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Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT_{1B} receptor

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1 Using a combination of RT–PCR and inverse-PCR techniques, we amplified, cloned and sequenced a full-length porcine 5-HT_{1B} receptor cDNA derived from porcine cerebral cortex. Sequence analysis revealed 1170 bp encoding an open reading frame of 390 amino acids showing a 95% similarity with the human 5-HT_{1B} receptor.

2 The recombinant porcine 5-HT_{1B} cDNA was expressed in monkey Cos-7 cells and its pharmacological profile was determined by radioligand binding assay using [^{3}H]-GR125743. The affinities of several agonists (L694247 > ergotamine $\geqslant$ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan) and putative antagonists (GR127935 > methiothepin > SB224289 > > ritanserin > ketanserin $\geqslant$ BRL15572) correlated highly with those described for the recombinant human 5-HT_{1B} receptor.

3 In membranes obtained from cells co-expressing the porcine 5-HT_{1B} receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein, 5-HT and zolmitriptan increased, while the 5-HT_{1B} receptor antagonist SB224289 decreased basal [^{35}S]-GTPγS binding, thus showing inverse agonism. The potency of zolmitriptan in the [^{35}S]-GTPγS binding assay (pEC_{50}: 7.64 ± 0.04) agreed with its affinity in displacing the antagonist [^{3}H]-GR125743 (pK_i: 7.36 ± 0.07).

4 The 5-HT_{1B} receptor mRNA was observed by RT-PCR in several blood vessels, cerebral cortex, cerebellum and trigeminal ganglion. *In situ* hybridization performed in frontal cerebral cortex sections revealed the expression of 5-HT_{1B} receptor mRNA in pyramidal cells.

5 In conclusion, we have cloned and established the amino acid sequence, ligand binding profile and location of the porcine 5-HT_{1B} receptor. This information may be useful in exploring the role of 5-HT_{1B} receptor in pathophysiological processes relevant for novel drug discovery in diseases such as migraine.

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Abbreviations: 5-HT, 5-Hydroxytryptamine; BRL15572, [1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl)piperazine] hydrochloride; CP122638, N-methyl-3-[pyrrolidin-2(R)-ylmethyl]-1H-indol-5-ylmethyl sulphonamide; GR125743, N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide; GR127935, (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 hydrochloride; L694247, 2-[5-[3-(4-methylsulphonylamino) benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine; RT-PCR, Reverse transcription polymerase chain reaction; SB224289, 2,3,6,7-tetrahydro-1′-methyl-5-[2′-methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carbonyl] furo [2,3-f] indole-3-spiro-4′-piperidine hydrochloride

Introduction

Over a decade and a half ago, Bradley *et al*. (1986) provided a general framework for the classification and nomenclature of 5-hydroxytryptamine (5-HT; serotonin) receptors recognising three main types: '5-HT_1-like', 5-HT_2 and 5-HT_3 receptors. The term '5-HT_1-like' represented a heterogeneous group of receptors mediating a variety of responses, including the contraction of cranial blood vessels, prejunctional inhibition of neuronal transmitter release, smooth muscle relaxation and tachycardia in the cat. The antimigraine drug sumatriptan was described as a selective and potent agonist at 5-HT_1-like receptors (Humphrey *et al*., 1988; 1989). However, subsequent studies showed that sumatriptan had a poor affinity for receptors mediating smooth muscle relaxation and tachycardia in the cat (now called 5-HT_7 receptors, Villalón *et al*., 1997; Saxena *et al*., 1998; De Vries *et al*., 1999a), but possessed a high affinity for 5-HT_{1D} recognition sites (Waeber *et al*., 1989; Hoyer *et al*., 1990). Molecular biological studies revealed that 5-HT_{1D} recognition sites consisted of two distinct receptors, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (Weinshank *et al*., 1992) that, on alignment with the human genome, were renamed 5-HT_{1D} and 5-HT_{1B} receptors, respectively (Hartig *et al*., 1996). Interestingly, the pharmacology of the human 5-HT_{1B} and 5-HT_{1D} receptors is much

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closer than that of the human and rat 5-HT$_{1B}$ receptors; the difference in the pharmacological profile of the two 5-HT$_{1B}$ receptors is due to just one amino acid in the seventh transmembrane domain (T355N, Metcalf *et al*., 1992; Oksenberg *et al*., 1992; Hoyer *et al*., 1994). In particular, some β-adrenoceptor antagonists are more potent, while sumatriptan is less potent at the rodent than at the human 5-HT$_{1B}$ receptor (Hoyer *et al*., 1994).

Sumatriptan as well as other triptans inhibit dural plasma protein extravasation, suppress action potentials in trigeminal nucleus caudalis, constrict isolated cranial blood vessels and decrease carotid arteriovenous anastomotic blood flow in anaesthetized animals (Moskowitz, 1992; Goadsby & Knight, 1997; Saxena & Tfelt-Hansen, 2000). Although the trigeminal neural effects of triptans, mediated mainly by the 5-HT$_{1D}$ receptor, may be involved to some extent in their anti-migraine action (De Vries *et al*., 1999b; Hargreaves & Shepheard, 1999), the efficacy of triptans is primarily attributed to the 5-HT$_{1B}$ receptor-mediated cranial vasoconstriction (De Vries *et al*., 1999b; Saxena & Tfelt-Hansen, 2000). Previous investigations from our laboratory have established that constriction of carotid arteriovenous anastomoses in the anaesthetized pig can serve as a predictive model for the antimigraine efficacy of 5-HT-based drugs (Saxena, 1995; De Vries *et al*., 1999b). To gain further insight into the mechanisms involved in drug actions as well as the disease, it is important to study the trigeminal neural control of porcine arteriovenous anastomoses and its potential modification by 5-HT receptor ligands. However, one of the difficulties in undertaking such studies is the limited knowledge of the molecular biology of porcine 5-HT receptors. We recently cloned the porcine 5-HT$_{1D}$ receptor and found that its amino acid sequence and ligand binding profile were very similar to that of the human 5-HT$_{1D}$ receptor, but a striking exception was the low affinity of BRL15572 ([1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl)piperazine] hydrochloride) at the porcine (Bhalla *et al*., 2000) compared to human (Price *et al*., 1997; Schlicker *et al*., 1997) receptor.

In the present investigation, we describe the molecular cloning and characterization of the porcine 5-HT$_{1B}$ receptor (receptor code: 2.1.5HT.01B). Using the total RNA isolated from the pig cerebral cortex, a full-length cDNA encoding 5-HT$_{1B}$ receptor was PCR-amplified and the deduced amino acid sequence was compared with that in other species. The ligand binding profile of the porcine 5-HT$_{1B}$ receptor was evaluated after transient transfection in Cos-7 cells and its distribution in the porcine tissues was explored using reverse transcription polymerase chain reaction (RT–PCR) and *in situ* hybridization.

Methods

RNA isolation and RT–PCR

As described in detail previously (Bhalla *et al*., 2000), total RNA was extracted from a pig (Yorkshire × Landrace, female, 12 kg) cerebral cortex (Chomczynski & Sacchi, 1987; Sharma *et al*., 1996) and processed for the purification of poly(A$^+$) mRNA using an Oligotex mRNA purification Kit (Qiagen GmbH, Hilden, Germany). Poly(A$^+$) mRNA (0.5 μg) was denatured at 65°C and the first strand of cDNA was synthesized in a reaction volume of 20 μl by adding sequentially the following reagents: reverse transcription buffer (in mM: Tris-HCl 25; pH 8.3, KCl 50, $MgCl_2$ 5, DTT 2), dNTPs 1.0, ribonuclease inhibitor (1 u μl^{-1}), random hexamer (150 ng μg^{-1} mRNA) and, finally, AMV reverse transcriptase (14 u μg^{-1} of mRNA; Pharmacia-LKB, Uppsala, Sweden). A parallel control without AMV reverse transcriptase was prepared to check the genomic contamination. The reactions were carried out for 90 min at 42°C, extended for another 10 min at 75°C and then cooled to 4°C. The cDNA thus synthesized was diluted to 50 μl and stored at −20°C until used as a PCR template. The quality of cDNA was checked by PCR amplification of porcine β-actin using human specific oligonucleotide primers (Ponte *et al*., 1984).

Porcine specific 5-HT$_{1B}$ receptor cDNA was amplified using a combination of RT–PCR and inverse-PCR techniques. Initially, we attempted to obtain the full-length 5-HT$_{1B}$ receptor cDNA employing 5′ and 3′ end oligonucleotide primers designed from the consensus sequences of other species (Demchyshyn *et al*., 1992; Maroteaux *et al*., 1992; Weinshank *et al*., 1992; Harwood *et al*., 1995; Zgombick *et al*., 1997). Unlike the cloning of porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000), we were unsuccessful in this case and, therefore, an alternative strategy, as depicted in Figure 1, was employed. New forward (5′-CCTGCCCTGGAAAGTAG-TAC-3′; nucleotides 4–23; GenBank accession number Z47984) and reverse (5′-TCAACTTGTGCACTTAAAAC-3′; nucleotides 1772–1791; GenBank accession number M75128) oligonucleotide primers (Figure 1A, 1 and 2) were designed from the porcine (partial) and human 5-HT$_{1B}$ receptor sequence, respectively. For PCR amplification, a 20 μl reaction mixture containing the following components was

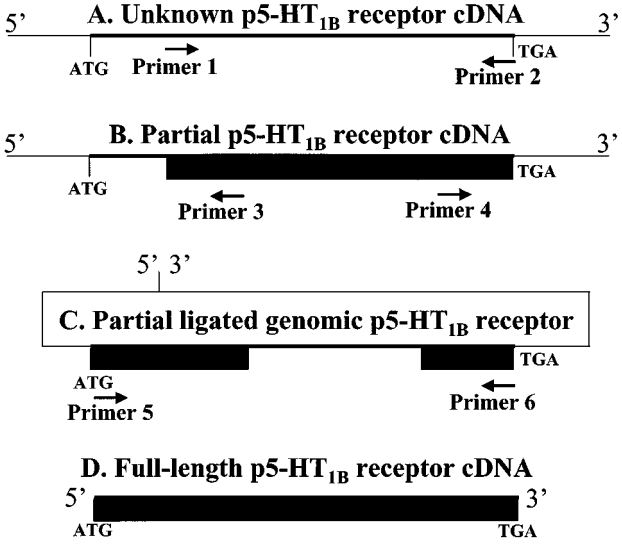

Figure 1 Strategy for cloning full-length porcine 5-HT$_{1B}$ receptor and position of various primers used for PCR amplification. (A) Unknown full-length porcine 5-HT$_{1B}$ receptor cDNA with primers derived from porcine (1) and human (2) nucleotide sequences. (B) The amplified partial sequence of porcine 5-HT$_{1B}$ receptor cDNA was used to design inverse porcine-specific primers (3) and (4). (C) The inverse-PCR amplified partial genomic sequence enabled us to design full-length porcine specific primers (5) and (6). (D) The full-length amplified product of porcine 5-HT$_{1B}$ receptor cDNA. Arrows and black rectangles denote the direction of the primers and amplified products, respectively.

prepared: 250 μM of each dNTP, 1.5 mM $MgCl_2$, PCR buffer: (1 × PCR buffer, 10 mM; Tris-HCl, pH 8.3; 50 mM KCl), Ampli Taq Gold™ enzyme (0.5 u), 0.5 μM each of the forward and reverse primer and 5 μl of cDNA template. After brief centrifugation, the enzyme was first activated for 10 min at 94°C in a PCR thermocycler (model PTC-100™, M.J. Research Inc, Watertown, U.S.A.). The timing of PCR was 1 min at 94°C, 30 s at 55°C and 90 s at 72°C with total of 36 cycles. Finally, the reaction was extended for additional 10 min at 72°C. The PCR amplified product of expected size was purified using a PCR purification kit (Promega Benelux b.v., Leiden, The Netherlands) and ligated into a pCR™ II vector using TA cloning kit (Invitrogen BV., Groningen, The Netherlands). The ligated vector was transformed into competent TOP10 cells and grown overnight on LB agar plates containing kanamycin (50 μg ml^{-1}) with IPTG and X-gal at 37°C. White over blue colonies were selected to identify positive clones containing the DNA insert of expected size. Three insert positive clones (namely, pHTB-2, pHTB-4, pHTB-19) were further processed for the plasmid DNA isolation (mini-prep, Promega Benelux b.v., Leiden, The Netherlands) and sequenced by the dideoxy nucleotide chain termination method using an automated fluorescence based DNA sequencer (ABI Prism™ 310 Genetic analyser, Perkin Elmer Applied Biosystem Benelux, Nieuwerkerk a/d IJssel, The Netherlands). The nucleotide sequences were compared and a consensus sequence was derived (DNAMAN sequence analysis program, Version 3.2, Lynnon Biosoft© 1994–1997). The final partial cDNA sequence (Figure 1B) was compared with those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, U.S.A.).

Analysis of 5′ and 3′ ends of porcine 5-HT$_{1B}$ receptor by Inverse-PCR

Inverse-PCR was performed to establish the porcine specific sequence of 5′ and 3′ ends (Ochman *et al*., 1988). Porcine genomic DNA was digested with *Bgl* II restriction enzyme, because the cloned human 5-HT$_{1B}$ receptor cDNA did not show any restriction site for this enzyme. After purification, the restricted DNA was ligated overnight at 16°C in the presence of T_4-DNA ligase in order to obtain DNA circles. The ligated DNA circles were subjected to inverse-PCR using primers specific for porcine 5-HT$_{1B}$ receptor (5′-GAGGC-GATCAGGTAGTTGGC-3′ for 5′ end and 5′-GATGCC-TGCTGGTTCCACC-3′ for 3′ end; Figure 1B, 3 and 4). The amplified products were separated on a 1% agarose gel, purified, cloned and sequenced, as described above.

Amplification and cloning of full-length porcine 5-HT$_{1B}$ receptor

For the amplification of full-length porcine 5-HT$_{1B}$ receptor cDNA, forward and reverse oligonucleotide primers (5′-ATGGAGGAAGCGGGCGCTCAG-3′ and 5′-TCAGCT-TGTGCACTTAAAGCG-3′) were designed from the sequences generated from inverse-PCR (Figure 1C, 5 and 6). After PCR amplification (see above), a product of expected size was purified, ligated into the pGEMT-Easy vector (Promega Benelux b.v., Leiden, The Netherlands), transformed into competent JM 109 cells and plasmid DNA was isolated. Four insert positive clones (pHTB-11, pHTB-12, pHTB-14, pHTB-17) were further processed for the plasmid DNA purification and sequencing. The full-length cDNA sequence of porcine 5-HT$_{1B}$ receptor was derived from two independent PCR amplified products and further verified by multiple partial sequences derived from cDNA (RT–PCR) as well as genomic DNA (inverse-PCR) amplified products. In sporadic cases showing nucleotide discrepancy in the sequence, the nucleotide having a clear majority in clones was preferred for establishing the final full-length cDNA sequence, using the DNAMAN sequence analysis program (Version 3.2, Lynnon Biosoft© 1994–1997). The final sequence (Figure 1D) was translated as a peptide sequence and compared with those in the GenBank (BLAST search at National Centre for Biotechnology Information, Bethesda, MD, U.S.A.). The hydrophobic regions (indicating putative transmembrane domains) and sequence homology with known 5-HT$_{1B}$ receptors from other species were established.

Transient transfection and radioligand binding assay

The purified full-length 5-HT$_{1B}$ receptor cDNA insert was subcloned into dephosphorylated eukaryotic expression vector, pcDNA3 and transformed into TOP10 competent cells. The plasmid DNA was purified according to maxi prep protocol using a commercially available kit (Qiagen SA, Courtaboeuf, France). Monkey Cos-7 cells were transiently transfected with the plasmid using a gene pulser transfection apparatus (Bio-Rad S.A., Ivry Sur Seine, France), as described earlier by Pauwels *et al*. (1996). After transfection, the cells were incubated for 48 h in Dulbecco modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal calf serum and antibiotics at 37°C in a humidified chamber containing 5% CO_2.

The transfected cells were washed twice with phosphate buffer saline (in mM: KCl 2.7, KH_2PO_4 1.5, NaCl 140, Na_2HPO_4 8; pH 7.2), and kept at −80°C for 10 min. The cells were scrapped from the petri-dish in ice-cold Tris-buffer (pH: 7.7) and homogenized. The homogenate was centrifuged at 1000 r.p.m. for 5 min at 4°C and the supernatant was collected and centrifuged again at 13,000 r.p.m. for 20 min. The membrane pellet was resuspended into 50 mM Tris-HCl buffer (pH: 7.7) containing 4 mM $CaCl_2$, 10 μM pargyline and 0.1% ascorbic acid, as described before (Pauwels & Colpaert, 1996). The membrane protein concentration was measured by dye binding assay (Bradford, 1976) using the Bio-Rad Kit and bovine serum albumin was used as a standard.

Binding assays to membranes obtained from transfected Cos-7 cells were performed using 1.0 nM [^{3}H]-GR125743 ([^{3}H]-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide) as radioligand. Incubation mixtures consisted of 0.4 ml of cell membrane preparation (30–50 μg of protein), 0.05 ml of the radioligand and 0.05 ml of compounds for inhibition or 10 μM 5-HT to determine non-specific binding. The reaction was terminated by filtration with ice-cold Tris-buffer and radioactivity on the filter paper was measured by using a liquid scintillation counter (Pauwels *et al*., 1996; Wurch *et al*., 1997). The filtration was performed over 0.2% polyethyleneimine-treated Whatman (Clifton, NJ, U.S.A.) GF/B glass fibre filters. Data were analysed graphically with inhibition curves, and IC_{50} values were derived. K_i values were calculated according to the equation

$K_i = IC_{50}/(1 + C/K_D)$, where C is the concentration and K_D is the equilibrium dissociation constant of the radioligand. Ligand saturation binding curves were analysed by the nonlinear least square curve-fitting programme to determine K_d and B_{max} values (Munson & Rodbard, 1980). Control binding experiments were run with nontransfected cells and they did not display specific [^{3}H]-GR125743 binding.

[^{35}S]-GTPγS binding response

CHO-K1 cells transiently co-expressing the cloned porcine 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein (Dupuis *et al*., 1999) were collected in phosphate-buffered-saline (pH 7.4) and centrifuged for 20 min at 48,000 × *g* and the pellet containing the membrane fraction was stored at −80°C. [^{35}S]-GTPγS binding was measured using the method previously described by Pauwels *et al*. (1997). Briefly, the pellet was thawed and diluted in 20 mM HEPES buffer (pH 7.4) containing (in mM): GDP 0.03, NaCl 100, $MgCl_2$ 3 and ascorbic acid 0.2. Incubation mixtures were prepared in glass tubes and consisted of 0.4 ml of membrane preparation (containing 5 μg protein) with 5-HT (10 μM), SB224289 (2,3,6,7-tetrahydro-1′-methyl-5-[2′-methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carbonyl] furo [2,3-f] indole-3-spiro-4′-piperidine hydrochloride; 1 μM) or zolmitriptan (0.1 nM–10 μM) in a volume of 0.05 ml. After an incubation period of 30 min at 25°C, 0.05 ml [^{35}S]-GTPγS (0.5 nM) was added for an additional period of 30 min. The reaction was stopped by adding 3 ml of ice-cold 20 mM HEPES (pH 7.4) containing 3 mM $MgCl_2$ and rapid filtration over Whatmann GF/B glass fibre filters with a Brandel harvester. The filters were rinsed three additional times with 3 ml HEPES buffer, placed in scintillation vials and the radioactivity was extracted in 4 ml of Emulsifier-Safe. Maximal stimulation of [^{35}S]-GTPγS binding was defined in the presence of 10 μM 5-HT. E_{max} values were expressed as a percentage of the maximal response obtained with 10 μM 5-HT. EC_{50} values were defined as the concentration of compound at which 50% of its own maximal stimulation was obtained.

Detection of 5-HT$_{1B}$ receptor mRNA by RT–PCR

RT–PCR was used to detect 5-HT$_{1B}$ receptor mRNA in the following tissues obtained from pigs (Yorkshire × Landrace, female, 12–15 kg): brain cortex, cerebellum, trigeminal ganglion, left cardiac ventricle, left anterior descending coronary, pulmonary, common carotid, superior mesenteric and femoral arteries and saphenous vein. The tissue samples were dissected and cleaned and the total RNA was isolated as described above. The residual DNA contamination was removed by treatment with RNase-free DNase (10 u 6 μg^{-1} RNA) for 25 min at 37°C as per instruction (Promega Benelux b.v., Leiden, The Netherlands). The purified total RNA samples were reverse transcribed into cDNA in presence of reverse transcriptase enzyme. A control reaction was always prepared in absence of reverse transcriptase to monitor the DNA contamination. For the PCR amplification of porcine 5-HT$_{1B}$ receptor, porcine specific sense (5′-CCTGCCCTGGAAAGTAGTAC-3′, nucleotides 135–154) and antisense (5′-TGATGGGCATCACCAGGATG-3′, nucleotides 297–316) primers were used along with other components of PCR exactly as described earlier, except that the annealing temperature was kept at 60°C instead of 55°C. The PCR amplified products (12 μl each) were separated on 3% agarose gel by electrophoretic separation and photographed.

Localization of 5-HT$_{1B}$ receptor mRNA by in situ *hybridization*

A piece of frontal cerebral cortex, obtained from a pig (Yorkshire × Landrace, female, 14 kg), was fixed using phosphate buffer saline containing 4% paraformaldehyde. After dehydration and embedding in paraffin, 5 μm thick sections were cut (Microtome, Microm Type HM325, Walldorf, Germany) and layered on superfrost plus® glass slides (Menzel–Glaser, Braunschweig, Germany). For *in situ* hybridization, a digoxigenin-labelled cRNA probe was prepared from recombinant porcine 5-HT$_{1B}$ receptor cDNA containing 470 bp (nucleotides 135–695). The cloned plasmid DNA was linearized with either *Bam*HI or *Xba*I, purified and transcribed to synthesise sense and antisense cRNA probes, using T_7 or SP_6 RNA polymerase. The cRNA probes were quantified by dot blotting as per protocol described for DIG RNA labelling kit (Roche Diagnostics Nederland B.V., Almere, The Netherlands). *In situ* hybridization was performed following the method described by de Boer *et al*. (1998). The purple coloured hybrids representing the 5-HT$_{1B}$ receptor mRNA in the tissue sections was visualized under a light microscope (Leica DM RBE, GmbH, Wetzlar, Germany).

Materials

All chemicals used in this study were of molecular biology and/or culture grade. The oligonucleotide primers were commercially procured from Life Technologies b.v. (Breda, The Netherlands) and the sources of the kits used have been identified in the text.

The compounds used in pharmacological assays were: 5-HT creatinine sulphate (Sigma Chemicals, St. Louis, MO, U.S.A.), BRL15572 (gift: Dr A.A. Parsons, SmithKline Beecham Pharmaceuticals, Harlow, Essex, U.K.), 5-carboxamidotryptamine, CP122638 (N-methyl-3-[pyrrolidin-2(R)-ylmethyl]-1H-indol-5-ylmethyl sulphonamide), [^{3}H]-GR125743 (83.0 Ci mmol^{-1}; Amersham, Les Ulis, France), GR127935 ((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 hydrochloride, [^{35}S]-GTPαS (1000 Ci mmol^{-1}; Amersham, Les Ulis, France), ketanserin (Sigma Chemicals, St. Louis, MO, U.S.A.), L694247 (2-[5-[3-(4-methylsulphonylamino) benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl] ethylamine; Tocris Cookson, Bristol, U.K.), methiothepin, ritanserin, sumatriptan, SB224289 and zolmitriptan. Except those specified above, all other compounds were synthesized at Centre de Recherche Pierre Fabre (Castres, France).

Results

Cloning of 5-HT$_{1B}$ receptor cDNA derived from porcine cerebral cortex

As described in Methods (Figure 1), using RT–PCR technique, we amplified a partial fragment (1038 bp) from

porcine brain cortex cDNA. Upon sequencing, this fragment revealed a high homology with the human 5-HT$_{1B}$ receptor (data not shown). On the basis of this porcine specific partial sequence of 5-HT$_{1B}$ receptor, inverse primers were designed and used on porcine ligated genomic DNA to establish the sequence of 5′ and 3′ ends of the porcine 5-HT$_{1B}$ receptor (Figure 2).

To amplify the full-length porcine 5-HT$_{1B}$ receptor, the cDNA template derived from poly(A$^+$) mRNA of porcine brain cortex was used with porcine specific 5′ and 3′ end primers. Agarose gel electrophoresis of RT–PCR products (Figure 3) shows amplification of the porcine β-actin cDNA (625 bp), which ensures that the quality of cDNA samples was adequate for amplification of other products. The presence of genomic DNA contamination was excluded by performing a parallel reaction in the absence of reverse transcriptase. The expected band ($\sim$1200 bp) of porcine cDNA, observed in presence of porcine specific primers, was cloned (Figure 3).

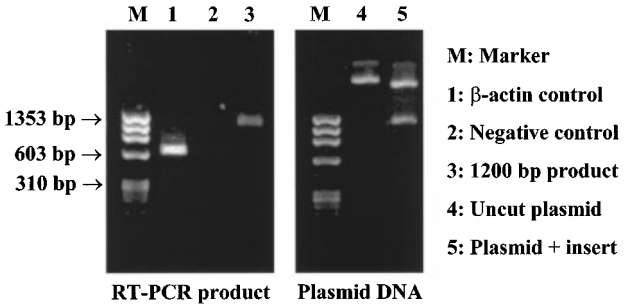

Figure 3 Agarose gel electrophoresis of RT-PCR products of cDNA synthesized from porcine cerebral cortex (left panel) and recombinant plasmid with insert cDNA (right panel). The different lanes marked on top denote: $\phi \times 174$ DNA/*Hae*III marker (M), positive control showing RT–PCR product of 625 bp using β-actin primers (1), negative control, i.e. a sample without reverse transcriptase to monitor genomic and/or PCR contamination (2), RT–PCR product of approximately 1200 bp obtained using forward and reverse primers of porcine specific 5-HT$_{1B}$ receptor (3), recombinant uncut plasmid DNA vector (4) and plasmid DNA vector restricted with *Eco*RI enzyme and showing a DNA insert of approximately 1200 bp (5). The size of three marker bands is indicated in the left margin.

Sequence analysis of cloned porcine cDNA

Sequencing of the recombinant plasmid revealed 1170 bp, starting with ATG codon and ending with TGA codon. DNAMAN analysis showed that this full-length porcine cDNA encoded a 390 amino acid protein exhibiting features of a typical G-protein coupled receptor with predicted seven transmembrane domains and putative N-glycosylation and phosphorylation sites (Figure 4). A BLAST search at GenBank of the 1170 bp nucleotide sequence revealed resemblance with the sequence of 5-HT$_{1B}$ receptors from other species.

Figure 5 compares the amino acid sequence of the porcine 5-HT$_{1B}$ receptor with those of other mammalian species (human, rabbit, mouse, rat and guinea-pig). Across the species, there was an 88–95% similarity in the overall sequence and the transmembrane domains 2–5 in porcine and human receptors were identical. However, it may be noted that some amino acids were unique to the porcine 5-HT$_{1B}$ receptor: Ala[4], Ser[15,21] and Arg[19] towards the N-terminal end, Val[51] and Ile[54] in the first transmembrane domain, Leu[277] in the third intracellular loop, Ala[336] in the sixth transmembrane domain as well as Phe[372] in the seventh transmembrane domain.

Inverse-PCR (5'end)

```
          5'end
1   ATGGAGGAACCGGGTGCTCAGTGCGCTCCACCGCCGCCCGCGGGCTCCGAGACCTGGGTT
142 ATGGAGGAAGCGGGCGCTCAGTGCGCCCCGCCGCTGCCCGCGAGCTCCCAGACCCGGCTT

61  CCTCAAGCCAACTTATCCTCTGCTCCCTCCCAAAACTGCAGCGCCAAGGACTACATTTAC
202 TCTCAAGCCAACCTCTCCGCGGCTCCCTCCCAAAACTGCAGCGCCGAGGGCTACATTTAC

121 CAGGACTCCATCTCCCTACCCTGGAAAGTACTGCTGGTTATGCTATTGGCGCTCATCACC
262 CAGGACTCCATCGCCCTGCCCTGGAAAGTAGTACTGGTCATTCTGCTGGCTCTCTTCACC

181 TTGGCCACCACGCTCTCCAATGCCTTTGTGATTGCCACAGTGTACCGGACCCGGAAACTG
322 TTGGCCACCACGCTCTCCAATGCGTTTGTGATCGCCACTGTGTACCGGACGCGGAAGCTC

241 CACACCCCGGCTAACTACCTGATCGCCTC
382 CATACCCCCGCCAACTACCTGATCTCCTC  3'end
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Inverse-PCR (3'end)

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          5'End
1024 GATGCCTGCTGGTTCCACCTAGCCATCTTTGACTTCTTCACATGGCTGGGCTATCTCAAC
1    GATGCCTGCTGGTTCCACCTGGCCATCTTTGACTTCTTCACGTGGCTAGGTTATCTCAAC

1084 TCCCTCATCAACCCCATAATCTATACCATGTCCAATGAGGACTTTAAACAAGCATTCCAT
61   TCCCTCATCAACCCCATCATCTATACCATGTTCAATGAGGACTTCAAACAAGCGTTCCAT

1144 AAACTGATACGTTTTAAGTGCACAAGTTGA  3'end
121  AAACTGATACGCTTTAAGTGCACAAGCTGA
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Figure 2 Sequence of inverse-PCR amplified product from porcine genomic DNA circles (in bold letters) showing high homology with the sequence of human 5-HT$_{1B}$ receptor (in normal letters). The deduced 5′ (N) and 3′ (C) terminal sequences of porcine 5-HT$_{1B}$ receptor are shown double underlined in boxes, while the inverse-PCR primers are thick underlined.

Pharmacological characterization of recombinant porcine 5-HT$_{1B}$ receptor

Membrane preparations from COS-7 cells transfected with porcine 5-HT$_{1B}$ receptor showed a high affinity for [^{3}H]-GR125743. The equilibrium dissociation constant (K_d) and total number of binding sites (B_{max}) for [^{3}H]-GR125743 were found to be 0.66 ± 0.05 nM and 2.70 ± 0.36 pmol mg^{-1} protein ($n = 3$ each), respectively.

The affinity constants (pK_i values) of 14 serotonergic compounds (eight agonists and six putative antagonists) for the displacement of [^{3}H]-GR125743 from membranes obtained from COS-7 cells expressing porcine 5-HT$_{1B}$ receptor are shown in Table 1. The rank order of potency of agonists was L694247 > ergotamine $\geqslant$ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan, while that of the putative antagonists was GR127935 > methiothepin > SB224289 > > ritanserin > ketanserin $\geqslant$ BRL15572.

In Figure 6, pK_i values of the above compounds obtained in the present experiments with membranes from cells expressing the cloned pig 5-HT$_{1B}$ receptor (Table 1) have

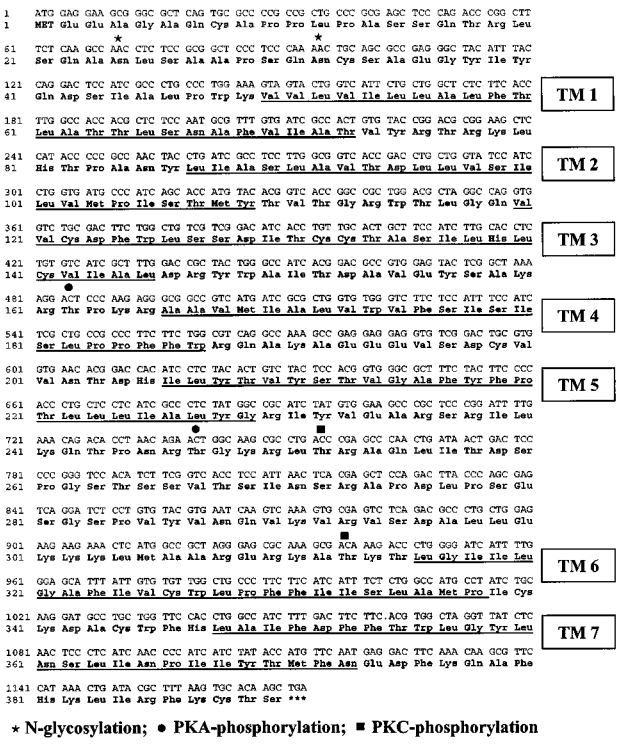


Figure 4 Nucleotide and deduced amino acid (in bold) sequences of the recombinant cDNA derived from porcine cerebral cortex. Numbering of nucleotide and amino acids is shown on the left. Computer analysis (software DNAMAN, version 3.2, Lynnon Biosoft©) predicted a typical G-protein coupled receptor with transmembrane (TM) 1–7 domains (underlined) as well as the putative N-glycosylation and protein kinase A (PKA) and protein

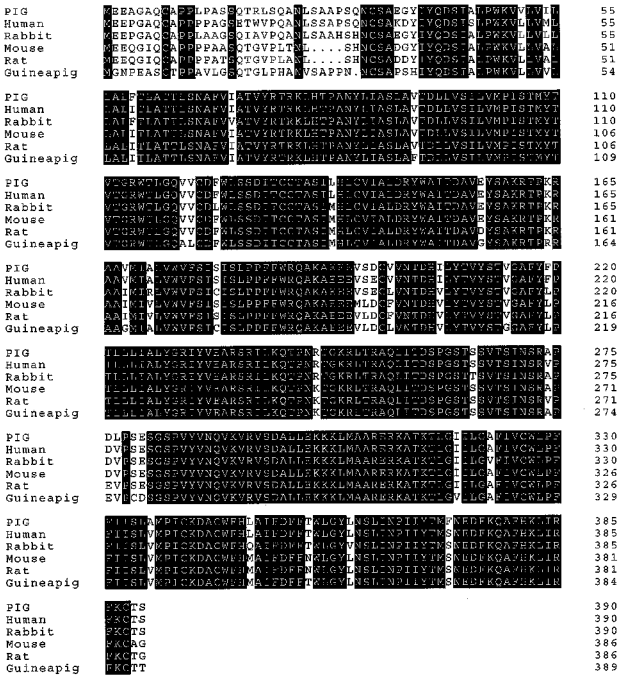


Figure 5 Comparison of amino acid sequences of the pig 5-HT$_{1B}$ receptor (GenBank accession number: AF188626) with the human (SwissProt accession number P28222), rabbit (P49144), mouse (P28334), rat (P28564) and guinea-pig (O08892) 5-HT$_{1B}$ receptor.

been plotted against pK_i values obtained earlier with the same compounds using membranes from cells expressing the cloned human (Wurch *et al*., 1998 and 2001 unpublished results) or rat (Beer *et al*., 1998) 5-HT$_{1B}$ receptor as well as the cloned porcine (Bhalla *et al*., 2000) or human (Wurch *et al*., 1998 and 2001 unpublished results) 5-HT$_{1D}$ receptor. The affinity constants at the porcine 5-HT$_{1B}$ receptor showed the highest correlation with those at the human 5-HT$_{1B}$ receptor ($r_s = 0.988$; $b = 0.985$). This was closely followed by that at the rat 5-HT$_{1B}$ receptor ($r_s = 0.808$), although the slope ($b = 0.455$) was conspicuously lower. The correlation at the porcine ($r_s = 0.662$) or human ($r_s = 0.460$) 5-HT$_{1D}$ receptors

Table 1 Inhibition by serotonergic compounds of [^{3}H]-GR125743 (1 nM) binding to membranes derived from Cos-7 cells transiently expressing the pig 5-HT$_{1B}$ receptor cDNA

Agonists	pK_i	*Putative antagonists*	pK_i
L694247	9.07±0.02	GR127935	8.50±0.03
Ergotamine	8.06±0.00	Methiothepin	8.29±0.04
5-Carboxamidotryptamine	8.06±0.18	SB224289	7.88±0.07
Dihydroergotamine	8.00±0.10	Ritanserin	6.67±0.01
5-HT	7.84±0.07	Ketanserin	5.78±0.12
CP122638	7.37±0.05	BRL15572	5.58±0.07
Zolmitriptan	7.36±0.07		
Sumatriptan	7.25±0.15		

Data are mean±s.e.mean ($n = 3$)

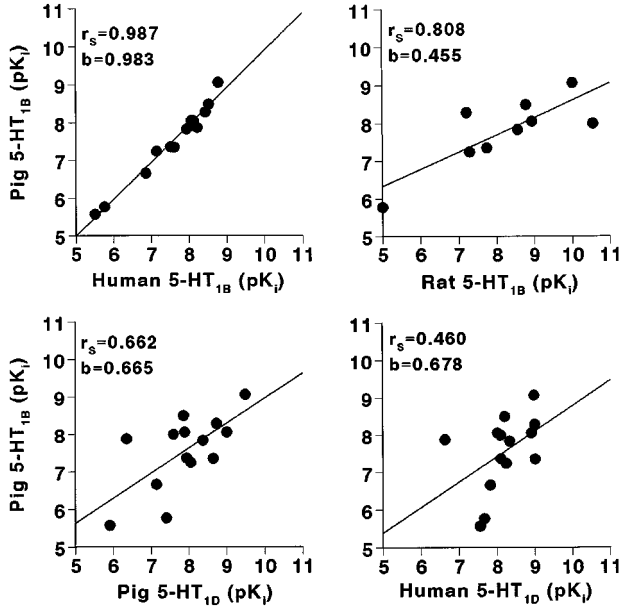


Figure 6 Regression analysis of pK_i values (affinity constants) of 5-HT receptor ligands obtained with the cloned pig 5-HT$_{1B}$ receptor (see Table 1) with those reported with cloned 5-HT$_{1B}$ (human and rat; upper panels) and 5-HT$_{1D}$ (pig and human; lower panels) receptors (for references, see text). In each case [^{3}H]-GR125743 was used as radioligand, except for the rat 5-HT$_{1B}$ receptor, where [^{3}H]-5-HT was used and pK_i values of some compounds included in Table 1 (ergotamine, CP122638, SB224289, ritanserin and BRL15572) were not available. The Spearman correlation coefficient (r_s) and the corresponding values of the slope (b), calculated with SlideWrite plus for Windows© (Advanced Graphics Software, Encinitas, CA,

was much weaker. It may be noted that the selective 5-HT$_{1B}$ receptor antagonist SB224289 (Schlicker *et al*., 1997; Gaster *et al*., 1998; Selkirk *et al*., 1998) showed a high affinity (pK_i: 7.88±0.07, Table 1), while the selective 5-HT$_{1D}$ receptor antagonist BRL15572 (Schlicker *et al*., 1997; Gaster *et al*., 1998) a low affinity (pK_i: 5.58±0.07; Table 1) at the porcine 5-HT$_{1B}$ receptor.

[^{35}S]-GTPγS binding response

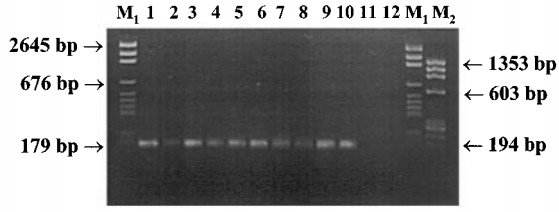
The basal [^{35}S]-GTPγS binding to membranes obtained from CHO-K1 cells transiently co-expressing the cloned porcine 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}Cys^{351}Ile$ protein was 181±34 fmol [^{35}S]-GTPγS mg^{-1} protein (n=5). 5-HT (10 μM) increased [^{35}S]-GTPγS binding by 123±10% (n=5) over the basal level; no effect was observed in membranes from cells transfected with empty plasmid. Zolmitriptan caused a concentration-dependent increase in [^{35}S]-GTPγS binding and elicited a full agonist response at the receptor (100±2% increase as compared to 10 μM 5-HT; Figure 7). The potency (pEC$_{50}$ value) of zolmitriptan was 7.64±0.04 nM (n=5), which is close to its binding affinity at the porcine 5-HT$_{1B}$ receptor (pK_i: 7.36±0.07 nM, n=3; Table 1). The selective 5-HT$_{1B}$ receptor antagonist SB224289 (1 μM) decreased basal [^{35}S]-GTPγS binding by −27±4% (n=5), showing that SB224289 exhibits a negative efficacy (inverse agonism).

5-HT$_{1B}$ receptor mRNA expression in various porcine tissues

RT-PCR technique was used to assess the expression of 5-HT$_{1B}$ receptor in various porcine tissues. As shown in Figure 8, a fragment of expected size (approximately 180 bp), representing porcine 5-HT$_{1B}$ receptor mRNA, was detected in the brain (cortex and cerebellum), trigeminal ganglion, heart (left ventricle) and blood vessels (left anterior descending coronary, pulmonary, common carotid, superior mesenteric and femoral arteries and saphenous vein). No such fragment was found in the negative controls (autoclaved water or brain cortex in the absence of reverse transcriptase step) run simultaneously during PCR, thus ruling out the possibility of genomic DNA and/or PCR contamination.

In situ *hybridization*

In situ hybridization technique was used to localize the expression pattern of 5-HT$_{1B}$ receptor mRNA in porcine frontal cerebral cortex. Using the antisense DIG-labelled cRNA probe, the 5-HT$_{1B}$ receptor mRNA was clearly visualised in the pyramidal cells and weak signals were also observed in the outer molecular layer (Figure 8, left panel). The specificity of the mRNA signals was confirmed with the sense probe, which did not show any signal in the cerebral cortex (Figure 8, right panel).



M$_1$: pGEM marker	**5:** LAD coronary artery	**10:** Saphenous vein
1: Brain cortex	**6:** Pulmonary artery	**11:** Brain cortex without RT
2: Cerebellum	**7:** Carotid artery	**12:** Autoclaved water
3. Trigeminal ganglion	**8:** Mesenteric artery	**M$_2$:** ϕx174 DNA/Hae III marker
4: Left ventricle	**9:** Femoral artery	

Figure 8 Agarose gel electophoresis of PCR amplified products derived from cDNA obtained from a number of porcine tissues. The size of marker bands is indicated in margins. LAD, left anterior descending; RT, reverse transcriptase.

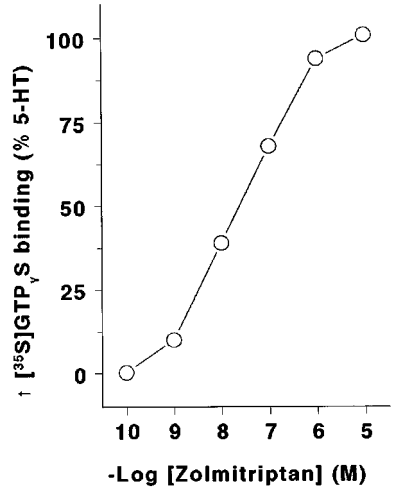


Figure 7 Increase in [^{35}S]-GTPγS binding, as percentage of the response to 10 μM 5-HT, by zolmitriptan in CHO-K$_1$ cells transiently co-expressing the cloned pig 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}$ Cys^{351} Ile protein (pEC$_{50}$ zolmitriptan: 7.64±0.04). Data are mean±s.e.mean (n=5); s.e.mean values fall within the symbol. The basal [^{35}S]-GTPγS binding (181±34 fmol mg^{-1} protein; n=5) was increased by 10 μM 5-HT to 390±62 fmol mg^{-1} protein (123±10% of the basal value; n=5) and decreased by 1 μM SB224289 to 136±29 fmol mg^{-1} protein (−27±4% of the basal value; n=5).

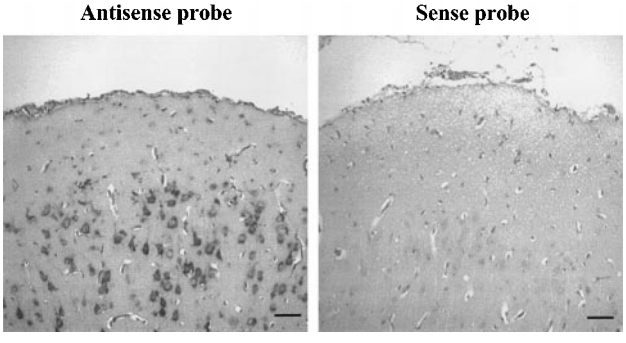


Figure 9 Localization of 5-HT$_{1B}$ receptor mRNA in the frontal part of porcine cerebral cortex by *in situ* hybridization. Left panel: photomicrograph showing the expression of 5-HT$_{1B}$ receptor mRNA in pyramidal cells with the antisense DIG-labelled cRNA probe (dark spots). Right panel: photomicrograph showing no hybridization signals with the sense probe. Scale bar = 50 μm.

Discussion

Sequence analysis of porcine 5-HT$_{1B}$ receptor

The specific primer sequences of the full-length porcine 5-HT$_{1B}$ receptor were identified by inverse PCR on ligated genomic DNA and used for amplification of the full-length porcine 5-HT$_{1B}$ receptor cDNA from cerebral cortex. The nucleotide sequence of porcine 5-HT$_{1B}$ receptor cDNA, which completely matched with a partial sequence submitted to GenBank by T. Wurch and colleagues (accession number: Y11867), revealed 1170 bp encoding an open reading frame of 390 amino acid peptide. This peptide showed a high homology (88–95%) with the sequence of 5-HT$_{1B}$ receptors in other species (Voigt *et al*., 1991; Hamblin *et al*., 1992; Jin *et al*., 1992; Maroteaux *et al*., 1992; Harwood *et al*., 1995; Zgombick *et al*., 1997). The homology between the cloned porcine and human 5-HT$_{1B}$ receptors (Hamblin *et al*., 1992; Jin *et al*., 1992) was the highest (95%) and there was a total identity in transmembrane domains 2–5. It is to be noted that the presence of the amino acid threonine at position 355 within the seventh transmembrane domain of the porcine 5-HT$_{1B}$ receptor, rather than asparagine at the corresponding position 351 in the mouse (Maroteaux *et al*., 1992) and rat (Voigt *et al*., 1991), was identical to the human (Hamblin *et al*., 1992; Jin *et al*., 1992), rabbit (Harwood *et al*., 1995) and guinea-pig (Zgombick *et al*., 1997) 5-HT$_{1B}$ receptors. Furthermore, the presence of putative asparagine-linked glycosylation and protein kinase A and C phoshorylation sites were consistent with the previously cloned receptors from other species (Jin *et al*., 1992; Harwood *et al*., 1995).

As observed in the porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000), with which there was an overall 61% amino acid identity, the porcine 5-HT$_{1B}$ receptor showed some unique amino acids (Ala^{4}, Ser^{15,21} and Arg^{19} towards the N-terminal end, Val^{51} and Ile^{54} in the first transmembrane domain, Leu^{277} in the third intracellular loop, Ala^{336} in the sixth transmembrane domain as well as Phe^{372} in the seventh transmembrane domain). These divergent amino acids were reconfirmed in the inverse PCR sequence derived from genomic DNA. Furthermore, it was most likely the presence of Ala^{4} that interfered with the amplification of full-length porcine 5-HT$_{1B}$ receptor cDNA based on primers designed from other species and necessitated the alternative cloning strategy used here (see Methods).

Ligand binding properties of porcine 5-HT$_{1B}$ receptor

It is well known that the pharmacological profile of the rodent and human 5-HT$_{1B}$ receptor differs substantially (Metcalf *et al*., 1992; Oksenberg *et al*., 1992; Hoyer *et al*., 1994). Our recent investigation also revealed that BRL15572, a selective antagonist at human 5-HT$_{1D}$ receptor (Price *et al*., 1997; Schlicker *et al*., 1997), unexpectedly exhibited a low affinity at the recombinant porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000). Thus, for a pathophysiological animal model to be of value in the evaluation of new drugs it is imperative that the pharmacology of the candidate receptor in the specific animal species must be comparable to that of the human receptor homologue.

Membranes prepared from COS-7 cells transfected with the porcine 5-HT$_{1B}$ receptor showed a high affinity and saturable binding for the 5-HT$_{1B/1D}$ receptor radioligand [^{3}H]-GR125743. Ligand displacement studies established that the pharmacological profile of the porcine 5-HT$_{1B}$ receptor and the affinity rank order for agonists (L694247 > ergotamine $\geqslant$ 5-carboxamidotryptamine = dihydroergotamine = 5-HT > CP122638 = zolmitriptan > sumatriptan) and putative antagonists (GR127935 > methiothepin > SB224289 > > ritanserin > ketanserin $\geqslant$ BRL15572) were close to those described for the recombinant human 5-HT$_{1B}$ receptor (Pauwels & Colpaert, 1996). It may be emphasized that, unlike the low affinity of BRL15572 at the porcine 5-HT$_{1D}$ receptor (Bhalla *et al*., 2000), the selective 5-HT$_{1B}$ receptor antagonist SB224289 (Hagan *et al*., 1997; Gaster *et al*., 1998; Selkirk *et al*., 1998) did have a high affinity at the recombinant porcine 5-HT$_{1B}$ receptor. As expected, BRL15572 showed a low affinity for the porcine 5-HT$_{1B}$ receptor, but this compound cannot be used to differentiate between porcine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes. In addition, ketanserin differentiated between porcine 5-HT$_{1B}$ (pK_i: 5.78 $\pm$ 0.12) and 5-HT$_{1D}$ (pK_i: 7.42 $\pm$ 0.04) receptors showing a 40 fold selectivity for the 5-HT$_{1D}$ over 5-HT$_{1B}$ receptor (present results, Bhalla *et al*., 2000). It is known that ketanserin differentiates between recombinant human, guinea-pig, rabbit and rat 5-HT$_{1B}$ and 5-HT$_{D}$ receptor subtypes (Kaumann *et al*., 1994; Harwood *et al*., 1995; Bard *et al*., 1996; Zgombick *et al*., 1997; Beer *et al*., 1998), but not the canine receptors (Zgombick *et al*., 1991; Wurch & Pauwels, 2000).

The correlation between the affinity constants (as well as the associated slope) for the porcine and human 5-HT$_{1B}$ receptors was higher than for the porcine and rat 5-HT$_{1B}$ receptors (Figure 5). Although we recognize that the affinity constants for the rat 5-HT$_{1B}$ receptor were obtained using [^{3}H]-5-HT (not [^{3}H]-GR125743 as in pig) and β-adrenoceptor antagonists were not evaluated, its molecular basis may be the presence of threonine at position 355 in the seventh transmembrane domain of porcine and human 5-HT$_{1B}$ receptors, rather than asparagine at the corresponding position in rodent receptors (Oksenberg *et al*., 1992). The correlation between the porcine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors for binding affinity of ligands was not so high, as observed earlier for the cloned human and guinea-pig receptors (Weinshank *et al*., 1992; Zgombick *et al*., 1997).

Functional characterization of porcine 5-HT$_{1B}$ receptor

Functional properties of recombinant 5-HT$_{1B}$ receptors have been established using predominantly cellular responses employing cyclic AMP and [^{35}S]-GTPγS binding assays (Pauwels *et al*., 1996, 1997). Using membranes from cells transiently co-expressing the porcine 5-HT$_{1B}$ receptor and a mutant $G_{\alpha o}$Cys^{351}Ile protein, we showed that 5-HT (10 μM) increased basal [^{35}S]-GTPγS binding by 123% over the basal levels and that zolmitriptan behaved as a near full agonist in this respect. This result is in agreement with earlier observations in the recombinant human 5-HT$_{1B}$ receptor (Pauwels *et al*., 1997). Moreover, as can be expected, the potency of zolmitriptan in the [^{35}S]-GTPγS binding assay (pEC$_{50}$: 7.64 $\pm$ 0.04; Figure 7) closely agreed with its affinity in the ligand binding assay (pK_i: 7.36 $\pm$ 0.07; Table 1). Finally, the 5-HT$_{1B}$ receptor antagonist SB224289 (Hagan *et al*., 1997; Gaster *et al*., 1998; Selkirk *et al*., 1998) inhibited basal [^{35}S]-GTPγS binding, thus exhibiting a negative efficacy

(inverse agonism), as noticed earlier using the recombinant human 5-HT$_{1B}$ receptor (Selkirk *et al*., 1998).

Localization of porcine 5-HT$_{1B}$ receptor

Ullmer *et al*. (1995) have previously reported that the 5-HT$_{1B}$ receptor mRNA is expressed in all blood vessels examined, both in the rat (aorta, renal, artery, vena cava and portal, femoral and jugular veins) and pig (coronary, cerebral and pulmonary arteries and cerebral vein). The present study also shows the ubiquitous presence of the 5-HT$_{1B}$ receptor mRNA in various porcine blood vessels. The presence of 5-HT$_{1B}$ receptor mRNA indicates that the vasoconstrictor property of 5-HT$_{1B}$ receptor ligands, such as the triptans, is mediated by the 5-HT$_{1B}$ receptor. Indeed, sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses is antagonized by the selective 5-HT$_{1B}$ receptor antagonist SB224289 (De Vries *et al*., 1999c). However, it may be pointed out that despite the expression of 5-HT$_{1B}$ receptor mRNA, sumatriptan fails to contract the porcine coronary artery (Humphrey *et al*., 1988; 1990). Interestingly, sumatriptan can reduce coronary blood flow in pigs fed a high cholesterol diet (P.R. Saxena, 2001, unpublished observations). It is therefore possible that hypercholesterolaemia either increases the density of 5-HT$_{1B}$ receptors or improves their coupling with G-proteins.

The 5-HT$_{1B}$ receptor mRNA was also observed in porcine trigeminal ganglion, as is the case in the human trigeminal ganglion (Bruinvels *et al*., 1992; Bouchelet *et al*., 1996). Since no studies exploring neural effects of triptans have been performed in the pig, we do not know whether or not the neural effects of triptans (Goadsby & Knight, 1997; Saxena & Tfelt-Hansen, 2000) are mediated by this receptor. Porcine frontal cortex and cerebellum also showed the presence of the 5-HT$_{1B}$ receptor mRNA and *in situ* hybridization studies revealed mRNA expression mainly in the pyramidal neurones of the frontal cerebral cortex. The 5-HT$_{1B}$ receptor is widely distributed in the brain and it may serve as an autoreceptor inhibiting transmitter release (Bonaventure *et al*., 1998; Marcoli *et al*., 1999).

In conclusion, we have established the cDNA sequence of recombinant porcine 5-HT$_{1B}$ receptor, which shows a high homology with other species homologues. The pharmacological profile of the recombinant porcine 5-HT$_{1B}$ receptor is quite similar to that of the human 5-HT$_{1B}$ receptor. The cloned porcine 5-HT$_{1B}$ receptor is also functionally active as observed by [^{35}S]-GTPγS binding and is ubiquitously expressed in blood vessels and brain tissues.

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GenBank accession number: AF188626

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