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Identification of an amino acid residue important for binding of methiothepin and sumatriptan to the human 5-HT_{1B} receptor

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Abstract

Site-directed mutagenesis of the human 5-HT_{1B} receptor was performed to investigate the role of the amino acid residues cysteine 326 and tryptophan 327 in transmembrane region VI and aspartic acid 352 in transmembrane region VII in ligand binding. Binding studies were performed with the antagonist radioligand [^{3}H]GR125743 on mutant and wild-type receptors stably expressed in Chinese hamster ovary cells (CHO)-K1 cells. Substitution of tryptophan 327 by alanine resulted in decreased affinities of all ligands tested. The most prominent changes in affinity were observed for the antagonist methiothepin and the antimigraine drug sumatriptan, which were reduced approximately 300- and 60-fold, respectively. Nevertheless, the affinity of 5-HT remained the same. Replacement of the aspartic acid 352 by alanine reduced high-affinity binding of 5-HT. Substitution of cysteine 326 by alanine had minor effects on ligand binding. Some of these results agree with the results from mutagenesis studies of the corresponding amino acids in other receptors. However, some notable differences also emerge showing that functional roles of individual amino acid residues must be tested experimentally in each receptor subtype.

Keywords: 5-HT_{1B} receptor; Site-directed mutagenesis; CGS12066; GR125743; GR127935; GR55562; L-694,247; Methiothepin; 5-Nonyloxytryptamine; Sumatriptan

1. Introduction

The detailed three-dimensional structure of G protein-coupled receptors and the molecular interactions involved in ligand binding and signal transduction are still only partially known. Nevertheless, all G protein-coupled receptors are predicted to share a common topology with seven α-helical transmembrane regions joined by alternating intracellular and extracellular loops (Suryanarayana et al., 1992; Zhou et al., 1994; Liu et al., 1995; Elling and Schwartz, 1996; Mizobe et al., 1996). In the absence of crystallographic data most structural information is derived from site-directed mutagenesis studies and from three-dimensional models based on the high-resolution (3.5 Å) structure of bacteriorhodopsin (Henderson et al., 1990) or the low-resolution (9 Å) structure of bovine rhodopsin (Schertler et al., 1993). Small neurotransmitters bind in the pocket formed by the transmembrane regions and induce conformational changes within the transmembrane bundle, leading to recruitment of signal transducing G proteins which activate intracellular second messenger systems (Strader et al., 1995). The interactions with the G proteins appear to involve the intracellular loops and the cytoplasmic carboxy terminus of the receptor, as demonstrated by various mutational studies (Kobilka et al., 1988; Strader et al., 1989; Lefkowitz et al., 1993). The contribution of conserved amino acids to ligand binding has been found to differ even between closely related receptor subtypes (Cox et al., 1992; Ho et al., 1992; Mansour et al., 1992; Pollock et al., 1992; Grånäs et al., 1998a), therefore investigations of individual receptor subtypes are important to allow development of subtype-specific drugs.

The 5-hydroxytryptamine (5-HT)$_{1B}$ receptor is a member of the large heterogeneous family of 5-HT receptors, which have been divided into seven distinct receptor subclasses (5-HT_1 to 5-HT_7) based upon structural and functional criteria. The 5-HT_1 receptor subclass now comprises five receptor subtypes, i.e., the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors (Hoyer and Martin, 1997). All 5-HT_1 receptors couple to G_i proteins that inhibit

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cyclic AMP production. The closest relative of the 5-HT_{1B} receptor is the 5-HT_{1D} receptor. The human 5-HT_{1D} and 5-HT_{1B} receptors have very similar pharmacological profiles and were previously called 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$, respectively (Weinshank et al., 1992; Hoyer and Martin, 1997).

The 5-HT_{1B} receptor is broadly distributed throughout the central nervous system of humans and several other species. High densities of receptor mRNA are present in cortical areas, caudate–putamen, hippocampus and cerebellum (Bruinvels et al., 1994). The human 5-HT_{1B} receptor has also been detected in cerebral vasculature (Hamel et al., 1993). The antimigraine drug sumatriptan has been shown to contract the cerebral blood vessels at the surface of the brain (Miller et al., 1992; De Keyser et al., 1993; Hamel et al., 1993) and to prevent the increase in plasma levels of calcitonin gene-related peptide-like material in the external jugular vein (Goadsby and Edvinsson, 1993; Arvieu et al., 1996). The 5-HT_{1B} receptor is localized presynaptically on several types of nerve terminals (Engel et al., 1986; Boschert et al., 1994; Bühlen et al., 1996). It has been shown to function both as an auto- and a heteroreceptor, inhibiting the release of 5-HT, acetylcholine, noradrenaline, dopamine and γ-aminobutyric acid (GABA) (Maura and Raiteri, 1986; Göthert et al., 1987; Molderings et al., 1987; Cameron and Williams, 1994; Iyer and Bradberry, 1996). Therefore, the 5-HT_{1B} receptor may be involved in several disorders related to these neurotransmitter systems.

In the present study we have used receptor sequence alignments of the human 5-HT, muscarinic, dopamine, and adrenoceptor subtypes in order to identify highly conserved amino acid residues that may be involved in high-affinity ligand binding and receptor function and we have used site directed mutagenesis to investigate the role of selected amino acid residues in the 5-HT_{1B} receptor. Accordingly, we have mutated the amino acid residue C326 in transmembrane region VI which is conserved in all dopamine receptors, adrenoceptors and in most but not all 5-HT receptors, and W327 in transmembrane region VI which is conserved in all aligned receptors.

In addition, amino acid residues that vary between the closely related 5-HT_1 receptor subtypes and may contribute to the selectivity of ligands have been identified. We have mutated the amino acid residue D352 in transmembrane region VII which is present in the 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_6 and in the dopamine D_1 and D_5 receptor subtypes (see Figs. 1 and 2). The three amino acid

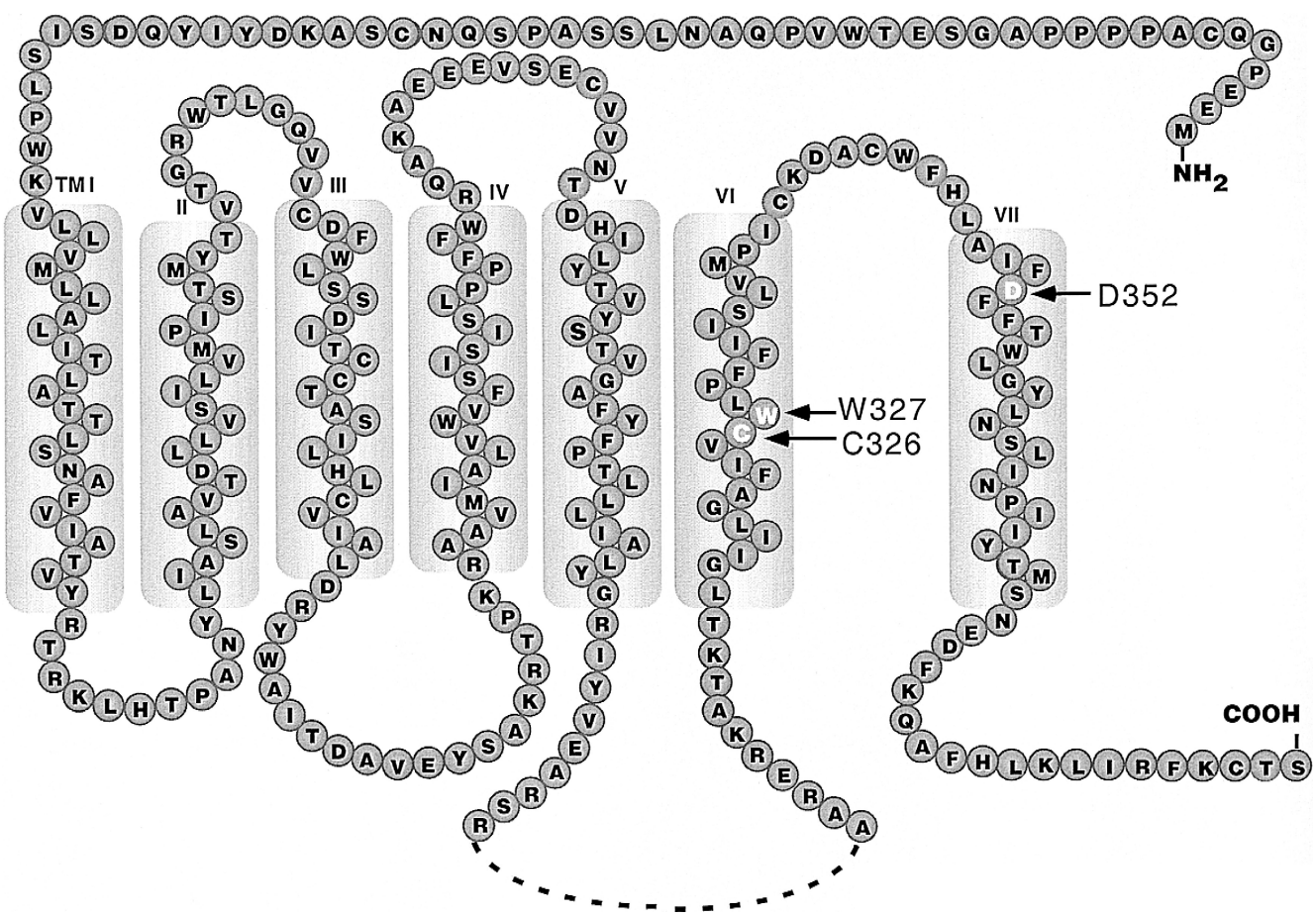

Fig. 1. Schematic representation showing positions of mutagenesis in the human 5-HT_{1B} receptor.

residues were replaced by alanine using site-directed mutagenesis. The role of these amino acids in ligand binding was investigated by radioligand binding studies using the antagonist radioligand [^{3}H]GR125743 which has high affinity for the 5-HT_{1B} and 5-HT_{1D} receptors.

2. Materials and methods

2.1. Materials

The U.S.E. Mutagenesis Kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The Chinese hamster ovary-K1 (dhfr-) cells were purchased from the American Type Culture Collection (Rockville, MD, USA). LIPOFECTIN, Iscove's modified Dulbecco's medium and most other cell culture reagents were purchased from Life Technologies (Gaithersburg, MD,USA). Polyethylenimine and most other chemical reagents were purchased from Sigma (St. Louis, MO, USA). The Ultima gold scintillation fluid was purchased from Packard Instrument (Groningen, The Netherlands). The radioligand [^{3}H]-GR125743 was purchased from Amersham Pharmacia Biotech. 7-Tri-fluoromethyl-4(4-methyl-1-piperazinyl)-pyrolo-(1,2-*a*)quinoxaline (CGS12066B), 2-[5-[3-(4-methylulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-l]ethanamine (L-694,247) and 5-nonyloxytryptamine were purchased from Tocris Cookson (Langford Bristol, UK). *N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)phenyl]-2′-mehyl-4′-(5-methyl-[1,2,4]oxadiazol-3-yl) biphenyl-4-carboamide (GR127935) and 3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide (GR55562) were provided by Glaxo Wellcome (Stevenage, UK). Methiothepin and 5-HT were purchased from RBI (Natick, MA, USA). Graph Pad PRISM version 2.0 was purchased from GraphPad (San Diego, CA).

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

The mutant 5-HT_{1B} receptors were constructed from the wild-type human 5-HT_{1B} receptors cDNA (Demchyshyn et al., 1992) using the U.S.E. Mutagenesis Kit. 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 the 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. 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): C326A, GCCTTTATTGTG**GC**TTGGCTACC-CTTC; W327A, GCCTTTATTGTGTGT**GC**-GCTACC-CTTC; D352A, CCTAGCCATCTTTG**C**CTTCTTCA-CATGG. After mutagenesis the nucleotide sequences of the mutant receptor clones were confirmed by DNA sequencing.

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

The mutated receptor genes were subcloned into the expression vector $pSVD_2$ containing a dihydrofolate reductase gene, after excision of the dopamine D_2 gene, using the restriction endonucleases *Hin*dIII and *Bgl*II. The $pSVD_2$ vector was a kind gift from Dr. P. Sokoloff (INSERM, Paris). After subcloning, the size of the insert

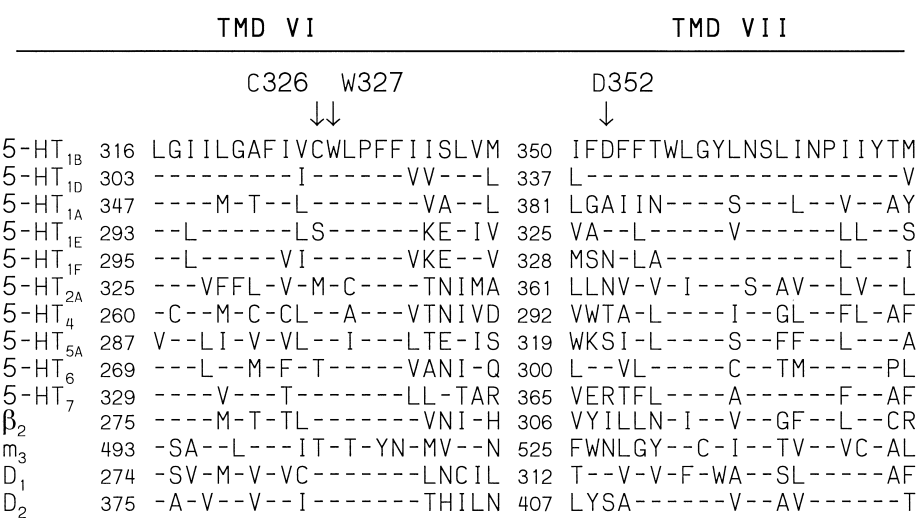

Fig. 2. Alignment of selected human receptor sequences in transmembrane regions VI and VII. Only amino acids that differ from the top 5-HT_{1B} sequence are shown. Dashes mean identities to the top sequence.

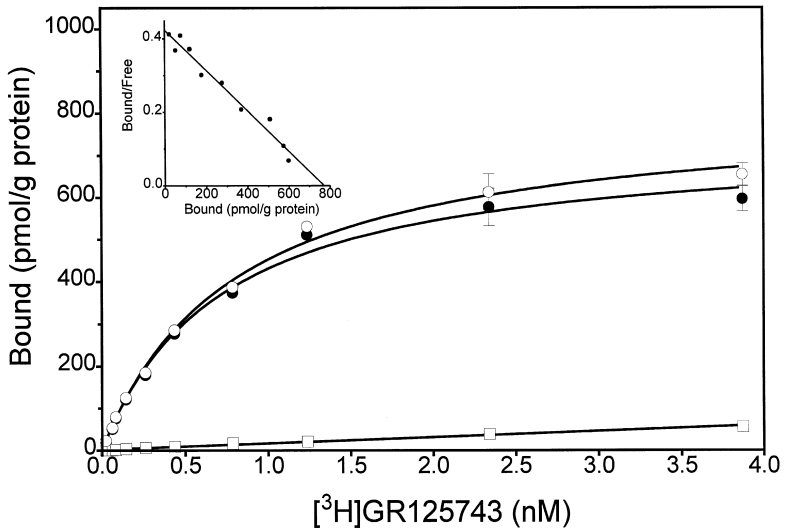

Fig. 3. [^{3}H]GR125743 binding to the h5-HT$_{1B}$ receptor. A representative saturation curve for [^{3}H]GR125743 binding to the h5-HT$_{1B}$ receptors expressed in CHO-K1 cells. Total binding (○). Specific binding (●). Nonspecific binding (□). Insert shows Scatchard plot of the specific [^{3}H]GR125743 binding. The K_d and B_{max} values obtained with the PRISM program (K_d = 0.71 nM, B_{max} = 740 pmol/g protein) were in good agreement with those obtained with the Scatchard analysis (K_d = 0.79 nM, B_{max} = 770 pmol/g protein). Where the standard error is not shown, it is smaller than the size of the symbol.

and the nucleotide sequence was analyzed by polymerase chain reaction and DNA sequencing. Stable cell-lines were produced by transfecting Chinese hamster ovary (CHO)-K1 cells deficient in dihydrofolate reductase using the LIPOFECTIN method (Felgner et al., 1987). Cells were grown to 30–50% confluence on 100-mm tissue culture plates in Iscove's modified Dulbecco's medium with 100 μM hypoxantine, 10 μM thymidine and 10% fetal calf serum. Prior to transfection, cells were washed once with 2-ml Opti-MEM I Reduced Serum Medium, a modification of Eagle's minimum essential medium. Two-microgram DNA was dissolved in 100-μl Opti-MEM I Reduced Serum Medium and was combined with a mixture 20 μl of 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 subcultured at a ratio of 1:5 and grown for selection in a media lacking hypoxantine and thymidine (i.e., Dulbecco's modification of Eagle's medium (DMEM) with 2-mM L-glutamine, 3.7 g/l sodium bicarbonate, 0.05 g/l streptomycin, 0.06 g/l penicillin G, 10% dialyzed fetal calf serum, 20 nM methotrexate and amino acid supplement for DMEM).

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 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 in binding buffer 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. In the experiments where the effects of Na^+ and GTPγS were studied, 120-mM NaCl (instead of 120-mM *N*-methyl-D-glucamine) and 0.1-mM GTPγS were added. 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 sonicator, and suspended in binding buffer to final concentration of 0.2–0.7-mg protein per sample, 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 ml at 30°C for 1 h. The incubations were terminated by

Table 1
K_d and B_{max} values of [^{3}H]GR125743 binding to wild-type and mutant h5-HT$_{1B}$ receptors

	K_d (nM)	B_{max} (pmol/g protein)
WT	0.71 ± 0.16	699 ± 79
C326A	0.38 ± 0.08	128 ± 14
W327A	2.51 ± 0.18[a]	87 ± 9
D352A	1.13 ± 0.52	342 ± 18

The experiments were performed and the K_d and B_{max} values calculated as described in Section 2. Data are expressed as mean ± S.E.M. from 2–3 independent experiments run in duplicate. [a] indicates a K_d value which is significantly different from the K_d value of the wild-type receptor.
[a] $P < 0.05$ (see Section 2.5 for data analysis).

rapid filtration on glass microfibre filters (GF-B, Whatman) pretreated in 0.3% polyethylenimine, 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 for at least 2 h before counting in a Packard (1900CA) liquid scintillation counter. Saturation experiments were done using 12 concentrations of [^{3}H]GR125743 ranging from 0.04 to 10 nM, (specific activities 74–76 Ci/mmol). Nonspecific binding was determined in the presence of 10-μM methiothepin or GR127935 (W327A mutant). The concentration of [^{3}H]GR125743 used in the competition experiments was 3 nM.

2.5. Data analysis

The data was analyzed by nonlinear regression using GraphPad PRISM. The K_d values used to calculate the K_i values were determined under corresponding assay conditions. Hill coefficients were calculated for each individual experiment. To normalize the data before statistical analysis, the individual K_d and K_i values were converted to pK_d and pK_i values. 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.

3. Results

3.1. Binding characteristics of [^{3}H]GR125743 to wild-type and mutant receptors

Fig. 3 shows a representative saturation curve of the antagonist [^{3}H]GR125743 binding to the wild-type 5-HT$_{1B}$

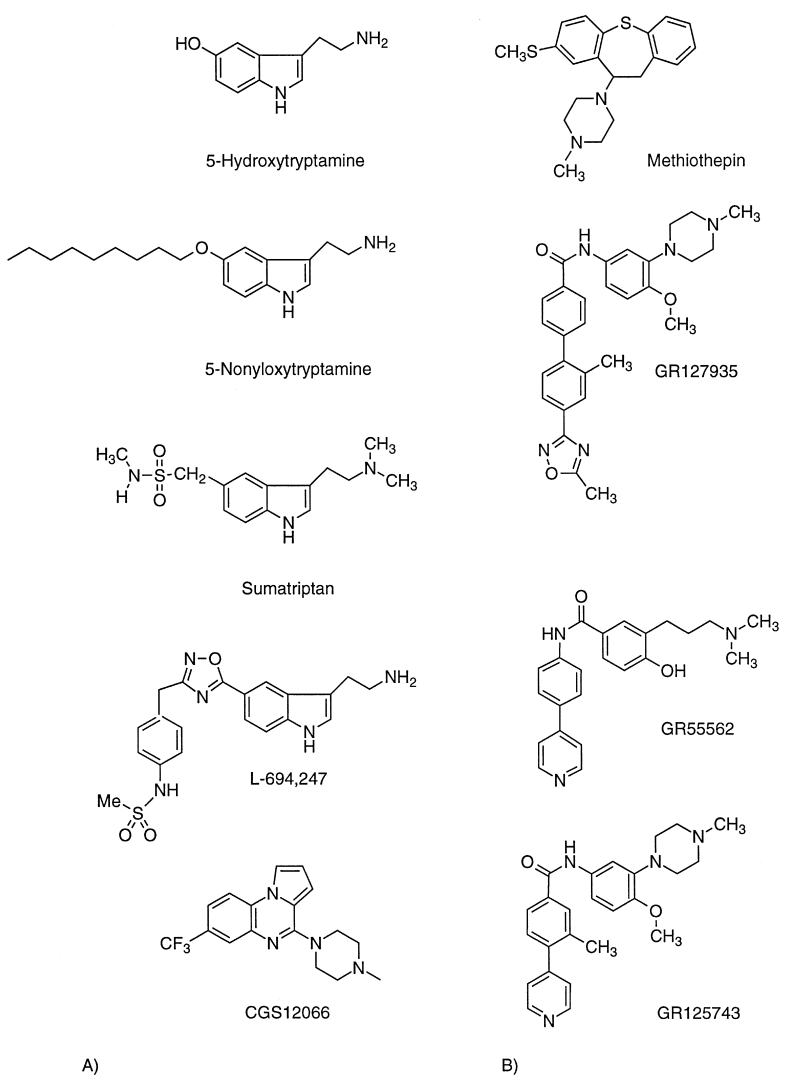

Fig. 4. Structures of (A) the agonists and (B) the antagonists included in the radioligand binding assays.

Table 2
Inhibition of [^{3}H]GR125743 binding to wild-type and mutant h5-HT$_{1B}$ receptors by various agonists and antagonists

	WT	C326A	W327A	D352A
		K_i (nM)		
Agonists				
5-Nonyloxytryptamine	6.7 ± 1.3	1.9 ± 0.2[b]	40.0 ± 6.8[b]	11.4 ± 2.2
Sumatriptan	150 ± 37	38.2 ± 2.8[b]	8417 ± 27[b]	169 ± 79
L-694,247	0.6 ± 0.1	0.2 ± 0.1[a]	4.4 ± 0.5[b]	2.4 ± 0.3[a]
CGS12066	8.0 ± 0.2	7.3 ± 2.2	140 ± 0.4[b]	25.8 ± 2.1[b]
Antagonists				
Methiothepin	14.3 ± 2.7	3.5 ± 0.2[b]	3947 ± 347[b]	7.5 ± 0.6[a]
GR127935	0.7 ± 0.3	0.8 ± 0.1	7.3 ± 1.3[b]	2.2 ± 0.1[a]
GR55562	32.3 ± 7.1	9.4 ± 2.0[a]	118 ± 41[a]	33.9 ± 7.7

The experiments were performed and the K_i values calculated as described in Section 2.5. Data are expressed as mean ± S.E.M. from 2–4 independent experiments run in duplicate. [a] and [b] indicate a K_i value which is significantly different from the K_i value of the wild-type receptor.
[a] $P < 0.05$
[b] $P < 0.01$.

receptor. As can be seen [^{3}H]GR125743 bound with high affinity, saturability and low non-specific binding. The Scatchard analysis of specific [^{3}H]GR125743 binding resulted in a linear plot consistent with a noncooperative, single class of binding sites (Fig. 3 insert).

Table 1 summarizes the results of saturation studies with [^{3}H]GR125743 to the wild-type and the various mutant receptors. The experiments were performed in a buffer that favors the conversion of receptors to the high-affinity agonist binding conformation, i.e., in the presence of Mg^{2+} and absence of Na^+ (Malmberg et al., 1998). [^{3}H]GR125743 bound with high affinity to both the wild-type and the mutant receptors. The K_d values of [^{3}H]GR125743 ranged from 0.38 to 2.51 nM and the B_{max} values from 87 to 699 pmol/g protein. The results in Table 1 further show that the C326A and D352A mutations did not cause any significant change in the affinity of [^{3}H]GR125743 as compared to the wild-type receptor. However, a small but statistically significant reduction in the affinity of the radioligand for the W327A mutant was observed. As expected, the addition of Na^+ and GTPγS did not have any effect on the binding of [^{3}H]GR125743 to the wild-type and mutant 5-HT$_{1B}$ receptors (data not shown). No specific binding could be detected in untransfected cells.

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

In the present study, we have used the antagonist [^{3}H]GR125743 in competition assays to investigate the effect of the alanine mutations on the affinity of several 5-HT$_{1B}$ receptor ligands with varying selectivity. In addition to the agonist 5-hydroxytryptamine, the 5-HT analogues 2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indol-3-yl]ethanamine (L-694,247) and 5-nonyloxytryptamine which have different structures in the 5-position were tested. L-694,247 and 5-nonyloxytryptamine binds with high affinity only to the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors but cannot differentiate between these receptor subtypes (Beer et al., 1993; Glennon et al., 1994). The agonist sumatriptan which is an antimigraine drug that has high affinity for the 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor subtypes (Weinshank et al., 1992; Adham et al., 1993) and a member of another class of agonists, 7-tri-fluoromethyl-4(4-methyl-1-piperazinyl)-pyrolo-(1,2-*a*)quinoxaline (CGS12066B) which has high affinity for 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1A}$ receptors, were also used in the study (Schoeffter and Hoyer, 1989).

In addition, three antagonists that bind with high affinity only to the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors were investigated, i.e., the radioligand (*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-3-methyl-4-(4-pyridyl)benzamide) (GR125743), the unlabeled compounds *N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)phenyl]-2′-methyl-4′-(5-methyl-[1,2,4]oxadiazol-3-yl)biphenyl-4-carboxamide (GR127935) and 3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide (GR55562) (Walsh et al., 1995). The three antagonists GR125743, GR127935 and

Table 3
Inhibition of [^{3}H]GR125743 binding to wild-type and mutant 5-HT$_{1B}$ receptors by 5-HT: effects of Na^+ and GTPγS

	5-HT					5-HT (GTPγS + Na^+)	n_H
	$K_{0.5}/K_i$ (nM)	n_H	K_{ih} (nM)	K_{il} (nM)	R_h (%)	K_i (nM)	
WT	26.7 ± 1.1	0.73 ± 0.05	8.3 ± 2.0	103 ± 31	53 ± 3.4	110 ± 2	0.89 ± 0.08
C326A	25.6 ± 9.3	0.88 ± 0.08	–	–		28.3 ± 0.1[b]	1.03 ± .03
W327A	126 ± 78[a]	0.59 ± 0.01	12.8 ± 4.8	1509 ± 109[b]	54 ± 11	745 ± 59[b]	0.91 ± 0.08
D352A	154 ± 38[b]	0.95 ± 0.02	–	–		268 ± 64[a]	0.90 ± 0.08

The experiments were performed and the K_i values and the Hill coefficients (n_H) calculated and data analyzed as described in Section 2.5. Data are expressed as mean ± S.E.M. from 2–3 independent experiments run in duplicate. [a] and [b] indicate a K_i value which is significantly different from the K_i value of the wild-type receptor.
The $K_{0.5}$ value is an approximation of the K_i value when binding is significantly ($p < 0.05$) better described by a two-site model than by a one-site model. R_h shows the percentage of receptors in the high-affinity state.
[a] $P < 0.05$.
[b] $P < 0.01$.

GR55562 cannot differentiate between 5-HT_{1B} and 5-HT_{1D} receptor subtypes. Thus, the antagonist methiothepin which binds to several receptors but differentiates between 5-HT_{1B} and 5-HT_{1D} receptor subtypes was included in the study (Wilkinson and Middlemiss, 1992). The structures of the ligands and the radioligand GR125743 are shown in Fig. 4.

The results from the competition assays with wild-type and mutant receptors are summarized in Table 2. The experiments were performed in a buffer that favors the agonist high-affinity conformation of the receptor. The competition curves of 5-HT binding to the wild-type receptor and W327A mutant were significantly better described by a two-site than a one-site model ($P < 0.05$), while competition curves with all other ligands were not. The affinities of the various ligands for the wild-type receptor are generally in good agreement with previous studies using agonist radioligands (Weinshank et al., 1992; Beer et al., 1993; Glennon et al., 1994; Doménech et al., 1997; Lamothe et al., 1997; Wurch et al., 1997; Grånäs et al., 1998b). However, the affinity of sumatriptan for the wild-type 5-HT_{1B} receptor in the competition assays with the antagonist radioligand [^{3}H]GR125743 was 17-fold lower than the affinity previously determined in competition studies with the agonist radioligand [^{3}H]5-HT (Grånäs et al., 1998b). The reason is probably that [^{3}H]5-HT selectively labels the high-affinity conformation of the receptor while [^{3}H]GR125743 labels both the high- and low-affinity conformation, even though the affinity of sumatriptan for the various states cannot be discriminated in these competition studies.

The results show that replacement of cysteine 326 with alanine resulted in a slight increase in the affinities of 5-nonyloxytryptamine, sumatriptan, L-694,247, methiothepin and GR55562 as compared to the wild-type receptor. No significant changes in the affinities of 5-HT, CGS12066 and GR127935 were observed. Replacement of tryptophan 327 with alanine resulted in statistically significant decreases in the affinities of all ligands tested. The affinities of 5-HT, 5-nonyloxytryptamine, L-694,247, GR127935 as well as GR55562 were reduced 4–10-fold and the affinity of CGS12066 was reduced 20-fold. The most prominent changes were in the affinities of methiothepin and sumatriptan, which were reduced approximately 300- and 60-fold, respectively. Replacement of aspartate 352 with alanine resulted in a six-fold reduction in the affinity of 5-HT. In addition, slight decreases in the affinities of L-694,247, CGS12066 and GR127935 were observed, whereas the affinity of methiothepin was somewhat increased.

3.3. Effect of Na^+ and GTPγS on 5-HT binding

The 5-HT_{1B} receptor as well as other G protein-coupled receptors display high- and low-affinity conformations for agonists. Agonists bind with high-affinity to the active G protein-coupled conformation and with low-affinity to the inactive G protein-uncoupled conformation. The conformational state of the receptor is regulated by guanine nucleotides and various ions (Kent et al., 1980; Horstman et al., 1990; Neve, 1991; Lahti et al., 1992). Thus, agonists usually display biphasic binding curves with two affinity constants, K_h (high) and K_l (low). The biphasic binding curves become uniphasic in the presence of guanine nucleotides and Na^+, i.e., all receptors uncouple from G proteins. This ''GTP-shift'' has been taken as an indication that the receptors couple to G proteins (De Lean et al., 1980; Kent et al., 1980). In the present study, we tested the effects of Na^+ and GTPγS on the 5-HT binding.

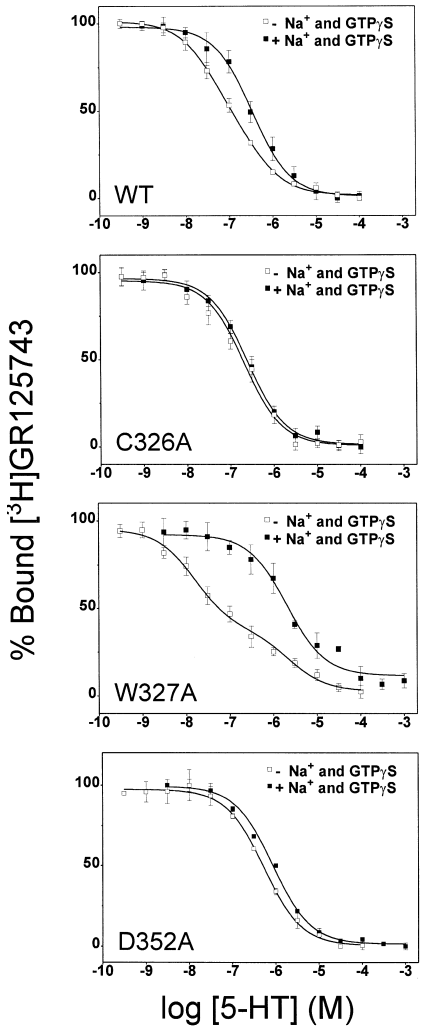

Fig. 5. Representative dose–response curves of 5-HT inhibition of [^{3}H]GR125743 at wild-type and mutant 5-HT_{1B} receptors in the presence of Na^+ and GTPγS (■) and in the absence of Na^+ and GTPγS (□). K_i values are presented in Table 3. Where the standard error is not shown, it is smaller than the size of the symbol.

The competition curves of 5-HT to the wild-type receptor and the W327A mutant in a buffer that favors the conversion of receptors to the high-affinity conformation were best described by a model of two binding-sites with the high-affinity sites corresponding to approximately 50%

of the total binding sites (Table 3 and Fig. 5). The affinities of 5-HT for the high- and low-affinity conformations of the wild-type receptor were 8.3 and 103 nM, respectively, and for the W327A mutant 12.8 and 1509 nM, respectively. In the presence of Na^+ and GTPγS, 5-HT binding to the wild-type receptor and W327A mutant was best described by a one binding-site model and the affinity was in good agreement with the low-affinity value of the two-site fit (Table 3 and Fig. 5). Competition curves of 5-HT to the C326A and D352A mutants were best described by a one binding-site model and no significant effect of Na^+ and GTPγS could be detected (Table 3 and Fig. 5).

4. Discussion

In the present study, we have investigated the involvement of three amino acid residues in transmembrane regions VI and VII of the 5-HT_{1B} receptor in binding of various ligands by site-directed mutagenesis. We have individually replaced the hydrophobic cysteine C326 and the aromatic W327 in transmembrane region VI, as well as the negatively charged aspartate D352 in transmembrane region VII by alanine residues. The mutant receptors were stably expressed in CHO-K1 cells and the binding affinities of several agonists and antagonists for wild-type and mutant receptors were investigated using the antagonist radioligand [^{3}H]GR125743 that identifies the total population of 5-HT_{1B} receptors. For the endogenous agonist 5-HT, radioligand binding experiments were performed in the presence and absence of the nonhydrolyzable GTP analogue, GTPγS.

The cysteine 326 in transmembrane region VI is well, although not perfectly conserved among G protein-coupled receptors that bind 5-HT, dopamine, noradrenaline and acetylcholine. The three 5-HT_2 receptor subtypes have a methionine in the corresponding position, the muscarinic and the 5-HT_6 receptors have a threonine and the 5-HT_{1E} receptor has a serine (Fig. 2). It has been suggested that the cysteine 326 in transmembrane region VI is not involved in disulfide bridging (Dohlman et al., 1990). We report here that substitution of cysteine 326 in the 5-HT_{1B} receptor by alanine resulted in a slight increase in the affinities of several ligands tested (Table 2). The affinity of the endogenous ligand 5-HT remained unchanged. In contrast to the wild-type receptor, 5-HT binding was best described by a one-site model and was not sensitive to the addition of Na^+ and GTPγS (Table 3 and Fig. 5), which indicates that C326 is important for the conformational changes of the receptor that are involved in receptor activation. It has been reported that replacement of the equivalent of C326 in the hamster β_2-adrenoceptor (C285) by serine (Fraser, 1989) or valine (Dohlman et al., 1990) did not affect the affinities of various adrenergic ligands. Substitution by serine reduced the ability of isoproterenol to stimulate cyclic AMP production, although agonist binding was as sensitive to guanine nucleotides as in the wild-type receptor. Furthermore, Gether et al. (1997) analyzed the movements of cysteine residues in the human β_2-adrenoceptor using fluorescence spectroscopy and suggested that the equivalent of C326 in the human β_2-adrenoceptor is involved in the movements of transmembrane region VI upon agonist binding. Thus, our results from the human 5-HT_{1B} receptor together with previous results from the β_2-adrenoceptor suggest that this cysteine residue is important for the conformational changes necessary for receptor activation.

From molecular models of G protein-coupled receptors, it has been predicted that conserved aromatic amino acid residues are involved in ligand binding (Hibert et al., 1991; Edvardsen et al., 1992; Kristiansen et al., 1993; Kristiansen and Dahl, 1996). The highly conserved tryptophan in transmembrane region VI has been shown to be directed towards the hydrophilic binding pocket by the substituted-cysteine accessibility method (Javitch et al., 1998). Replacement of tryptophan 327 in the 5-HT_{1B} receptor with alanine decreased the affinities of all ligands tested. The changes in affinity were most prominent for the antagonist methiothepin and the antimigraine drug sumatriptan, which were reduced approximately 300- and 60-fold, respectively (Table 2). W327 is conserved in all G protein-coupled 5-HT receptors but sumatriptan and methiothepin has low affinity for several of them suggesting that other amino acids also are involved in the binding of these substances. The loss of affinity for the W327A mutant may also be an indirect effect of structural changes in the mutant. The mutation may also be causing structural instability of the receptor protein since the expression level is low as compared to the wild-type receptor (Table 1).

Binding of 5-HT was best described by a two-site model for both the wild-type receptor and the W327A mutant (Table 3 and Fig. 5). The high-affinity binding of 5-HT to the W327A mutant was unchanged and it was sensitive to Na^+ and GTPγS, even though low-affinity binding of 5-HT was reduced 15-fold as compared to the wild-type receptor (Table 3). These results suggest that the structure and function of the receptors expressed are essentially maintained.

The corresponding tryptophan residue in the muscarinic M_3 receptor has been replaced by a phenylalanine (Wess et al., 1993). Agonists and antagonists displayed reduced affinity for this mutant but the mutant receptor nevertheless retained considerable functional activity (phosphatidylinositol hydrolysis) (Wess et al., 1993). The corresponding tryptophan residue in the 5-HT_{2A} receptor has been replaced by an alanine, which resulted in dramatic loss of agonist affinity (the affinity of 5-HT was reduced 900-fold) as well as reduced functional activity (phosphatidylinositol hydrolysis) (Roth et al., 1997). Thus, our results for the 5-HT_{1B} receptor show that the role of W327 in binding of the endogenous ligand seems to differ between receptors.

Site-directed mutagenesis studies in several G protein-coupled receptor classes have shown that aspartate residues are important for ligand binding and receptor activation (Fraser et al., 1988; Strader et al., 1988; Fraser et al., 1989; Ho et al., 1992; Wang et al., 1993; Sealfon et al., 1995). The aspartate 352 is conserved among 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_6, dopamine D_1 and dopamine D_5 receptors (Fig. 2) and has not to our knowledge previously been mutated. We replaced the aspartate 352 in transmembrane region VII of the 5-HT_{1B} receptor by alanine. The affinity of 5-HT for the D352A mutant was reduced 6-fold as compared to the affinity for the wild-type receptor and binding could best be described by a one binding-site model. No significant effect of Na^+ and GTPγS could be observed (Table 3 and Fig. 5). These data suggest that D352 is important for high-affinity binding of 5-HT.

Molecular dynamic simulations in a model of the 5-HT_{2A} receptor have suggested that amino acid residues in transmembrane region VII are important for ligand binding (Zhang and Weinstein, 1993) and previous mutagenesis studies in the 5-HT_{1B} receptor (Oksenberg et al., 1992) and the closely related 5-HT_{1A} receptor (Chanda et al., 1993) showed that amino acids in this region of transmembrane region VII are important for ligand selectivity. Furthermore, replacement of the corresponding amino acid residue in the dopamine D_2 receptor, which is a serine, by an alanine resulted in moderately decreased affinities of several of the agonists (Cox et al., 1992) in agreement with our results for the 5-HT_{1B} receptor.

In conclusion, these results suggest that cysteine 326 in transmembrane region VI of the human 5-HT_{1B} receptor is not crucial for high-affinity binding of any of the ligands tested in this study. Furthermore, our results suggest that W327 in transmembrane region VI is important for binding of several chemically diverse ligands including the antimigraine drug sumatriptan, but is not involved in high-affinity binding of the endogenous ligand 5-HT. In contrast, an equivalent mutation in the 5-HT_{2A} receptor resulted in a dramatic loss in affinity of 5-HT (Wess et al., 1993). Moreover, the amino acid D352 in transmembrane region VII of the 5-HT_{1B} receptor appears to contribute to high-affinity binding of 5-HT. These results emphasize that functional roles of individual amino acid residues cannot be inferred from sequence comparisons between receptor subtypes but must be tested experimentally.

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