Activation of constitutive 5-hydroxytryptamine_{1B} receptor by a series of mutations in the BBXXB motif: positioning of the third intracellular loop distal junction and its G_α protein interactions

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Constitutive activity of the recombinant human 5-hydroxytryptamine_{1B} (5-HT_{1B}) receptor (RC code 2.1.5HT.01.B) was investigated by mutagenesis of the BBXXB motif (in which B represents a basic residue and X a non-basic residue) located in the C-terminal portion of the third intracellular loop. In contrast with wild-type 5-HT_{1B} receptors, three receptor mutants (Thr³¹³ → Lys, Thr³¹³ → Arg and Thr³¹³ → Gln) increased their agonist-independent guanosine 5'-[γ-³⁵S]thio]triphosphate binding response by 26–41 %. This activity represented approx. 30 % of the maximal response induced by 5-HT and could be reversed by the inverse agonists methiothepin and 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyridin-4-yl phenyl)-benzenamide (GR 55562). Enhanced agonist-independent and agonist-dependent 5-HT_{1B} receptor activation was provided by co-expression of a pertussis toxin-resistant rat G_α Cys³⁵¹ → Ile protein. The wild-type 5-HT_{1B} receptor displayed a doubling in basal activity, whereas a spectrum of enhanced basal activities (313–571 %) was observed with a series of diverse amino acid substitutions (isoleucine,

glycine, asparagine, alanine, lysine, phenylalanine, glutamine and arginine) at the 5-HT_{1B} receptor position 313 in the presence of pertussis toxin (100 ng/ml). Consequently, the constitutive 5-HT_{1B} receptor activity can be modulated by the mutation of Thr³¹³, and displays a graded range between 11 % and 59 % of maximal 5-HT_{1B} receptor activation by 5-HT. No clear pattern is apparent in the framework of traditionally cited amino acid characteristics (i.e. residue size, charge or hydrophobicity) to explain the observed constitutive activities. The various amino acid substitutions that yielded enhanced activity are unlikely to make similar intramolecular interactions within the 5-HT_{1B} receptor. It is hypothesized that the positioning of the junction between the third intracellular loop and transmembrane domain VI is altered by mutation of Thr³¹³ in the BBXXB motif and thereby unmasks G_α-protein interaction points.

Key words: guanine nucleotide-binding protein-coupled receptors, inverse agonist, mutagenesis.

INTRODUCTION

Several lines of research have revealed that a number of guanine nucleotide-binding protein-coupled receptors (GPCR) demonstrate constitutive activity in the absence of agonist in their native state, by reconstitution in phospholipid vesicles, in recombinant heterologous receptor-expression systems and in transgenic mice [1–6]. This led to the concept that GPCR change their conformation spontaneously or by mutation and oscillate between active and inactive receptor conformations [7]. Receptors exist in an equilibrium between both conformations: an inactive conformation that is structurally constrained and unable to bind a G_α-protein subunit, and an active conformation that can interact productively with a GTP-bound G_α subunit. Certain ligands, termed inverse agonists or negative antagonists, seem capable of driving activated GPCR to the inactive conformation [8]. Negative antagonism is demonstrated when a ligand binds to a receptor that exhibits some basal activity, which is then decreased. If ligand efficacy represents the differential affinity of a ligand for the active and/or the inactive conformation of the receptor, then a zero efficacy, which would be required for a pure neutral antagonist, might be uncommon, because this would require the same ligand to exhibit a similar affinity for both

receptor states [9]. Numerous drugs previously classified as neutral antagonists might actually act as inverse agonists [9]; such a reclassification might have pharmacological and therapeutical implications [6].

A widely accepted model used to describe the activation of GPCR by agonists is the ternary complex model, which accounts for the co-operative interactions between receptor, G-protein and agonist [10]. This model has recently been extended to accommodate the observation that many receptors can activate G-proteins in the absence of agonist and that mutations in different structural domains of the GPCR can enhance the agonist-independent activity [8,11]. The extended ternary complex model also accounts for the effects of different classes of drug (full agonists, partial agonists, neutral antagonists and inverse agonists) on receptor signalling [12].

The C-terminal portion of the third intracellular loop has been suggested to be involved in constraining the GPCR in the inactive (G-protein-uncoupled) conformation [13]. Mutagenesis studies of the BBXXB motif (in which B represents a basic residue and X a non-basic residue) in the third intracellular loop of α_{2A}-adrenergic, α_{1B}-adrenergic, 5-hydroxytryptamine_{2A} (5-HT_{2A}) and 5-HT_{2C} receptors demonstrated constitutively active mutants [13–16]. Several anti-psychotic drugs displayed inverse

Abbreviations used: CHO, Chinese hamster ovary; GPCR, guanine-nucleotide-binding protein-coupled receptors; GR 55562, 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyridin-4-yl phenyl)-benzenamide; GR 125743, N-[4-methoxy-3-(4-methylpiperazin-1-yl)]-3-methyl-4-pyridin-4-yl-benzenamide; GR 127935, 2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]amide; [³⁵S]GTP[S], guanosine 5'-[γ-³⁵S]thio]triphosphate; 5-HT, 5-hydroxytryptamine (serotonin); PTX, *Bordetella pertussis* toxin; SB 224289, 1'-methyl-5-[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine]; TM, transmembrane domain.

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agonist activity by inhibiting the constitutive activation of phosphatidylinositol hydrolysis by mutant Cys³²² → Lys 5-HT_{2A} receptor [15]. Similarly, mianserin and mesulergine displayed inverse agonist activity by decreasing basal levels of phosphatidylinositol hydrolysis stimulated by mutant Ser³¹² → Lys 5-HT_{2C} receptors [16] in accordance with the observations for native 5-HT_{2C} receptors expressed in NIH 3T3 cells [17] or Sf₉ cells [18]. Otherwise, constitutively active mutant (Thr³⁷³ → Lys) α_{2A} -adrenergic receptors provide apparently opposite results (i.e. positive efficacy) for ligands that have so far been characterized as α_2 -adrenergic receptor antagonists [19]. The partial agonist activity of classical antagonists has also been reported for constitutively active mutant dopamine D₁ (Leu²⁸⁶ → Ala [20]), AT_{1A} angiotensin II (Asn¹¹¹ → Ala [21]) and m₅ muscarinic receptors (Phe⁴⁵¹ → Pro [22]). It is conceivable that structural alterations near the ligand-binding pocket might modify the nature of the ligand–receptor interactions, such that some antagonists are capable of stabilizing an active conformation at the new binding interface generated by the mutation.

The present paper reports on constitutively active mutant 5-HT_{1B} receptors. One particular amino acid (Thr³¹³) was exchanged in the BBXXB motif (Arg³¹⁰Lys-Ala-Thr-Lys) by each of the natural amino acids. Because the agonist-independent guanosine 5'-[γ -³⁵S]thio]triphosphate [³⁵S]GTP[S] binding responses were clearly measurable only with a few receptor mutants, analysis was also performed by co-expression with a G_o α Cys³⁵¹ → Ile protein resistant to *Bordetella pertussis* toxin (PTX) [23]. The local cellular G-protein concentration has been shown to regulate the constitutive receptor activity of 5-HT_{1A} and muscarinic receptors [24,25]. The 5-HT_{1B} receptor mutants were analysed in terms of their structure–activity relationship by measuring 5-HT_{1B} receptor-mediated agonist-independent and agonist-dependent activity. To avoid possible coupling of the 5-HT_{1B} receptor to endogenous G_{i/o} α -protein subtypes in Chinese hamster ovary (CHO)-K1 cells, functional responses were determined by measuring PTX-resistant binding of the stable GTP analogue [³⁵S]GTP[S]. The process of constitutive activation of the 5-HT_{1B} receptor is considerably favoured by co-expression of the G_o α Cys³⁵¹ → Ile protein and amplified by a series of diverse amino acid substitutions at the receptor position 313.

MATERIALS AND METHODS

Cloning of mutant 5-HT_{1B} receptors and rat G_o α Cys³⁵¹ → Ile protein

The recombinant human 5-HT_{1B} receptor (RC code: 2.1.5HT.01B, Genbank accession number M89478) was mutated at position Thr³¹³ (codon ACC) into the codons for the 19 natural possible amino acids by a modified overlap extension PCR technique [26]. In brief, linearized pCR3.1/human 5-HT_{1B} plasmid was amplified by PCR into two distinct fragments with the use of two primer sets P1s (5'-GCCATGGAGGAACCG-GGTGCTCAG-3'), P1r (5'-CCCTAGGGTCTTNNNGGCTT-TGCGCTCCCT-3') and P2s (5'-AGGGAGCGCAAACGGN-NNAAGACCCTAGGG-3'), P2r (5'-AAGTCAACTTGTGCA-CTTAAACG-3') (mutated codons are shown in bold). Both fragments were fused together in a second PCR step and the fusion product was subsequently amplified by using the primers P1s and P2r as described [26] to generate a full-length human 5-HT_{1B} receptor gene carrying the respective mutation. Each 5-HT_{1B} receptor Thr³¹³ mutant was cloned into 50 ng of a mammalian expression vector, pCR3.1. Sequencing was performed on an ABI Prism 310 Genetic analyser by using Big Dye terminator cycle sequencing, confirming the respective mutations in the 5-HT_{1B} receptor nucleotide sequence. The PTX-resistant

rat G-protein α subunit gene G_o α Cys³⁵¹ → Ile was cloned by PCR [23] with the use of specific primers [the reverse primer carried the Cys³⁵¹ → Ile mutation (TGT into ATT)], designed at respectively the start and stop codons of the gene in accordance with the published nucleotide sequence (GenBank accession number M17526).

Cell culture and transfection

The CHO-K1 cell line (ATCC, CCL 61) was cultured in Ham's F12 nutrient mixture supplemented with 10% (v/v) heat-inactivated foetal calf serum. Cells grown to 60–80% confluency were used for transfection with a Lipofectamine Plus kit. pCR3.1 plasmid (6 μ g) containing the wild-type or mutant 5-HT_{1B} receptor gene, or 3 μ g of wild-type or mutant 5-HT_{1B} receptor gene and 3 μ g of mutant G_o α Cys³⁵¹ → Ile protein gene, was mixed with 10 μ l of Lipofectamine Plus reagent in 0.2 ml of Opti-MEM and incubated at room temperature for 15 min. Subsequently, 20 μ l of Lipofectamine reagent diluted in 0.2 ml of Opti-MEM was added for 15 min and exposed with 5 ml of Opti-MEM to CHO-K1 cells for 3 h at 37 °C. Thereafter, cells were further incubated with 10 ml of complete growth medium and harvested 48 h after transfection. Treatment with PTX (100 ng/ml) was performed overnight before membranes were prepared.

Membrane preparation and radioligand binding experiments

Membrane preparation steps were performed at 4 °C. Cells were washed twice with PBS and stored at –80 °C. Cells were then scraped mechanically in 10 mM Tris/HCl, pH 7.5, supplemented with 0.1 mM EDTA and centrifuged for 10 min at 45000 g. The pellet was homogenized in the same buffer with a Polytron and centrifuged. The final pellet was dissolved in 0.5 ml of Tris/EDTA buffer (25–45 μ g/ml protein) and stored at –80 °C until use. Membrane preparations were diluted in 50 mM Tris/HCl, pH 7.7, containing 4 mM CaCl₂, 10 μ M pargyline and 0.1% ascorbic acid and used for binding experiments with *N*-[³H]-4-methoxy-3-(4-methyl-piperazin-1-yl)-3-methyl-4-pyridin-4-yl-benzenamide (GR 125743, 1 nM) and 5-[³H]carboxamido-tryptamine (1 nM) as described [27,28]. 5-HT (10 μ M) was used to determine non-specific radioligand binding.

[³⁵S]GTP[S] binding responses

Agonist-independent (basal) and agonist-dependent [³⁵S]GTP[S] binding responses were performed to membranes as described [29] in 20 mM Hepes, pH 7.4, supplemented with 30 μ M GDP, 100 mM NaCl, 3 mM MgCl₂ and 0.2 mM ascorbic acid. Control [³⁵S]GTP[S] binding responses were performed systematically in the presence of 1 μ M methiothepin to correct for enhanced basal [³⁵S]GTP[S] binding. Maximal stimulation of [³⁵S]GTP[S] binding was defined in the presence of 10 μ M 5-HT and calculated in comparison with that obtained with 1 μ M methiothepin, unless indicated otherwise. pEC₅₀ values were defined as the ligand concentration at which 50% of its own maximal stimulation of [³⁵S]GTP[S] binding was obtained. pIC₅₀ values represent the ligand concentration that showed 50% inhibition of its own maximal inhibition of basal [³⁵S]GTP[S] binding. In antagonist experiments, 1'-methyl-5-[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl]-2,3,6,7-tetrahydrospiro-[furo[2,3-f]indole-3,4'-piperidine] (SB 224289; 30 and 100 nM) was incubated together with 5-HT. pK_B values were calculated as $K_B = B/(A'/A - 1)$, where B is the concentration of the antagonist and A and A' are the EC₅₀ values of 5-HT

measured in the absence or the presence of antagonist respectively. Statistical analysis was performed with Student's *t* test.

Immunological G_oα protein detection

Membrane preparations of CHO-K1 cells containing either the wild-type 5-HT_{1B} or 5-HT_{1B} receptor mutants in the presence of the G_oα Cys³⁵¹ → Ile protein were prepared as described above. Total proteins were separated by SDS/PAGE [12.5 % (w/v) gel], as described [30]. Thereafter the proteins were blotted to a nylon membrane by semi-dry electrotransfer (23 V, 45 min) in 192 mM glycine/20 % (v/v) methanol/25 mM Tris/HCl buffer (pH 8.3). Proteins were probed with a polyclonal antibody raised against a peptide corresponding to residues 18–33 of the rat G_oα protein. The incubation was performed in PBS containing 0.1 % (w/v) Tween 20, 5 % (w/v) dried non-fat milk and the antibody at a dilution of 1:1000. Proteins were detected with an anti-rabbit IgG antibody coupled to alkaline phosphatase with the use of a colorimetric reaction [0.12 mM Nitro Blue Tetrazolium chloride monohydrate/0.12 mM 5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt/5 mM MgCl₂ in 100 mM diethanolamine (pH 9.6)].

Protein content

Membrane protein levels were estimated with a dye-binding assay by using a Bio-Rad kit; BSA was used as a standard [31].

Materials

The ABI Prism 310 Genetic Analyser and the Big Dye terminator cycle sequencing kit were from Perkin-Elmer (Foster City, CA, U.S.A.). The pCR3.1 expression vector was from Invitrogen (San Diego, CA, U.S.A.). CHO-K1 cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.). The G_oα and the anti-rabbit IgG antibodies were from Calbiochem (La Jolla, CA, U.S.A.). [³H]GR 125743 (47–80 Ci/mmol) and [³⁵S]GTP[S] (1035–1163 Ci/mmol) were obtained from Amersham (Les Ulis, France). The Lipofectamine Plus kit, cell culture media, foetal calf serum, culture plates and PTX (50 µg/ml) were obtained from Gibco Biocult Laboratories (Paisley, Renfrewshire, U.K.). The Emulsifier-Safe was obtained from Packard (Warrenville, PA, U.S.A.). 5-HT and the substrates for the colorimetric immunodetection were from Sigma (St. Louis, MO, U.S.A.). Methiothepin, ritanserin, ketanserin and 1-naphthylpiperazine were from R.B.I. (Natick, MA, U.S.A.). 3-(3-Dimethylaminopropyl)-4-hydroxy-*N*-(4-pyridin-4-yl-phenyl)-benzenamide (GR 55562), GR 125743, 2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]amide (GR 127935) and SB 224289 were synthesized at the Centre de Recherche Pierre Fabre. Stock solutions of ligands were prepared at 1 mM. Serial dilutions were made in the respective incubation buffers.

RESULTS

[³⁵S]GTP[S] binding responses by 5-HT_{1B} receptors

In contrast with plasmid-transfected CHO-K1 cells, 5-HT_{1B} receptors displayed, on transient expression, a 5-HT-dependent [³⁵S]GTP[S] binding response (Figure 1). The basal [³⁵S]GTP[S] binding response was enhanced 2.5-fold with 5-HT (10 µM), whereas it was virtually unaffected by the inverse agonist methiothepin (1 µM). Mutation of Thr³¹³ in the 5-HT_{1B} receptor to the basic residue arginine displayed a significantly (*P* < 0.05)

Figure 1 [³⁵S]GTP[S] binding responses of wild-type (wt) 5-HT_{1B} receptor and 5-HT_{1B} receptor mutant Thr³¹³ → Arg in CHO-K1 cells

[³⁵S]GTP[S] ([³⁵S]GTPγS) binding responses were performed with 0.5 nM [³⁵S]GTP[S] either in the absence (Basal), or the presence of 1 µM methiothepin (Methio.) or 10 µM 5-HT as described in the Materials and methods section. Results are means ± S.E.M. for six or seven independent transfection experiments, each performed in duplicate. The basal condition compared with methiothepin treatment was significantly (*P* < 0.05) different for the Thr³¹³ → Arg mutant.

enhanced basal response (44 ± 9 %) and a similar (*P* > 0.05) maximal 5-HT response (Figure 1). The enhanced basal [³⁵S]GTP[S] binding response was fully blocked by methiothepin (1 µM). To analyse further whether the 5-HT_{1B} receptor mutant Thr³¹³ → Arg was the most effective mutation to enhance constitutive 5-HT_{1B} receptor activity, a series of 18 other amino acid mutations at the Thr³¹³ position in the 5-HT_{1B} receptor was constructed and assayed for 5-HT_{1B} receptor expression and for agonist-independent and agonist-dependent [³⁵S]GTP[S] binding responses. Apart from the 5-HT_{1B} receptor mutant Thr³¹³ → Arg, none of the investigated mutants displayed a significantly enhanced basal [³⁵S]GTP[S] binding response. The amount of inhibition of basal [³⁵S]GTP[S] binding by methiothepin (1 µM) was 20–28 % for the mutants with lysine, arginine and glutamine. Similar results were obtained with GR 55562 (1 µM, results not shown). The enhanced constitutive receptor activity was not related to an increased 5-HT_{1B} receptor expression. Specific [³H]GR 125743 binding was, apart from the 5-HT_{1B} receptor mutant Thr³¹³ → His, either similar to or at least half that of the wild-type 5-HT_{1B} receptor (Figure 2B). The 5-HT_{1B} receptor mutant Thr³¹³ → His expressed a small amount of specific [³H]GR 125743 binding (7 % of wild-type 5-HT_{1B} receptor) and 5-HT was virtually inactive in stimulating the [³⁵S]GTP[S] binding response (Figure 2C). The 5-HT-mediated [³⁵S]GTP[S] binding response was for most of the 5-HT_{1B} receptor mutants either unchanged from that of the wild-type 5-HT_{1B} receptor or attenuated by 48–59 % (*P* < 0.05) for the mutants with either a proline, glutamate or aspartate residue (Figure 2C). Sumatriptan also behaved as an efficacious agonist, with *E*_{max} values close to those of 5-HT (results not shown). In contrast, GR 125743 (1 µM) clearly demonstrated properties of a partial agonist (Figure 2D); its maximal effect for the 5-HT_{1B} receptor mutants with an arginine or glutamine residue attained the response mediated by 5-HT at the wild-type 5-HT_{1B} receptor.

[³⁵S]GTP[S] binding responses by 5-HT_{1B} receptors on co-expression of the rat G_oα protein

Co-expression of the 5-HT_{1B} receptor with a rat G_oα protein was investigated to enhance the resolution of constitutive 5-HT_{1B}

Figure 2 $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding responses and expression levels of wild-type (wt) 5-HT_{1B} receptor and 5-HT_{1B} receptor mutants Thr³¹³ → Xaa in CHO-K1 cells

(A) $[^{35}\text{S}]\text{GTP}[\text{S}]$ ($[^{35}\text{S}]\text{GTP}\gamma\text{S}$) binding responses were obtained as the difference between the binding of $[^{35}\text{S}]\text{GTP}[\text{S}]$ (0.5 nM) performed in either the absence or the presence of 1 μM methiothepin as described in the Materials and methods section. Results are expressed as the net increase of basal $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding compared with the respective methiothepin-mediated $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding response for each mutant receptor. (B) The 5-HT_{1B} receptor density was estimated by measuring the specific binding of $[^3\text{H}]\text{GR 125743}$. This was obtained as the difference between total and non-specific (in the presence of 10 μM 5-HT) radioligand binding. (C) Maximal $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding responses were obtained as the difference between the responses mediated by 5-HT (10 μM) and by methiothepin (1 μM), and are expressed as percentages of that obtained with the 5-HT_{1B} receptor mutant Thr³¹³ → Arg (234 ± 35 fmol/mg of protein). (D) GR 125743-mediated stimulation of $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding responses was obtained as the difference between the response mediated by GR 125743 (1 μM) and methiothepin (1 μM), and are expressed as percentages of the 5-HT-mediated response as mediated by the 5-HT_{1B} receptor mutant Thr³¹³ → Arg. Results are means ± S.E.M. for five independent transfection experiments, each performed in duplicate.

receptor activation because the 5-HT_{1B} receptor-to-G-protein stoichiometry might be unfavoured in the overexpression of 5-HT_{1B} receptor protein in CHO-K1 cells. To ascertain the specific coupling of the 5-HT_{1B} receptor to a co-expressed G_oα protein rather than to endogenous G_{z1/o} proteins of CHO-K1 cells, a putative PTX-resistant G_oα Cys³⁵¹ → Ile protein was assayed

Figure 3 Effect of co-expression of rat G_oα Cys³⁵¹ → Ile protein and treatment with PTX on $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding responses of wild-type 5-HT_{1B} receptor and 5-HT_{1B} receptor mutant Thr³¹³ → Arg in CHO-K1 cells

$[^{35}\text{S}]\text{GTP}[\text{S}]$ ($[^{35}\text{S}]\text{GTP}\gamma\text{S}$) binding responses were determined with 0.5 nM $[^{35}\text{S}]\text{GTP}[\text{S}]$ either in the absence (Basal) or the presence of 1 μM methiothepin (Methio.) or 10 μM 5-HT as described in the Materials and methods section. (A) wild-type 5-HT_{1B}; (B) wild-type 5-HT_{1B} plus PTX (100 ng/ml); (C) wild-type 5-HT_{1B} plus G_oα Cys³⁵¹ → Ile; (D) wild-type 5-HT_{1B} plus G_oα Cys³⁵¹ → Ile plus PTX (100 ng/ml); (E) 5-HT_{1B} receptor mutant Thr³¹³ → Arg plus G_oα Cys³⁵¹ → Ile plus PTX (100 ng/ml). Results are means (B) or means ± S.E.M. for three (C), five (D, E) or seven (A) independent transfection experiments, each performed in duplicate. The basal condition compared with methiothepin treatment was significantly ($P < 0.05$) different in (D) and (E).

[23]. The basal $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding response was increased to 220 ± 45 fmol/mg of protein on co-expression of the wild-type 5-HT_{1B} receptor with a G_oα Cys³⁵¹ → Ile protein in the presence of PTX (100 ng/ml), and attenuated by methiothepin (−54 ± 6%) to the basal $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding level of the wild-type 5-HT_{1B} receptor protein in the absence of G_oα Cys³⁵¹ → Ile protein (Figure 3). The wild-type 5-HT_{1B} receptor displayed a PTX-sensitive 5-HT-mediated $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding response in the absence of G_oα Cys³⁵¹ → Ile protein (Figure 3B), whereas the 2.4-fold 5-HT-mediated increase and methiothepin-mediated decrease in $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding were fully resistant to PTX on co-expression of a G_oα Cys³⁵¹ → Ile protein (Figure 3D). A comparison with the 5-HT_{1B} receptor mutant Thr³¹³ → Arg plus the G_oα Cys³⁵¹ → Ile protein (Figure 3E) displayed a similar PTX-resistant 5-HT-mediated stimulus, a 2-fold increase in basal $[^{35}\text{S}]\text{GTP}[\text{S}]$ binding response and its blockade by methiothepin. Figure 4(A) shows dose-dependent G_oα Cys³⁵¹ → Ile protein expression in CHO-K1 membranes at different plasmid concentrations for the 5-HT_{1B} receptor mutant Thr³¹³ → Arg. Constitutive and 5-HT-dependent 5-HT_{1B} receptor activity were similar between co-expression with 0.1 μg and with 3 μg of the G_oα Cys³⁵¹ → Ile protein plasmid (results not shown). Subsequent experiments were therefore performed with 3 μg of either the wild-type 5-HT_{1B} or the mutant 5-HT_{1B} receptor plasmid and 3 μg of the G_oα Cys³⁵¹ → Ile plasmid to ensure sufficient G_oα protein ex-

Figure 4 Immunological detection of G_{α} protein expression in CHO-K1 cells co-expressing either wild-type 5-HT_{1B} or mutant 5-HT_{1B} receptors with the G_{α} Cys³⁵¹ → Ile protein

Total proteins (100 μ g) of CHO-K1 cells transfected with either 3 μ g of 5-HT_{1B} receptor mutant Thr³¹³ → Arg plasmid with the indicated amount of G_{α} Cys³⁵¹ → Ile protein plasmid (0.1–3 μ g) or 3 μ g of wild-type 5-HT_{1B} receptor plasmid with 3 μ g of empty plasmid (T⁻) (A), or 3 μ g of wild-type 5-HT_{1B} receptor plasmid with 3 μ g of empty plasmid (T⁻) or 3 μ g of G_{α} Cys³⁵¹ → Ile plasmid (T) or 3 μ g of 5-HT_{1B} receptor mutant with 3 μ g of G_{α} Cys³⁵¹ → Ile protein plasmid (B), were analysed by SDS/PAGE; immunodetection was performed as described in the Materials and methods section with an antibody raised against a peptide corresponding to the N-terminal portion of the G_{α} -protein.

pression for monitoring 5-HT_{1B} receptor activation. In contrast with co-expression with an empty plasmid (T⁻), G_{α} protein expression was present for the wild-type 5-HT_{1B} receptor and each of the 5-HT_{1B} receptor mutants (Figure 4B). However, some variation was apparent between several 5-HT_{1B} receptor mutants. This variation was not related to the observed rank order of 5-HT_{1B} receptor-mediated constitutive activity, as shown in Figure 5(A). In contrast to the histidine receptor mutant, each of the amino acid substitutions at position 313 in the 5-HT_{1B} receptor yielded, by co-expression of the G_{α} Cys³⁵¹ → Ile protein, an elevated basal [³⁵S]GTP[S] binding response ranging from 69% to 571%. The 5-HT_{1B} receptor mutants Thr³¹³ → Ile, Thr³¹³ → Gly, Thr³¹³ → Asn, Thr³¹³ → Ala, Thr³¹³ → Lys, Thr³¹³ → Phe, Thr³¹³ → Gln and Thr³¹³ → Arg displayed agonist-independent responses that were greater than that for the wild-type 5-HT_{1B} receptor. Methiothepin (1 μ M) reversed for each of the mutants the enhanced basal [³⁵S]GTP[S] binding response to the level of the wild-type 5-HT_{1B} receptor in the absence of the G_{α} Cys³⁵¹ → Ile protein (T⁻). The enhanced constitutive receptor activity was not a consequence of an increased 5-HT_{1B} receptor expression as estimated by measuring the specific binding of [³H]GR 125743 (Figure 5B). Maximal [³⁵S]GTP[S] binding responses as determined by 5-HT (10 μ M) were similar for most of the 5-HT_{1B} receptor mutants in comparison with the wild-type 5-HT_{1B} receptor. An attenuation of 26–57% in the 5-HT-mediated response ($P < 0.05$ compared with wild-type 5-HT_{1B} receptor) was observed with the receptor mutants containing a glutamate, a tryptophan or a cysteine residue. The partial agonist GR 125743 displayed a spectrum of activities between 26% and 95% of the response mediated by 5-HT at the 5-HT_{1B} receptor mutant Thr³¹³ → Arg (Figure 5D). Figure 6 compares a series of concentration–[³⁵S]GTP[S] response curves of ligands that have

Figure 5 [³⁵S]GTP[S] binding responses and expression levels of wild-type (wt) 5-HT_{1B} receptor and 5-HT_{1B} receptor mutants Thr³¹³ → Xaa on co-expression of a rat G_{α} Cys³⁵¹ → Ile protein in CHO-K1 cells

CHO-K1 cultures were treated overnight with PTX (100 ng/ml). Control CHO-K1 experiments were performed with the wild-type 5-HT_{1B} receptor plus empty plasmid (T⁻) instead of the rat G_{α} Cys³⁵¹ → Ile protein plasmid, then treated with PTX (100 ng/ml). (A) [³⁵S]GTP[S] ([³⁵S]GTP[S] binding responses were performed with 0.5 nM [³⁵S]GTP[S] either in the absence (hatched columns) or the presence of 1 μ M methiothepin (open columns) as described in the Materials and methods section. (B) The 5-HT_{1B} receptor density was estimated by measuring the specific binding of [³H]GR 125743 as described in the legend to Figure 2. (C) Maximal [³⁵S]GTP[S] binding responses were obtained as the difference between the responses mediated by 5-HT (10 μ M) and methiothepin (1 μ M), and are expressed as percentages of that obtained with the 5-HT_{1B} receptor mutant Thr³¹³ → Arg (719 ± 88 fmol/mg of protein). (D) GR 125743-mediated [³⁵S]GTP[S] binding responses were obtained as the difference between the responses mediated by GR 125743 (1 μ M) and methiothepin (1 μ M), and are expressed as percentages of the 5-HT-mediated response as mediated by the 5-HT_{1B} receptor mutant Thr³¹³ → Arg. Results are means ± S.E.M. for five independent transfection experiments, each performed in duplicate.

been previously reported to display antagonist properties at 5-HT_{1B} receptors. They yielded either stimulation or inhibition of [³⁵S]GTP[S] binding by wild-type 5-HT_{1B} receptors with the rat G_{α} Cys³⁵¹ → Ile protein. The corresponding $E_{\max}$, pEC₅₀ and pIC₅₀ values are summarized in Table 1 and are compared with their pK_i values for the wild-type 5-HT_{1B} receptor. Each

Figure 6 Ligand-mediated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses of the wild-type 5-HT_{1B} receptor co-expressed with a rat G_oα Cys³⁵¹ → Ile protein, and antagonism by SB 224289

Cultures were treated with PTX (100 ng/ml) and assayed for $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses as indicated in the legend to Figure 5. (A) Stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses is expressed as a percentage of that obtained with 5-HT (10 μM). Symbols: ●, 5-HT; ■, GR 127935; ▼, GR 125743; ◆, 1-naphthylpiperazine. (B) Inhibition of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses is expressed as a percentage of that obtained with methiothepin (1 μM). Symbols: □, methiothepin; ▽, SB 224289; ◇, GR 55562; △, ritanserin; ○, ketanserin. (C) Antagonism of the 5-HT response by SB 224289 (●, 0 nM; ○, 30 nM; □, 100 nM). Results are expressed as a percentage of the stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding as obtained by 5-HT (10 μM). The calculated pK_B value for SB 224289 was 8.22 ± 0.07 . Results are means $\pm$ S.E.M. for two or three independent experiments, each performed in duplicate. The E_{max} , pEC_{50} and pIC_{50} values of ligands are summarized in Table 1.

of the ligands under investigation displayed half-maximal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses at concentrations similar to their 5-[³H]carboxamidotryptamine binding affinity for the wild-type 5-HT_{1B} receptor. 1-Naphthylpiperazine, GR 125743 and GR 127935 behaved as partial agonists with potencies between 0.6 and 18 nM. Methiothepin and SB 224289 inhibited basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding by approx. 70% with nanomolar potencies. GR 55562 and ritanserin inhibited at submicromolar and micromolar potencies slightly less than basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. Ketanserin (10 μM) was virtually inactive towards the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response. SB 224289 (30 and 100 nM) antagonized the 5-HT-mediated concentration–response curve with a pK_B of 8.22, close to its affinity for the wild-type 5-HT_{1B} receptor (Figure 6C).

Table 1 E_{max} , pEC_{50} and pIC_{50} values of 5-HT ligands for wild-type 5-HT_{1B} receptor co-expressed with rat G_oα Cys³⁵¹ → Ile protein in CHO-K1 cells, and their pK_i values

$[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses were performed to CHO-K1 membranes with wild-type 5-HT_{1B} (Thr³¹³) receptors and the G_oα Cys³⁵¹ → Ile protein as described in the legend to Figure 6. E_{max} values of agonists are expressed as percentages of the 5-HT (10 μM)-mediated response (558 ± 85 fmol/mg of protein). E_{max} values of inverse agonists are expressed as percentages of the methiothepin (1 μM)-mediated inhibition (61–78%) of the basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response (220 ± 45 fmol/mg of protein). All values are means $\pm$ S.E.M. for two or three independent experiments. pK_i values were obtained by the binding of 5-[³H]carboxamidotryptamine ($[^3\text{H}]\text{5-CT}$) to either membranes of Cos-7 or C6-gial cells expressing 5-HT_{1B} receptors, as described [27].

Ligand	$[^{35}\text{S}]\text{GTP}[\text{S}]$ binding response			$[\text{}^3\text{H}]\text{5-CT}$ binding pK_i
	E_{max} (%)	$p\text{EC}_{50}$	$p\text{IC}_{50}$	
Agonist				
GR 127935	62.0 ± 5.7	8.83 ± 0.12	—	9.79 ± 0.12
GR 125743	62.5 ± 2.1	9.24 ± 0.03	—	9.45 ± 0.10
1-Naphthylpiperazine	63.5 ± 0.7	7.76 ± 0.05	—	8.00 ± 0.00
5-HT	100	8.16 ± 0.06	—	8.16 ± 0.03
Inverse agonist				
Ritanserin	-80.5 ± 7.8	—	6.05 ± 0.24	6.09 ± 0.13
GR 55562	-86.7 ± 2.3	—	7.27 ± 0.15	7.52 ± 0.23
SB 224289	-95.0 ± 3.5	—	8.10 ± 0.04	7.76 ± 0.01
Methiothepin	—100	—	8.15 ± 0.00	7.69 ± 0.05
Inactive				
Ketanserin	-8.0 ± 0	< 5.00		< 5.00

DISCUSSION

The present paper reports on the modulation of agonist-independent and agonist-dependent activation of the recombinant human 5-HT_{1B} receptor by mutation of Thr³¹³ in the BBXXB motif located in the C-terminal portion of the third intracellular loop. Wild-type 5-HT_{1B} receptors yielded a low (less than 10%) and variable level of constitutive activity in CHO-K1 cells. Mutation of Thr³¹³ in the 5-HT_{1B} receptor to either a lysine, an arginine or a glutamine residue increased constitutive activity by 26–41%, which was reversed by inverse agonists. Increased constitutive receptor activation was provided by co-expression of the 5-HT_{1B} receptor with a PTX-resistant rat G_oα Cys³⁵¹ → Ile protein. The wild-type 5-HT_{1B} receptor displayed a doubling of basal activity, whereas this response was amplified with a series of diverse amino acid substitutions (isoleucine, glycine, asparagine, alanine, lysine, phenylalanine, glutamine and arginine) at 5-HT_{1B} receptor position 313. Enhanced basal activity was maximal (approx. 500%) with the 5-HT_{1B} receptor mutants Thr³¹³ → Gln and Thr³¹³ → Arg; this activity represented approx. 50% of maximal receptor activation by 5-HT. Few or no modifications in the 5-HT_{1B} receptor expression levels were observed for the 5-HT_{1B} receptor mutations in either the absence or the presence of the G_oα Cys³⁵¹ → Ile protein, with the exception of the mutant Thr³¹³ → His. This suggests that the observed effects were caused primarily by changes in receptor–G-protein coupling. Specific [³H]GR 125743 binding to the 5-HT_{1B} receptor mutant Thr³¹³ → His was very low, suggesting improper protein folding. The other 5-HT_{1B} receptor mutants investigated were probably not abnormal structures but very probably mimicked transient states of the native mechanism of 5-HT_{1B} receptor activation. The various mutated residues at position 313 that yielded enhanced constitutive 5-HT_{1B} receptor activity are unlikely to make similar intramolecular interactions within the 5-HT_{1B} receptor. No clear

pattern is apparent in the framework of traditionally cited amino acid characteristics such as residue size, charge or hydrophobicity. The study also illustrates that each of the ligands previously characterized as 5-HT_{1B} antagonists behave, in the presence of the G_oα Cys³⁵¹ → Ile protein, either as partial agonists or inverse agonists.

Similar constitutively active mutations in the BBXXB motif have been reported for α_{1B}-adrenergic (Ala²⁹³ [13]), α_{2A}-adrenergic (Thr³⁷³ [14]), 5-HT_{2A} (Cys³²² [15]) and 5-HT_{2C} (Ser³¹² [16]) receptors. Replacement by the basic residue lysine in each of these receptor subtypes induces, as in the 5-HT_{1B} receptor at position 313, an enhanced constitutive activity. An arginine residue is also well tolerated in the α_{1B}-adrenergic and 5-HT_{1B} receptors, whereas a histidine residue is not readily admitted at this position. Otherwise, a glutamate residue has also been shown to increase basal activity of α_{1B}-adrenergic, α_{2A}-adrenergic and 5-HT_{2A} receptors in contrast with 5-HT_{1B} receptors. The other acidic residue, aspartate, does not improve constitutive activity for both α_{1B}-adrenergic and 5-HT_{1B} receptors.

The mutation of Thr³¹³ might disrupt interactions that hold the 5-HT_{1B} receptor in an inactive conformation and/or facilitate its interactions with G-proteins. Wild-type 5-HT_{1B} receptors exhibited a low level of constitutive activity, whereas the mutation of Thr³¹³ might selectively destabilize the inactive receptor conformation so that in the absence of ligands up to 28 % (i.e. mutant Thr³¹³ → Arg) of the receptors are in an active conformation and 72 % in an inactive conformation. Co-expression with the G_oα Cys³⁵¹ → Ile protein resulted in an enhanced proportion of active 5-HT_{1B} receptors, ranging from 19 % to maximally 59 % for the wild-type 5-HT_{1B} (Thr³¹³) receptor and its Thr³¹³ → Arg mutant respectively. It is possible that the hydroxy group of Thr³¹³ is directly involved in a constraining interaction or in critical post-translational modifications (e.g. phosphorylation), hydrogen-bonding or other intramolecular protein interactions, because these would be destroyed by mutation of Thr³¹³ to a glycine or an alanine residue. These substitutions produced a 5-HT_{1B} receptor phenotype that was less constrained. The mutants with a tyrosine or serine residue, which also contain a hydroxy group, showed similar activities to that of threonine. Otherwise, the hydroxy group of Thr³¹³ is unlikely to be involved in the agonist-activation mechanism of the 5-HT_{1B} receptor. With the exception of proline and the acidic residues aspartate and glutamate at position 313, which caused lower levels (48–59 %) of 5-HT_{1B} receptor activation, the other mutants either did not affect or affected only slightly the 5-HT-mediated receptor activation. Another argument against a direct involvement of the hydroxy group of the threonine residue in constraining the 5-HT_{1B} receptor interactions might be the presence of an alanine, a cysteine or a serine residue at the same position in α_{1B}-adrenergic, 5-HT_{2A} and 5-HT_{2C} receptor subtypes shown to yield a low degree of constitutive activity in their native states [13,15,16].

The junction between the third intracellular loop and transmembrane domain (TM) VI of GPCR has been proposed to fold into an α-helical amphiphilic domain that extends the α-helix of TM VI into the cytoplasm [32,33]. This region might either be embedded into the plasma membrane or protrude into the cytoplasm, depending on the structure of the GPCR. The basic residues of the BBXXB motif face towards one side of the putative α-helix; their hydrophilic character probably excludes them from TM VI. Mutation of Thr³¹³ in the 5-HT_{1B} receptor, facing the same side as the basic residues Arg³¹⁰, Lys³¹⁴ and Lys³¹¹, to a basic residue increases the positively charged character of this putative α-helix. This feature might release this helix from the plasma membrane and thereby facilitate interactions

with G-proteins. A similar model can be envisaged for the amino acids glutamine and asparagine. The constitutive activity of the 5-HT_{1B} receptor is also increased by exchanging Thr³¹³ for the non-polar residues phenylalanine and isoleucine. This might increase the hydrophobic character of the BBXXB motif and probably modify its positioning with regard to TM VI and consequently modify G-protein interactions. Alternatively, mutagenesis of the Thr³¹³ residue might modify intramolecular bonds (i.e. with the other intracellular domains) and thereby modify the receptor activation state.

Although the physiological implications of constitutively active 5-HT_{1B} receptor mutations remain unclear, the identification of mutations in thyrotropin receptors in patients with thyroid adenomas [3], rhodopsin receptors in retinitis pigmentosa [34], parathyroid hormone receptors in dwarfism [35] and luteinizing hormone receptors in precocious puberty [36] suggests a role for natural GPCR mutations in disease pathophysiology. In each case, the expression of these mutant receptors *in vitro* in mammalian cell lines resulted in the activation of appropriate second messenger systems in the absence of agonist, thereby confirming the constitutively active nature of the mutated receptor identified *in vivo*. Proliferation of human small-cell lung carcinoma cell lines has been reported to be mediated by 5-HT_{1B} receptors [37]. It would be interesting to test the hypothesis that mutations in the 5-HT_{1B} receptor might be involved in the pathophysiology of various disease states.

Ligands previously characterized as antagonists at the wild-type 5-HT_{1B} receptor demonstrated in this study either a positive (GR 125743, GR 127935 and 1-naphthylpiperazine) or a negative efficacy (ritanserin, GR 55562, SB 224289 and methiothepin) at concentrations relevant to their affinities for the wild-type 5-HT_{1B} receptor. The 5-HT_{1B} receptor model systems discussed here permit the unambiguous definition of ligands as partial agonists, neutral antagonists or inverse agonists. The lack of ligands that do not modify the basal [³⁵S]GTP[S] binding response at 5-HT_{1B} receptors suggests 5-HT_{1B}-neutral antagonists might be relatively uncommon. This is not surprising because this would require the same ligand to exhibit a similar affinity for both the active and the inactive receptor conformation.

In summary, the present study demonstrates that the 5-HT_{1B} receptor can be transformed to a constitutively activated form either by a single amino acid (Thr³¹³) mutation, by G_oα protein co-expression or by both. The constraint of 5-HT_{1B} receptor activation can be relieved by mutation with a series of various amino acids. It is likely that the positioning of the junction between the third intracellular loop and TM VI is altered by mutation of Thr³¹³ in the BBXXB motif, thereby unmasking G_α protein interaction points.

We thank Frédéric Finana and Stéphanie Tardif for excellent technical assistance, and Stéphanie Cecco for secretarial assistance.

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Received 17 May 1999/23 July 1999; accepted 12 August 1999