Cloning, Expression, and Pharmacology of Four Human 5-Hydroxytryptamine 4 Receptor Isoforms Produced by Alternative Splicing in the Carboxyl Terminus

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Cloning, Expression, and Pharmacology of Four Human 5-Hydroxytryptamine$_4$ Receptor Isoforms Produced by Alternative Splicing in the Carboxyl Terminus

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Abstract: We report here the molecular cloning of three new splice variants of the human serotonin 5-hydroxytryptamine$_4$ (h5-HT$_4$) receptor, which we named h5-HT$_{4(b)}$, h5-HT$_{4(c)}$, and h5-HT$_{4(d)}$. The sequence following the splicing site at Leu$_{358}$ in the C-terminal tail of h5-HT$_{4(b)}$ displays a 74% protein identity with the same region in the long form of the rat 5-HT$_4$ receptor (r5-HT$_{4L}$) but is shorter by 18 amino acids compared to its rat counterpart. The splice variants h5-HT$_{4(c)}$ and h5-HT$_{4(d)}$ are the first of their kind to be described in any animal species. The C terminus of h5-HT$_{4(c)}$ displays a high number of putative phosphorylation sites. The h5-HT$_{4(d)}$ isoform corresponds to an ultrashort form of the receptor, with a truncation two amino acids after the splicing site. Tissue distribution studies revealed some degree of specificity in the pattern of expression of the different isoforms within the human body. The four splice variants transiently expressed in COS-7 cells displayed an identical 5-HT$_4$ pharmacological profile and showed a similar ability to stimulate adenylyl cyclase activity in the presence of 5-HT. The stimulatory pattern of cyclic AMP formation in response to the 5-HT$_4$ agonist renzapride was found to be significantly different between h5-HT$_{4(a)}$ and the other h5-HT$_4$ isoforms, indicating that the splice variants may differ in the way they trigger the signal transduction cascade following receptor activation. **Key Words:** Human—Serotonin 5-HT$_4$ receptors—CNS—Heart—Gastrointestinal tract—Bladder.

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The neurotransmitter serotonin [5-hydroxytryptamine (5-HT)] is found in the CNS and periphery of vertebrates, where it serves various physiological roles mediated by distinct receptor subtypes (Saxena, 1995). The 5-HT$_4$ receptor is a member of the seven transmembrane-spanning (7TM) G protein-coupled family of receptors, which is positively coupled to adenylyl cyclase (for review, see Hegde and Eglen, 1996). 5-HT$_4$ receptors are expressed in a wide variety of tissues, including rodent and human brain (Eglen et al., 1995), rodent, pig, dog, and human gastrointestinal tract, and human and pig heart (Hegde and Eglen, 1996). In the mammalian brain, 5-HT$_4$ receptors contribute to the control of dopamine secretion (Bonhomme et al., 1995) and regulate learning and long-term memory through modulation of acetylcholine release (Marchetti-Gauthier et al., 1997). In peripheral tissues, 5-HT$_4$ receptors have been shown to regulate gastrointestinal tract motility, intestinal electrolyte secretion, adrenal corticosteroid secretion, bladder contraction, and atrial contractility (for review, see Hegde and Eglen, 1996).

5-HT$_4$ receptors are thought to be involved in various central and peripheral disorders, including cardiac arrhythmias (Kaumann, 1994) and neurodegenerative diseases (Reynolds et al., 1995; Wong et al., 1996). Moreover, the development of 5-HT$_4$ receptor agonists

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Abbreviations used: βARK, β-adrenergic receptor kinase; BIMU1, (*endo-N*-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide; cAMP, cyclic AMP; GR113808, [1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate; h5-HT$_4$, m5-HT$_4$, and r5-HT$_4$ receptor, human, mouse, and rat 5-HT$_4$ receptor, respectively; 5-HT, 5-hydroxytryptamine (serotonin); 5-MeOT, 5-methoxytryptamine; ML10302, 2-(1-piperidinyl)ethyl 4-amino-5-chloro-2-methoxybenzoate; ML10375, 2-(*cis*-3,5-dimethylpiperidino)ethyl 4-amino-5-chloro-2-methoxybenzoate; RACE, rapid amplification of cDNA ends; renzapride, (±)-*endo*-4-amino-5-chloro-2-methoxy-*N*-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride (BRL24924); 7TM, seven transmembrane-spanning; zacopride, 4-amino-5-chloro-2-methoxy-*N*-(1-azabicyclo[2.2.2]oct-3-yl)benzamide monohydrochloride.

and antagonists may have therapeutic applications in the CNS for the treatment of neuropsychiatry disorders related to central dopamine system dysfunction such as Parkinson's disease (Bonhomme et al., 1995) or the treatment of memory deficits as displayed by patients with Alzheimer's disease (Marchetti-Gauthier et al., 1997). Such drugs could also be useful in the treatment of peripheral disorders such as irritable bowel syndrome, gastroparesis, urinary incontinence, and cardiac arrhythmias (for review, see Hegde and Eglen, 1996).

5-HT_4 receptors have a unique pharmacology, which is clearly different from that of the other members of the 5-HT receptor family (Ford and Clarke, 1993). Most of the pharmacological and transductional studies on 5-HT_4 receptors have been performed in the rodent CNS and gastrointestinal tract and in human and pig heart (Eglen et al., 1995; Hegde and Eglen, 1996). Although the pharmacology of 5-HT_4 receptors present in these preparations is very similar, some unexplained differences exist. For instance, benzamides, such as renzapride (BRL 24924) {(±)-*endo*-4-amino-5-chloro-2-methoxy-*N*-(1-azabicyclo[3.3.1]non-4-yl)-benzamide monohydrochloride} and cisapride, behave as potent and full agonists of the 5-HT_4 receptors in mouse colliculi neurons but are less potent and only partial agonists in human heart (Ford and Clarke, 1993; Hoyer et al., 1994) and detrusor muscle isolated from human bladder (Ford and Clarke, 1993; Hoyer et al., 1994; Candura et al., 1996). Human detrusor muscle also showed an unusually low potency for the 5-HT_4 agonist 5-methoxytryptamine (5-MeOT) compared with other tissues (Candura et al., 1996). The 5-HT_4 receptor agonist ML10302 [2-(1-piperidinyl)ethyl 4-amino-5-chloro-2-methoxybenzoate], which mimics the effect of 5-HT on the relaxation of rat esophagus as well as on the electrically evoked contraction of the guinea pig ileum (Langlois et al., 1994), displayed a poor agonistic effect combined with a net antagonism to 5-HT on the cyclic AMP (cAMP) response generated by the human 5-$HT_{4(a)}$ [h5-$HT_{4(a)}$] receptor cloned from human atrium (Blondel et al., 1997) and an antagonist effect in colliculi neurons (Ansanay et al., 1996). Moreover, desensitization mechanisms for 5-HT_4 receptors are also tissue-dependent. Indeed, a rapid and full homologous (cAMP-independent) desensitization of 5-HT_4 receptors is observed in mouse colliculi neurons (Ansanay et al., 1996) and rat esophagus (Rondé et al., 1995), whereas this type of receptors desensitize to a lesser extent in human atrium (Kaumann et al., 1991).

Whether this variability in the regulation of 5-HT_4 receptor function is due to the existence of different 5-HT_4 receptor subtypes mediating the functional response to 5-HT in brain, gastrointestinal tract, and heart remains unknown. The first 5-HT_4 receptor was cloned from rat brain (Gerald et al., 1995), and two splice variants of the rat 5-HT_4 (r5-HT_4) receptor (r5-HT_{4L} and r5-HT_{4S}) have been identified. These variants differ in the length and sequence of their C termini. The long form (r5-HT_{4L}), which has also been cloned recently in mouse colliculi neurons (m5-HT_{4L}), has transcripts present in about every part of the brain (Claeysen et al., 1996). An interesting observation comes from the peripheral distribution of r5-HT_{4L} and r5-HT_{4S} transcripts in the rat. Although both forms are expressed in gastrointestinal tract (ileum and colon), only the r5-HT_{4S} transcript is found in the heart (Gerald et al., 1995). Moreover, r5-HT_{4S} was found to be located exclusively in the atrium (Gerald et al., 1995). More recently, we have reported the molecular cloning and functional characterization of a 5-HT_4 receptor cloned from human atrium, h5-$HT_{4(a)}$ (Blondel et al., 1997), that corresponds to the human counterpart of the rodent 5-HT_{4S} isoform (Gerald et al., 1995). Expression of this receptor was restricted to the brain, intestine, and atrium. h5-$HT_{4(a)}$ transiently expressed in COS-7 cells displayed a classical 5-HT_4 pharmacological profile. However, affinities of the cloned h5-$HT_{4(a)}$ receptor for agonists such as ML10302, BIMU1 {(*endo*)-*N*-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide}, renzapride, or zacopride {4-amino-5-chloro-2-methoxy-*N*-(1-azabicyclo[2.2.2]oct-3-yl)benzamide monohydrochloride} were lower than the ones found in brain. Moreover, the stimulatory patterns of cAMP formation by h5-$HT_{4(a)}$ in response to the 5-HT_4 agonists ML10302 and renzapride were very similar to the patterns of stimulation of L-type calcium current by 5-HT obtained in response to these two compounds in isolated human atrial myocytes (Blondel et al., 1997).

These previous studies led us to propose that the cardiac effects of 5-HT are mediated by the short splice variant, whereas the long form determines most of the neuronal effects of 5-HT. Modification of the C termini of receptors by alternative splicing can induce changes in the potency of various agonists and/or antagonists to bind to the receptor, as suggested by differences in binding affinity observed between the long and short form of the receptor (Gerald et al., 1995). Modifications of the C-terminal tails of 7TM receptors following alternative splicing have also been shown to determine G protein specificity and hence to affect signal transduction pathways in the 7TM receptor family (Namba et al., 1993; Spengler et al., 1993).

In the present study, we searched for alternative splice variants of the h5-HT_4 receptor in tissues other than the heart. Reverse-transcribed RNAs from human brain and intestine were pooled and used as templates for the amplification of h5-HT_4 cDNA ends. We report here the molecular cloning of three new splice variants of h5-HT_4, which we named h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$. Splice variants h5-$HT_{4(c)}$ and h5-$HT_{4(d)}$ are the first of their kind to be described in any animal species.

MATERIALS AND METHODS

Rapid amplification (RACE) and cloning of cDNA ends

The sequence of the recently characterized h5-$HT_{4(a)}$ receptor (Blondel et al., 1997) was used to synthesize the two

primers $HHT_4$5 (5′-CGGTGCTTATTTCCTGTAATG-3′) and HTS3 (5′-ATGGTCAACAAGCCCTAC-3′) corresponding to the beginning of the receptor sequence and the fifth transmembrane domain, respectively. To obtain cDNAs from the different 5′ ends generated by the h5-HT_4 gene, the anchored-RACE extension technique was applied as described (Newton and Graham, 1994). Fifty nanograms of total RNA from human brain and ileum was reverse-transcribed using an oligo(dT) primer containing two anchor sequences and Superscript reverse transcriptase (GibcoBRL). Reaction products from the two tissues were then pooled and used as template for a RACE reaction using primer $HHT_4$5 together with the first anchor. The product of this first PCR procedure was then used as template for a nested-RACE reaction using primer HTS3 (modified to include a *Hin*dIII restriction sequence) together with the second anchor (containing an *Eco*RI restriction sequence). Both PCR procedures were performed using 25 cycles of amplification and the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 54°C, and extension for 2 min at 72°C with the final extension for 8 min. The annealing temperatures were 52°C for the RACE reaction and 54°C for the nested-RACE reaction. DNA fragments were separated on a 1.5% agarose gel, cloned into pGEM-7Z (*Hin*dIII/*Eco*RI cut), and sequenced. The cDNAs characterized in this study were amplified from at least two independent PCR procedures, and all fragments were sequenced to detect and correct any potential mutations that could have been generated during the PCR amplification process.

Cloning of the full-length cDNAs

Total cDNA corresponding to the three new splice variants of the h5-HT_4 receptor characterized in this study [h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$] were amplified using the original pool of reverse transcription products from human brain and ileum and a nested-PCR strategy. Primer $HHT_4$5 (modified to include a *Hin*dIII restriction site) was used as forward primer in all PCR procedures. Reverse primers for the first round of amplification were F81 (5′-GCCTCAGGTGAAGAGAAT-3′), F61 (5′-TGGCATTAGGATGGTTTGGTCA-3′), and F71 (5′-GCAATAAGAATTGGCCAC-3′) for h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$, respectively. Reverse primers for the second round of amplification, all containing an *Eco*RI restriction site in their 5′ end, were F82 (5′-GTCTTCTGGGTC-ATTGTC-3′), F62 (5′-TTAGGATGGTTTGGTCA-3′), and F72 (5′-CTCAAGGAGCTCAAAATC-3′) for h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$, respectively. PCR conditions were the same as for the two rounds of RACE-PCR amplification. Fragments corresponding to the full-length cDNAs were purified on a 1.5% agarose gel, subcloned into pGEM-7Z (*Hin*dIII/*Eco*RI cut), and sequenced to confirm the integrity of the coding sequence.

Tissue localization studies

Total RNA were prepared from human peripheral tissues using the Trizol RNA purification system (GibcoBRL). Total RNA from human brain was from Clontech. cDNA was prepared from mRNA using oligo(dT) primers and Superscript reverse transcriptase (GibcoBRL). The cDNAs specific for the four isoforms of the 5-HT_4 receptor were detected using a nested PCR amplification. A first reaction was performed using 50 ng of cDNA together with the specific primer HHT45, designed to the 5′ end and common to all isoforms, and 3′-end primers HHT43D (5′-TGAATGCGAATGAATGGCTA-3′), F81, F61, and F71, specific for h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$, respectively. Products of this first reaction were used as templates for a nested PCR amplification with a primer corresponding to the central region common to all receptor subtypes, HTS5 (5′-GGCTGCTGGGTCATC-CCCAC-3′), and 3′-end primers HHT43C (5′-GTTGTG-AGCCATGTCCTCAATCA-3′), F82, F62, and F72, specific for h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$, respectively. The PCR products were electrophoresed on a 1.5% agarose gel and transferred to nitrocellulose membranes. Filters were hybridized with a ^{32}P-5′-end-labeled internal primer common to all splice variants, HTS4 (5′-CGAGAGCAGGCC-TCAGTCGG-3′), and washed under high stringency. To assess relative quantities of cDNA from different tissue sources, a single PCR amplification was performed using reverse and forward primers specific for the rat/human β-actin: 5′-CAC-CTTCTACAATGAGCTGCGTGTGGC-3′ and 5′-TGTTTG-CTGATCCACATCTGCTGGAAGGTGGA-3′, respectively. All PCR procedures in tissue localization studies were performed as follows: 25 cycles (1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C) and a final elongation (8 min at 72°C). The expected fragment sizes for these amplifications were 749, 764, 714, and 664 bp for h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$, respectively. PCR fragments amplified from the different human tissues were subcloned and sequenced to confirm identity with the cDNA sequences previously characterized in this study.

DNA transfection

The full coding region of the h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$ cDNAs were subcloned in the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA, U.S.A.). Transfections were performed using the vector polyethylenimine as described (Boussif et al., 1995). Cells were transfected using a mixture of DNA and polyethylenimine at a ratio of 20 nmol of polyethylenimine/mg of DNA in 0.9% NaCl. For radioligand binding assays, COS-7 cells were seeded 1 day before transfection into 1.5×10^4 mm^2 culture vials at a density of 1.5×10^7 cells per vial, incubated for 6 h with plasmid DNA (150 μg per vial), and harvested 48 h after transfection. For measurement of cAMP formation, COS-7 cells were seeded 1 day before transfection into 12-well plates at a density of 5×10^5 cells per well, incubated for 6 h with plasmid DNA (4–8 μg per well depending on experiments), and assayed 24 h after transfection. Cells transfected with the h5-HT_4 cDNA constructs were compared with mock-transfected cells that were only exposed to the crude pRC/CMV plasmid.

Membrane preparation

Each vial of cells for use in radioligand binding assays was washed twice with phosphate-buffered saline. Cells were scraped, collected, and centrifuged at 300 *g* for 5 min. The pellet was resuspended in 2.5 ml of ice-cold HEPES buffer (50 m*M*, pH 7.4) and homogenized by an Ultraturax tissue grinder. The lysate was subsequently centrifuged at 40,000 *g* for 20 min at 4°C. The resulting pellet was resuspended in 15 volumes of HEPES buffer (50 m*M*, pH 7.4). Membrane preparations were kept on ice and used within 2 h for the radioligand binding assays. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Radioligand binding assays

Radioligand binding studies were performed in 500 μl of buffer (50 m*M* HEPES, pH 7.4); 20 μl of competing agent

(for drug competition studies), ML10375 [2-(*cis*-3,5-dimethylpiperidino)ethyl 4-amino-5-chloro-2-methoxybenzoate] to give a final concentration of 10 μM (for determination of nonspecific binding), or buffer (for determination of total binding); 20 μl of ^{3}H-labeled GR113808 {[1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate} to give a final concentration of 50% of K_D values; and 50 μl (100–200 μg) of membrane preparation. Saturation studies were conducted using [^{3}H]-GR113808 at nine different concentrations ranging from 0.01 to 3.5 nM. Tubes were incubated at 25°C for 30 min. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filter paper using the Brandel model 48R cell harvester. Filters were presoaked in a solution of polyethylenimine (0.1%) to reduce binding to filters. Filters were subsequently washed with ice-cold buffer (50 mM Tris-HCl, pH 7.4) and placed overnight in 4 ml of Ready-Safe scintillation cocktail (Beckman, Fullerton, CA, U.S.A.). Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data were analyzed by computer-assisted nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA).

Measurement of cAMP formation

For measurement of intracellular cAMP accumulation, transiently transfected COS-7 cells were incubated 24 h after transfection in Dulbecco's modified Eagle's medium containing 5 mM theophylline, 10 mM HEPES, and 10 μM pargyline for 15 min at 37°C in 5% CO_2. 5-HT (1 μM), other serotoninergic agents (1 μM), or forskolin (10 μM) was added and incubated for an additional 15 min at 37°C in 5% CO_2. The reaction was stopped by aspiration of the medium and addition of 500 μl of ice-cold ethanol. After 1 h at room temperature, the ethanol fraction was collected and lyophilized. The pellet was reconstituted, and cAMP was quantified using a radioimmunoassay (E.R.I.A. Diagnostics Pasteur radioimmunoassay kit 79830). Student's t tests were performed using the QuickTTest software.

Materials

Polyethylenimine (MW 800,000) was from Fluka (L'Isle d'Abeau Chesnes, France). Dulbecco's modified Eagle's medium was obtained from GibcoBRL. Pertussis toxin was from Calbiochem. ML10302 and ML10375 were synthesized as recently described (Langlois et al., 1994; Yang et al., 1997). BIMU1 and zacopride were synthesized in our laboratory. GR113808 was a generous gift from Glaxo Research Group (Ware, Hertfordshire, U.K.), and [^{3}H]-GR113808 was from Amersham (Arlington Heights, IL, U.S.A.). Renzapride was a generous gift of SmithKline Beecham (U.K.). 5-HT and 5-MeOT were from Aldrich (L'Isle d'Abeau Chesnes), and all other drugs were from Sigma (L'Isle d'Abeau Chesnes). PCR was performed on a GeneAmp model 2400 apparatus (Perkin Elmer). HiTaq DNA polymerase and related reaction buffer were from Bioprobe Systems. In all PCR procedures, deoxynucleotide triphosphates and specific primers were at a final concentration of 200 and 1 μM, respectively. Doubled-stranded DNA was sequenced with a T7 DNA polymerase sequencing kit (Pharmacia) according to the manufacturer's instructions.

RESULTS

Primary structure of 5-HT$_4$ receptor splice variants

Four DNA fragments were isolated when human brain and ileum cDNA were pooled and used as templates in a nested RACE-PCR amplification using oligonucleotide primers derived from the 5′ end and central region of the h5-HT$_{4(a)}$ receptor subtype (see Materials and Methods). The nucleotide sequences of all the amplified fragments were found to be identical up to the codon coding for Leu^{358} (for the complete amino acid sequence of the common region, see Blondel et al., 1997), but four alternative coding sequences could be identified following this site. The deduced amino acid sequences of these different splice variants are shown in Fig. 1, starting from the splicing site at Leu^{358}. Within this region of the receptor, h5-HT$_{4(a)}$ shares 93% protein identity with the short form of the r5-HT$_4$ receptor (r5-HT$_{4S}$), as previously reported (Blondel et al., 1997). The region between Leu^{358} and the last amino acid of h5-HT$_{4(b)}$ displays 74% protein identity with the corresponding region in the long form of the r5-HT$_4$ receptor, r5-HT$_{4L}$. However, the carboxyl terminus of h5-HT$_{4(b)}$ was found to be 18 amino acids shorter than its rat counterpart and lacked the consensus site for protein kinase C phosphorylation described in the C terminus of the r5-HT$_{4L}$ receptor (Gerald et al., 1995). The h5-HT$_{4(c)}$ and h5-HT$_{4(d)}$ splice variants have not been described in any species to date. It is interesting that the carboxyl terminus of h5-HT$_{4(c)}$ displays an unusually high number of putative phosphorylation sites: two casein kinase II sites, a protein kinase C site, and a consensus sequence for protein kinase A/protein kinase G phosphorylation. The h5-HT$_{4(d)}$ isoform corresponds to an ultrashort form of the receptor, with a truncation of the carboxyl terminus only two amino acids after the splicing site on Leu^{358}.

Tissue-specific expression of h5-HT$_4$ receptor splice variants

Expression of the different h5-HT$_4$ transcripts was analyzed by amplification of cDNA derived from RNA isolated from various human tissues using a nested RT-PCR technique. We were able to examine the tissue distribution by using two pairs of primers specific for each type of variants and two successive rounds of PCR amplification. The amplified products were identified using a specific internal oligonucleotide probe (Fig. 2). The h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, and h5-HT$_{4(c)}$ isoforms were all expressed in atrium, brain, and intestine. It is interesting that bladder and kidney each expressed detectable levels of only one receptor subtype [h5-HT$_{4(a)}$ and h5-HT$_{4(b)}$, respectively]. Expression of h5-HT$_{4(d)}$ was only detected in the intestine. Finally, ventricle and lung did not express detectable amounts of any of the four h5-HT$_4$ isoforms. We also demonstrated the presence in all tissues of cDNA corresponding to the constitutively expressed β-actin gene, as well as the absence of β-actin PCR products in control experiments without reverse transcriptase (Fig. 2).

Pharmacological characterization of h5-HT$_4$ receptor splice variants

The cDNAs encoding the four h5-HT$_4$ receptor isoforms were transiently expressed in COS-7 cells for

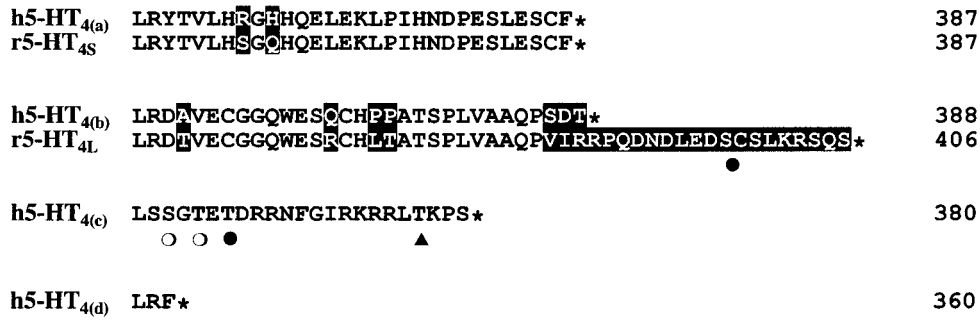

FIG. 1. Deduced C-terminal amino acid sequences of the 5-HT_4 receptor splice variants in the rat and the human. In both species, the sequences diverge after Leu^{358}. Changes in human amino acids versus rat are shaded in dark gray. Open circles correspond to a protein kinase C consensus site, black circles to casein kinase II consensus sites, and the black triangle to a protein kinase A/protein kinase G consensus site. The asterisk corresponds to the terminal stop codon. The EMBL accession numbers for h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$ are Y08756, Y12505, Y12506, and Y12507, respectively.

pharmacological evaluation. Saturation analysis using [^{3}H]GR113808 revealed single saturable sites of high affinities for the four splice variants of the receptor (Fig. 3). Similar K_D values for GR113808 were found for the four isoforms: h5-$HT_{4(a)}$, 0.23 ± 0.06; h5-$HT_{4(b)}$, 0.62 ± 0.05; h5-$HT_{4(c)}$, 0.30 ± 0.08; and h5-$HT_{4(d)}$, 0.14 ± 0.05 nM. However, the density of receptors transiently expressed in COS-7 cells (B_{max}) varied greatly in transfection experiments: h5-$HT_{4(a)}$, 214 ± 10; h5-$HT_{4(b)}$, 1,411 ± 55; h5-$HT_{4(c)}$, 77.5 ± 5.8; and h5-$HT_{4(d)}$, 31.4 ± 2.8 fmol/mg of protein. Nonspecific binding increased linearly with increasing ligand concentration (Fig. 3). A range of 5-HT_4 receptor agonists and antagonists completely inhibited the specific binding of [^{3}H]GR113808 to all the cloned h5-HT_4 receptor isoforms (Fig. 4). All the displacement curves were monophasic, giving a Hill coefficient of 0.6–1.1. The data summarized in Table 1 demonstrate that the pharmacological profiles of all the cloned h5-HT_4 receptor isoforms, in terms of rank order of potencies of the ligands tested, are very similar to the one found for 5-HT_4 receptors as studied in situ [human (Kaumann et al., 1996) and piglet (Kaumann et al., 1995) atria, human striatum (Reynolds et al., 1995) and caudate (Waeber et al., 1993), rat (Langlois et al., 1994; Yang et al., 1997) and guinea pig (Ansanay et al., 1996) striatum, and mouse colliculi (Ansanay et al., 1996)] or after expression of cloned isoforms in cultured fibroblasts [r5-HT_{4L} (Gerald et al., 1995; Adham et al., 1996) and m5-HT_{4L} (Claeysen et al., 1996)].

Stimulation of cAMP production by h5-HT_4 receptor splice variants

To examine and compare the ability of cells expressing h5-HT_4 receptor splice variants to couple to adenylyl cyclase, cAMP synthesis was assayed in COS-7 cells transiently transfected with h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$ cDNAs (Fig. 5). Basal cAMP values were not significantly different in mock-transfected cells and cells expressing h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, and h5-$HT_{4(d)}$ receptors, indicating that these

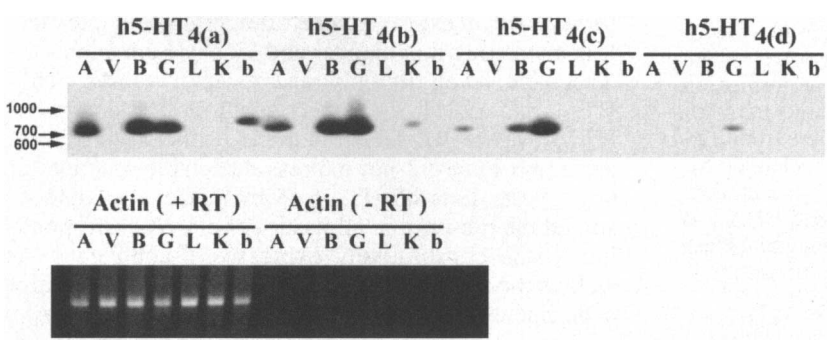

FIG. 2. Reverse transcription-PCR analysis performed with 50 ng of mRNA from various human tissues. The PCR products were separated on a 1.5% agarose gel and analyzed by Southern blotting using a ^{32}P-5′-end-labeled internal primer common to the h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$ isoforms. An 8-h exposure of the autoradiogram is shown. A positive control was performed using rat actin primers on mRNA samples treated with (+RT) or without (−RT) reverse transcriptase. The PCR products for this control experiment were analyzed on a 1.5% agarose gel, and a photograph of the ethidium bromide-stained gel is shown. PCR primers used for this analysis and expected length of the amplified products are described in Materials and Methods. Positions of three molecular weight markers are indicated in bp. A, atrium; V, ventricle; B, brain; G, gut; L, liver; K, kidney; b, bladder.

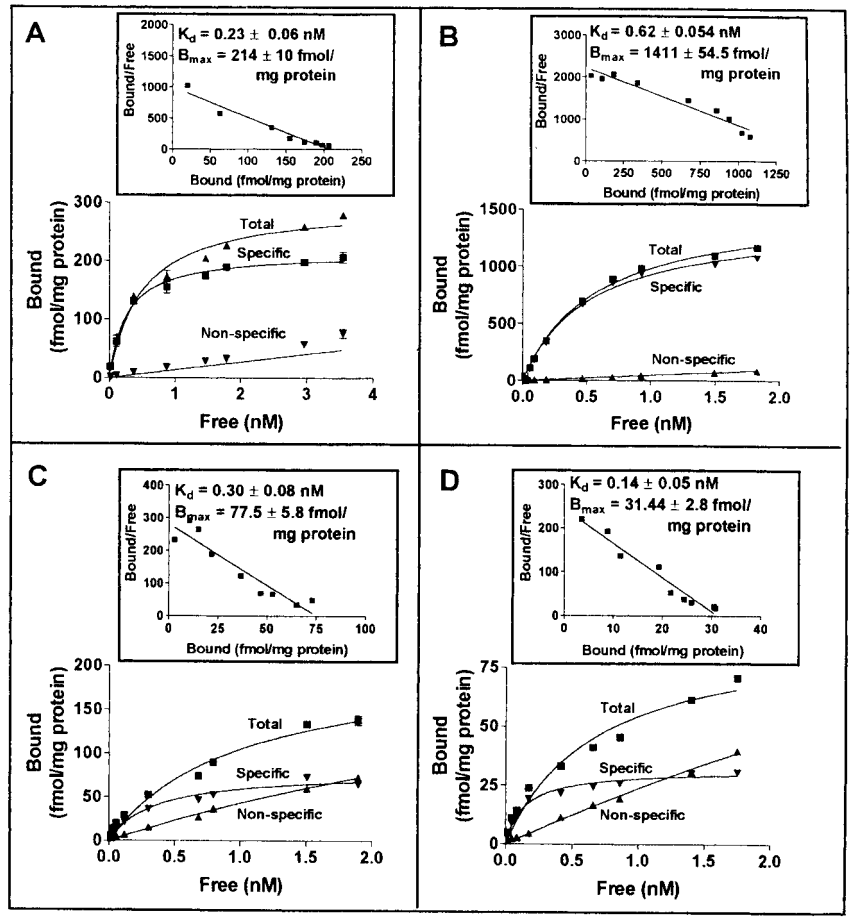

FIG. 3. Saturation analysis of [^{3}H]-GR113808 binding on membrane preparations from cells expressing h5-$HT_{4(a)}$ (**A**), h5-$HT_{4(b)}$ (**B**), h5-$HT_{4(c)}$ (**C**), and h5-$HT_{4(d)}$ (**D**) receptor isoforms. Membranes harvested from transiently transfected COS-7 cells were incubated with eight concentrations of [^{3}H]GR113808 (0.02–3.5 n*M*) for 30 min at 25°C. Nonspecific binding was defined by 10 μM ML10375. Results are from single experiments but are representative of three such experiments. K_D and B_{max} values were determined by computer-assisted nonlinear regression analysis (Prism; GraphPad).

expressed isoforms of the receptor had no intrinsic activity on cAMP formation in transiently transfected cells in the absence of agonists (Fig. 5A). However, transient expression of the h5-$HT_{4(c)}$ isoform resulted in a significant increase in basal adenylyl cyclase activity in the absence of 5-HT_4 agonists (Fig. 5A). This last result indicates that expression of the h5-$HT_{4(c)}$ splice variant in our system generated a spontaneously active receptor state. 5-HT (1 μM) had no effect on basal adenylyl cyclase activity in mock-transfected COS-7 cells (Fig. 5A), indicating that endogenous adenylyl cyclase-coupled 5-HT receptors are not present in these cells. In cells expressing the h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$ isoforms, addition of 5-HT (1 μM) significantly increased basal cAMP concentration by 82, 85, 64, and 77%, respectively (Fig. 5A). Forskolin (10 μM), a direct activator of adenylyl cyclase, induced similar increases in cAMP concentrations in cells expressing h5-HT_4 receptor isoforms and in mock-transfected COS-7 cells (Fig. 5A), indicating that the potential for maximal activation of adenylyl cyclase was not impaired in cells expressing h5-HT_4 receptors. The 5-HT_4 receptor agonists ML10302 and renzapride had no significant effect on basal cAMP levels in mock-transfected COS-7 cells (Fig. 5B). In cells expressing h5-HT_4 receptor isoforms, ML10302 behaved as a poor 5-HT_4 agonist and displayed only 28–34% of the 5-HT stimulatory effect (Fig. 5C), despite a high affinity for the receptor (Table 1). Furthermore, preincubation of cells with 1 μM ML10302 before addition of 5-HT (1 μM) antagonized significantly the ability of 5-HT to increase basal cAMP levels (Fig. 5C). As previously reported

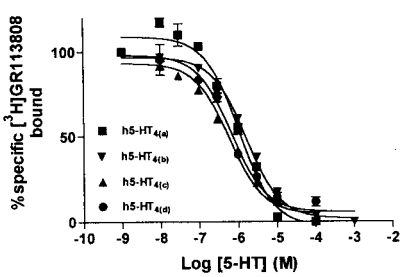

FIG. 4. Inhibition of specific [^{3}H]GR113808 binding to cloned h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$ receptor isoforms by 5-HT. Membranes from transiently transfected COS-7 cells were incubated with a concentration of [^{3}H]GR113808 equal to 50% of the K_D value for each receptor isoform. Nonspecific binding was defined by 10 μM ML10375. Results are presented as a percentage of specific binding in the absence of 5-HT. Data were analyzed by computer-assisted nonlinear regression analysis (Prism; GraphPad).

TABLE 1. *Comparison of the binding affinities of various 5-HT$_4$ receptor agonists and antagonists on cloned h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, h5-HT$_{4(c)}$, and h5-HT$_{4(d)}$ receptors*

	K_i (nM)			
	5-HT$_{4(a)}$	5-HT$_{4(b)}$	5-HT$_{4(c)}$	5-HT$_{4(d)}$
5-HT	772 ± 278	1,151 ± 170	481.9 ± 112	330 ± 113
5-MeOT	2,080 ± 508	2,443 ± 510	1,020 ± 236	553 ± 149
ML10302	8.4 ± 1.5	10.72 ± 2.9	7.98 ± 2.77	3.69 ± 1.25
BIMU1	373 ± 119	123.4 ± 9.0	66 ± 8.37	38.85 ± 12
Renzapride	635 ± 148	1,179 ± 142	636 ± 91	173 ± 40
Zacopride	7,750 ± 1,615	11,490 ± 950	10,630 ± 2,160	3,599 ± 1,835
GR113808	0.33 ± 0.033	0.53 ± 0.10	0.41 ± 0.13	0.078 ± 0.24
ML10375	0.56 ± 0.11	1.62 ± 0.82	0.61 ± 0.08	0.41 ± 0.10

Experiments correspond to competition of the various compounds for [^{3}H]GR113808 binding to membranes of transiently transfected COS-7 cells. For each h5-HT$_4$ receptor isoform, the concentration of [^{3}H]GR113808 was adjusted to 50% of the K_D value. Affinity estimates are given as K_i values in nM and were determined from IC_{50} values obtained by computer-assisted nonlinear curve analysis (Prism; GraphPad). K_i values are representative of at least two determinations. Rank order of potency of the drugs tested here was identical for the four h5-HT$_4$ receptor isoforms and was GR113808 > ML10375 > ML10302 > BIMU1 > renzapride = 5-HT > 5-MeOT > zacopride.

(Blondel et al., 1997), renzapride also behaved as a poor 5-HT$_4$ agonist in cells expressing the h5-HT$_{4(a)}$ receptor isoform and increased cAMP formation by only 56% in these cells (Fig. 5D), despite an affinity for the h5-HT$_{4(a)}$ receptor similar to that of 5-HT (Table 1). However, renzapride behaved as a full agonist in cells expressing the h5-HT$_{4(b)}$, h5-HT$_{4(c)}$, and h5-HT$_{4(d)}$ isoforms, as the renzapride-induced cAMP formation mediated by these receptor splice variants was not significantly different from the 5-HT-induced cAMP formation (Fig. 5D).

DISCUSSION

The molecular structure and functional characterization of four splice variants of the human 5-HT$_4$ receptor are described and compared in this study. The splice variants have been named h5-HT$_{4(a)}$ (already charac-

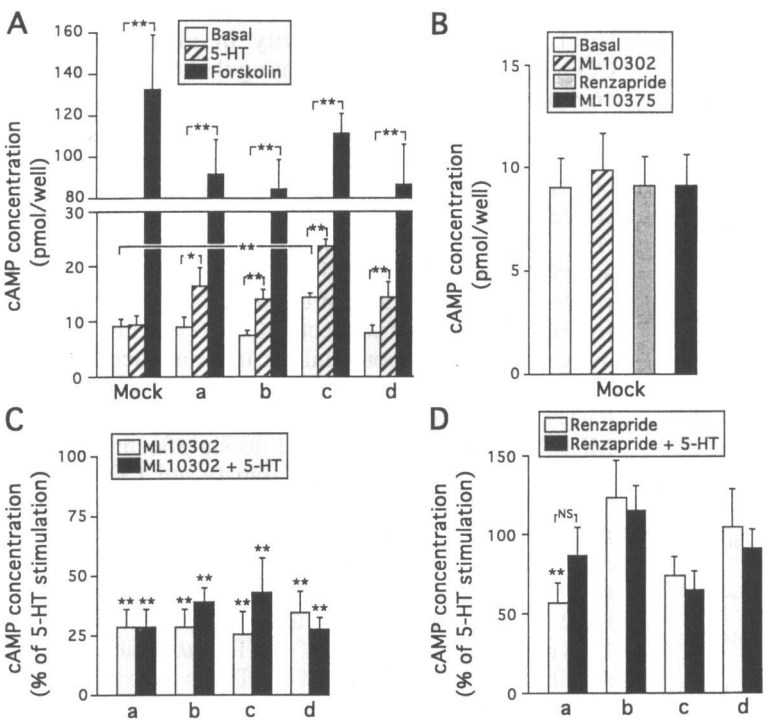

FIG. 5. cAMP responses to various 5-HT$_4$ receptor agonists and antagonist using h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, h5-HT$_{4(c)}$, and h5-HT$_{4(d)}$ receptors transiently expressed in COS-7 cells or mock-transfected cells as control. Cells were preincubated with 5 mM theophylline and 10 μM pargyline for 15 min and then incubated with 1 μM agonist—5-HT (**A, C,** and **D**), ML10302 (**B** and C), or renzapride (B and D) or 10 μM forskolin (A) for 15 min. The effect of agonist on 5-HT-induced cAMP accumulation was assayed by addition of the agonist during the 15-min preincubation period, followed by addition of 5-HT for 15 min. Data are mean ± SEM (bars) values of seven to 12 experiments. NS, not significant. $^*p < 0.05$, $^{**}p < 0.01$ versus indicated values by t test.

terized by Blondel et al., 1997), h5-$HT_{4(b)}$, h5-$HT_{4(c)}$, and h5-$HT_{4(d)}$, respectively, in accordance with the nomenclature recently proposed for 5-HT receptor classification (Hoyer and Martin, 1997). The four variants are generated by splicing events that occur in the C terminus of the h5-HT_4 receptor, just after the amino acid Leu^{358}. This alternative splicing site has already been described in the r5-HT_4 receptor, where it generates a short (r5-HT_{4S}) and long (r5-HT_{4L}) form of the receptor (Gerald et al., 1995). Long (m5-HT_{4L}) and short (m5-HT_{4S}) forms of the receptor generated from the same splicing site have also been described in the mouse (Claeysen et al., 1996). Moreover, four C-terminus splice variants of 5-HT_7, another 5-HT receptor positively coupled to adenylyl cyclase, have also been described (Heidmann et al., 1997). Sequence homology analysis suggests that h5-$HT_{4(a)}$ and h5-$HT_{4(b)}$ are the human counterparts of the r5-HT_{4S} and r5-HT_{4L} isoforms, respectively, whereas h5-$HT_{4(c)}$ and h5-$HT_{4(d)}$ represent two newly described isoforms of the receptor.

h5-$HT_{4(c)}$ displayed a strikingly high number of putative phosphorylation sites (one for protein kinase C, one for protein kinase A/protein kinase G, and two for casein kinase II), all contained within the last 25 residues of its amino acid sequence. Coexpression studies of h5-$HT_{4(c)}$ with the putative regulatory kinases, together with in vitro receptor phosphorylation studies, will be needed in the future to determine if these regulatory sites are indeed functional in the h5-HT_4 receptor. It is interesting that an alternative splice variant of the human 5-HT_7 receptor, 5-HT_{7d}, also contains phosphorylation sites in its C terminus, one for protein kinase C and one for casein kinase II, whereas the three other 5-HT_7 splice variants do not show any known consensus phosphorylation sites in their C terminus (Heidmann et al., 1997). Phosphorylation in the C terminus of 7TM receptors by protein kinase A has been shown to regulate receptor desensitization and receptor–G protein association in response to increased cAMP concentrations (for review, see Dohlman et al., 1991). Moreover, receptor phosphorylation by non–cAMP-dependent protein kinases, such as the β-adrenergic receptor kinases (βARK1 and βARK2) or the rhodopsin kinase, allows substrate-activated homologous desensitization in 7TM receptors such as the β_1- and β_2-adrenergic receptors (Pei et al., 1994; Freedman et al., 1995) and the 5-HT_{2C} receptor (Westphal et al., 1995). The number and the nature of the phosphorylation sites present in the C terminus of 5-HT_4 receptor splice variants are therefore likely to influence the negative regulation of the receptor function. Tissue-dependent differences in the desensitization mechanisms of 5-HT_4 receptors have been reported (for review, see Ford and Clarke, 1993) and could be related to the restricted patterns of expression of 5-HT_4 receptor isoforms in the different tissues (see below). For example, the mechanism of desensitization of 5-HT_4 receptors in mouse colliculi neurons resembles the one described for β-adrenergic receptors and appears to be independent of the cyclic AMP pathways. It has been proposed that a "βARK-like" kinase mediates specific phosphorylation of the 5-HT_4 receptor in these neuronal cells (Ford and Clarke, 1993). If the h5-$HT_{4(c)}$ receptor isoform was found to mediate most of the 5-HT stimulation in neurons like colliculi, C-terminus phosphorylation by either casein kinase II or protein kinase C could account for the cAMP-independent desensitization observed in these cells.

h5-$HT_{4(d)}$ is characterized by a very short C-terminal end, with a coding sequence ending just two amino acids after Leu^{358}. This represents another striking similarity with the 5-HT_7 receptor family, as the protein sequence of one of the 5-HT_7 receptor splice variants, 5-HT_{7b}, also ends two amino acids after the splicing site (Heidmann et al., 1997). Structure similarities between the C termini of 5-HT_4 and 5-HT_7 receptor splice variants (same number of variants, including one isoform rich in phosphorylation sites and one isoform truncated near the splicing site), despite the absence of any clear sequence homology, suggest similarities in the regulation of functional activity among the 5-HT receptors positively coupled to adenylyl cyclase.

The expression pattern of each h5-HT_4 splice variant was not restricted to any given tissue, except for h5-$HT_{4(d)}$, which was found only in intestine. Some tissues (heart atrium, brain, and intestine) expressed three or four different h5-HT_4 isoforms, whereas others (bladder and kidney) expressed only one isoform. In two tissues (heart ventricle and liver), no transcript encoding any of the h5-HT_4 splice variants could be amplified. The physiological relevance and the possible pathological implications of these restricted patterns of expression remain to be determined. For example, the unusually low potency of 5-MeOT in the human detrusor muscle has been proposed as evidence for the existence of a specific form of 5-HT_4 receptor in the bladder, which cannot be easily distinguished pharmacologically from the other members of the family using conventional 5-HT_4 agonists or antagonists (Candura et al., 1996). According to our tissue distribution studies, this specific isoform could well be h5-$HT_{4(a)}$. Functional studies leading to a further characterization of the 5-MeOT-induced cAMP production in cells expressing the h5-$HT_{4(a)}$ receptor will be necessary to test this hypothesis.

All h5-HT_4 receptor subtypes expressed in COS-7 cells displayed a classical 5-HT_4 receptor profile in terms of rank order of potency of the various serotoninergic ligands tested (Hoyer et al., 1994). Besides, no major differences could be found among the receptor isoforms as far as the affinity constants of 5-HT_4 agonists and antagonists were concerned. However, important differences were found in the total number of binding sites (B_{max}), indicating variations in the levels of functional receptors expressed following different transfection experiments. Because the plasmid con-

structs and the noncoding sequences present in the 5′ regions were identical for all the expressed receptor isoforms, we attribute this variability to differences in transfection efficacy between experiments rather than to intrinsic properties of each receptor isoform. Changeable transfection efficacy is also likely to explain the relatively high variability observed in cAMP synthesis assays (see below).

Although all h5-HT_4 receptor subtypes expressed in COS-7 cells displayed a similar ability to couple to adenylyl cyclase when exposed to 5-HT, expression of the h5-$HT_{4(c)}$ isoform gave a constitutive activation of adenylyl cyclase that resulted in an increased basal cAMP level. The degree of constitutive coupling was further increased by overexpressing the h5-$HT_{4(c)}$ receptor isoform using more plasmid DNA for transcription (data not shown). Such constitutive coupling has been described in several other situations, e.g., (a) in the metabotropic glutamate receptor family, some splice variants are spontaneously coupled to G proteins (Prezeau et al., 1996); (b) specific pathological or experimental mutations lead to a constitutive activity in some receptors (Coughlin, 1994); and (c) overexpression of 7TM receptors (for review, see Kenakin, 1996) such as the m5-HT_{4L} receptor (Claeysen et al., 1996) leads to a constitutive activation of G_s. Although in our hands the h5-$HT_{4(c)}$ isoform was always spontaneously coupled to adenylyl cyclase, we also obtained constitutive coupling of the h5-$HT_{4(a)}$, h5-$HT_{4(b)}$, and h5-$HT_{4(d)}$ receptors to adenylyl cyclase when overexpressing these isoforms (data not shown).

Some interesting features were found with the effects of the 5-HT_4 agonists ML10302 and renzapride. (a) ML10302 behaved like a partial agonist on all h5-HT_4 receptor subtypes, with a stimulatory effect that was only 25–34% of the corresponding 5-HT-induced cAMP synthesis and an inhibitory effect on the 5-HT stimulation of adenylyl cyclase. Thus, the differences found in the literature on the physiological effects of ML10302, which acts as a full agonist in rat esophagus and guinea pig ileum (Langlois et al., 1994) and as a partial agonist in human atrium (Blondel et al., 1997), cannot be explained solely by structural differences in the 5-HT_4 receptor C termini. (b) Renzapride was as potent as 5-HT in activating adenylyl cyclase in cells expressing the h5-$HT_{4(b)}$ and h5-$HT_{4(d)}$ receptor isoform. On the h5-$HT_{4(c)}$ isoform, renzapride produced only 72% of the effect of 5-HT, but this difference was not statistically significant. In contrast, renzapride produced a significantly lower stimulation of cAMP synthesis as compared with 5-HT in cells expressing the h5-$HT_{4(a)}$ receptor. It is interesting that renzapride behaves as a potent and full agonist of the 5-HT_4 receptors in mouse colliculi neurons and is less potent and only a partial agonist (compared with 5-HT) in human heart (Ford and Clarke, 1993; Hoyer et al., 1994).

Comparison of recombinant expression experiments with in vivo physiological studies may help to understand tissue-dependent specificities in 5-HT_4-mediated signal transduction. For example, differences observed between 5-HT_4 receptors from colliculi neurons (full response to renzapride and full homologous desensitization) and cardiac cells (weak response to renzapride and partial desensitization) may well be explained by the functional dominance of a given receptor subtype in these tissues [in the present case, h5-$HT_{4(c)}$ and h5-$HT_{4(a)}$, respectively]. However, detection of further functional differences among the four h5-HT_4 isoforms may be limited by our expression system. For example, reliable dose–response studies on the regulation of adenylyl cyclase activity by 5-HT_4 agonists and antagonists could not be achieved using our transient transfection protocol owing to the variability in transfection efficiency and the poor stimulatory effects following expression of functional exogenous receptors in only a portion of the total cell population. Stable expression of the four h5-HT_4 receptor isoforms will be needed in the future to assess potential differences in receptor–ligand interactions and receptor–G protein coupling. Production of such stable cell lines is in progress.

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