Molecular Cloning, Functional Expression, and Pharmacological Characterization of 5-Hydroxytryptamine$_3$ (5-HT$_3$) 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 carboxy-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$_3$A inotropic receptor subunit (5-HT$_3$R-A) was cloned 6 years ago from the NCB20 neuroblastoma cell line (Marić 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 $\alpha$ or $\beta$ 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 (Marić 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-

ABBREVIATIONS: 5-HT$_3$R, 5-hydroxytryptamine$_3$ receptor; 5-HT, 5-hydroxytryptamine; PBG, 1-phenylbiguanide; mPBG, m-chlorophenylbiguanide; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; RT, reverse transcriptase; EGTA, ethylene glycol bis-$\beta$-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.
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-HT₃Rs 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-HT₃R is expressed predominantly in neurons in the area postrema and mesolimbic system (Kilpatrick et al., 1987, 1988; Tecott et al., 1993). Thus, 5-HT₃Rs seem to be a potential target for the development of drugs for the treatment of nausea and behavioral disorders (Aput, 1993). 5-HT₃R antagonists prevent emesis induced by cytostatic drugs that are mediated by 5-HT₃Rs (Peters et al., 1992).

Thus, 5-HT₃Rs have been considered useful targets for the development of antiemetic drugs. A recent pharmacological study provided new insights into the role of 5-HT₃Rs in the modulation of emesis induced by cytostatic drugs. The study showed that the 5-HT₃R antagonist ondansetron (ZD 12333) significantly reduced the incidence of emesis induced by the cytostatic drug cisplatin in female rats. The results of this study suggest that 5-HT₃Rs in the area postrema may play a role in the modulation of emesis induced by cisplatin.

However, the exact role of 5-HT₃Rs in the modulation of emesis remains to be determined. Further studies are needed to clarify the role of 5-HT₃Rs in the modulation of emesis and other pharmacological effects.

**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 or a gene-specific primer (P4 CAGGAGCTCCCAC/TTCICCC/GAT20) or a gene-specific primer (P5, 50 pmol of T7), cloned and sequenced as described. 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 P2 at 94° for 1 min, 50° for 1 min, 72° for 1 min, 2.5 units of Taq-polymerase, followed by 30 cycles with 50 pmol of primer P6 (ACAGAATTCTG1ACA/GTCGA/GAAGG/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, Madison, WI) 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 pmol of primer P3 and T7). The specific reaction product was purified with Streptavidin-Paramagnetic Particles (Promega) and biotinylated internal primer P1, cloned blunt end into pBluescript II KS⁺ (Stratagene), and sequenced.

**Construction of recombinant plasmids p5-HT³GP₂, p5-HT³GP₁, and p5-HT³H.** The recombinant plasmids p5-HT³GP₂ and p5-HT³H, carrying the entire protein coding sequence of guinea pig 5-HT₃R splice variants were constructed as follows. PCR was done with intestine P7 primed cDNA as template and the primers P6 (CCCCAGGTTGCGCACCAGTTGCGTGCCTCCAGCTG) containing a Hind III 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-HT₃R and P8 (TACCTTGCGAACATCCTATCTCCAGT) containing a Hind III 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 PuⅢ-Polymerase (Stratagene). The reaction product was digested with Hind III and Xhol, subcloned into an eucaryotic expression vector (pReCMV; Invitrogen, San Diego, CA), and sequenced on both strands. The expression plasmid p5-HT₃H for the human 5-HT₃R was constructed in the same manner as above using oligo(dT)-primed cDNA from human colon and primers P9 (5' CCAACGGTCGGTCGATCTGCTCTCCTAGCAAGTTGCCATGGC ATGAGGAGCAGTAGAGCGCTGACAACTGCTGACGAGT CAGT) and P10 (5' CAGTGGATCCCTAGCAAGTTGCCATGGC ATGAGGAGCAGTAGAGCGCTGACAACTGCTGACGAGT CAGT) (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-HT₃GP₂ (P6) and the 3'-region of 5-HT₃H cDNA (P10). For the reverse chimeras, we used primers P9 and P8 fitting the 3'-region of 5-HT₃GP₂ and the 5'-region of 5-HT₃H 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 PuⅢ-Polymerase (Stratagene) and 2.5 units of Taq-polymerase and a mixture of 1 ng of Hind III cut p5-HT₃GP₂ and p5-HT₃H as the template. The reaction product was digested with Hind III and Xhol and subcloned into an eucaryotic expression vector (pReCMV; Invitrogen). The “switch-point” was mapped by restriction digestion with PstⅠ and chimeric cDNAs of interest were sequenced on both strands. The resulting pE4 contained the 5'-end of 5-HT₃GP₂ up to position 792 (Fig. 1) fused to the 3'-end of 5-HT₃H.
Fig. 1. Nucleotide and amino acid sequence of cloned cDNA encoding the 5-HT₃R 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-HT₃H₅₉-end up to position 724 and 5-HT₃GPs 3₉-end beginning at position 876. The switch-point was defined as the first detectable nucleotide of B in an AxB 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% CO₂.
accomplished by mixing 15 μg of expression vector and 250 μl of 250 mM CaCl2. The material was added dropwise to 250 μl of 1.5 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 KH2PO4, 8.0 g/liter NaCl, 1.15 g/liter Na2HPO4). Stable cell lines were established by selection with 50 μg/ml G418.

Electrophysiology and solutions. Transfected HEK 293 cells stably expressing the recombinant 5-HT3Rs [human (H), mouse (M), guinea pig (GP, and GP-i)] 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-HT3-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-HT3, 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 [3H]GR65630 to membrane fractions of cells expressing the 5-HT3-R. HEK 293 cells stably expressing the human or guinea pig 5-HT3-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), benzamidene (0.1 mM), phenylmethylsulfonyl fluoride (0.5 mM), and trans-epoxyoxycinnyl-1-leucylamido-(4-guanidino)-butane (1 μM) as described previously (Mariq 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°C for 30 min with the indicated concentrations of [3H]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/F filters with a Tittertek 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 EDBA 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-HT3-R. A. Using primers P1 and P2 (Fig. 1) deduced from conserved regions of known 5-HT3-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-HT3-R (Mariq 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-HT3-R 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 adenine 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-HT3-R, 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-HT3-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-HT3-R encodes for a protein of 484 amino acids (5-HT3-R) or 490 amino acids (5-HT3-R), respectively. Both encode the same mature polypeptide except for a deletion of 6 amino acids in 5-HT3-R. Amino acid sequence comparison reveals 81% homology to the mouse and rat 5-HT3-R splice variants and 86% homology to the human 5-HT3-R (Fig. 2). The structural features correspond to other ligand-gated ion channels (Stroud et al., 1990). The guinea pig 5-HT3-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 cysteine bridge spanning 13 amino acids, which are typical sequence features of nicotinic acetylcholine, glycine, and γ-aminobutyric acidA 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 cytoplasmic 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...
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-HT₃R 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-HT₃R₃ has a lower level of expression compared with 5-HT₃R₂. This finding is supported by inves-
tigation of subcloned PCR fragments (p5-HT3GP plasmids). Restriction analysis revealed that only 4 of 40 clones contain cDNA for the 5-HT3R.

**Electrophysiological Recordings**

HEK 293 cells expressing 5-HT3Rs from mouse (M), human (H), or guinea pig (GP1 or GP2), 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-HT3Rs 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-HT3Rs 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-HT3Rs but slower in cells expressing guinea pig 5-HT3Rs (Table 1).

**Current/voltage relationship.** The 5-HT-induced currents of murine, human, and guinea pig 5-HT3Rs 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-HT3Rs 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-HT3Rs 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-HT3Rs induced currents that declined with time constants of τfast = 155 ± 60 msec and τslow = 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-HT3Rs declined with time constants of τfast = 280 ± 49 msec and τslow = 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-HT3Rs 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-HT3Rs, in contrast to only slight desensitization of both types of the guinea pig 5-HT3Rs. In addition, we investigated the presensitization characteristics of the human and GP2 5-HT3Rs. We evaluated the amplitude of the response to application of 300 μM 5-HT in various background concentrations of 5-HT. The presensitization EC50 value (IC50) was 0.2 ± 0.002 μM for human and 0.5 ± 0.008 μM for GP2 5-HT3Rs (n = 5).

Desensitization kinetics of guinea pig 5-HT3Rs (GP1 and GP2) showed no consistent voltage dependence, whereas about half of the cells expressing human 5-HT3Rs (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).

**Pharmacology of Human, Murine, and Guinea Pig 5-HT3Rs**

**Agonists at 5-HT3Rs.** We investigated the potencies of the 5-HT3R agonists 5-HT, 2-Me-5-HT, PBG, and mCPBG. The expressed 5-HT3Rs of all species responded to 5-HT and the 5-HT3R agonist 2-Me-5-HT in a dose-dependent way and with very similar apparent affinities (Fig. 6; for EC50 values, see Table 2). The agonists PBG and mCPBG discriminated between the 5-HT3Rs of the various species. In murine 5-HT3Rs, mCPBG in nanomolar concentration induced marked responses, whereas in human 5-HT3Rs, micromolar concentration of mCPBG is required. At least, the guinea pig 5-HT3Rs GP1 and GP2 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-HT3R had a four times higher apparent affinity for PBG than did the human 5-HT3R. The guinea pig 5-HT3Rs (GP1 and GP2) did not respond to PBG, even in millimolar concentrations. We also found that PBG did not antagonize guinea pig 5-HT3Rs (data not shown).

**Antagonists at 5-HT3Rs.** We also tested the effectiveness of the competitive 5-HT3R antagonists metoclopramide and tropisetron (Fig. 7). The murine and human 5-HT3Rs were
most sensitive to tropisetron and metoclopramide, whereas the guinea pig 5-HT3Rs had 10 times lower apparent affinities (for IC50 values, see Table 2).

Radioligand Binding Studies

Radioligand binding studies of recombinant human and guinea pig 5-HT3Rs were performed with membrane preparations of HEK 293 cells stably transfected with receptor cDNA. The 5-HT3R-selective radioligand [3H]GR65630 specifically bound to membranes from cells expressing human 5-HT3R with a Kd value of 2.56 ± 1.2 nM and a Bmax value of 4915 ± 1632 fmol/mg of protein and bound to membranes from cells expressing 5-HT3GPl with a Kd value of 3.08 ± 1.2 nM and a Bmax value of 324 ± 612 fmol/mg of protein (data not shown). To assess the binding potency of 5-HT3R 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-HT3R with KI values of 4.82 ± 1.3 nM, 989 ± 412 nM, 22 ± 4 μM, and 243 ± 112 nM, respectively. The specific binding of [3H]GR65630 to membranes from cells expressing 5-HT3GPl was displaced by tropisetron, 2-Me-5-HT, and mCPBG with KI values of 23 ± 7 nM, 1.2 ± 0.4 μM, and 6.2 ± 1 μM. PBG in concentrations of <100 μM did not displace [3H]GR65630 (Fig. 8B).

Chimeric 5-HT3Rs

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-HT3R 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-

TABLE 1

<table>
<thead>
<tr>
<th>5-HT3 receptor</th>
<th>Time to peak</th>
<th>Erev</th>
<th>Normal amplitude after 2 sec of 5-HT application</th>
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<tr>
<td>H</td>
<td>83 ± 10</td>
<td>-4.9 ± 1.4</td>
<td>0.33 ± 0.04</td>
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<tr>
<td>M1</td>
<td>100 ± 12</td>
<td>2.3 ± 2</td>
<td>0.42 ± 0.04</td>
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<td>GP1</td>
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<td>0.84 ± 0.04</td>
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<tr>
<td>GP2</td>
<td>200 ± 12</td>
<td>-5.5 ± 3.5</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>C1</td>
<td>194 ± 14</td>
<td>-1.8 ± 0.3</td>
<td>0.9 ± 0.02</td>
</tr>
<tr>
<td>E4</td>
<td>52 ± 3</td>
<td>-4.2 ± 0.4</td>
<td>0.31 ± 0.04</td>
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Fig. 4. Whole-cell currents of HEK 293 cells expressing the human (first row), murine (second row) or guinea pig (GP1, third row; GP2, fourth row) 5-HT3Rs. 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; M1, 100 μM), respectively. The guinea pig 5-HT3Rs 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).
spectively (Fig. 9, E and F). The “switch points” are indicated in Fig. 2 (5-HT3GP numbering: E4, amino acid 220; C1, amino acid 248). Both the E4 and C1 5-HT3Rs contained a human 5-HT3R-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-HT3R channels, which were sensitive to 5-HT and PBG (Fig. 9). The dose-response relationship yielded EC50 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-HT3R. Recently, a splice variant of the murine 5-HT3R-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 (Mariq 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-HT3R-A cDNA and show evidence for alternative splicing in different tissues of guinea pig as a method of generating two different 5-HT3R-A mRNAs coding for long (5-HT3R-A1) and short (5-HT3R-A2) 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-HT3R-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-HT3R-A cDNAs reported here confirm the ligand-gated ion channel features found previously in the 5-HT3R-A subunit cloned from NCB20 cells (Mariq 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-HT3R does not define a novel class of 5-HT3Rs in terms of homology classification. The electrophysiological and pharmacological data for 5-HT3R 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.
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$_3$R than for that of human and mouse. The derivative PBG is neither agonistic nor antagonistic for guinea pig 5-HT$_3$Rs stably expressed in HEK 293 cells. These data are confirmed by the observation that PBG failed to bind to 5-HT$_3$Rs in isolated membranes. PBG showed no effect on 5-HT$_3$Rs 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$_3$R 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$_3$Rs in guinea pig muscle myenteric plexus and vagus nerve preparations (Butler et al., 1990), in which

<table>
<thead>
<tr>
<th>5-HT$_3$ receptor</th>
<th>5-HT</th>
<th>2-Me-5-HT</th>
<th>PBG</th>
<th>mCPBG</th>
<th>Metoclopramide</th>
<th>Tropisetron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>H</td>
<td>2.3 ± 0.2</td>
<td>2.8 ± 0.5</td>
<td>41.2 ± 4</td>
<td>1.9 ± 0.2</td>
<td>0.29 ± 0.01</td>
<td>0.59 ± 0.07</td>
</tr>
<tr>
<td>M$_1$</td>
<td>2.9 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>12.5 ± 1.1</td>
<td>0.55 ± 0.18</td>
<td>0.11 ± 0.01</td>
<td>0.19 ± 0.003</td>
</tr>
<tr>
<td>GP$_1$</td>
<td>2.9 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>&gt;1000</td>
<td>13.3 ± 0.1</td>
<td>3.4 ± 0.3</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>GP$_2$</td>
<td>3.8 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>&gt;1000</td>
<td>15.1 ± 0.3</td>
<td>5.6 ± 0.5</td>
<td>9.3 ± 0.5</td>
</tr>
<tr>
<td>C1</td>
<td>1.3 ± 0.08</td>
<td>2.1 ± 1.4</td>
<td>19.3 ± 0.06</td>
<td>21.8 ± 1.4</td>
<td>15.1 ± 0.3</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>E4</td>
<td>1.28 ± 0.06</td>
<td>19.3 ± 7.2</td>
<td>19.3 ± 0.06</td>
<td>21.8 ± 1.4</td>
<td>15.1 ± 0.3</td>
<td>5.6 ± 0.5</td>
</tr>
</tbody>
</table>
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$_3$R 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$_3$R is a homo-oligomer; a similar molecular structure is suggested for the neuronal $\alpha_7$ 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$_3$Rs 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$_3$R 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$_3$R-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$_3$R sequences. Molecular cloning produced two chimeric receptors, E4 and C1, which consisted of the guinea pig amino terminus and the human carboxy-terminal domain (E4) or the human amino-terminal domain and the guinea pig carboxy terminus (C1), respectively. Both the E4 and C1 5-HT$_3$R s contained a human 5-HT$_3$R-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$_3$R 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 $\mu$M 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 carboxy-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$_3$R for all antagonists tested in comparison to its normal sensitivity for 5-HT
is caused by partially overlapping sites for agonist and antagonist binding.

Our data show that the 5-HT\textsubscript{3}Rs 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\textsubscript{3} agonists or antagonists with clinical relevance.

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