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Site-directed mutagenesis of the human 5-HT_{1B} receptor

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Abstract

Site-directed mutagenesis was used to investigate the molecular interactions involved in ligand binding to the human 5-HT_{1B} receptor. Six mutants were constructed at four positions and expressed in Chinese hamster ovary cells. Substitution of the amino acid F185 in transmembrane region IV by an alanine increased the affinities of sumatriptan, methysergide and 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) 3–4-fold and substitution by a methionine increased the affinities of methysergide and methiothepin 2- and 3-fold, respectively. Substitution of amino acid S334 in transmembrane region VI by an alanine increased the affinity of 8-OH-DPAT 5-fold. In accordance with this, the EC_{50} value of 8-OH-DPAT was decreased 7-fold. This suggests that the serine at position 334 causes steric hindrance for 8-OH-DPAT binding that is lost in the S334A mutant. Mutation of F354 in transmembrane region VII, which differs between receptor subtypes, increased the affinity of methiothepin 2–3-fold but the affinities of the other compounds tested were essentially unchanged.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); Mutagenesis, site-directed; Methiothepin; Sumatriptan; 5-Carboxamidotryptamine; L-694,247; 5-HT_{1B} receptor

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) has many and diverse effects both in the central and peripheral nervous systems. It influences temperature regulation, sexual behavior, feeding behavior, pain, and cognition (Branchek, 1993; Julius, 1991). Several antidepressant and anxiolytic drugs influence the serotonergic transmission in the brain indicating involvement of the serotonergic system in these psychiatric diseases (Glennon, 1990; Murphy, 1990).

In view of the many and varied effects of 5-HT, the discovery of 13 G protein-coupled 5-HT receptor subtypes in mammals is not surprising (Branchek, 1993). Cloning of these receptors enables sequence comparisons and simplifies pharmacological characterization. The present investigation describes mutagenesis studies of the human 5-HT_{1B} (previously-called the 5-$HT_{1D\beta}$) receptor which inhibits adenylyl cyclase via coupling to G_i proteins (Hamblin and Metcalf, 1991; Weinshank et al., 1992). It is mainly located presynaptically and stimulation of the 5-HT_{1B} receptor inhibits the release of 5-HT (Boschert et al., 1994). Therefore, a selective 5-HT_{1B} receptor antagonist should be able to increase the level of 5-HT in the synaptic cleft. Such a drug could be useful in the treatment of diseases that are thought to be due to too low levels of 5-HT, as for example depression. The serotonergic agonist sumatriptan has antimigraine effects. The drug is shown to contract cerebral blood vessels at the surface of the brain (De Keyser et al., 1993; Hamel et al., 1993; Miller et al., 1992) and prevents the increase in plasma levels of calcitonin gene-related peptide-like material in the external jugular vein, acting through 5-HT_{1B} receptors (Arvieu et al., 1996; Goadsby and Edvinsson, 1993).

Investigations of the ligand-binding interactions of 5-HT receptor subtypes are important to allow the development of selective drugs for various disorders. In order to characterize the molecular interactions involved in ligand binding to the human 5-HT_{1B} receptor, we have used a combination of sequence comparisons, computer-based modelling and site-directed mutagenesis. A three-dimensional model based on the high-resolution structure of bacteriorhodopsin

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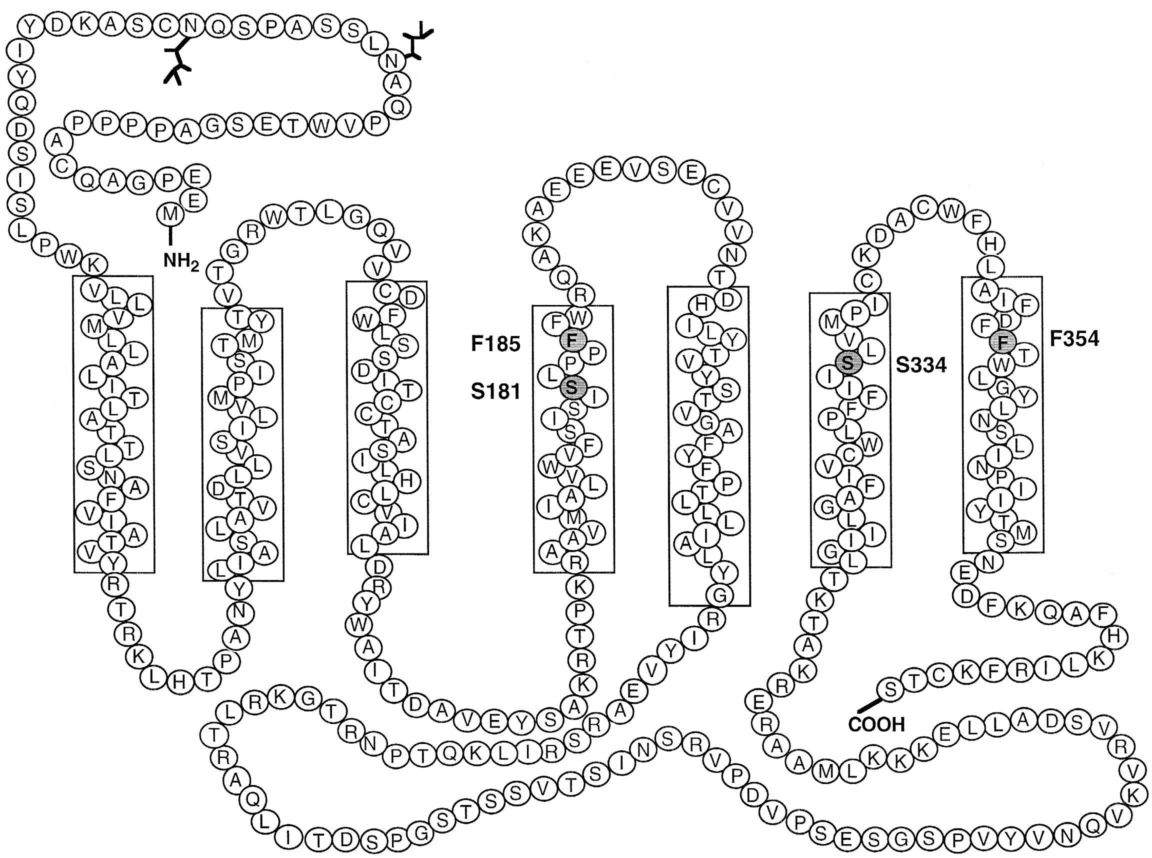

Fig. 1. Schematic representation showing positions of mutagenesis in the human 5-HT_{1B} receptor.

was constructed and used to suggest possible ligand interaction points. In addition, sequence alignments were used to identify amino acids that are invariant between the 5-HT receptor subtypes as well as amino acids that differ between subtypes. Invariant amino acids may play important roles in the overall folding and thereby influence high affinity ligand binding and receptor function. Residues that vary between subtypes may contribute to the differences in affinity to 5-HT and exogenous ligands. Six mutant receptors were generated (see Figs. 1 and 2). We have investigated two polar residues, S181 and S334, located in the transmembrane regions IV and VI (see Fig. 1). We have also mutated two residues, F185 in transmembrane region IV and F354 in transmembrane region VII, which differ between 5-HT_1 receptor subtypes and may contribute to ligand selectivity (see Figs. 1 and 2). The amino acids S181 and S334 were replaced by alanines, F185 was replaced by either alanine or methionine and F354 was replaced by alanine or tyrosine. In two of the mutants, F185M and S334A, the amino acid of the 5-HT_{1A} receptor was introduced (see Fig. 2) in order to investigate if 1A/1B ligand selectivity could be influenced. We report here binding studies on wild-type and mutant 5-HT_{1B} subtypes with different serotonergic ligands.

	TM IV (S181, F185)		TM VI (S334)		TM VII (F354)	
5-HT_{1B}	166	AAVMIALVWVFSISISLPPFF	316	LGIILGAFIVCWLPFFIISLVMPI	350	IFDFFTWLGYLNSLINPIIYTMSN
5-HT_{1D}	155	--T---I--AI--C--I--L-	303	---------I------VV---L--	337	L----T---------------VF-
5-HT_{1A}	153	--AL-S-T-LIGFL--I--ML	347	----M-T--L-------VA--L-F	381	LGAIIN----S---L--V--AYF-
5-HT_{1E}	139	--L--LT--TI--F--M--L-	293	--L------LS------KE-IVGL	325	VA--L-----V------LL--SF-
5-HT_{1F}	140	-GI--TI--II-VF--M--L-	295	--L-----VIC-----VKE--VNV	328	MSN-LA-----------L---IF-
5-HT_{2A}	192	-FLK-IA--TI-VG--M-IPV	325	---VFFL-V-M-C----TNIMAV-	361	LLNV-V-I---S-AV--LV--LF-
5-HT_{2B}	172	-FIK-TV--LI--G-AI-VPI	326	---VFFL-LLM-C----TNITLVL	361	LLEI-V-I--VS-GV--LV--LF-
5-HT_{2C}	171	-IMK--I--AI--GV-V-IPV	313	---VFFV-LIM-C----TNILSVL	349	LLNV-V-I--VC-G---LV--LF-

Fig. 2. Alignment of 5-HT receptor sequences in transmembrane regions IV, VI and VII. Only amino acids that differ from the top 5-HT_{1B} sequence are shown. Dashes mean identities to the top sequence.

2. Materials and methods

2.1. Receptor modelling

The model of the human 5-HT_{1B} receptor was constructed using Sybyl 5.5 (TRIPOS Associates, 1699 S. Hanley Road, Suite 303, St. Louis, MO). The amino acid sequence for the human 5-HT_{1B} receptor was obtained from the published sequence (Demchyshyn et al., 1992). The receptor model of the human 5-HT_{1B} receptor was constructed according to a strategy previously described for the muscarinic m1 receptor using bacteriorhodopsin as a template (Henderson et al., 1990; Nordvall and Hacksell, 1993).

The receptor model was used for the manual docking in Sybyl of several 5-HT_{1B} receptor ligands. The compounds used in the docking were 5-HT, sumatriptan, methysergide, and methiothepin. The aim of the docking study was to identify residues that could be directly involved in the ligand binding. We assume that the aspartic acid in transmembrane region III (D129) is of importance for the binding of agonists as well as antagonists. This is in analogy with other G protein-coupled receptors where the endogenous ligand has a protonated/quaternary nitrogen, e.g., the adrenoceptors and muscarinic receptors (Fong and Strader, 1994). Therefore, D129 (transmembrane region III) served as an anchoring point in the docking with the protonated nitrogen of the ligands interacting via a hydrogen bond-reinforced ionic interaction with one of the oxygens of the aspartate.

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

Six mutants of the human 5-HT_{1B} receptor (Demchyshyn et al., 1992) were constructed using a modified version of the U.S.E. Mutagenesis Kit (Pharmacia P-L Biochemicals). The method is based on the unique site-elimination procedure where a two-primer system is used to generate site-specific mutations (Deng and Nickoloff, 1992). One primer introduces the desired mutation into a known sequence of the cloned gene. A second primer eliminates a unique non-essential restriction enzyme site in the plasmid DNA. This site serves as the basis for elimination of unmutated plasmids from the mutated plasmids through restriction enzyme cleavage. Both primers anneal to the same strand of the denatured plasmid and a new strand of DNA is synthesized containing both mutations, using T4 DNA polymerase. Some changes of the mutagenesis kit were made consisting of alterations of the primer:plasmid ratio, which was raised by 100-fold to improve the annealing efficiency, and an additional cleavage-transformation step was introduced to eliminate unmutated plasmids. The restriction site used for selection was *Xmn*I, since no such sites are present in the 5-HT_{1B} receptor insert. The vector used during mutagenesis was pSP73 (Promega). For constructing the mutants the following oligonucleotide primers were used (bold style = mutant bases): S181A, ATCTCTATC**G**CGCTGCCGCC-CTTCTTC; F185A, CTGCCGCCC**GC**CTTCTG-GCGTCAGGCT; F185M, CTGCCGCCCTT**G**TTCTG-GCGTCAGGCT; S334A, TTC-ATCATC**G**CCCTAGT-GATGCCTATC; F354A, TTTGACTTC**GC**CACATG-GCTGGGCTAT; F354Y, TTTGACTTCT**A**CACATG-GCTGGGCTAT. The mutated receptor genes were, after digestion with the restriction endonucleases *Hin*dIII and *Eco*RV, 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 were confirmed by DNA sequencing. The size of the insert and the nucleotide sequence were also analyzed by PCR and DNA sequencing after subcloning.

2.3. 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 with L-glutamine, without sodium bicarbonate), 10% fetal calf serum, sodium bicarbonate 3.7 g/l, streptomycin 0.05 g/l, penicillin G 0.06 g/l. Prior to transfection cells were washed once with 2 ml Opti-MEM I Reduced Serum Medium. Afterwards, 2 μg DNA was dissolved in Opti-MEM I Reduced Serum Medium to a total volume of 100 μl and was combined with a mixture of 20 μl of LIPOFECTIN Reagent and 80 μl of 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. Then the cells were subcultured at a ratio of 1:5 and grown in media containing geneticin at 0.35 g/l for selection.

2.4. 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 scrape and pooled in serum-free medium and subsequently lysed 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 $\times$ *g* for 10 min. The cell membranes were washed in a binding buffer promoting the binding of ligands to the high-affinity

Table 1
K_d and B_{max} values of [^{3}H]5-HT binding to wild-type and mutant human 5-HT_{1B} receptors

	K_d (nM)	B_{max} (pmol/g protein)
WT	3.1 ± 0.1	287 ± 12
S181A	2.0 ± 0.5	126 ± 15
F185A	2.5 ± 0.3	48 ± 2
F185M	1.7 ± 0.2	45 ± 5
S334A	3.0 ± 0.3	52 ± 8
F354A	1.8 ± .0.1	281 ± 24
F354Y	1.6 ± 0.3	58 ± 8

K_d and B_{max} values were calculated as described in materials and methods. Data are expressed as mean ± S.E.M. from 3–5 independent experiments run in duplicate.

agonist binding site consisting of 50 mM Tris–HCl, 4 mM $MgCl_2$, 120 mM *N*-methyl-D-glucamine, 1 mM EDTA, 10 μM pargyline (pH 7.4) and centrifuged. *N*-methyl-D-glucamine was added to the buffer to compensate for the high-ionic strength that raises from adding Na^+ to a buffer promoting the binding of ligands to the low-affinity agonist binding site (data not shown). The membranes were resuspended in binding 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 using bovine serum albumin as standard (Markwell et al., 1978).

The frozen membranes were thawed, homogenized with a Branson 450 sonifier, and suspended in binding buffer to final concentration of 0.4–2.4 mg protein/ml, depending on the receptor density. The receptor binding assays were initiated by the addition of membranes and carried out in a volume of 0.5–2 ml at 37°C for 1 h. The incubations were terminated by rapid filtration on glass microfibre filters (GF-B, Whatman) 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 ranging from 0.6 to 20 nM, (specific activities 24.5–112 Ci/mmol (Amersham or NEN)). Non-specific binding was determined in the presence of 10 μM methiothepin. Radioligand binding was performed with duplicate samples in at least three independent experiments. Competition studies were done using [^{3}H]5-HT as radioligand. Twelve different concentrations of the competing ligands were used ranging from: 1 mM to 3.1 nM for 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT), and 10 μM to 31 pM for 5-HT, methiothepin, methysergide and sumatriptan, and 1 μM to 3.1 pM for 5-carboxamidotryptamine (5-CT) and 2-[5-[3-(4-methylsulphonylamino) benzyl -1,2,4 - oxadiazol -5-yl] -1 *H*- indol-3-yl]ethanamine (L-694,247). The substances were dissolved and diluted in 0.1% ascorbic acid. 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. The Hill coefficients were calculated for each individual experiment. Student's *t*-test (unpaired) was used for statistical comparisons.

2.5. 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 suspended 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 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). The cell density was determined using a Bürker chamber. 450 μl aliquots of the cell suspension were added to the test tubes containing forskolin (final concentration 10 μM) in all tubes except for measuring the basal cyclic AMP level. Forskolin alone was used to achieve a maximal cyclic AMP level. The final assay

Table 2
Inhibition of [^{3}H]5-HT binding to wild-type and mutant human 5-HT_{1B} receptors

Compound	K_i (nM)						
	WT	S181A	F185A	F185M	S334A	F354A	F354Y
5-HT	3.4 ± 0.5	3.8 ± 0.9	3.1 ± 0.4	4.2 ± 0.9	3.8 ± 0.6	1.9 ± 0.9	2.5 ± 0.3
5-CT	1.5 ± 0.2	1.9 ± 0.7	1.1 ± 0.0[a]	1.1 ± 0.4	0.6 ± 0.1[b]	1.0 ± 0.2	0.9 ± 0.1[a]
Sumatriptan	8.7 ± 0.7	12.3 ± 1.2	2.4 ± 0.7[a]	3.2 ± 1.2	5.8 ± 0.6[a]	8.4 ± 0.1	10.5 ± 2.5
L-694,247	0.20 ± 0.03	0.14 ± 0.03	0.17 ± 0.03	0.34 ± 0.07	0.25 ± 0.07	0.12 ± 0.04	0.42 ± 0.12
Methysergide	18.7 ± 2.6	22.0 ± 0.6	5.5 ± 0.9[b]	9.9 ± 1.1[a]	13.7 ± 3.8	21.1 ± 3.6	18.0 ± 1.0
Methiothepin	6.5 ± 0.1	5.9 ± 0.5	4.6 ± 0.4[a]	2.4 ± 0.5[a]	4.8 ± 0.6	3.2 ± 0.8[a]	2.3 ± 0.2[c]
8-OH-DPAT	747 ± 118		291 ± 22[b]	425 ± 164	151 ± 5[c]		

K_i values were calculated as described in materials and methods. Data are expressed as mean ± S.E.M. from 3–6 independent experiments run in duplicate. a, b or c indicate a K_i value which is significantly different from the K_i value of the wild-type receptor. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$.

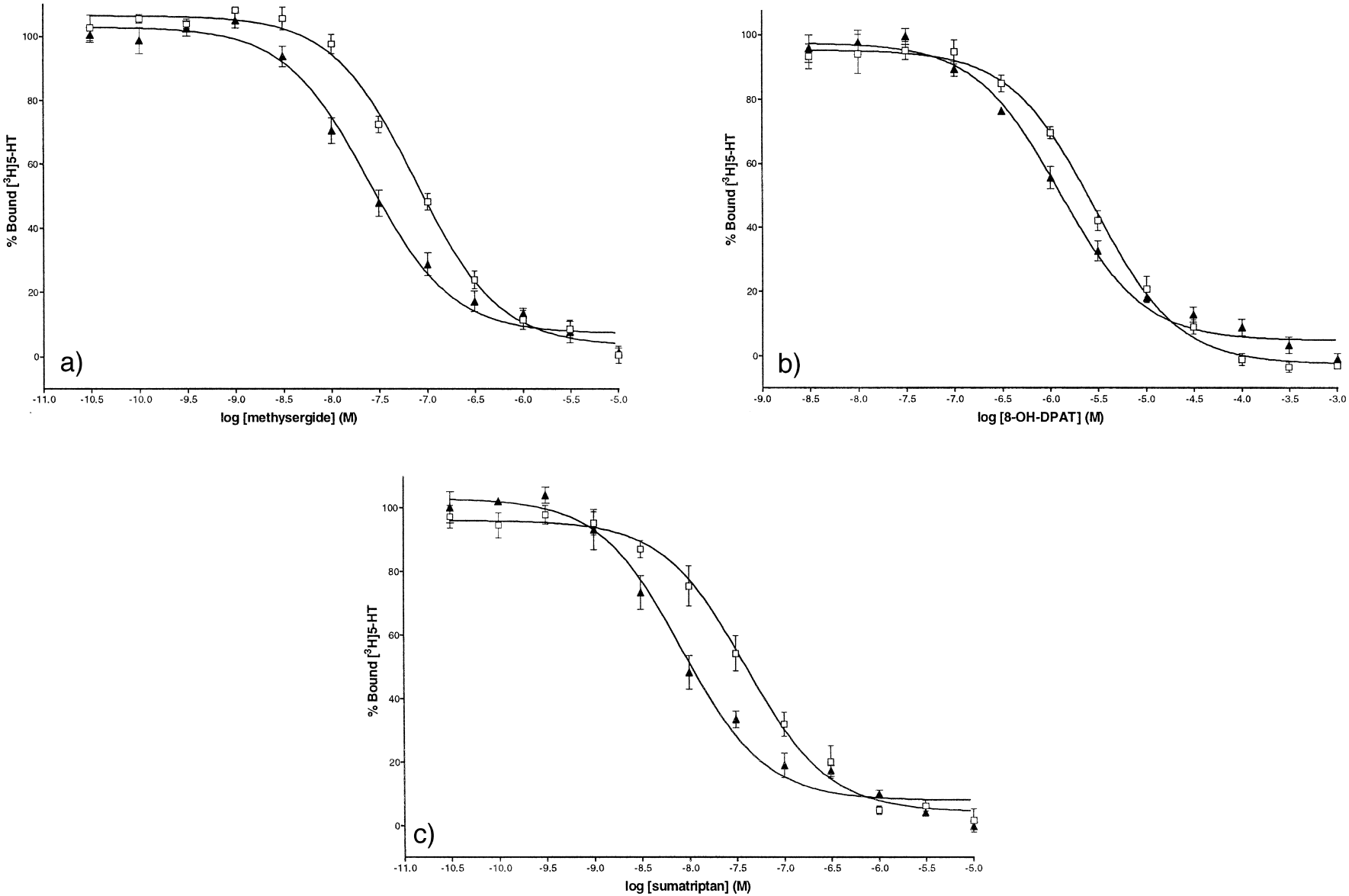

Fig. 3. Inhibition of [^{3}H]5-HT binding by (a) methysergide; (b) 8-OH-DPAT; (c) sumatriptan. □, wild-type receptor; ▲, F185A mutant. K_i values are presented in Table 2.

volume was 500 μl. All samples were done in duplicate and incubated for 20 min at 37°C. Reactions were terminated by adding 50 μl 4.4 M $HClO_4$. The samples were neutralized by adding 100 μl of alkaline neutralization buffer consisting of 5 M KOH and 1 M Tris. The lysates were centrifuged at $9000 \times g$ for 5 min and 50 μl of the supernatant was used for cyclic AMP measurements. Cyclic AMP concentrations were measured using the Cyclic AMP [^{3}H] assay system from Amersham UK and a Packard (1900CA) liquid scintillation counter. The data were analyzed by nonlinear regression using PRISM (GraphPad, San Diego, CA). Student's *t*-test (unpaired) was used for statistical comparisons.

3. Results

3.1. The binding characteristics of [^{3}H]5-HT to wild-type and mutant receptors

Wild-type and mutant receptor plasmid constructs were transfected into CHO-K1 cells and shown to be transcribed by Northern blot analysis (data not shown). Saturation experiments with [^{3}H]5-HT confirmed the expression of receptor proteins. Receptor binding studies were performed in a buffer that favors the conversion of receptors to the high affinity conformation, i.e., in the presence of Mg^{2+} and absence of Na^+. [^{3}H]5-HT bound with high affinity and saturability to wild-type and mutant receptors with K_d values ranging from 1.6 to 3.1 nM (see Table 1). Under the buffer conditions, used 5-HT selectively-labelled the high agonist affinity state of the receptor as represented by a one-site binding curve. The cell-lines expressing the mutant and wild-type receptors displayed B_{max} values ranging from 45 to 287 pmol/g protein (see Table 1).

3.2. The affinities of various ligands for mutant and wild-type receptors

Five 5-HT_{1B} receptor agonists, i.e., 5-HT, 5-carboxamidotryptamine (5-CT), sumatriptan, methysergide, L-694,247, and one antagonist, methiothepin, were tested. In addition, the affinity of the 5-HT_{1A} receptor agonist 8-OH-DPAT was determined for the F185A, F185M, and S334A mutants. The K_i values derived from competition studies of [^{3}H]5-HT binding with human 5-HT_{1B} receptor

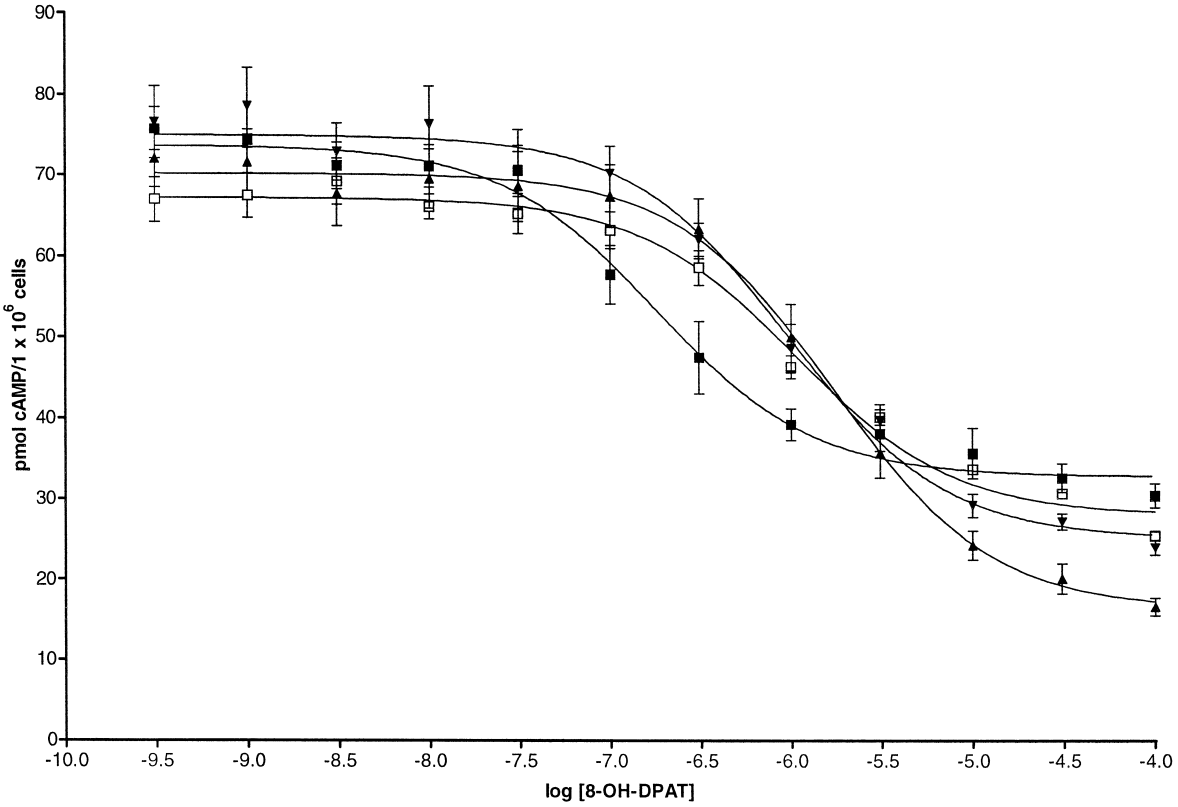

Fig. 4. Concentration-response curves for 8-OH-DPAT-induced inhibition of forskolin-stimulated cyclic AMP accumulation in cells expressing the following receptors: □, wild-type receptor; ▲, F185A mutant; ▼, F185M mutant and ■, S334A mutant. Data are expressed as mean ± S.E.M. from 4 independent experiments run in duplicate. EC_{50} values are presented in Table 3. The basal and forskolin-stimulated cyclic AMP levels, respectively, in cell lines expressing wild-type or mutant receptors were as follows: wild-type, 10 ± 1 and 71 ± 3 pmol/1.0×10^6 cells; F185A mutant, 9 ± 2 and 73 ± 4 pmol/1.0×10^6 cells; F185M mutant, 8 ± 1 and 68 ± 3 pmol/1.0×10^6 cells; S334A mutant, 9 ± 2 and 70 ± 4 pmol/1.0×10^6 cells.

ligands are presented in Table 2. All competition curves were significantly better described by a one-site model than a two-site model. Sumatriptan, methysergide and 8-OH-DPAT displayed a 3–4-fold increase in affinity for the F185A mutant (see Table 2, Fig. 3a–c). Also, 5-CT and methiothepin showed a minor increase in affinity for this mutant. When methionine was introduced at this position, methysergide and methiothepin displayed a 2–3-fold increase in affinity (see Table 2). 8-OH-DPAT displayed a 5-fold increase in affinity for the S334A mutant as compared to the wild-type receptor (see Table 2) and 5-CT displayed a 2.5-fold increase in affinity. Also, sumatriptan showed a minor increase in affinity for this mutant. When we replaced F354 with alanine or tyrosine, the affinity of methiothepin was increased 2–3-fold and a minor increase in the affinity of 5-CT was observed when tyrosine was introduced (see Table 2) while no change in affinity was seen for any of the other compounds tested. No changes in affinities of 5-HT or any of the other compounds were observed for the S181A mutant.

3.3. Effect of mutations on 8-OH-DPAT-induced inhibition of forskolin-stimulated cyclic AMP accumulation

Concentration-response curves for 8-OH-DPAT-induced inhibition of forskolin-stimulated cyclic AMP accumulation were determined in cells expressing the wild-type receptor or the F185A, F185M or S334A mutants, respectively (see Fig. 4). 8-OH-DPAT was 7-fold more potent at the S334A mutant than at the wild-type receptor (see Table 3 and Fig. 4). No significant changes in the potency of 8-OH-DPAT at the F185A or F185M mutants could be observed. The basal and maximal levels of cyclic AMP in the cell lines are shown in legend to Fig. 4. No significant changes in basal or maximal cyclic AMP levels were seen in cells expressing the mutant receptors as compared to in cells expressing the wild-type receptor. None of the mutations markedly altered the efficacy of 8-OH-DPAT. No 8-OH-DPAT-induced inhibition of forskolin-stimulated cyclic AMP accumulation was seen in untransfected cells (data not shown).

Table 3
EC_{50} values for 8-OH-DPAT inhibiting forskolin-stimulated cyclic AMP accumulation in cells expressing wild-type and mutant human 5-HT_{1B} receptors

	EC_{50} (nM)
WT	1098 ± 181
F185A	1730 ± 393
F185M	1034 ± 67
S334A	164 ± 29[a]

EC_{50} values were calculated as described in Section 2. Data are expressed as mean ± S.E.M. from 4 independent experiments run in duplicate. [a]Indicates an EC_{50} value which is significantly different from the EC_{50} value of the wild-type receptor. $P < 0.001$.

4. Discussion

Most models of G protein-coupled receptors suggest that amino acids in transmembrane regions III, V, VI, and VII are the most important for ligand binding (Hibert et al., 1991, 1993; Trumpp et al., 1992). Transmembrane region IV may also be involved in ligand binding but data from the low resolution structure of rhodopsin suggests a somewhat different helical arrangement as compared to bacteriorhodopsin in that transmembrane region IV may be localized more outside of the transmembrane bundle than in bacteriorhodopsin (Baldwin, 1993).

Our 5-HT_{1B} model suggested a spatial clustering of serine and threonine residues contributed by transmembrane region IV (S177, S181), transmembrane region V (T209, S212, T213), and transmembrane region VI (S334). Mutational studies in other G protein-coupled amine receptors, e.g., dopamine receptors and adrenoceptors, have demonstrated that the serine residues in transmembrane region V contribute to the binding of agonists (Cox et al., 1992; Ho et al., 1992; Mansour et al., 1992; Pollock et al., 1992; Strader et al., 1989b). We therefore chose to investigate the two polar residues S181 and S334, located in the adjacent transmembrane regions (IV and VI) (see Fig. 1). S181 in transmembrane region IV is a highly conserved residue in the 5-HT receptors (see Fig. 2), dopamine receptors, and adrenoceptors. In the hamster β-adrenoceptor, this serine residue was mutated to alanine but the mutant receptor protein failed to be expressed (Strader et al., 1989a). The proximity of S181 in transmembrane region IV to S212 in transmembrane region V suggests that it could be involved in a hydrogen bond interaction with either the hydroxyl group of 5-HT or intramolecularly to S212 in transmembrane region V. The mutant S181A was successfully expressed but no changes in affinities of 5-HT or any of the other compounds were observed. This suggests that S181 does not participate in the binding of any of these compounds.

In transmembrane region VI, a serine is present at position 334 in the 5-HT_{1B} receptor and at the corresponding position in the 5-HT_{1D} receptor whereas all other 5-HT_1 subtypes differ (see Fig. 2). Our initial hypothesis was that S334 could be in a position suitable for interaction with the sulphonamide group of sumatriptan. However, after mutating this amino acid to alanine, no decrease in affinity for sumatriptan was seen. Sumatriptan was initially reported as a selective 5-$HT_{1D/B}$ receptor agonist but it has subsequently been shown to possess high affinity also for the 5-HT_{1F} receptor (Adham et al., 1993). The 5-HT_{1F} receptor has a glutamic acid residue in the position corresponding to S334 as does the 5-HT_{1E} receptor for which sumatriptan has low affinity (Gudermann et al., 1993; Zgombick et al., 1992). This is in agreement with our finding that S334 does not contribute to sumatriptan specificity. The corresponding residue in the 5-HT_{1A} receptor is an alanine and the 5-HT_{1A} receptor agonist,

8-OH-DPAT displayed a 5-fold increase in affinity for the mutant. Furthermore, the EC_{50} value of 8-OH-DPAT was decreased 7-fold for the S334A mutant as compared to the wild-type receptor (see Table 3). This suggests that the serine at position 334 causes steric hindrance for 8-OH-DPAT binding which is lost in the S334A mutant. The dopamine D_2 receptor has a histidine at the corresponding position (H394) and this residue has been replaced by a leucine by Woodward et al. (1994). The mutant D_2 receptor showed no changes in affinity for the selective D_2 antagonist [^{3}H]spiperone but displayed both increases and decreases in affinities of certain members of the substituted benzamide class of antagonists. Thus, also the D_2 receptor involves this position in the binding of exogenous ligands.

The phenylalanine at position 185 is only found in the 5-HT_{1B} receptor among 5-HT receptors (see Fig. 2). Because of this and its location in the upper part of the proposed binding pocket, we hypothesized that it might be responsible for some of the unique binding properties of the 5-HT_{1B} receptor (Demchyshyn et al., 1992; Hamblin et al., 1992; Jin et al., 1992; Weinshank et al., 1992). F185 was mutated to methionine, which is the corresponding residue in the 5-HT_{1A} receptor, or to alanine. Methysergide and methiothepin displayed a 2–3-fold increase in affinity for the F185M mutant (see Table 2). When alanine was introduced at this position, sumatriptan, methysergide and 8-OH-DPAT displayed a 3–4-fold increase in affinity (see Table 2). The increase in the affinity of these compounds for the mutant may be explained by an elimination of a blocking interaction when F185 is substituted. However, the increase in affinity of 8-OH-DPAT for the F185A mutant did not result in an increase in potency of 8-OH-DPAT, but rather in a decrease which, however, is not statistically significant.

The phenylalanine at position 354 in transmembrane region VII is not conserved between 5-HT receptor subtypes. The adjacent position 355 differs between the human (threonine) and rat (asparagine) 5-HT_{1B} receptors (Oksenberg et al., 1992). The rat receptor binds β-adrenoceptor antagonists, such as (−)-propranolol with high affinity (Oksenberg et al., 1992). Introduction of an asparagine at this position in the human 5-HT_{1B} as well as the 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors gives β-adrenoceptor antagonists high affinity also for these receptor subtypes, which shows that amino acids in this region are important for receptor selectivity (Adham et al., 1994). The amino acid T355 has been postulated to contribute to the selectivity by an indirect mechanism rather than a direct interaction with sumatriptan (Smolyar and Osman, 1993). When we replaced F354 with alanine or tyrosine, no change in affinity of sumatriptan was seen. Sumatriptan also has a high affinity for the 5-HT_{1F} receptor (Adham et al., 1993) which has very different amino acids in the outermost part of transmembrane region VII compared to the 5-$HT_{1D/B}$ receptors (see Fig. 2). This suggests that sumatriptan is unlikely to derive its receptor subtype selectivity from interactions with amino acids in transmembrane region VII.

In conclusion, these data show that mutagenesis of the phenylalanines F185 and F354 results in minor increments in the affinity of several of the ligands tested. The most pronounced effect is observed when the amino acid S334 is substituted by an alanine which increases the affinity of 8-OH-DPAT 5-fold. In agreement with this, the potency of 8-OH-DPAT was increased 7-fold for this mutant. Surprisingly, mutation of S181 which is highly-conserved, did not alter the receptor binding characteristics of any of the compounds tested. Thus, we have identified one position in the human 5-HT_{1B} receptor (S334) that influences the affinity and second-messenger response of the 5-HT_{1A} receptor agonist 8-OH-DPAT and may contribute to the difference in 8-OH-DPAT affinity between the 5-HT_{1A} and 5-HT_{1B} receptors. The results obtained from the receptor mutants in this study may help to improve the structural models of all five receptors of the 5-HT_1 receptor subfamily.

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