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Differences in Signal Transduction of Two 5-HT_4 Receptor Splice Variants: Compound Specificity and Dual Coupling with Gαs- and Gαi/o-Proteins

ARMELLE PINDON,[1] GEERT VAN HECKE, PAUL VAN GOMPEL, ANNE S. LESAGE,[1] JOSÉE E. LEYSEN, and MIREK JURZAK

Department of Receptor Pharmacology, Janssen Research Foundation, B-2340 Beerse, Belgium

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ABSTRACT

This study documents differences in ligand binding and signal transduction properties between the human (h) 5-hydroxytryptamine (5-$HT)_{4a}$ and h5-HT_{4b} receptor splice variants stably expressed in human embryonic kidney 293 cells. The fraction of the [^{3}H]5-HT high-affinity site relative to the whole receptor population measured with [^{3}H]GR113808 was higher for the h5-HT_{4a} isoform (around 0.4) than for the 5-HT_{4b} isoform (around 0.2) and was independent of the level of expression. The potency and efficacy of reference compounds tested for the cAMP response differed slightly but significantly between both variants. Most remarkably, 5-methoxytryptamine and prucalopride were found more potent on the 5-HT_{4b} variant, whereas SDZ-HTF 919 and SB204070 were more potent on the 5-HT_{4a} variant. Guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding on membranes and cAMP assays in whole cells revealed that only the h5-HT_{4b} isoform coupled to Gαi/o-proteins in addition to its well-documented Gαs coupling. In contrast, the h5-HT_{4a} receptor coupled only to Gαs-proteins, however, was able to trigger an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$). The observed $[Ca^{2+}]_i$ increase did not occur through inositol phosphate formation and was not sensitive to *Bordatella pertussis* toxin, forskolin, or 3-isobutyl-1-methylxanthine (pre)treatment but was due to Ca^{2+} influx from the extracellular environment. Interestingly, the Ca^{2+} pathway was dependent on high receptor expression levels and was compound-specific, because benzamide-like compounds triggered two to three times higher responses than indoleamines. Taken together, these data provide the first evidence for fine functional differences between C-terminal splice variants of the h5-HT_4 receptor, which may contribute to a better understanding of the functional diversity of this receptor class.

The ubiquitous neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) has so far been shown to interact with seven receptor classes, classified as 5-HT_1 to 5-HT_7 receptors. Three of them were defined as Gαs-protein-coupled receptors: the 5-HT_4, 5-HT_6, and 5-HT_7 receptors. In this study, we focus on the functional properties of the 5-HT_4 receptor class, in which a new level of structural diversity has been highlighted in recent years.

The wide distribution of 5-HT_4 receptors, from the central nervous system to the peripheral tissues, suggests a wide range of functional roles. In the central nervous system, 5-HT_4 receptors have been localized in rat basal ganglia, hippocampus, and olfactory tubercule (Compan et al., 1996; Vilaro et al., 1996). In human brain, radioligand binding (Domenech et al., 1994; Reynolds et al., 1995) and in situ hybridization studies (Bonaventure et al., 2000) have shown predominant expression of 5-HT_4 receptors in basal ganglia and limbic structures. Functionally, central 5-HT_4 receptors have been implicated in diverse processes such as anxiety, memory, and cognition (Silvestre et al., 1996; Fontana et al., 1997). 5-HT_4 receptors have also been shown to be widely distributed in peripheral organs such as the gastrointestinal tract, the myocardium, the urinary bladder, and the adrenal glands (for review, see Ford and Clarke, 1993). Furthermore, pharmacological differences have been identified in various tissues and species, suggesting a heterogeneity of 5-HT_4 receptors (Ford and Clarke, 1993; Leung et al., 1996). Indeed,

[1] Present address: CNS Discovery Research, Janssen Research Foundation, B-2340 Beerse, Belgium.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; h5-HT_4 receptor, human 5-hydroxytryptamine$_4$ receptor; AC, adenylyl cyclase; DMEM, Dulbecco's modified Eagle's medium; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PBS, phosphate-buffered saline; PTX, *Bordatella pertussis* toxin; HEK, human embryonic kidney; FLIPR, fluorometric imaging plate reader; IBMX, 3-isobutyl-1-methylxanthine; 5-MeOT, 5-methoxytryptamine; IP, inositol phosphate; RFU, relative fluorescence units; SB204070, 1,4-benzodioxin-5-carboxylic acid, 8-amino-7-chloro-2,3-dihydro-(1-butyl-4-piperidinyl)methyl ester; GR113808, 1*H*-indole-3-carboxylic acid, 1-methyl-(1-(2-((methylsulfonyl)-amino)ethyl)-4-piperidinyl)-methyl ester; SDZ205,557, benzoic acid, 4-amino-5-chloro-2-methoxy-2-diethylamino)ethyl ester; SDZ-HTF919 hydrazinecarboximidamide 2-((5-methoxy-1*H*-indol-3-yl)methylene)-*N*-pentyl-(*Z*)-2-butenedioate.

Gerald et al. (1995) have initially cloned two alternative splice variants coding for short (5-HT_{4S}) and long (5-HT_{4L}) isoforms of the receptor, which could be the structural basis for functional diversity. So far, seven receptor variants that differ in their C termini have been identified: 5-HT_{4a} (previously named 5-HT_{4S}), 5-HT_{4b} (previously named 5-HT_{4L}), 5-HT_{4c}, 5-HT_{4d}, 5-HT_{4e}, 5-HT_{4f}, and 5-HT_{4g} (Claeysen et al., 1997; Blondel et al., 1998; Bender et al., 2000). Moreover, by cloning the human 5-HT_4 receptor gene, our group has recently shown the existence of an additional site of alternative splicing (5-HT_{4h}), leading to an extended second extracellular loop that could theoretically combine with each C-terminal variant (Bender et al., 2000). Interestingly, the only experimentally isolated h5-HT_{4hb}-receptor variant showed an altered functional pharmacology because the prototypic 5-HT_4 receptor antagonist GR113808 exhibited partial agonistic properties. This finding supported the hypothesis of the existence of a functional diversity among 5-HT_4 receptor splice variants. In addition, we have recently shown a differential tissue distribution of the h5-HT_4 receptor isoforms (Bender et al., 2000), which may also contribute to tissue-specific functional differences. To date, all isoforms have been shown to activate adenylyl cyclase (AC) in vitro, and no difference in signal transduction between C-terminal 5-HT_4 receptor variants has been demonstrated. Although the functional significance for the existence of 5-HT_4 receptor splice variants is currently not well understood, studies on other receptors suggest that different C termini may discriminate between signal transduction pathways. G-protein selectivity was shown to be different for the C-terminal splice variants of the prostaglandin E receptor EP3 (Satoh et al., 1999), whereas somatostatin receptor isoforms have been found to differ in the coupling efficiency and desensitization events (Vanetti et al., 1993). To better understand the functional significance of 5-HT_4 receptor isoforms, we investigated the pharmacological profile and signal transduction of h5-HT_{4a} and h5-HT_{4b} receptors, two initially cloned C-terminal splice variants.

We have found major differences between the two variants, not only in the pharmacology of the cAMP response and the fractions of agonist high-affinity sites, but also in the selection of the G-proteins activated by the receptors and the second messengers triggered by agonists. Interestingly, this differential signaling was receptor variant and compound specific. These data provide further evidence for functional differences between 5-HT_4 receptor isoforms, underscoring a physiological relevance for the existence of 5-HT_4 receptor splice variants.

Experimental Procedures

Materials. [^{3}H]GR113808 (specific activity of 3.7 TBq/mmol), 5-hydroxy[^{3}H]tryptamine triacetate ([^{3}H]5-HT, specific activity of 4.44 TBq/mmol), and [^{35}S]GTPγS (specific activity of 37 MBq/ml) were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). The mammalian expression vector pcDNA3 was obtained from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), sodium butyrate, Geneticin (G-418), and calf serum were from Invitrogen. The Bradford protein assay was performed with the reagent supplied by Bio-Rad (Nazareth Eke, Belgium). The Adenylyl Cyclase Activation FlashPlate kit was supplied by PerkinElmer Life Sciences (Brussels, Belgium). The *Bordatella pertussis* toxin (PTX) was from Calbiochem (La Jolla, CA). The liquid scintillation spectrometer, the scintillation fluids Ultima Gold MV and Ultimaflo AF, and the Unifilter-96 GF/B plates were from Packard (Meriden, CT). GDP and dilithium salt were from Hoffman-La Roche (Basel, Switzerland). Fluo3-acetoxymethyl ester was purchased from Molecular Probes (Leiden, The Netherlands). Forskolin and probenecid were from Sigma (St Louis, MO). Cisapride and prucalopride are proprietary compounds from Janssen Pharmaceutica (Antwerp, Belgium). Renzapride, SB204070, GR113808, SDZ205070, and SDZ HTF919 were synthesized by Janssen Pharmaceutica for its own purposes. 5-HT and 5-MeOT were from Acros Organics (Geel, Belgium). For pharmacological testing, compounds were dissolved and diluted in dimethyl sulfoxide except for indoleamines, which were dissolved in water and protected from light throughout the experiment. The final dimethyl sulfoxide concentration never exceeded 0.5% (v/v). The GraphPad Prism program was from GraphPad Software, Inc. (San Diego, CA).

Stable Transfections and Selection of Monoclonal Cell Lines. The h5-HT_{4a} and h5-HT_{4b} receptors were stably expressed in HEK 293 cells. Expression was under the control of the constitutive cytomegalovirus promoter provided by the pcDNA3 vector. The calcium phosphate transfection method was used with modifications as described previously (Lesage et al., 1998). Stable monoclonal cell lines were created by limited dilution and selected by [^{3}H]GR113808 radioligand binding after culture under Geneticin (G-418) selection (800 μg/ml). Receptor expression levels of a range of isolated monoclonal cell lines were investigated with the antagonist [^{3}H]GR113808 in radioligand binding experiments; nontransfected HEK 293 cells did not show specific [^{3}H]GR113808 binding (data not shown). Of the 39 monoclonal cell lines obtained after transfection with the h5-HT_{4a} receptor, six had expression levels of about 2000 fmol/mg of protein after treatment with sodium butyrate (see below). Clone 8 (maximal expression levels ~3100 fmol/mg) was used for further studies and characterization. Another clone (clone 1) with a lower expression level (maximal ~1500 fmol/mg) was used to investigate the contribution of different receptor expression levels in the functional tests performed in this study. Of the 33 monoclonal cell lines expressing the h5-HT_{4b} receptor, cell clone 9 had an expression level of about 7000 fmol/mg of protein. Clone 9 was used for further study and characterization, and as for the other receptor splice variant, a clone of lower expression level (clone 4, maximal ~2000 fmol/mg) was used to investigate the effect of different receptor expression levels in the functional tests performed in this study.

Cell Culture and Treatments. Cells were grown in DMEM containing 10% dialyzed calf serum, 10^5 IU/l penicillin G, 0.1 g/l streptomycin, 0.1 g/l pyruvate, and 0.292 g/l L-glutamine, under 5% CO_2 at 37°C. Cells were grown under selection (800 μg/ml G418) for 48 h every 2 weeks. Culture plates were coated with PBS containing 10 μg/ml poly(L-lysine) for 45 min at 37°C and washed once with PBS before the cells were seeded.

In certain experiments, receptor expression levels were also manipulated using sodium butyrate, which is a histone deacetylase inhibitor found to arrest growth and to induce differentiation in various cell types by modulating gene expression (Archer et al., 1998). Therefore, sodium butyrate is a "differentiating agent" able to change expression levels of various proteins. The observed and described differences between both isoforms have been obtained using the same conditions in sodium butyrate-treated or nontreated cells. In addition, we provide data from clones exhibiting lower receptor levels whenever appropriate to understand the effects. In our experience, short-term overnight sodium butyrate treatments can be used to boost receptor expression without altering the pharmacological properties of the receptor. For sodium butyrate treatment, cells were incubated in medium containing 5 mM sodium butyrate for 16 to 18 h before experiments to boost receptor expression before membrane preparation for radioligand saturation binding, [^{35}S]GTPγS binding, and calcium measurements. PTX was used to prevent the coupling of Gαi/o-proteins to the receptor and was dissolved in 10

mM sodium phosphate, pH 7.0, and 50 mM NaCl and added to the cell medium overnight before the experiment at a concentration of 100 ng/ml.

Membrane Preparations and Radioligand Binding. Cells were cultured on 150-mm Petri dishes and washed twice with ice-cold PBS. The cells were then scraped from the plates with a cell scraper, suspended in 50 mM Tris-HCl buffer, pH 7.4, and harvested by centrifugation at 16,000*g* for 10 min. The pellet was re-suspended in 5 mM Tris-HCl, pH 7.4, and homogenized with an Ultra Turrax homogenizer (IKA Labortecnik, Staufen, Germany). The resulting membranes were collected by centrifugation at 25,000*g* for 20 min and stored at −70°C in 50 mM Tris-HCl buffer, pH 7.4, at a protein concentration of approximately 1 mg/ml. The Bradford protein assay was used for protein determination with bovine serum albumin as a standard.

For radioligand binding, assay mixtures (0.5 ml) contained 50 μl of the tritiated ligand (either the 5-HT$_4$ antagonist [^{3}H]GR113808 or the agonist [^{3}H]5-HT) and 0.4 ml of membrane preparation (at 0.010 mg/ml protein for [^{3}H]GR113808 binding or 0.1 mg/ml for [^{3}H]5-HT). Furthermore, 50 μl of solvent was added for total binding or either 50 μl of 10 μM SB204070 for [^{3}H]GR113808 or 50 μl of 10 μM GR113808 for [^{3}H]5-HT binding, for determination of nonspecific binding. The [^{3}H]GR113808 assay buffer was 50 mM HEPES-NaOH, pH 7.5. The [^{3}H]5-HT assay buffer was 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 1 μM pargyline (monoamine oxidase inhibitor), and 1 μM paroxetine (5-HT transporter inhibitor). The mixtures were incubated for 1 h at 25°C. The incubation was terminated by rapid filtration over Whatman GF/B filters presoaked in 0.15% polyethylenimine, followed by three washing steps with 3 ml of 50 mM HEPES-NaOH, pH 7.5, for [^{3}H]GR113808 binding, or 3 ml of 50 mM Tris-HCl, pH 7.4, for [^{3}H]5-HT binding. Ligand concentration isotherms were obtained with eight concentrations of [^{3}H]GR113808 ranging from 0.02 to 1 nM. For [^{3}H]5-HT saturation curves, eight concentrations ranging from 0.2 to 6 nM were used to characterize the high-affinity site, whereas 14 concentrations from 0.2 to 40 nM were chosen for the determination of the low-affinity site.

Ligand concentration binding isotherms (rectangular hyperbola) were calculated by nonlinear regression analysis as described previously (Lesage et al., 1998). The maximal number of binding sites (B_{max}) and equilibrium dissociation constant (K_D) of the radioligand were derived from the curve fitting. Graphs were prepared with the GraphPad Prism program.

Measurements of cAMP Formation. After 2 days of growth, cells were detached from the Petri dishes with 3 ml of EDTA (0.04% w/v in PBS) and resuspended in PBS without Ca^{2+} and Mg^{2+}. The cells were centrifuged at 500*g* for 5 min. The pellet was resuspended in the "stimulation buffer" provided with the PerkinElmer kit and diluted to a concentration of 10^6 cells/ml, and 50 μl was added per well of the Flashplate (50,000 cells/well). Compounds were diluted in PBS without Ca^{2+} and Mg^{2+} containing 1 μM pargyline and 1 μM paroxetine. Fifty microliters of the compound solution was added per well, followed by an incubation for 20 min at 37°C. After the incubation period, a direct radioimmunoassay using ^{125}I-cAMP was performed by the addition of 100 μl of detection mix per well according to the supplier's instructions. After incubation for 18 h at room temperature, counting was performed in a Topcount HTS9912V counter (Packard).

[^{35}S]GTPγS Binding Assays. For further functional testing, the [^{35}S]GTPγS binding assay was performed on membrane preparations. Membranes were incubated (105 μg/ml) in 20 mM HEPES-NaOH, pH 7.4, containing 10 mM $MgCl_2$, 1 μM GDP, 1 μM pargyline, and 1 μM paroxetine, in the presence of 0.25 nM [^{35}S]GTPγS and compounds. The final volume was 250 μl and the incubation was performed in polypropylene tubes (PPN-tube-96; Micronic bv, Lelystad, The Netherlands) at 37°C for 20 min. The incubation was stopped by filtration on Unifilter-96 GF/B plates with ice-cold phosphate buffer, 10 mM Na_2HPO_4, adjusted to pH 7.4 with a solution of 10 mM NaH_2PO_4. The filter plates were dried at room temperature, 30 μl of Microscint was added per well, and plates were counted in a Topcount HTS9912V counter (Packard).

Measurements of Intracellular Calcium Concentration. The intracellular $[Ca^{2+}]_i$ was measured with a fluorometric imaging plate reader (FLIPR; Molecular Devices, Crawley, England). Cells were seeded at 40,000 cells/well in 96-well plates (Cluster plates, black with clear bottom; Costar; Merck, Overijse, Belgium) for 2 days until they reached confluence. Cells were loaded with 2 μM Fluo3-acetoxymethyl ester in DMEM supplemented with 20 mM HEPES, pH 7.4, and 2.5 mM probenecid, for 1 h at 37°C under 5% CO_2. The plates were washed three times with 5 mM HEPES, pH 7.4, 1.25 mM $CaCl_2$, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, and 10 mM glucose, containing 2.5 mM probenecid (to prevent extrusion of Fluo-3), 1 μM pargyline, and 1 μM paroxetine. $[Ca^{2+}]_i$ was measured by FLIPR, and the peak of the calcium transient was considered as the relevant signal. Treatment of the cells with 10 μM ionomycin (Ca^{2+}-inophore) was performed as a control for dye loading and cell viability.

To investigate the source of the $[Ca^{2+}]_i$ increase, we performed the same assay as described above, using several modifications. We performed the assay in the absence of extracellular calcium, where the Fluo3-loaded cells were washed two times with the above buffer without calcium. The final washing step and the measurements were performed in Ca^{2+}-free buffer containing 2 mM EGTA. Ionomycin (10 μM) and 1% Triton X-100 were used as controls to estimate the maximal signals from extracellular and intracellular compartments, respectively. $[Ca^{2+}]_i$ measurements were also performed on cells

TABLE 1

Saturation binding analysis of membrane preparations from HEK 293 cells stably transfected with 5-HT$_4$ receptor isoforms

Saturation binding was performed using [^{3}H]GR113808 at concentrations from 0.02 to 1 nM and [^{3}H]5-HT from 0.2 to 6 nM. The membranes were prepared from monoclonal cell lines either treated or nontreated with 5 mM sodium butyrate. B_{max} and K_D values were obtained from saturation curve fitting using nonlinear regression analysis. Values are presented as means (± S.D.) obtained from *n* experiments performed in duplicate. Fraction of [^{3}H]5-HT high-affinity sites (agonist fraction) is the ratio of B_{max} obtained with [^{3}H]5-HT and [^{3}H]GR113808, respectively.

Receptor (Clone)	h5-HT$_{4a}$ (8)					h5-HT$_{4b}$ (9)				
	B_{max}		K_D		*n*	B_{max}		K_D		*n*
	Mean	± S.D.	Mean	± S.D.		Mean	± S.D.	Mean	± S.D.	
	fmol/mg		*nM*			*fmol/mg*		*nM*		
+ Sodium butyrate										
[^{3}H]GR113808	3100	500	0.07	0.05	5	6800	560	0.09	0.01	6
[^{3}H]5-HT	1200	170	3.2	0.8	5	1600	520	2.7	0.9	17
Agonist fraction	0.39					0.24				
− Sodium butyrate										
[^{3}H]GR113808	1800	100	0.05	0.02	3	4100	300	0.1	0.01	3
[^{3}H]5-HT	750	70	2.7	0.4	3	860	140	2.7	0.9	3
Agonist fraction	0.42					0.21				

pretreated with 100 ng/ml PTX (overnight), 200 μM bepridil (15 min), 1 mM 3-isobutyl-1-methylxanthine (IBMX; 30 min), and 1 μM forskolin (15 min).

Results

Characterization of Cell Lines in Ligand Concentration Binding. The two human 5-HT_4 receptor isoforms investigated in this study, h5-HT_{4a} and h5-HT_{4b}, were stably transfected into HEK 293 cells. In this study, we have primarily used a high-level expressing clone of each splice variant, the h5-HT_{4a} clone 8 and the h5-HT_{4b} clone 9. In addition, clones with lower expression levels, h5-HT_{4a} clone 1 and h5-HT_{4b} clone 4, were employed in certain experiments to investigate the effect of receptor expression on the functional properties of each receptor variant. The isolated monoclonal cell lines were characterized by saturation binding, using two radioligands, an antagonist, [^{3}H]GR113808, and the natural agonist, [^{3}H]5-HT. The experiments were performed on membrane preparations from cells treated with sodium butyrate and from untreated cells. Independent of expression levels, the concentration binding isotherms of the antagonist [^{3}H]GR113808 to h5-HT_{4b} and h5-HT_{4a} receptors expressed in different clones showed rectangular hyperbolae. The resulting linear Scatchard plots revealed a single high-affinity binding site (K_D values ranging between 0.05 and 0.1 nM for both 5-HT_4 receptor isoforms). Furthermore, whereas treatment with sodium butyrate increased all B_{max} values by almost 70%, it did not alter mean K_D values. The calculated K_D and B_{max} values are presented in Table 1. For comparison, the B_{max} values of the two lower level expressing clones, treated with sodium butyrate, were 1500 (±160) and 2087 (±513) fmol/mg of protein for the h5-HT_{4a} clone 1 and the h5-HT_{4b} clone 4, respectively.

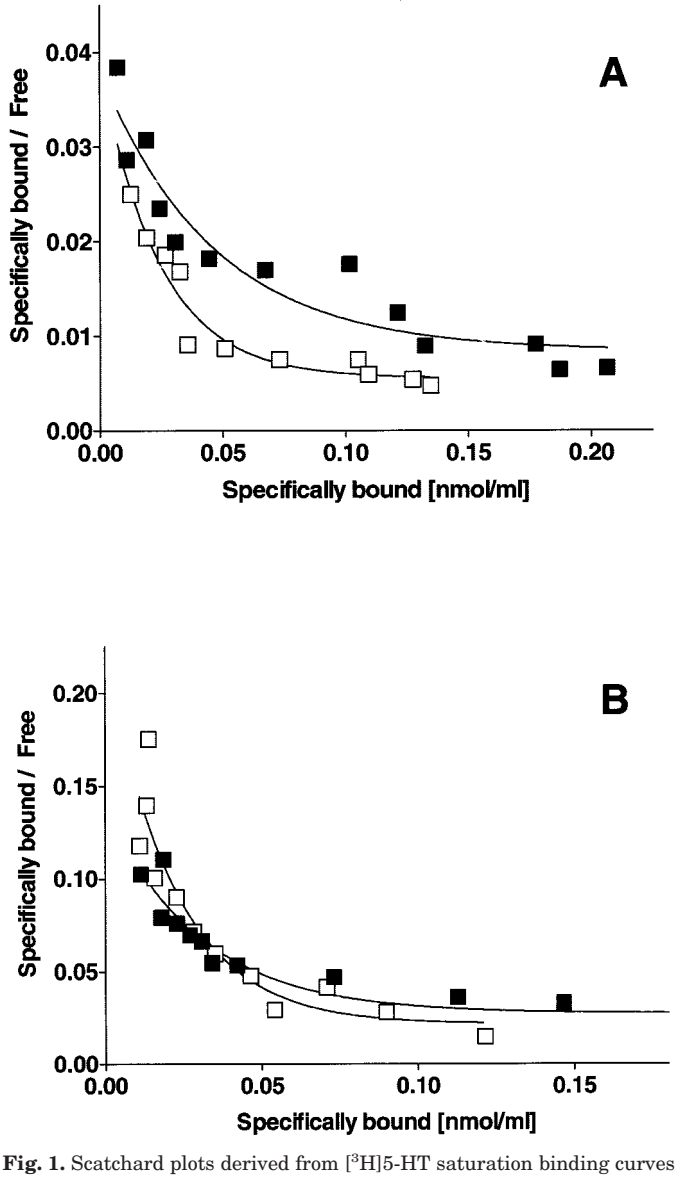

Fig. 1. Scatchard plots derived from [^{3}H]5-HT saturation binding curves performed on membrane preparations from HEK 293 cells stably transfected with h5-HT_4 receptor isoforms. Saturation binding was performed with [^{3}H]5-HT concentrations ranging from 0.2 to 40 nM to visualize the low-affinity sites. The membranes were prepared from h5-HT_{4a} (A) or h5HT_{4b} (B) expressing cells under sodium butyrate-treated (■) or non-treated conditions (□). The parabolic Scatchard plots show the two affinity sites for both h5-HT_4 receptor isoforms. The data shown represent a typical example out of three independent experiments performed in duplicate.

The same comparison of the derived K_D and B_{max} values with and without sodium butyrate treatment, and between the two receptor splice variants, was performed with the agonist [^{3}H]5-HT at eight concentrations ranging from 0.2 to 6 nM (Table 1). Interestingly, in agreement with the findings of the antagonist binding experiments, sodium butyrate treatment up-regulated B_{max} values for [^{3}H]5-HT also by 60 to 86%, indicating that the ratio of G-protein-coupled to total receptors was not affected. Furthermore, sodium butyrate did not affect K_D values for [^{3}H]5-HT, which were found to be in the range of 1.2 to 3.0 nM. However, in contrast to the results obtained with [^{3}H]GR113808, the Scatchard plots of [^{3}H]5-HT binding indicated the existence of a low-affinity site when slightly higher concentrations were used.

When [^{3}H]5-HT concentrations up to 40 nM were employed Scatchard analysis clearly showed two affinity sites for both splice variants (Fig. 1). The low-affinity site K_D values computed from the saturation curve were 34 ± 12 nM for the h5-HT_{4a} receptor and 18 ± 5 nM for the h5HT_{4b} receptor in preparations treated with sodium butyrate. Without the treatment, the low-affinity site K_D values were 42 ± 19 and 26 ± 5 nM for the h5-HT_{4a} and 5-HT_{4b} receptor, respectively.

As an indication for the proportion of receptors that are in the conformation to bind an agonist with high affinity, we calculated the fraction [^{3}H]5-HT B_{max} value of the [^{3}H]GR113808 B_{max} value (Table 1). Interestingly, this fraction of [^{3}H]5-HT high-affinity sites was approximately two times higher for the h5-HT_{4a} than for the h5-HT_{4b} receptor splice variant and was not modified by sodium butyrate treatment and thus by receptor expression levels. This different ratio of receptor states observed for the two receptor isoforms could be a consequence of a higher G-protein coupling of h5-HT_{4a} receptors, which could result in functional differences of the two receptor isoforms. These findings prompted us to investigate signal transduction events triggered by the two variants.

Adenylyl Cyclase Activation. The activity of AC after h5-HT_4 receptor stimulation with various reference ligands was estimated on living cells, which were not treated with sodium butyrate to elevate receptor expression levels. An increase of basal cAMP levels, found in the high expressing clones compared with wild-type HEK 293 cells, revealed the

constitutive activity of both 5-HT$_4$ receptors, which is consistent with previous reports (Claeysen et al., 1999, 2000). Compared with the basal values measured in the wild-type HEK 293 cells, the measured constitutive activity was 482% (±135) and 881% (±156) (mean ± S.D., $n = 3$) in 5-HT$_{4a}$ and 5-HT$_{4b}$ receptor-expressing cells, respectively.

The investigated agonists were the natural agonist 5-HT, its natural derivative 5-methoxytryptamine (5-MeOT), the two benzamides cisapride and renzapride, the benzofuran prucalopride, and the indole-derivative SDZ-HTF919 (Appel-Dingemanse et al., 1999). The reference antagonists used were GR113808, an indole carboxylate analog, SDZ205,557, a benzoate analog, and SB204070, an imidazole amide. All compounds were tested for agonistic efficacy, and the results were expressed as a percentage of maximal 5-HT response (5-HT$_{max}$); i.e., 100% of stimulation was the maximum of the fitted plots obtained with 5-HT. The derived pEC$_{50}$ and 5-HT$_{max}$ values are presented in Table 2 and the concentration-response curves are shown in Fig. 2. All tested agonists were found to be full agonists for both h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors. The antagonist GR113808 did not induce cAMP formation via h5-HT$_{4a}$ or via h5-HT$_{4b}$ receptors.

The putative reference antagonist SB204070 acted as partial agonists inducing 41 ± 3 and 50 ± 3% of the 5-HT-induced response on h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors, respectively. SDZ205,557 also exhibited partial agonistic properties leading to 31 ± 7 and 30 ± 11% of maximal stimulation on h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors, respectively. For SB204070 this difference in efficacy and in potency (9.5 ± 0.5 versus 8.8 ± 0.1) was found to be statistically significant. Another compound that showed a significant difference in efficacy of cAMP stimulation was cisapride, which behaved as a more efficacious agonist on the h5-HT$_{4b}$-receptor isoform. In terms of potency 5-MeOT and prucalopride were significantly more potent on the 5-HT$_{4b}$-variant, whereas SDZ-HTF919 and SB204070 were more potent on the h5-HT$_{4a}$ receptor isoform. Therefore, the stronger G-protein coupling of the h5-HT$_{4b}$ indicated in the saturation binding assays was not reflected in a general shift in pharmacology of the cAMP assay but was rather a specific property of tested compounds.

Changes in Intracellular Calcium Concentration. To investigate other potential signal transduction pathways, we measured the modulations of $[Ca^{2+}]_i$ after h5-HT$_{4a}$ and h5-HT$_{4b}$ receptor activation. The same reference compounds as described for the cAMP experiments were employed. The h5-HT$_{4a}$ receptor showed fast increase in $[Ca^{2+}]_i$ after exposure to all tested agonists, independent of the expression level found in both investigated clones. Interestingly, the Ca^{2+} response induced by the h5-HT$_{4a}$ receptor was dependent on the chemical nature of the agonists. For the two agonistic benzamide-like compounds, cisapride and prucalopride, the peaks of the calcium transients were two and three times higher than for the natural agonist 5-HT and showed a different time course (Fig. 3). Both cisapride- and prucalopride-stimulation needed a longer time (>100 s) to reach

TABLE 2

cAMP formation in HEK 293 cells stably transfected with h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors

The pEC$_{50}$ values and percentage maximal responses were calculated by nonlinear regression analysis of each concentration-response curve. The maximal response of each compound was normalized to the maximal stimulation obtained with 5-HT (% 5-HT$_{max}$). Results are expressed as means (± S.D.) of n independent experiments performed in duplicate.

Receptor (Clone)	h5-HT$_{4a}$ (8)					h5-HT$_{4b}$ (9)				
	% 5-HT$_{max}$		pEC$_{50}$		n	% 5-HT$_{max}$		pEC$_{50}$		n
	Mean	± S.D.	Mean	± S.D.		Mean	± S.D.	Mean	± S.D.	
5-HT	100		8.2	0.2	7	100		8.3	0.1	13
5-MeOT	115	11	7.7[b]	0.1	6	111	3	8.3	0.2	6
Cisapride	98[b]	7	7.8	0.3	8	114	4	7.9	0.1	8
Prucalopride	118	7	8.2[b]	0.1	11	117	3	8.5	0.1	11
SDZ-HTF919	114	15	8.7[a]	0.1	3	134	6	8.3	0.1	3
Renzapride	111	6	7.7	0.2	4	103	12	7.6	0.1	4
SB204070	41[b]	3	9.5[a]	0.5	6	50	3	8.8	0.1	8
SDZ205,557	31	7	7.6	0.2	3	30	11	7.5	0.1	3

[a] $p < 0.001$.
[b] $p < 0.0001$.

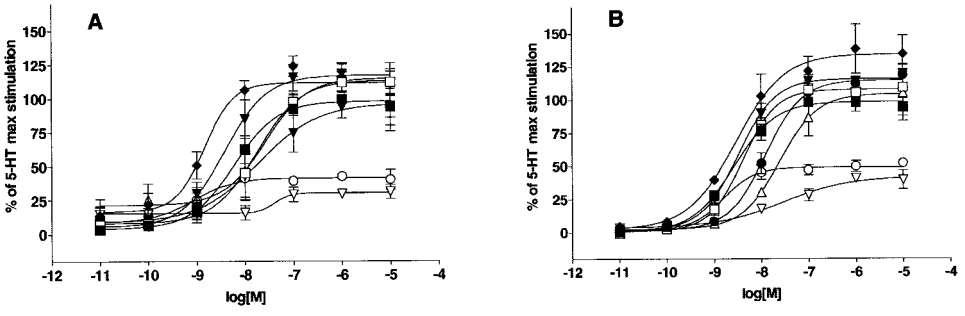

Fig. 2. cAMP formation investigated in HEK 293 cells stably transfected with h5-HT$_4$ receptor isoforms. Concentration-response curves of cAMP accumulation were performed on whole cells expressing h5-HT$_{4a}$ (A) or h5-HT$_{4b}$ (B) receptors stimulated with 5-HT (■), prucalopride (▼), cisapride (●), SDZ-HTF919 (◆), renzapride (△), 5-MeOT (●), SB204070 (○), and SDZ205,557 (▽). The maximal response for each compound was normalized to the maximal stimulation obtained with 5-HT (% of 5-HT$_{max}$). Results are expressed as fitted curves to the mean (±S.D.) of independent experiments performed in duplicate, as summarized in Table 2.

maximal $[Ca^{2+}]_i$ than was observed with indoleamines (<50 s).

In contrast, the h5-HT_{4b} receptor variant showed a totally different pharmacological response pattern despite a higher expression level. Specifically, only the natural indoles 5-HT and 5-MeOT could induce weak and slow increases in $[Ca^{2+}]_i$ (Figs. 3 and 4). The pEC_{50} values derived from fitted concentration-response curves are presented in Table 3. No response could be detected with the antagonist GR113808 and the partial agonist SDZ205,557. However, SB204070 was found to act as a weak partial agonist for the calcium response on the highly expressing h5-HT_{4a} clone only and induced variable responses with a mean of 23% (±24) of the 5-HT-induced maximal response. This indicates that this partial agonist exhibits a lower efficacy in the calcium signaling than in the AC pathway. Several findings provide evidence that unlike in cAMP tests, the intensity of the calcium responses was dependent on the receptor expression level. Specifically, the higher expressing clone of the h5-HT_{4a} receptor (clone 8) showed a higher response than the lower expressing h5-HT_{4a} clone (clone 1). On average clone 8 responded with a 2- to 3-fold higher Ca^{2+}-peak than clone 1, which is consistent with its 2-fold higher B_{max}. In addition, the comparison of cells treated with sodium butyrate versus untreated cells showed a decrease in the signal intensity for h5-HT_{4a} receptor cell lines and a disappearance of the signal for the h5-HT_{4b} receptor variant in untreated cells (Fig. 4). Although sodium butyrate has been reported to up-regulate Na-channels (Kunzelmann et al., 1995), we believe that its enhancing effect on the Ca^{2+}-signaling is rather linked to its up-regulating effect of receptor levels, because the treatment is not a prerequisite to measure a response (Fig. 4, D versus C). Taken together this results indicate that the calcium pathway is relatively h5-HT_{4a} receptor-specific, dependent on receptor expression level, and more responsive to benzamide-like compounds in comparison to indoleamines.

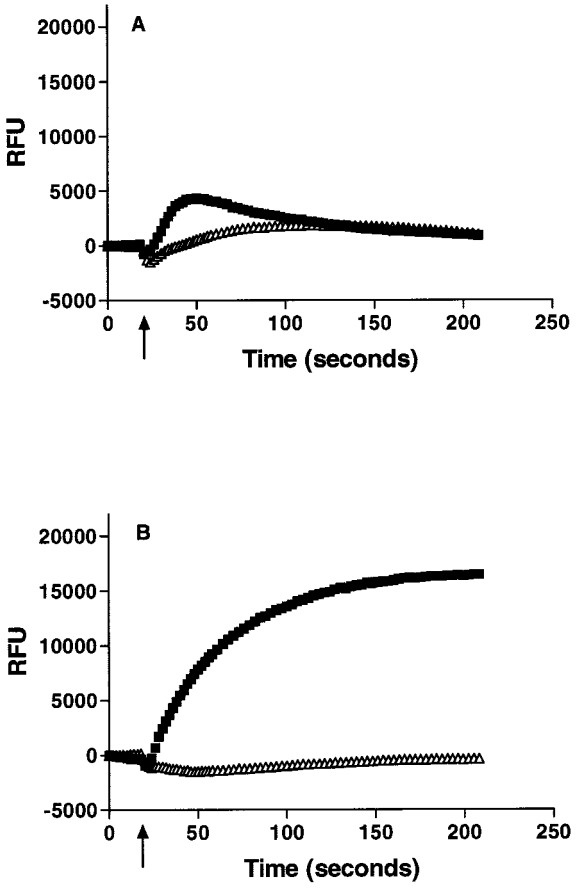

Fig. 3. Transients of $[Ca^{2+}]_i$ on HEK 293 cells stably transfected with h5-HT_4 receptor isoforms. Time course of changes in $[Ca^{2+}]_i$ triggered via the h5-HT_{4b} (△) or the h5-HT_{4a} (■) receptor were studied by the calcium-sensitive fluorescence of Fluo3 measured with FLIPR on sodium butyrate-treated cells. Ordinates represent the increase of RFU measured at the peak of the Ca^{2+} transient. The receptors were stimulated by 10^{-6} M 5-HT (A) or 10^{-6} M prucalopride (B). The arrows indicate the time when the compound was added to cells. The results were normalized to the basal fluorescence levels of the cells before compound addition.

To further investigate the molecular mechanism of the calcium response, we investigated whether the changes in $[Ca^{2+}]_i$ were caused by an influx from the extracellular milieu and/or resulting from mobilization of intracellular stores.

Inositol Phosphates or Extracellular Calcium Influx. Mobilization of calcium from intracellular stores would classically involve the activation of phospholipase C and was investigated by means of IP measurements. Assays were performed after h5-HT_{4a} receptors had been stimulated with the natural agonist 5-HT and the benzofuran prucalopride. No increase of IP concentrations could be detected (data not shown) in contrast to a parallel control performed with CHO cells expressing h5-HT_{2A} receptors. Our results, therefore, indicate that the h5-HT_{4a}-induced calcium mobilization does not involve the phospholipase C/IP_3 pathway.

When calcium was removed from the extracellular environment before h5-HT_{4a} receptor stimulation, no response

TABLE 3

$[Ca^{2+}]_i$ measurements on HEK293 cells stably transfected with h5-HT_{4a} and h5-HT_{4b} receptors and treated with sodium butyrate

Changes in $[Ca^{2+}]_i$ were estimated by the calcium-sensitive fluorescence of Fluo3 measured with FLIPR. Results are the means of *n* independent experiments performed in triplicate. The maximal response of each compound was normalized to the maximal stimulation obtained with 5-HT (% 5-HT_{max}). The pEC_{50} values and percentage maximal responses were calculated by nonlinear regression analysis of each concentration-response curve.

Receptor (Clone)	h5-HT4a (1)					h5-HT4a (8)					h5-HT4b (9)				
	% 5-HT_{max}		pEC_{50}		*n*	% 5-HT_{max}		pEC_{50}		*n*	% 5-HT_{max}		pEC_{50}		*n*
	Mean	± S.D.	Mean	± S.D.		Mean	± S.D.	Mean	± S.D.		Mean	± S.D.	Mean	± S.D.	
5-HT	100		7.8	0.6	7	100		7.6	0.4	9	100		7.9	0.3	9
5-MeOT	91	14	7.2	0.6	4	75	9	7.5	0.3	4	106	9	7.4	0.4	4
Cisapride	219	66	7.4	0.8	4	319	83	6.8	0.7	6	N.A.		N.A.		6
Prucalopride	223	93	7.4	0.8	4	317	117	7.3	0.1	6	N.A.		N.A.		6
Renzapride	85	10	6.7	0.7	3	143	76	6.8	0.4	3	N.A.		N.A.		3
SB204070	N.A.		N.A.		3	23	24	9.6	0.5	3	N.A.		N.A.		3

N.A., not active.

could be triggered with 1 μM 5-HT or 1 μM prucalopride (Fig. 5A). These results indicate that the h5-HT$_{4a}$ receptor-induced increase in $[Ca^{2+}]_i$ was caused by opening of calcium channels located in the plasma membrane. This hypothesis could be confirmed by pretreating the cells with 200 μM bepridil, which resulted in a complete blockade of the Ca^{2+}-signal (Fig. 5B). In addition, the response was not dependent on the contribution of Gαi-proteins, because it was insensitive to PTX pretreatment (Fig. 5A). IBMX treatment (not shown) and forskolin addition (Fig. 5B) did not modify or induce the Ca^{2+} transients, which speaks against a direct contribution of the cAMP pathway.

The difference in signal transduction between the two variants, 5-HT$_{4a}$ and 5-HT$_{4b}$, showing an apparent stronger coupling of the 5-HT$_{4a}$ isoform to the Ca^{2+}-pathway, is in parallel to the higher fraction of high-affinity agonist sites of the 5-HT$_{4a}$ variant. This prompted us to investigate the signal transduction at the direct level of G-protein activation.

[^{35}S]GTPγS Binding. G-protein coupling to receptors was investigated by measuring [^{35}S]GTPγS binding to membrane preparations from cells expressing either h5-HT$_4$ receptor isoform. Results were expressed as percentage increase in [^{35}S]GTPγS binding over basal levels (Fig. 6). The levels of stimulation were found to differ between h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors, and were in the range of 50 and 100% of stimulation, respectively. To investigate potential differences in pharmacology, the same six agonists and three putative antagonists were used as described above. The pEC$_{50}$ values derived from fitted concentration-response curves are presented in Table 4. We were not able to detect any stimulatory effects on [^{35}S]GTPγS binding with the two antagonists GR113808 and SDZ205,557. However, SB204070 triggered a weak response via h5-HT$_{4b}$ receptors. Despite the higher levels of stimulation triggered via the h5-HT$_{4b}$ receptor, no significant differences in the potency of the agonistic compounds were detected between the both receptor variants.

To investigate whether the difference in levels of stimulation was triggered by the involvement of the Gαi/o-proteins, we performed similar [^{35}S]GTPγS binding experiments on membranes from PTX-pretreated cells (Fig. 6; Table 4). We observed, for all agonists used, a decrease in the maximal levels of [^{35}S]GTPγS binding in the h5-HT$_{4b}$ receptor preparation treated with PTX. The percentages of inhibition induced by PTX on the h5-HT$_{4b}$-receptor are presented in Table 5. Therefore, the uncoupling of the receptor from the Gαi/o-proteins by PTX pretreatment results in lower levels of [^{35}S]GTPγS binding stimulation via the h5-HT$_{4b}$ receptor. Interestingly, the pEC$_{50}$ values were not significantly modified, although they tended to be higher on PTX-treated membranes (Table 4). In contrast, no significant modulation of [^{35}S]GTPγS binding was observed on the h5-HT$_{4a}$ receptor preparation. Because the h5-HT$_{4b}$ cell line used for these experiments had higher receptor expression levels than the h5-HT$_{4a}$ clone, we investigated whether the coupling to Gαi/o-proteins was not a consequence of the high receptor levels. To this end, we performed the same experiments on the lower-expressing h5-HT$_{4b}$ clone (i.e., clone 4, expressing approximately one-third of receptors). We observed the same decrease in [^{35}S]GTPγS binding efficacy after PTX pretreatment (data not shown). Taken together, these results indicate that in addition to Gαs, the h5-HT$_{4b}$ receptor splice variant is also coupled to Gαi/o-proteins and that this is independent of the expression levels. In contrast, the lack of sensitivity to PTX of [^{35}S]GTPγS binding indicates that h5-HT$_{4a}$ receptors do not couple to Gαi/o-proteins. We also investigated the contribution of Gαi/o-proteins to the other h5-HT$_{4b}$ receptor-mediated signal transduction pathways.

Adenylyl Cyclase Activation after PTX Pretreatment. AC stimulation was investigated on h5-HT$_{4b}$- and, as a control, on h5-HT$_{4a}$ receptors on cells pretreated with PTX and on untreated cells. Because the basal levels of cAMP were increased after PTX pretreatment the results were expressed as a percentage of the maximal stimulation obtained on nontreated cells. Consistent with the findings in

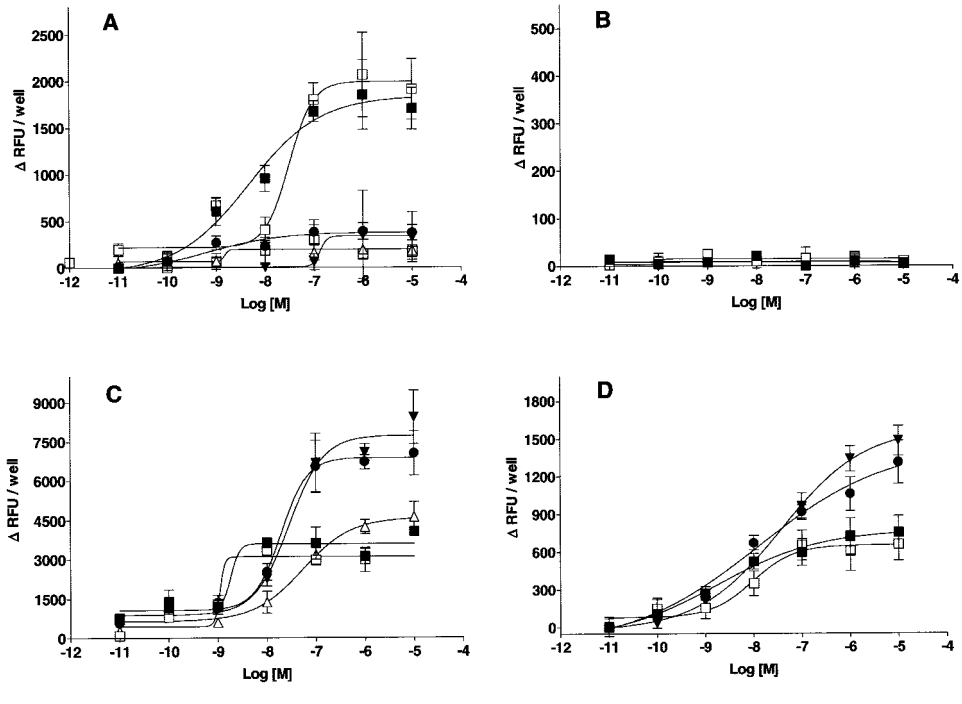

Fig. 4. $[Ca^{2+}]_i$ measurements on HEK 293 cells stably transfected with h5-HT$_4$ receptor isoforms. Changes in $[Ca^{2+}]_i$ in cells expressing the h5-HT$_{4b}$ (A and B) or the h5-HT$_{4a}$ (C and D) receptor under either sodium butyrate-treated (A and C) or nontreated (B and D) conditions measured with FLIPR as indicated in Fig. 3. The compounds used for receptor stimulation were 5-HT (■), prucalopride (▼), cisapride (●), renzapride (△), and 5-MeOT (□). Data are expressed as fitted curves to the mean (±S.D.) peak values (in RFU) of triplicate determinations and represent a typical experiment out of independent experiments as summarized in Table 3. Note that sodium butyrate treatment is not necessary to obtain a Ca^{2+} response.

[^{35}S]GTPγS binding experiments, cAMP formation induced by 5-HT or prucalopride was found to be increased by PTX pretreatment in the h5-HT$_{4b}$-variant indicating a des-inhibition of the cAMP-pathway (Fig. 7). The increase in maximally accumulated cAMP was 27% (±5) for 5-HT and 29% (±6) for prucalopride stimulation. In contrast, no significant modulation of the AC response by PTX treatment was found with the h5-HT$_{4a}$ receptor variant (Fig. 7).

Discussion

This study uses the initially cloned and widely distributed 5-HT$_{4a}$ and 5-HT$_{4b}$ receptors to investigate the pharmacological and functional properties of these receptor splice variants in stably transfected HEK 293 cells. Analysis of saturation binding performed with [^{3}H]GR113808 revealed K_D values that were consistent with previous findings on transiently transfected cells (Ansanay et al., 1996; Van den Wyngaert et al., 1997) and different brain tissues (Grossman et al., 1993; Waeber et al., 1994; Arranz et al., 1998). We confirm that C-terminal variations of h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors did not modify binding K_D values, consistent with the findings on EP3 and somatostatin receptors (Tsunehisa et al., 1993; Vanetti et al., 1993) and very recently also 5-HT$_4$ receptors (Bach et al., 2001). Detailed saturation binding performed with [^{3}H]5-HT, revealed the presence of two affinity sites for both h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors. Previous studies could only show a single affinity site for [^{3}H]5-HT of 20 (±7) nM for the rat 5-HT$_{4b}$ receptor expressed in COS7 cells, although the shallow competition binding curves and partial GTPγS sensitivity indicated the existence of two affinity sites (Adham et al., 1996). The high concentrations of [^{3}H]5-HT used in that study (5–100 nM) may have precluded the detection of the high-affinity site described in this report (K_D values ranging from 1.3 to 3.2 nM).

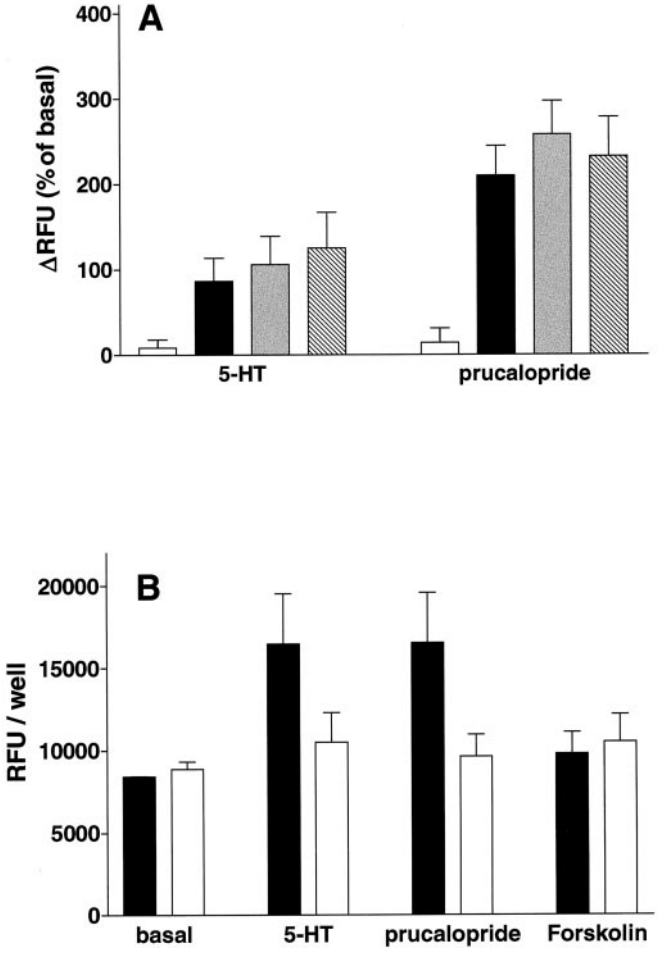

Fig. 5. Characterization of the h5-HT$_{4a}$ receptor-mediated Ca^{2+} response. h5-HT$_{4a}$ cells were stimulated by vehicle (basal), 10^{-6} M 5-HT, 10^{-6} M prucalopride, or 10^{-6} M forskolin. A, the experiments were performed using a modification of the standard buffer containing 1.25 mM extracellular calcium (black bars) by replacing calcium with 2 mM EGTA (□) or in the standard buffer with a 24-h preincubation with 100 ng/ml PTX (▨) or vehicle (▧). The ordinate represents the mean RFU measured at the peak of the Ca^{2+} transient normalized to the mean RFU obtained before the stimulation (% of basal). Data are expressed as the mean (±S.D.) of three independent experiments performed in triplicate. B, the experiments were performed in standard buffer containing 1.25 mM extracellular calcium after a 15-min preincubation with 200 μM bepridil (□) or vehicle (■). The ordinate represents the mean RFU measured at the peak of the Ca^{2+} transient. Data are expressed as the mean (±S.D.) of nine determinations and represent a typical experiment out of three independent experiments.

The pharmacological profile obtained for cAMP formation was partially divergent compared with previously reported results. We have shown that benzamide-like compounds in our system were full agonists on both variants, in contrast to reports classifying renzapride and cisapride as partial agonists (Ouadid et al., 1992; Blondel et al., 1998). Moreover, SB204070 and SDZ205,557, described as antagonists, were found to be partial agonists on both h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors. In addition, small but statistically significant differences in efficacy and/or potency of agonists were found between the two splice variants. Direct extrapolation of these results to tissue-based studies should bear in mind that functional responses found in tissues may reflect not only different expression levels but also be the result of the stimulation of several 5-HT$_4$ receptor splice variants expressed in a given tissue.

A very recent study of Bach et al. (2001) investigating the cAMP response in h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors could also not match the pharmacological pattern found on cells to measurement on right atrium membranes, although the 5-HT$_{4a}$ isoform is thought to be predominant in that tissue (Blondel et al., 1997). In contrast to our study, they have found partial agonism of renzapride and cisapride. However, their pharmacological comparison is focused on antagonists, and the study uses [α-^{32}P]ATP-loaded membranes for mea-

surements of the cAMP, whereas our study is performed on intact cells. At similar expression levels, our EC_{50} for 5-HT is approximately 10-fold lower than the ones reported by Bach et al. (2001). Therefore, the membrane based assay used by Bach et al., which allows a direct comparison to measurements performed on tissue samples, may suffer from impaired coupling and may not be able to reveal the subtle differences seen in this study on intact cells.

In addition to AC activation, 5-HT$_4$ receptors have also been shown to trigger other signal transduction pathways, such as reduction of potassium currents in mouse colliculi neurons (Fagni et al., 1992), calcium influx in adrenocortical cells (Contesse et al., 1996), and atrial myocytes (Ouadid et al., 1992). Our results revealed a clear functional difference between the two h5-HT$_4$ receptor isoforms in their ability and pharmacology to increase $[Ca^{2+}]_i$. The h5-HT$_{4a}$ receptor was able to trigger strong $[Ca^{2+}]_i$ increase, in contrast to h5-HT$_{4b}$ receptors, which responded with a weaker signal and only to indoleamines. This signal transduction pathway was dependent on receptor expression levels, however, also detected the lowest expression level of the h5-HT$_{4a}$ receptor [i.e., clone 1 expressing 1500 (±160) fmol/mg]. Interestingly, the calcium response was not only variant-specific but also dependent on compound structure, because benzamide-like compounds triggered higher responses than indoleamines.

The absence of IP pathway activation, the elimination of the response after removal of extracellular calcium, and the inhibition of the response by bepridil suggest that h5-HT$_{4a}$ receptors were able to activate calcium channels. Nevertheless, the question of the cascade events triggering the calcium channel activation has yet to be assessed. The phosphodiesterase inhibitor IBMX and forskolin, a direct AC activator did not enhance the increase of $[Ca^{2+}]_i$ (data not shown), suggesting that, in contrast to the h5-HT$_4$-induced $[Ca^{2+}]_i$ response in adrenocortical cells (Contesse et al., 1996), the h5-HT$_{4a}$ calcium response in HEK 293 cells was not dependent on the cAMP pathway. Moreover, PTX treatment did not modify the calcium response, which excluded

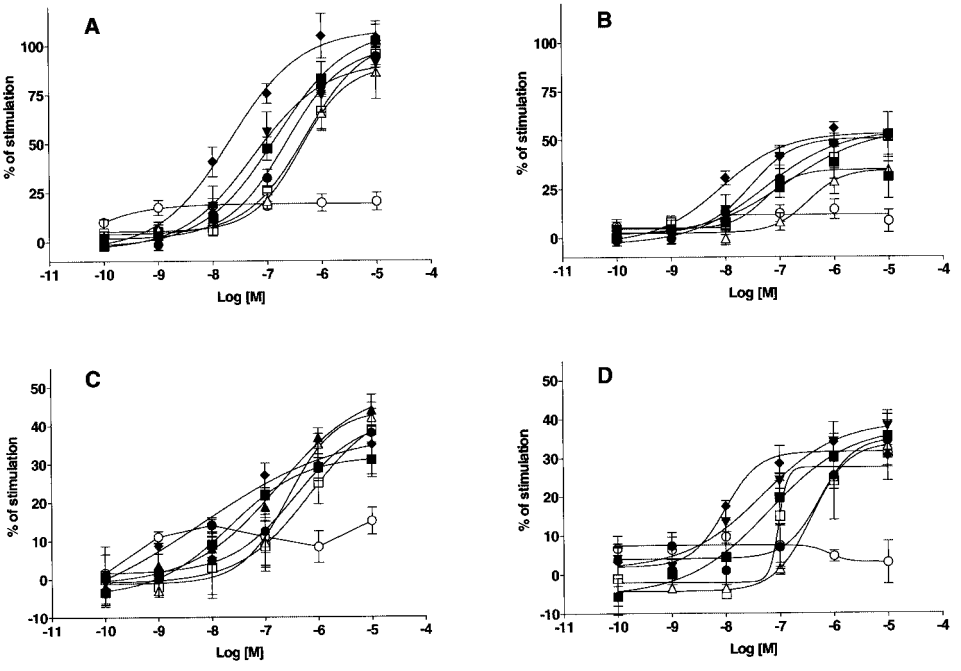

Fig. 6. Effects of PTX pretreatment on [^{35}S]GTPγS binding on membranes from HEK 293 cell line stably transfected with h 5-HT$_4$ receptor isoforms. The h5-HT$_{4b}$ (A and B) and h5-HT$_{4a}$ (C and D) receptor-expressing membranes were employed in [^{35}S]GTPγS binding using a series of agonistic compounds. The membranes in B and D are treated with 100 ng/ml PTX, whereas A and C show membranes from vehicle-treated cells. Results are expressed as percent stimulation over basal levels. The nonstimulated (basal) [^{35}S]GTPγS binding levels were 4150 ± 274 and 3711± 977 dpm for the 5-HT$_{4a}$ and 5-HT$_{4b}$ receptor preparations, respectively. The compounds used for receptor stimulation were 5-HT (■), prucalopride (▼), cisapride (●), SDZ-HTF919 (♦), renzapride (△), 5-MeOT (□), SB204070 (○), and SDZ205,557 (▽). Data are expressed as fitted curves to the mean (±S.D.) of three independent experiments performed in duplicate.

TABLE 4

Effects of PTX-pretreatment on [^{35}S]GTPγS binding on membranes from HEK 293 stably transfected with h5-HT$_{4a}$ and h5-HT$_{4b}$ receptors

PTX stands for membrane preparations from cells treated with 100 ng/ml PTX. Presented results are the means of three independent experiments performed in duplicate. The pEC_{50} values were calculated by nonlinear regression analysis of each concentration-response curve.

Receptor (Clone)	h5-HT$_{4a}$ (8)				h5-HT$_{4b}$ (9)			
	Control		PTX		Control		PTX	
	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.
5-HT	7.6	0.4	7.2	0.4	6.8	0.2	7.3	0.4
5-MeOT	6.1	0.6	7.0	0.8	6.3	0.2	6.8	0.3
Cisapride	6.4	0.7	6.3	0.3	6.7	0.1	7.2	0.3
Prucalopride	6.7	0.3	7.3	0.3	7.2	0.3	7.5	0.2
SDZ-HTF 919	8.2	0.9	8.0	0.2	7.7	0.2	8.1	0.2
Renzapride	6.6	0.3	6.4	0.4	6.4	0.2	6.5	0.3

the hypothesis of Gαi/o-protein involvement in the mechanism. However, because it has been shown that after activation of G-protein heterotrimers, the βγ-subunits are able to activate calcium channels (for review, see Holler et al., 1999), such a pathway has to be considered for the 5-HT_{4a} receptor. In addition, opening of Ca^{2+}-channels subsequent to a βγ-subunit-mediated activation of potassium channels could be another mechanism.

In regard to the G-protein coupling to h5-HT_{4a} and h5-HT_{4b} receptors, we have found a difference between the level of G-protein stimulation induced by h5-HT_{4a} and h5-HT_{4b} receptors. PTX pretreatment induced a reduction in h5-HT_{4b} receptor-mediated [^{35}S]GTPγS binding to the level found for the h5-HT_{4a} receptor, suggesting the existence of an additional Gαi/o component in the h5-HT_{4b} receptor G-protein coupling. This was confirmed by an enhanced increase of cAMP formation induced via the h5-HT_{4b} variant after a PTX pretreatment, which was not found for the h5-HT_{4a} receptor. Taken together, these results indicate that the h5-HT_{4b} receptor is able to activate both Gαi/o- and Gαs-proteins, in contrast to the h5-HT_{4a}-receptor, which couples only to Gαs-proteins. The pEC_{50} values of both PTX-pretreated and nontreated cells in [^{35}S]GTPγS binding and cAMP formation were similar, suggesting that unlike for the D_2 receptor, 5-HT_{4b} receptor dual coupling was not dependent on the agonist concentration (Chang et al., 1997). Until now, dual G-protein coupling is hypothesized to have two main functions. On the one hand, it has been shown for other receptors that these were sequential events contributing to desensitization mechanisms (Lefkowitz, 1998). On the other hand, Gαs- and Gαi/o-proteins have been shown to couple simultaneously to the α_2 adrenergic receptor (Eason et al., 1992), the functional net result of cAMP production representing the integration of the two G-protein-mediated effects.

Taken together, these results show that the h5-HT_{4a} and h5-HT_{4b}-receptor variants do activate partially different intracellular processes (Fig. 8, A and B). The characteristic sum of these differences may result in pharmacological differences of compounds as described in different tissues (Dumuis et al., 1988; Bockaert et al., 1997). Although possible differences in the localization of 5-HT_{4a} and 5-HT_{4b} receptors in brain tissue are still controversial (Gerald et al., 1995; Bender et al., 2000), saturation binding experiments performed on membranes from different human brain regions have revealed a higher fraction of agonist binding sites in the substantia nigra (0.54) than in other brain regions such as the frontal cortex (0.27), the striatum (0.25), or the hippocampus (0.16) (Bonaventure et al., 2000). These values parallel very closely the findings in the present cellular study. Although further studies on cell- and tissue-specific 5-HT_4 receptor expression patterns are required; the above findings together with the reported tissue-specific differences in agonist efficacy provide a physiological context of our study. We have evaluated the influence of receptor levels on the observed responses, however, for the Ca^{2+}-response, promiscuous coupling cannot be completely ruled out. However, we have shown that it is not cAMP-dependent in HEK cells and has different thresholds and pharmacology on both receptor isoforms. It is important to state that a direct comparison of tissue expression levels (guinea pig striatum ~200 fmol/mg; Van den Wyngaert et al., 1997) to data derived on recombinant cells is problematic, because the physiological concentration of neurotransmitter receptors in the synaptic

TABLE 5

Inhibition of maximal [^{35}S]GTPγS binding by 100 ng/ml PTX on membranes from HEK 293 stably transfected with h5-HT_{4b} receptors

Experiments were performed as described for Table 4. Results are means of three independent experiments performed in duplicate and are based on fitted concentration-response curves as presented in Fig. 6. Only the calculated differences between the fitted maximal responses from nontreated and PTX-treated membranes are presented.

	Inhibition of Response by PTX (Mean ± S.D.)
	%
5-HT	68 ± 5
5-MeOT	47 ± 10
Cisapride	44 ± 7
Prucalopride	44 ± 4
SDZ-HTF 919	51 ± 4
Renzapride	61 ± 5

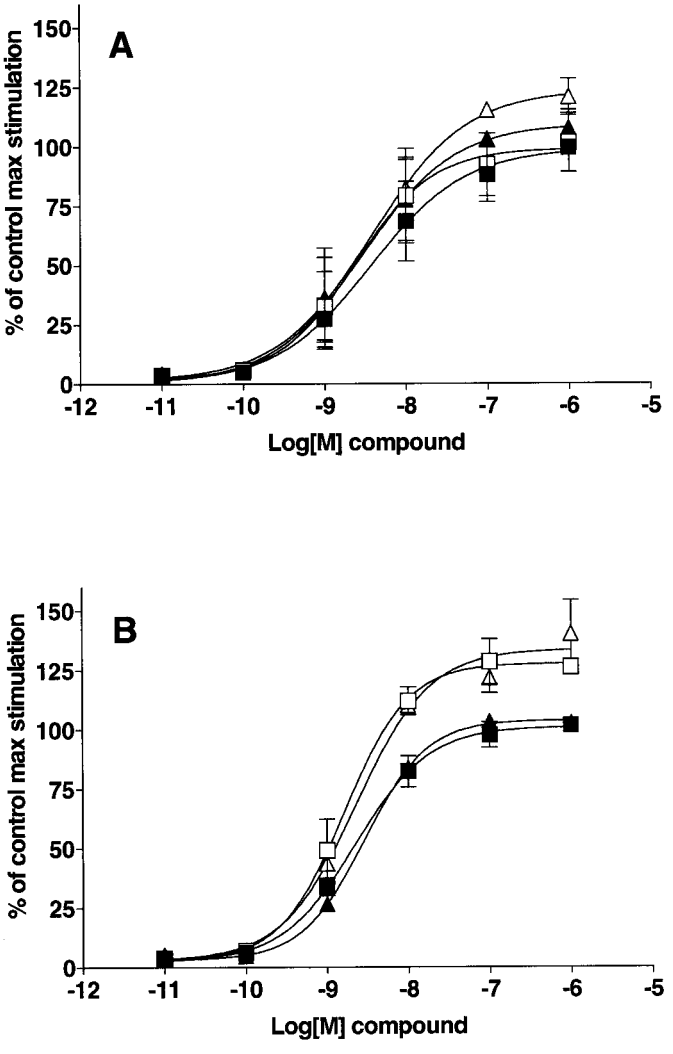

Fig. 7. Effects of PTX pretreatment on cAMP formation in HEK 293 cell lines stably transfected with h5-HT_4 receptor isoforms. The h5-HT_{4a} (A) and the h5-HT_{4b} (B) receptor-expressing cells were stimulated by 5-HT (■ and □) or prucalopride (▲ and △). Cells were treated with 100 ng/ml PTX (open symbols) or vehicle (solid symbols) 24 h before the experiment. Concentration-response curves of cAMP formation were derived from cells as mentioned in Fig. 2. The maximal response of a compound was normalized to the maximal stimulation obtained with 5-HT in nontreated cells (% of 5-HT_{max}). Results are expressed as fitted curve to the mean (±S.D.) of three independent experiments performed in duplicate.

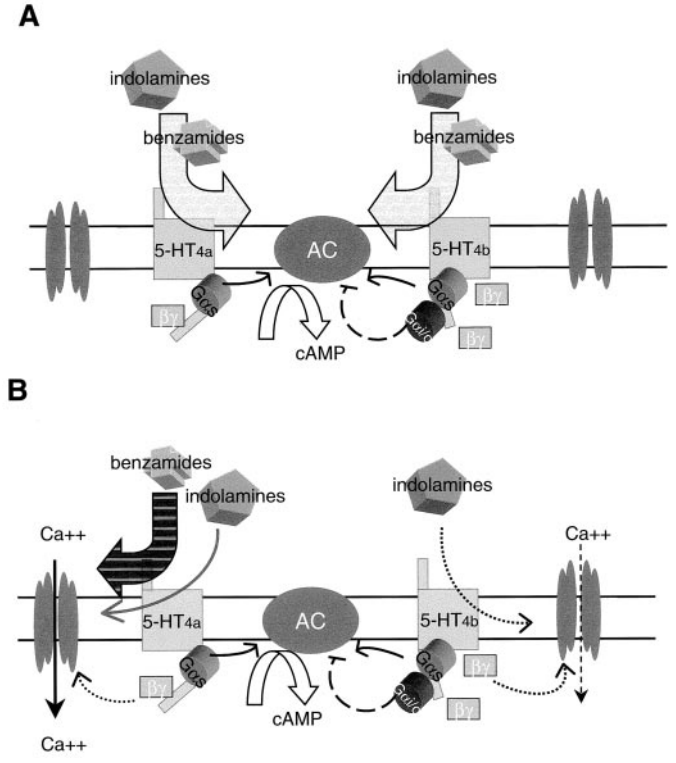

Fig. 8. Model of the signal transduction pathways of h5-HT$_{4a}$ and h-5HT$_{4b}$ receptor splice variants. A summarizes the AC activation pathway. h5-HT$_{4b}$ receptor-induced cAMP formation results from a balance between stimulatory (Gαs) and inhibitory (Gαi/o) G-protein activation, whereas h5-HT$_{4a}$ receptors induce AC activation only via Gαs-proteins. For both splice variants, AC activation is only slightly dependent on the chemical class of agonists. In contrast, calcium influx (B) is largely specific for the h5-HT$_{4a}$ receptor, and its efficacy is strongly dependent on the compound structure, preferring benzamide-like structures to indoleamines. Pointed arrows represent putative pathways; dashed arrows represent inhibitory effects.

cleft is certainly higher than the tissue average observed in binding studies.

On the receptor level the differences in the functional profile of the investigated reference compounds in cAMP and Ca^{2+} measurements favor different forms of R* for each receptor isoform and signaling pathway, which can be stabilized to a different degree by the compounds investigated. In general, further understanding of the fine-tuning of G-protein-coupled receptor isoform-associated signaling and its compound specificity could, in the future, lead to G-protein-coupled receptor isoform-specific (i.e., "super") drugs with potentially broader safety margins.

References

Adham N, Gerald C, Schechter L, Vaysse P, Weinshank RL, and Branchek T (1996) [^{3}H]5-hydroxytryptamine labels the agonist high affinity state of the cloned rat 5-HT4 receptor. *Eur J Pharmacol* **304:**231–235.

Ansanay H, Sebben M, Bockaert J, and Dumuis A (1996) Pharmacological comparison between [^{3}H]GR 113808 binding sites and functional 5-HT4 receptors in neurons. *Eur J Pharmacol* **298:**165–174.

Appel-Dingemanse S, Lemarechal MO, Kumle A, Hubert M, and Legangneux E (1999) Integrated modelling of the clinical pharmacokinetics of SDZ HTF 919, a novel selective 5-HT4 receptor agonist, following oral and intravenous administration. *Br J Clin Pharmacol* **47:**483–491.

Archer S, Meng S, Wu J, Johnson J, Tang R, and Hodin R (1998) Butyrate inhibits colon carcinoma cell growth through two distinct pathways. *Surgery* **124:**248–253.

Arranz B, Rosel P, San L, Sarró S, Navarro MA, and Marcusson J (1998) Characterization of the 5-HT$_4$ binding site in human brain. *J Neural Transm* **105:**575–586.

Bach T, Syversveen T, Kvingedal AM, Krobert KA, Brattelid T, Kaumann AJ, and Levy FO (2001) 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors have nearly identical pharmacology and are both expressed in human atrium and ventricle. *Naunyn-Schmiedeberg's Arch Pharmacol* **363:**146–160.

Bender E, Pindon A, van Oers I, Zhang YB, Gommeren W, Verhasselt P, Leysen J, and Luyten W (2000) Structure of the human serotonin 5-HT4 receptor gene and cloning of a novel 5-HT4 splice variant. *J Neurochem* **74:**478–489.

Blondel O, Gastineau M, Dahmoune Y, Langlois M, and Fischmeister R (1998) Cloning, expression, and pharmacology of four human 5-hydroxytryptamine4 receptor isoforms produced by alternative splicing in the carboxyl terminus. *J Neurochem* **70:**2252–2261.

Blondel O, Vandecasteele G, Gastineau M, Leclerc S, Dahmoune Y, Langlois M, and Fischmeister R (1997) Molecular and functional characterization of a 5-HT$_4$ receptor cloned from human atrium. *FEBS Lett* **412:**465–474.

Bockaert J, Fagni L, and Dumuis A (1997) 5-HT$_4$ receptors: an update, in *Handbook of Experimental Pharmacology: Serotonergic Neurons and 5-HT Receptors in the CNS* (Baumgarten HG and Göthert M eds) pp 439–465, Springer-Verlag, Berlin.

Bonaventure P, Hall H, Gommeren W, Cras P, Langlois X, Jurzak M, and Leysen JE (2000) Mapping of serotonin 5-HT(4) receptor mRNA and ligand binding sites in the post-mortem human brain. *Synapse* **36:**35–46.

Chang A, Shin SH, and Pang SC (1997) Dopamine D2 receptor mediates both inhibitory and stimulatory actions on prolactin release. *Endocrine* **7:**177–182.

Claeysen S, Faye P, Sebben M, Lemaire S, Bockaert J, and Dumuis A (1997) Cloning and expression of human 5-HT4S receptors. Effect of receptor density on their coupling to adenylyl cyclase. *NeuroReport* **8:**3189–3196.

Claeysen S, Sebben M, Becamel C, Bockaert J, and Dumuis A (1999) Novel brain-specific 5-HT4 receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol Pharmacol* **55:**910–920.

Claeysen S, Sebben M, Becamel C, Eglen RM, Clark RD, and Bockaert J (2000) Pharmacological properties of 5-hydroxytryptamine$_4$ receptor antagonists on constitutively active wild-type and mutated receptors. *Mol Pharmacol* **58**:136–144.

Compan V, Daszuta A, Salin P, Sebben M, Bockaert J, and Dumuis A (1996) Lesion study of the distribution of serotonin 5-HT$_4$ receptors in rat basal ganglia and hippocampus. *Eur J Neurosci* **8:**2591–2598.

Contesse V, Hamel C, Lefebvre H, Dumuis A, Vaudry H, and Delarue C (1996) Activation of 5-hydroxytryptamine4 receptors causes calcium influx in adrenocortical cells: involvement of calcium in 5-hydroxytryptamine-induced steroid secretion. *Mol Pharmacol* **49:**481–493.

Domenech T, Beleta J, Fernandez AG, Gristwood RW, Cruz Sánchez F, and Palacios JM (1994) Identification and characterization of serotonin 5-HT4 receptor binding sites in human brain: comparison with other mammalian species. *Brain Res Mol Brain Res* **21:**176–180.

Dumuis A, Bouhelal R, Sebben M, Cory R, and Bockaert J (1988) A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* **34:**880–887.

Eason MG, Kurose H, Holt BD, Raymond JR, and Liggett SB (1992) Simultaneous coupling of alpha 2-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of alpha 2C10, alpha 2C4, and alpha 2C2 adrenergic receptors to Gi and Gs. *J Biol Chem* **267**:15795–15801.

Fagni L, Dumuis A, Sebben M, and Bockaert J (1992) The 5-HT4 receptor subtype inhibits K^+ current in colliculi neurones via activation of a cyclic AMP-dependent protein kinase [published erratum appears in *Br J Pharmacol* 1992;**106:**756]. *Br J Pharmacol* **105:**973–979

Fontana DJ, Daniels SE, Wong EH, Clark RD, and Eglen RM (1997) The effects of novel, selective 5-hydroxytryptamine (5-HT)4 receptor ligands in rat spatial navigation. *Neuropharmacology* **36:**689–696.

Ford APDW and Clarke DE (1993) The 5-HT4 receptor. *Med Res Rev* **13:**633–662.

Gerald C, Adham N, Kao HT, Olsen MA, Laz TM, Schechter L, Bard JA, Vaysse JJ, Hartig PR, Branchek TA, et al. (1995) The 5-HT$_4$ receptor: molecular cloning and pharmacological characterization of two splice variants. *EMBO* (*Eur Mol Biol Organ) J* **14:**2806–2815.

Grossman CJ, Kilpatrick GJ, and Bunce KT (1993) Development of a radioligand binding assay for 5-HT4 receptors in guinea-pig and rat brain. *Br J Pharmacol* **109:**618–624.

Holler C, Freissmuth M, and Nanoff C (1999) G proteins as drug targets. *Cell Mol Life Sci* **55:**257–270.

Kunzelmann K, Kathöfer S, Hipper A, Gruenert DC, and Greger R (1995) Culture-dependent expression of Na^+ conductances in airway epithelial cells. *Pfleug Arch Eur J Physiol* **431:**578–586.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* **273:** 18677–18680.

Lesage AS, Wouters R, Van Gompel P, Heylen L, Vanhoenacker P, Luyten WH, and Leysen JE (1998) Agonistic properties of alniditan, sumatriptan and dihydroergotamine on human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors expressed in various mammalian cell lines. *Br J Pharmacol* **123:**1655–1665.

Leung E, Pulido-Rios MT, Bonhaus DW, Perkins A, Zeitung KD, Hsu SAO, Clark RD, Wong EH, and Eglen RM (1996) Comparison of 5-HT$_4$ receptors in guinea-pig colon and rat oesophagus: effects of novel agonists and antagonists. *Naunyn-Schmiedeberg's Arch Pharmacol* **354:**145–156.

Ouadid H, Seguin J, Dumuis A, Bockaert J, and Nargeot J (1992) Serotonin increases calcium current in human atrial myocytes via the newly described 5-hydroxytryptamine4 receptors. *Mol Pharmacol* **41:**346–351.

Reynolds GP, Mason SL, Meldrum A, De Keczer S, Parnes H, Eglen RM, and Wong EH (1995) 5-Hydroxytryptamine (5-HT)$_4$ receptors in post mortem human brain tissue: distribution, pharmacology and effects of neurodegenerative diseases. *Br J Pharmacol* **114:**993–998.

Satoh S, Chang CS, Katoh H, Hasegawa H, Nakamura K, Aoki J, Fujita H, Ichikawa A, and Negishi M (1999) The key amino acid residue of prostaglandin EP3 receptor for governing G protein association and activation steps. *Biochem Biophys Res Commun* **255:**164–168.

Silvestre JS, Fernandez AG, and Palacios JM (1996) Effects of 5-HT4 receptor

antagonists on rat behaviour in the elevated plus-maze test. *Eur J Pharmacol* **309:**219–222.
Tsunehisa N, Sugimoto Y, Negishi M, Irie A, Ushikubi F, Kakizuka A, Ito S, Ichikawa A, and Narumiya S (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature (Lond)* **365:**166–170.
Van den Wyngaert I, Gommeren W, Verhasselt P, Jurzak M, Leysen JE, Luyten W, and Bender E (1997) Cloning and expression of a human serotonin 5-HT_4 receptor cDNA. *J Neurochem* **69:**1810–1819.
Vanetti M, Vogt G, and Höllt V (1993) The two isoforms of the mouse somatostatin receptor (mSSTR2A and mSSTR2B) differ in coupling efficiency to adenylate cyclase and in agonist-induced receptor desensitization. *FEBS Lett* **331:**260–266.
Vilaro MT, Cortes R, Gerald C, Branchek TA, Palacios JM, and Mengod G (1996) Localization of 5-HT4 receptor mRNA in rat brain by in situ hybridization histochemistry. *Brain Res Mol Brain Res* **43:**356–360.
Waeber C, Sebben M, Nieoullon A, Bockaert J, and Dumuis A (1994) Regional distribution and ontogeny of 5-HT4 binding sites in rodent brain. *Neuropharmacology* **33:**527–541.

Address correspondence to: Dr. Mirek Jurzak, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium. E-mail: mjurzak@janbe.jnj.com