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Molecular and functional characterization of a 5-HT_4 receptor cloned from human atrium

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Abstract 5-Hydroxytryptamine (5-HT) has been shown to exert positive inotropic, chronotropic, and lusitropic effects and to stimulate the L-type calcium channel current (I_{Ca}) in human atrial tissue through activation of the pharmacologically defined 5-HT_4 receptor subtype. However, the molecular nature of the receptor(s) involved in these effects is still unknown. In the present study, we report the molecular nature of a 5-HT_4 receptor cloned from human atrium, h5-HT_{4A}. Sequence analysis reveals that h5-HT_{4A} displays a 93% protein identity with the short form of the 5-HT_4 receptor recently isolated from rat brain. h5-HT_{4A} mRNA is expressed in human atrium but not ventricle, and is also found in brain and GI tract. h5-HT_{4A} transiently expressed in COS-7 cells displays a classical 5-HT_4 pharmacological profile. However, affinities of the h5-HT_{4A} receptor for agonists such as ML10302, BIMU1, renzapride or zacopride were 4–10-fold lower than the ones found in brain. Moreover, the stimulatory patterns of cAMP formation by h5-HT_{4A} in response to the 5-HT_4 agonists ML10302 and renzapride were very similar to the patterns of stimulation of I_{Ca} obtained in response to these compounds in human atrial myocytes. We conclude that h5-HT_{4A} likely mediates the effects of 5-HT in human atrium and may differ from 5-HT_4 receptor isoforms present in the brain and GI tract.

Key words: Human heart; Serotonin receptor; 5-HT_4 receptor; Calcium channel current; Human cardiac myocyte; 5-HT_4 receptor agonist; 5-HT_4 receptor antagonist

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) exerts strong positive chronotropic, inotropic and lusitropic effects in human and pig atrium [1,2]. These effects are due to (i) 5-HT binding to the pharmacologically defined 5-HT_4 receptor [3,4], leading to (ii) stimulation of adenylyl cyclase activity, (iii) activation of cAMP-dependent protein kinase and (iv) phosphorylation of several key proteins involved in the excitation–contraction coupling. One of these proteins is the L-type Ca^{2+} channel [3,5]. Indeed, in isolated cells from human right atrial appendage, 5-HT induces a stimulation of the cardiac L-type Ca^{2+} current (I_{Ca}) [3] similar to the effect of β-adrenergic agonists such as isoprenaline [5]. However, unlike β-adrenergic agonists, 5-HT exerts its effects exclusively on the atrial tissue and has no functional effects on ventricles [1,6]. Moreover, while the cardiac effects of β-adrenergic agonists are universally present in the animal kingdom, the cardiac effects of 5-HT are restricted to only few mammalian species (human, pig, monkey), and are absent in a large number of laboratory animals, such as rat, guinea pig, rabbit and frog [2,3]. In human heart, the unique location of 5-HT_4 receptors in the atrium, together with its stimulatory effect on I_{Ca}, makes it reasonable to assume that 5-HT_4 receptors may participate in the control of cardiac rhythmic and/or arrhythmic activity [7]. Endogenous serotonin, which is released from platelets in human heart, may contribute to the occurrence of atrial fibrillation and stroke [7]. These possible pathophysiological implications of human cardiac 5-HT_4 receptors make it urgent to define the molecular nature of the receptor(s) involved and to synthesize appropriate ligands which would bind selectively to these receptors.

However, to our knowledge, the molecular identity of the human cardiac 5-HT_4 receptor(s) remains unknown. Although initial physiological experiments suggested that these receptors were similar to the 5-HT_4 receptor subtype that has been pharmacologically characterized in primary cultures of fetal mouse colliculi neurons, a number of results suggest that the human atrial 5-HT_4 receptor differs from the rodent neuronal 5-HT_4 receptor [2,4,8]. The main differences concern the sensitivities of cardiac and neuronal 5-HT_4 receptors to benzamides, such as renzapride and cisapride. While these compounds behave as potent and full agonists of the 5-HT_4 receptors in mouse colliculi neurons, they are less potent and only partial agonists (compared to 5-HT) in human heart [4,8]. Other differences exist between the functional response and sensitivities to 5-HT_4 receptor agonists and antagonists in brain and GI tract [4,8]. Whether these differences are due to species differences of the same receptor or to different 5-HT_4 receptor subtypes mediating the functional response to 5-HT in brain, GI tract and heart remains unknown. A 5-HT_4 receptor was recently cloned from rat brain [9] and two splice variants (r5-HT_{4L} and r5-HT_{4S})

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 5-MeOT, 5-methoxytryptamine; BIMU1, endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide; GI, gastrointestinal tract; GR113808, [1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate; I_{Ca}, L-type calcium current; ML10302, 2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride; ML10375, 2-(*cis*-3,5-dimethylpiperidino)ethyl 4-amino-5-chloro-2-methoxybenzoate; PEI, polyethylenimine; renzapride, (±)-endo-4-amino-5-chloro-2-methoxy-*N*-(1-azabicyclo[3.3.1]non-4-yl)benzamide monhydrochloride (BRL 24924); zacopride, 4-amino-5-chloro-2-methoxy -*N*-(1-azabicyclo[2.2.2]oct-3-yl)benzamide monhydrochloride

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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}, [10]), has transcripts present in about every part of the brain. However, according to Gerald et al. [9], the short form (r5-HT_{4S}) is only present in the striatum while, according to Claeysen et al. [10], both splice variants are ubiquitously present in every part of the mouse and rat brain. An interesting observation comes from the peripheral distribution of r5-HT_{4L} and r5-HT_{4S} transcripts in the rat. While both forms are expressed in GI tract (ileum and colon), only the r5-HT_{4S} transcript is found in the heart [9]. Moreover, r5-HT_{4S} was found to be located exclusively in the atrium [9]. Thus, it is tempting to hypothesize that the cardiac effects of 5-HT are mediated by the short splice variant, while the long form may determine most of the neuronal effects of 5-HT. This hypothesis is supported by the identity between the pharmacological profiles of the cloned mouse m5-HT_{4L} receptor and the native 5-HT_4 receptor in mouse colliculi neurons [10]. However, a serious drawback in this hypothesis is that although 5-HT_4 receptor transcripts are present in rat atrium, no cardiac effect of 5-HT related to 5-HT_4 receptors has been reported to date in this animal species [3].

These observations prompted us to characterize the molecular nature of the 5-HT_4 receptors expressed in the human heart where their functional role has been well documented. We report here the molecular nature of a 5-HT_4 receptor cloned from human atrium. This receptor is the first receptor of this kind characterized in human, and we therefore named it h5-HT_{4A}. h5-HT_{4A} is expressed in atrium but not in ventricle. h5-HT_{4A} displays a 93% protein identity with the short splice variant of the rat 5-HT_4 receptor, r5-HT_{4S}. h5-HT_{4A} transiently expressed in COS-7 cells displays a classical 5-HT_4 pharmacological profile. However, affinities of the cloned h5-HT_{4A} receptor for agonists such as ML10302, BIMU1, renzapride or zacopride were lower than the ones found in brain. Moreover, the stimulatory patterns of cAMP formation by h5-HT_{4A} in response to the 5-HT_4 receptor agonists ML10302 and renzapride were very similar to the patterns of stimulation of I_{Ca} by 5-HT obtained in response to these two compounds in isolated human atrial myocytes. Thus, h5-HT_{4A} likely mediates the effects of 5-HT in human atrium, and may differ from the 5-HT_4 receptor isoform(s) responsible for the neuronal effects of 5-HT.

2. Materials and methods

2.1. Surgery

All protocols for obtaining human cardiac tissue were approved by the Ethics Committee of our institution (GREBB, Hôpital de Bicêtre, Université de Paris-Sud). Specimens of right atrial appendages were obtained from nine patients (aged 11–74 years) undergoing heart surgery for congenital defects, coronary artery diseases or valve replacement at the Hôpital Marie-Lannelongue, Le Plessis-Robinson, France. Most patients received a pharmacological pretreatment (Ca-channel blockers, β-adrenergic antagonists, ACE-inhibitors, NO-donors, and/or anti-arrhythmic drugs). In addition, all patients received sedatives, anesthesia, and, antibiotics. Dissociation of the cells was realized immediately after surgery. Human atrial mRNAs used for the PCR cloning of the receptor were prepared from the left atrium that was harvested postmortem on a patient at the Hôpital Marie-Lannelongue.

2.2. PCR cloning and rapid amplification of cDNA ends

The sequence of the recently characterized rat 5-HT_4 receptor [9] was used to synthesize two pairs of degenerate primers corresponding to a region comprised between the third and the seventh transmembrane domains of the receptor. Primers HT41 [5′-ATA(C/T)T(A)C(G)IC(T/A)TIGAC(T)C(A)GA(T/G/C)TAC(T)-3′] and HT46 [5′-GAC(T)CCA(T/G/C)TTC(T)ATA(T/C)GAC(T)TAC(T)AC-3′] were used to amplify 50 ng of total RNA from human atrium that was reverse transcribed using oligo(dt) primers and Superscript reverse transcriptase (Gibco-BRL). Products of this first PCR reaction were used as templates for a second PCR amplification using degenerate primers HT42 [5′-AGCTACCGGAATTCATGC(T)TIGGA(T/G/C)GGA(T/G/C)TGC(T)TGG-3′] and HT43 [5′-GCTAGCCCAAGCTTC(T)-TTC(T)GTA(T/G/C)ACA(T/G/C)AAC(T)ATA(C/T)-3′]. Both PCR reactions were performed using the following cycle conditions: denaturation for 1 min at 94°C, annealing for 1.5 min, and extension for 2 min at 72°C with the final extension for 8 min. The annealing temperatures of cycles 1-2 and 3-30 were 37°C and 54°C, respectively. DNA fragments of about 430 bp, which are the expected size of 5-HT_4 receptor-related sequences, were cloned into pGEM7Z (*Eco*RI–*Hin*dIII cut) and sequenced. To obtain the cDNA from 3′- and 5′-ends, the anchored-RACE extension method was applied as described [11], using primers which were specific for the central part of the human 5-HT_4 cDNA. For the amplification of the 3′-end of the gene, the RACE reaction was performed using primer HTS1 [5′-GAACTCTAACTCTACGTA-3′] together with the first anchor, and the nested RACE reaction using primer HTS3 [5′-ATGGTCAA-CAAGCCCTAC-3′] together with the second anchor. To obtain cDNA from the 5′-end of the gene, a reverse transcription using specific primer HTS2 [5′-CCCATGATGATGCACAGG-3′] was followed by the addition of a polyA tail to the 3′ of the cDNA as described [11] using the Terminal Deoxynucleotidyl Transferase according to the manufacturer instructions (Pharmacia). The RACE reaction was then performed using specific primer HTS2 together with the first anchor, and the nested RACE reaction using primer HTS4 [5′-CCGACTGAGGCCTGCTCTCG-3′] together with the second anchor. All reactions used in this RACE-PCR strategy were as follows: 30 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). PCR fragments generated by this method were cloned and sequenced. After determination of the complete cDNA sequence, total cDNA was amplified by PCR using specific primers HHT45 [5′-CGGTGCTTATTTCCTGTAATG-3′] and HHT43D [5′-TGAATGCGAATGAATGGCTA-3′].

2.3. Tissue localization studies

Total RNA were prepared from human peripheral tissues using the Trizol RNA purification system (Gibco-BRL). Total RNA from human brain was from Clontech. cDNA was prepared from mRNA using oligo(dT) primers and Superscript reverse transcriptase (Gibco-BRL). cDNA specific for the human 5-HT_4 receptor was detected using a nested PCR amplification. A first reaction was performed using 50 ng of cDNA together with specific primers HHT45 and HHT43D, designed to the 5′- and 3′-end of the cloned cDNA, respectively. Products of this first reaction were used as templates for a nested PCR amplification with specific primers HTS13 [5′-ACATCT-CTGGACGTCCTGCT-3′] and HHT43C [5′-GTTGTGAGCCATG-TCCTCA ATCA-3′]. The PCR products were run on a 1.5% agarose gel and transferred to nitrocellulose membranes. Filters were hybridized with the ^{32}P-5′-end-labeled internal primer HTS5 [5′-GGCTGC-TGGGTCATCCCCAC-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′-CACCTTCTACAAT-GAGCTGCGTGTGGC-3′] and [5′-TGTTTGCTGATCCACATCT-GCTGGAAGGTGGA-3′], respectively. All PCR reactions 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).

2.4. DNA transfection

The full coding region of the h5-HT_{4A} gene was subcloned in the mammalian expression vector pRC/CMV (Invitrogen, Carlsbad, CA). Transfections were performed using the vector Polyethylenimine (PEI) as described [12]. Cells were transfected using a mixture of DNA and PEI at a ratio of 20 nmol PEI/μg 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/

vial, incubated for 6 h with plasmid DNA (150 μg/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/well, incubated for 6 h with plasmid DNA (16 μg/well) and assayed 24 h after transfection. Cells transfected with the h5-HT_{4A} cDNA construct were compared with mock-transfected cells that were exposed to the crude pRC/CMV plasmid.

2.5. Membrane preparation

Each vial of cells for use in radioligand binding assays was washed twice with phosphate-buffered saline (PBS). Cells were scraped, collected and centrifuged at $300\times g$ for 5 min. The pellet was re-suspended in 2.5 ml of ice-cold HEPES buffer (50 mM, pH 7.4), and homogenized by an Ultraturax tissue grinder. The lysate was subsequently centrifuged at $40\,000\times g$ for 20 min at 4°C. The resulting pellet was re-suspended in 15 vol. of HEPES buffer (50 mM, pH 7.4). Membrane preparations were kept on ice and utilized within 2 h for the radioligand binding assays.

2.6. Radioligand binding assays

Radioligand binding studies were performed in 500 μl of buffer (HEPES, 50 mM, pH 7.4), 20 μl of either competing agent (for drug competition studies), ML10375 to give a final concentration of 10 μM (for determination of non-specific binding) or buffer (for determination of total binding), 20 μl of [^{3}H]GR113808 (Amersham, Arlington Heights, IL) to give a final concentration of 0.1 nM 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 48R cell Brandel Harvester. Filters were pre-soaked in a solution of polyethylenimine (PEI, 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). Radioactivity was measured using a Beckman LS 6500C liquid scintillation counter. Binding data were analyzed by computer-assisted non-linear regression analysis (Graph Pad PRISM Program, Graph Pad Software Inc., San Diego, CA).

2.7. 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, 5% CO_2. 5-HT (1 μM), other serotoninergic agents (1 μM) or forskolin (10 μM) were added and incubated for an additional 15 min at 37°C, 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 (kit 79830, ERIA, Diagnostics Pasteur, Marnes La Coquette, France).

2.8. Human atrial cell dissociation

Myocytes were isolated as described previously [13,14]. Human atrial cell suspension (100–200 μl) was put in a Petri dish containing control external solution.

2.9. Electrophysiological experiments

The whole-cell configuration of the patch-clamp technique was used to record the high-threshold calcium current (I_{Ca}) on Ca^{2+}-tolerant human atrial myocytes [14]. In the routine protocols the cells were depolarized every 8 s from a holding potential of −80 mV to 0 mV for 200 or 400 ms after a short prepulse (50 ms) to −50 mV. The prepulse together with the application of tetrodotoxin (30 μM) was used to eliminate fast sodium currents. In some experiments all sodium ions were substituted for tetraethylammonium (TEA) ions to prevent sodium currents, without any detectable incidence on the results compared to those obtained with sodium-containing solutions. K^+ currents were blocked by replacing all K^+ ions with intracellular Cs^+ and extracellular Cs^+ or TEA^+. All experiments were done at room temperature (19–25°C).

2.10. Solutions

Control external solution contained (in mM): 107.1 NaCl, 10 HEPES, 40 CsCl, 4 $NaHCO_3$, 0.8 NaH_2PO_4, 1.8 $CaCl_2$, 1.8 $MgCl_2$, 5 D-glucose, 5 sodium pyruvate, 0.03 tetrodotoxin, pH 7.4 adjusted with NaOH. TEA external solution contained (in mM): 136.9 TEACl, 10 HEPES, 5 D-glucose, 1.8 $CaCl_2$, 1.8 $MgCl_2$, pH 7.4 adjusted with TEAOH. Control or drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 250 μm inner diameter capillary tubings flowing at a rate of ≈10 μl/min. Patch electrodes (0.8–1.5 MΩ) were filled with control internal solution that contained (in mM): 119.8 CsCl, 5 EGTA (acid form), 4 $MgCl_2$, 5 creatine phosphate disodium salt, 3.1 Na_2-ATP, 0.42 Na_2-GTP, 10 HEPES, 62 μM $CaCl_2$ (p*Ca* 8.5), pH 7.3 adjusted with CsOH.

2.11. Data analysis

The maximal amplitude of I_{Ca} was measured as the difference between the peak inward current and the leak current, which was the current amplitude at the end of the 400 ms duration pulse [14]. Currents were not compensated for capacitive and leak currents. The results are expressed as mean ± SEM. The variations in I_{Ca} induced by 5-HT or ML10302 were tested for statistical significance by Student's *t* test.

2.12. Materials

Collagenase type IV and protease type XXIV were purchased from Sigma (L'Isle d'Abeau Chesnes, France). PEI (MW 800 kDa) was from Fluka (L'Isle d'Abeau Chesnes, France). DMEM was obtained from Gibco-BRL. Tetrodotoxin was from Latoxan (Rosans, France). All other drugs were from Sigma. ML10302 and ML10375 were synthetized as recently described [15,16]. All drugs were dissolved in ionic aqueous solution. PCR were performed on a GeneAmp 2400 apparatus (Perkin Elmer). HiTaq DNA polymerase and related reaction buffer was from Bioprobe Systems (Montreuil-sous-Bois, France). In all PCR reactions, dNTPs and specific primers were at a final concentration of 200 μM and 1 μM, respectively. Doubled-stranded DNA was sequenced with a T7 DNA Polymerase sequencing kit (Pharmacia) according to the manufacturer instructions.

3. Results

3.1. Primary structure of human 5-HT_4 receptor

A 430 bp DNA fragment was isolated when human atrial RNA was used as template in a nested RT-PCR amplification with two pairs of degenerate oligonucleotide primers derived from the central region of the rat 5-HT_4 receptor subtype. The corresponding full-length cDNA was then isolated from the same atrial RNA preparation using an anchored-RACE PCR strategy (see Section 2). The deduced amino acid sequence are shown in Fig. 1. The nucleotide and peptide sequences were 90% and 93% identical, respectively, to the short form of the rat 5-HT_4 receptor, r5-HT_{4S} [9]. The sequences from the two species display a common length of 387 amino acids. The region of the receptor isolated from human atrium that precedes the splicing site described in the r5-HT_{4S} receptor also shares 94% protein identity with the m5-HT_{4L} receptor recently cloned from mouse colliculi neurons [10]. We have therefore isolated the first human 5-HT_4 receptor subtype, that we propose to name h5-HT_{4A}. The hydrophobicity plot displayed seven hydrophobic putative membrane-spanning regions, labelled TM1–TM7 in Fig. 1. Finally, h5-HT_{4A} displayed all the potential regulatory sites (two N-linked glycosylation sites, a palmitoylation site, three protein kinase C consensus sites) already described for r5-HT_{4S} and m5-HT_{4L} [9,10].

3.2. Tissue-specific expression of the h5-HT_{4A} receptor

The expression of h5-HT_{4A} transcripts was analyzed by amplification of cDNA derived from RNA isolated from various human tissues using a nested RT-PCR technique. We

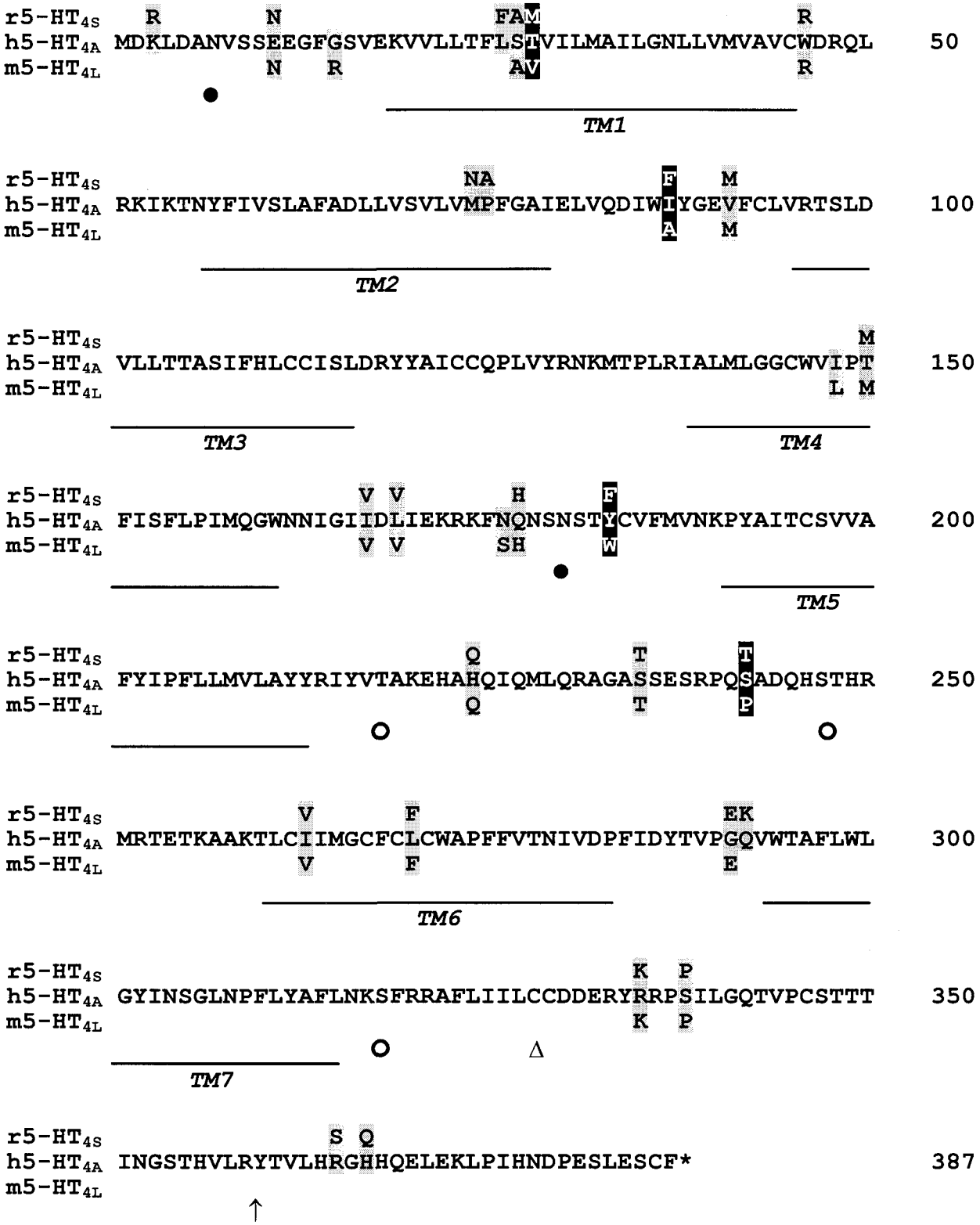

Fig. 1. Comparison of the amino acid sequences of the h5-HT_{4A} receptor cloned from human atrium with the r5-HT_{4S} receptor cloned from rat brain, and with the region of the m5-HT_{4L} receptor cloned from mouse colliculi that precedes the splicing site described in the rat 5-HT_4 receptor. The seven putative transmembrane domains are underlined and labelled TM1 to TM7. Changes in human amino acids versus rat and/or mouse are indicated: differences common to two species are shaded in grey, and absolute differences are shaded in black. ●, Potential N-linked glycosylation sites; ○, protein kinase C consensus phosphorylation sites; △, potential palmitoylation site; ↑, site of alternative splicing described for the rat 5-HT_4 receptor; *, terminal stop codon. The Genebank accession number for h5-HT_{4A} is (pending).

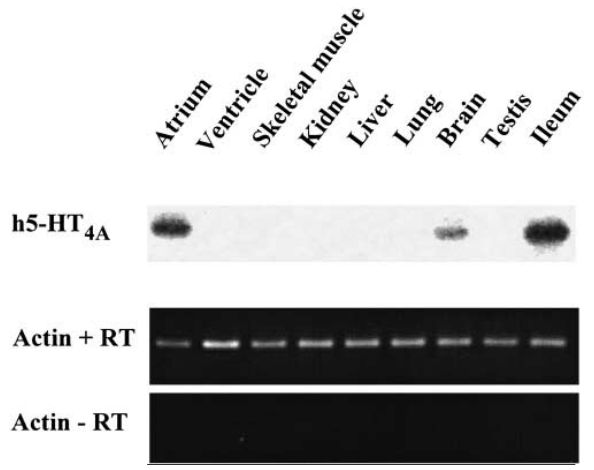

Fig. 2. RT-PCR analysis performed with 50 ng of mRNA from various human tissues. The PCR products were analyzed by Southern blotting using a ^{32}P-5′-end-labeled internal primer. The PCR primers used for this analysis are described in Section 2. An 8 h exposure of the autoradiogram is shown. A positive control was performed using rat actin primers on mRNA samples treated with (+RT) or without (−RT) reverse transcriptase. The PCR products were analyzed on a 1.5% agarose gel and a photograph of the ethidium bromide stained gel is shown.

were able to examine the tissue distribution by using two pairs of specific primers and two successive rounds of PCR amplification. The amplified products were identified using a specific internal oligonucleotide probe (Fig. 2). We also demonstrated the presence in all tissues of cDNA corresponding to the constitutively expressed β-actin gene, as well as the absence of actin PCR product in a minus reverse transcriptase control (Fig. 2). Interestingly, h5-HT$_{4A}$ in the human heart was expressed at high levels in the atrium, but not in the ventricle. h5-HT$_{4A}$ was also found in whole brain preparations, and at high levels in the ileum (Fig. 2). These hybridization signals were not due to any contaminating genomic DNA since no bands were observed when RNA was directly amplified (−RT control).

3.3. Pharmacological characterization of the h5-HT$_{4A}$ receptor

The cDNA encoding the h5-HT$_{4A}$ receptor was transiently expressed in COS-7 cells for pharmacological evaluation. Saturation analysis revealed a single saturable site of high affinity ($K_d = 0.23 \pm 0.06$ nM) using [^{3}H]GR113808 (Fig. 3A). The h5-HT$_{4A}$ receptor expressed in COS-7 cells displayed a B_{max} of 0.21 ± 0.01 pmol/mg of protein. Non-specific binding increased linearly with increasing ligand concentration. A range of 5-HT$_4$ receptor agonists and antagonists completely inhibited the specific binding of [^{3}H]GR113808 to the cloned h5-HT$_{4A}$ receptor (Fig. 3B). All the displacement curves were monophasic, giving a Hill coefficient not different from 1. The degree of non-specific binding at 0.1 nM of [^{3}H]GR113808 ranged from 5 to 11% depending on experiments. The data summarized in Table 1 demonstrate that the pharmacological profile of the cloned h5-HT$_{4A}$ receptor, in terms of rank order of potencies of the ligands tested, is very similar to the one found for 5-HT$_4$ receptors as studied in situ (human [17] and piglet [18] atria, human striatum [19] and caudate [20], rat [15,16] and guinea pig [21] striatum, mouse colliculi [21]) or after expression of cloned isoforms in cultured fibroblasts (r5-HT$_{4L}$ [9,22] and m5-HT$_{4L}$ [10]). However, the h5-HT$_{4A}$ receptor displayed lower affinities for all the agonists and partial agonists tested when compared to human brain preparations, or cultured fibroblasts expressing the r5-HT$_{4L}$ and m5-HT$_{4L}$ receptors. When affinity estimates for 5-HT$_4$ agonists in piglet or human heart tissue were available in the literature, these values were much closer to the one found in COS-7 cells expressing the h5-HT$_{4A}$ receptor than affinity values found in neuronal tissues or in cells expressing the long form of the 5-HT$_4$ receptor (r5-HT$_{4L}$ or m5-HT$_{4L}$). Binding data concerning the short form of the 5-HT$_4$ receptor cloned in rat are not available in the literature.

3.4. Stimulation of cAMP production by the h5-HT$_{4A}$ receptor

To examine the ability of cells expressing h5-HT$_{4A}$ to couple to adenylyl cyclase, cAMP synthesis was assayed in COS-7 cells transiently transfected with the h5-HT$_{4A}$ cDNA. Basal cAMP values were not significantly different in cells expressing the h5-HT$_{4A}$ receptor and in mock-transfected cells (9.94 ± 1.61 and 7.60 ± 1.54 pmol/well, respectively), indicating that the h5-HT$_{4A}$ receptor had no intrinsic activity on cAMP formation in transiently transfected cells in the absence of agonists. cAMP responses to various 5-HT$_4$ receptor agonists and to forskolin are represented in Fig. 4 as % increase over basal cAMP concentration. 5-HT (1 μM) had no effect on basal adenylyl cyclase activity in mock-transfected COS-7 cells, indicating that endogenous adenylyl cyclase-coupled serotonin receptors are not present in these cells. In h5-HT$_{4A}$ clones, addition of 5-HT (1 μM) increased cAMP concentration by 99%. The 5-HT$_4$ agonist ML10302 (1 μM: [16]) behaved as a poor 5-HT$_4$ agonist in h5-HT$_{4A}$ clones, and increased cAMP concentration by only 28%, despite an affinity for the receptor that was found ≈90 times higher than the one of 5-HT (Table 1). Furthermore, pre-incubation of h5-HT$_{4A}$ clones with 1 μM ML10302 prior to addition of 5-HT antagonized significantly the ability of 5-HT (1 μM) to increase basal cAMP levels (Fig. 4). Renzapride also behaved as a poor 5-HT$_4$ agonist, and increased cAMP formation by only 53%, despite an affinity for the h5-HT$_{4A}$ receptor similar to the one of 5-HT (Table 1). Forskolin (10 μM), a direct activator of adenylyl cyclase, induced a similar increase in cAMP concentration in h5-HT$_{4A}$ clones and in mock-transfected COS-7 cells, indicating that the potential for maximal activation of adenylyl cyclase was not impaired in cells expressing the h5-HT$_{4A}$ receptor.

3.5. 5-HT regulation of calcium current in human atrial myocytes

To examine whether h5-HT$_{4A}$ may mediate the cardiac effects of 5-HT in human atrium, we have re-examined the 5-HT$_4$ receptor mediated regulation of I_{Ca} in isolated human atrial myocytes. Since the most striking results obtained with the cAMP response mediated by the cloned h5-HT$_{4A}$ concerned the agonist/antagonist properties of ML10302, we compared the effects of ML10302 and 5-HT on I_{Ca}. Basal I_{Ca} amplitude, measured at 0 mV membrane potential, was on average 192.3 ± 33.9 pA ($n = 21$), and I_{Ca} density, which represents the ratio of I_{Ca} amplitude to membrane capacitance, was 3.0 ± 0.45 pA/pF ($n = 21$). Like on the cAMP measurements, the effects of ML10302 and 5-HT were first tested at a 1 μM concentration. As shown in Fig. 5A, application of 1 μM ML10302 produced a small and reversible stimulation of basal I_{Ca} amplitude. The individual current traces shown in

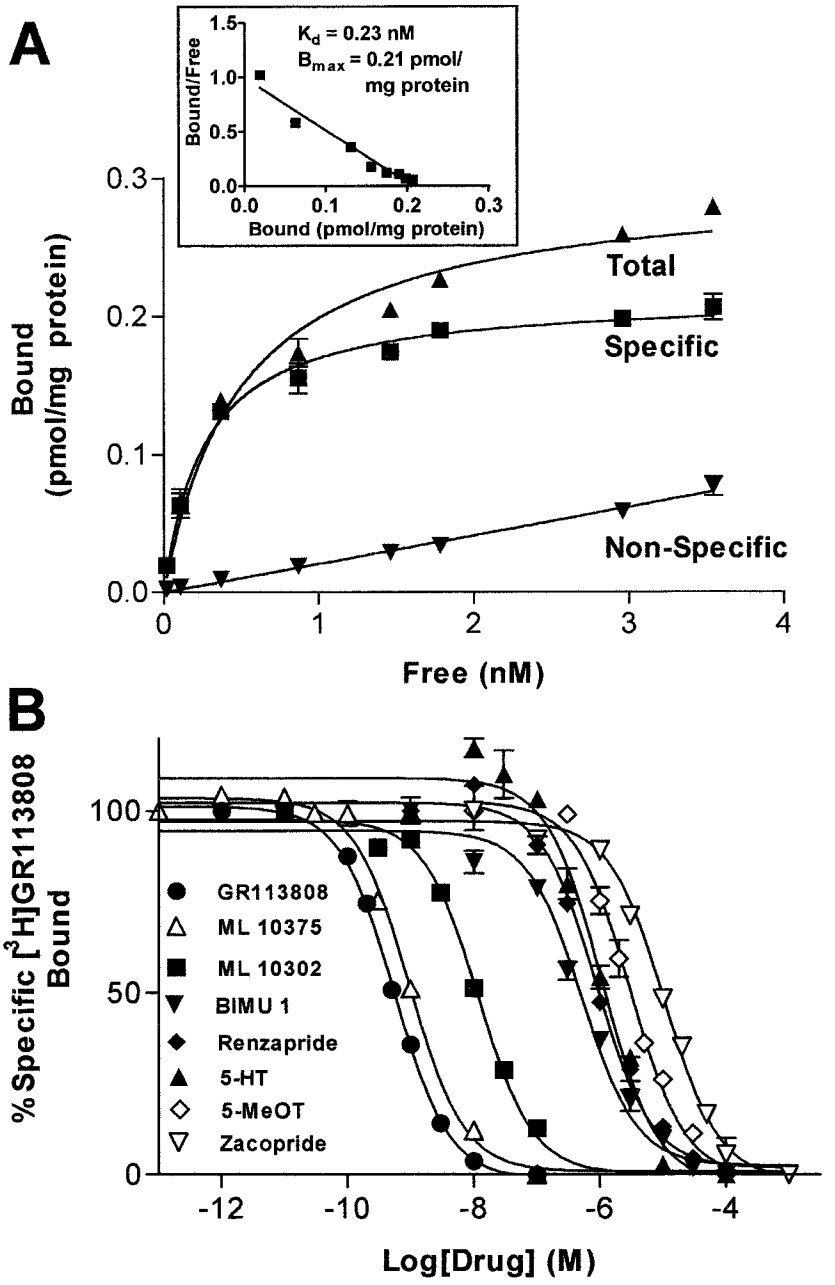

Fig. 3. A: Saturation analysis of [^{3}H]GR113808 binding on h5-HT$_{4A}$ clones. Membranes harvested from transiently transfected COS-7 cells were incubated with 8 concentrations of [^{3}H]GR113808 (0.02–3.3 nM) for 30 min at 25°C. Non-specific binding was defined by 10 μM ML10375. Results are from a single experiment but are representative of three such experiments. K_D and B_{max} values were determined by computer-assisted non-linear regression analysis (GraphPad, Prism Software). B: Inhibition of specific [^{3}H]GR113808 binding to the cloned h5-HT$_{4A}$ receptor. Membranes from transiently transfected COS-7 cells were incubated with 0.4 nM [^{3}H]GR113808. Non-specific binding was defined by 10 μM ML10375. Results are presented as a percentage of specific binding in the absence of a competing agent. Results are from a single experiment but are representative of three such experiments. Data were analyzed by computer-assisted non-linear regression analysis (GraphPad, Prism Software).

the top panel of Fig. 5A demonstrate that the effect of ML10302 was not accompanied by any significant modification in the kinetics of I_{Ca}. This suggests that ML10302 did not modify the voltage dependence of the Ca channel gating. While ML10302 had little effect on basal I_{Ca} amplitude, application of 5-HT after washout of ML10302 resulted in a large stimulation of I_{Ca}. In 14 experiments in which both ML10302 and 5-HT were successively tested on the same cells (like in Fig. 5A), 1 μM ML10302 and 1 μM 5-HT increased I_{Ca} by, respectively, 17.4 ± 8.0% and 157.0 ± 40.6% over a control amplitude of 117.5 ± 24.5 pA. Thus, similarly to the cAMP response mediated by the cloned h5-HT$_{4A}$, ML10302 behaved as a poor activator compared to 5-HT of the L-type calcium current in human atrial myocytes.

To examine whether ML10302 behaved as an antagonist of the native human cardiac 5-HT$_4$ receptors, the effect of ML10302 on I_{Ca} was tested in a different series of experiments by adding the compound in the presence of 5-HT. Fig. 5B shows that application of 1 μM ML10302 in the presence of 1 μM 5-HT resulted in a reduction of the stimulatory effect of

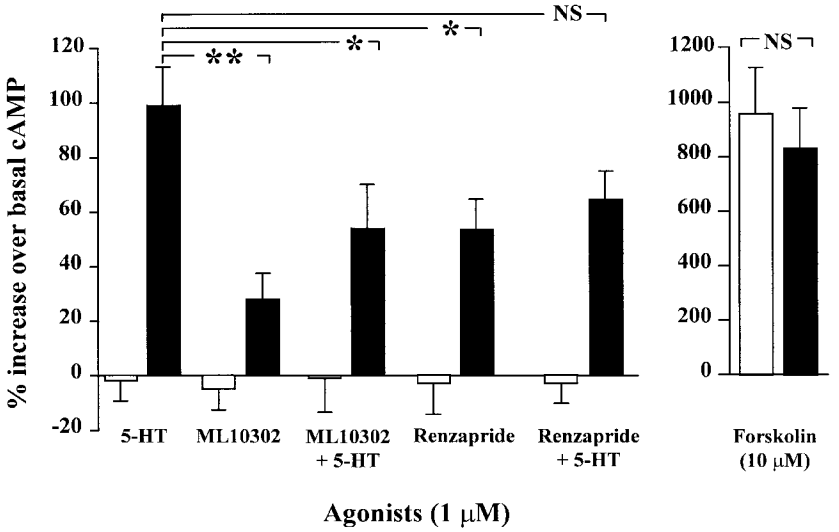

Fig. 4. cAMP responses to various 5-HT_4 receptor agonists and antagonist using the h5-HT_{4A} receptor transiently expressed in COS-7 cells (filled bars) or mock transfected cells (empty bars) as control. cAMP measurements were performed as described in Section 2. Cells were pre-incubated with 5 mM theophylline and 10 μM pargyline for 15 min, then incubated with 1 μM of agonist or antagonist, or 10 μM forskolin for 15 min. Effect of agonists or antagonist on 5-HT-induced cAMP accumulation was assayed by addition of the agonist or the antagonist during the 15 min pre-incubation period, followed by addition of 5-HT for 15 min. Values are mean ± SEM of 6–10 experiments. $^{*}P<0.05$; $^{**}P<0.001$ vs. indicated values (*t*-test).

5-HT on I_{Ca}. In four similar experiments in which 5-HT (1 μM) was applied first and ML10302 (1 μM) was added later on top of 5-HT, 5-HT alone increased I_{Ca} by 121.3 ± 42.4% over basal level, and ML10302 reduced this effect to 60.0 ± 16.0%. To further characterize the inhibitory effect of ML10302 on I_{Ca}, a cumulative concentration-response curve was obtained by adding increasing concentrations of ML10302 (1–100 nM) to cells initially exposed to 100 nM 5-HT. As shown in Fig. 5C, 100 nM 5-HT alone increased I_{Ca} by 195.9 ± 26.0% ($n=13$) over its basal amplitude. Increasing concentrations of ML10302 progressively reduced this effect to 71.8 ± 12.8% ($n=6$) at the maximal concentration tested (100 nM). Thus, ML10302 antagonized the response of I_{Ca} to 5-HT in a concentration-dependent manner. At a 100 nM 5-HT concentration, the IC_{50} for ML10302 was 17 nM and the maximal effect was ≈70% inhibition of the 5-HT response (Fig. 5C). Thus, similarly to the cAMP response mediated by the cloned h5-HT_{4A}, ML10302 behaved as an antagonist of the 5-HT mediated response in human atrial myocytes.

4. Discussion

In the present study, we have used degenerate oligonucleotide primers designed from the sequence of the rat 5-HT_4 cDNA and a PCR-based strategy to isolate and clone 5-HT_4 receptor-related sequences from human atrium. We have identified the sequence of a protein cDNA, that we have named h5-HT_{4A}. We believe that h-5HT_{4A} is the first reported sequence of a human 5-HT_4 receptor cDNA since

Table 1
Comparison of the binding affinities of various 5-HT_4 receptor agonists and antagonists on cloned and native 5-HT_4 receptors

Compounds	K_i (nM)							
	h5-HT_{4A} in COS-7	Human atrium[17]	Piglet atrium[18]	Human brain[19,20]	Rodent striatum [15,16,21]	Mouse colliculi[21]	r5-HT_{4L} in COS-7[9]	m5-HT_{4L} in LLCPK1[10]
Agonists								
ML10302	8.4	-	-	-	1.07[c]	-	-	-
5-HT	772	1585	251	30.2 [a]	187[c]	79	145	56
5-MeOT	2080	-	6309	501[b]	2580[c]	524	401	229
Partial agonists								
BIMU1	373	-	-	12.3[a]	15.8[d]	53	-	28
Renzapride	635	398	-	251[b]	127[c]	97	243	131
Zacopride	7750	-	-	692[a]	1510[c]	223	808	190
Antagonists								
GR113808	0.33	-	-	0.06[a]	0.15[c]	0.32	-	0.20
ML10375	0.56	-	-	-	0.26[c]	-	-	-

The first column indicates the affinities of various compounds that compete for 0.4 nM [^{3}H]GR113808 binding to membranes of COS-7 cells transiently transfected with the human h5-HT_{4A} gene. Affinity estimates are given as K_i values in nM and were determined from IC_{50} values obtained by computer-assisted non-linear curve analysis. K_i values are representative of at least two determinations. These results are compared with data from the literature (see references in columns 2–8). [^{3}H]GR113808 was used as radioligand in binding studies on the native 5-HT_4 receptor in human striatum, rat striatum, mouse colliculi neurons, and on the cloned r5-HT_{4L} (rat) and m5-HT_{4L} (mouse) receptors expressed in fibroblasts. [^{125}I]SB207710 was used as radioligand in binding studies on the native 5-HT_4 receptor in human and piglet atria. Binding assays on human brain were performed on striatum ([a], ref. [19]) and caudate ([b], ref. [20]). Binding assays in rodent stratum were from rat ([c], ref. [15,16]) and guinea pig ([d], ref. [21]). Blanks correspond to data that are not available in the literature.

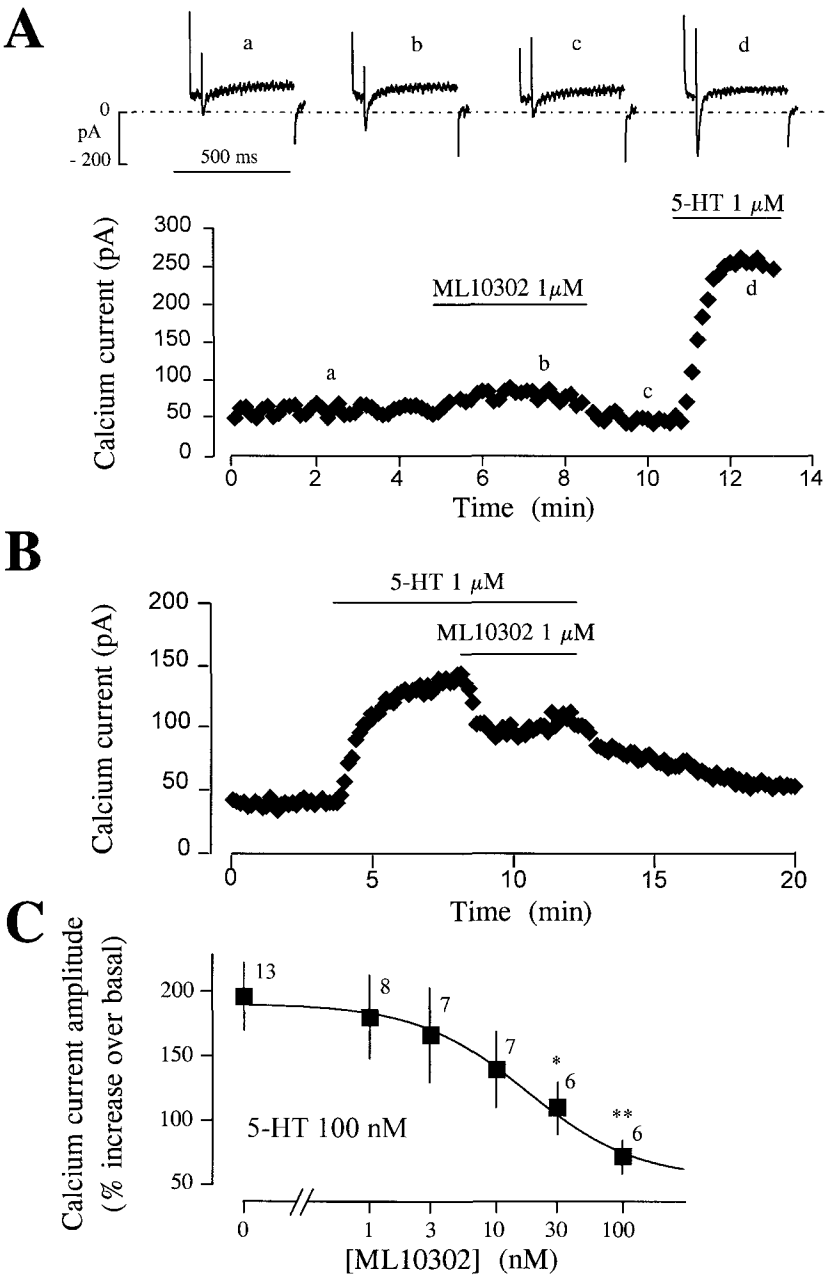

Fig. 5. A,B: Time course of the effects of ML10302 and serotonin on basal I_{Ca} in isolated human atrial myocytes. Each symbol corresponds to a measure of I_{Ca} at 0 mV obtained every 8 s. A: The cell was first superfused with control solution and then exposed to 1 μM ML10302 or 10 μM serotonin during the periods indicated by the solid lines. The individual current traces shown on the upper part were obtained at the times indicated by the corresponding letters in the bottom graph. The dotted line indicates the zero current level. B: The cell was first superfused with control solution and then exposed to 1 μM 5-HT alone or to 5-HT+ML10302 (1 μM) during the periods indicated by the solid lines. After washout of the drugs, the current returned to control amplitude. C: Cumulative dose–response curve for the inhibitory effect of ML10302 on the response of I_{Ca} to 100 nM 5-HT. The points indicate the means and the lines the S.E.M. of the number of experiments indicated near the points. The continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation: %increase in $I_{Ca} = 190.4 - 135.5 \times [ML10302]/([ML10302]+17.1)$. Thus, ML10302 antagonized the effect of 100 nM 5-HT with an IC_{50} of 17.1 nM to a maximal inhibitory level which was 55% above basal I_{Ca}. Statistically significant differences between the data in 5-HT+ML10302 and in 5-HT alone are indicated as: $^{*}P < 0.05$; $^{**}P < 0.01$ (*t*-test).

our results demonstrate that: (i) the deduced amino acid sequence of h5-HT_{4A} displays a 93% identity with the short form of the 5-HT_4 receptor isolated from rat brain (r5-HT_{4S}, [9]), and a 94% identity with the region preceding the splicing site in the m5-HT_{4L} receptor recently cloned from mouse colliculi neurons [10]; (ii) transient expression of h5-HT_{4A} in COS-7 cells displays a classical 5-HT_4 pharmacological profile in terms of rank order of potency of the various serotoninergic ligands tested [8]; (iii) the cloned h5-HT_{4A} receptor is positively coupled to adenylyl cyclase. For the reasons detailed below, we believe that h5-HT_{4A} is the 5-HT_4 receptor that mediates the functional response of human atrium to 5-HT.

(1) h5-HT_{4A} mRNA is expressed in human atrium but not

in ventricle. This is in agreement with a number of functional studies showing positive inotropic and chronotropic effects of 5-HT which are located exclusively in the human atrial tissue [1,6]. This is also in agreement with an expression pattern of the 5-HT_4 receptor restricted to the atrium in rat heart [9].

(2) Transient expression of h5-HT_{4A} in COS-7 cells revealed significantly lower binding affinities for all the agonists and partial agonists used as compared to the affinity constants found in the literature concerning the endogenous 5-HT_4 receptors in neuronal tissues, or in cultured fibroblasts expressing the long form of either the rat or the mouse 5-HT_4 receptor (Table 1). However, there was a good correlation between the affinity constants of 5-HT, 5-MeOT and renzapride for the cloned h5-HT_{4A} receptor and those obtained for the same agonists when binding to the native cardiac 5-HT_4 receptors in human and pig atria (Table 1). This suggests that h5-HT_{4A} is the receptor accounting for the 5-HT binding sites found in human atrium. The differences in binding affinities for 5-HT_4 agonists observed between heart and brain may be due to the presence of different 5-HT_4 receptor isoforms in these tissues.

(3) We have examined the ability of h5-HT_{4A} receptors to couple to adenylyl cyclase in COS-7 transfected cells. We found that 1 μM 5-HT produced a ≈2-fold stimulation of cAMP production. This was much less than what was found for the cloned rat and mouse 5-HT_4 receptors. Indeed, overexpression of the rat r5-HT_{4L} receptors in COS-7 cells produced a ≈20-fold stimulation of basal cAMP release in response to 1 μM 5-HT, similar to the stimulation induced by 10 μM forskolin [9]. The most likely reason for this discrepancy is a difference in the level of protein expression. The level of expression of the h5-HT_{4A} receptor in our system appeared to be within the physiological range, since the h5-HT_{4A} receptor density in transiently transfected COS-7 cells was similar to the density of endogenous 5-HT_4 receptors reported in human brain [20]. However, expression of the cloned rat 5-HT_4 receptors also in COS-7 cells led to a receptor density of 2.5 pmol/mg protein [9], i.e. >10-fold higher than the density found in our system or in human substantia nigra reticulata [20]. Over-expression of the mouse m5-HT_{4L} receptor in LLCPK1 cells led to an even higher receptor density (≈8 pmol/mg protein) which resulted in a substantial increase in basal adenylyl cyclase activity observed even in the absence of 5-HT_4 agonists [10].

(4) Renzapride produced only a moderate stimulation of cAMP synthesis in COS-7 cells expressing h5-HT_{4A}. When both renzapride and 5-HT were used at a saturating concentration of 1 μM, renzapride produce only ≈50% of the effect of 5-HT (Fig. 4). This suggests that renzapride behaves as a partial agonist (compared to 5-HT) of h5-HT_{4A} receptors. However, this partial agonistic activity of renzapride is a general feature of native cardiac 5-HT_4 receptors [4,8]. For example, in isolated human atrial myocytes, the maximal stimulatory effect of renzapride on I_{Ca} was only ≈40% of that of 5-HT [3]. By contrast, renzapride behaves as 'superagonist' in mouse colliculi neurons, with an intrinsic activity greater than 5-HT [4]. Thus, the cloned h5-HT_{4A} receptors and the native human atrial 5-HT_4 receptors shares a clear similitude with respect to their sensitivities to renzapride.

(5) A striking finding in our study was the poor agonistic effect, combined with a net antagonism to 5-HT, of ML10302 on the cAMP response generated by the cloned h5-HT_{4A} receptor. ML10302 was shown recently to bind to 5-HT_4 receptors in the rat striatum with a nanomolar affinity [15,16]. Moreover, ML10302 mimics the effect of 5-HT, with an EC_{50} of ≈4-6 nM, on the relaxation of rat esophagus as well as on the electrically evoked contraction of the guinea pig ileum [15]. While ML10302 binds to the h5-HT_{4A} receptor with a nanomolar affinity (Table 1), it increased cAMP by only 30% at a 1 μM concentration. Moreover, pre-incubation of transfected cells with ML10302 significantly reduced the 5-HT stimulation of adenylyl cyclase activity. Again, these features appeared representative of the native human atrial 5-HT_4 receptors, since ML 10302, unlike 5-HT, has no stimulatory effect on I_{Ca} in human atrial myocytes and exerted a dose-dependent inhibition of the stimulatory effect of 5-HT on I_{Ca} (Fig. 5). Although the effects of ML10302 were only examined in rodent CNS and GI tract, it is likely that ML10302 also acts as an agonist in human preparations. Thus, the absence of stimulatory effect of ML10302 in human atrium would suggest that h5-HT_{4A} does not play a major role in the response of the CNS and GI tract 5-HT, although this receptor is expressed in these tissues (Fig. 2). Further studies are needed to examine whether other member(s) of the 5-HT_4 receptor family participate(s) in the effect of 5-HT in these tissues.

Taken altogether, our data strongly suggest that (i) the h5-HT_{4A} receptor is similar to the native 5-HT_4 receptor which mediates the positive inotropic, chronotropic and lusitropic effects of 5-HT in human atrium, and (ii) differs from 5-HT_4 receptors previously characterized in neurons and the GI tract. The simplest hypothesis to explain this tissue-specificity of 5-HT_4 receptors is the existence of alternative-spliced variants differentially expressed in the CNS and peripheral tissues. Modification of the C-termini of receptors by alternative splicing could induce changes in the potency of various agonists and/or antagonists to bind to the receptor. Modifications of the C-terminal tails of seven-transmembrane-domain receptors following alternative splicing has also been shown to determine G-protein specificity and hence to affect signal transduction pathways [23]. Search for alternative splice-variants of the h5-HT_{4A} receptor and comparative functional studies will be needed in the future to test these hypotheses.

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