



# Isolation of the serotonergic 5-HT<sub>4(e)</sub> receptor from human heart and comparative analysis of its pharmacological profile in C6-glia and CHO cell lines

<sup>1,3</sup>Jeanne Mialet, <sup>2,3</sup>Isabelle Berque-Bestel, <sup>4</sup>Pierre Eftekhari, <sup>1,3</sup>Monique Gastineau, <sup>2,3</sup>Mireille Giner, <sup>2,3</sup>Yamina Dahmoune, <sup>5</sup>Patrick Donzeau-Gouge, <sup>4</sup>Johan Hoebeke, <sup>2,3</sup>Michel Langlois, <sup>2,3</sup>Sames Sicsic, <sup>\*,1,3</sup>Rodolphe Fischmeister & <sup>1,3</sup>Frank Lezoualc'h

<sup>1</sup>Laboratoire de Cardiologie Cellulaire et Moléculaire, INSERM U-446, Université de Paris-Sud, Faculté de Pharmacie, F-92296 Châtenay-Malabry, France; <sup>2</sup>Laboratoire de Reconnaissance Moléculaire et Cellulaire, BIOCIS UPRES A CNRS 8076, France; <sup>3</sup>Institut de Signalisation et Innovation Thérapeutique (IFR-ISIT), Université de Paris-Sud, Faculté de Pharmacie, F-92296 Châtenay-Malabry, France; <sup>4</sup>CNRS UPR 9021, F-67084 Strasbourg, France; <sup>5</sup>Institut Hospitalier Jacques Cartier, Service de Chirurgie Cardiaque, Avenue du Noyer Lambert, F-91349 Massy Cedex, France

**1** RT-PCR technique was used to clone the human 5-HT<sub>4(e)</sub> receptor (h5-HT<sub>4(e)</sub>) from heart atrium. We showed that this h5-HT<sub>4(e)</sub> receptor splice variant is restricted to brain and heart atrium.

**2** Recombinant h5-HT<sub>4(e)</sub> receptor was stably expressed in CHO and C6-glia cell lines at 347 and 88 fmol mg<sup>-1</sup> protein, respectively. Expression of h5-HT<sub>4(e)</sub> receptors at the cell membrane was confirmed by immunoblotting.

**3** The receptor binding profile, determined by competition with [<sup>3</sup>H]-GR113808 of a number of 5-HT<sub>4</sub> ligands, was consistent with that previously reported for other 5-HT<sub>4</sub> receptor isoforms. Surprisingly, we found that the rank order of potencies (EC<sub>50</sub>) of 5-HT<sub>4</sub> agonists obtained from adenylyl cyclase functional assays was inversely correlated to their rank order of affinities (K<sub>i</sub>) obtained from binding assays. Furthermore, EC<sub>50</sub> values for 5-HT, renzapride and cisapride were 2 fold lower in C6-glia cells than in CHO cells.

**4** ML10302 and renzapride behaved like partial agonists on the h5-HT<sub>4(e)</sub> receptor. These results are in agreement with the reported low efficacy of these two compounds on L-type Ca<sup>2+</sup> currents and myocyte contractility in human atrium.

**5** A constitutive activity of the h5-HT<sub>4(e)</sub> receptor was observed in CHO cells in the absence of any 5-HT<sub>4</sub> ligand and two 5-HT<sub>4</sub> antagonists, GR113808 and ML10375, behaved as inverse agonists.

**6** These data show that the h5-HT<sub>4(e)</sub> receptor has a pharmacological profile which is close to the native h5-HT<sub>4</sub> receptor in human atrium with a functional potency which is dependent on the cellular context in which the receptor is expressed.

*British Journal of Pharmacology* (2000) **129**, 771–781

**Keywords:** Human; atrial arrhythmia; serotonergic receptors; 5-HT<sub>4</sub> ligands; inverse agonism; G-protein coupled receptors; adenylyl cyclase; benzamides

**Abbreviations:** 5-HT, 5-hydroxytryptamine; 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; C6 cells, rat glioma cells; CHO cells, chinese hamster ovary cells; cisapride, *cis*-4-amino-5-chloro-N-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide; GR113808, [1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl-1-methyl-1H-indole-3-carboxylate; GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-bibhenyl]-4-carboxamide; h5-HT<sub>4</sub>, human 5-HT<sub>4</sub> receptor; I<sub>Ca</sub>, L-type Ca<sup>2+</sup> channel current; I<sub>f</sub>, pacemaker current; ML 10302, 2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride; ML10375, 2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate; renzapride (BRL 24924), (±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride; RS23597, 3-(piperidine-1-yl)-propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride RS39604, 1-(5-chloro-2(3,5-dimethoxy)benzyloxy-4-aminophenyl)-3-(N-(methylsulfamido)ethyl-4-piperidyl)propanone; RS67333, 1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl)-1-propanone; SB204070, 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride

## Introduction

Several receptors mediate the action of serotonin [5-hydroxytryptamine (5-HT)] both in the central nervous system and the periphery (Saxena, 1995). The 5-HT<sub>4</sub> receptor is a member of the seven transmembrane-spanning G protein-coupled family of receptors which is positively coupled to adenylyl

cyclase (Bockaert & Pin, 1999). Functional responses of the 5-HT<sub>4</sub> receptor have been described in a wide variety of vertebrate tissues including brain, heart, gastrointestinal tract, bladder and adrenal gland where they produce many physiological effects (Eglen *et al.*, 1995; Hedge & Eglen, 1996). In addition, 5-HT<sub>4</sub> receptors are thought to be involved in a variety of central and peripheral human disorders including gastroparesis (Hedge & Eglen, 1996) and neurodegenerative disorders such as Alzheimer's disease (Reynolds *et al.*, 1995; Wong *et al.*, 1996).

\*Author for correspondence at: INSERM U-446, Faculté de Pharmacie, F-92296 Châtenay-Malabry Cedex, France; E-mail: Fisch@vjf.inserm.fr

With respect to the heart, activation of 5-HT<sub>4</sub> receptors can induce strong positive chronotropic, inotropic and lusitropic effects in human and pig atrium (Kaumann, 1991; Kaumann *et al.*, 1991). These cardiac effects of 5-HT<sub>4</sub> receptors are associated with an increase in intracellular cyclic AMP leading to an activation of cyclic AMP-dependent protein kinase which phosphorylates several key proteins involved in the excitation-contraction coupling such as the L-type Ca<sup>2+</sup> channel (Ouadid *et al.*, 1991; Hove-Madsen *et al.*, 1996; Blondel *et al.*, 1997). Furthermore, Pino and colleagues (1998) have recently reported that 5-HT<sub>4</sub> receptor stimulation can increase the pacemaker current I<sub>f</sub> in human isolated atrial myocytes. Therefore, given the ability of 5-HT<sub>4</sub> receptors to activate two potential arrhythmogenic currents, the L-type Ca<sup>2+</sup> channel current I<sub>Ca</sub> and I<sub>f</sub>, it has been suggested that these receptors may be involved in the genesis of atrial arrhythmias (Kaumann, 1994; Workman & Rankin, 1998). In support of this hypothesis, serotonin-induced arrhythmic contractions have been demonstrated in isolated human atrial strips (Kaumann & Sanders, 1994) and these effects were abolished by specific 5-HT<sub>4</sub> antagonists indicating mediation through 5-HT<sub>4</sub> receptors. Thus, 5-HT<sub>4</sub> receptors may represent a new potential therapeutic target for the treatment of cardiac arrhythmias as well as other 5-HT<sub>4</sub> receptor associated disorders. In order to design novel potent and selective 5-HT<sub>4</sub> ligands an expeditious pharmacological characterization of 5-HT<sub>4</sub> receptors is necessary.

A large number of pharmacological studies have been performed in a wide variety of tissues and species including rat oesophagus, rat and human brain, human and porcine heart (Ford & Clarke, 1993; Eglen *et al.*, 1995). 5-HT<sub>4</sub> receptors have a unique pharmacology which is clearly different from that of the other members of the 5-HT receptor family (Ford & Clarke, 1993). Interestingly, despite the existence of a typical 5-HT<sub>4</sub> pharmacological profile, heterogeneity has been reported on the basis of differences in the potency and intrinsic activity of 5-HT<sub>4</sub> ligands in different biological models. For instance, the benzoate ML10302 is a highly potent, selective, and partial 5-HT<sub>4</sub> agonist in guinea pig and rat oesophagus (Langlois *et al.*, 1994). In contrast, it displays a poor agonistic effect on I<sub>Ca</sub> in human atrial myocytes (Blondel *et al.*, 1997) and behaves as an antagonist in mouse colliculi neurones (Ansanay *et al.*, 1996). Yet, one of the main pharmacological differences between 5-HT<sub>4</sub> receptors in different tissues and species, comes from the observation that benzamides behave either as full agonists in mouse colliculi neurones (Dumuis *et al.*, 1989) or as partial agonists in human heart, rat distal colon and oesophagus (Ouadid *et al.*, 1991; Wardle & Sanger, 1993; Bockaert *et al.*, 1998). Taken together, these findings suggest the existence of several 5-HT<sub>4</sub> receptors which may account for the apparent pharmacological discrepancies regarding the efficiency of 5-HT<sub>4</sub> ligands tested in different tissues.

Recently, remarkable progress in understanding the pharmacological behaviour of 5-HT<sub>4</sub> receptors has been made with the molecular identification of four splice variants of the human 5-HT<sub>4</sub> receptor (h5-HT<sub>4</sub>) (Blondel *et al.*, 1997; Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997; Blondel *et al.*, 1998a). These splice variants have been named h5-HT<sub>4(a)</sub>, h5-HT<sub>4(b)</sub>, h5-HT<sub>4(c)</sub>, and h5-HT<sub>4(d)</sub> and are generated by splicing events that occur in the C-terminus of the 5-HT<sub>4</sub> receptor. Tissue distribution studies revealed some degree of specificity in the pattern of expression of the different human isoforms with h5-HT<sub>4(a)</sub>, h5-HT<sub>4(b)</sub> and h5-HT<sub>4(c)</sub> being expressed in cardiac atria and brain (Blondel *et al.*, 1998a). This distinct spatial distribution may explain the difference in efficacy of 5-HT<sub>4</sub> ligands on 5-HT<sub>4</sub> receptor-mediated responses in various

tissues. Alternatively, the coupling efficiency of each 5-HT<sub>4</sub> receptor isoform may be influenced by the cellular environment. In support of this hypothesis, alternative splicing in the C-terminal sequence of other G-protein coupled receptors was shown to contribute to the coupling specificity between receptor and G proteins and hence to determine the specificity of the signalling pathway of a given receptor (Namba *et al.*, 1993; Spengler *et al.*, 1993).

To further understand the pharmacology of the h5-HT<sub>4</sub> receptor and its pathological implication in atrial arrhythmias, we searched for other splice variants in human heart. In this study, we report the molecular identification and the tissue expression of an additional h5-HT<sub>4</sub> splice variant cloned from human atrium. Sequence analysis revealed that this isoform corresponds to the recently identified h5-HT<sub>4(e)</sub> receptor isolated from human brain (Claeysen *et al.*, 1999). Tissue distribution studies showed that this h5-HT<sub>4(e)</sub> receptor splice variant is restricted to brain and heart atrium. In order to provide a pharmacological profile of this newly described h5-HT<sub>4(e)</sub> receptor and to analyse the influence of the cellular environment on its signalling pathway, we stably introduced h5-HT<sub>4(e)</sub> receptor expression vectors into Chinese hamster ovary cells (CHO) and rat glioma cells (C6). Development of specific antibodies against the second extracellular loop of the h5-HT<sub>4</sub> receptor allowed us to demonstrate the presence of the h5-HT<sub>4(e)</sub> receptor in these cellular clones. Finally, binding studies and the functional profile of the h5-HT<sub>4(e)</sub> were compared in these two cellular systems using a number of standard 5-HT<sub>4</sub> agonists and antagonists.

## Methods

### Surgery

All protocols for obtaining human tissue were approved by the ethics committee of our institution (GREBB, Hôpital de Bicêtre, Université de Paris-Sud, France). Specimens of right atrial appendages were obtained from patients undergoing heart surgery for coronary artery diseases or valve replacement at the Institut Hospitalier Jacques Cartier, Massy, France.

### PCR cloning of the human 5-HT<sub>4(e)</sub> cDNA

Primers HHT45 and HHT43C (Blondel *et al.*, 1998a; accession number Y08756) corresponding to the beginning of the receptor sequence and the 3' untranslated part of the splice variant h5-HT<sub>4(a)</sub> respectively, were used to amplify 2 µg of total RNA from human atrium. This RNA was then reverse transcribed with oligo(dt) primers and Superscript reverse transcriptase (Life Technologies Inc., Cergy-Pontoise, France). Products of this first reaction were used as templates for a nested PCR amplification using primers HTS5 and HHT43D, a 3' primer specific for h5-HT<sub>4(a)</sub> isoform (Blondel *et al.*, 1998a; accession number Y08756). Both PCR reactions were performed using the following cycle conditions: denaturation for 1 min at 94°C, annealing for 45 s at 55°C, and extension for 1 min 30 s at 72°C with the final extension for 8 min. The PCR products were electrophoresed on a 2% agarose gel containing 0.01% ethidium bromide and photographed under u.v. irradiation at 320 nm. Photograph analysis of the gel revealed two DNA fragments of about 760 and 850 bp, which were cloned into the pGEMT-easy vector (Promega, Charbonnières, France) and sequenced. We found that the 760 bp PCR product corresponds to the 3' end of the h5-HT<sub>4(a)</sub> variant and the 850 bp DNA fragment is the 3' end of the h5-HT<sub>4(e)</sub>

recently isolated by Claeysen *et al.*, (1999) (accession number AJ011371) from human brain. To obtain the full cDNA of the h5-HT<sub>4(e)</sub> receptor, a PCR reaction was performed using primers HHT45 and G2S (5'-GCAGAACGGTGTACAGAGCA-3', accession number AJ011371). PCR was performed with Taq DNA polymerase (Boehringer Mannheim, Meylan, France), 94°C for 3 min, followed by 35 cycles of denaturation (30 s at 94°C), annealing (40 s at 52°C) and elongation (1 min at 72°C). These conditions yielded a band of approximately 1180 bp that was subcloned into the pGEMT-easy vector (Promega, Charbonnières, France) and sequenced with a T7 DNA Polymerase sequencing kit (Amersham Pharmacia Biotech, Orsay, France) according to the manufacturer instructions.

#### Tissue localisation studies

Total RNA was prepared from human brain and peripheral tissues using the Trizol RNA purification system (Life Technologies Inc., Cergy-Pontoise, France). cDNA was prepared using the same procedure as described above and cDNA specific for h5-HT<sub>4(e)</sub> receptor was detected using a nested PCR amplification. A first reaction was performed using 100 ng of cDNA together with specific primers HHT45 and G1rev (5'-GCAGAAGAGCAGGAGGAAGC-3'; accession number AJ011371) designed to the 5'- and 3'-end of the cloned h5-HT<sub>4(e)</sub> receptor, respectively. Products of this first reaction were used as templates for a nested PCR amplification with specific primers HTS5 (Blondel *et al.*, 1998a; accession number Y08756) and G2rev (5'-GAGACAGGGGAACAGCACT-3'; accession number AJ011371). The PCR products were run on a 1.5% agarose gel. 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  $\beta$ -actin (Blondel *et al.*, 1997). All PCR reactions in tissue localization studies were performed as follows: 28 cycles (30 s at 94°C, 45 s at 55°C and 1.5 min at 72°C) and a final elongation (8 min at 72°C).

#### Cell culture

Cell culture materials and reagents were obtained from Life Technologies (Cergy Pontoise, France). CHO, a Chinese hamster ovary cell line and C6, a rat glioma cell line were purchased from ATCC (Rockville, U.S.A.). Stock cultures of CHO and C6-glia cells were grown at 37°C and 5% CO<sub>2</sub> in HamsF12 medium and DMEM medium respectively, supplemented with 10% foetal calf serum, 10 mM HEPES (pH 7.4) and antibiotics.

#### Stable expression of the h5-HT<sub>4(e)</sub> receptor in CHO and C6-glia cell lines

The full coding region of the h5-HT<sub>4(e)</sub> receptor was subcloned into the expression vector pRC/CMV containing the neomycin selection gene (Invitrogen, Carlsbad, CA, U.S.A.). Briefly, confluent cells were transfected with 10  $\mu$ g of the expression vector by electroporation using a gene pulser transfection apparatus (Biorad, Ivry sur Seine, France; setting 960  $\mu$ F, 250 V). Forty-eight hours after the transfection period, neomycin (1.25 mg ml<sup>-1</sup>) was added to the dishes for selection. The antibiotic-containing medium was replaced every 2–3 days over 2 weeks. Isolated colonies were selectively trypsinized for further selection, subcloning and propagation of cell clones. h5-HT<sub>4(e)</sub> expressing clones were detected both by their ability to stimulate cyclic AMP production after

treatment with 5-HT and to bind a specific 5-HT<sub>4</sub> antagonist, [<sup>3</sup>H]-GR 113808.

#### Cyclic AMP radioimmunoassay

For measurement of intracellular cyclic AMP accumulation, stably transfected cells were grown to confluence and were incubated with serum-free medium 4 h before the beginning of the assay. Then, the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline, 10  $\mu$ M pargyline and 1  $\mu$ M GR127935 in CHO cells to block the activity of endogenous 5-HT<sub>1B</sub> receptors. 5-HT or other serotonergic ligands were then added for an additional 15 min. The reaction was stopped by aspiration of the medium and addition of 500  $\mu$ l of ice-cold ethanol. After 30 min incubation at room temperature, the ethanol fraction was collected and evaporated under vacuum. The pellet was reconstituted and cyclic AMP was quantified using a radioimmunoassay kit (cyclic AMP competitive radioimmunoassay, Immunotech, Marseille, France). Student's *t*-tests were performed using the QuickTTest software.

#### Membrane preparation and radioligand binding assays

Membrane preparation and radioligand binding assays were performed as previously described (Blondel *et al.*, 1998a). Briefly, cells grown at confluence were washed twice with Phosphate-Buffered Saline (PBS) and centrifuged at 300  $\times$  *g* for 5 min. The resulting pellet was resuspended in 1 ml of ice-cold HEPES buffer (50 mM, pH 7.4), centrifuged at 40,000  $\times$  *g* for 15 min at 4°C. The final pellet containing intracellular membrane component as well as plasma membranes was resuspended in 1 ml HEPES buffer and protein concentrations were determined by the method of Bradford (1976).

Radioligand binding assays were performed in 500  $\mu$ l buffer (50 mM HEPES, pH 7.4) containing 20  $\mu$ l of [<sup>3</sup>H]-GR113808, 50  $\mu$ g of membrane preparation and 20  $\mu$ l of displacing drug. Saturation experiments were performed using [<sup>3</sup>H]-GR113808 at nine concentrations ranging from 0.01 nM to 4 nM. Non specific binding was measured in the presence of 10  $\mu$ M ML10375 and subtracted from total binding to determine the affinity of [<sup>3</sup>H]-GR113808 for its receptor ( $K_d$ , nM) and the total number of receptors ( $B_{max}$ , fmol mg<sup>-1</sup> protein). At a concentration of [<sup>3</sup>H]-GR113808 corresponding to  $K_d$ , the total radioactivity was >500 d.p.m. and non specific binding <30% of total binding for the saturation experiment to be considered as valid. Competition assays were performed in the presence of nine concentrations of the displacing ligands (10<sup>-12</sup>–10<sup>-4</sup> M) and a concentration of [<sup>3</sup>H]-GR113808 corresponding to its  $K_d$  for the receptor. Incubations were performed at 25°C for 30 min and the reaction was terminated by rapid filtration through Whatman GF/B filter paper using the Brandel model 48R cell harvester. Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data were analysed by computer-assisted nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA, U.S.A.).

#### Antibody production and immunoblotting

A peptide (sequence: G I I D L I E K R K F N Q N S N S T Y C V) corresponding to the second extracellular loop of the 5HT<sub>4</sub> receptor (G21V) was synthesized in an automated synthesizer using the Fmoc technique (Neimark & Briand, 1993). The peptide was then purified by HPLC and checked by mass spectrophotometry.

Rabbits were immunized subcutaneously with a mix of 0.25 mg peptide and 3 mg methylated bovine serum albumin emulsified in complete Freund's adjuvant. Three booster injections were given at the same concentration but in incomplete Freund's adjuvant. Sera were collected 1 week after the last booster injection. Rabbit sera were precipitated at 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation and dissolved in PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) at 1:1 dilution.

The G21V peptide was coupled to activated EAH-Sepharose (Pharmacia diagnostics AB, Uppsala, Sweden) according to the standard procedure. The rabbit immunoglobulin fractions were diluted ten times with PBS and centrifuged at 200 × *g* for 5 min. They were then passed through the column with a flow of 6 mlh<sup>-1</sup> for 3 h at 4°C. Finally the adsorbed antibodies were eluted with 3 ml of 3 M KSCN and dialysed directly against 6 l PBS overnight at 4°C. Specificity of antibodies was checked by enzyme immunoassay as described previously (Lebesgue *et al.*, 1998).

Western blotting was performed as previously described (Lezoualc'h *et al.*, 1998). Briefly, membrane proteins (30 µg) from CHO and C6-glia cells, prepared as described above, were mixed with SDS sample buffer containing 4% β-mercaptoethanol and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a membrane (Hybond-P, Amersham Pharmacia Biotech, Orsay, France) and incubated with the anti-5-HT<sub>4</sub> receptor antibody (see below) overnight at 4°C. The primary antibody was detected by counterstaining with a horseradish peroxidase-linked antibody and visualized by the ECL detection kit (Amersham Pharmacia Biotech, Orsay, France). A part of the SDS gel was stained with Coomassie blue to verify whether equal amounts of proteins had been used.

### Drugs

GR113808 ([1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate) and GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide) were gifts from Glaxo Research Group (Ware, Hertfordshire, U.K.). [<sup>3</sup>H]-GR113808 was purchased from Amersham (Orsay, France). ML10302 (2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride) and ML10375 (2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate) were synthesized as previously

described (Langlois *et al.*, 1994; Yang *et al.*, 1997). 5-HT (5-hydroxytryptamine) and 5-MeOT (5-methoxytryptamine) were from Aldrich (L'Isle d'Abeau Chesnes, France). BIMU1 (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide) and cisapride (*cis*-4-amino-5-chloro-N-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide) were synthesized in our laboratory. Renzapride (BRL 24924) ((±)-endo-4-amin-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride) and SB204070 (8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride) were generously given by SmithKline Beecham (U.K.). RS23597 (3-(piperidine-1-yl)propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride), RS39604 (1-(5-chloro-2(3,5-dimethoxy)benzyloxy-4-aminophenyl)-3-(N-(methylsulfamido)ethyl-4-piperidyl)propanone) and RS67333 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl)-1-propanone) were from Tocris Interchim (Montluçon, France).

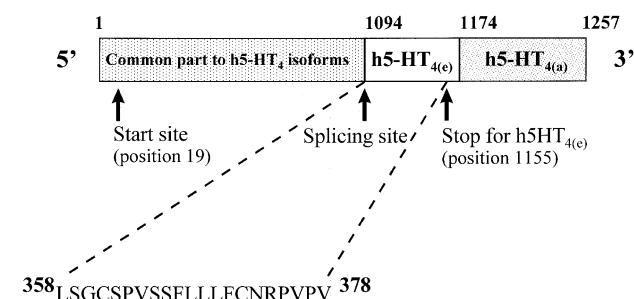
## Results

### Primary structure of the h5-HT<sub>4(e)</sub> receptor

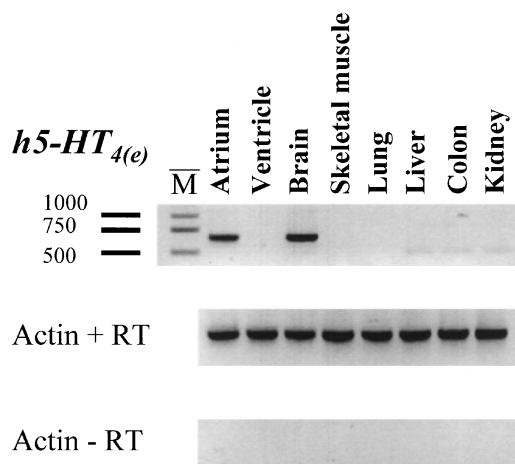
Two DNA fragments of approximately 760 and 850 bp, were isolated when human heart cDNA was used as a template in a nested RACE-PCR amplification using oligonucleotide primers derived from the central region and the 3'-end of the h5-HT<sub>4(a)</sub> receptor subtype (see Methods). The nucleotide sequences of the two amplified fragments were found to correspond to the h5-HT<sub>4(a)</sub> isoform and to the novel h5-HT<sub>4</sub> receptor named h5-HT<sub>4(e)</sub> receptor. It is suggested that this new h5-HT<sub>4(e)</sub> isoform is the orthologue of the corresponding mouse 5-HT<sub>4(e)</sub> [m5-HT<sub>4(e)</sub>] and rat 5-HT<sub>4(e)</sub> [r5-HT<sub>4(e)</sub>] variants (Claeyen *et al.*, 1999). The h5-HT<sub>4(e)</sub> receptor is identical to the other h5-HT<sub>4</sub> splice variants up to the codon coding for Leu<sub>358</sub> (Blondel *et al.*, 1998a; Claeyen *et al.*, 1999). A schematic representation of the h5-HT<sub>4(e)</sub> receptor and its deduced amino acid sequence starting from the splicing site at Leu<sub>358</sub> are shown in Figure 1. Interestingly, we found that the 3' untranslated part of h5-HT<sub>4(e)</sub> receptor cDNA corresponds to the coding region of h5-HT<sub>4(a)</sub> cDNA (Figure 1).

### Tissue-specific expression of the 5-HT<sub>4(e)</sub> receptor

The expression of h5-HT<sub>4(e)</sub> transcripts was analysed by amplification of cDNA derived from RNA isolated from various human tissues using a nested RT-PCR technique with specific primers for the h5-HT<sub>4(e)</sub> isoform (see Methods). PCR resulted in a fragment of around 664 bp as predicted from the nucleotide sequence of the splice variant. Gel electrophoresis of these PCR products revealed that h5-HT<sub>4(e)</sub> transcripts are expressed both in human atrium and brain (Figure 2). In contrast, we found no detectable level of h5-HT<sub>4(e)</sub> mRNA in other 5-HT<sub>4</sub> receptor target tissues such as the kidney and the colon (Figure 2). Interestingly, the h5-HT<sub>4(e)</sub> in the human heart was expressed at high levels in the atrium, but not in the ventricle (Figure 2). We also demonstrated the presence in all tissues of cDNA corresponding to the constitutively expressed β-actin gene (Figure 2), as well as the absence of actin PCR product in a minus reverse transcriptase control (Figure 2). Therefore, signals obtained in our study were not due to any contaminating genomic DNA since no bands were observed when RNA was directly amplified.



**Figure 1** Schematic representation of the h5-HT<sub>4(e)</sub> receptor cDNA and its C terminal amino acid sequence. The numbers indicate the position of base pairs in the cDNA sequence (accession number AJ011371). In all of the cloned h5-HT<sub>4</sub> receptors, the sequence diverges after Leu<sub>358</sub>.



**Figure 2** Expression analysis of h5-HT<sub>4(e)</sub> transcripts in human tissues. Reverse Transcription-PCR analysis was performed with mRNA extracted from various human tissues. The PCR products were analysed on a 1.7% agarose gel and photographs of the ethidium bromide stained gels are shown. The PCR primers used for this analysis and expected length of the PCR products are described in Methods. A positive control was performed using rat/human actin primers on mRNA samples treated with (+ RT) or without (- RT) reverse transcriptase. Positions of three molecular weight markers are indicated in bp. This figure is representative of three separate determinations of h5-HT<sub>4(e)</sub> mRNA expressions obtained by RT-PCR. M, molecular weight markers.

#### Pharmacological characterisation of the h5-HT<sub>4(e)</sub> receptor

The coding region of the h5-HT<sub>4(e)</sub> receptor was cloned in an expression vector containing the neomycin selection gene and was stably expressed in CHO and C6-gliial cells at  $347 \pm 7$  fmol mg<sup>-1</sup> protein and  $88 \pm 7$  fmol mg<sup>-1</sup> protein respectively (Figure 3, Table 1). The expression of the receptor at the plasma membrane was monitored by Western blotting with an affinity rabbit purified polyclonal antibody directed against the second extracellular loop of the h5-HT<sub>4</sub> receptor (Figure 4). Figure 4 shows that CHO and C6-gliial cells transfected with the h5-HT<sub>4(e)</sub> receptor displayed a single specific band migrating around 60 kDa. No specific signal was detected in the control cell clones transfected with the control vector.

Saturation analysis revealed a single saturable binding site of high affinity for [<sup>3</sup>H]-GR113808 in each cellular clone (Figure 3).  $K_d$  values were  $0.22 \pm 0.02$  and  $0.08 \pm 0.03$  nM in CHO and C6 clones expressing h5-HT<sub>4(e)</sub> receptor, respectively (Figure 3, Table 1).  $K_d$  values are similar to the other h5-HT<sub>4</sub> isoforms previously cloned (Claeyen *et al.*, 1997; Van den Wyngaert *et al.*, 1997; Blondel *et al.*, 1998a). For each cell line, non-specific binding increased linearly with increasing ligand concentration (Figure 3). In addition, no detectable binding was found in non transfected CHO and C6-gliial cells (data not shown).

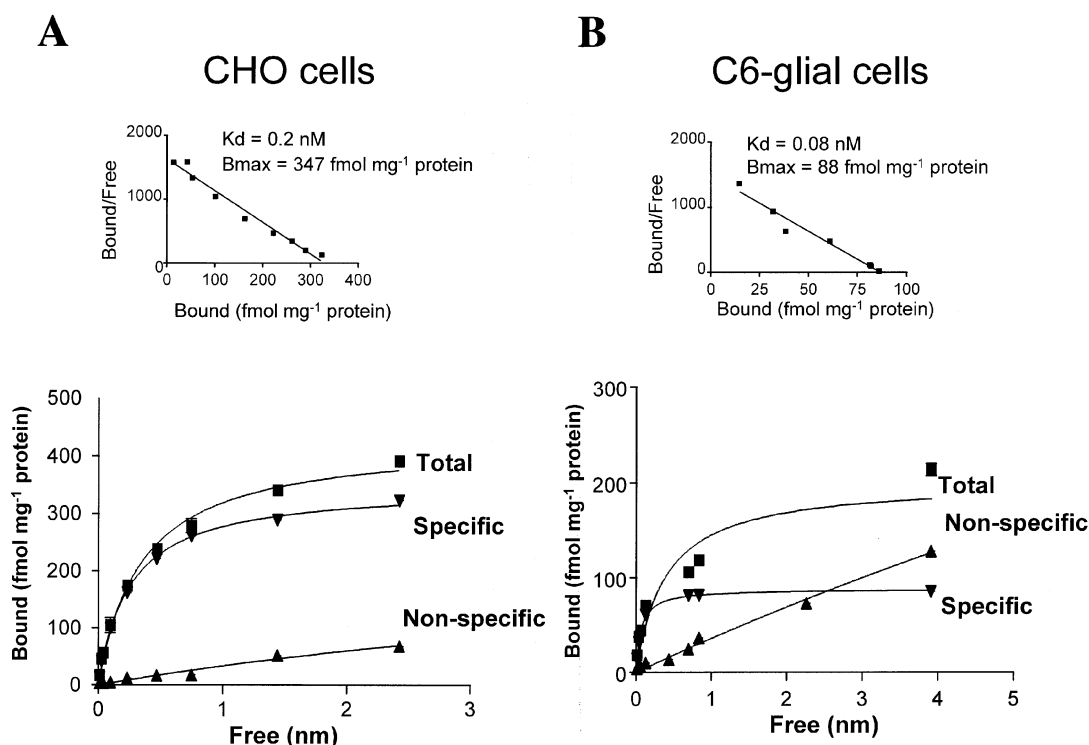
In order to determine and to compare the pharmacological properties of h5-HT<sub>4(e)</sub> receptor in both cell lines, various selective 5-HT<sub>4</sub> agonists and antagonists were tested for the inhibition of [<sup>3</sup>H]-GR113808 binding in CHO and C6 membranes. All the displacement curves were monophasic, giving a Hill coefficient of 0.9 to 1.1. Inhibition curves of radioligand binding in membranes from transfected CHO and C6-gliial cells are shown in Figure 5. Comparison analysis

**Table 1** Affinities of various 5-HT<sub>4</sub> ligands that compete with the binding of 0.2 and 0.08 nM [<sup>3</sup>H]-GR113808 in CHO and C6-gliial cells respectively, stably expressed with h5-HT<sub>4(e)</sub> receptor

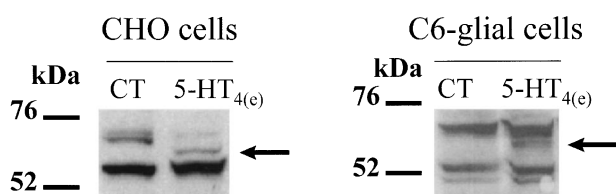
<sup>3</sup> [H] GR113808	CHO cells	C6-gliial cells
$K_d$ (nM)	$0.22 \pm 0.02$	$0.08 \pm 0.03$
$B_{max}$ (fmol mg <sup>-1</sup> prot)	$347 \pm 7$	$88 \pm 7$
<b>AGONISTS</b>		
	$K_i$ (nM) (95% confidence intervals)	
5-HT	629	1046
	(433–914)	(557–1965)
5-MeOT	1132	6920
	(780–1640)	(4970–9640)
ML10302	4.2	5.6
	(3–5.8)	(3.3–9.6)
Renzapride	238	1723
	(130–290)	(1064–2789)
Cisapride	248	173
	(176–348)	(129.7–232)
RS67333	6.1	15.2
	(4.7–7.8)	(10.3–22.4)
BIMUI	3.8	76
	(29–51)	(53–109)
<b>ANTAGONISTS</b>		
ML10375	0.11	0.34
	(0.06–0.18)	(0.24–0.47)
RS23597	29	42
	(20–43)	(29–61)
RS39604	2.99	5.2
	(2.15–4.15)	(3.4–7.9)
SB204070	0.059	0.095
	(0.04–0.08)	(0.06–0.16)

$K_d$  and  $B_{max}$  values were obtained from saturation binding experiments with [<sup>3</sup>H]-GR113808 in CHO and C6-gliial cells stably transfected with the h5-HT<sub>4(e)</sub> receptor. Non specific binding was determined with 10 μM ML10375.  $K_i$  values were obtained from competition binding assays with 0.2 nM (CHO cells) or 0.08 nM (C6-gliial cells) [<sup>3</sup>H]-GR113808. IC<sub>50</sub> values correspond to a displacement of 50% of specific radio ligand binding and were converted to  $K_i$  values according to the equation:  $K_i = IC_{50} / (1 + (\text{concentration of } [^3\text{H}]\text{-GR113808}/K_d))$ . The data were obtained using nine concentrations of ligand and all experiments were performed in triplicate.

of the binding affinities ( $K_i$ ) between CHO and C6-gliial cells expressing the h5-HT<sub>4(e)</sub> receptor revealed a similar rank order of potency for all the ligands tested in each cell line (Table 1). The data summarized in Table 1 demonstrate that the pharmacological profile of the h5-HT<sub>4(e)</sub> receptor in terms of rank order of potencies of the different ligands tested, is similar to those found for native 5-HT<sub>4</sub> receptors as studied *in vivo* in human atria (Kaumann *et al.*, 1996), rat striatum (Langlois *et al.*, 1994; Yang *et al.*, 1997), mouse colliculi (Ansanay *et al.*, 1996), or after expression of cloned human, mouse or rat 5-HT<sub>4</sub> receptor isoforms in cell lines (Gerald *et al.*, 1995; Adham *et al.*, 1996; Claeyen *et al.*, 1996; Blondel *et al.*, 1997; 1998a). The rank order of apparent antagonist and agonists affinities were, respectively, SB204070 > ML10375 > RS39604 > RS23597 and ML10302 > RS67333 > BIMUI > renzapride > cisapride > 5-HT > 5-MeOT (Table 1). However, the benzamide derivative renzapride had a lower affinity for the h5-HT<sub>4(e)</sub> receptor than 5-HT in C6-gliial cells (Table 1). Furthermore, we found a slightly better affinity (about 2 fold) for all the ligands tested in CHO cells compared to C6-gliial cells, to the exception of the gastroprokinetic agent, cisapride (Table 1).



**Figure 3** Saturation analysis of [<sup>3</sup>H]-GR113808 binding to the h5-HT<sub>4(e)</sub> receptor stably expressed in CHO and C6-gial cells. Membranes harvested from stably transfected CHO (A) and C6-gial cells (B) were incubated with nine concentrations of [<sup>3</sup>H]-GR113808 for 30 min at 25°C. Non-specific binding was determined with 10 μM ML10375. Results are from single experiments but are representative of three such experiments.



**Figure 4** Western blot detection of the h5-HT<sub>4(e)</sub> receptor in h5-HT<sub>4(e)</sub> receptor transfected subclones. CHO and C6-gial cells were transfected either with the h5-HT<sub>4(e)</sub> receptor neo-vector or the corresponding control (CT) and selected for their resistance to the antibiotic. Membrane protein extracts were separated on a polyacrylamide gel and analysed by immunoblotting with antiserum raised against the second extracellular loop of the h5-HT<sub>4(e)</sub> receptor (see Methods). In CHO and C6-gial cells stably transfected with the h5-HT<sub>4(e)</sub> receptor, a specific band migrating to the level of 60 kDa was detected whereas in control cells no labelling was detected at this position. This immunoblot is representative of three independent experiments. Molecular weight marker positions are indicated in kilodaltons.

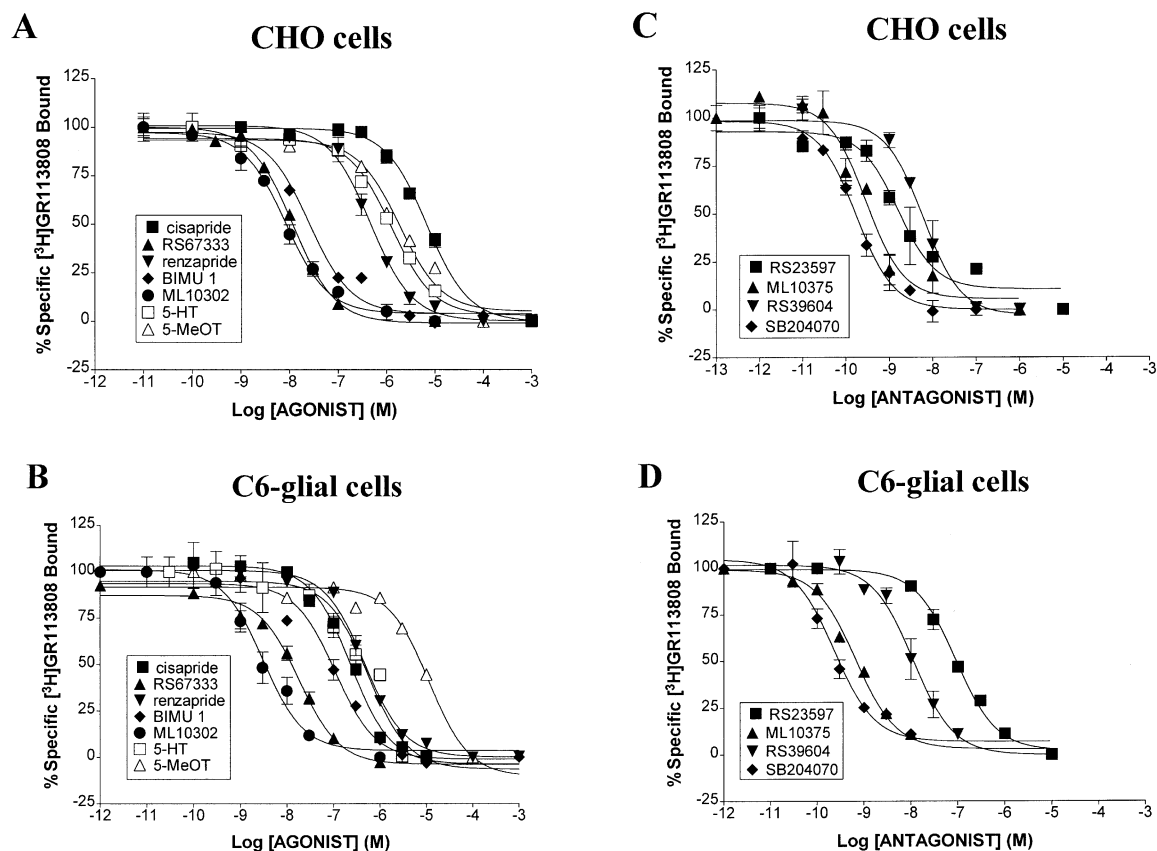
#### Constitutive activity of the h5-HT<sub>4(e)</sub> receptor

The ability of the h5-HT<sub>4(e)</sub> receptor to stimulate adenylyl cyclase activity was analysed by measuring cyclic AMP production. A constitutive activity of the h5-HT<sub>4(e)</sub> receptor was observed in the absence of any 5-HT<sub>4</sub> ligand (Figure 6). Indeed, we found that basal cyclic AMP levels were increased about 3 fold in CHO cells expressing the h5-HT<sub>4(e)</sub> receptor as compared to non transfected cells (Figure 6). These data indicate that expression of h5-HT<sub>4(e)</sub> splice variant induced a spontaneously active receptor state. In addition, two selective 5-HT<sub>4</sub> antagonists, GR113808 (1 μM) and ML10375 (1 μM), significantly decreased basal cyclic AMP production to values which

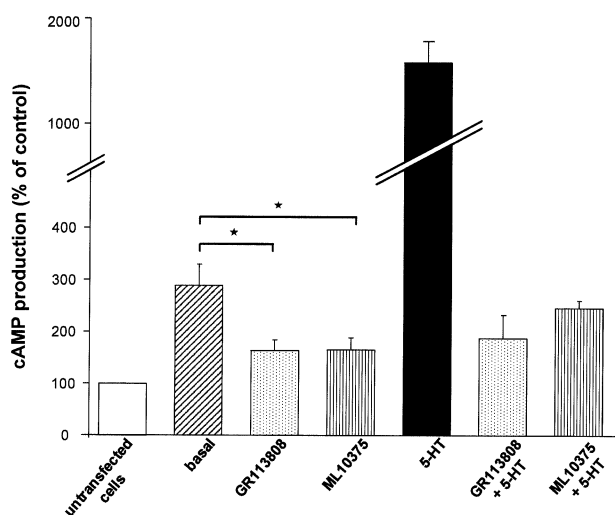
were only 64 ± 21% and 65 ± 24% above the level of cyclic AMP production in non transfected cells, respectively (Figure 6). These results show that these two antagonists behaved as inverse agonists.

#### Functional effects of 5-HT<sub>4</sub> agonists

5-HT (1 μM) had no effect on cyclic AMP production in non transfected cells (data not shown). In vector transfected cells, 5-HT significantly induced stimulation of basal cyclic AMP up to 8 fold in both cell lines (Figure 6). Dose response curves using the most representative 5-HT<sub>4</sub> agonists are shown in Figure 7A,B (see Ford & Clarke, 1993; Eglén *et al.*, 1995; for details and bibliography on the compounds used). All 5-HT<sub>4</sub> agonists used in these experiments stimulated cyclic AMP production in a dose dependent manner, both in CHO (Figure 7A) and C6-gial cells (Figure 7B). However, depending on the cell line we found some differences in their rank order of potency in stimulating cyclic AMP formation (Table 2). Rank orders based on mean *EC*<sub>50</sub> values were as follows: 5-MeOT > 5-HT > ML10302 = BIMU1 > renzapride > cisapride for h5-HT<sub>4(e)</sub> expressed in CHO cells and 5-HT > renzapride > 5-MeOT > BIMU1 > cisapride for h5-HT<sub>4(e)</sub> expressed in C6-gial cells (Table 2). Most importantly *EC*<sub>50</sub> values for 5-HT, renzapride and cisapride were twice lower in C6-gial cells than in CHO cells, while the *EC*<sub>50</sub> for the indoleamine 5-MeOT was 10 fold higher in C6 gial than in CHO cells indicating that the cellular environment can influence the coupling efficiency of the h5-HT<sub>4(e)</sub> receptor to its effector. With regard to agonist activities, 5-HT and 5-MeOT displayed full agonist properties, whereas BIMU1, ML10302, renzapride and cisapride acted as partial agonists (Figure 7A,B, Table 2). In addition, the rank order of affinities obtained with the agonists from binding assays was



**Figure 5** Inhibition of specific [<sup>3</sup>H]-GR113808 binding to the h5-HT<sub>4(e)</sub> receptor in CHO and C6-gliial cells. Membranes from stable CHO (A,C) and C6-gliial (B,D) cells expressing the h5-HT<sub>4(e)</sub> receptor were incubated with 0.2 and 0.08 nM of [<sup>3</sup>H]-GR113808, respectively, in the presence or absence of increasing concentrations of 5-HT<sub>4</sub> agonists (A,B) or antagonists (C,D). Non-specific binding was defined by 10 μM ML10375. Data are presented as a percentage of specific binding in the absence of displacing drug. Results are from single experiments but are representative of three such experiments using a range of nine concentrations of ligands. Data were analysed by computer-assisted non-linear regression analysis (GraphPad, Prism Software). The corresponding *K<sub>i</sub>* values are presented in Table 1.

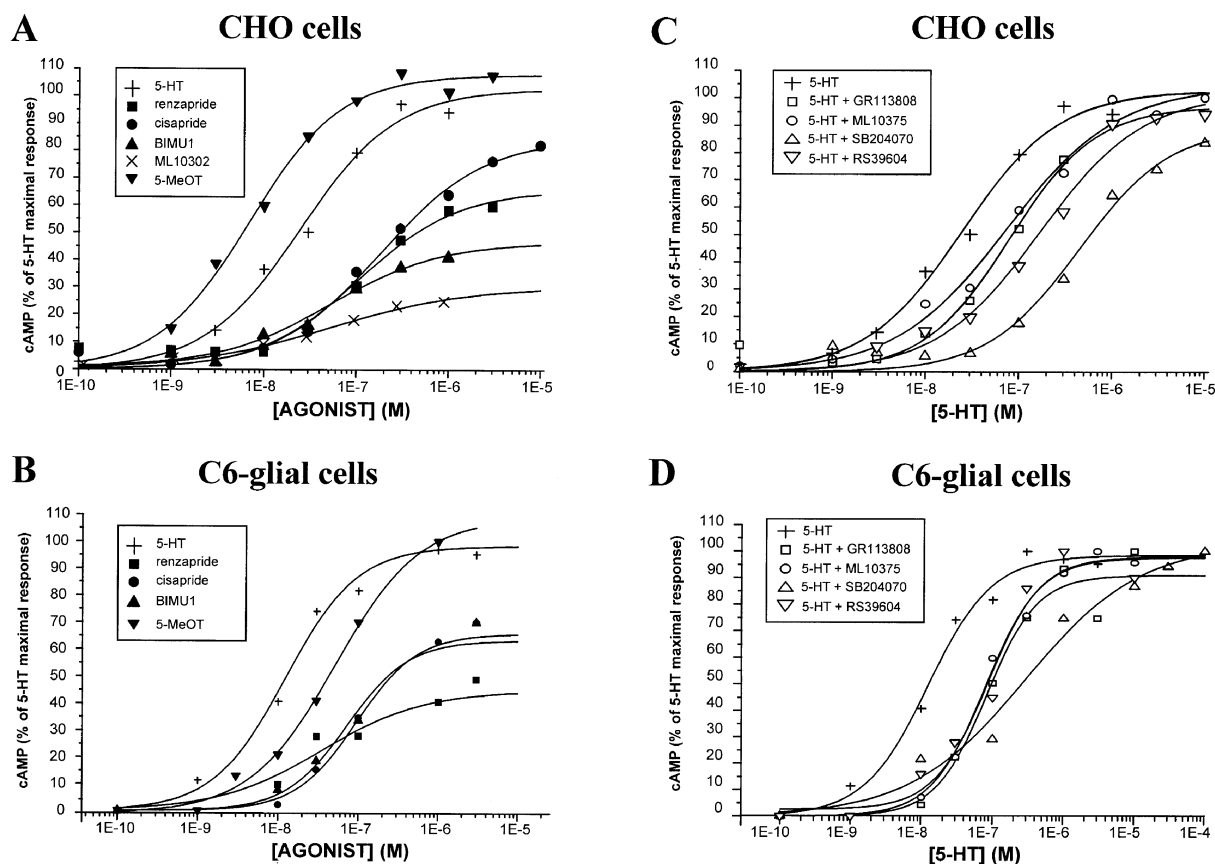


**Figure 6** Inverse agonist effects of GR113808 and ML10375 in CHO cells expressing the h5-HT<sub>4(e)</sub> receptor. The effects of 5-HT, GR113808 and ML10375 in the absence or in the presence of 5-HT on cyclic AMP production are expressed as percentage of control (untransfected CHO cells). GR113808, ML10375 and 5-HT were used at a concentration of 1 μM. Absolute values for cyclic AMP production in untransfected cells and under basal conditions were 4.3 ± 0.8 pmoles well<sup>-1</sup> and 13.0 ± 1.6 pmoles well<sup>-1</sup>, respectively. Values are mean ± s.e.mean of three independent experiments performed in duplicate. \**P* < 0.05 versus indicated values by *t*-test.

inversely correlated to their rank order of potencies obtained from functional studies in both cell lines (compare Tables 1 and 2). For instance, 5-MeOT had the lowest affinity for competing with [<sup>3</sup>H]-GR113808 binding (Table 1) whereas, in functional studies, 5-MeOT exhibited the highest coupling efficiency at the cloned h5-HT<sub>4(e)</sub> (Table 2). BIMU1 behaved as a highly potent 5-HT<sub>4</sub> agonist and displayed only 47 ± 5% and 63 ± 5% of the 5-HT stimulatory effect in CHO and C6-gliial cells, respectively (Table 2).

#### Functional effects of 5-HT<sub>4</sub> antagonists

Antagonists were tested at a concentration of ten times *K<sub>i</sub>* and caused a parallel concentration-dependent rightward displacement of the 5-HT curve without depression of the maximum response (Figure 7C,D). As previously observed for *EC*<sub>50</sub> values obtained from functional studies with 5-HT<sub>4</sub> agonists, estimated *K<sub>b</sub>* values were twice lower in C6-gliial cells compared to CHO cells (Table 2). Among the different 5-HT<sub>4</sub> antagonists tested in our system, SB204070 was the most potent antagonist in inhibiting 5-HT-induced cyclic AMP production (Table 2). In addition, rank orders of *K<sub>b</sub>* values obtained for the different antagonists tested from functional studies in our cellular systems are comparable with those obtained with *K<sub>i</sub>* values derived from binding experiments (Tables 1 and 2).



**Figure 7** cyclic AMP responses to various 5-HT<sub>4</sub> ligands in CHO and C6-gliial cells expressing the h5-HT<sub>4(e)</sub> receptor. cyclic AMP measurements were performed as described in Methods. In (A) and (B) the cells were incubated for 15 min with increasing concentrations of agonists and cyclic AMP production was then quantified. In (C) and (D), the cells were preincubated for 15 min with a concentration of antagonist corresponding to 10 fold the  $k_i$  value as measured in binding experiments (see Table 1); increasing concentrations of 5-HT were then added for an additional 15 min before cyclic AMP was measured. Values are expressed as the percentage of 5-HT maximal response. Each point is the mean of at least three independent experiments, each performed in triplicate.  $EC_{50}$  and  $E_{max}$  values are presented in Table 2.

## Discussion

In this paper, we report the cloning of a novel h5-HT<sub>4</sub> receptor from human heart atrium. Sequence analysis revealed that this isoform corresponds to the recently identified h5-HT<sub>4(e)</sub> receptor isolated from human brain which is considered to be the human counterpart of rat and mouse isoforms (Claeyens *et al.*, 1999). This splice variant together with previously cloned h5-HT<sub>4(a)</sub>, h5-HT<sub>4(b)</sub>, h5-HT<sub>4(c)</sub> and h5-HT<sub>4(d)</sub> isoforms is generated by splicing events occurring in the C terminus of the h5-HT<sub>4</sub> receptor, just after the amino acid Leu<sub>358</sub> (Blondel *et al.*, 1997; 1998a; Claeyens *et al.*, 1997; Van den Wyngaert *et al.*, 1997). Interestingly, we found that the 3' untranslated part of the h5-HT<sub>4(e)</sub> cDNA is represented by the h5-HT<sub>4(a)</sub> as a non coding sequence (Figure 1). This molecular event is not only restricted to the h5-HT<sub>4</sub> receptor since m5-HT<sub>4(a)</sub> is also found in the 3' untranslated parts of m5-HT<sub>4(e)</sub> and m5-HT<sub>4(f)</sub> (Claeyens *et al.*, 1999). Although an analysis of the exon-intron organization of this receptor gene has still to be performed, these observations suggest that the organization of the 5-HT<sub>4</sub> receptor gene is well conserved among animal species.

The h5-HT<sub>4(e)</sub> receptor isoform is not restricted to the brain as reported for the m5-HT<sub>4(e)</sub> and m5-HT<sub>4(f)</sub> variants (Claeyens *et al.*, 1999) but we found in this study that this receptor is also expressed in human heart atrium. However, as reported for the h5-HT<sub>4(a)</sub>, h5-HT<sub>4(b)</sub> and h5-HT<sub>4(c)</sub> isoforms (Blondel *et al.*, 1998a), h5-HT<sub>4(e)</sub> 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 (Kaumann, 1991; Schoemaker *et al.*, 1993). Concerning the distribution pattern of 5-HT<sub>4</sub> receptor messengers, some differences exist between species. For instance, m5-HT<sub>4(b)</sub> mRNA is present in the bladder and kidney whereas h5-HT<sub>4(b)</sub> messenger is not detectable in these organs (Blondel *et al.*, 1998a; Claeyens *et al.*, 1999). Only the development of specific antibodies against 5-HT<sub>4</sub> subtypes will enable us to assess 5-HT<sub>4</sub> receptor expression at the protein level and will confirm 5-HT<sub>4</sub> isoform differential distributions between species.

A major issue is to understand the physiological relevance of the restricted expression of 5-HT<sub>4</sub> receptors. Four 5-HT<sub>4</sub> receptor isoforms have been shown to be expressed in human atria. Detailed analysis of 5-HT<sub>4</sub> receptor pharmacology is therefore crucial for the elucidation of their pathological implication in atrial arrhythmias. We have characterized in this study the radioligand binding and functional properties of recombinant h5-HT<sub>4(e)</sub> receptor stably expressed in two different cellular systems, CHO and C6-gliial cells. To assess the expression of the h5-HT<sub>4(e)</sub> receptor in the transfected cell lines, polyclonal antibodies were produced against a peptide corresponding to the second extracellular loop of the h5-HT<sub>4</sub> receptor. The anti-5-HT<sub>4</sub> receptor antibody recognised the transfected h5-HT<sub>4(e)</sub> receptor as shown by Western blotting experiments (Figure 4). We anticipate that this antibody which is the first anti-5-HT<sub>4</sub> receptor antibody available will be a



**Table 2** Pharmacological profile of cyclic AMP response using the h5-HT<sub>4(e)</sub> receptor stably transfected in CHO and C6 glial cells in response to 5-HT<sub>4</sub> agonists and antagonists

	CHO cells		C6-glia cells	
	EC <sub>50</sub> (nM)	E <sub>max</sub> (% maximal response to 5-HT)	EC <sub>50</sub> (nM)	E <sub>max</sub> (% maximal response to 5-HT)
<b>AGONISTS</b>				
5-HT	25 ± 6	100	12 ± 2	100
5-MeOT	7.0 ± 0.7	108 ± 2	50 ± 4	106 ± 10
renzapride	107 ± 41	66 ± 6	35 ± 19	45 ± 5
cisapride	180 ± 35	85 ± 4	94 ± 17	66 ± 3
BIMU1	52 ± 23	47 ± 5	72 ± 22	63 ± 5
ML 10302	51 ± 23	30 ± 3	ND	ND
<b>ANTAGONISTS</b> (concentration)				
	EC <sub>50</sub> (nM)	K <sub>b</sub> (nM)	EC <sub>50</sub> (nM)	K <sub>b</sub> (nM)
GR113808	79 ± 12 (3 nM)	1.38	81 ± 22 (0.88 nM)	0.53
ML 10375	74 ± 17 (1 nM)	1.01	79 ± 9 (3 nM)	0.52
SB204070	443 ± 133 (1 nM)	0.06	285 ± 157 (1 nM)	0.036
RS39604	166 ± 45 (30 nM)	5.3	106 ± 6 (50 nM)	6.25

cyclic AMP production was measured in CHO and C6-glia expressing the h5-HT<sub>4(e)</sub> receptor. EC<sub>50</sub> values correspond to the concentration of agonists required to obtain half-maximal stimulation of adenylyl cyclase. The maximum response produced by each drug was normalised to the 5-HT induced maximum response. For the antagonists, EC<sub>50</sub> values correspond to the concentration of 5-HT required to produce half-maximal stimulation of adenylyl cyclase in the presence of the indicated concentration of antagonists. K<sub>b</sub> values were calculated with the formula:  $K_b = [B]/[(A'/A)-1]$  where [B] is the concentration of the agonists and A' and A the EC<sub>50</sub> values of 5-HT measured respectively in the presence and absence of antagonists. Values are the means ± s.e.mean of three experiments performed in triplicate. They were analysed by non linear regression analysis. ND, non determined.

useful tool for studying the anatomic distribution, regulation and function of h5-HT<sub>4</sub> receptors.

Using the radiolabelled specific antagonist [<sup>3</sup>H]-GR113808, we found that densities of specific binding sites in CHO and C6 cells were 347 ± 7 fmol mg<sup>-1</sup> protein and 88 ± 7 fmol mg<sup>-1</sup> protein, respectively (Figure 3). These values are comparable to those found in rat and human brains where levels of native 5-HT<sub>4</sub> receptor expression have been reported to vary between 20 and 400 fmol mg<sup>-1</sup> protein depending on the considered brain region (Waeber *et al.*, 1993; 1994). On the contrary, the density of human atrial binding sites with 5-HT<sub>4</sub> receptor characteristics have been found to be around 4 fmol mg<sup>-1</sup> protein which is much lower than in our cellular systems and in the central nervous system (Kaumann *et al.*, 1996).

Binding experiments with [<sup>3</sup>H]-GR113808 showed a typical 5-HT<sub>4</sub> profile for the h5-HT<sub>4(e)</sub> receptor expressed in CHO and C6-glia cells (Figure 3). K<sub>i</sub> values (Figure 3, Table 1) measured in our study are in good agreement with those reported in human atria (Kaumann *et al.*, 1996), in human brain membranes (Waeber *et al.*, 1993), and in COS-7 cells transiently transfected with other h5-HT<sub>4</sub> receptor splice variants (Blondel *et al.*, 1997; 1998a; Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997). The rank order of affinity of compounds used for the competition of [<sup>3</sup>H]-GR113808 binding on CHO and C6 membranes matched the expected pharmacological profile. The ligand found to have the lowest K<sub>i</sub> values was SB204070 (Table 1). This benzodioxane derivative is the most potent and selective 5-HT<sub>4</sub> antagonist described so far (Gaster & Sanger, 1994). On the other hand, 5-MeOT had the lowest affinity for competing with [<sup>3</sup>H]-GR113808 binding in both cell lines (Table 1). This latter

observation correlates well with that obtained in binding studies using preparations such as human brain membranes (Waeber *et al.*, 1993). Furthermore, we found a good correlation between the affinity constants of 5-HT and 5-MeOT for the cloned h5-HT<sub>4(e)</sub> receptor and those obtained for the same agonists when binding to the native cardiac 5-HT<sub>4</sub> receptors in human and pig atria (Kaumann *et al.*, 1995; 1996).

Interestingly, we found that the rank order of affinities obtained with the agonists from binding assays was inversely correlated to their rank order of potencies obtained from functional studies in both cell lines (Tables 1 and 2). A similar observation has already been reported for r5-HT<sub>4(a)</sub> and r5-HT<sub>4(b)</sub> receptors when expressed in recombinant systems and it was suggested that some ligands might differentially activate a receptor reserve (Gerald *et al.*, 1995). Clearly, these pharmacological differences between the affinity of a given ligand and its functional effects will have to be taken into consideration in the future when designing novel 5-HT<sub>4</sub> drugs.

A constitutive activation was found with the h5-HT<sub>4(e)</sub> receptor expressed in CHO cells. Such constitutive coupling has already been described for other 5-HT<sub>4</sub> receptor splice variants such as h5-HT<sub>4(c)</sub>, m5-HT<sub>4(e)</sub> and m5-HT<sub>4(f)</sub> receptors (Blondel *et al.*, 1998b, Claeysen *et al.*, 1999). In addition we report that two 5-HT<sub>4</sub> antagonists, GR113808 and ML10375, significantly decreased basal cyclic AMP values indicating that these antagonists behave as inverse agonists. This was not due to the presence of 5-HT in the culture medium since cells were deprived of serum (see Methods). ML10375 also reduced basal adenylyl cyclase activity in COS cells transfected with the h5-HT<sub>4(e)</sub> receptor (Blondel *et al.*, 1998b). Therefore, these inverse agonistic properties are likely to be of therapeutic relevance in

the treatment of disorders involving 5-HT<sub>4</sub> receptors, particularly if these disorders are directly related to constitutive h5-HT<sub>4</sub> receptor activation.

Since the h5-HT<sub>4(e)</sub> receptor had similar ligand binding properties in CHO and C6-glia cell lines and a higher expression level ( $B_{max}$ ) in CHO cells, one would have expected to obtain lower  $EC_{50}$  values and higher maximal stimulation ( $E_{max}$ ) of adenylyl cyclase activity induced by 5-HT<sub>4</sub> agonists in C6-glia as compared to CHO cells. However, 5-HT induced a similar 8 fold maximal stimulation of adenylyl cyclase activity in both cell lines. Moreover, with the exception of 5-MeOT and BIMU1, all other 5-HT<sub>4</sub> agonists had a higher potency in activating adenylyl cyclase (lower  $EC_{50}$  values) in C6-glia cells than in CHO cells (Table 2). This is in contradiction with the previously reported inverse relationship between receptor number and  $EC_{50}$  value observed for the  $\beta_2$ -adrenergic receptor-mediated cyclic AMP accumulation in several heterologous expression systems (Bouvier *et al.*, 1988; Whaley *et al.*, 1994). Since functional interactions between G protein-coupled receptors and G proteins are strongly influenced by their relative expression level (Kenakin, 1996), one may speculate that C6-glia cells have a higher level of G<sub>s</sub> protein expression than CHO cells. Alternatively, C6-glia and CHO cells may possess different isoforms of G<sub>s</sub> proteins with different coupling efficiency to 5-HT<sub>4</sub> receptors and/or adenylyl cyclase. Indeed, two splice variants of G<sub>s</sub> have been described, a short (G<sub>s</sub> $\alpha_s$ ) and a long isoform (G<sub>s</sub> $\alpha_L$ ) (Bray *et al.*, 1986; Robishaw *et al.*, 1986), with G<sub>s</sub> $\alpha_s$  being more efficiently coupled to adenylyl cyclase than G<sub>s</sub> $\alpha_L$  (Seifert *et al.*, 1998). Expression analysis of G<sub>s</sub> $\alpha$  splice variants in CHO and C6-glia cell lines will be necessary to test these hypotheses.

Some interesting features were found with the effects of the 5-HT<sub>4</sub> agonists, cisapride and 5-MeOT. Human detrusor muscle showed an unusually low potency for 5-MeOT compared to other tissues (Candura *et al.*, 1996). The fact that 5-MeOT behaved as a full agonist in our cellular models excludes the possibility that the h5-HT<sub>4(e)</sub> receptor mediates 5-HT<sub>4</sub> effects in the bladder. Cisapride was a partial and not highly potent agonist whereas 5-MeOT displayed full agonist properties in stable cell lines expressing the h5-HT<sub>4(e)</sub> receptor

(Table 2). *In vivo* studies have shown that cisapride acts as partial agonist in human heart whereas it behaves as a full and highly potent agonist in brain (reviewed in Eglen *et al.*, 1995). Additional data show that the pharmacological behaviour of the h5-HT<sub>4(e)</sub> is close to the cardiac native h5-HT<sub>4</sub> receptor. Indeed, ML10302 and renzapride behaved like partial agonists on the h5-HT<sub>4(e)</sub> with stimulatory effects that were 30 and 66% of the maximal 5-HT-induced cyclic AMP synthesis in CHO cells, respectively (Table 2). These results are in agreement with the reported low efficacy of these compounds in activation of I<sub>Ca</sub> currents or myocyte contractility in human atrium (Sanders *et al.*, 1995; Blondel *et al.*, 1997). However, previous results from our laboratory have shown that the pharmacological profile of the h5-HT<sub>4(a)</sub> receptor in recombinant systems is also very similar to the native h5-HT<sub>4</sub> receptor in human atrium (Blondel *et al.*, 1997). Thus, it is crucial in the future to determine the relative contribution of each h5-HT<sub>4</sub> receptor isoform to the positive inotropic, chronotropic and lusitropic effects of 5-HT in human atrium and to the genesis of pathological events such as cardiac arrhythmias.

In conclusion, we have isolated the h5-HT<sub>4(e)</sub> receptor from human atrium and characterized its pharmacological profile in two cellular systems, CHO and C6-glia cell lines. We did not observe any major differences in the binding affinity of the different ligands tested between the h5-HT<sub>4(e)</sub> receptor, other 5-HT<sub>4</sub> receptor splice variants and the two cell lines used in the study. However, the rank order of affinities obtained with the agonists from binding assays was inversely correlated to their rank order of potencies obtained from functional studies. Furthermore, functional potency of h5-HT<sub>4(e)</sub> receptor ligands was dependent on the cellular context in which the receptor was expressed. Finally, we found that the h5-HT<sub>4(e)</sub> receptor has a pharmacological profile which is very close to that of the native h5-HT<sub>4</sub> receptor in human atrium.

We wish to thank Drs Jean-Jacques Mercadier (Hôpital Bichat, Paris, France), Christian Brechot (Hôpital Necker-Enfants Malades, Paris), and Pierre Peillac (Hôpital St-Louis, Paris) for providing human tissues. Frank Lezoualc'h was a recipient of a grant from the Fondation pour la Recherche Médicale.

## References

- ADHAM, N., GERALD, C., SCHECHTER, L., VAYASSE, P., WEINSHANK, R. & BRANCHEK, T. (1996). [<sup>3</sup>H]5-hydroxytryptamine labels the agonist high affinity state of the cloned rat 5-HT<sub>4</sub> receptor. *Eur. J. Pharmacol.*, **304**, 231–235.
- ANSANAY, H., SEBBEN, M., BOCKAERT, J. & DUMUIS, A. (1996). Pharmacological comparison between [<sup>3</sup>H]GR113808 binding sites and functional 5-HT<sub>4</sub> receptors in neurons. *Eur. J. Pharmacol.*, **298**, 165–174.
- BLONDEL, O., GASTINEAU, M., DAHMOUNE, Y., LANGLOIS, M. & FISCHMEISTER, R. (1998a). Cloning, expression and pharmacology of four human 5-HT<sub>4</sub> receptor isoforms produced by alternative splicing in the carboxyl terminus. *J. Neurochem.*, **70**, 2252–2261.
- BLONDEL, O., GASTINEAU, M., LANGLOIS, M. & FISCHMEISTER, R. (1998b). The 5-HT<sub>4</sub> receptor antagonist ML10375 inhibits the constitutive activity of human 5-HT<sub>4(e)</sub> receptor. *Br. J. Pharmacol.*, **125**, 595–597.
- BLONDEL, O., VANDECASTEELE, G., GASTINEAU, M., LECLERC, S., DAHMOUNE, Y., LANGLOIS, M. & FISCHMEISTER, R. (1997). Molecular and functional characterization of a 5-HT<sub>4</sub> receptor cloned from human atrium. *FEBS Lett.*, **412**, 465–474.
- BOCKAERT, J., CLAEYSEN, S., SEBBEN, M. & DUMUIS, A. (1998). 5-HT<sub>4</sub> receptors: gene, transduction and effects on olfactory memory. *Ann. N.Y. Acad. Sci.*, **861**, 1–15.
- BOCKAERT, J. & PIN, J.P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.*, **18**, 1723–1729.
- BOUVIER, M., HNATOWICH, M., COLLINS, S., KOBILKA, B.K., DEBLASI, A., LEFKOWITZ, R.J. & CARON, M.G. (1988). Expression of a human cDNA encoding the  $\beta_2$ -adrenergic receptor in Chinese hamster fibroblasts (CHW): functionality and regulation of the expressed receptors. *Mol. Pharmacol.*, **33**, 133–139.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
- BRAY, P., CARTER, A., SIMONS, C., GUO, V., PUCKETT, C., KAMHOLZ, J., SPIEGEL, A. & NIRENBERG, M. (1986). Human cDNA clones for four species of G $\alpha_s$  signal transduction protein. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8893–8897.
- CANDURA, S.M., MESSORI, E., FRANCESCHETTI, G.P., D'AGOSTINO, G., VICINI, D., TAGLIANI, M. & TONINI, M. (1996). Neural 5-HT<sub>4</sub> receptors in the human isolated detrusor muscle: effects of indole, benzimidazolone and substituted benzamide agonists and antagonists. *Br. J. Pharmacol.*, **118**, 1965–1970.

- CLAEYSEN, S., FAYE, P., SEBBEN, M., LEMAIRE, S., BOCKAERT, J. & DUMUIS, A. (1997). Cloning and expression of human 5-HT<sub>4s</sub> receptors. Effect of receptor density on their coupling to adenylyl cyclase. *Neuroreport*, **8**, 3189–3196.
- CLAEYSEN, S., SEBBEN, M., BECAMEL, C., BOCKAERT, J. & DUMUIS, A. (1999). Novel brain-specific 5-HT<sub>4</sub> receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol. Pharmacol.*, **55**, 910–920.
- CLAEYSEN, S., SEBBEN, M., JOURNOT, L., BOCKAERT, J. & DUMUIS, A. (1996). Cloning, expression and pharmacology of the mouse 5-HT<sub>4L</sub> receptor. *FEBS Lett.*, **398**, 19–25.
- DUMUIS, A., SEBBEN, M. & BOCKAERT, J. (1989). The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT<sub>4</sub>) positively coupled to adenylyl cyclase in neurons. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 403–410.
- EGLÉN, R.M., WONG, E.H.F., DUMUIS, A. & BOCKAERT, J. (1995). Central 5-HT<sub>4</sub> receptors. *Trends Pharmacol. Sci.*, **16**, 391–397.
- FORD, A.P. & CLARKE, D.E. (1993). The 5-HT<sub>4</sub> receptor. *Med. Res. Rev.*, **13**, 633–662.
- GASTER, L.M. & SANGER, G.J. (1994). SB204070 : 5-HT receptor antagonists and their potential therapeutic utility. *Drugs Future*, **19**, 1109–1121.
- GERALD, C., ADHAM, N., KAO, H.T., OLSEN, M.A., LAZ, T.M., SCHECHTER, L.E., BARD, J.E., VAYSSE, P.J.J., HARTIG, P.R., BRANCHEK, T.A. & WEINSHANK, R.L. (1995). The 5-HT<sub>4</sub> receptor: molecular cloning and pharmacological characterization of two splice variants. *EMBO J.*, **14**, 2806–2815.
- HEDGE, S. & EGLÉN, R. (1996). Peripheral 5-HT<sub>4</sub> receptors. *FASEB J.*, **10**, 1398–1407.
- HOVE-MADSEN, L., MÉRY, P.-F., JUREVICIUS, J., SKEBERDIS, A.V. & FISCHMEISTER, R. (1996). Regulation of myocardial calcium channels by cyclic AMP metabolism. *Basic Res. Cardiol.*, **91**, 1–8.
- KAUMANN, A.J. (1991). 5-HT<sub>4</sub>-like receptors in mammalian atria. *J. Neural Transm.*, **34**, 195–201.
- KAUMANN, A.J. (1994). Do human atrial 5-HT<sub>4</sub> receptors mediate arrhythmias? *Trends Pharmacol. Sci.*, **15**, 451–455.
- KAUMANN, A.J., LYNHAM, J.A. & BROWN, A.M. (1995). Labelling with [<sup>125</sup>I]-SB207710 of a small 5-HT<sub>4</sub> receptor population in piglet right atrium: functional relevance. *Br. J. Pharmacol.*, **115**, 933–936.
- KAUMANN, A.J., LYNHAM, J.A. & BROWN, A.M. (1996). Comparison of the densities of 5-HT<sub>4</sub> receptors,  $\beta$ <sub>1</sub>- and  $\beta$ <sub>2</sub>-adrenoceptors in human atrium: functional implications. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353**, 592–595.
- KAUMANN, A.J. & SANDERS, L. (1994). 5-Hydroxytryptamine causes rate-dependent arrhythmias through 5-HT<sub>4</sub> receptors in human atrium: facilitation by chronic  $\beta$ -adrenoceptor blockade. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **349**, 331–337.
- KAUMANN, A.J., SANDERS, L., BROWN, A.M., MURRAY, K.J. & BROWN, M.J. (1991). A 5-HT<sub>4</sub>-like receptor in human right atrium. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **344**, 150–157.
- KENAKIN, T. (1996). The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol. Rev.*, **48**, 413–463.
- LANGLOIS, M., ZHANG, L., YANG, D., BRÉMONT, B., SHEN, S., MANARA, L. & CROCI, T. (1994). Design of a potent 5-HT<sub>4</sub> receptor agonist with nanomolar affinity. *Biomed. Chem. Lett.*, **4**, 1433–1436.
- LEBESGUE, D., WALLUKAT, G., MIJARES, A., GRANIER, C., ARGIBAY, J. & HOEBEKE, J. (1998). An agonist-like monoclonal antibody against the human  $\beta$ <sub>2</sub>-adrenoceptor. *Eur. J. Pharmacol.*, **348**, 123–133.
- LEZOUALC'H, F., SAGARA, Y., HOLSBOER, F. & BEHL, C. (1998). High constitutive NF- $\kappa$ B activity mediates resistance to oxidative stress in neuronal cells. *J. Neurosci.*, **18**, 3224–3232.
- NAMBA, T., SUGIMOTO, Y., NEGISHI, M., IRIE, A., USHIKUBI, F., KAKIZUKA, A., ITO, S., ICHIKAWA, A. & NARUMIYA, S. (1993). Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature*, **365**, 166–170.
- NEIMARK, J. & BRIAND, J.P. (1993). Development of a fully automated multichannel peptide synthesizer with integrated TFA cleavage capability. *Peptide Res.*, **6**, 219–228.
- OUADID, H., SEGUIN, J., DUMUIS, A., BOCKAERT, J. & NARGEOT, J. (1991). Serotonin increases calcium current in human atrial myocytes via the newly described 5-hydroxytryptamine 4 receptors. *Mol. Pharmacol.*, **41**, 346–351.
- PINO, R., CERBAI, E., CALAMAI, G., ALAJMO, F., BORGIOLO, A., BRACONI, L., CASSAI, M., MONTESI, G.F. & MUGELLI, A. (1998). Effect of 5-HT<sub>4</sub> receptor stimulation on the pacemaker current I<sub>f</sub> in human isolated atrial myocytes. *Cardiovasc. Res.*, **40**, 516–522.
- REYNOLDS, G.P., MASON, L., MELDRUM, A., DE KECZER, S., PARNES, H. & WONG, E.H.F. (1995). 5-hydroxytryptamine (5-HT) receptors in post mortem human brain tissue: distribution, pharmacology and effects of neurodegenerative diseases. *Br. J. Pharmacol.*, **114**, 993–998.
- ROBISHAW, J.D., SMIGEL, M.D. & GILMAN, A.G. (1986). Molecular basis for two forms of the G protein that stimulates adenylyl cyclase. *J. Biol. Chem.*, **261**, 9587–9590.
- SANDERS, L., LYNHAM, J.A., BOND, B., DEL MONTE, F., HARDING, S.E. & KAUMANN, A.J. (1995). Sensitization of human atrial 5-HT<sub>4</sub> receptors by chronic  $\beta$ -blocker treatment. *Circulation*, **92**, 2526–2539.
- SAXENA, P.R. (1995). Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol. Ther.*, **66**, 339–368.
- SCHOEMAKER, R.G., DU, X.Y., BAX, W.A., BOS, E. & SAXENA, P.R. (1993). 5-Hydroxytryptamine stimulates human isolated atrium but not ventricle. *Eur. J. Pharmacol.*, **230**, 103–105.
- SEIFERT, R., WENZEL-SEIFERT, K., LEE, T.W., GETHER, U., SANDERS-BUSH, E. & KOBILKA, B.K. (1998). Different effects of Gs alpha splice variants on  $\beta$ <sub>2</sub>-adrenoceptor-mediated signaling. The beta<sub>2</sub>-adrenoceptor coupled to the long splice variant of Gs alpha has properties of a constitutively active receptor. *J. Biol. Chem.*, **273**, 5109–5116.
- SPENGLER, D., WAEBER, C., PANTALONI, C., HOLSBOER, F., BOCKAERT, J., SEEBURG, P.H. & JOURNOT, L. (1993). Differential signal transduction by five splice variants of the PACAP receptor. *Nature*, **365**, 170–175.
- VAN DEN WYNGAERT, I., GOMMEREN, W., VERHASSELT, P., JURZAK, M., LEYSEN, J., LUYTEN, W. & BENDER, E. (1997). Cloning and expression of a human serotonin 5-HT<sub>4</sub> receptor cDNA. *J. Neurochem.*, **69**, 1810–1819.
- WAEBER, C., SEBBEN, M., GROSSMAN, C., JAVOY-AGID, F., BOCKAERT, J. & DUMUIS, A. (1993). [<sup>3</sup>H]-GR113808 labels 5-HT<sub>4</sub> receptors in the human and guinea-pig brain. *Neuroreport*, **4**, 1239–1242.
- WAEBER, C., SEBBEN, M., NIEOULLON, A., BOCKAERT, J. & DUMUIS, A. (1994). Regional distribution and ontogeny of 5-HT<sub>4</sub> binding sites in rodent brain. *Neuropharmacology*, **33**, 527–541.
- WARDLE, K.A. & SANGER, G.J. (1993). The guinea-pig distal colon: a sensitive preparation for the investigation of 5-HT<sub>4</sub> receptor-mediated contractions. *Br. J. Pharmacol.*, **110**, 1593–1599.
- WHALEY, B.S., YUAN, N., BIRNBAUMER, L., CLARK, R.B. & BARBER, R. (1994). Differential expression of the  $\beta$ -adrenergic receptor modifies agonist stimulation of adenylyl cyclase: a quantitative evaluation. *Mol. Pharmacol.*, **45**, 481–489.
- WONG, E.H., REYNOLDS, G.P., BONHAUS, D.W., HSU, S. & EGLÉN, R.M. (1996). Characterization of [<sup>3</sup>H]GR113808 binding to 5-HT<sub>4</sub> receptors in brain tissues from patients with neurodegenerative disorders. *Behav. Brain Res.*, **73**, 249–252.
- WORKMAN, A.J. & RANKIN, A.C. (1998). Serotonin, I(f) and human atrial arrhythmia. *Cardiovasc. Res.*, **40**, 436–437.
- YANG, D., SOULIER, J.L., SICSIC, S., MATHÉ-ALLAINMAT, M., BRÉMONT, B., CROCI, T., CARDAMONE, R., AUREGGI, G. & LANGLOIS, M. (1997). New esters of 4-amino-5-chloro-2-methoxybenzoic acid as potent agonists and antagonists for 5-HT<sub>4</sub> receptors. *J. Med. Chem.*, **40**, 608–621.

(Received July 19, 1999

Revised November 1, 1999

Accepted November 16, 1999)