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Chimeric Receptor Analysis of the Ketanserin Binding Site in the Human 5-Hydroxytryptamine$_{1D}$ Receptor: Importance of the Second Extracellular Loop and Fifth Transmembrane Domain in Antagonist Binding

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ABSTRACT

The 5-hydroxytryptamine (5-HT)$_{1B/1D}$ receptor subtypes are involved in the regulation of 5-HT release and have gained particular interest because of their apparent role in migraine. Although selective antagonists for both receptor subtypes recently have been developed, the receptor domains involved in the pharmacological specificity of these antagonists are defined poorly. This was investigated with a chimeric 5-HT$_{1B/1D}$ receptor analysis and using ketanserin as a selective antagonist of h5-HT$_{1D}$ (h5-HT$_{1D}$) K_i = 24–27 nM) as opposed to h5-HT$_{1B}$ (K_i = 2193–2902 nM) receptors. A domain of the h5-HT$_{1D}$ receptor encompassing the second extracellular loop and the fifth transmembrane domain is necessary and sufficient to promote higher affinity binding (K_i = 65–115 nM) for ketanserin to the h5-HT$_{1B}$ receptor. The same domain of the h5-HT$_{1B}$ receptor, when exchanged in the h5-HT$_{1D}$ receptor, abolished high affinity binding of ketanserin (K_i = 364-1265 nM). A similar observation was made with the antagonist ritanserin and seems specific because besides the unmodified binding affinities for 5-HT and zolmitriptan, only minor modifications (2–4-fold) were observed for the agonists L 694247 and sumatriptan and the antagonists GR 127935 and SB 224289. Generating point mutations of divergent amino acids compared with the h5-HT$_{1B}$ receptor did not demonstrate a smaller peptide region related to a significant modification of ketanserin binding. The antagonists ketanserin and ritanserin are likely to bind the h5-HT$_{1D}$ receptor by its second extracellular loop, near the exofacial surface of the fifth transmembrane domain, or both.

Dysregulation of serotonin receptor function may contribute to various peripheral and central nervous system disorders (Glennon and Westkaemper, 1993). The 5-HT$_{1B/1D}$ receptor subtypes are, among other 5-HT receptors, involved in the regulation of 5-HT release and have gained particular interest because of their potential role in migraine, depression, and diseases involving the basal ganglia (Hoyer *et al.*, 1994). These receptors may serve a presynaptic autoreceptor function inasmuch as their activation acts to inhibit 5-HT release (Middlemiss *et al.*, 1988; Hamblin *et al.*, 1992). They also seem to function as heteroreceptors as indicated by studies of nonserotonergic neurons in which 5-HT inhibits the release of acetylcholine, glutamate, dopamine, norepinephrine, and γ-aminobutyric acid (Hen, 1992). Similarly, 5-HT$_{1B/1D}$ receptors have been suggested to inhibit peptide release from trigeminal nerve endings in the dura mater (Buzzi *et al.*, 1991). The precise function of each receptor subtype is still controversial. Available data favor the view that vasoconstriction is mediated primarily by 5-HT$_{1B}$ receptors, whereas neuroinhibition in the trigeminovascular system involves predominantly the 5-HT$_{1D}$ receptor subtype (Longmore *et al.*, 1997). It can be put forward that the expression of the 5-HT$_{1D}$ receptor is less abundant than the 5-HT$_{1B}$ receptor. Molderings *et al.* (1996) suggested norepinephrine release to be inhibited by 5-HT$_{1D}$ receptors located on the noradrenergic axon terminals in human atrial appendages. Regulation of [^{3}H]5-HT release in raphé nuclei of 5-HT$_{1B}$ receptor gene knockout mice seems to be mediated by a 5-HT$_{1D}$-like receptor (Piñeyro *et al.*, 1995). In these mice, 5-HT$_{1B}$, but not 5-HT$_{1D}$, autoreceptors inhibit 5-HT release at nerve terminals located in the frontal cortex and ventral hippocampus (Trillat *et al.*, 1997).

The h5-HT$_{1B}$ and h5-HT$_{1D}$ receptor subtypes show a relatively low (63%) overall amino acid identity with 77% identity within the TMDs (Weinshank *et al.*, 1992). TMD I is most divergent (59% identity), whereas the six other TMDs share between 71% (TMD V) to 96% (TMD III) amino acid identity. Notwithstanding this low homology, the h5-HT$_{1D}$ and h5-HT$_{1B}$ receptor subtypes first were reported to display similar binding profiles (Weinshank *et al.*, 1992). Both receptor sub-

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); 5-CT, 5-carboxamidotryptamine; TMD, transmembrane domain; ECL, extracellular loop; PCR, polymerase chain reaction; WT, wild-type; h, human.

types can be pharmacologically differentiated using the 5-HT$_2$ receptor antagonists ketanserin and ritanserin (Peroutka, 1994; Pauwels *et al.*, 1996). Both compounds show potent binding affinity for and are silent antagonists at cloned 5-HT$_{1D}$ receptors of human, rat, and guinea pig (Wurch *et al.*, 1997b). Surprisingly, they show micromolar affinity for canine 5-HT$_{1D}$ receptors (Zgombick *et al.*, 1997). A series of benzanilides, such as GR 125743 (*N*-[4-methoxy-3-(4-methyl-piperazin-1-yl)phenyl]3-methyl-4-(4-pyridyl)-benzamide) and GR 127935 (*N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5- methyl-1,2,4-oxadiazol-3-yl)[1,1′-biphenyl]-4-carboxamide), have been reported as examples of mixed 5-HT$_{1B/1D}$ receptor antagonists (Clitherow *et al.*, 1994). However, they also display agonist properties both *in vitro* and *in vivo* (Pauwels, 1997). Recently, some antagonists have been communicated as selective for 5-HT$_{1B}$ receptors [SB 216641 (*N*-[3-(2-dimethylamino) ethoxy-4-methoxyphenyl]2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-(1,1′-biphenyl)-4-carboxamide) (Price *et al.*, 1997) and SB 224289 (1′-methyl-5-(2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl)-2,3,6,7-tetrahydrospiro [furo[2,3*f*]indole-3,4′-piperidine) (Roberts *et al.*, 1997)] and for 5-HT$_{1D}$ receptors [BRL-15572 (3-[4-(3-chlorophenyl)piperazin-1-yl]1,1-diphenyl-2-propanol) (Price *et al.*, 1997)]. Hence, several ligands are available that distinguish between 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes.

We are interested in the ligand binding divergence between h5-HT$_{1D}$ and h5-HT$_{1B}$ receptors and the molecular correlates that underlie their pharmacological specificity. Therefore, a study was undertaken to identify the domain or domains of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors that determine ligand binding specificity. This was performed using a chimeric receptor approach by combining different parts of h5-HT$_{1D}$ and h5-HT$_{1B}$ receptors. The binding profile of the various chimeric receptors was determined on transient expression in Cos-7 cells with two different radioligands (i.e., the agonist [^{3}H]5-CT and the putative antagonist [^{3}H]GR 125743). The current report summarizes our findings on the selective interaction of ketanserin and ritanserin with a 5-HT$_{1D}$ receptor domain restricted to the second ECL and the fifth TMD.

Experimental Procedures

Construction of chimeric 5-HT$_{1D/1B}$ receptors and point-mutated 5-HT$_{1D}$ receptors. Chimeric 5-HT$_{1D/1B}$ receptors and point-mutated 5-HT$_{1D}$ receptors were constructed by a modified PCR-based overlap extension technique. It allows the construction of chimeric receptors without generating a frame-shift or insertion or deletion of amino acids. Briefly, each chimeric receptor is realized by a three-step PCR-based method that allows the fusion of two or three PCR fragments corresponding to the respective segments that represent the chimeric receptor. The first PCR step corresponds to the amplification of the different fragments of the chimeric receptor, which will be fused together in a second PCR step. A third PCR step amplifies the obtained fusion product. The corresponding PCR-primers were designed according to the reported h5-HT$_{1B}$ and h5-HT$_{1D}$ receptor gene sequences (Weinshank *et al.*, 1992) such that they possess a 5′ extension that is complementary to the adjacent PCR fragment that has to be fused. These primer sequences are listed in Table 1. For each series of primers, the first PCR mixture (50 μl each) contained 10 ng of purified full-length receptor gene fragment, 25 μM concentration of each dNTP, 400 nM concentration of each primer, and 1.25 units of *Taq* DNA polymerase in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris·HCl, pH 8.3). The PCR program consisted of 10 repetitive cycles with a strand separation step at 94° for 30 sec, an annealing step at 58° for 1, min and an elongation step for 1 min at 72°. The PCR products were separated by 2% agarose gel electrophoresis and purified using a Geneclean II kit. For the second PCR step, an equimolecular amount of each fragment (~10 nmol

TABLE 1

Primers used for the construction of chimeric 5-HT$_{1D/1B}$ receptor genes

The sequence of the PCR primers located at the junctions of the receptor domains in the chimeric 5-HT$_{1D/1B}$ receptors is shown. The underlined portion of the primers corresponds to the nonhomologous regions necessary for the fusion of the PCR products as detailed in the text. The 5-HT$_{1D/1B}$ receptor splice sites are indicated: the sequence in bold corresponds to the amino acids from the h5-HT$_{1B}$ receptor.

Chimer	PCR primer	Upstream splice site	Downstream splice site
D/BI-IV	P1 CCAGGCAGTACCTTGAGCGCCTGGAGGGTCCT P2 GGCGCTCAAGGTACTGCTGGTTATGCTATTGGCGC P3 CCTTGGCCTGACGCCAGAAGAAGGGCGGCAGCGAG P4 CTTCTGGCGTCAGGCCAAGGCCCAGGAGGAGATGT	LQALK**VLLVM**	**PFFWR**QAKAQ
D/BI-V	P1 and P2 P5 GGTAGATCCGGCCATAGAGGGCGATGAGGAGCAGGGT P6 CCTCTATGGCCGGATCTACCGGGCTGCCCGGAACCGCA	LQALK**VLLVM**	**IALYG**RIYRA
D/BVI-VII	P7 CTTCTGGCGTCAGGCCAAGGCCCAGGAGGAGATGTCG P8 CCTATATGGCCGCATCTACGTAGAAGCCCGCTCCCGG	IILYG**RIYVE**	Carboxyl-terminal end of the h5-HT$_{1B}$ receptor
D/B2ECL+V	P9 CCTTAGCCTGCCGCCAGAAGAGCGGGGGGATGGA P10 CTTCTGGCGGCAGGCTAAGGCCGAAGAGGAGGTG P5 and P6	PFFWR**QAKAE**	**IALYG**RIYRA
B/D2ECL+V	P11 CCTTGGCCTGACGCCAGAAGAAGGGCGGCAGCGAGAT P12 CTTCTGGCGTCAGGCCAAGGCCCAGGAGGAGATGTCG P13 CGTAGATGCGGCCATATAGGATGATGAGCAACACCGA P14 CCTATATGGCCGCATCTACGTAGAAGCCCGCTCCCGG	**PLFRW**QAKAQ	IILYG**RIYVE**
D/BV	P15 CGTGTAGAGGATCTGAGAGGTGTTCACCAGACAGTCCG P16 GAACACCTCTCAGATCCTCTACACGGTCTACTCCACGGT P5 and P6	VNTSQ**ILYTV**	**IALYG**RIYRA
B/DV	P17 TGTAGGAGATGTGGTCGGTGTTCACCACGCATTCCGA P18 CACCGACCACATCTCCTACACCATCTACTCCACCTGTGG P13 and P14	**VNTDH**ISYTI	IILYG**RIYVE**
D/B2ECL	P9 and P10 P19 GTAGGAGATGTGGTCGGTGTTCACCACGCATTCCGA P20 ACCGACCACATCTCCTACACCATCTACTCCACCTGT	PFFWR**QAKAE**	**VNTDH**ISYTI

each) was mixed with 50 μM concentration of each dNTP and 2.5 units of *Taq* DNA polymerase in the absence of additional primers (each DNA strand serves as a primer) in PCR buffer. The PCR program consisted of 10 repetitive cycles with a denaturation step at 94° for 30 sec, an annealing step at 50° for 25 min (to improve base-pairing between the complementary ends of each fragment that must be fused), and an elongation step for 5 min at 72°. The fusion product was subsequently amplified: 0.5 or 5 μl of the PCR reaction was mixed with 25 μM concentration of each dNTP, 400 nM concentration of primers located at the start and stop codons of the h5-HT_{1B} or h5-HT_{1D} receptor gene sequences (Weinshank *et al.*, 1992), and 1.25 units of *Taq* DNA polymerase in PCR buffer. The PCR program was identical to that of the first PCR reaction, except that the elongation step was prolonged to 1.5 min at 72°. The PCR products were separated and purified as described above before ligation into 50 ng of a pCR3.1 expression vector. Sequencing was performed manually on denatured double-stranded plasmid DNA using a Sequenase quick-denature sequencing kit.

Chimeric receptor nomenclature. Extramembrane loops and TMDs were determined according to the hydrophobicity plots of the h5-HT_{1D} and h5-HT_{1B} receptors (Weinshank *et al.*, 1992). Constructs were defined by two letters: the first letter (D for h5-HT_{1D} or B for h5-HT_{1B} receptor) corresponds to the receptor that represents the majority of the chimeric receptor, and the second letter denotes the exchanged TMD of the second receptor indicated by a roman number, the exchanged ECL indicated by an arabic number, or both.

Expression of receptor genes. Ten micrograms of plasmid was used to transfect 5×10^6 Cos-7 cells by electroporation using a BioRad (Hercules, CA) gene pulser apparatus at 250 mV and 250 μF. Cells were grown for 48 hr in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Each transfection experiment with chimeric receptor plasmids was performed in parallel with WT h5-HT_{1D} and h5-HT_{1B} receptor plasmids.

Radioligand binding experiments to membrane preparations. Membrane preparations of Cos-7 cells were prepared in 50 mM Tris·HCl, pH 7.7, containing 4 mM $CaCl_2$, 10 μM pargyline, and 0.1% ascorbic acid as described previously (Pauwels *et al.*, 1996). Binding assays were performed with either 3.0 nM [^{3}H]5-CT or 1.0 nM [^{3}H]GR 125743. Incubation mixtures consisted of 0.4 ml of cell membranes, 0.05 ml of radioligand, and 0.05 ml of compound for inhibition or 10 μM 5-HT to determine nonspecific binding. The reactions were stopped after a 30-min incubation at 25° by adding 3.0 ml of ice-cold 50 mM Tris·HCl, pH 7.7, and rapid filtration over Whatman GF/B glass-fiber filters with a Brandel (Montreal, Quebec, Canada) harvester, washed, and counted as described previously (Pauwels *et al.*, 1996). In the case of [^{3}H]GR 125743, 50 mM Tris·HCl, pH 7.7, was used, and filtration was performed over 0.2% polyethyleneimine-treated Whatman (Clifton, NJ) GF/B glass-fiber filters. Data were analyzed graphically with inhibition curves, and IC_{50} values (concentration of the compounds producing 50% inhibition of specific binding) were derived. K_i values were calculated according to the equation $K_i = IC_{50}/(1 + C/K_D)$, where C is concentration and K_D is the equilibrium dissociation constant of the radioligand. For ligand saturation binding curves, [^{3}H]5-CT and [^{3}H]GR 125743 were used at concentrations of 0.07–20 nM and 0.15–10 nM, respectively. The curves were analyzed by the nonlinear least-squares curve-fitting program Ligand (Biosoft, Cambridge, UK). Control binding experiments were run with nontransfected cells and did not display specific [^{3}H]5-CT or [^{3}H]GR 125743 binding.

Membrane protein levels were estimated with a dye-binding assay using the BioRad protein assay kit and bovine serum albumin as a standard.

Statistical analysis. Statistical analysis was performed on the K_i values of the chimeric 5-$HT_{1D/1B}$ receptors versus the K_i values of their respective WT receptors using a Student's *t* test.

Materials. Oligonucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer (Foster City, USA). Cos-7 cells were from American Type Culture Collection (Rockville, MD). The pCR3.1 vector was from InVitrogen (San Diego, CA). The Geneclean II kit was purchased from Bio 101 (Vista, CA). *Taq* DNA polymerase and dNTP were from Gibco-Life Technologies (Paisley, UK). The Sequenase quick denature sequencing kit was from Amersham (Les Ulis, France). The protein assay kit was from BioRad Laboratories. [^{3}H]5-CT (61.3 Ci/mmol) and [^{3}H]GR 125743 (69 Ci/mmol) were obtained from New England Nuclear (Les Ulis, France) and Amersham (Les Ulis, France), respectively. 5-HT and ketanserin were from Sigma Chemical (St. Louis, MO). Ritanserin was obtained from RBI (Natick, MA). L 694247 (2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1*H*-indol-3-yl]ethanamine) was purchased from Tocris Cookson (Bristol, UK). Zolmitriptan ([4(*S*)-[3- [2-(dimethylamino)ethyl]-1*H*-indol-5-yl-methyl]oxazolidin-2-one]), sumatriptan, GR 127935, and SB224289 were synthesized at the Center de Recherche Pierre Fabre. Stock solutions (1 mM) of compounds were prepared in water, ethanol, or dimethylsulfoxide.

Results

Construction and expression of chimeric 5-$HT_{1D/1B}$ receptors compared with WT h5-HT_{1B} and h5-HT_{1D} receptors in Cos-7 cells. In a first approach, different parts of the WT h5-HT_{1D} and WT h5-HT_{1B} receptor were combined and evaluated for expression of [^{3}H]5-CT and [^{3}H]GR 125743 binding compared with their parental receptors on transient expression in Cos-7 cells. Fig. 1 shows a schematic representation of eight chimeric 5-$HT_{1D/1B}$ receptors being investigated. The PCR primers designed at the junction of selected TMD sequences (TMDs I, IV, and V) are indicated for each chimeric receptor in Table 1. Sequencing of each chimeric receptor demonstrated full identity with the predicted chimera sequence. Each of these chimeric receptors displayed specific binding for [^{3}H]5-CT and [^{3}H]GR 125743 as observed with the WT h5-HT_{1B} and h5-HT_{1D} receptors. With the exception of some variation in the K_D values for [^{3}H]5-CT of the chimeric receptors D/BV and B/DV, very similar values were obtained compared with the WT h5-HT_{1B} and h5-HT_{1D} receptors. The K_D values for [^{3}H]GR 125743 ranged between 0.54 and 2.38 nM, in agreement with those obtained for the WT h5-HT_{1B} (0.65 nM) and h5-HT_{1D} (1.22 nM) receptors (Fig. 1). Maximal expression levels of [^{3}H]5-CT and [^{3}H]GR 125743 binding for each of the chimeric receptors were in the same range as for the WT h5-HT_{1B} and h5-HT_{1D} receptors: 0.51–2.17 and 0.22–2.67 pmol/mg protein, respectively.

Inhibition of [^{3}H]5-CT and [^{3}H]GR 125743 binding to WT h5-HT_{1D} and h5-HT_{1B} receptors and chimeric 5-$HT_{1D/1B}$ receptors by 5-HT and ketanserin. Table 2 summarizes the affinity constants of 5-HT and ketanserin for WT h5-HT_{1D} and h5-HT_{1B} receptors and eight chimeric 5-$HT_{1D/1B}$ receptors. 5-HT displaced [^{3}H]5-CT binding with a similar affinity for both WT h5-HT_{1D} and 5-HT_{1B} receptors. 5-HT exhibited an 8-fold selectivity for the WT h5-HT_{1D} over the WT h5-HT_{1B} receptor when [^{3}H]GR 125743 was used as a radioligand. The 5-HT_{2A} receptor antagonist ketanserin yielded an affinity of 24–27 nM for the WT h5-HT_{1D} receptor and a low binding affinity (K_i = 2193 to 2902 nM) for the WT h5-HT_{1B} receptor, regardless of whether [^{3}H]5-CT or [^{3}H]GR 125743 was used as a radioligand. Replacement of TMDs I–IV of the WT h5-HT_{1D} receptor by the equivalent amino acid region of the WT h5-HT_{1B} receptor (chimera D/BI-IV, Fig. 1) did not modify ($p > 0.05$) the binding affinity of ketanserin compared with the WT h5-HT_{1D} receptor. The 5-HT binding affinity was not modified for the D/BI-IV chi-

mera when [^{3}H]5-CT was used as a radioligand, but a 4-fold decrease ($p < 0.001$) in binding affinity was measured with [^{3}H]GR 125743. The addition of TMD V (chimera D/BI-V) promoted a low affinity for ketanserin (K_i = 3,428–>10,000 nM), being statistically similar to that of the WT h5-HT$_{1B}$ receptor when [^{3}H]GR 125743 was used as a radioligand. Otherwise, the binding affinity of 5-HT was similar to the WT h5-HT$_{1D}$ receptor when measured with [^{3}H]5-CT but significantly decreased (2.5-fold, $p < 0.01$) when analyzed with [^{3}H]GR 125743. Exchange of TMDs VI and VII of the h5-HT$_{1B}$ receptor into the h5-HT$_{1D}$ receptor (chimera D/BVI-VII) did not modify ($p > 0.05$ versus the WT h5-HT$_{1D}$ receptor) the binding affinities of ketanserin and 5-HT. Ketanserin showed a significantly lower binding affinity (15–46-fold, $p < 0.001$ versus the WT h5-HT$_{1D}$ receptor) in the chimera D/B2ECL+V when only the second ECL and the fifth TMD of the h5-HT$_{1D}$ receptor were exchanged by the equivalent amino acid domain of the h5-HT$_{1B}$ receptor. The affinity of 5-HT remained unmodified ($p > 0.05$) compared with the WT h5-HT$_{1D}$ receptor by using both radioligands. The reverse chimera (B/D2ECL+V) promoted a 25–34-fold higher affinity ($p < 0.01$ versus the WT h5-HT$_{1B}$ receptor) for ketanserin but still differed (2–5-fold, $p < 0.01$) from that of the WT h5-HT$_{1D}$ receptor. The affinity of the chimeric receptor B/D2ECL+V for 5-HT was not affected ($p > 0.05$ versus the WT h5-HT$_{1B}$ receptor).

Hence, the fifth TMD and the second ECL seemed to be necessary to yield high affinity for ketanserin. Further analysis demonstrated that the exchange of the fifth TMD without the second ECL of the h5-HT$_{1D}$ receptor by its h5-HT$_{1B}$

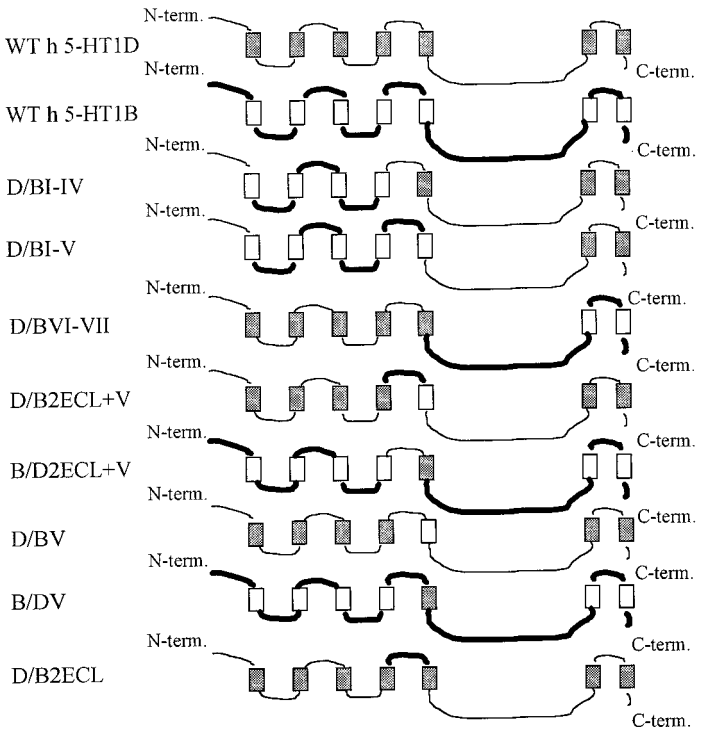

Fig. 1. Schematic representation of the WT h5-HT$_{1D}$, WT h5-HT$_{1B}$, and chimeric 5-HT$_{1D/1B}$ receptors. *Shaded rectangles*, TMDs derived from the h5-HT$_{1D}$ receptor. *Open rectangles*, TMDs derived from the h5-HT$_{1B}$ receptor. Extramembrane regions are drawn as either a thin (h 5-HT$_{1D}$) or a thick (h5-HT$_{1B}$) line. *N-term. and C-term.*, amino- and carboxyl-terminal ends of the receptor, respectively. The nomenclature of each chimeric receptor is explained in the text. The equilibrium dissociation constants (K_D, nM) were determined for each WT and chimeric receptor as described in the text and are expressed as mean ± standard error values of two to four independent experiments.

TABLE 2

K_i values of 5-HT and ketanserin for inhibition of [^{3}H]5-CT and [^{3}H]GR 125743 binding to WT h5-HT$_{1D}$, WT h5-HT$_{1B}$ receptors, and various chimeric 5-HT$_{1D/1B}$ receptors expressed in Cos-7 cells

Radioligand binding was performed with 3.0 nM [^{3}H]5-CT or 1.0 nM [^{3}H]GR 125743 for Cos-7 cellular membranes as described in the text. Results are expressed as mean ± standard error values from three to eight independent experiments, with each one performed in duplicate.

Receptor	K_i			
	[^{3}H]5-CT		[^{3}H]GR 125743	
	5-HT	Ketanserin	5-HT	Ketanserin
	nM			
WT h5-HT$_{1D}$	9.21 ± 1.10	27.37 ± 4.99	5.65 ± 0.66	24.02 ± 5.89
WT h5-HT$_{1B}$	9.61 ± 1.09	2193 ± 302	46.18 ± 3.69	2902 ± 422
D/BI-IV	10.57 ± 0.57	43.14 ± 5.71	21.83 ± 4.28^c	44.36 ± 3.07
D/BI-V	9.20 ± 0.84	>10,000^c	14.43 ± 4.16^b	3428 ± 1097^c
D/BVI-VII	7.55 ± 1.68	14.24 ± 5.84	6.12 ± 0.24	32.39 ± 1.77
D/B2ECL+V	7.57 ± 1.41	1265 ± 439^c	5.22 ± 0.89	364 ± 56^c
B/D2ECL+V	10.67 ± 1.67	65.02 ± 5.14^b	39.00 ± 9.54	115 ± 25^c
D/BV	8.16 ± 1.50	16.08 ± 2.85	2.96 ± 0.11^b	10.37 ± 3.07
B/DV	11.00 ± 1.88	733 ± 156^a	11.13 ± 1.35^c	344 ± 26^b
D/B2ECL	5.00 ± 1.03^a	157 ± 33^c	2.73 ± 0.48^b	112 ± 16^b

Statistical analysis was performed using Student's *t* test: $^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$ versus K_i values of their respective WT receptors, indicated by the first letter of each chimeric receptor as described in the text.

receptor equivalent (chimera D/BV) did not modify ($p > 0.05$) the ketanserin binding affinity compared with the parental h5-HT_{1D} receptor. Nevertheless, a statistically significant 3-fold ([^{3}H]5-CT) to 8-fold ([^{3}H]GR 125743) increased affinity ($p < 0.05$) was observed for ketanserin with the reverse chimera B/DV compared with the WT h5-HT_{1B} receptor. Exchange of the second ECL between the h5-HT_{1D} and h5-HT_{1B} receptors (chimera D/B2ECL) induced a 5-fold decrease ($p < 0.01$) in the binding affinity of ketanserin compared with the WT h5-HT_{1D} receptor. 5-HT yielded a 2–4- fold increased binding affinity ($p < 0.01$) for each of these three constructs compared with their parental receptors when [^{3}H]GR 125743 was used as a radioligand.

A comparison between the second ECL and fifth TMD of the h5-HT_{1B} and h5-HT_{1D} receptors reveals 13 different amino acids (Fig. 2). All of them have been replaced in the h5-HT_{1D} receptor sequence by their h5-HT_{1B} receptor counterparts by either point mutations or chimeric receptors to determine which amino acid or acids of the second ECL and/or fifth TMD may be involved in the modification of the ketanserin binding affinity. The point mutations h5-HT_{1D}/Ser210Thr, h5-HT_{1D}/Val211Leu, and h5-HT_{1D}/Ile215Ala could not be analyzed because they did not express detectable radioligand binding. Otherwise, the point mutations h5-HT_{1D}/Ser196Leu, h5-HT_{1D}/Ile199Val, h5-HT_{1D}/Cys203Val, and h5-HT_{1D}/Ile208Phe and the double mutant h5-HT_{1D}/SerGln193–194AspHis showed maximally a 7-fold decreased affinity for ketanserin compared with the WT h5-HT_{1D} receptor (Table 3). Hence, none of these substituted residues were able to alter the ketanserin binding affinity to the same level as D/B2ECL+V. Therefore, seven other combinations [D/BV(Ser196-Ile199), D/BV(Cys203-Ile208), D/BV(Ser210-Ile215), D/BV(Ile199-Val211), D/BV(Ser196-Val211), D/BV(Ile199-Ile215), and D/BV(Gln182-Leu189)] were tried by exchanging small regions of the h5-HT_{1B} receptor going from four to eight amino acids inside the second ECL and TMD V of the h5-HT_{1D} receptor (Fig. 2). Although the chimeric receptor D/BV(Cys203-Ile208) did not show detectable ligand binding, the other combinations showed only minor modifications (maximally 8-fold decrease) of the ketanserin binding affinity compared with the WT h5-HT_{1D} receptor (Table 3). In addition, four chimeric 5-$HT_{1D/1B}$ receptors were constructed, starting from the chimeric receptor D/BV and gradually exchanging one amino acid of the h5-HT_{1D} receptor second ECL with its equivalent residue in the WT h5-HT_{1B} receptor, generating D/BV(Ser193-Ile215), D/BV(Leu189-Ile215), D/BV(Asp187-Ile215), and D/BV(Met185-Ile215). The ketanserin binding affinity of these four chimeric receptors was almost similar (maximally 7-fold decrease) to the value obtained for the WT h5-HT_{1D} receptor (Table 3).

Binding profile of the WT h5-HT_{1D} and h5-HT_{1B} receptors and chimeric receptors D/B2ECL+V and B/D2ECL+V. A series of 5-HT receptor ligands were tested on the chimeric receptors D/B2ECL+V and B/D2ECL+V for inhibition of [^{3}H]GR 125743 binding and compared with the binding profile obtained with the WT h5-HT_{1B} and h5-HT_{1D} receptors (Table 4). Representative binding curves are illustrated in Fig. 3. The mixed 5-$HT_{1A/1B/1D}$ agonist L 694247 yielded a slight increase (1.6-fold, $p < 0.01$) in its binding affinity for the chimeric receptor B/D2ECL+V compared with the WT h5-HT_{1B} receptor, although no difference was apparent between D/B2ECL+V and the WT h5-HT_{1D} receptor. The agonists zolmitriptan and 5-HT displayed 8- and 25-fold selectivity for the WT h5-HT_{1D} over h5-HT_{1B} receptor. The chimeric receptors are not significantly different ($p > 0.05$) from their parental receptors in regard to these agonists; 8- and 33-fold selectivity was observed. The chimera B/D2ECL+V yielded a 4-fold decreased binding affinity ($p < 0.01$) for the agonist sumatriptan compared with the WT h5-HT_{1B} receptor, whereas the K_i values remained unmodified for D/B2ECL+V and WT h5-HT_{1D} receptors. The mixed 5-$HT_{1B/1D}$ antagonist GR 127935 yielded similar binding affinities for the chimeric receptors compared with their respective WT receptors. The 5-HT_{1B} inverse agonist SB 224289 showed 35-fold selectivity for the WT h5-HT_{1B} receptor; 11-fold selectivity was conserved between the chimeric receptors B/D2ECL+V and D/B2ECL+V. The 5-HT_2 antagonist ritanserin displayed 8-fold selectivity for the WT h5-HT_{1D} over h5-HT_{1B} receptor. The chimeric receptors B/D2ECL+V and D/B2ECL+V yielded similar binding affin-

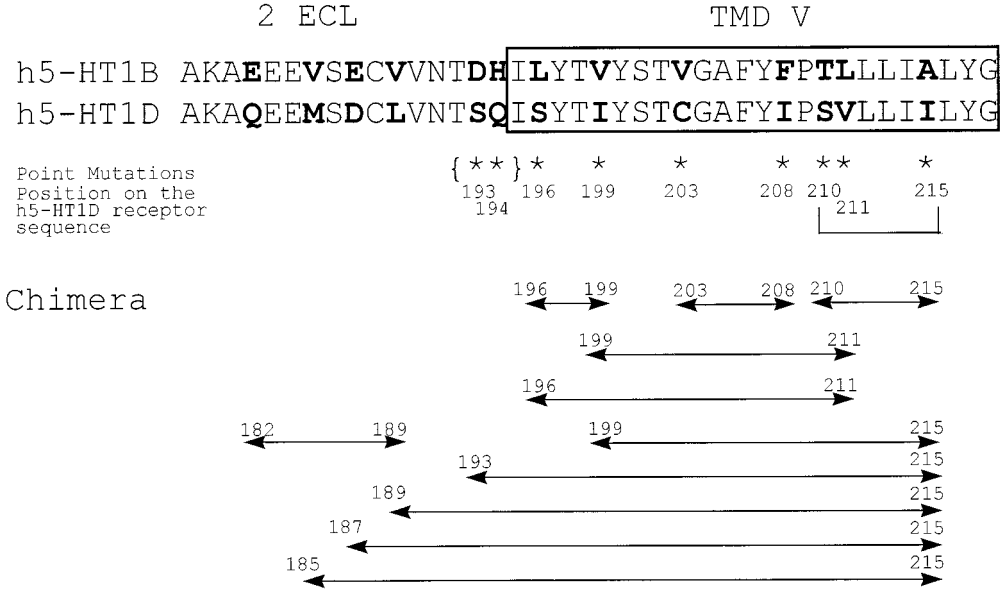

Fig. 2. Schematic representation of point mutations and chimeric receptors created within the second ECL and the fifth TMD of the h5-HT_{1D} receptor. *Bold amino acid residues*, diverge between the h5-HT_{1D} and h5-HT_{1B} receptors. The point mutations (*PM*) that have been constructed are shown (*), and their position in the h5-HT_{1D} receptor sequence is indicated. *Arrows*, constructed chimeric receptors. *Left and right arrowheads*, the first and last amino acid of the h5-HT_{1D} receptor replaced by the homologous region of the h5-HT_{1B} receptor, respectively. Each chimeric receptor and point mutation was constructed by the modified overlap extension PCR-based technique using appropriate complementary mutagenic primers as described in the text. The corresponding IC_{50} values for ketanserin are summarized in Table 3.

ities for ritanserin as for the WT h5-HT$_{1D}$ and 5-HT$_{1B}$ receptors, respectively. Experiments conducted with [^{3}H]5-CT yielded similar results for the comparison between the chimeric receptor B/D2ECL+V and the WT h5-HT$_{1B}$ receptor (Table 5). Otherwise, slight variations were observed with L 694247, sumatriptan, GR 127935, and SB 224289 when comparing the chimeric receptor D/B2ECL+V with the WT h5-HT$_{1D}$ receptor.

Discussion

The major finding of this study concerns the involvement of the second ECL and the fifth TMD in the high affinity binding of ketanserin and ritanserin to the h5-HT$_{1D}$ receptor. This effect seems to be specific because the other ligands, including 5-HT, various 5-HT$_{1B/1D}$ agonists, the mixed 5-HT$_{1B/1D}$ antagonist GR 125743, and the selective 5-HT$_{1B}$ inverse agonist SB 224289, displayed either no or only slight modifications in their ligand binding affinities. Moreover, these minor modifications were, in contrast to those of ritanserin and ketanserin, not observed by analysis of both [^{3}H]5-CT and [^{3}H]GR 125743 binding. These results point to a specific binding site for ketanserin and ritanserin, distinct from that for the diverse agonists, and also from the inverse agonist SB 224289. The observed changes in the binding profile of the chimeric receptors are likely to result from differences in certain amino acids that interact directly with ketanserin and ritanserin. It is unlikely to be a consequence of a global change in the receptor conformation because the binding affinities of the other ligands were either not or only slightly modified. Although ketanserin is a key compound that differentiates between recombinant 5-HT$_{1D}$ versus 5-HT$_{1B}$ receptors of human (Pauwels *et al.*, 1996), guinea pig (Wurch *et al.*, 1997b; Pauwels *et al.*, 1998), and rat (Bach *et al.*, 1993), displaying >100-fold selectivity for the 5-HT$_{1D}$ receptor subtype, the affinity of ketanserin for the 5-HT$_{1D}$ receptor is species dependent (Table 6). The antagonist is weakly selective (11-fold) at the rabbit 5-HT$_{1D}$ receptor relative to the rabbit 5-HT$_{1B}$ receptor (Wurch *et al.*, 1997a) and apparently recognizes neither the canine 5-HT$_{1D}$ nor the canine 5-HT$_{1B}$ receptor subtypes (Zgombick *et al.*, 1997). Amino acid alignment of a domain encompassing the second ECL and the fifth TMD of human, rat, guinea pig, rabbit, and dog 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors and the h5-HT$_{2A}$ receptor does not immediately point to amino acids that may be specifically involved in this differential ketanserin-binding profile (Table 6). The lack of amino acid homology between receptors with either a high or a low affinity for ketanserin, especially between the canine 5-HT$_{1D}$ versus the 5-HT$_{1B}$ receptors and the h5-HT$_{2A}$ versus the 5-HT$_{1D}$ receptors, suggests a complex ligand/receptor interaction.

Segregation between agonist and antagonist binding sites in G protein-coupled receptors. Although the h5-HT$_{1D}$ receptor, like other G protein-coupled receptors (Strader *et al.*, 1988), binds agonists and antagonists in a competitive manner (Pauwels *et al.*, 1996), a structural overlap of agonist and antagonist binding sites has not been clearly demonstrated. Alternatively, distinct binding sites for agonists and antagonists may be involved, but they influence the receptor configuration in such a way that agonist and antagonist cannot be bound at the same time. Our results with the chimeric receptors D/BI-V, D/B2ECL+V, and B/D2ECL+V could differentiate between a 5-HT- and a ketanserin-binding site. The data suggest that the binding sites of ketanserin and ritanserin and of the agonists for the h5-HT$_{1D}$ receptor are not identical. Thus, the competitive antagonism by ketanserin and ritanserin does not imply that

TABLE 3

IC$_{50}$ values of ketanserin for inhibition of [^{3}H]5-CT and [^{3}H]GR 125743 binding to WT h5-HT$_{1D}$, WT h5-HT$_{1B}$ receptors, and various point mutations and chimeric 5-HT$_{1D/1B}$ receptors expressed in Cos-7 cells

Radioligand binding was performed with 3.0 nM [^{3}H]5-CT and/or 1.0 nM [^{3}H]GR 125743 for Cos-7 cellular membranes as described in the text. Each of the point mutations and chimeric receptors is explained in the legend to Fig. 2. Results are expressed in IC$_{50}$ values (nM) as mean or as mean ± standard error of one or two independent experiments, with each one performed in duplicate. The point mutations D/Ser210Glu, D/Val211Leu, and D/Ile215Ala and the chimeric receptor D/BVal203-Phe208 did not reveal specific [^{3}H]5-CT and [^{3}H]GR 125743 binding.

Receptor	Ketanserin (IC$_{50}$)	
	[^{3}H]5-CT	[^{3}H]GR 125743
	nM	
WT h5-HT$_{1D}$	58	43
WT h5-HT$_{1B}$	7450	7366
D/SerGln193-194AspHis	280	110
D/Ser196Leu	420	120
D/Ile199Val	125	45
D/Cys203Val	240	70
D/Ile208Phe	170	57
D/BLeu196-Val199	435 ± 81	130 ± 7.0
D/BThr210-Ala215	185 ± 25	80 ± 3.5
D/BVal199-Leu211	130 ± 14	51 ± 6.4
D/BLeu196-Leu211	280	130
D/BGlu182-Val189	74 ± 25	5.3 ± 2.6
D/BVal199-Ala215	255 ± 18	74 ± 11
D/BAsp193-Ala215	430 ± 120	115 ± 26
D/BVal189-Ala215	ND	170
D/BGlu187-Ala215	ND	280 ± 14
D/BVal185-Ala215	ND	255 ± 32

ND, not determined.

TABLE 4

K_i values of 5-HT ligands for inhibition of [^{3}H]GR 125743 binding to WT h5-HT$_{1B}$, h5-HT$_{1D}$, and the chimeric receptors D/B2ECL+V and B/D2ECL+V expressed in Cos-7 cells

Radioligand binding was performed with 1.0 nM [^{3}H]GR 125743 as described in the text. Results (K_i, nM) are expressed as mean ± standard error values from three to six independent experiments, each one performed in duplicate.

Ligand	WT h5-HT$_{1B}$	B/D2ECL+V	D/B2ECL+V	WT h5-HT$_{1D}$
L 694247	1.92 ± 0.05	1.20 ± 0.15[b]	0.46 ± 0.16	1.08 ± 0.43
Zolmitriptan	20.58 ± 4.81	39.64 ± 9.32	1.19 ± 0.44	0.82 ± 0.21
5-HT	46.18 ± 3.69	39.00 ± 9.54	5.22 ± 0.89	5.65 ± 0.66
Sumatriptan	78.79 ± 8.33	302 ± 59[b]	5.26 ± 1.19	6.05 ± 2.08
GR 127935	1.97 ± 0.11	0.96 ± 0.23	2.41 ± 0.91	4.85 ± 1.70
SB 224289	6.70 ± 0.48	10.66 ± 3.46	116 ± 21	233 ± 71
Ritanserin	133 ± 25	22.37 ± 7.61[a,d]	70.13 ± 23.57[a,e]	16.85 ± 8.16
Ketanserin	2902 ± 422	115 ± 25[c]	364 ± 56[c]	24.02 ± 5.89

Statistical analysis was performed using Student's t test: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ versus K_i values of their respective WT receptors and [d] not significantly different compared with the WT h5-HT$_{1D}$ receptor, [e] not significantly different compared with the WT h5-HT$_{1B}$ receptor.

they share the same molecular interaction points with the receptor as the agonist. A similar hypothesis has been put forward for the competitive antagonism of substance P by several nonpeptide antagonists at the neurokinin NK1 receptor (Cascieri *et al.*, 1995). Site-directed mutagenesis studies of α-adrenergic receptors (Chung *et al.*, 1988; Strader *et al.*, 1988), dopamine D_2 receptors (Mansour *et al.*, 1992), and chimeric 5-HT_{2A}/5-HT_{2C} receptors (Roth *et al.*, 1993) also have demonstrated a differential implication of receptor domains in agonist compared with antagonist binding.

Importance of the second ECL and fifth TMD in ligand binding to G protein-coupled receptors. TMD V of several G protein-coupled receptors is likely to play an important role in agonist and antagonist binding, especially via the ability of its hydroxylated residues to form hydrogen bonds. TMD V of the h5-HT_{1B} receptor previously has been reported to participate in the binding of the agonist sumatriptan; it may form a hydrogen bond with a Ser507 residue of the WT h5-HT_{1B} receptor (Table 6; Granäs *et al.*, 1995). Replacement of Thr508 (Table 6) by a serine residue in the h5-HT_{1B} receptor induced a 7.5- and 13-fold decrease in 5-HT affinity and potency, respectively (Veldman and Bienkowski, 1994). This Thr508 residue (or Ser508 in h5-HT_{2A}) also may be involved in hydrogen bonds to the hydroxyl group of the aromatic ring of 5-HT (Trumpp-Kallmeyer *et al.*, 1992). Similar hydroxylated amino acid residues are found at the same location in TMD V of other serotonergic, α-adrenergic, and β-adrenergic receptors; in particular, the same amino acid residues (Ser507-Thr508; Table 6) are present in the h5-HT_{1D} receptor. A hydroxylated threonine residue (Thr196, position 511 in Table 6) in TMD V of the rat 5-ht_6 receptor has been shown to interact with the N1 position of N1-unsubstituted ergolines and tryptamines, probably forming a hydrogen bond with the hydroxyl moiety of threonine (Boess *et al.*, 1997). Johnson *et al.* (1994) reported that Ala242 (position 511 in Table 6) in TMD V of the rat 5-HT_{2A} receptor plays an important role in the structure-activity relationship for ergolines and tryptamines. This single amino

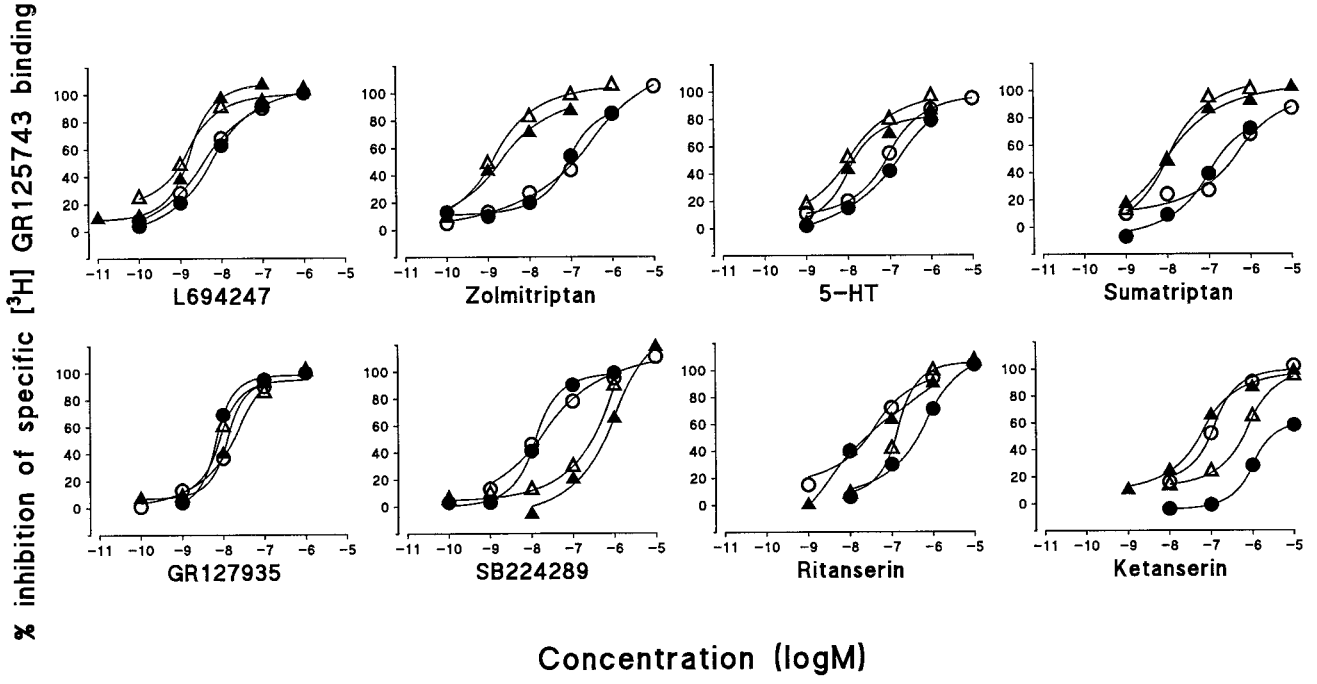

Fig. 3. Inhibition curves of 5-HT receptor ligands for [^{3}H]GR 125743 binding to a membrane preparation of Cos-7 cells expressing either WT h5-HT_{1B} (●), WT h5-HT_{1D} (▲), chimeric D/B2ECL+V (△), or B/D2ECL+V (○) receptors. Radioligand binding was performed as described in Table 3. Curves were constructed using the mean values from one representative experiment of three to six independent experiments. Mean K_i values are summarized in Table 3.

TABLE 5

K_i values of 5-HT ligands for inhibition of [^{3}H]5-CT binding to WT h5-HT_{1D} and h5-HT_{1B} receptors and the chimeric receptors D/B2ECL+V and B/D2ECL+V expressed in Cos-7 cells

Radioligand binding was performed with 3.0 nM [^{3}H]5-CT as described in the text. Results (K_i, nM) are expressed as mean ± standard error values from three to six independent experiments, with each one performed in duplicate.

Ligand	WT h5-HT_{1B}	B/D2ECL+V	D/B2ECL+V	WT h5-HT_{1D}
L 694247	0.25 ± 0.07	0.57 ± 0.27	0.28 ± 0.07[a]	1.05 ± 0.30
Zolmitriptan	6.02 ± 0.46	2.93 ± 1.28	0.68 ± 0.16	1.04 ± 0.11
5-HT	9.61 ± 1.09	10.67 ± 1.67	7.57 ± 1.41	9.21 ± 1.10
Sumatriptan	29.66 ± 3.69	16.12 ± 5.50	6.06 ± 0.69[b]	12.41 ± 1.24
GR 127935	0.22 ± 0.03	1.15 ± 0.58	2.72 ± 0.50[b]	1.09 ± 0.14
SB 224289	9.84 ± 2.92	6.77 ± 1.69	148 ± 27[b]	317 ± 26
Ritanserin	80.61 ± 6.22	14.90 ± 2.77[c,d]	56.45 ± 17.65[b,e]	5.01 ± 1.82
Ketanserin	2193 ± 302	65.02 ± 5.14[b]	1265 ± 439[c]	27.37 ± 4.99

Statistical analysis was performed using Student's t test: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ versus K_i values at their respective WT receptors and [d] $p < 0.05$ versus the WT h5-HT_{1D} receptor, [e] not significantly different compared with the WT h5-HT_{1D} receptor.

acid is implied in the high affinity of the antagonist mesulergine, a N1-methylergoline, for the rat 5-HT$_{2A}$ receptor (Kao *et al.*, 1992). The h5-HT$_{2A}$ receptor, for which this amino acid is replaced by a serine residue (Ser511; Table 6), is unable to bind mesulergine. The alanine residue, present at the position 511 (Table 6) in TMD V of both the h5-HT$_{1B}$ and h5-HT$_{1D}$ receptor subtypes, seems not to be critical for ketanserin binding.

A role of TMD V in binding of ketanserin to the h5-HT$_{1D}$ receptor cannot be excluded because of its intermediate affinity (K_i = 344–733 nM) for the chimeric receptor B/DV compared with the WT h5-HT$_{1B}$ (K_i = 2193–2902 nM) and h5-HT$_{1D}$ receptors (K_i = 24–27 nM). Otherwise, the affinity of ketanserin for the reverse chimeric receptor D/BV was not statistically different from that of the WT h5-HT$_{1D}$ receptor. None of the point mutations inside the fifth TMD of the WT h5-HT$_{1D}$ receptor (Fig. 2) modified the binding profile of 5-HT and ketanserin compared with the h5-HT$_{1D}$ receptor. Two of these point mutations (Ser193Asp and Ser196Leu) replaced serine residues, thereby excluding the existence of an H-bond between serine and ketanserin. Two other residues seem not to be important in ketanserin binding to the h5-HT$_{1D}$ receptor: Cys203 and Ile208. Hence, the involvement of the thiol group of Cys203 in maintaining the tridimensional receptor structure can be minimized. These data suggest either a complex interaction between ketanserin and residues of TMD V of the h5-HT$_{1D}$ receptor or that this TMD participates in putative long-distance intramolecular contacts that are conserved in the various point-mutated receptors. The involvement of the second ECL in ketanserin binding to the h5-HT$_{1D}$ receptor was unexpected. Although its precise role in ligand binding could not be clearly determined, the chimeric receptor D/B2ECL yielded an altered ketanserin-binding profile with a 5-fold lower affinity ($p < 0.01$) compared with the WT h5-HT$_{1D}$ receptor.

Two hypotheses can be proposed in regard to the ketanserin/h 5-HT$_{1D}$ receptor interaction: (1) the ECL is folded away from the TMD and the binding of ketanserin may be positioned above the receptor's surface. Such a situation has been described for agonist and antagonist binding to various peptide and chemokine receptors (Fong *et al.*, 1992) and has been proposed for the antagonist binding of phentolamine and WB 4110 to the α_{1A}-adrenergic receptor (Zhao *et al.*, 1996). (2) The ECL is folded toward the agonist-binding pocket such that the antagonist interacts with other TMD amino acid residues located near the extracellular surface. Three adjacent amino acid residues (Gln196, Ile197, Asn198), which are located on the exofacial surface of the fifth TMD in the α_{1A}-adrenergic receptor, are implied in the high affinity of the antagonists phentolamine and WB4110 to the α_{1A}-adrenergic compared with the α_{1B}-adrenergic receptor (Zhao *et al.*, 1996). In view of our results, the involvement of the second ECL and the fifth TMD in the binding of ketanserin and ritanserin to the h5-HT$_{1D}$ receptor is highlighted. This favors a receptor model in which the second ECL folds down close to the receptor surface, allowing dual interactions with this ECL and TMD V. On the other hand, the second ECL also can subtly influence the helical packing of the two neighboring domains (TMDs IV and V) and thus alter the binding pocket that is exposed to ketanserin and ritanserin. Recently, the involvement of ECL has been proposed for the control of helical packing of TMD; a chimeric β_2-adrenergic receptor containing the third ECL derived from an α_{1A}-adrenergic receptor yields an increased agonist potency (Zhao *et al.*, 1998).

Molecular modeling of ketanserin/5-HT$_{2C}$ receptor interactions revealed contacts with 22 different residues within TMDs III–VII for ketanserin. Strong aromatic interactions with Phe and Trp residues and a hydrogen bond with an Asn residue in TMD VI have been proposed (Kristiansen and Dahl, 1996). On the other hand, in the case of chimeric rat 5-HT$_{2A/2C}$ receptors, ketanserin binding has been found to depend on TMDs I–III and an amino acid region between the third intracellular loop and TMD VII (Choudhary *et al.*, 1992). In both studies, a region encompassing the second ECL and the fifth TMD has been shown to be involved in the ketanserin/receptor interaction. Our results with the chimeric receptor D/BI-IV, exchanging TMDs I–IV, did not modify the affinity of ketanserin compared with the parental h5-HT$_{1D}$ receptor, thus minimizing the importance of this region of the h5-HT$_{1D}$ receptor for ketanserin binding. However, it cannot be excluded that critical interaction points

TABLE 6

Amino acid sequences of the putative second ECL and fifth TMD of canine, guinea pig, rabbit, and h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors and 5-HT$_{2A}$ receptor

Bold, divergent amino acids between h5-HT$_{1D}$ and h5-HT$_{1B}$ receptors. For convenience, the first and last residues (*arrows*) of TMD V are numbered as 501 and 524 as referred to in Discussion. The corresponding K_i values for the inhibition of radioligand binding by ketanserin to each of these receptor subtypes were obtained using [^{3}H]5-HT, [^{3}H]5-CT, or [^{3}H]ketanserin as a radioligand.

	2 ECL / TMD V	Ketanserin K_i (nM)	Reference
ca5-HT$_{1B}$	AKAEEE**VSE**CVVNT**DHIL**YT**V**YST**V**GAFY**FPTL**LLI**A**LYG	5,248 [a]	Zgombick *et al.,* 1997
gp5-HT$_{1B}$	AKAEEE**VLD**CLVNT**DHV**LYT**V**YST**G**GAFY**LPTL**LLI**A**LYG	3,697 [b]	Pauwels *et al.,* 1998
rb5-HT$_{1B}$	AKAEEE**VSE**CLVNT**DHV**LYT**V**YST**V**GAFY**LPTL**LLI**A**LYG	251 [b]	Wurch *et al.,* 1997a
h5-HT$_{1B}$	AKA**E**EE**VSE**CVVNT**DHIL**YT**V**YST**V**GAFY**FPTL**LLI**A**LYG	>10,000 [b]	Pauwels *et al.,* 1996
h5-HT$_{1D}$	AKA**Q**EE**MSD**CLVNT**SQIS**YT**I**YST**C**GAFY**IPSV**LLI**I**LYG	10.9[b]	Pauwels *et al.,* 1996
rb5-HT$_{1D}$	AKAHEE**VSD**CLVNT**SQIS**YT**I**YST**C**GAFY**IPSV**LLI**V**LYG	21.8[a]	Bard *et al.,* 1996
gp5-HT$_{1D}$	AQAQEE**MSD**CLVNT**SQIS**YT**I**YST**C**GAFY**IPSV**LLI**I**LYS	16.2[b]	Wurch *et al.,* 1997b
ca5-HT$_{1D}$	AKAEED**MSD**CQVNT**SQIS**YT**I**YST**C**GAFY**IPSV**LLI**I**LYG	3,020 [a]	Zgombick *et al.,* 1997
h5-HT$_{2A}$	SKVFKE**G**S-CLLAD**DN**——FV**L**IGS**F**VSFF**IPLT**IMV**I**TYF	3.2[c]	Bonhaus *et al.,* 1995
	↑ 501 ↑ 524		

ca, canine; rb, rabbit; gp, guinea pig.
[a] [^{3}H]5-HT.
[b] [^{3}H]5-CT.
[c] [^{3}H]ketanserin.

between ketanserin and the receptor are conserved by exchanging h5-HT_{1D} receptor domains by equivalent h5-HT_{1B} receptor regions. Whether such interactions exist between ketanserin and the 5-HT_{1D} receptor requires further molecular modeling studies. Interestingly, ketanserin-5-HT_{2C} receptor simulations oriented either the *p*-fluoro-benzoyl or the quinazolindione groups of ketanserin toward the extracellular opening of the 5-HT_{2C} receptor model (Kristiansen and Dahl, 1996).

In conclusion, the results provided in this study imply that the process of ligand binding to receptors is more complex than generally assumed. It seems that the 5-HT receptor antagonist ketanserin, and probably also ritanserin, binds to the 5-HT_{1D} receptor by its second ECL and/or near the exofacial surface of the TMD V of the receptor and not down into the binding pocket formed by the seven TMDs, as determined for agonist binding. This further supports the hypothesis that there are multiple binding sites for ligands within the h5-HT_{1D} receptor. Targeting of these different binding sites may open new possibilities to selectively stimulate, block, or otherwise manipulate the receptor.

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