Novel Brain-Specific 5-HT_4 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-HT_4) receptor splice variants from mouse (m5-HT_4(a)R and m5-HT_4(b)R), rat (r5-HT_4(a)R), and human brain tissue (h5-HT_4(a)R) which differ, as do the previously described 5-HT_4 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-HT_4 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(a)R and m5-HT_4(b)R) was higher than that of the long C-terminal sequence variants (m5-HT_4(c)R and m5-HT_4(d)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-HT_4R 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-HT_4(R), 5-HT_6(R), and 5-HT_7R) are positively coupled to G protein that stimulates adenylate cyclase (G_s) (Hoyer and Martin, 1997). 5-HT_4 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-HT_4 receptor agonists such as metoclopramide and cisapride are used therapeutically as prokinetic drugs. A role for 5-HT_4 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-HT_4 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-HT_4 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-HT_4 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_4(a)R and r5-HT_4(b)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)R and m5-HT_4(b)R 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(a)R and h5-HT_4(b)R) also displaying divergent sequences after L358. In this study, we have cloned two additional splice variants in mouse tissue (m5-HT_4(a)R and m5-HT_4(b)R). The (a) form has also been cloned in rat and human brain tissue (r5-HT_4(a)R and h5-HT_4(a)R). All of these variants share the same sequence up to L358, followed by specific C termini. Splice variants of

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 (Journet 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-Leibovich 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-HT$_4$ receptors like TSHRs showed constitutive activity when expressed at low physiological densities. At a similar receptor density, the newly cloned 5-HT$_4$ receptor splice variants (m5-HT$_{4v,R}$ and m5-HT$_{4a,R}$) have, in their native conformation, an agonist-independent activity level above the constitutively active mutant β$_a$-adrenergic receptor (CAM β$_a$-AR).

### Materials and Methods

#### Isolation and Sequencing of the Mouse 5-HT$_{4a,R}$, 5-HT$_{4v,R}$, and 5-HT$_{4f,R}$ 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)$_{25}$ (Dynal Inc., Compiegne, 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 (Claeyssen et al., 1996; accession number Y09586; position 1–24), 5'-TAATGGGACAACTTGTGATGTAAGT-3', and ON2, designed on the sequences of the rat brain 5-HT$_4$ receptor, a novel human variant, has been cloned by RT-PCR on total human brain poly(A)$^+$RNA (Clontech, Oxyme, Montigny le Bretonneux, France) by the same procedures as described above. ON1 was used as a control, we cloned ON9 (Claeyssen et al., 1997a) (accession number Y0586; position 1–24) and ON2.

#### Isolation and Sequencing of the Human 5-HT$_{4e,R}$ Receptor cDNA by RT-PCR.

cDNA encoding human brain 5-HT$_{4e,R}$ 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 (Claeyssen 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'-CCAGGGCCGAGGACT-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'-TTGCTCTAGCTGCTTGGAA-3', for the amplification of the C-terminus of the (b) form. For the amplification of the β-actin as a control, we used ON9 (J00691; position 2252–2271), 5'-GACTCCGGGAGCGGTTAC-3' and ON10 (J00691; position 2482–2501) 5'-CGACCTCTTTCTCTCCCTGG-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$_{4a,R}$, 503 bp for m5-HT$_{4v,R}$, 527 bp for m5-HT$_{4f,R}$ (primer couple ON7/ON2), 528 bp for m5-HT$_{4a,R}$ (primer couple ON7/ON8), and 450 bp for β-actin (primer couple ON9/ON10). PCR products were run in 3% MetaPhor agarose (FMC Corp., Tebu, Le Perray-en-Yvelines, France) and stained with ethidium bromide gels and photographed. Positive and negative controls (with or without reverse transcriptase) were performed on the β-actin amplification. Southern blotting was done after transfer of the DNA to charged nylon membranes (Qiaobond; Qiagen S.A., Courtaboeuf, France). The hybridization probe was an RT-PCR fragment obtained in the same PCR conditions with r5-HT$_{4v,R}$ as a template (a generous gift from Dr. Christophe Geral, 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, Oxyme, Montigny le Bretonneux, France).

### β-Adrenergic Receptor and CAM β$_a$-AR cDNAs.

The human β$_a$-adrenergic receptor cDNA (hβ$_a$-AR) and the CAM β$_a$-AR cDNA were obtained from Susanna Cotecchia (Institut de Pharmacologie et Toxicologie, Université de Lausanne, Switzerland).

#### Construction of Truncated 5-HT$_{4e,R}$ Receptor cDNA.

Constrains were obtained by inserting a stop codon after residue 327,
The homogenate was centrifuged at 20,000 g with a rubber policeman, harvested in PBS, and centrifuged at 4°C. The cells were washed twice in PBS, scraped, trypsinized, centrifuged, and resuspended in EP Buffer 1 (50 mM K$_2$HPO$_4$, 20 mM H$_3$CO$_2$K, 26.7 mM MgSO$_4$; pH 7.4) for 20 h in DMEM without dFBS, as previously described on 15-cm dishes and grown in DMEM with 10% dFBS for 6 h and et al. (1988).

To perform radioligand binding studies with [H]$^3$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 pargylene and 0.01% ascorbic acid and was incubated at 20°C for 30 min with 100 μl of [H]$^3$GR 113808 and 50 μl of buffer or competing drugs. For saturation analysis assays, various concentrations of [H]$^3$GR 113808 (0.001–1 nM) were used. For competition binding experiments, the [H]$^3$GR 113808 concentration was 0.15 nM. Receptor expression was determined with a saturating concentration of [H]$^3$GR 113808 (0.3–0.5 nM). 5-HT (0.5 μM) was used to determine nonspecific binding.

For determination of β$_2$-adrenergic and CAM β$_2$-adrenergic receptor expression in COS-7 cells, membrane preparation and binding studies were performed as described previously (Chidiac et al., 1994). The specific radioligand ([125]I)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 MgCl$_2$, 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% polyethyleneimine 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.

**Results**

**Primary Structure of Different 5-HT$_4$R Splice Variants.** We have published the isolation of m5-HT$_{4\alpha}$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-HT$_4$ 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-HT$_4$R splice variants, (c) and (d) (Fig. 1). Taking into account the already published human variants, we named the present new variants m5-HT$_{4\alpha}$R and m5-HT$_{4\beta}$R (Fig. 1). We also cloned a r5-HT$_{4\alpha}$R receptor identical to m5-HT$_{4\alpha}$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-HT₄(e)R. Whatever the species, mouse, human or rat, the different splice variants have an identical sequence up to Leu358, 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-HT₄(e), m5-HT₄(e), and m5-HT₄(f)R, as well as the h5-HT₄(e)R 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-HT₄(e)R and m5-HT₄(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-HT₄(a)R and m5-HT₄(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-HT₄(e)R and h5-HT₄(e)R mRNAs have been detected only in brain tissue (data not shown).

5-HT₄(a) Receptors Have a High Constitutive Activity. Because we previously observed that native m5-HT₄(a)R and h5-HT₄(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-HT₄(a)R receptor density (150–200 fmol/mg protein) comparable to those found in brain tissues (Waebel 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-HT₄(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 lane, a 2-fold increase in cAMP production was observed for a receptor density of 100 fmol/mg protein. Figure 3A shows that native 5-HT₄(a)R receptors had a much higher constitutive activity than the native h₂AR 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...
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-HT₄(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-HT₄(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-μl 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 μl of a 50-μl 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.](image)

![Fig. 3. Basal levels of cAMP in COS-7 and LLC-PK1 cells transiently expressing increasing concentrations of human, rat, and mouse 5-HT₄ receptor splice variants. A, COS-7 cells were transfected with increasing concentrations of human, rat, or mouse 5-HT₄R and hβ₂-AR cDNA (25–1000 ng of cDNA/10⁶ cells). The total amount of DNA was kept constant at 15 μg/10⁶ cells with wild-type pRK5 vector. The percentage of conversions of [³H]ATP to [³H]cAMP in mock-transfected cells was 0.12 ± 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-HT₄(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 ± 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 hβ₂-AR densities were determined with a saturating concentration of [³H]CYP (0.5 nM). Each point is the mean ± S.E.M. of three independent experiments.](image)
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, hβ2-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-HT₄(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-HT₄140R 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-HT₄140R, whereas a larger deletion mutant (deletion of 13 residues, 5-HT₄Δ346 receiver) 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 hβ2-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 β2-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 β₂-AR that produced the CAM hβ2-AR (Sammaa 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 hβ2-AR (S. Cotecchia, personal communication). Therefore, one possible explanation for the highest constitutive activity of native 5-HT₄ receptors as compared with native β₂-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 β₂-AR. For this reason, we mutated this A258 to L in the 5-HT₄(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-HT₄(e) receptors was as high as the constitutive activity of CAM hβ2-AR.

Basal and 5-HT-Stimulated cAMP Production in Cells Transfected with 5-HT₄(a) and 5-HT₄(e) Receptors and 5-HT₄ Mutated Receptors. The short C-terminal sequence 5-HT₄(e) receptors had a higher basal constitutive activity than the long C-terminal sequence 5-HT₄(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-HT₄(a) and 5-HT₄(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

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Fig. 4. Effect of two potent 5-HT₄R antagonists, RS 100235 and GR 125487, on basal and 5-HT-stimulated 5-HT₄(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-HT₄(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-HT₄(a), m5-HT₄(b), m5-HT₄(e), m5-HT₄(f), or hβ2-AR as a function of receptor density. COS-7 cells were transfected with increasing concentrations of m5-HT₄(a), m5-HT₄(b), m5-HT₄(e), m5-HT₄(f), or hβ2-AR receptors’ cDNA (10–1000 ng of cDNA/10⁶ 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 hβ2-AR receptor densities were determined as indicated in Fig. 3. Data for each of the splice variants represent the means ± 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 (Δ346 and Δ327) of m5-HT₄(e) Receptors Increases the Affinity of 5-HT and Its Potency in Stimulating cAMP. The extended ternary complex (Samaama 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-HT₄ₐₑ R A258L and C-terminal-deleted (Δ346 and Δ327) 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 Kᵣ values for 5-HT were 47 ± 12 nM for the wild-type 5-HT₄ₐₑ R, 0.53 ± 0.6 nM and 1.34 ± 0.15 nM for the truncated 5-HT₄ₐₑ R Δ346 and 5-HT₄ₐₑ R Δ327, respectively, and 0.85 ± 0.10 nM for the mutated 5-HT₄ₑ 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 μM) did not modify this difference (data not shown). In contrast to these results, we found no differences 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-HT₄ₑ R, 5-HT₄ R Δ346, 5-HT₄ R Δ327, and 5-HT₄ₑ R A258L, respectively.

Discussion

We report the cloning of new 5-HT₄ R splice variants (m5-HT₄ₑ R, m5-HT₄₁ R, r5-HT₄ₑ R, and h5-HT₄ₑ R) that possess original C-terminal domains compared with already cloned 5-HT₄ R splice variants (Gerald et al., 1995; Claeyesen et al., 1996, 1997a; Blondel et al., 1997, 1998; Van den Wynngaert 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...
these splice variants with proteins similar to PDZ-domain-containing proteins. Because of the roles of 5-HT4 receptors in learning and memory (Ansany 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.,

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**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-HT4a, 5-HT4b, 5-HT4a, A258L, 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.

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**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-HT4(e)R, m5-HT4R Δ346, m5-HT4R Δ327 or m5-HT4R 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 adenylyl 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.
matic increase in 5-HT4 receptor constitutive activity was not the case and, in contrast, a further and dra-
In brief, the specific sequences of the C-terminal domain of the i3 of 5-HT4R and hβ2-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 leucine in hβ2-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). Mutations at this position are typical for agonists (able to reverse the isomerization because of their higher affinity for R than for R* on this receptor.

Native 5-HT4(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-HT4(a) receptors produced a 2-fold increase in cAMP production compared with mock transfected cells. In comparison, hβ2-AR did not modify cAMP production when expressed at this 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 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
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 $\beta_2$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-HT$_4$ receptor splice variants ($\Delta$359) gave a constitutive activity similar to those of the shortest splice variants (5-HT$_{\Delta 346}$R and 5-HT$_{\Delta 359}$R). Because the 5-HT$_{4(c)}$ $\Delta$359 receptor is identical with the 5-HT$_{4(a)}$ 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 346 and 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-HT$_4$ receptor protein, up to residue 346, is mainly a constitutive active receptor coupled to G$_s$; 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$_{4(c)}$ and 5-HT$_{4(d)}$ 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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