

Novel Brain-Specific 5-HT₄ Receptor Splice Variants Show Marked Constitutive Activity: Role of the C-Terminal Intracellular Domain

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ABSTRACT

We have cloned new 5-Hydroxytryptamine 4 (5-HT₄) receptor splice variants from mouse (m5-HT_{4(e)}R and m5-HT_{4(f)}R), rat (r5-HT_{4(e)}R), and human brain tissue (h5-HT_{4(e)}R) which differ, as do the previously described 5-HT₄ receptor variants, in the length and composition of their intracellular C termini after the common splicing site (L358). These new variants have a unique C-terminal sequence made of two PV repeats and are only expressed in brain tissue. All of the 5-HT₄ receptor splice variants have a high constitutive activity when expressed at low and physiological densities (<500 fmol/mg protein). At similar density, they showed a much higher constitutive activity than

the native and the mutated β_2 -adrenergic receptors. The constitutive activity of the new splice variants with short C-terminal sequences (m5-HT_{4(e)}R and m5-HT_{4(f)}R) was higher than that of the long C-terminal sequence variants (m5-HT_{4(a)}R and m5-HT_{4(b)}R). This may indicate that the short variants have a higher capacity for isomerization from the inactive to the active conformation. Moreover, we further identified a sequence within the C-terminal tail upstream of L358, rich in serine and threonine residues, that played a crucial role in maintaining 5-HT₄R under its inactive conformation.

The physiological functions of 5-hydroxytryptamine (5-HT) in both the central nervous system and the periphery of vertebrates are mediated by a large number of G protein-coupled receptors (GPCRs). Among the 13 known genes coding for 5-HT GPCR, only three (5-HT₄R, 5-HT₆R, and 5-HT₇R) are positively coupled to G protein that stimulates adenylate cyclase (G_s) (Hoyer and Martin, 1997). 5-HT₄ receptors are expressed in a wide variety of tissues of vertebrates including brain, esophagus, ileum, colon, adrenocortical cells, urinary bladder, and human and porcine heart (Eglen et al., 1995; Bockaert et al., 1997). Benzamide 5-HT₄ receptor agonists such as metoclopramide and cisapride are used therapeutically as prokinetic drugs. A role for 5-HT₄ receptors in learning and memory has been demonstrated in brain tissue (Eglen et al., 1995; Letty et al., 1997; Marchetti-Gauthier et al., 1997). Although the pharmacology of 5-HT₄ receptors is similar in the different tissues studied, some unexplained differences still remain. For example, benzamides are superagonists (efficacy greater than that of 5-HT)

in colliculi neurons, the preparation on which 5-HT₄ receptors were first described, whereas in many systems they are partial agonists or even antagonists (Dumuis et al., 1988; Bockaert et al., 1997). A difference in structure may explain some of the observed differences and, in particular, the superagonism of benzamides in colliculi neurons. This is the reason we have persevered in our cloning effort of 5-HT₄ receptors, especially in brain tissues, and further analyzed the coupling efficiency of variants cloned in different species. Gerald et al. (1995) first reported the cloning of two rat variants (r5-HT_{4S}R and r5-HT_{4L}R, now called r5-HT_{4(a)}R and r5-HT_{4(b)}R) that differ in their C-terminal tail after the splicing site at L358. We and others have cloned the human and mouse m5-HT_{4(a)} and m5-HT_{4(b)} receptor homologs (Claeysen et al., 1996; Blondel et al., 1997; Claeysen et al., 1997a). However, Blondel et al. (1998) reported the existence of two different human variants (h5-HT_{4(c)}R and h5-HT_{4(d)}R) also displaying divergent sequences after L358. In this study, we have cloned two additional splice variants in mouse tissue (m5-HT_{4(e)}R and m5-HT_{4(f)}R). The (e) form has also been cloned in rat and human brain tissue (r5-HT_{4(e)}R and h5-HT_{4(e)}R). All of these variants share the same sequence up to L358, followed by specific C termini. Splice variants of

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ABBREVIATIONS: GPCRs, G protein coupled receptors; 5-HT, 5-hydroxytryptamine; TSHR, thyrotropin receptor; RT-PCR, reverse transcription polymerase chain reaction; β_2 -AR, β_2 -adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium; dFBS, dialyzed fetal bovine serum; R, inactive receptor conformation; R*, active receptor conformation; ON, oligonucleotide; CAM, constitutively active mutant; TM, transmembrane domain; i3, third intracellular loop; G_s, G protein that stimulates adenylate cyclase.

GPCRs generally differ in their intracellular domains in particular, within the third intracellular loop (i3) and the C-terminal tail. These two regions are involved in the fine tuning of the coupling of GPCRs to G proteins (Journot et al., 1994). The role of the C-terminus in maintaining the receptor in an inactive conformation (R) in the absence of an agonist has been highlighted by recent studies (Parker and Ross, 1991; Weiss et al., 1994; Matus-Leibovitch et al., 1995; Hasegawa et al., 1996; Mary et al., 1998). These data support the hypothesis of a GPCR structure that was likely selected during evolution to constrain allosteric transition from the inactive (R) to the active (R*) forms in the absence of an agonist (Cotecchia et al., 1990, 1992; Kjelsberg et al., 1992; Kenakin, 1995). This transition is governed by the allosteric transition constant *J*, which is an index of the magnitude of receptor constraint (Samama et al., 1993). The lower the constant, the higher the signal-to-noise ratio.

The aptitude for a given receptor to convert from R to R* in the absence of an agonist is also an important physiopathological parameter to take into account because of the increasing number of pathologies associated with constitutively active receptors (under the R* form in the absence of agonist; Spiegel et al., 1993) and the potential therapeutic use of inverse agonists. Not all receptors have the same propensity to isomerize from R to R* in the absence of a ligand. Thyrotropin receptor (TSHR) is one of the GPCRs that has the highest propensity to isomerize. This is probably the reason that mutations of residues localized throughout the TSHR structure lead to the constitutively active states found in thyroid hyperplasia (Van Sande et al., 1995). Here, we report that 5-HT₄ receptors like TSHRs show marked constitutive activity when expressed at low physiological densities. At a similar receptor density, the newly cloned 5-HT₄ receptor splice variants (m5-HT_{4(e)}R and m5-HT_{4(f)}R) have, in their native conformation, an agonist-independent activity level above the constitutively active mutant β_2 -adrenergic receptor (CAM β_2 -AR).

Materials and Methods

Isolation and Sequencing of the Mouse 5-HT_{4(a)}, 5-HT_{4(e)}, and 5-HT_{4(f)} Receptors' cDNA by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from whole adult Swiss mouse brain. poly(A)⁺ RNA was extracted with Dynabeads Oligo(dT)₂₅ (Dyna Inc., Compiègne, France) and reverse transcribed by Superscript II RNase H Reverse Transcriptase (Life Technologies Inc., Cergy-Pontoise, France) and random nonamers. RT-PCR was performed with the following oligonucleotides: ON1 (Claeyens et al., 1996; accession number Y09585; position 1–24), 5'-TAATGGACAACTTGATGCTAATG-3'; and ON2, designed on International Patent WO 94/14957 (Sequence 1; position 1302–1325), 5'-TAGTAACCTGTTCATGCAGACACA-3'. ON1 is located in the 5'-translated region of m5-HT_{4(b)}R and ON2 is located in the 3'-untranslated part of the r5-HT_{4S}R cDNA. PCR was performed with the *Taq* DNA polymerase (Eurobio, Les Ulis, France), 94°C for 3 min, followed by 30 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec), and elongation (72°C for 1 min). These conditions yielded a large band of nearly 1228 bp that was subcloned into pCR2.1 vector with the Original TA Cloning Kit (Invitrogen, Leek, the Netherlands) and plasmid DNA was prepared for insert sequencing. Nucleotide sequencing was performed with the Sanger dideoxynucleotide chain-termination method on denatured double-stranded plasmid templates with T7 DNA polymerase (Amersham Pharmacia Biotech, Orsay, France). Three different clones were iso-

lated: the m5-HT_{4(a)}R cDNA that is the equivalent of the previously cloned r5-HT_{4S}R in the rat (Gerald et al., 1995) and two other cDNA sequences presenting additional cassettes at position 1079 that we named m5-HT_{4(e)}R and m5-HT_{4(f)}R, taking into account the other published 5-HT₄ receptors and the order of cloning. To confirm the sequence of the 5' part of the receptor around the ATG codon, we performed a PCR reaction with the following primers: ON3 (Patent; Sequence 1; position 21–41), 5'-GGTTGGAAGGAGGAGGATGCT-3', in the 5' noncoding sequence and ON4 (Patent; Sequence 1; position 699–721), 5'-GAGGAGAAACGGGATGTAGAAGG-3', in the middle of the receptor sequence. The PCR conditions with the *Taq* DNA Polymerase were the same as above. Fragments of the expected size (701 bp) were subcloned with the Original TA Cloning Kit. To define the exact sequence of all of the 5-HT₄ receptor splice variant cDNAs, the sequences were performed for each cDNA on three clones obtained from three independent PCR reactions.

Isolation and Sequencing of the Rat 5-HT_{4(e)} Receptor cDNA by RT-PCR. cDNA encoding rat brain 5-HT_{4(e)} receptor, a novel rat variant, has been cloned by RT-PCR on total rat brain poly(A)⁺ RNA (Clontech, Ozyme, Montigny le Bretonneux, France) by the same procedures as described above. Primers used were ON5 (Gerald et al., 1995; accession number U20906; position 19–42) and ON2.

Isolation and Sequencing of the Human 5-HT_{4(e)} Receptor cDNA by RT-PCR. cDNA encoding human brain 5-HT_{4(e)} receptor, a novel human variant, has been cloned by RT-PCR on total human brain poly(A)⁺ RNA (Clontech) by the same procedures as described above. Primers used were ON6 (Claeyens et al., 1997a) (accession number Y0586; position 1–24) and ON2.

Tissue Localization Studies. Brain areas and organs were dissected from adult Swiss mice. cDNA was obtained as described above from poly(A)⁺ RNA. Primers designed to carry out PCR amplification were one sense primer in the common part of the variant sequences, ON7 (Patent WO 94/14957; Sequence 1; position 864–880), 5'-CCAAGGCAGCCAAGACT-5', and two antisense primers, ON2 for the amplification of the C termini of the (a), (e), and (f) forms and ON8 (Patent WO 94/14957; Sequence 3; position 1322–1340), 5'-TTGCCTCATGCTCTTGAA-3', for the amplification of the C-terminus of the (b) form. For the amplification of β -actin as a control, we used ON9 (J00691; position 2252–2271), 5'-GACTCCG-GAGACGGGGTCAC-3' and ON10 (J00691; position 2482–2501) 5'-CGCATCTCTTCTCCCTGG-3'. The PCR conditions were those described above. The template cDNA used per tube was retrotranscribed from 250 or 500 ng of poly(A)⁺ RNA. The expected sizes of the PCR fragments were 463 bp for m5-HT_{4(a)}R, 503 bp for m5-HT_{4(f)}R, 527 bp for m5-HT_{4(e)}R (primer couple ON7/ON2), 528 bp for m5-HT_{4(b)}R (primer couple ON7/ON8), and 450 bp for β -actin (primer couple ON9/ON10). PCR products were run on 3% MetaPhor agarose (FMC Corp., Tebu, Le Perray-en-Yvelines, France)/ethidium bromide gels and photographed. Positive and negative controls (with or without reverse transcriptase) were performed on the β -actin amplification (data not shown). Southern blotting was done after transfer of the DNA to charged nylon membranes (QiaBond; Qiagen S.A., Courtaboeuf, France). The hybridization probe was an RT-PCR fragment obtained in the same PCR conditions with r5-HT_{4(a)}R cDNA as a template (a generous gift from Dr. Christophe Gerald, Synaptic Pharmaceutical Corporation, Paramus, NJ) and the two primers, ON7 and ON2. The probe was labeled with the Prime-It II Random Primer Labeling Kit (Stratagene, Ozyme, Montigny le Bretonneux, France).

β_2 -Adrenergic Receptor and CAM β_2 -AR cDNAs. The human β_2 -adrenergic receptor cDNA (h β_2 -AR) and the CAM β_2 -AR cDNA were obtained from Susanna Cotecchia (Institut de Pharmacologie et Toxicologie, Université de Lausanne, Switzerland).

All of the receptors were subcloned in the eucaryotic expression vector pRK5, a CMV-based expression vector (Schall et al., 1990).

Construction of Truncated 5-HT_{4(a)} Receptor cDNA. Constructs were obtained by inserting a stop codon after residue 327,

346, or 359 in the 5-HT_{4(a)}R cDNA sequence, with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The sense primers used were: Δ327, 5'-CATCCTCTGAAGTGATGATGAGCG-3'; Δ346, 5'-TCCCCTGTTAAACCAACCATT-3'; and Δ359, 5'-CATGTAC-TAAGGTAGACAGTTTTGCAC-3'.

Construction of Mutated m5-HT_{4(e)} Receptor cDNA. The mutant A258L was obtained by exchanging the endogenous A258 to L in the m5-HT_{4(e)}R cDNA sequence with the QuikChange Site-Directed Mutagenesis Kit. The sense primer used was 5'-AGACCAAG-GCACTCAAGACTTTATGTGT-3'.

Construction of Epitope-Tagged m5-HT₄R Splice Variants. The cDNA of all of the 5-HT₄R variants cloned was subcloned into pRK5, a CMV-based expression vector (Schall et al., 1990). We then tagged the m5-HT_{4(a)}, m5-HT_{4(b)}, m5-HT_{4(e)}, and m5-HT_{4(f)} receptors with a stretch of nucleotides coding for a 10-amino acid epitope (EQKLI-SEEDL) of the human c-Myc protein (Ramsay et al., 1984) in the N-terminal extremity with the QuikChange Site-Directed Mutagenesis Kit. The sense primer used was 5'-GATTGAATTCTAATGGAA-CAAAAGCTTATTTCTGAAGAAGACTTGGACAACTTGATGC-3'. Bold letters indicate a start codon and underlined letters indicate a c-Myc epitope.

Cell Culture and Transfection. The cDNAs, subcloned into pRK5, were introduced into LLC-PK1 or in COS-7 cells by electroporation as described by Claeysen et al. (1996). Briefly, cells were trypsinized, centrifuged, and resuspended in EP Buffer 1× (50 mM K₂HPO₄, 20 mM CH₃CO₂K, 20 mM KOH, 26.7 mM MgSO₄; pH 7.4) with 25 to 2000 ng of receptor cDNA. The total amount of cDNA was kept constant at 15 μg per transfection with wild-type pRK5 vector. After 15 min at room temperature, 300 μl of cell suspension (10⁷ cells) were transferred to a 0.4-cm electroporation cuvette (Bio-Rad Ivry sur Seine, France) and pulsed with a gene pulser apparatus (setting 1000 μF, 270 V). Cells were diluted in Dulbecco's modified Eagle's medium (DMEM; 10⁶ cells/ml) containing 10% dialyzed and decplemented fetal bovine serum (dFBS) and plated on 10-cm Falcon Petri dishes or into 12-well clusters at the desired density. With epitope-tagged 5-HT₄ receptors, we estimated the percentage of cells that expressed the receptors.

Immunocytochemical Fluorescence Labeling of Epitope-Tagged 5-HT₄ Receptors in Cells. 5-HT₄ receptors on intact cells were visualized with the anti-c-Myc primary antibody and a rhodamine-conjugated secondary anti-mouse antibody. Cells were grown on coverslips and fixed 24 h after transient transfection in 4% paraformaldehyde diluted in PBS (pH 7.4) for 30 min at room temperature, washed three times in a glycine buffer (0.1 M, pH 7.4), and incubated for 2 h at 37°C with monoclonal antibody 9E10; ascites fluid was diluted 1:250 in PBS-gelatin 0.2%. The cells were then washed and incubated for 1 h with a rhodamine-conjugated secondary anti-mouse antibody (1:40) in PBS-gelatin (0.2%). Coverslips were washed and mounted on glass slides with Gel/Mount (Biomedica Corp., Foster City, CA.). Cells were viewed on a Zeiss Axioplan 2 microscope (Carl Zeiss SARRL, Le Pecq, France).

Determination of cyclic AMP (cAMP) Production in Intact Cells. Six hours after transfection, cells were incubated overnight in DMEM without dFBS with 2 μCi [³H]adenine/ml to label the ATP pool and cAMP accumulation was measured as described by Dumuis et al. (1988).

Membrane Preparation and Radioligand Binding Assay. Membranes were prepared from transiently transfected cells plated on 15-cm dishes and grown in DMEM with 10% dFBS for 6 h and then for 20 h in DMEM without dFBS, as previously described (Ansanay et al., 1996). The cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4°C (900g for 4 min). The pellet was resuspended in buffer containing 10 mM HEPES (pH 7.4), 5 mM EGTA, 1 mM EDTA, and 0.32 M sucrose and homogenized 10 times with a glass-Teflon homogenizer at 4°C. The homogenate was centrifuged at 20,000g for 20 min and the membrane pellet was resuspended in 50 mM HEPES (pH 7.4; 5 mg of protein in 1 ml of solution) and stored at -80°C until used.

To perform radioligand binding studies with [³H]GR 113808 (specific activity, 83 Ci/mmol), 100 μl of membrane suspension, prepared as described above, was diluted 5 times with 50 mM HEPES (pH 7.4; 2–20 μg of protein) containing 10 mM pargyline and 0.01% ascorbic acid and was incubated at 20°C for 30 min with 100 μl of [³H]GR 113808 and 50 μl of buffer or competing drugs. For saturation analysis assays, various concentrations of [³H]GR 113808 (0.001–1 nM) were used. For competition binding experiments, the [³H]GR 113808 concentration was 0.15 nM. Receptor expression was determined with a saturating concentration of [³H]GR 113808 (0.3–0.5 nM). 5-HT (0.5 μM) was used to determine nonspecific binding.

For determination of β₂-adrenergic and CAM β₂-adrenergic receptor expression in COS-7 cells, membrane preparation and binding studies were performed as described previously (Chidiac et al., 1994). The specific radioligand [¹²⁵I]cyanopindolol was used at saturating concentrations (300–500 pM; K_d = 21 nM) and propranolol at 10 μM was added to determine nonspecific binding. For binding experiments, membranes were incubated in a buffer containing 7 mM Tris-HCl (pH 7.4), 12.5 mM MgCl₂, 2 mM EDTA, and proteins (5–20 μg) for 90 min at 20°C.

In both assays, the incubation was terminated by vacuum filtration over Whatman GF/B filters presoaked with 0.1% polyethylenimine with a Brendel 48-well cell harvester; the filters were then washed 3 times with 4 ml of ice-cold buffer (10 mM HEPES). Filtration was performed in less than 10 s. Radioactivity was counted with a complete phase combining system for liquid scintillation counting (Amersham, Les Ulis, France). Protein concentration in the samples was determined with the Bio-Rad protein assay.

Drugs. GR 113808 ([1-[2(methylsulfonyl-amino)ethyl]4-piperidinyl]methyl-1-methyl-indole-3 carboxylate, maleate) and GR 125487 ([1-[2(methylsulfonyl-amino)ethyl]4-piperidinyl]methyl-5-fluoro-2-methoxy-1H-indole-3-carboxylate, hydrochloride) were generously donated by Glaxo (Ware, Herts, U.K.); [³H]GR 113808 was purchased from Amersham Pharmacia Biotech; 5-HT (serotonin) and isoproterenol were purchased from Sigma Chemical Co. (St. Louis, MO). RS 100235 (1-(8-amino-7-chloro-1,4-benzodioxan-5-yl)-3-[[3,4-dimethoxyphenyl]prop-1-yl]piperidin-4-yl]propan-1-one) was generously donated by Roche-Syntex Laboratories (Palo Alto, CA).

Results

Primary Structure of Different 5-HT₄R Splice Variants. We have published the isolation of m5-HT_{4(b)}R, previously named long form (Claeysen et al., 1996), which has also been isolated in rat and human tissue (Gerald et al., 1995; Van den Wyngaert et al., 1997). We performed RT-PCR experiments with poly(A)⁺ RNA prepared from mouse brain with primers designed to clone the variant (a) (described in *Materials and Methods*). We obtained three different full-length cDNA fragments. One of them corresponded to variant (a), previously cloned in rat and human tissue (Gerald et al., 1995; Blondel et al., 1997; Claeysen et al., 1997a), whereas two new clones generated from the same RT-PCR corresponded to new 5-HT₄ receptor cDNAs, coding for variants significantly shorter than the (a) and (b) forms. Blondel et al. (1998) have reported the existence of two unrelated h5-HT₄R splice variants, (c) and (d) (Fig. 1). Taking into account the already published human variants, we named the present new variants m5-HT_{4(e)}R and m5-HT_{4(f)}R (Fig. 1). We also cloned a r5-HT_{4(e)} receptor identical to m5-HT_{4(e)}R in length and amino acid sequence after the splicing site by performing RT-PCR experiments with poly(A)⁺ RNA prepared from rat brain. From human brain tissue, we have also isolated a new splice variant very close but not identical in sequence to the (e) form. When comparing its nucleotide

and amino acid sequences to those of rat and mouse (e) forms, we can suggest that this new human variant produced by alternative splicing is the human ortholog of the mouse and rat (e) forms. Therefore, we called it h5-HT_{4(e)}R. Whatever the species, mouse, human or rat, the different splice variants have an identical sequence up to L358, whereas the length as well as the composition of the C-terminal end is specific for each splice variant (Fig. 1). Note the specific characteristics of the last four residues of the C termini of the new clones described here. The r5-HT_{4(e)}, m5-HT_{4(e)}, and m5-HT_{4(f)}, as well as the h5-HT_{4(e)} receptors contain two PV repeats.

Novel Mouse 5-HT₄R Splice Variants are Brain-Specific. With pairs of primers designed as described in *Materials and Methods*, the expression of the different m5-HT₄R transcripts (Fig. 2A) was assayed in both brain and in the peripheral tissues. Note that our RT-PCRs performed either with 250 or 500 ng of poly(A)⁺ RNA are nonsaturating, i.e., the ratio between the different bands amplified remain the same whatever the quantity used (Fig. 2B). The most interesting observation was the total absence of m5-HT_{4(e)}R and m5-HT_{4(f)}R mRNA expression in peripheral tissues. In brain tissue, we detected a high level of the long form (a) and (b) mRNAs in all of the structures studied (Fig. 2C). The two new variants, (e) and (f), were expressed in the colliculi, the striatum, and the hippocampus. In the cerebellum and the cortex, the (e) form was not detected. We observed a relatively high level of the m5-HT_{4(a)}R and m5-HT_{4(b)}R mRNA in heart and kidney tissue, but also in bladder, ileum, and colon tissue. The esophagus and the adrenal gland showed very low levels of signal, and liver tissue did not express detectable amounts of any of the four splice variants. r5-HT_{4(e)}R

and h5-HT_{4(e)}R mRNAs have been detected only in brain tissue (data not shown).

5-HT_{4(a)} Receptors Have a High Constitutive Activity. Because we previously observed that native m5-HT_{4(a)}R and h5-HT_{4(b)}R expressed high basal constitutive activities in the absence of an agonist (Claeysen et al., 1997a,b), we decided to investigate the capacity of 5-HT₄ receptors to convert from R to R* by studying their constitutive activity as a function of receptor density. In COS-7 cells, in the absence of 5-HT, a 2-fold increase in cAMP production as compared with mock transfected cells was obtained for a 5-HT_{4(a)} receptor density (150–200 fmol/mg protein) comparable to those found in brain tissues (Waeber et al., 1994; Fig. 3A). Because comparison of receptor density is rather complex, we have determined the percentage of cells expressing the 5-HT₄ receptors. Cells were transiently transfected with the epitope-tagged receptor cDNA and visualized with the anti c-Myc primary antibody. We found that 57 ± 16% (n = 6) of the cells expressed the transfected epitope-tagged 5-HT₄ receptor isoforms, whatever the splice variant considered.

The constitutive activity of 5-HT_{4(a)}R was almost linearly proportional to the receptor density and independent of the species origin of the receptor (Fig. 3A). Such a constitutive activity was not specific to COS-7 cells because similar results were obtained in LLC-PK1 cells (Fig. 3B). In this cell line, a 2-fold increase in cAMP production was observed for a receptor density of 100 fmol/mg protein. Figure 3A shows that native 5-HT_{4(a)} receptors had a much higher constitutive activity than the native hβ₂-AR when expressed in COS-7 cells.

Although our experiments were performed with dialyzed serum, it was important to check whether some contamination by 5-HT, which is known to stick to plastic surfaces, was

C-termini of 5-HT₄R splice variants

	358		
m5-HT ₄ R	(a)	LRYTVLHSGHHQELEKLP IHN DPESLESCF	387 *
	(b)	LRDAVECGGQWESRCHLTATSPLVAAQPSDT	388 *
	(e)	LSFPLLFRNRPVPV	371 *
	(f)	LRPVPV	363 *
r5-HT ₄ R	(a)	LRYTVLHSGQHQELEKLP IHN DPESLESCF	387 †
	(b)	LRDTVECGGQWESRCHLTATSPLVAAQPSDT	388 †
	(e)	LSFPLLFCNRPVPV	371 *
h5-HT ₄ R	(a)	LRYTVLHRGHHQELEKLP IHN DPESLESCF	387 *
	(b)	LRDAVECGGQWESQCHPPATSPLVAAQPSDT	388 §
	(c)	LSSGTETDRNFGIRKRLTKPS	380 ‡
	(d)	LRF	360 ‡
	(e)	LSGCSPVSSFLLLFCNRPVPV	378 *

Fig. 1. C-terminal amino acid sequences of the 5-HT₄ receptor splice variants. All of the cloned 5-HT₄ receptors share the same amino acid sequence up to Leu 358 and differ from Leu 358 on (boxed). *Receptors cloned by our group (Claeysen et al., 1996; Claeysen et al., 1997a). †Receptors cloned by Gerald et al. (1995). ‡Receptors previously cloned by Blondel et al. (1998). §Receptors cloned by Van den Wyngaert et al. (1997). Note that the amino acid sequences after Leu 358 of newly cloned m5-HT_{4(e)}, m5-HT_{4(f)}, r5-HT_{4(e)}, and h5-HT_{4(e)} receptors are shaded. The EMBL Nucleotide Sequence Database accession numbers for mouse 5-HT_{4(a)}, 5-HT_{4(b)}, 5-HT_{4(e)} and 5-HT_{4(f)} receptors are Y09587, Y09585, Y09588, and AJ011369, respectively. For r5-HT_{4(a)}, r5-HT_{4(b)}, and r5-HT_{4(e)} receptors, the numbers are U20906, U20907, and AJ011370, respectively, and for h5-HT_{4(a)}, h5-HT_{4(b)}, h5-HT_{4(c)}, h5-HT_{4(d)} and h5-HT_{4(e)} receptors, Y09586, Y10437, Y12506, Y12507, and AJ011371, respectively.

responsible for the observed intrinsic activity. Therefore, we used highly potent selective 5-HT₄R antagonists. Some, like RS 100235, were neutral antagonists and had no effect on basal 5-HT_{4(a)} receptor-induced constitutive activity (Fig. 4A). However, other antagonists, like GR 125487, behaved as inverse agonists because they reduced this basal constitutive activity (Costa and Hertz, 1989). In addition, both antagonists used in Fig. 4A were highly potent in inhibiting the

5-HT-induced cAMP production in transfected COS-7 cells expressing 5-HT_{4(a)} receptors (Fig. 4B). These experiments indicated that the observed constitutive activity was not due to 5-HT contamination.

Mouse 5-HT₄ Receptor Splice Variants Exhibit Different Constitutive Activities. It was of particular interest to check if the difference in the structure of the C-termini of the different m5-HT₄ receptor splice variants influence

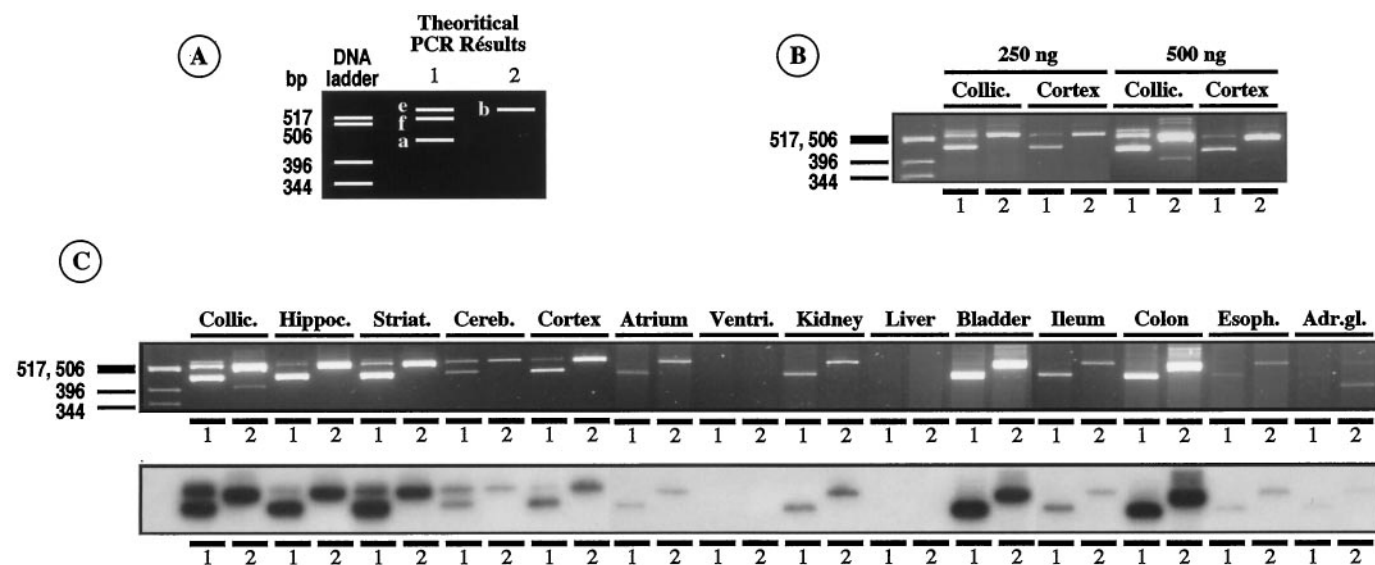


Fig. 2. Regional distribution of the four mouse 5-HT₄ receptor splice variants. A, theoretical PCR results. Lane 1, primer couple ON7/ON2 (see *Materials and Methods*) amplifying the mouse (a), (e), and (f) forms. Lane 2, primer couple ON7/ON8 (see *Materials and Methods*) amplifying the mouse (b) form. B, Distribution of mouse 5-HT₄R transcripts. RT-PCR analysis was performed with 250 and 500 ng of poly(A)⁺ RNA from the colliculus (collic.) and the cortex of adult Swiss mice with primer couple ON7/ON2 (Lane 1) or primer couple ON7/ON8 (Lane 2) (see *Materials and Methods*). Thirty-five microliters of a 50- μ l reaction was loaded and separated on 3% agarose gels. A photograph of the ethidium bromide-stained gels is shown. C, distribution of mouse 5-HT₄R transcripts. RT-PCR analysis was performed with 500 ng of poly(A)⁺ RNA from various structures of adult Swiss mice with primer couple ON7/ON2 (Lane 1) or primer couple ON7/ON8 (Lane 2) (see *Materials and Methods*). Top, PCR products were separated on 3% agarose gels and 25 μ l of a 50- μ l reaction was loaded. A photograph of the ethidium bromide-stained gels is shown. Bottom, Southern blot of the PCR results. A 2-h exposure of the autoradiogram is shown. Collic., colliculus; Hippoc., hippocampus; Striat., striatum; Cereb., cerebellum; Ventri., ventricle; Esoph., esophagus; Adr.gl., adrenal gland.

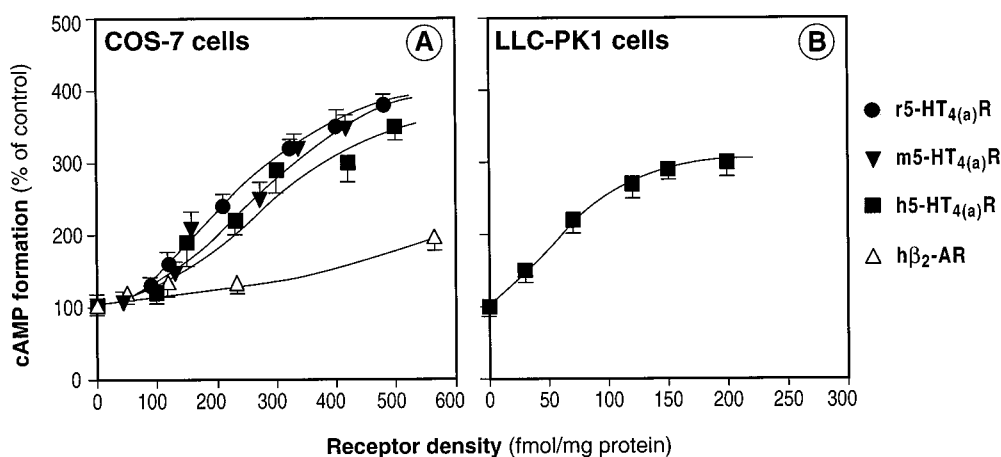


Fig. 3. Basal levels of cAMP in COS-7 and LLC-PK1 cells transiently expressing increasing concentrations of human, rat, and mouse 5-HT_{4(a)} receptor splice variants. A, COS-7 cells were transfected with increasing concentrations of human, rat, or mouse 5-HT_{4(a)}R and h β_2 -AR cDNA (25–1000 ng of cDNA/10⁷ cells). The total amount of DNA was kept constant at 15 μ g/10⁷ cells with wild-type pRK5 vector. The percentage of conversions of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.12 \pm 0.03 (control). Basal levels of cAMP productions at different receptor concentrations, measured after a 30-min incubation, are expressed as a percentage of control. B, LLC-PK1 cells were transfected with increasing concentrations of h5-HT_{4(a)} receptor cDNA/10⁶ cells (25–1000 ng of cDNA) or with equivalent concentrations of pRK5 plasmid (mock-transfected cells). The percentage of conversion of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.115 \pm 0.021 (control). Basal levels of cAMP production, measured at different receptor concentrations after a 30-min incubation, are expressed as a percentage of control. In A and B, 5-HT₄ receptor densities were determined with a saturating concentration of [³H]GR 113808 (0.4–0.5 nM) and h β_2 -AR densities were determined with a saturating concentration of [³H]CYP (0.5 nM). Each point is the mean \pm S.E.M. of three independent experiments.

their propensity to induce high basal constitutive activity. We actually found that although the (a) and (b) forms gave a similar constitutive activity, when studied as the function of receptor density, the shortest forms, (e) and (f), presented a two-fold higher basal constitutive activity (Fig. 5). In comparison, in this experiment, h β_2 -AR did not modify cAMP production when expressed at this density (Fig. 5).

A C-Terminal Domain of 5-HT₄ Receptors Inhibits Constitutive Activity. Because we found that the shortest splice variants had the highest constitutive activity (Fig. 5), we wondered if the C-terminal domain was important for constraining 5-HT₄ receptors in an inactive state. This turned out to be true because progressive deletions of the C-terminal part of the 5-HT_{4(a)} receptor (Fig. 6A) resulted in a progressive increase in constitutive activity for a similar density of wild-type and truncated receptors (Fig. 6B). The truncated 5-HT₄Δ359 receptor, identical in length and sequence to the 5-HT_{4(f)}R variant without its PVPV sequence, represents the common region of the four splice variants. This truncated receptor displayed a constitutive activity similar to that of 5-HT_{4(f)}R, whereas a larger deletion mutant (deletion of 13 residues, 5-HT₄Δ346 receptor) dramatically activated the receptor. A larger deletion of the C-terminal (Δ327) did not further increase the constitutive activity compared to Δ346. Both deletions (Δ346 and Δ327) induced a 5-fold activation of basal cAMP production for receptor densities below 200 fmol/mg protein.

The C-Terminal Part of the i3 Constrains the 5-HT₄ Receptor in its Inactive Form. A comparison between the sequences of the C-terminal part of i3 of m5-HT₄ receptors on one hand and of the h β_2 -AR on the other hand indicated that among the last seven residues, four of them were identical. One of them, alanine in 5-HT₄ receptor and leucine in β_2 -AR at position -3 upstream of transmembrane domain (TM)VI, was interesting because the mutation of leucine to alanine

was one of the four mutations in i3 of β_2 -AR that produced the CAM h β_2 -AR (Samama et al., 1993; Fig. 7A). It is also this mutation (leucine to alanine) which is the most potent mutation in inducing the constitutive activity of CAM h β_2 -AR (S. Cotecchia, personal communication). Therefore, one possible explanation for the highest constitutive activity of native 5-HT₄ receptors as compared with native β_2 -AR was the presence, at this homologous position, of an alanine (A258) in the wild-type 5-HT₄ receptor (Fig. 7A) instead of a leucine in β_2 -AR. For this reason, we mutated this A258 to L in the 5-HT_{4(e)} receptor. Instead of reducing the constitutive activity of the receptor, we obtained a dramatic increase (Fig. 7B). For comparison, we note that the constitutive activity of the native 5-HT_{4(e)} receptors was as high as the constitutive activity of CAM h β_2 -AR.

Basal and 5-HT-Stimulated cAMP Production in Cells Transfected with 5-HT_{4(a)} and 5-HT_{4(e)} Receptors and 5-HT₄ Mutated Receptors. The short C-terminal sequence 5-HT_{4(e)} receptors had a higher basal constitutive activity than the long C-terminal sequence 5-HT_{4(a)} receptors as already described in Fig. 5. However, they had identical activity in the presence of 5-HT (Fig. 8A). In this experiment, we expressed about 1000 fmol/mg protein of 5-HT_{4(a)} and 5-HT_{4(e)} receptors. The identical 5-HT-stimulated activity observed with these two 5-HT₄ variants was not due to a limiting step in cAMP formation because increasing the number of expressed receptors from 1000 to 4000 fmol/mg protein increased the 5-HT-stimulated activity proportionally (data not shown). Figure 8B illustrates the relative efficacy of 5-HT to stimulate over the basal constitutive activity of the wild-types and of the mutated receptors. The higher the basal constitutive activity, the lower the relative efficacy of 5-HT. Note that the 5-HT maximum response was slightly lower in severely truncated receptors (Fig. 8A). A decrease in the mutated receptor stability during incubation or an increase

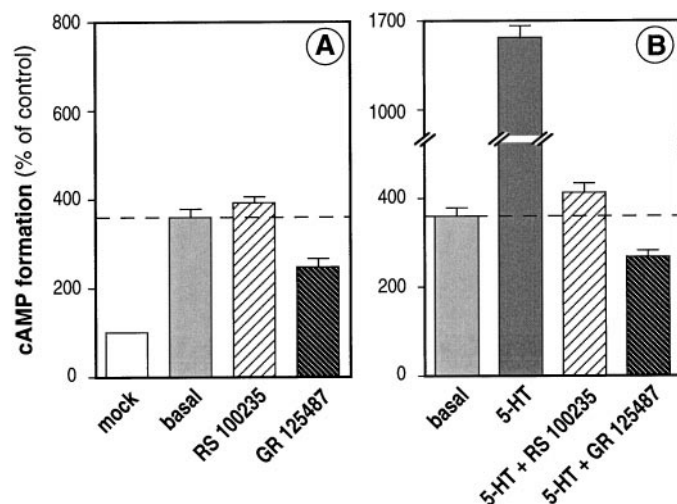


Fig. 4. Effect of two potent 5-HT₄R antagonists, RS 100235 and GR 125487, on basal and 5-HT-stimulated 5-HT_{4(a)} receptor. The effects of RS 100235 and GR 125487 on cAMP production, in the absence (A) and presence (B) of 5-HT (10⁻⁷ M) were evaluated on h5-HT_{4(a)} receptor splice variant transiently transfected in COS-7 cells expressing 320 ± 45 fmol/mg protein. Levels of cAMP accumulation were measured after a 15-min incubation and expressed as a percentage of the control. The percentage of conversion of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.14 ± 0.018 (control). Each value is the means ± S.E.M. of three independent experiments, each performed in triplicate.

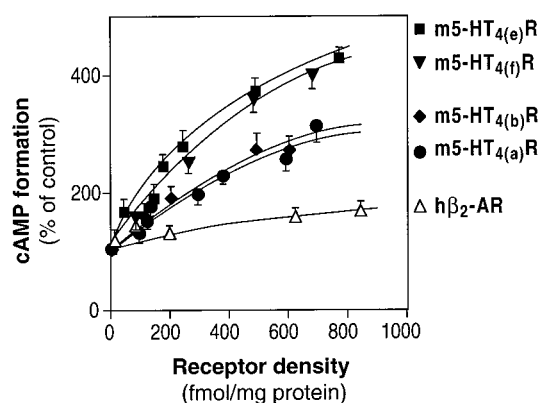


Fig. 5. Mouse 5-HT₄ receptor splice variants exhibit different basal activities. The activity was measured by the increase in cAMP production in COS-7 cells, expressed in m5-HT_{4(a)}, m5-HT_{4(b)}, m5-HT_{4(e)}, m5-HT_{4(f)}, or h β_2 -A receptor as a function of receptor density. COS-7 cells were transfected with increasing concentrations of m5-HT_{4(a)}, m5-HT_{4(b)}, m5-HT_{4(e)}, m5-HT_{4(f)} and h β_2 -A receptors' cDNA (10–1000 ng of cDNA/10⁷ cells). Basal levels of cAMP accumulation were measured after a 30-min incubation. The percentage of conversion of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.12 ± 0.03 (control). Basal cAMP production measured at different concentrations of receptor is expressed as a percentage of the control. 5-HT₄ and h β_2 -A receptor densities were determined as indicated in Fig. 3. Data for each of the splice variants represent the mean ± S.E.M. for at least three independent experiments, each performed in triplicate.

in desensitization can be proposed as an explanation (Pei et al., 1994; Gether et al., 1997).

Substitution of A258 by L within the i3 and C-Terminal Truncations ($\Delta 346$ and $\Delta 327$) of m5-HT_{4(e)} Receptors Increases the Affinity of 5-HT and Its Potency in Stimulating cAMP. The extended ternary complex (Samama et al., 1993), as well as the cubic ternary complex (Kenakin and Weiss, 1996), introduced for the modeling of the G protein receptor isomerization from R to R* and for their interaction with G proteins, indicated that the observed affinity of the agonist increased as the function of the isomerization constant *J*. Indeed, we observed that the affinity of 5-HT for both the mutated 5-HT_{4(e)}R A258L and C-terminal-deleted ($\Delta 346$ and $\Delta 327$) 5-HT₄ receptors expressed at similar densities, increased by almost 2 logs₁₀ when compared with that of the native receptor. 5-HT binding was measured by competition. The *K_d* values for 5-HT were 47 ± 12 nM for the wild-type 5-HT_{4(e)}R, 0.53 ± 0.6 nM and 1.34 ± 0.15 nM for the truncated 5-HT₄R $\Delta 346$ and 5-HT₄R $\Delta 327$, respectively, and 0.85 ± 0.10 nM for the mutated 5-HT_{4(e)}R A258L

(Fig. 9A). The difference in affinity observed was certainly not due to a difference in the coupling to G proteins (different proportion of the high affinity state) because a high GppNHp concentration (10 μ M) did not modify this difference (data not shown). In contrast to these results, we found no difference between the affinity of 5-HT for the different natural splice variants. The EC₅₀ of 5-HT to stimulate cAMP was about 10 times lower when determined in mutant receptor-transfected cells rather than in native receptor-transfected cells (Fig. 9B). The EC₅₀ values were 8 ± 2 nM, 0.3 ± 0.10 nM, 0.7 ± 0.10 nM, and 0.9 ± 0.15 nM for 5-HT_{4(e)}R, 5-HT₄R $\Delta 346$, 5-HT₄R $\Delta 327$, and 5-HT_{4(e)}R A258L, respectively.

Discussion

We report the cloning of new 5-HT₄R splice variants (m5-HT_{4(e)}R, m5-HT_{4(f)}R, r5-HT_{4(e)}R, and h5-HT_{4(e)}R) that possess original C-terminal domains compared with already cloned 5-HT₄R splice variants (Gerald et al., 1995; Claeysen et al., 1996, 1997a; Blondel et al., 1997, 1998; Van den Wyngaert et al., 1997). They have in common an intriguing and unique PVPV C-terminal sequence that is also found in the C termini of some EPH/ELK family receptor tyrosine kinases (Wicks et al., 1992). We can only speculate on the possible roles for this sequence, but one could be the interaction of

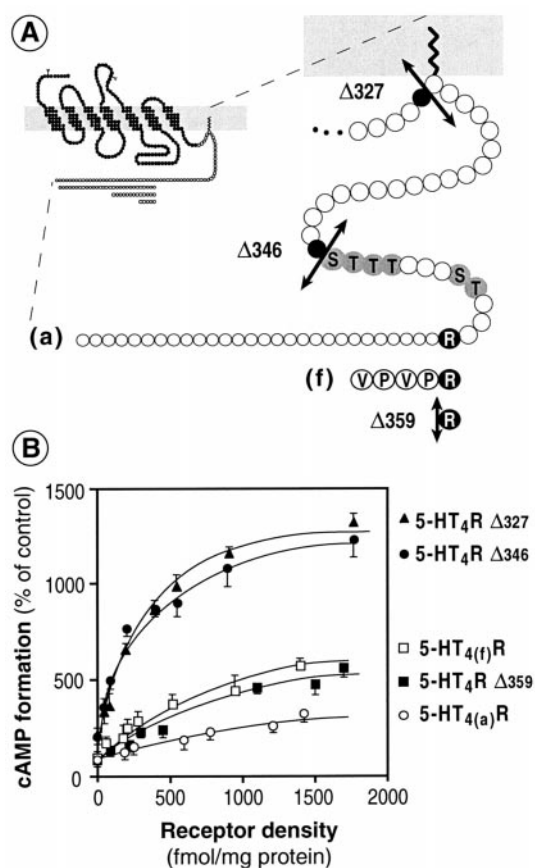


Fig. 6. A common sequence in the C-terminal domain of 5-HT₄ receptor temporizes its basal activity. A, schematic representation of the C-terminal tail of the m5-HT_{4(a)} and m5-HT_{4(f)} receptor splice variants. The arrows indicate the sites substituted by a stop codon to produce the truncated receptors (5-HT₄R $\Delta 359$, 5-HT₄R $\Delta 346$, and 5-HT₄R $\Delta 327$). Serine-threonine clusters are shaded. B, COS-7 cells were transfected with increasing concentrations of m5-HT_{4(a)}R, m5-HT_{4(f)}R, m5-HT₄R $\Delta 359$, m5-HT₄R $\Delta 346$, or m5-HT₄R $\Delta 327$ receptor cDNA/10⁷ cells (10–2000 ng of cDNA). The percentage of conversion of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.15 ± 0.03 (control). cAMP productions were measured at different concentrations of receptor are expressed as a percentage of the control. Each point is the mean \pm S.E.M. of three independent experiments, each performed in triplicate.

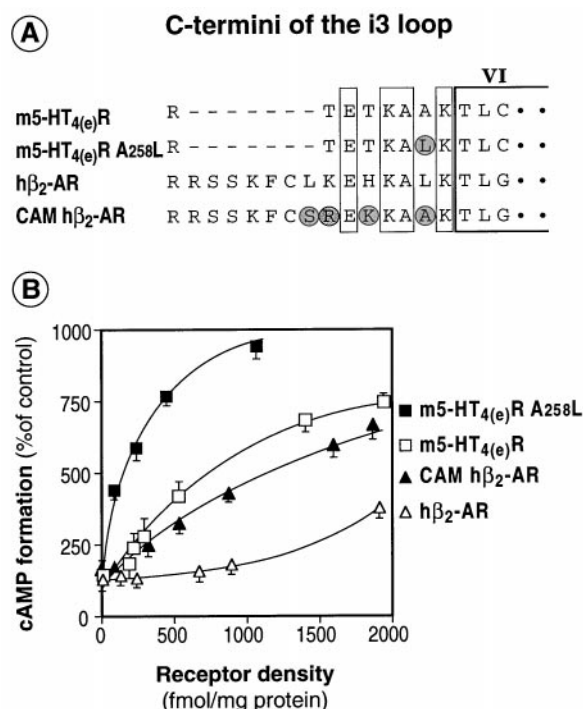


Fig. 7. Mutations in the C-terminal part of the i3 loop of 5-HT_{4(e)} and β ₂-A receptors increase constitutive activity. The relationship between receptor density and basal levels of cAMP production is measured in COS-7 cells transfected with m5-HT_{4(e)}R or m5-HT_{4(e)}R A258L or h β ₂ or CAM h β ₂ receptors. A, amino acid sequences of the C-termini of the i3 in m5-HT₄R and h β ₂-AR, mutated residues in m5-HT_{4(e)}R A258L and CAM h β ₂-AR. The mutated residues are shaded. B, different receptor densities were obtained by transfecting COS-7 cells by electroporation with varying amounts of cDNA (10–2000 ng/10⁷ cells). The total amount of DNA was kept constant at 15 μ g/transfection with wild-type pRK5 vector. 5-HT₄ receptor densities were determined with a saturating concentration of [³H]GR 113808 (0.4–0.5 nM), h β ₂-AR and CAM h β ₂-AR densities were determined with a saturating concentration of [³H]CYP (0.5 nM). The percentage of conversion of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.14 ± 0.03 (control).

these splice variants with proteins similar to PDZ-domain-containing proteins. Because of the roles of 5-HT₄ receptors in learning and memory (Ansanay et al., 1995; Eglen et al., 1995; Letty et al., 1997; Marchetti-Gauthier et al., 1997), it is interesting to note that the newly cloned PVPV-containing receptors are only expressed in brain tissue.

In view of the large number of 5-HT₄ receptor variants that differ only in their C-termini domains, it was of particular interest to analyze the role of these domains in G protein coupling. Over the past 10 years, our view on how GPCRs are activated has been reassessed based on the pioneering work of Costa and Lefkowitz (Costa and Hertz, 1989; Cotecchia et al., 1990). They demonstrated that GPCRs can isomerize

from an inactive form (R) to an active form (R*) in the absence of an agonist. GPCRs are no longer considered as shifting from an inactive to an active conformation when interacting with the agonist but it is now proposed that the agonist is only stabilizing the R* form. Thus, GPCRs can be considered as allosteric molecules, which already include ionotropic receptors such as nicotinic receptors (Monod et al.,

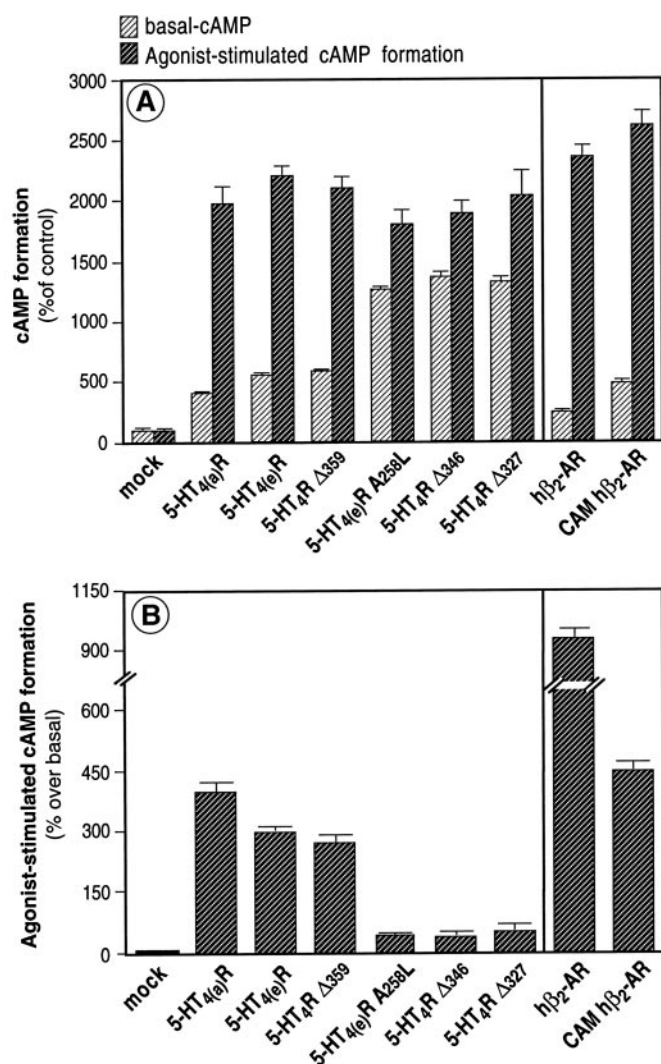


Fig. 8. Mutations in 5-HT₄ receptor decrease responsiveness to an agonist proportionally to increasing constitutive activity. A, COS-7 cells were incubated for 15 min at 37°C with vehicle (basal) or with 10⁻⁶ M 5-HT for cells expressing wild-type or mutated 5-HT₄R and with vehicle (basal) or with 10⁻⁶ M isoproterenol for those expressing wild-type or mutated hβ₂-AR. The expression levels of 5-HT_{4(e)}R, 5-HT_{4(e)}R Δ258L, 5-HT₄Δ346, 5-HT₄Δ327, hβ₂-A, and CAM hβ₂-A receptors were 940 ± 56, 1060 ± 126, 1180 ± 170, 1090 ± 165, 827 ± 89, 860 ± 35, and 980 ± 105 fmol/mg protein, respectively. The percentage of conversion of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.12 ± 0.03 (control). Results are expressed as a percentage of the control. Data are means ± S.E.M. of four experiments. B, levels of agonist-stimulated cAMP production in the experiment reported in (A) are expressed as the percentage over basal cAMP formation.

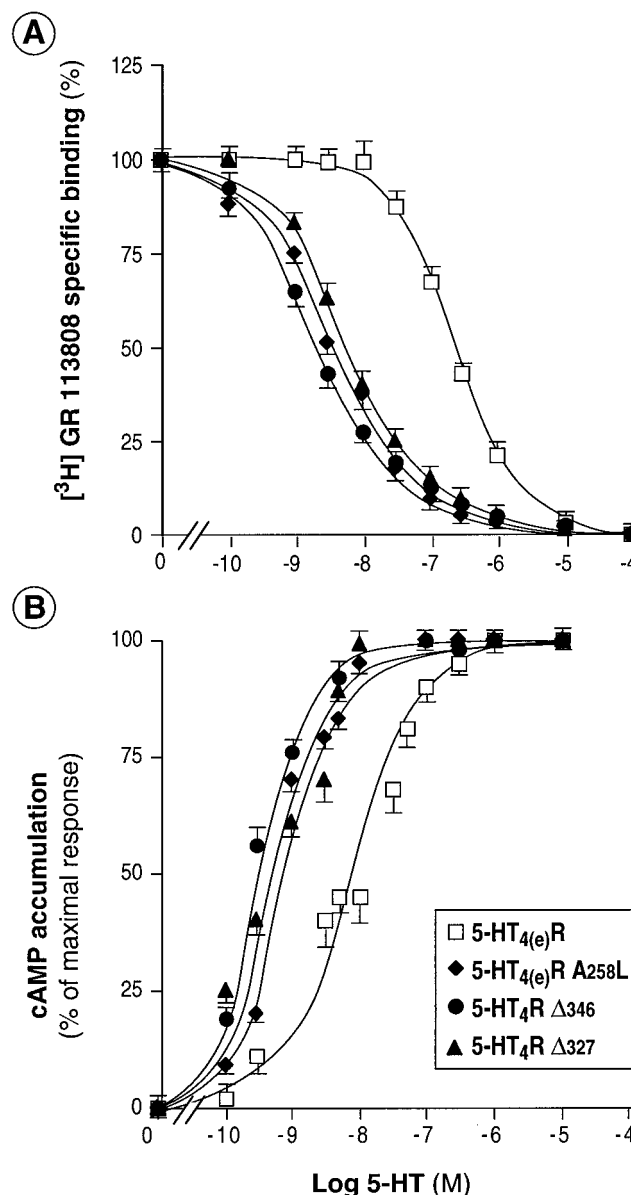


Fig. 9. Competition binding and 5-HT-induced cAMP formation in COS-7 cells expressing wild-type or mutated 5-HT₄ receptors. A, competition of 5-HT for [³H]GR 113808 binding on membranes derived from COS-7 cells expressing comparable receptor densities (between 827 and 1180 fmol/mg protein as in experiments reported in Fig. 8) of m5-HT_{4(e)}R, m5-HT₄R Δ346, m5-HT₄R Δ327 or m5-HT_{4(e)}R Δ258L. The results expressed as a percentage of the specific binding in the absence of a competing agent are from a representative experiment of three to six independent experiments. B, intracellular cAMP levels were measured at increasing concentrations of 5-HT in the same transfected COS-7 cells as for binding experiments and experiments reported in Fig. 8. The 100% maximal increase above basal differs between receptors. The basal and 5-HT maximal adenyl cyclase stimulation values expressed as a percentage of the control, are provided in Fig. 8. The results are expressed as a percentage of stimulation and are representative of four independent experiments performed in triplicate.

1965; Changeux and Edelstein, 1998). Among the numerous theoretical consequences of this view is the idea that the structure of the receptor has been selected to constrain the receptor under the R form in the absence of an agonist. The higher the constraint (low isomerization J constant), the higher the signal-to-noise ratio in the presence of agonist.

The capacity of a native receptor to spontaneously isomerize from R to R* is certainly a very important pharmacological characteristic because this may explain part of its physiological and possible pathological behavior, as well as the effect of drugs classified as inverse agonists (able to reverse the isomerization because of their higher affinity for R than for R*) on this receptor.

Native 5-HT_{4(a)} receptors of different species (mouse, rat, and human) have a much higher capacity to convert from R to R* than most of the other GPCRs studied so far. Indeed, when expressed at low density (100–200 fmol/mg), 5-HT_{4(a)} receptors produced a 2-fold increase in the cAMP production compared with mock transfected cells. In comparison, h β_2 -AR did not modify cAMP production when expressed at this density (Fig. 3 and Samama et al., 1993). Indeed, in COS-7 cells, a less than 2-fold increase in cAMP production was obtained when native β_2 -AR was expressed at a density of 20 pmol/mg protein (Samama et al., 1993). Increasing native 5-HT_{4(a)} receptor density to 400 fmol/mg protein was sufficient to increase cAMP production by 4-fold as compared with mock transfected cells. This high capacity to isomerize was not restricted to expression of the receptor in COS-7 cells because in LLC-PK1 cells, when the receptor density reached a level as low as 150 fmol/mg protein, the cAMP produced was 3 times that found in mock transfected cells.

The C-terminal domains of the i3 of 5-HT₄R and h β_2 -AR are remarkably similar. Upstream of the TM VI, four of the last seven residues are identical. One of the divergent positions is an alanine residue in 5-HT₄ receptor that is a leucine in h β_2 -AR (localized at residue -3 upstream of TM VI). This position is critical for keeping GPCR in an inactive form (Kjelsberg et al., 1992; Samama et al., 1993). Mutation of this position in α_{1B} -AR (Kjelsberg et al., 1992) and TSHRs (Parma et al., 1993) increases their constitutive activity. In CAM β_2 -AR, in which four residues of the C-terminal part of the i3 were mutated (Samama et al., 1993), this position is crucial for constitutive activation (S. Cotecchia, personal communication). Because native 5-HT₄ receptors have a much higher constitutive activity than native h β_2 -AR and have, as CAM h β_2 -AR, an alanine at this position, we mutated the alanine of 5-HT₄ receptor to leucine to see if the constitutive activity would drop to the level of native h β_2 -AR. This was not the case and, in contrast, a further and dramatic increase in 5-HT₄ receptor constitutive activity was observed. However, this further indicates that the C-terminal part of the i3 is also crucial to keep 5-HT₄ receptor in the inactive R state and that the native primary sequence is the one that probably gives the conformation having the lowest constitutive activity.

The highest basal constitutive activity of the short C-terminal sequence splice variants (5-HT_{4(e)}R and 5-HT_{4(f)}R) compared with the long C-terminal sequence variants (5-HT_{4(a)}R and 5-HT_{4(b)}R) can be explained by either one of the following hypotheses. In the first one, the shortest splice variants have a greater ability to isomerize from R to R* (and an equal efficacy of their R* form to stimulate G proteins). In

the second one, the efficacy of their R* forms to stimulate G proteins is different and their ability to isomerize is identical. It is difficult to completely exclude the second hypothesis. However, the observation that the 5-HT-induced maximum responses were identical whatever the splice variant considered argues in favor of a higher capacity of short C-terminal sequence splice variants to isomerize from R to R*.

One important issue is to know whether such a constitutive activity is present or not in neurons or other peripheral tissues (atria, colon, ileum, etc.) in which 5-HT₄ receptors are expressed at comparable density (Waeber et al., 1994; Eglen et al., 1995). Comparison of receptor density is rather complex because although approximately 57% of the cells expressed the transfected tagged 5-HT₄ receptors (whatever the splice variant considered), the quantity of cells expressing this receptor in heterogeneous tissues, such as brain, for example, remains unknown. In addition, the distribution of 5-HT₄ receptors in COS-7 cell and neuronal membranes are completely different. In preliminary experiments, we did not find a reduction of basal cAMP production in primary colliculi neurons expressing 5-HT₄ receptors with the inverse agonist GR 125487 (data not shown). Because the isomerization constant depends on environmental conditions, it is also possible that proteins that interact or chemically modify the C-termini (one may think of a phosphorylation of the serine-threonine-rich domain upstream of the splicing site) reduce

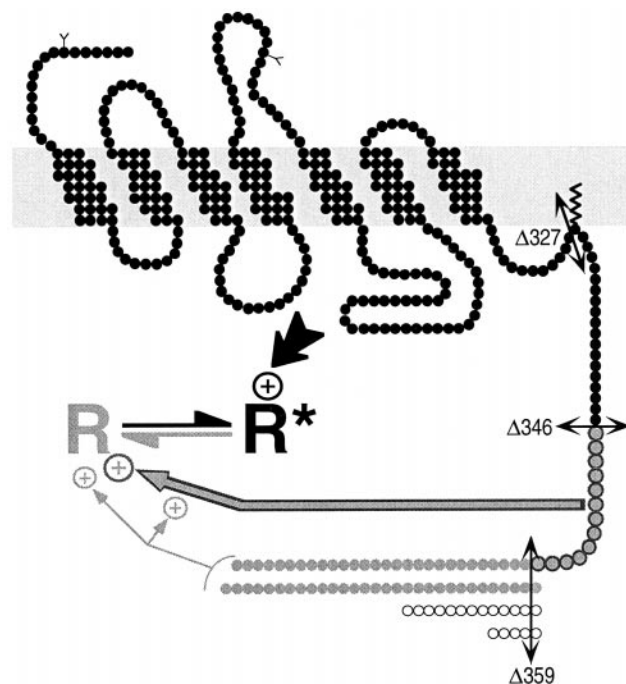


Fig. 10. Proposed mechanism for modulation of the constitutive activation of 5-HT₄ receptor splice variants by common and specific carboxy-terminal domains. The 5-HT₄ receptor up to residue 346, which is common to all four of the 5-HT₄R splice variants, is mainly a constitutive receptor coupled to G_s. The common sequence in the C-terminal domain between residues 346 and 359 imposes conformational constraints maintaining the receptor under its inactive form. The specific sequences of the (a) and (b) forms reduce its constitutive activation to an even greater extent, either by increasing the inhibitory effect of the C-terminal common sequence or by a direct negative effect on the isomerization. The specific sequences of (e) and (f) splice variants, which share the same PVPV C-terminal sequence, do not add supplementary constraint to that imposed by the amino acid sequence 346 to 359.

the *J* constant in neurons. The search for such putative interacting proteins will certainly be of interest.

There are increasing data to indicate that specific sequences of the C-terminal end of GPCRs modulate the isomerization of these receptors from R to R*. A cluster of basic residues present in the C-terminal tail of the short variants of mGluRs (mGluR1b, mGluR1c, and mGluR1d), impairs their ability to isomerize from R to R*. Removing this cluster by mutation reveals their constitutive activity (Mary et al., 1998). In addition, the effect of this inhibitory cluster is suppressed by a long C-terminal domain of mGluR1a which, in fact, has a high constitutive activity (Mary et al., 1998). Truncation of the last residues of thyrotropin-releasing hormone receptor and prostaglandin E receptors causes constitutive activity (Matus-Leibovitch et al., 1995; Hasegawa et al., 1996). The last 12 residues of bovine rhodopsin have also been proposed to be involved as negative regulator of GTP exchange (Weiss et al., 1994). Removal of the extended C-terminal domain of avian β_2 -AR increases its activity (Parker and Ross, 1991).

We found that truncation of the C-terminal domain consisting in the removal of the sequences that are divergent in individual 5-HT₄ receptor splice variants ($\Delta 359$) gave a constitutive activity similar to those of the shortest splice variants (5-HT_{4(e)}R and 5-HT_{4(f)}R). Because the 5-HT_{4(a)} $\Delta 359$ receptor is identical with the 5-HT_{4(f)} receptor with its PVPV sequence removed, the PVPV sequence is not responsible for the intrinsic activity of the shortest splice variants (Fig. 6). In contrast, truncation of the last 13 residues ($\Delta 346$), 12 of which are common in the C-terminal tails of all of them, resulted in a dramatic release on the receptor constraint and the basal activity increased 10-fold when as few as 500 fmol/mg protein of receptors are expressed. A further truncation ($\Delta 327$) did not exacerbate this constitutive activity. This indicates that within the 13 residues comprised between $\Delta 346$ and $\Delta 359$, one set of residues is highly potent in maintaining the receptor under the R form. Interestingly, this sequence contains 6 serine and threonine residues susceptible to being phosphorylated, a post-translational modification that may modulate the receptor intrinsic activity. Removal of this inhibitory sequence rendered the receptor so active that it became less sensitive to 5-HT.

In conclusion, we can propose a model in which (Fig. 10): 1) the 5-HT₄ receptor protein, up to residue 346, is mainly a constitutive active receptor coupled to G_i; 2) the last common sequence in the C-terminal domain (around 13 residues between 346 and 359) is mainly engaged in the constraint of the receptor under its inactive form; 3) the specific sequences of 5-HT_{4(e)} and 5-HT_{4(f)} receptors are neutral in the modulation of the isomerization because their constitutive activity did not differ from that of $\Delta 359$; and 4) the specific sequences of the 5-HT_{4(a)} and 5-HT_{4(b)} receptors reduce the isomerization either by reducing the inhibitory effect of the C-terminal common sequence or by a direct negative effect on the isomerization.

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