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Recombinant saphenous vein 5-HT_{1B} receptors of the rabbit: comparative pharmacology with human 5-HT_{1B} receptors

Thierry Wurch, Christiane Palmier, Francis C. Colpaert & [1]Petrus J. Pauwels

Centre de Recherche Pierre Fabre, Laboratory of Cellular & Molecular Neurobiology, 17, avenue Jean Moulin, 81106 Castres Cédex, France

1 The rabbit recombinant saphenous vein 5-hydroxytryptamine$_{1B}$ (rb 5-HT_{1B}) receptor stably transfected in rat C6-glial cells was characterized by measuring adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation upon exposure to various 5-HT receptor ligands. The effects of agonists and antagonists were compared with their effects determined previously at the human cloned 5-HT_{1B} (h 5-HT_{1B}) receptor under similar experimental conditions.

2 Intact C6-glial cells expressing rb 5-HT_{1B} receptors exhibited [^{3}H]-5-carboxamidotryptamine (5-CT) binding sites with a K_d of 0.80 ± 0.13 nM and a B_{max} between 225 to 570 fmol mg^{-1} protein. The binding affinities of a series of 5-HT receptor ligands determined in a membrane preparation with [^{3}H]-5-CT or [^{3}H]-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide (GR 125,743) were similar. With the exception of ketanserin, ligand affinities were comparable to those determined at the cloned h 5-HT_{1B} receptor site.

3 rb 5-HT_{1B} receptors were negatively coupled to cyclic AMP formation upon stimulation with 5-HT agonists. Of the several 5-HT agonists tested, 5-CT was the most potent, the potency rank order being: 5-CT > 5-HT > zolmitriptan > naratriptan > rizatriptan > sumatriptan > **R**(+)-8-(hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT). The maximal responses of these agonists were similar to those induced by 5-HT. The potency of these agonists showed a positive correlation ($r^2 = 0.87$; $P < 0.002$) with their potency at the cloned h 5-HT_{1B} receptor subtype.

4 2′-Methyl-4-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-amide (GR 127,935), methiothepin and ketanserin each behaved as silent, competitive antagonists at rb 5-HT_{1B} receptors; pK_B values were 8.41, 8.32 and 7.05, respectively when naratriptan was used as an agonist. These estimates accorded with their binding affinities and the potencies found on 5-HT and/or sumatriptan-mediated contraction of isolated rabbit saphenous vein segments.

5 In conclusion, the recombinant saphenous vein 5-HT_{1B} receptor of the rabbit shares important pharmacological similarities with the cloned h 5-HT_{1B} receptor. However, ketanserin is a more potent antagonist of rb 5-HT_{1B} receptors.

Keywords: Rabbit recombinant saphenous vein 5-HT_{1B} receptor; human 5-HT_{1B} receptor; cyclic AMP; ketanserin; rat C6-glial cell line

Introduction

Seven families of 5-hydroxytryptamine (serotonin, 5-HT) receptors have been identified: 5-HT_1 to 5-HT_7 (Hoyer *et al.*, 1994). 5-HT_1-like receptors constitute a group of related receptors that have not yet been definitively equated with any of the 5-HT_1 binding site subtypes that have been identified in the central nervous system (Hoyer *et al.*, 1994). They mediate a number of functional responses which include smooth muscle contraction, a decrease in noradrenaline release from sympathetic nerves and certain central effects such as the inhibition of 5-HT and glutamate release (see Hoyer *et al.*, 1994). These receptors can be clearly distinguished from 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} binding sites and show similarities with 5-HT_{1D} binding sites. This latter binding site is complex since it is encoded by a subfamily of two distinct genes (see Hartig *et al.*, 1996), 5-HT_{1D} (previously designated 5-$HT_{1D\alpha}$) and 5-HT_{1B} (previously designated 5-$HT_{1D\beta}$), as has been clearly shown for man (Weinshank *et al.*, 1992) and, recently, for the dog and rabbit (Branchek *et al.*, 1995; Harwood *et al.*, 1995).

The precise function of these receptor subtypes in man remains to be determined. Using *in situ* hybridization Hamel *et al.* (1993) have shown that mRNA for the 5-HT_{1B} receptor, but not the 5-HT_{1D} receptor, is present in human and bovine cerebral arteries. Reverse transcription - polymerase chain reaction (RT-PCR) experiments with primers designed according to the dog 5-HT_{1D} receptor gene sequence resulted in the specific amplification of a 632 base pair sequence in both canine coronary artery and saphenous vein (Cushing *et al.*, 1994). Otherwise, Sgard *et al.* (1996) obtained evidence for 5-HT_{1B} but not 5-HT_{1D} receptor subtype expression in canine large coronary arteries and saphenous vein. Hence, both 5-HT_{1B} and 5-HT_{1D} receptor subtypes are probably involved in the contraction of blood vessels. Recently, we cloned and identified a 5-HT_{1B} receptor gene in rabbit saphenous vein (rb 5-HT_{1B}; Wurch *et al.*, 1996a). With the exception of one amino acid (Ala 13) the amino acid sequence of this receptor gene is identical to the 5-HT_{1B} receptor gene cloned by Harwood *et al.* (1995) using a genomic DNA library from rabbit liver. Its receptor binding profile together with its amino acid sequence suggest a pharmacological profile close to that of the human cloned 5-HT_{1B} (h 5-HT_{1B}) receptor.

In the present study, we investigated the functional pharmacological properties of the rabbit cloned saphenous vein 5-HT_{1B} receptor under similar experimental conditions as previously described for the cloned h 5-HT_{1B} receptor (Pauwels *et al.*, 1996). Stably transfected rat C6-glial cells were used to determine the agonist and/or antagonist activities of various 5-HT receptor ligands by measuring their potency to inhibit forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation. The cyclic AMP data were compared with the binding affinities of the investigated 5-HT receptor ligands and their characteristics at the cloned h 5-HT_{1B} receptor. Special attention was paid to the results with the 5-HT_2

[1] Author for correspondence

receptor antagonist, ketanserin, because it discriminates between recombinant h 5-HT$_{1D}$ and h 5-HT$_{1B}$ receptor-mediated responses (Pauwels & Colpaert, 1995; Zgombick *et al*., 1995) and has been described to be an antagonist of both 5-HT and sumatriptan-induced contraction of the rabbit saphenous vein, albeit with a blocking potency less than at 5-HT$_{2A}$ receptors (Martin & MacLennan, 1990; Van Heuven-Nolsen *et al*., 1990; Razzaque *et al*., 1995).

Methods

PCR-reactions and sequencing

Polymerase-chain-reaction (PCR) mixtures were performed as previously described (Wurch *et al*., 1996a) with 250 ng of DNase-treated, reverse-transcribed RNA extracted from New Zealand white rabbit saphenous vein. Sense primers P1A: 5′CGGTGCGCCCCACCGCTTGCGGCG and P1B: 5′CGGTGCGCCCCACCGCTTGCG were used with the reverse P1 primer: 5′TGGGAGTCCTTTTAGCTGAGT as described by Wurch *et al*. (1996a). The PCR products were purified by agarose gel electrophoresis with the Genclean II kit as described by the supplier and further ligated into 50 ng of pCR II plasmid. The cloned PCR products were sequenced manually by use of Sequenase 2.0.

Transfection of monkey Cos-7 and rat C6-glial cells with a rabbit 5-HT$_{1B}$ receptor gene

Cos-7 and C6-glial cells were transiently and permanently transfected with a pZeo/SV/rb 5-HT$_{1B}$ plasmid (Wurch *et al*., 1996a) by a gene pulser transfection apparatus (Bio-Rad; 10 μg plasmid per 0.5×10^7 cells at 250 mV and 250 μF; Pauwels *et al*., 1995). Control experiments were performed with non-transfected cells and cells transfected with a pZeo/SV plasmid without the receptor gene. Transfected Cos-7 cells were harvested after 48 h for radiogand binding experiments. Transfected C6-glial cells were subcultured after 48 h, trypsinised and diluted 50, 500 and 2500-times and grown in the presence of 0.45 mg zeocin ml^{-1}. Individual colonies were isolated after about 10 days and subcultured in the presence of 0.45 mg zeocin ml^{-1}. Radioligand binding and cyclic AMP experiments were performed with one clone selected on the basis of its ability to inhibit forskolin-stimulated cyclic AMP formation by 1 μM sumatriptan (>80%) and an expressed receptor density of approximately 0.5 pmol. mg^{-1} protein on intact cells.

5-HT$_{1B}$ receptor binding on intact cells and derived membrane preparations

Transfected cells were washed twice with 1 ml physiological salt solution (PSS (mM): NaCl 120, KCl 5.4, $MgCl_2$ 0.8, glucose 5 and Tris-HCl 25, pH 7.4) and incubated for 30 min at 25°C with 0.5 ml PSS containing 10 μM chloroquine (to avoid ligand trapping) and [^{3}H]-5-carboxamidotryptamine (5-CT, 0.05 to 20 nM) in either the absence or presence of 10 μM 5-HT. The incubation was stopped by washing the cultures three times with 1 ml ice-cold PSS. The cells were lysed and [^{3}H]-5-CT binding was measured as previously described (Pauwels *et al*., 1996). Membrane preparations were prepared in 50 mM Tris-HCl pH 7.7 for [N-methyl-^{3}H]-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide (GR 125,743) binding, or 50 mM Tris-HCl pH 7.7 containing 4 mM $CaCl_2$, 10 μM pargyline and 0.1% w/v ascorbic acid for [^{3}H]-5-CT binding (see Pauwels *et al*., 1996). Incubation mixtures consisted of 0.4 ml cell membrane preparation (10 to 25 μg protein), 0.05 ml of radioligand (0.3 to 0.6 nM) and 0.05 ml of inhibitor compound, or 10 μM 5-HT to determine non-specific binding. The reactions were stopped after 30 min incubation as described previously (Pauwels *et al*., 1996). Filtration was performed over Whatman GF/B glass fibre filters (0.2% polyethyleneimine-treated for [^{3}H]-GR 125,743 measurements). Specific binding of [^{3}H]-5-CT and [^{3}H]-GR 125,743 was defined as the portion of total binding which was inhibited by 10 μM 5-HT. Data were analysed graphically as inhibition curves and IC_{50} values (concentration of the compound producing 50% inhibition of specific binding) were derived. K_i-values were calculated according to the equation $K_i = IC_{50}/(1 + C/K_d)$ with C the concentration and K_d the equilibrium dissociation constant of the [^{3}H]ligand. For ligand saturation binding curves, [^{3}H]-5-CT and [^{3}H]-GR 125,743 were used at concentrations between 0.02 to 10 nM. Ligand saturation binding curves were analysed by the non-linear least square curve fitting program (Munson & Rodbard, 1980); two-site versus a single-site analysis was performed. Cellular and membrane protein concentrations were estimated with the dye-binding assay using the Bio-Rad kit (Bradford, 1976). Bovine serum albumin was used as a standard.

5-HT$_{1B}$ receptor-mediated inhibition of forskolin-stimulated cyclic AMP formation

Cyclic AMP formation was measured as described previously (Pauwels *et al*., 1996). Cultures were washed with 1 ml PSS and incubated for 5 min at 37°C with 1 ml PSS containing 1 mM isobutylmethylxanthine in the presence of 100 μM forskolin either in the absence or presence of test compound or 1 μM 5-HT to determine maximal cyclic AMP inhibition. Basal accumulation of cyclic AMP was measured in the absence of forskolin and test compound. Antagonist potency was assessed after a 15 min pre-incubation. The cellular cyclic AMP content was assayed by use of a radioimmunoassay kit as described by the supplier. Inhibition of forskolin-induced cyclic AMP formation was calculated as the percentage of that obtained with 1 μM 5-HT. EC_{50} values (concentration of test agent yielding 50% inhibition of its own maximum response) were derived. The dissociation equilibrium constant (K_B) of each antagonist was calculated according to the equation $K_B = (B)/(A'/A^A) - 1$, where B is the concentration of the antagonist and A and A′ are the EC_{50} values of agonist concentration measured in the absence and presence of antagonist, respectively. This was assumed when no differences were apparent between E_{max} values of agonist in the absence or presence of antagonist. Control experiments performed with non-transfected C6-glial cells did not show inhibition of forskolin-stimulated cyclic AMP formation by sumatriptan (Pauwels *et al*., 1996).

Materials

New Zealand white rabbits were obtained from Elevage Scientifique des Dombes (Chatillon-sur-Chalaronne, France). Cos-7 and C6-glial cells were obtained from ATCC (Rockville, U.S.A.). Cell culture media, foetal calf serum and 24-well tissue culture plates were obtained from Gibco Biocult. Laboratories (Paisley, U.K.). The expression vector pZeo/SV and zeocin were obtained from Invitrogen (San Diego, U.S.A.). The geneclean II kit was purchased from Bio101 Inc. (La Jolla, U.S.A.). The sequence 2.0 kit was from Amersham (Les Ulis, France). The radioimmunoassay kit for cyclic AMP was from Immunotech (Marseille, France). [^{3}H]-5-CT (15–30 Ci mmol^{-1}, batch 3241-023) was from New England Nuclear (Les Ulis, France). [N-methyl-^{3}H]-GR 125,743 (69 Ci mmol^{-1}, batch 1) was from Amersham (Les Ulis, France), 1-naphthylpiperazine and 2′-methyl-4-(5-methyl-[1,2,4]oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)-phenyl]amide (GR 127,935) were prepared internally, courtesy of Drs S. Halazy and C. Jorand, Zolmitriptan, sumatriptan, naratriptan and rizatriptan were synthetized internally, courtesy of Mr J-L. Maurel. 5-HT creatinine sulphate was from Sigma (St Louis, U.S.A.). Methiothepin was obtained from Tocris Cookson (Bristol, U.K.). **R**(+)-8-(hydroxy-2-(di-n-propylamino)tetralin [R(+)-8-OH-DPAT], ke-

tanserin and 5-CT were from RBI (Natick, U.S.A.). (±)-Cyanopindolol was from Tebu (Le Perray-en-Yvelines, France). Stock solutions of compounds were prepared in water or ethanol. The final concentration of ethanol (0.1% v/v) did not interfere with the binding and cyclic AMP experiments.

Results

Re-evaluation of the rabbit saphenous vein 5-HT$_{1B}$ receptor nucleotide sequence

The rb 5-HT$_{1B}$ receptor gene published by Harwood *et al.* (1995) shows 8 nucleotide differences (G13, G14, T16, 37GCG, A798, C1147) with our reported gene sequence (Wurch *et al.*, 1996a). These differences are mainly located in the 5′ terminal region of the coding sequence; we found an extra-$_{37}$GCG triplet. In order to re-analyse this GC-rich region ($_{37}$GCGGCGGGC$_{45}$), two PCR products were amplified in which this triplet was either present (P1A sense, see Methods) or absent (P1B sense) in the sequence of the primer. The approximately 500 bp long PCR products were sequenced on both coding and complementary strands, together with the original PCR product obtained with rabbit saphenous vein cDNA using the P1 primer set as well as the rabbit genomic clone (Wurch *et al.*, 1996a). Sequence analysis clearly showed that the original sequence corresponds to the PCR product obtained with the P1B sense primer. We therefore conclude that the $_{37}$GCG-triplet is not present in the nucleotide sequence of the rb 5-HT$_{1B}$ receptor gene. The corresponding amino acid sequence of the rabbit saphenous vein 5-HT$_{1B}$ receptor is 100% identical to the rb 5-HT$_{1B}$ receptor gene identified by Harwood *et al.* (1995), although two differences exist at the nucleotide level (A$_{798}$ to T and C$_{1147}$ to T).

Binding properties of 5-HT receptor ligands at recombinant rabbit saphenous vein 5-HT$_{1B}$ receptor sites

In contrast to control C6-glial cells, saturation binding experiments with [^{3}H]-5-CT and [^{3}H]-GR 125,743 on a membrane preparation of C6-glial cells stably transfected with a rb 5-HT$_{1B}$ receptor gene indicated the presence of a single high affinity binding site for [^{3}H]-5-CT (K_d: 0.28±0.12 nM, $P<0.001$, $n=3$) and for [^{3}H]-GR 125,743 (K_d: 0.45±0.19, $P<0.001$, $n=3$). The maximal binding capacity for both radioligands varied between 1.4 to 2 pmol mg^{-1} protein. Binding to rb 5-HT$_{1B}$ receptors on intact C6-glial cells was only specific with [^{3}H]-5-CT and showed a lower affinity (K_d: 0.80±0.13 nM, $n=3$) and B$_{max}$ of 225 to 570 fmol mg^{-1} protein. This suggests that only a fraction (16 to 29%) of the total 5-HT$_{1B}$ receptor population in transfected C6-glial cells binds [^{3}H]-5-CT at the plasma membrane, probably as a consequence of its physiochemical properties. It can be assumed that 5-CT, being structurally very similar to 5-HT, does not penetrate the plasma membrane. Therefore, 5-CT is likely to label only functionally active rb 5-HT$_{1B}$ receptor sites on intact C6-glial cells. In contrast, GR 125,743 did not reveal specific binding on intact cells as a consequence of massive trapping of this ligand in the intact cells. The trapping was non-saturable and masked the presence of receptor sites.

A series of 11 5-HT receptor ligands was tested in parallel for inhibition of [^{3}H]-5-CT or [^{3}H]-GR 125,743 binding to rb 5-HT$_{1B}$ receptors in a C6-glial cell membrane preparation. Similar binding affinities were obtained with both radioligands (Table 1). The compounds methiothepin, GR 127,935 and ketanserin showed a slightly higher (1.7 to 4 fold) binding af-

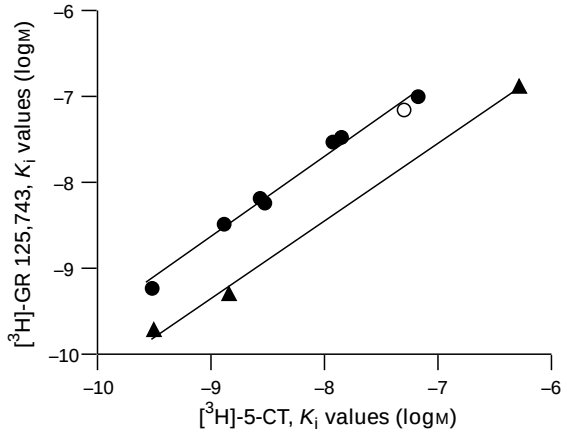

Figure 1 5-HT$_{1B}$ receptor binding affinities for 5-hydroxytryptamine (5-HT) receptor ligands measured at rabbit saphenous vein 5-HT$_{1B}$ receptor sites in C6-glial cells by either [^{3}H]-5-carboxamidotryptamine (5-CT) or [^{3}H]-GR 125,743 as a radioligand. Mean K_i values were taken from Table 1. (●) Compounds (5-CT, zolmitriptan, naratriptan, rizatriptan, sumatriptan and **R**(+)-8-OH-DPAT) with agonist efficacy like 5-HT; (○) (±)-cyanopindolol with partial and weak agonist efficacy; (▲) silent antagonists such as methiothepin, GR 127,935 and ketanserin. The correlation coefficient r^2 was 0.88 ($P<0.001$) when taken into account all the plotted values, 0.99 ($P<0.001$) considering the values of the compounds with intrinsic activity, and 1.00 ($P<0.040$) for the values of the corresponding silent antagonists.

Table 1 pK_i values of 5-HT receptor ligands for inhibition of [^{3}H]-5-carboxamidotryptamine (5-CT) or [^{3}H]-GR 125,743 binding to recombinant rb 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptor sites

	rb 5-HT$_{1B}$ binding affinities (pK_i; 95% cl)			*h 5-HT$_{1B}$ binding affinities (pK_i; 95% cl)*	
Cell type	*C6-glial*	*C6-glial*	*Cos-7*	*Cos-7*	*C6-glial*
Radioligand	*[^{3}H]-5-CT*	*[^{3}H]-GR 125,743*	*[^{3}H]-5-CT*	*[^{3}H]-5-CT*	*[^{3}H]-GR 125,743*
5-CT	9.53 (9.52–9.53)	9.24 (9.06–9.41)	9.38*		8.96 (8.62–9.30)
5-HT	8.89 (8.82–8.95)	8.52 (8.38–8.65)	8.92*		8.48 (8.04–8.91)
Zolmitriptan	8.53 (8.46–8.60)	8.28 (8.12–8.44)	8.33*		8.65 (8.51–8.80)
Naratriptan	8.57 (8.47–8.66)	8.21 (8.20–8.21)	8.40*		8.93 (8.92–8.95)
Rizatriptan	7.85 (7.84–7.86)	7.50 (7.41–7.59)	7.68*		7.74 (7.66–7.82)
Sumatriptan	7.93 (7.89–7.97)	7.54 (7.36–7.71)	7.59*		8.12 (8.08–8.16)
R(+)-8-OH-DPAT	7.18 (7.10–7.26)	7.01 (6.98–7.04)	6.84*	6.53 (6.14–6.91)	–
(±)-Cyanopindolol	7.31 (7.26–7.35)	7.19	7.00 (6.90–7.09)	6.97 (6.83–7.12)	–
Methiothepin	8.85 (8.70–8.99)	9.32	8.56*		8.53 (8.47–8.59)
GR 127,935	9.51 (9.43–9.59)	9.74 (9.73–9.76)	9.79 (9.75–9.83)		9.37 (9.36–9.37)
Ketanserin	6.29 (6.20–6.38)	6.91 (6.74–7.08)	6.60 (6.39–6.82)		<5

Radioligand binding was performed with 0.3 nM [^{3}H]-5-CT or [^{3}H]-GR 125,743 as described in Methods. rb 5-HT$_{1B}$ receptor: C6-glial cells: [^{3}H]-5-CT total binding: 1924±123 d.p.m., non-specific binding: 56±30 d.p.m., C6-glial cells: [^{3}H]-GR 125,743 total binding: 3085±124 d.p.m., non-specific binding: 146±24 d.p.m., Cos-7 cells: [^{3}H]-5-CT total binding: 2361±58 d.p.m., non-specific binding: 91±22 d.p.m., h 5-HT$_{1B}$ receptor: C6-glial cells: [^{3}H]-GR 125,743 (K_d:0.12±0.03 nM, B$_{max}$: 538±120 fmol mg^{-1} protein, $n=3$), total binding: 4465±212 d.p.m., non-specific binding: 206±43. *Values were taken from Wurch *et al.* (1996a).

finity with [^{3}H]-GR 125,743 as a radioligand, whereas slightly lower (1.3 to 2.4 fold) affinities were measured for the other compounds tested with this radioligand compared to [^{3}H]-5-CT. This is further illustrated in Figure 1. The [^{3}H]-5-CT binding data of transfected C6-glial cells are in agreement with those measured in Cos-7 cells transfected with a rb 5-HT$_{1B}$ receptor gene (Table 1). With the exception of ketanserin, similar binding affinities were found for these compounds determined with [^{3}H]-GR 125,743 in C6-glial cells stably transfected with a h 5-HT$_{1B}$ receptor gene (Table 1).

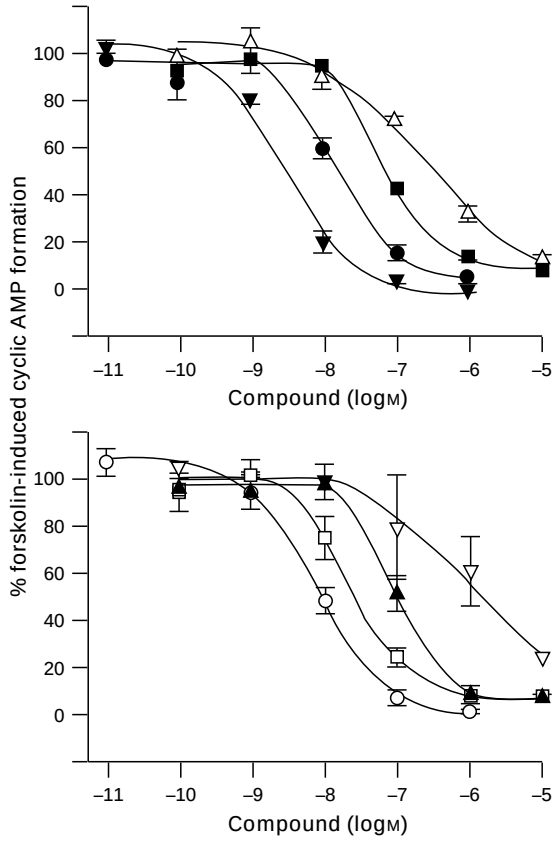

Figure 2 Concentration-response curves of 5-HT receptor ligands for inhibition of stimulated cyclic AMP formation in C6-glial cells stably transfected with a rabbit saphenous vein 5-HT$_{1B}$ receptor gene. Cyclic AMP formation was stimulated with 100 μM forskolin (305 ± 52 pmol/well, $n=6$) and inhibited by 5-HT receptor ligands as described in Methods and results expressed relative to values obtained with 1 μM 5-HT (83 ± 6% inhibition of forskolin-induced cyclic AMP formation, $n=6$). Curves were constructed with mean values ± s.e.mean (vertical lines) from three to six independent experiments, each one performed in triplicate. Mean pEC$_{50}$ values are summarized in Table 1. (a) 5-CT (▼), zolmitriptan (●), rizatriptan (■) and **R**(+)-8-OH-DPAT (△); (b) 5-HT (○), naratriptan (□), sumatriptan (▲) and (±)-cyanopindolol (▽).

Cyclic AMP responses of 5-HT receptor ligands at recombinant rabbit saphenous vein 5-HT$_{1B}$ receptor sites

5-HT produced concentration-dependent [pEC$_{50}$: 8.09 (7.89–8.30)] inhibition of 100 μM forskolin-stimulated cyclic AMP formation with a maximal effect of 83 ± 6% ($n=6$). The compounds 5-CT, zolmitriptan, naratriptan, rizatriptan, sumatriptan and R(+)-8-OH-DPAT inhibited forskolin-stimulated cyclic AMP formation to virtually the same extent as 1 μM 5-HT (Figure 2). Their corresponding potencies and E$_{max}$ values to inhibit cyclic AMP formation versus that of 1 μM 5-HT are summarized in Table 2, and compared with the respective agonist potencies measured at cloned h 5-HT$_{1B}$ receptor sites. 5-CT was the most potent compound, three-times more active than 5-HT. Correlation analysis performed between the agonist potencies at the cloned rb 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptor subtype shows a positive significant correlation ($r^2=0.87$; $P<0.002$). (±)-Cyanopindolol inhibited forskolin-stimulated cyclic AMP formation with a maximal effect of 76% at 10 μM; its EC$_{50}$/K_i ratio was between 32 to 43. Methiothepin, GR 127,935 and ketanserin (each at 10 μM) did not inhibit the stimulated cyclic AMP formation. In contrast, they antagonized rb 5-HT$_{1B}$ receptor-mediated cyclic AMP responses. Figure 3 shows the concentration-dependent antagonism of naratriptan or sumatriptan-induced inhibition of cyclic AMP formation. Parallel displacement of the concentration-effect curves of naratriptan, without depression of the maximum responses, was observed with 0.1 to 1 μM methiothepin, 0.01 to 0.1 μM GR 127,935, and 1 to 10 μM ketanserin, respectively. The calculated pK_B values for methiothepin (100 nM, $n=4$), GR 127,935 (10 nM, $n=4$) and ketanserin (1 μM, $n=4$) are 8.32 (8.14–8.50), 8.14 (8.10–8.71) and 7.05 (6.78–7.32), respectively. A similar result [(pK_B value: 6.79 (6.50–7.07)] was obtained for ketanserin (1 μM, $n=2$) with the agonist sumatriptan (Figure 3d).

Discussion

Analysis of the rabbit saphenous vein 5-HT$_{1B}$ receptor gene sequence was undertaken in order to clarify nucleotide sequence divergences between our previously obtained sequence (Wurch *et al.*, 1996a) and the rb 5-HT$_{1B}$ receptor gene sequence of Harwood *et al.* (1995). The present rabbit saphenous vein 5-

Table 2 pEC$_{50}$ values of 5-HT receptor ligands for inhibition of forskolin-induced cyclic AMP formation in C6-glial cells stably transfected with a recombinant rb 5-HT$_{1B}$ or h 5-HT$_{1B}$ receptor gene

	rb 5-HT$_{1B}$		*h 5-HT$_{1B}$*
	Cyclic AMP, pEC$_{50}$ (95% cl)	*E$_{max}$ (%)*	*Cyclic AMP, pEC$_{50}$ (95% cl)*
5-CT	8.49 (8.42–8.56)	101 ± 1.0	8.55*
5-HT	8.09 (7.89–8.30)	100 ± 1.0	7.81 (7.60–8.02)
Zolmitriptan	7.83 (7.67–7.99)	95.8 ± 1.4	7.51 (7.30–7.73)
Naratriptan	7.50 (7.36–7.64)	93.5 ± 2.7	7.77**
Rizatriptan	7.16 (7.00–7.32)	93.0 ± 2.0	6.94 (6.73–7.14)
Sumatriptan	6.96 (6.76–7.16)	94.0 ± 2.0	7.29*
R(+)-8-OH-DPAT	6.42 (6.34–6.51)	89.0 ± 2.7	6.38***
(±)-Cyanopindolol	5.68	76.0	<5*
Methiothepin	<5	−8.5 ± 7.5	<5*
GR 127,935	<5	23.5 ± 12.5	<5*
Ketanserin	<5	−1.0 ± 7.8	<5*

Inhibition of 100 μM forskolin-induced cyclic AMP formation in stably transfected glial cell lines was measured as described in Methods. Values are taken from 3 to 5 independent experiments, each one performed in triplicate. E$_{max}$ values (means ± s.e.mean) are expressed versus maximal inhibition obtained by 1 μM 5-HT. Values were taken from *Pauwels *et al.* (1996); **Pauwels & Colpaert (1996a); ***Pauwels & Colpaert (1996b).

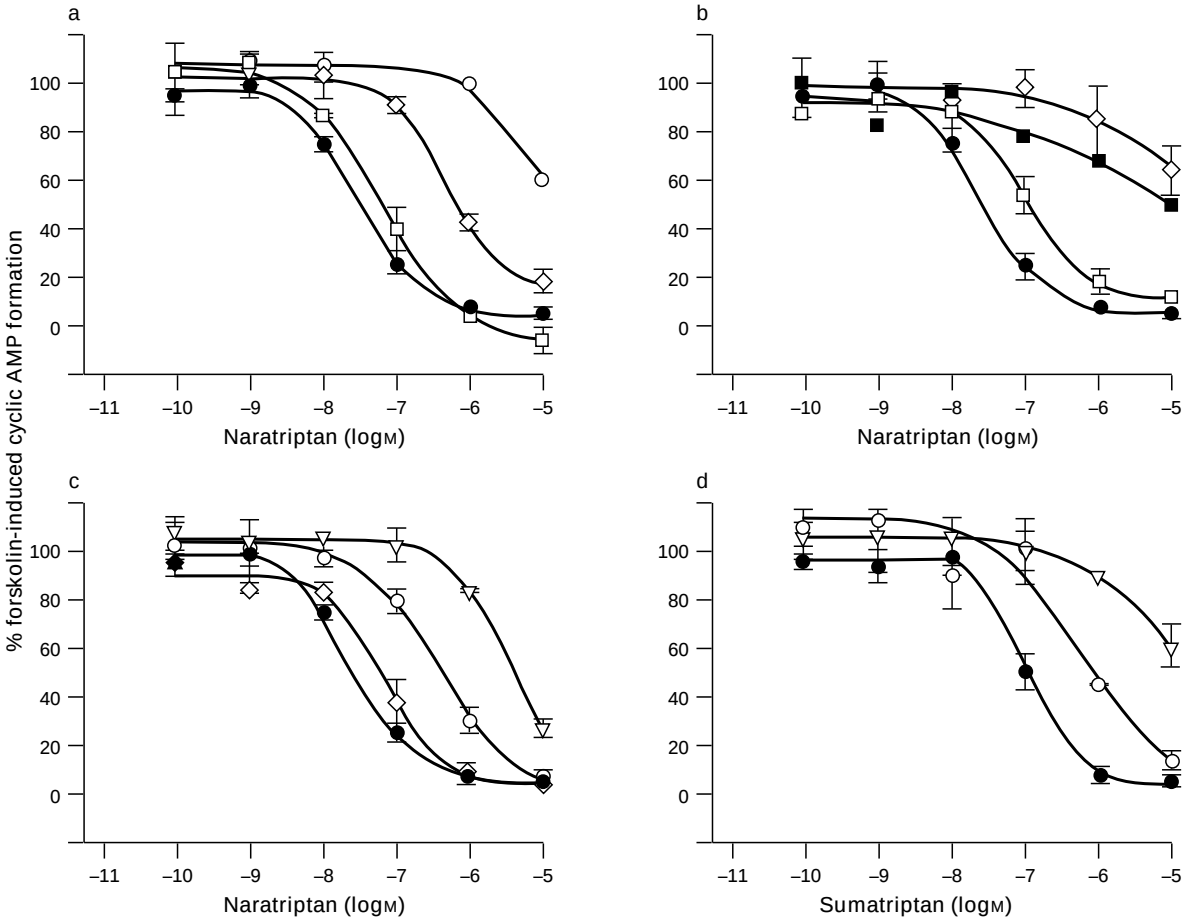

Figure 3 Antagonist effects of methiothepin, GR 127,935 and ketanserin against naratriptan- or sumatriptan-mediated inhibition of forskolin-stimulated cyclic AMP formation in C6-glial cells stably transfected with a rabbit saphenous vein 5-HT$_{1B}$ receptor gene. Cells were preincubated with antagonist for 15 min and subsequently exposed for 5 min to 100 μM forskolin and the indicated concentrations of naratriptan (pEC$_{50}$: 8.57 (8.47–8.66, $n=6$) or sumatriptan (pEC$_{50}$: 7.93 (7.98–7.97), $n=5$) in the absence (●) or presence of increasing concentrations of either methiothepin [a; 10 nM, pEC$_{50}$: 7.2 (6.81–7.59) ($n=2$); 100 nM, pEC$_{50}$: 6.14 (6.05–6.24) ($n=3$); 1000 nM, pEC$_{50}$: <5 ($n=1$)]; GR 127,935 [b; 10 nM, pEC$_{50}$; 6.94 (6.60–7.28) ($n=4$); 30 nM, pEC$_{50}$: 5 ($n=1$); 100 nM, pEC$_{50}$: <5 ($n=4$)] or ketanserin [c; 100 nM, pEC$_{50}$: 7.17 (6.89–7.44) ($n=4$); 1000 nM, pEC$_{50}$: 6.40 (6.14–6.67) ($n=4$); 10000 nM, pEC$_{50}$: 5.36 (5.21–5.52) ($n=4$) and d; 1000 nM, pEC$_{50}$: 6.07 (6.02–6.12) ($n=4$); 10000 nM. pEC$_{50}$: <5 ($n=2$)]. Inhibition of forskolin-induced cyclic AMP formation is expressed as a percentage of that obtained with 1 μM 5-HT. Concentration-response curves were constructed with mean values ± s.e.mean (vertical lines) from a single to four independent experiments, each one performed in triplicate. Symbols represent (□) 0.01, (■) 0.03, (◇) 0.1, (○) 1 and (▽) 10 μM of antagonist.

HT$_{1B}$ receptor is 100% identical at the amino acid level to the rb 5-HT$_{1B}$ receptor clone of Harwood *et al.* (1995). Two nucleotide differences were observed (A$_{798}$ to T and C$_{1147}$ to T). They may represent silent genetic variations in the rb 5-HT$_{1B}$ receptor gene, as has already been shown for the h 5-HT$_{1B}$ receptor gene (Nöthen *et al.*, 1994).

The cyclic AMP results demonstrate stable and functional expression of recombinant rb saphenous vein 5-HT$_{1B}$ receptors in rat C6-glial cells. This 5-HT$_{1B}$ receptor is negatively coupled to cyclic AMP formation as described for the cloned h 5-HT$_{1B}$ receptor (Weinshank *et al.*, 1992). The magnitude of receptor expression and the amount of inhibition of forskolin-stimulated cyclic AMP formation by 5-HT was similar for both rb 5-HT$_{1B}$ (this study) and h 5-HT$_{1B}$ receptors (Pauwels & Colpaert, 1995) expressed in C6-glial cells. Under these conditions, a similar pharmacological profile was obtained with the 5-HT receptor agonists being studied (i.e.; 5-CT, 5-HT, zolmitriptan, naratriptan, rizatriptan, sumatriptan and **R** (+)-8-OH-DPAT), as well as with the non-selective 5-HT receptor antagonist methiothepin and the 5-HT$_{1B}$/5-HT$_{1D}$ receptor antagonist GR 127,935. This was evident from both binding and cyclic AMP data. The pK_B values of methiothepin and GR 127,935 were close to their binding affinities for the rb 5-HT$_{1B}$ receptor. Furthermore, these values fit the reported blocking potencies of methiothepin (pA$_2$: 8.25 to 9.4; Martin *et al.*, 1990; Van Heuven-Nolsen *et al*,. 1990; Valentin *et al.*, 1996) and GR 127,935 (pA$_2$: 9.0 to 9.4; Razzaque *et al.*, 1995; Valentin *et al.*, 1996) on 5-HT and/or sumatriptan-mediated contraction of rabbit isolated saphenous vein segments. The moderate binding affinity of (±)-cyanopindolol for rb 5-HT$_{1B}$ receptors together with its micromolar affinity for CP 93129 (Wurch *et al.*, 1996a) and the presence of a Thr-354 in transmembrane domain VII (Harwood *et al.*, 1996; Wurch *et al.*, 1996a) strongly suggest that the pharmacology of the rabbit saphenous vein 5-HT$_{1B}$ receptor is related more to the h 5-HT$_{1B}$ than to the rodent 5-HT$_{1B}$ receptor subtype (Guan *et al.*, 1992; Oksenberg *et al.*, 1992; Adham *et al.*, 1994).

Interestingly, rabbit saphenous vein 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors appear to have a different affinity for the 5-HT$_2$ receptor antagonist ketanserin. This apparently silent antagonist at cloned rb 5-HT$_{1B}$ receptors is up to 40-times more potent than at cloned h 5-HT$_{1B}$ receptors (pK_B: 5.43 to 5.74; Zgombick *et al.*, 1995; Pauwels & Colpaert, 1996a). The rabbit pK_B value found for ketanserin is in accordance with its respective binding affinity of two different transfected cell types (Cos-7 and C6-glial) and two radioligands ([^{3}H]-5-CT and [^{3}H]-GR 125,743). In addition, this pK_B value was found to fit the pA$_2$ value of ketanserin (pA$_2$: 7.51 to 7.76) measured with 5-HT

and/or sumatriptan-mediated contraction of isolated rabbit saphenous vein segments (Martin *et al*., 1990; Van Heuven-Nolsen *et al*., 1990; Razzaque *et al*., 1995). Nevertheless, the ketanserin binding affinity and antagonist potency differed from the low binding affinity of ketanserin (pIC_{50}: 5.4) for cloned rb 5-HT$_{1B}$ receptor sites obtained by Harwood *et al*. (1995). We have no explanation for this binding difference assuming that the same 5-HT$_{1B}$ receptor sequence was transfected in the same host cell. The latter authors also cloned the rb 5-HT$_{1D}$ receptor gene which shows a higher binding affinity for ketanserin (pIC_{50}: 7.4). Ketanserin seems to show various affinities for 5-HT$_{1B}$/5-HT$_{1D}$ receptor sites depending on the species that is being investigated. It is of interest that ketanserin is unable to differentiate between the dog (ca) 5-HT$_{1D}$ and ca 5-HT$_{1B}$ receptor subtypes and that it has low affinity for them (pK_i of 5.5 and 5.3, respectively; Branchek *et al*., 1995). In contrast, ketanserin has binding affinity for and is apparently a silent antagonist at cloned h 5-HT$_{1D}$ (pK_B: 7.28 to 7.76), rat (r) 5-HT$_{1D}$ (pK_B: 7.92) and guinea-pig (gp) 5-HT$_{1D}$ (pK_B: 7.51) receptor sites (Zgombick *et al*., 1995; Pauwels & Colpaert., 1996a,b; Wurch *et al*., 1996b). Its low affinity for h 5-HT$_{1B}$ and r 5-HT$_{1B}$ (pK_i: <5; Van Wijngaarden *et al*., 1990) receptor sites demonstrates ketanserin only differentiates between 5-HT$_{1D}$ and 5-HT$_{1B}$ receptor subtypes from man and rat. Sequence analysis of these various 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes does not immediately suggest a particular amino acid domain explaining this differential ketanserin binding affinity. However, it should be noted that the highly basic arginine residue at the 5′ end of transmembrane domain IV of the ca 5-HT$_{1D}$ receptor as well as the rb 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors is replaced by a weakly basic histidine residue in the h 5-HT$_{1D}$ receptor. It also appears that the non-polar valine or isoleucine residues found in transmembrane domain IV of the ca 5-HT$_{1D}$, rb 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors are replaced by an unchanged polar threonine residue in the h 5-HT$_{1D}$ receptor. Construction of chimaeric receptors and site-directed mutagenesis experiments may provide further insight into the molecular basis for the observed ketanserin-5-HT$_{1B}$/5-HT$_{1D}$ receptor interactions. Hoyer *et al*. (1994) considered the blocking potency of ketanserin to be a species-specific characteristic of rb 5-HT$_1$-like receptors that mediate contraction. We cannot exclude, at the present time, the involvement of 5-HT$_{1D}$ receptors in the contraction of rabbit saphenous vein. Preliminary RT-PCR experiments suggest at least the presence of 5-HT$_{1D}$ receptor mRNA in the rabbit saphenous vein RNA preparation (unpublished results). The rb 5-HT$_{1B}$ receptor is likely to be involved in this contraction and appears to be sensitive to ketanserin blockade.

In conclusion, the recombinant saphenous vein 5-HT$_{1B}$ receptor of the rabbit shares strong pharmacological similarities with the cloned h 5-HT$_{1B}$ receptor, although ketanserin is a more potent antagonist at rabbit saphenous vein 5-HT$_{1B}$ receptors. This study also confirms that the receptor characteristics for a ligand may vary considerably between species.

Note added in proof

During the review of this manuscript another publication has appeared on the cloning of the rabbit 5-HT$_{1B}$ receptor gene (J.A. Bard *et al*. (1996), *Naunyn-Schmiedeberg's Arch. Pharmacol*., **354,** 237–244).

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