*	
H	83 ± 10	−4.9 ± 1.4	0.33 ± 0.04
M_1	100 ± 12	2.3 ± 2	0.42 ± 0.04
GP_1	230 ± 28	−1.9 ± 2.5	0.84 ± 0.04
GP_2	200 ± 12	−5.5 ± 3.5	0.86 ± 0.05
C1	194 ± 14	−1.8 ± 0.3	0.9 ± 0.02
E4	52 ± 3	−4.2 ± 0.4	0.31 ± 0.04

most sensitive to tropisetron and metoclopramide, whereas the guinea pig 5-HT_3Rs had 10 times lower apparent affinities (for IC_{50} values, see Table 2).

Radioligand Binding Studies

Radioligand binding studies of recombinant human and guinea pig 5-HT_3R were performed with membrane preparations of HEK 293 cells stably transfected with receptor cDNA. The 5-HT_3R-selective radioligand [^{3}H]GR65630 specifically bound to membranes from cells expressing human 5-HT_3R with a K_D value of 2.56 ± 1.2 nM and a B_{max} value of 4915 ± 1632 fmol/mg of protein and bound to membranes from cells expressing 5-HT_3GP_l with a K_D value of 3.08 ± 1.2 nM and a B_{max} value of 324 ± 112 fmol/mg of protein (data not shown). To assess the binding potency of 5-HT_3R agonists and antagonists, we performed competition studies. As shown in Fig. 8A, the antagonist tropisetron and the agonists 2-Me-5-HT, PBG, and mCPBG displaced from human 5-HT_3R with K_I values of 4.82 ± 1.3 nM, 989 ± 412 nM, 22 ± 4 μM, and 243 ± 112 nM, respectively. The specific binding of [^{3}H]GR65630 to membranes from cells expressing 5-HT_3GP_l was displaced by tropisetron, 2-Me-5-HT, and mCPBG with K_I values of 23 ± 7 nM, 1.2 ± 0.4 μM, and 6.2 ± 1 μM. PBG in concentrations of <100 μM did not displace [^{3}H]GR65630 (Fig. 8B).

Chimeric 5-HT_3Rs

To investigate the molecular determinants for the species differences in desensitization kinetics and ligand binding properties, we constructed chimeric receptors between human and GP 5-HT_3R sequences. Molecular cloning produced, among others, two chimeric receptors, E4 and C1, which consisted of the guinea pig amino terminus and the human carboxyl-terminal domain (E4) and the human amino-terminal domain and the guinea pig carboxyl terminus (C1), re-

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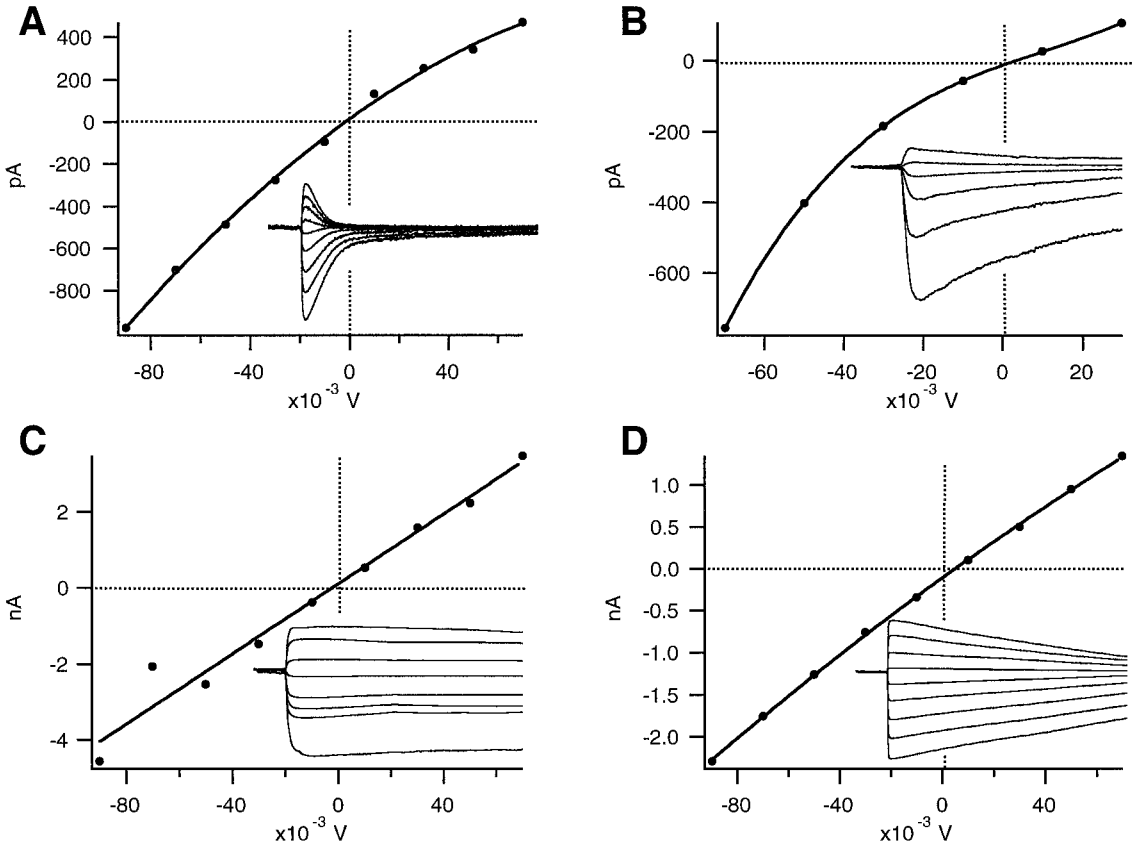

Fig. 5. Representative current/voltage relationships (current/voltage curves) of 5-HT (10 μM)-induced currents of transfected HEK 293 cells expressing human (A), murine (B), GP$_s$ (C), or GP$_l$ (D) 5-HT$_3$Rs. *Insets*, records with a total length of 4 sec (A, C, and D) or 2 sec (B).

spectively (Fig. 9, E amd F). The "switch points" are indicated in Fig. 2 (5-HT$_3$GP$_s$ numbering: E4, amino acid 220; C1, amino acid 248). Both the E4 and C1 5-HT$_3$Rs contained a human 5-HT$_3$R-derived 28-amino-acid-spanning sequence adjacent to the M1 domain. Transient expression of the E4 or C1 receptor plasmids in HEK 293 cells produced functional 5-HT$_3$R channels, which were sensitive to 5-HT and PBG (Fig. 9). The dose-response relationship yielded EC$_{50}$ values of 1.3 ± 0.08 μM 5-HT and 21.8 ± 1.4 μM PBG for C1 receptors and 1.28 ± 0.06 μM 5-HT and 19.3 ± 7.2 μM PBG for E4 receptors, respectively. In addition, the application of 10 μM 5-HT induced fast desensitizing (human-like) currents in E4 and slow desensitizing (guinea pig-like) currents in C1 receptor-expressing HEK cells (Table 1).

Discussion

Significant efforts by workers in several laboratories using cloning (by homology or expression) and/or purification have not revealed more than one subunit of the 5-HT$_3$R. Recently, a splice variant of the murine 5-HT$_3$R-A was identified in N1E-115 mouse neuroblastoma cells (Hope *et al.*, 1993) showing a deleted region of six amino acids within the putative cytoplasmic loop between M3 and M4 compared with the original NCB20 clone (Maricq *et al.*, 1991). RT-PCR experiments performed with primers flanking this region showed that both isoforms occur in all murine cell lines (N1E-115, NCB20, NG108–15; Hope *et al.*, 1993; Werner *et al.*, 1994). Analysis of a mouse genomic clone suggested that these isoforms are generated by the alternative use of acceptor splice sites (Uetz *et al.*, 1994). We report here the corresponding sequences of the guinea pig 5-HT$_3$R-A cDNA and show evidence for alternative splicing in different tissues of guinea pig as a method of generating two different 5-HT$_3$R-A mRNAs coding for long (5-HT$_3$R-A$_l$) and short (5-HT$_3$R-A$_s$) forms. The physiological relevance of the two alternative spliced subunits in rodents still is unclear. Besides the action of the partial agonist 2-Me-5-HT, significant differences could not be detected between the pharmacological properties of the murine splice variants. Recent investigations suggest the involvement of the splice variants in neuronal development (Miquel *et al.*, 1995). The relative expression of the long form of 5-HT$_3$R-A mRNA in the hippocampus and cerebral cortex of rat was found to be significantly higher prenatally than postnatally.

The full-length sequences of the guinea pig 5-HT$_3$R-A cDNAs reported here confirm the ligand-gated ion channel features found previously in the 5-HT$_3$R-A subunit cloned from NCB20 cells (Maricq *et al.*, 1991). The high homology (81% and 86%) to the murine and human receptor, respectively, indicates that despite the electrophysiological and pharmacological differences, the guinea pig 5-HT$_3$R does not define a novel class of 5-HT$_3$Rs in terms of homology classification.

The electrophysiological and pharmacological data for 5-HT$_3$R from guinea pig, human, and mouse were determined in the same cellular background to avoid artifacts resulting from the expression system (*e.g.*, oocytes versus mammalian cells), different modifications, or specific subunit composition characteristic for a given tissue.

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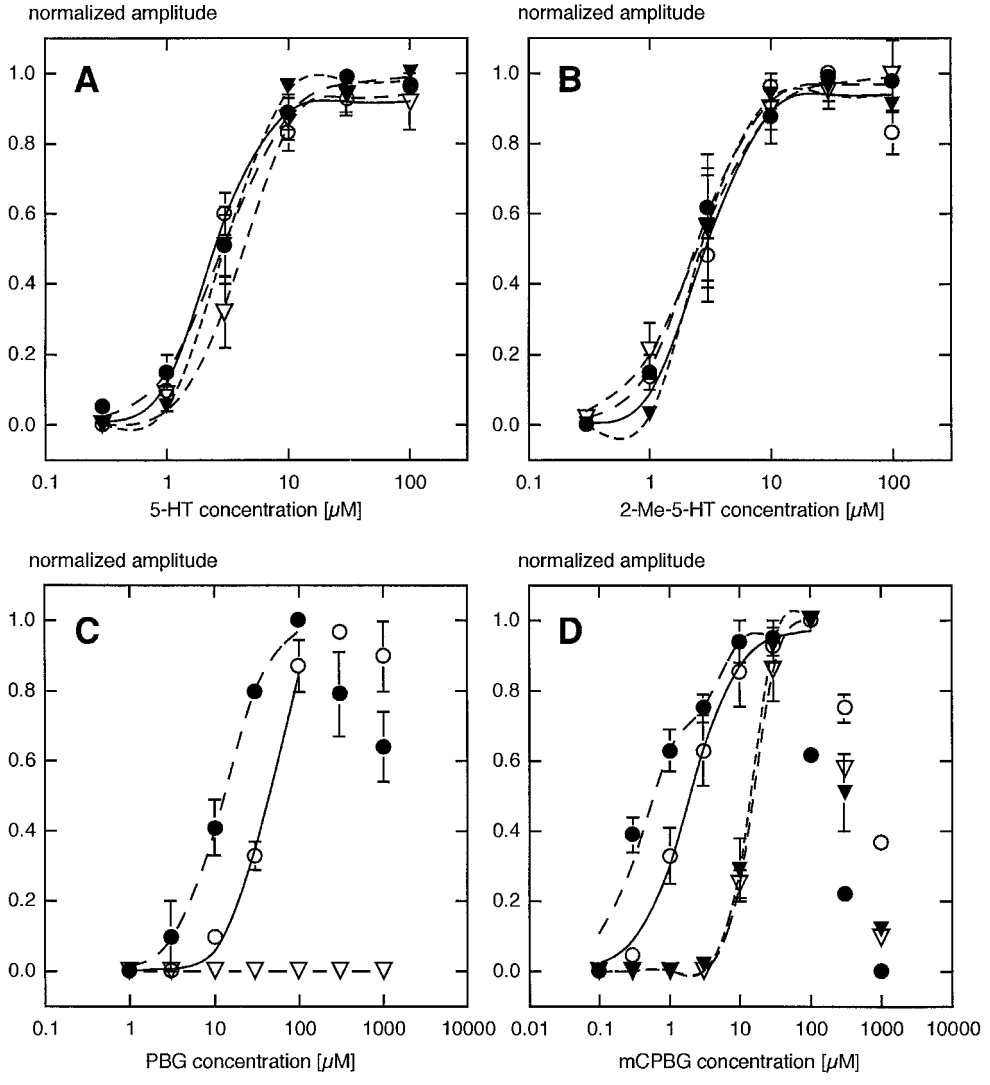

Fig. 6. Dose-response relationship of 5-HT- (A), 2-Me-5-HT- (B), PBG- (C), and mCPBG- (D) induced currents in HEK 293 cells expressing human (○), murine (●), GP_1 (△), and GP_s (▲) 5-HT_3R. Agonists at the concentrations indicated were applied using a fast superfusion system. The peak amplitude of the response was normalized to the maximum response induced by the respective agonist. C, None of the cells expressing guinea pig 5-HT_3Rs responded to PBG, even in millimolar concentrations. The symbols represent the data points and are shown as mean ± standard error. *Lines*, Hill fits of the data drawn as spline functions of the fit results. Data were obtained from 4–11 independent experiments.

TABLE 2

EC_{50} and IC_{50} values of recombinant and chimeric 5-HT_3 receptors for various agonists and antagonists

Antagonists were tested in the presence of 10 μM 5-HT. Transfected HEK 293 cells were recorded at a holding potential of −50 mV. The data were obtained from 4 to 11 independent experiments. Values are presented as mean ± standard error.

5-HT_3 receptor	5-HT	2-Me-5-HT	PBG	mCPBG	Metoclopramide	Tropisetron
			μM			*nM*
H	2.3 ± 0.2	2.8 ± 0.5	41.2 ± 4	1.9 ± 0.2	0.29 ± 0.01	0.59 ± 0.07
M_1	2.9 ± 0.2	2.3 ± 0.2	12.5 ± 1.1	0.55 ± 0.18	0.11 ± 0.01	0.19 ± 0.003
GP_1	2.9 ± 0.1	2.7 ± 0.1	>1000	13.3 ± 0.1	3.4 ± 0.3	9.2 ± 0.4
GP_2	3.8 ± 0.3	2.5 ± 0.1	>1000	15.1 ± 0.3	5.6 ± 0.5	9.3 ± 0.5
C1	1.3 ± 0.08		21.8 ± 1.4			
E4	1.28 ± 0.06		19.3 ± 7.2			

The K_I values obtained from radioligand competition studies qualitatively support the EC_{50}/IC_{50} values determined through patch-clamp experiments. The quantitative differences between affinity and apparent affinity may be due in part to methodological reasons. Electrophysiological studies determine receptor function in the living cells, whereas binding studies carried out with membrane preparations measure strictly receptor/ligand affinities.

Our data show that mCPBG is less potent for the guinea pig 5-HT_3R than for that of human and mouse. The derivative PBG is neither agonistic nor antagonistic for guinea pig 5-HT_3Rs stably expressed in HEK 293 cells. These data are confirmed by the observation that PBG failed to bind to 5-HT_3R protein in isolated membranes. PBG showed no effect on 5-HT_3Rs of guinea pig in functional assays (Butler *et al.*, 1990; Blier and Bouchard, 1993). The antagonists metoclopramide and tropisetron are less active on the guinea pig 5-HT_3R that on the receptors of mouse and human in electrophysiological and radioligand binding assays. These findings correspond to the data obtained by functional characterization of 5-HT_3Rs in guinea pig muscle myenteric plexus and vagus nerve preparations (Butler *et al.*, 1990), in which

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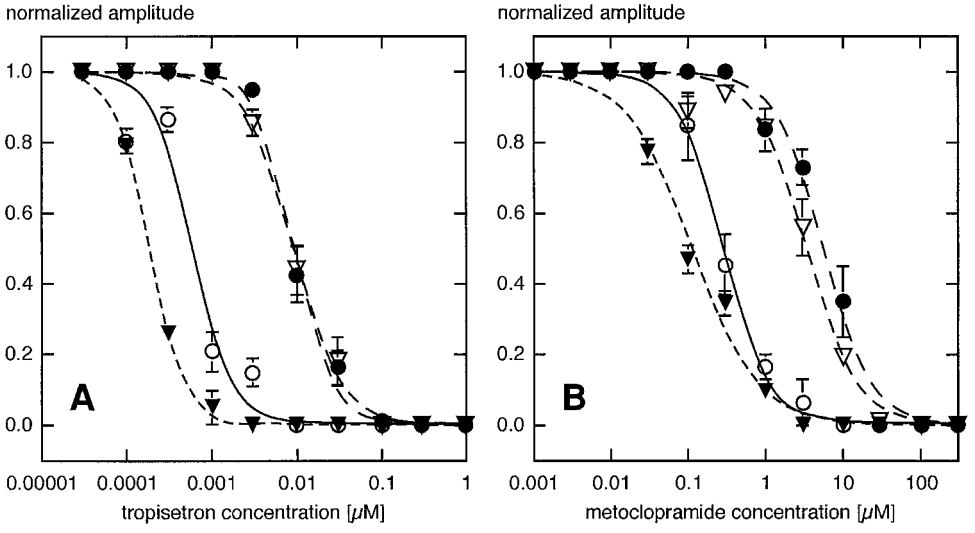

Fig. 7. Dose-response relationship of the competitive antagonism of tropisetron (A) and metoclopramide (B) on human (○), murine (▲), GP_l (△), and GP_s (●) 5-HT_3Rs stably expressed in HEK 293 cells. 5-HT (10 μM) and the antagonists at the indicated concentrations were applied via a fast superfusion system. The antagonists were present before and during the 5-HT application. The peak amplitude of the response to 10 μM 5-HT in the absence of antagonist was set at 1. Data were obtained from 4–11 independent experiments.

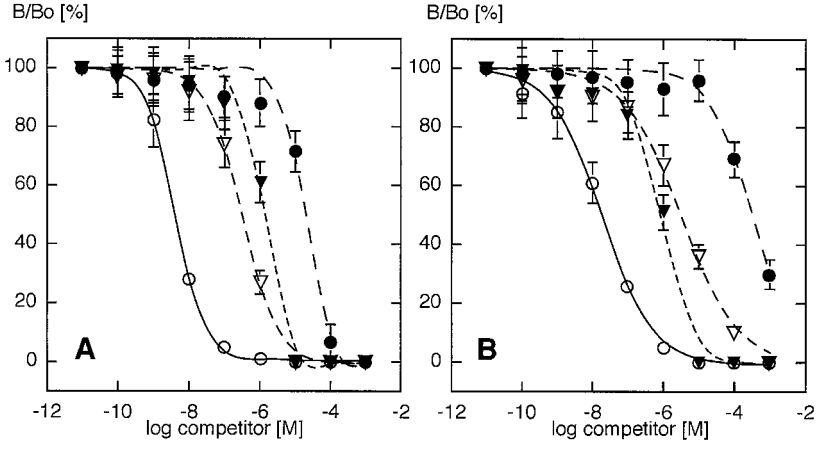

Fig. 8. Competition of tropisetron (○), PBG (●), mCPBG (△), and 2-Me-5-HT (▲) for ^{3}[H]GR65630 binding to membrane fractions of HEK 293 cells expressing the human 5-HT_3R (A) or the long splice variant of the guinea pig 5-HT_3R (B). Membranes were incubated in the presence of 2 nM ^{3}[H]GR65630 and increasing concentrations of unlabeled competitor. Binding data are expressed as the percentage of specific binding remaining after inclusion of 10^{-11} to 10^{-3} M competitor (*B*). A value of 100% represents the amount of specific binding of 2 nM [^{3}H]GR65630 in the absence of competitor (*Bo*). Results are expressed as the mean ± standard error of three independent experiments. A, K_I (mean ± standard deviation) was 4.82 ± 1.3 nM for tropisetron, 22 ± 4 μM for PBG, 243 ± 112 nM for mCPBG, and 989 ± 412 nM for 2-Me-5-HT. B, K_I (mean ± standard deviation) were 23 ± 7 nM for tropisetron, 6.2 ± 1 μM for mCPBG, and 1.2 ± 0.4 μM for 2-Me-5-HT. PBG (<100 μM) did not displace ^{3}[H]GR65630.

all 11 antagonists exhibited a markedly lower affinity for guinea pig than for rat receptors.

Binding and electrophysiological studies revealed that the properties of the recombinantly expressed 5-HT_3R from guinea pig do not significantly differ from those in native tissues (Butler *et al.*, 1990; Kilpatrick and Tyers, 1992). These data are in line with the assumption that the native 5-HT_3R is a homo-oligomer; a similar molecular structure is suggested for the neuronal α7 acetylcholine receptor (Sargent, 1993). This does not contradict the findings that cloned and native receptors differ in single-channel conductances; modulation of single-channel conductances in 5-HT_3Rs in N1E-115 cells has been shown by the action of protein kinase C (Van Hooft and Vijverberg, 1995).

Binding sites for agonists of the 5-HT_3R are postulated to occur on the large extracellular amino-terminal domain from homology to the nicotinic acetylcholine receptor (Barnard, 1992). Comparison of the guinea pig with human and mouse 5-HT_3R-A sequences reveals that only few amino acids are unique to guinea pig.

To investigate the molecular determinants for the species differences in desensitization kinetics and ligand binding properties, we constructed chimeric receptors between human and guinea pig 5-HT_3R sequences. Molecular cloning produced two chimeric receptors, E4 and C1, which consisted of the guinea pig amino terminus and the human carboxyl-terminal domain (E4) or the human amino-terminal domain and the guinea pig carboxyl terminus (C1), respectively. Both the E4 and C1 5-HT_3Rs contained a human 5-HT_3R-derived 28-amino-acid-spanning sequence adjacent to the M1 domain. Functional expression of the E4 or C1 receptor plasmids in HEK 293 cells produced 5-HT_3R channels that were sensitive to 5-HT and PBG. Accepting the hypothesis that the ligand binding site is located at the amino-terminal domain (Eisele *et al.*, 1993), the apparent PBG sensitivity of the E4 receptor (guinea pig/human) suggests that at least parts of the PBG binding site are located between the switching points of E4 and C1.

The application of 10 μM 5-HT-induced fast desensitizing (human-like) currents in E4 and slow desensitizing (guinea pig-like) currents in C1 receptor-expressing HEK 293 cells indicates that the desensitization kinetics might be delegated to the carboxyl-terminal part of the receptor subunit. Others, however, found the tertiary and quaternary structures of the whole receptor molecule were responsible for the kinetics of the current (Eisele *et al.*, 1993).

Chimeric receptors from guinea pig and human therefore are a suitable tool for detailed mapping of agonist and antagonist binding sites. It is tempting to speculate that the reduced sensitivity of the guinea pig 5-HT_3R for all antagonists tested in comparison to its normal sensitivity for 5-HT

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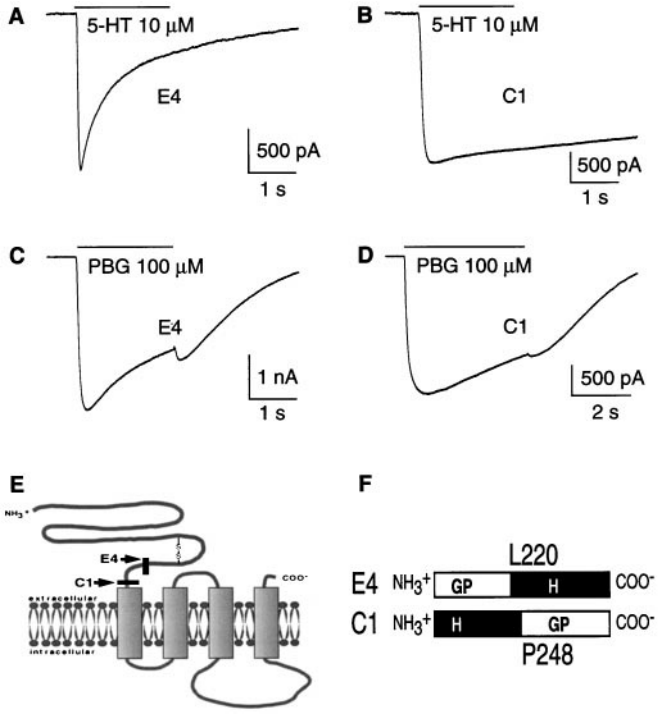

Fig. 9. Whole-cell recordings of agonist-induced currents of transfected HEK 293 cells expressing chimeric 5-HT_3Rs. 5-HT (10 μM) induced currents of HEK 293 cells expressing E4 (A) or C1 (B) chimeric 5-HT_3Rs. C and D, PBG (100 μM) induced currents of HEK 293 cells expressing E4 (C) or C1 (D) chimeric 5-HT_3Rs. *Bar*, application of agonist via a fast superfusion device. Cells were recorded at −50 mV. E, Schematic drawing of the chimeric 5-HT_3R subunit. *Arrows*, location of the "switch points" of the amino acid sequence. F, Schematic drawing of the chimeric 5-HT_3R subunits. The E4 receptor consists of the guinea pig (white) amino terminus and the human (black) carboxyl terminus, whereas the C1 receptor consists of the human (black) amino terminus and the guinea pig (white) carboxyl terminus. Both the E4 and C1 receptors share the human receptor-derived sequence of ≈28 amino acids adjacent (amino-terminal) to the M1 region.

is caused by partially overlapping sites for agonist and antagonist binding.

Our data show that the 5-HT_3Rs from human and guinea pig differ markedly in their pharmacological properties and suggest that the guinea pig is not a suitable experimental animal for the development of new 5-HT_3 agonists or antagonists with clinical relevance.

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