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Site-directed mutagenesis of the 5-HT_{1B} receptor increases the affinity of 5-HT for the agonist low-affinity conformation and reduces the intrinsic activity of 5-HT

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Abstract

The antagonist radioligand [^{3}H]GR125743 and the agonist radioligand [^{3}H]5-HT were used to investigate the pharmacological characteristics of the G protein uncoupled agonist low-affinity and G protein coupled agonist high-affinity conformations of the wild-type and mutant human 5-hydroxytryptamine$_{1B}$ (5-HT_{1B}) receptors. We found that substitution of phenylalanine 185 in transmembrane region IV by alanine or methionine resulted in a reduced number of receptors in the coupled conformation, as well as a reduced affinity of 5-HT for the uncoupled conformation. In contrast, substitution of phenylalanine 331 in transmembrane region VI by alanine increased the affinity of 5-HT for the uncoupled conformation 11-fold thus reducing the agonist low-affinity to agonist high-affinity (K_{il}/K_{ih}) ratio 5-fold. This reduced ratio was correlated with a significantly reduced intrinsic activity of 5-HT previously determined by its ability to inhibit forskolin-stimulated cAMP production. In conclusion, these results show that single amino acid substitutions can selectively change the affinity of 5-HT for the G protein uncoupled conformation of the 5-HT_{1B} receptor and alter the intrinsic activity of the ligand.

Keywords: 5-HT; 5-Hydroxytryptamine; 5-HT_{1B} receptor; Site-directed mutagenesis; GR125743

1. Introduction

The 5-hydroxytryptamine$_{1B}$ (5-HT_{1B}) receptor is a member of the large family of 5-HT receptor that has seven receptor subclasses. The 5-HT_1 receptor subclass consists of five homologous receptor subtypes that all couple to G_i proteins and inhibit cyclic AMP production (Hoyer and Martin, 1997). The closest relative of the 5-HT_{1B} receptor is the 5-HT_{1D} receptor. The overall amino acid identity between the 5-HT_{1B} and 5-HT_{1D} receptors is 63% and within the transmembrane spanning region, the identity is 77%. Both 5-HT_{1B} and 5-HT_{1D} receptors are broadly distributed throughout the central nervous system in mammalian species. They are presynaptically localized on several types of nerve terminals and have been shown to function both as auto- and heteroreceptors (Boschert et al., 1994; Bühlen et al., 1996; Engel et al., 1986). High density of 5-HT_{1B} receptor mRNA has been detected in the cerebellum, hippocampus, caudate putamen and cortical areas (Bruinvels et al., 1994). The 5-HT_{1B} receptor co-distributes with the 5-HT_{1D} receptor in many brain regions but it is expressed at higher levels (Bruinvels et al., 1994).

The 5-HT_{1B} receptor is also expressed in cerebral vasculature (Hamel et al., 1993). The anti-migraine drug sumatriptan is believed to act through the 5-HT_{1B} receptor and contract cerebral blood vessels at the surface of the brain (De Keyser et al., 1993; Hamel et al., 1993; Miller et al., 1992). Sumatriptan has also been suggested to inhibit neurogenic inflammation, acting through 5-HT_{1D} receptors (Yu et al., 1997). Identification of the physiological role of the 5-HT_{1B} receptor as well as the other 5-HT_1 receptor subtypes has been hampered by the lack of selective ligands. However, some agonist ligands that discriminate between the 5-HT_{1B} and 5-HT_{1D} receptor subtypes have been reported (Price et al., 1997).

Most radioligand-binding studies on 5-HT_1 receptors have been performed using radiolabeled agonists, which preferentially identify the agonist high-affinity conformation of the receptor and thus do not label the total receptor

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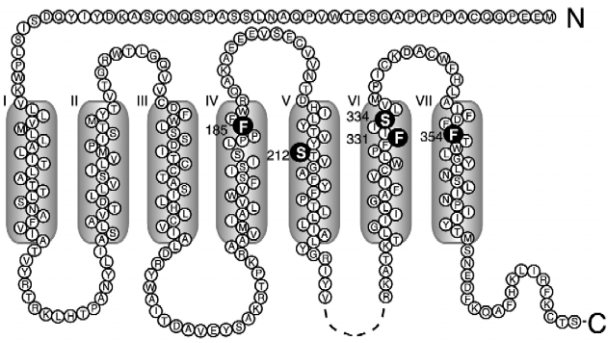

Fig. 1. Schematic representation of the human 5-HT_{1B} receptor, which shows the amino acid residues that are mutated in white letters on filled black circles. Dashed line symbolizes the large third intracellular loop.

population. In our previous studies, we have constructed several mutant 5-HT_{1B} receptors to investigate the subtype specific ligand binding properties of the 5-HT_{1B} receptor. Several serines or phenylalanines in transmembrane regions IV–VII, which either differ between 5-HT receptor subtypes or are highly conserved, were substituted (Fig. 1). We observed that the number of agonist high-affinity binding sites labeled with the agonist radioligand [^{3}H]5-HT varied considerably between the cell lines expressing the various 5-HT_{1B} receptor mutants (Grånäs et al., 1998a,b). In the present study, we have used the 5-$HT_{1B/1D}$ receptor-selective antagonist [^{3}H]GR125743 that detects the whole receptor population (Doménech et al., 1997), to study the effects of the mutations on the total receptor population. Thus, we have determined the affinity of 5-HT for the inactive agonist low-affinity conformation that is G protein uncoupled (K_{il}, hereafter called "uncoupled conformation") using [^{3}H]GR125743 (in the presence of the nonhydrolyzable GTP analogue Gpp(NH)p and Na^+) and compared it with the affinity of 5-HT for the active, agonist high-affinity conformation that is predominantly G protein coupled (K_{ih}, hereafter called "coupled conformation"). The K_{il}/K_{ih} ratio of 5-HT was calculated and correlated with the 5-HT-induced inhibition of forskolin-stimulated cyclic AMP accumulation in intact cells.

2. Materials and methods

2.1. Mutagenesis of the human 5-HT_{1B} receptor

The human 5-HT_{1B} receptor mutants were constructed using a modified version of the USE Mutagenesis Kit (Pharmacia P-L Biochemicals) as described previously (Grånäs et al., 1998a,b). After digestion with the restriction endonucleases *Hin*dIII and *Eco*RV, the mutated receptor genes were subcloned into the expression vector pcDNAI/Neo containing a Neomycin resistance gene for selection of transfected cells (Invitrogen, San Diego, CA). After mutagenesis, the nucleotide sequence of the mutant receptor clones was confirmed by DNA sequencing. After subcloning, the size of the insert and the nucleotide sequence was analyzed by polymerase chain reaction (PCR) and DNA sequencing, respectively.

2.2. Expression of wild-type and mutant 5-HT_{1B} receptors

Stable cell lines were produced by transfecting Chinese hamster ovary (CHO) K1 cells with each of the mutant receptor clones or the wild-type receptor clone using the LIPOFECTIN method (Life Technologies, Gaithersburg, MD) (Felgner et al., 1987). Cells were grown to 30–50% confluence on 100-mm tissue plates in media consisting of Dulbecco's modification of Eagle's medium (DMEM) with L-glutamine supplemented with 10% fetal calf serum, 0.05 g/l streptomycin and 0.06 g/l penicillin G. Prior to transfection, cells were washed once with 2 ml Opti-MEM I Reduced Serum Medium. 2 μg DNA was dissolved in 100 μl Opti-MEM I Reduced Serum Medium and was combined with a mixture of 20 μl LIPOFECTIN Reagent and 100 μl Opti-MEM I Medium and was incubated at room temperature for 15 min. The LIPOFECTIN–DNA complexes were mixed with 1.8 ml Opti-MEM I Reduced Serum Medium and added to the cells. After incubating for 5 h, the DNA solution was aspirated and growth medium was added. The cells were incubated for 48 h and then split at a ratio of 1:5 and grown in media containing geneticin at 0.35 g/l for selection.

2.3. Radioligand-binding assays

Receptor binding studies were done on membrane preparations from cell clones after large-scale cultivation. Confluent cells were harvested using a rubber scraper and pooled in serum-free medium and subsequently dissolved in 10 mM Tris–HCl buffer with 5 mM $MgSO_4$ (pH 7.4) and homogenized with a Dounce homogenizer (15 strokes). Membranes were collected by centrifugation at 10,000 × *g* for 10 min. The cell membranes were washed with a buffer consisting of 50 mM Tris–HCl, 4 mM $MgCl_2$, 120 mM *N*-methyl-D-glucamine, 1 mM EDTA and 10 μM pargyline (pH 7.4) and centrifuged. The membranes were resuspended in the same buffer and frozen at −70°C in 1-ml aliquots until use. All the membrane preparation steps were done at 4°C. Protein concentrations were determined by the method of Markwell et al. (1978) using bovine serum albumin as standard.

On the day of the experiment, the frozen membranes were thawed, homogenized with a Branson 450 sonifier, and resuspended in a buffer to a final concentration of 0.4–2.2 mg protein per milliliter, depending on the receptor density. Determination of the affinities of ligands for the agonist high-affinity coupled conformation of the receptors was performed in a buffer consisting of 50 mM Tris–HCl, 4 mM $MgCl_2$, 120 mM *N*-methyl-D-glucamine, 1 mM EDTA and 10 μM pargyline (pH 7.4). Determina-

tion of the affinities of the ligands for the agonist low-affinity uncoupled conformation of the receptor was performed in the presence of 120 mM NaCl (instead of 120 mM *N*-methyl-D-glucamine) and 0.1 mM Gpp(NH)p.

The receptor binding assays were initiated by the addition of membranes and carried out in a volume of 0.5–2.0 ml at 30°C for 1 h. The incubations were terminated by rapid filtration on glass microfibre filters (GF-B, Whatman) pre-treated in 0.3% polyethylenimine (Sigma) using a Brandel M-24S cell harvester, and subsequent washing in cold buffer (50 mM Tris–HCl, pH 7.4). Filters were soaked in scintillation fluid (Ultima gold, Packard) for at least 2 h before counting in a Packard (1900CA) liquid scintillation counter.

Saturation experiments were done using 12 concentrations of [^{3}H]5-HT (specific activities 106–112 Ci/mmol, Amersham) or [^{3}H]GR125743 (specific activities 74–76 Ci/mmol, Amersham). Competition studies were done using 12 concentrations of competing unlabeled ligand. The substances were dissolved and diluted in 0.1% ascorbic acid. Nonspecific binding was determined in the presence of 10 μM methiothepin. The data were analyzed by nonlinear regression using PRISM (GraphPad, San Diego, CA). The K_d values used to calculate the K_i values were determined under corresponding assay conditions. To normalize the data before statistical analysis, the individual K_d and K_i values were converted to pK_d and pK_i values. The Hill coefficients were calculated for each individual experiment. One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used for statistical comparisons. One- and two-site curve fittings were tested in all experiments. The two-site model was accepted when it significantly improved the curve fit ($P < 0.05$; *F*-test) and when each site accounted for $> 20\%$ of the receptors.

2.4. Determination of agonist-induced inhibition of forskolin-stimulated cyclic AMP accumulation

CHO-K1 cells expressing wild-type or mutant 5-HT$_{1B}$ receptors were grown as described above. When confluent, medium was removed and cells were rinsed in DMEM containing 20 mM HEPES at pH 7.4 and detached from the plates using 2 ml Earle's Balanced Salt Solution (EBSS) without Mg^{2+} and Ca^{2+}, containing 1 mM EDTA and 20 mM HEPES, pH 7.4. The cells were harvested and resuspended in 10 ml DMEM containing 20 mM HEPES, pH 7.4. The suspension was centrifuged at $110 \times g$ for 6 min at room temperature. The cells were resuspended in 10 ml DMEM containing 10 mM HEPES, 10 μM pargyline and 1 mM 3-isobutyl-1-methylxanthine (IBMX), pH 7.4, and incubated for 30 min at 37°C. After diluting the cells to a density of 3×10^6 cells ml^{-1}, 425-μl aliquots of the cell suspension were added to Eppendorf tubes containing 25 μl forskolin (final concentration 10 μM) in all tubes except for measuring the basal cyclic AMP level. The 5-HT-induced functional response was determined by adding 25 μl 5-HT (final concentration 10 μM). Forskolin (10 μM) in the absence of 5-HT was used to achieve a maximal cyclic AMP level. Volumes were adjusted to 500 μl with buffer. All samples were done in duplicate and incubated for 20 min at 37°C. Reactions were terminated by boiling for 5 min. The lysates were centrifuged at $9000 \times g$ for 2 min and 50 μl of the supernatant was used for cyclic AMP measurements, done in duplicate. Cyclic AMP concentrations were measured by the Cyclic AMP [^{3}H] assay system from Amersham, UK. The data were analyzed using PRISM (GraphPad). ANOVA followed by Dunnett's post-test was used for statistical comparisons.

3. Results

3.1. Comparison of the characteristics of [^{3}H]5-HT and [^{3}H]GR125743 binding to the human wild-type 5-HT$_{1B}$ receptor

In previous radioligand-binding studies of the wild-type and mutant human 5-HT$_{1B}$ receptors, we used the agonist [^{3}H]5-HT as radioligand and a binding buffer that promotes the G protein coupled conformation of the receptor (i.e. in the presence of Mg^{2+} and absence of guanine nucleotides and Na^+). We found that the total number of high-affinity agonist binding sites varied considerably between the various cell lines (Grånäs et al., 1998a,b). The present study was undertaken to further characterize the receptor–ligand interactions of wild-type and mutant 5-HT$_{1B}$ receptors using the antagonist [^{3}H]GR125743 that labels both the coupled and uncoupled conformation of the receptor with the same affinity, thus identifying the total receptor population. Fig. 2 shows representative saturation curves of [^{3}H]5-HT and [^{3}H]GR125743 binding to the wild-type receptor. The experiments were performed in parallel and on receptors from the same membrane preparation. As can be seen, both [^{3}H]5-HT and [^{3}H]GR125743 bound with high affinity and saturability to the wild-type receptor. The Scatchard analysis of specific [^{3}H]5-HT and [^{3}H]GR125743 binding resulted in linear plots consistent with a noncooperative single class of binding sites. The K_d and B_{max} values of [^{3}H]5-HT binding were 3.5 ± 0.3 nM and 285 ± 12 pmol/g protein, respectively ($n = 5$) and of [^{3}H]GR125743 binding 0.7 ± 0.2 nM and 599 ± 115 pmol/g protein, respectively ($n = 4$) (Table 1). Thus, [^{3}H]5-HT identified 48% of the total receptor population labeled by [^{3}H]GR125743.

3.2. The effect of various mutations on [^{3}H]5-HT and [^{3}H]GR125743 binding

The results from the saturation studies with [^{3}H]GR125743 and [^{3}H]5-HT of the wild-type and the various 5-HT$_{1B}$ receptor mutants are summarized in Table 1. The affinities of [^{3}H]5-HT for the S212A and F331A

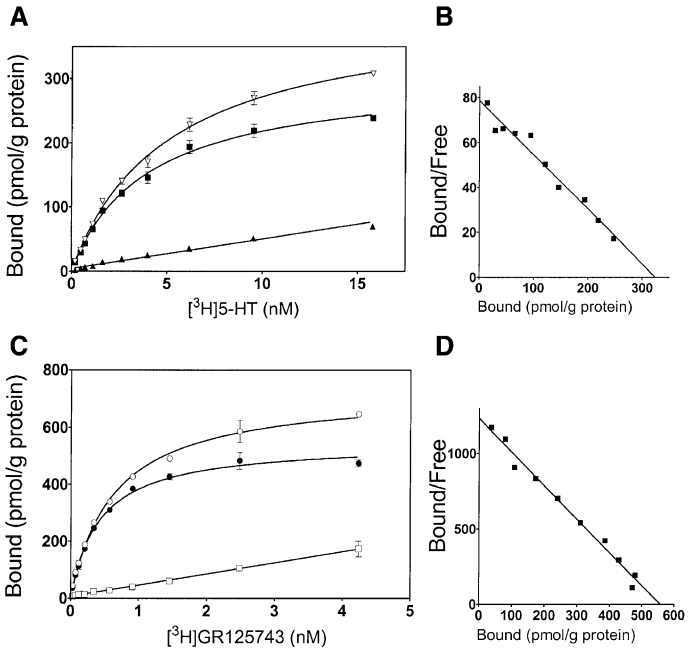

Fig. 2. [^{3}H]GR125743 and [^{3}H]5-HT binding to the wild-type 5-HT$_{1B}$ receptor. The experiments were performed in a buffer that favors the conversion of receptors to the G protein coupled conformation. K_d and B_{max} values are presented in Table 1. Representative saturation curve of [^{3}H]5-HT binding (A), total binding (▽), specific binding (■) and nonspecific binding (▲). Scatchard plot of the specific [^{3}H]5-HT binding (B). The K_d and B_{max} values determined from the Scatchard analysis were 4.2 nM and 326 pmol/g protein, respectively. Representative saturation curve of [^{3}H]GR125743 binding (C), total binding (○), specific binding (●) and nonspecific binding (□). Scatchard plot of the specific [^{3}H]GR125743 binding (D). The K_d and B_{max} values determined from the Scatchard analysis were 0.45 nM and 554 pmol/g protein, respectively.

mutants were slightly increased as compared to the wild-type receptor. No statistically significant changes of the K_d values of [^{3}H]5-HT binding to the other mutants were observed. In contrast, the B_{max} values as determined by [^{3}H]5-HT binding varied considerably between the 5-HT$_{1B}$ receptor mutants (Table 1). These results are in agreement with our previous studies (Grånäs et al., 1998a,b) and show that the receptor densities determined by the agonist radioligand [^{3}H]5-HT vary between the cell lines.

The affinity of [^{3}H]GR125743 for the S334A mutant was slightly increased as compared to the wild-type receptor; however, no statistically significant changes of the K_d values of [^{3}H]GR125743 binding to the other mutants were observed (Table 1). In addition, we determined the affinity

Table 1
K_d and B_{max} values of [^{3}H]GR125743 and [^{3}H]5-HT binding to wild-type and mutant human 5-HT$_{1B}$ receptors
The experiments were performed and the K_d and B_{max} values were calculated as described in Section 2. The saturation studies with both radioligands were performed in a buffer that promotes the G protein coupled conformation of the receptor. The results are expressed as means ± standard errors from n independent experiments run in duplicate.

	[^{3}H]GR125743			[^{3}H]5-HT			Percentage of high-affinity sites
	K_d (nM)	B_{max} (pmol/g protein)	n	K_d (nM)	B_{max} (pmol/g protein)	n	
WT	0.7 ± 0.2	599 ± 115	4	3.5 ± 0.3	285 ± 12	5	48
F185A	1.0 ± 0.2	505 ± 80	2	3.3 ± 0.5	66 ± 9	6	13
F185M	1.2 ± 0.4	667 ± 15	2	2.4 ± 0.5	67 ± 16	5	10
S212A	0.6 ± 0.04	412 ± 18	2	2.2 ± 0.2[a]	83 ± 5	4	20
F331A	0.3 ± 0.04	319 ± 33	3	1.2 ± 0.1[b]	170 ± 30	5	53
S334A	0.2 ± 0.04[a]	203 ± 28	4	3.6 ± 0.4	57 ± 7	8	28
F354A	0.9 ± 0.1	1563 ± 155	2	3.0 ± 0.7	391 ± 77	5	25
F354Y	1.2 ± 0.2	135 ± 36	3	2.2 ± 0.6	60 ± 6	4	43

[a] and [b] indicate a K_d value that is significantly different from the K_d value of the wild-type receptor.
[a] $P < 0.01$.
[b] $P < 0.001$.

of [^{3}H]GR125743 for the wild-type and mutant receptors in a buffer with Na^+ and Gpp(NH)p. The change of buffer did not significantly alter the affinity of [^{3}H]GR125743 for the receptors (data not shown). Interestingly, determination of the B_{max} values by [^{3}H]GR125743 show that the relative proportion of receptors in the coupled conformation identified by the agonist [^{3}H]5-HT as compared with the total receptor population labeled with [^{3}H]GR125743 varies considerably between the wild-type receptor and the various mutant receptors (Table 1). In the cells expressing the F185A or F185M mutants, the proportions of receptors labeled by [^{3}H]5-HT were 13% and 10%, respectively, as compared to 48% for the wild-type receptor.

3.3. Various mutations selectively change the affinity of 5-HT for the uncoupled conformation of the receptor

The affinity of 5-HT for the uncoupled conformation (K_{il}) of the wild-type and mutant receptors was determined by competition studies in the presence of Na^+ and the nonhydrolyzable GTP analogue Gpp(NH)p using the antagonist [^{3}H]GR125743 as radioligand. Table 2 summarizes the results and compares them with the affinity of 5-HT for the coupled conformation of the receptors (K_{ih}) previously determined using the agonist radioligand [^{3}H]5-HT in the absence of Na^+ and Gpp(NH)p (Grånäs et al., 1998a,b).

As can be seen in Table 2, the K_{il} values of 5-HT binding to the uncoupled conformation of the F331A, F354A and F354Y mutants were significantly reduced as compared the wild-type receptor. Furthermore, the K_{il} values of 5-HT binding to the uncoupled conformation of the F185A, F185M and S334A mutants were significantly increased as compared the wild-type receptor. No significant difference in the K_{il} value of 5-HT binding to the uncoupled conformation of the S212A mutant was observed. All the competition curves were best described by a model of one binding-site as also indicated by Hill coefficients close to 1.

The ratios of K_{il} to K_{ih} of 5-HT binding to the wild-type and the various mutant receptors were calculated and are shown in Table 2. The K_{il}/K_{ih} ratio of 5-HT binding to the wild-type receptor was 29. Thus, a 29-fold rightward shift of the 5-HT-inhibition curve was observed in the presence of Na^+ and Gpp(NH)p as illustrated by Fig. 3A. The K_{il}/K_{ih} ratios of 5-HT binding to the F185A, F185M and S334A mutants increased several-fold. The K_{il}/K_{ih} ratio of 5-HT binding to the F331A mutant

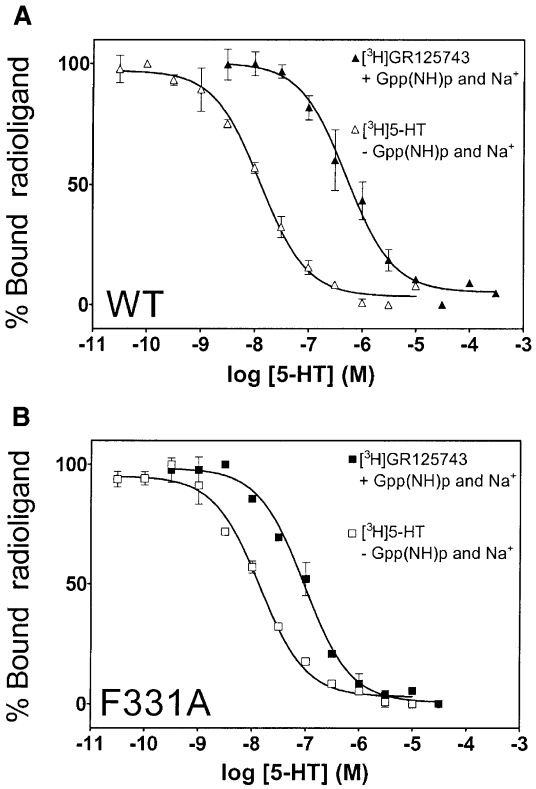

Fig. 3. Concentration–response curves of 5-HT inhibition of [^{3}H]GR125743 and [^{3}H]5-HT binding to the wild-type 5-HT$_{1B}$ receptor. Representative dose–response curves of 5-HT inhibition of [^{3}H]5-HT (△), and [^{3}H]GR125743 (▲), binding to the wild-type 5-HT$_{1B}$ receptor (A). Representative dose–response curves of 5-HT inhibition of [^{3}H]5-HT (□), and [^{3}H]GR125743 (■), binding to the F331A mutant (B). The competition experiments with [^{3}H]5-HT were performed in a buffer that promotes the G protein coupled receptor conformation, while the competition experiments with [^{3}H]GR125743 were performed in a buffer that promotes the uncoupled receptor conformation. Where the standard error is not shown, it is smaller than the size of the symbol.

Table 2
The affinity of 5-HT for the G protein uncoupled conformation (K_{il}) of the wild-type and mutant 5-HT$_{1B}$ receptors determined by [^{3}H]GR125743 in comparison with the previously determined affinity for the G protein coupled conformation (K_{ih}) determined by [^{3}H]5-HT
The experiments were performed and the K_i values were calculated as described in Section 2. The results are expressed as means ± standard errors from three to four independent experiments run in duplicate. The K_d values used to calculate the K_i values were determined under corresponding assay conditions.

	[^{3}H]GR125743	[^{3}H]5-HT	Ratio
	5-HT K_{il} (nM)	5-HT K_{ih} (nM)	K_{il}/K_{ih}
WT	100 ± 10	3.4 ± 0.5[a]	29
F185A	565 ± 72[b]	3.1 ± 0.4[c]	182
F185M	279 ± 12[b]	4.2 ± 0.9[c]	66
S212A	97.3 ± 16	3.3 ± 0.3[a]	29
F331A	9.3 ± 0.9[b]	1.6 ± 0.2[a,d]	5.8
S334A	414 ± 64[e]	3.8 ± 0.6[c]	109
F354A	60.1 ± 2.7[d,f]	1.9 ± 0.9[c]	32
F354Y	46.5 ± 6.5[e]	2.5 ± 0.3[c]	19

[a, b] and [c] indicate K_{ih} or K_{il} values, which are significantly different from the K_{ih} or K_{il} values of the wild-type receptor.
[a] Data from Grånäs et al. (1998a).
[b] $P < 0.001$.
[c] Data from Grånäs et al. (1998b).
[d] $P < 0.05$.
[e] $P < 0.01$.
[f] $n = 2$.

Table 3
5-HT-induced inhibition of forskolin-stimulated cyclic AMP accumulation in cells expressing wild-type and mutant 5-HT_{1B} receptors
Forskolin and 5-HT were used at a final concentration of 10 μM. The results are expressed as the means ± standard errors from four to seven independent experiments run in duplicate.

	Cyclic AMP levels expressed as pmol/1.0×10^6 cells			
	Basal	Forskolin	Forskolin + 5-HT	Percentage of inhibition
WT	6.2 ± 0.8	111.0 ± 5.0	14.9 ± 2.2	87
F185A	10.7 ± 1.2	91.5 ± 5.2	17.9 ± 1.0	80
F185M	10.0 ± 0.6	93.9 ± 3.5	24.3 ± 1.9	74
S212A[a]	12.3 ± 1.2[b]	101.9 ± 12.2	32.4 ± 3.3[b]	68
F331A[a]	15.3 ± 1.2[c]	158.3 ± 8.7[c]	73.7 ± 3.3[c]	53
S334A	10.5 ± 2.2	90.8 ± 4.6	25.0 ± 2.7	72
F354A	24.4 ± 1.1[c]	93.5 ± 0.8	55.0 ± 8.8[c]	59
F354Y	19.9 ± 1.9[c]	88.5 ± 7.0	37.1 ± 4.3[c]	58

[a] and [b] indicate significant differences as compared to the wild-type receptor.
[a] Data from Grånäs et al. (1998a).
[b] $P < 0.01$.
[c] $P < 0.001$.

decreased 5-fold (Table 2). This 5-fold rightward shift of the 5-HT-inhibition curve in the presence of Na^+ and Gpp(NH)p is shown in Fig. 3B. The K_{il}/K_{ih} ratio of 5-HT binding to the F354Y mutant was also slightly decreased, whereas no changes in the K_{il}/K_{ih} ratios of the S212A and F354A mutants as compared to the wild-type receptor were observed.

3.4. 5-HT-induced inhibition of forskolin-stimulated cyclic AMP accumulation

The intrinsic activities of 5-HT at wild-type and mutant receptors were determined by measuring the receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation in intact cells stably expressing each receptor construct. In addition, the basal cyclic AMP levels and the maximal cyclic AMP levels in forskolin-stimulated cells were determined. The results from these studies are summarized in Table 3. As can be seen, the addition of 5-HT (10 μM) inhibited the forskolin-stimulated cyclic AMP production in cells expressing the F185A, F185M, S334A, F354A and F354Y mutant receptors. However, the inhibition by 5-HT was significantly reduced at the F354A and F354Y mutants as compared to the wild-type receptor (Table 3). The basal cyclic AMP levels were also significantly increased (3-fold) for the F354A and F354Y mutants as compared to the wild-type receptor (Table 3). Moreover, the basal cyclic AMP levels in cells expressing the wild-type receptor or the F185A, F185M or S334A mutants were somewhat reduced in comparison with the basal cyclic AMP levels in untransfected cells which was 15.9 ± 2.5 pmol/1.0×10^6 cells. No significant change in the cyclic AMP level in forskolin-stimulated untransfected CHO-K1 cells was evoked by 5-HT.

Results from a previous study of the S212A and F331A mutants are included in the table (Grånäs et al., 1998a). This study showed that the inhibition of forskolin-stimulated cyclic AMP accumulation by 5-HT (10 μM) was significantly reduced in cells expressing the F331A mutant and somewhat reduced for the S212A mutant. The basal cyclic AMP levels were also significantly increased for the S212A and F331A mutants as compared to the wild-type receptor. Moreover, the forskolin-stimulated cyclic AMP accumulation was significantly increased only for the F331A mutant (Table 3).

4. Discussion

4.1. Binding of agonists and antagonists to G protein coupled receptors

According to the prevailing model of G protein coupled receptors, they exist in an equilibrium between an inactive G protein-uncoupled conformation and an isomerized conformation that has high affinity for the G protein and readily forms the signaling G protein coupled conformation (Lefkowitz et al., 1993; Samama et al., 1993). Most agonists bind with high affinity (K_{ih}) to the isomerized and signaling G protein coupled conformations and with low affinity (K_{il}) to the inactive G protein uncoupled conformation. "Neutral" antagonists bind to all conformations of the receptor with the same affinity.

In our previous study on the dopamine D_2 receptor, we demonstrated that the affinity of an agonist for the uncoupled conformation of the receptor (K_{il}) can be determined in the presence of Na^+ and guanine nucleotides (which convert the receptors to the uncoupled conformation) using an antagonist as radioligand (Malmberg et al., 1998). The affinity of an agonist for the coupled conformation of the receptor (K_{ih}) could be determined in the presence of Mg^{2+} and absence of Na^+ using an agonist as radioligand.

Using this approach, we showed that the K_{il}/K_{ih} ratio for various ligands correlated well with the intrinsic activity determined by cyclic AMP measurements.

In the present study, we have used an agonist radioligand and an antagonist radioligand in appropriate assay conditions to study the effect of various mutations on the interaction of 5-HT with the high- and low-affinity conformations of the receptor.

4.2. Reduced number of agonist high-affinity binding sites in the F185A and F185M mutants

Saturation assays with the antagonist [^{3}H]GR125743 labeling the total receptor population in parallel with the agonist [^{3}H]5-HT that selectively labels the coupled conformation showed that in cells expressing the wild-type 5-HT_{1B} receptor, the proportion of receptors in the coupled conformation was 48%. Interestingly, in cells expressing the F185A or F185M mutant receptors, the proportion of receptors in the coupled conformation was considerably reduced, i.e. 13% and 10%, respectively (Table 1). The changed proportion of coupled and uncoupled receptors as compared to the wild-type receptor was observed in different cell clones for each receptor. However, no significant change in the affinity of 5-HT for coupled conformation could be seen (Table 2) and no significant reduction in the 5-HT-induced second messenger response was observed (Table 3). Thus, the reduced number of coupled receptors probably results from an impaired ability of the mutants to adopt the agonist high-affinity conformation. The lack of reduction in second messenger response may be the result of the substantial receptor reserve, often yielded when expressing receptors in cell lines (Adham et al., 1993), compensating for the reduced proportion of receptors in the coupled conformation. A changed ability of mutant receptors to adopt different receptor conformations has also been observed for the tachykinin NK_1 receptor (Ciucci et al., 1998; Riitano et al., 1997; Rosenkilde et al., 1994). In contrast, the affinity of 5-HT for uncoupled conformation of the F185A and F185M mutants was significantly reduced as compared to the wild-type receptor. Accordingly, the K_{il}/K_{ih} ratio was increased (Table 2). However, the 5-HT-induced second messenger response was not increased. The finding, that a K_{il}/K_{ih} ratio higher than the ratio sufficient to obtain full agonism does not correlate with increased intrinsic activity, has previously been observed by Malmberg et al. (1998), suggesting that rate-limiting steps are influencing the correlation between the K_{il}/K_{ih} ratio and intrinsic activity.

4.3. Increased affinity of 5-HT for the uncoupled conformation of the F331A mutant

In previous studies, we found that 5-HT displayed a significantly reduced second messenger response in cells expressing the F331A mutant although the affinity of 5-HT for the F331A mutant was increased as compared to the wild-type receptor (Grånäs et al., 1998a). Thus, we suggested that this amino acid is involved in the conformational changes necessary for signal transduction. In the present study, we show that 5-HT also displays significantly increased affinity for the uncoupled conformation of the mutant receptor as compared to the wild-type receptor (Table 2).

The affinity of 5-HT for the uncoupled conformations of the F331A mutant was increased 11-fold, which results in a 5-fold decrement in the K_{il}/K_{ih} ratio as compared to the wild-type receptor (Table 2 and Fig. 3). Hence, these results show that the reduced intrinsic activity of 5-HT at the F331A mutant correlates with a decreased K_{il}/K_{ih} ratio of 5-HT binding.

4.4. Reduced intrinsic activity of 5-HT at the F354A and F354Y mutants

5-HT also displayed significantly reduced second messenger response in cells expressing the F354A and F354Y mutants as compared to cells expressing the wild-type receptor and the basal cyclic AMP levels were significantly increased in cells expressing the mutants (Table 3). In addition, the affinity of 5-HT for the uncoupled conformations of the F354A and F354Y mutants was significantly increased as compared to the wild-type receptor resulting in increased K_{il}/K_{ih} ratios. These data suggest that phenylalanine 354 is important for the conformational changes necessary for signal transduction. However, the increase in the affinity of 5-HT for uncoupled conformations of the F354A and F354Y mutants was not as pronounced as for the F331A mutant (Table 2).

In conclusion, we show that single amino acid mutations may selectively change the affinity of 5-HT for the uncoupled conformation of the receptor, which results in altered intrinsic activity of the ligand.

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