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Molecular cloning, sequence analysis and pharmacological properties of the porcine 5-HT_{1D} receptor

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1 A cDNA encoding the full-length 5-HT_{1D} receptor derived from porcine cerebral cortex was amplified, cloned and sequenced, using guinea-pig 5-HT_{1D} receptor coding sequence oligonucleotide primers in reverse transcription-polymerase chain reaction (RT–PCR). The 5′ and 3′ ends of the porcine 5-HT_{1D} receptor cDNA were verified by inverse PCR.

2 Sequence analysis of porcine 5-HT_{1D} receptor cDNA revealed an open reading frame of 1134 nucleotides encoding a polypeptide of 377 amino acids having 92% homology with the human 5-HT_{1D} receptor and 88–90% homology with other species homologues.

3 The porcine 5-HT_{1D} receptor cDNA was further subcloned into a mammalian expression vector pcDNA3 and expressed in monkey Cos-7 cells. Radioligand binding assays using either [^{3}H]-5-CT or [^{3}H]-GR125743 on Cos-7 cell membranes showed that pK_i values of 14 serotonin ligands were highly correlated with those obtained with the human 5-HT_{1D} receptor. Nonetheless, a selective antagonist at the human 5-HT_{1D} receptor, BRL15572, only poorly recognized the porcine homologue.

4 Using membranes from cells co-expressing the porcine 5-HT_{1D} receptor and rat $G_{\alpha i}Cys^{351}$ Ile protein, it was shown that 5-HT and zolmitriptan increased, while ketanserin decreased basal [^{35}S]-GTPγS binding. The potency of zolmitriptan in the [^{35}S]-GTPγS binding assay (pEC_{50}: 8.46 ± 0.08) agreed with its affinity in displacing the radioligands [^{3}H]-5-CT and [^{3}H]-GR125743 (pK_i: 8.38 ± 0.15 and 8.67 ± 0.08, respectively).

5 In conclusion, we have established the cDNA sequence and pharmacology of the cloned porcine 5-HT_{1D} receptor. This information would be useful in exploring the role of divergent amino acid residues in the receptor-ligand interaction as well as the role of 5-HT_{1D} receptor in pathophysiological processes relevant for novel drug discovery in diseases such as migraine.
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Abbreviations: 5-CT, 5-Carboxamidotryptamine; 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; 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

5-Hydroxytryptamine (5-HT; serotonin) has long been suspected to be involved in the pathophysiology of migraine, but the exact nature of its role is even today not clear (Hamel & Saxena, 2000). Nevertheless, pharmacological characterization of 5-HT receptors was instrumental in the design of the antimigraine drug sumatriptan (Humphrey *et al*., 1988; 1990; Saxena & Ferrari, 1992), an agonist at a novel, but heterogeneous group of 5-HT receptors, then called '5-HT_1-like' (Bradley *et al*., 1986). The term '5-HT_1-like' has since become redundant (Saxena *et al*., 1998), because this group has been shown to comprise of receptors where sumatriptan has either a high (5-HT_{1B}, 5-HT_{1D} and 5-ht_{1F}) or low (5-HT_7) affinity (Peroutka & McCarthy, 1989; Waeber *et al*., 1990; Hoyer *et al*., 1994).

Sumatriptan as well as the second-generation triptans potently constrict isolated cranial blood vessels and decrease arteriovenous anastomotic fraction of carotid blood flow in anaesthetized animals. These drugs also inhibit dural plasma protein extravasation and action potentials in trigeminal nucleus caudalis following stimulation of the trigeminal ganglion and superior sagittal sinus, respectively (Moskowitz, 1992; Edvinsson & Goadsby, 1998; Saxena & Tfelt-Hansen, 2000). Molecular and pharmacological studies have convincingly shown that the vasoconstrictor effect of triptans is mediated *via* 5-HT_{1B}, but not 5-HT_{1D} or 5-ht_{1F} receptors (Bouchelet *et al*., 1996; 2000; De Vries *et al*., 1998; 1999b; Verheggen *et al*., 1998; Cohen & Schenck, 1999; Nilsson *et al*., 1999). On the other hand, the trigeminal neural effects of triptans seem to involve primarily the 5-HT_{1D} receptor, although 5-HT_{1B} and 5-ht_{1F} receptors have also been implicated (Hoyer *et al*., 1990; Longmore *et al*., 1997; McCall, 1997; Wainscott *et al*., 1998; De Vries *et al*., 1999a; Hargreaves & Shepheard, 1999; Mitsikostas *et al*., 1999). The antimigraine efficacy of triptans has, therefore, been attributed to their

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ability to constrict large intracranial arteries and arteriovenous anastomoses *via* the 5-HT$_{1B}$ receptor and to the inhibition of peripheral trigeminal sensory nerve terminals in the meninges and central terminals in brain stem sensory nuclei *via* the 5-HT$_{1D}$ receptor (Hargreaves & Shepheard, 1999; 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*., 1999a,c). 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$_{1D}$ receptor ligands. However, one of the difficulties in undertaking such studies is the lack of knowledge of molecular biology of porcine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Although we (De Vries *et al*., 1999b) have employed 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) and BRL15572 ([1-(3-chlorophenyl)-4-[3,3-diphenyl (2-(S,R) hydroxypropanyl)piperazine] hydrochloride), that have been shown to be selective antagonists for the human 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors (Price *et al*., 1997; Schlicker *et al*., 1997; Gaster *et al*., 1998; Selkirk *et al*., 1998), respectively, it is difficult to be sure that these compounds also have a high and selective affinity at the respective porcine receptor.

In the present investigation, we describe the molecular cloning and characterization of the porcine 5-HT$_{1D}$ receptor (R.C.: 2.1.5HT.01D). Using the total RNA derived from the pig cerebral cortex, a full-length cDNA encoding 5-HT$_{1D}$ receptor was amplified and the deduced amino acid sequence was compared with those in the other species. The pharmacological profile of the porcine 5-HT$_{1D}$ receptor was evaluated after transient transfection in Cos-7 cells and compared with that of the recombinant human 5-HT$_{1D}$ receptor. A part of these results has been presented to the British Pharmacological Society (Bhalla *et al*., 2000).

Methods

mRNA isolation and cDNA synthesis

Brain cortex obtained from a pig (Yorkshire × Landrace, female, 12 kg) killed after an acute haemodynamic experiment was snap frozen in liquid nitrogen and stored at −80°C. The frozen tissue was transferred to guanidium thiocyanate buffer, homogenized (Ultra-Turrax homogenizer, model T8, Janke & Kunkel GmbH, Staufen, Germany) and the total RNA was extracted as described earlier (Chomczynski & Sacchi, 1987; Sharma *et al*., 1996). The RNA concentration was measured by UV absorbance at 260 nm using a Gene Quant RNA/DNA calculator (Pharmacia-LKB, Biochrom, UK) and the quality of RNA was assessed by OD_{260}/OD_{280} ratio of >1.8 as well as by formaldehyde-agarose gel electrophoresis. Subsequently, poly(A)$^+$ mRNA was purified from the total RNA 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 (25 mM Tris-HCl, pH 8.3; 50 mM KCl; 5.0 mM $MgCl_2$, 2.0 mM DTT), 1.0 mM dNTPs, ribonuclease inhibitor (1 u μl^{-1}), random hexamer (150 ng μg^{-1} mRNA) and, finally, AMV reverse transcriptase (14 u μg^{-1} mRNA). A control was similarly prepared, except that the AMV reverse transcriptase was omitted. 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 β-actin using specific oligonucleotide primers (Ponte *et al*., 1984).

PCR amplification and cloning of 5-HT$_{1D}$ receptor cDNA

Oligonucleotide primers were designed according to the start and stop codon regions of the guinea-pig 5-HT$_{1D}$ receptor gene (Wurch *et al*., 1997; Zgombick *et al*., 1997). The forward and reverse primers were 5′-ATGTCCCCGCCAAACCAGTC-3′ and 5′-CTAGGAGGCTTTCCGGAAATG-3′, respectively. The following components were added in a reaction volume of 20 μl: 250 μM of each dATP, dTTP, dGTP and dCTP, 1.5 mM $MgCl_2$, PCR buffer (1 × PCR buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl), Ampli Taq Gold™ (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.). cDNA was denatured for 1 min at 94°C and annealed to the primers for 30 s at 60°C with the reaction extended for 90 s at 72°C and this procedure was repeated for 36 cycles. Finally, the reaction was extended for an additional 10 min at 72°C. Several independent PCR reactions were performed to exclude possible misincorporation of nucleotides by Ampli Taq for sequence analysis.

The amplified PCR products were separated on 1% agarose gel in TBE buffer (90 mM Tris-HCl, pH 8.0, 90 mM boric acid, 2 mM EDTA) containing ethidium bromide (0.5 $\mu g\ ml^{-1}$), visualized under UV light and photographed. Subsequently, the PCR product of expected size was purified using a PCR purification kit (Promega Benelux b.v., Leiden, The Netherlands) and the amount of DNA was measured spectrophotometrically. An aliquot of purified PCR product was ligated into the pGEMT-Easy vector (Promega Benelux b.v., Leiden, The Netherlands). The ligated vector was transformed into competent JM 109 cells and grown on IPTG/X-gal-treated ampicillin resistant LB-Agar plates at 37°C. White over blue colonies were selected to identify positive clones, which were further characterized by the presence of an expected size insert in them. Four insert positive clones (namely, pHTD-11, pHTD-13, pHTD-25, pHTD-27) were further processed for the plasmid DNA isolation and sequencing.

Plasmid purification and sequencing

Bacterial colonies harbouring recombinant plasmids were grown overnight at 37°C in LB medium containing ampicillin (100 $\mu g\ ml^{-1}$). Bacteria were harvested by centrifugation and the plasmid DNA was isolated using a commercially available midi-prep plasmid isolation kit (Promega Benelux b.v., Leiden, The Netherlands). Purified plasmid DNA from all four clones (pHTD-11, pHTD-13, pHTD-25, pHTD-27) was sequenced by the dideoxy nucleotide chain termination method, using universal forward and reverse sequencing primers. Sequencing reactions were loaded on an automated fluorescence based DNA sequencer (ABI Prism™ 310 Genetic analyser, Perkin Elmer Applied Biosystem Benelux, Nieuwerkerk a/d Ijssel, The Netherlands) and the raw sequence data were processed and analysed. The nucleotide sequences thus obtained were compared and a consensus sequence was derived using the

DNAMAN sequence analysis program (version 3.2, Lynnon Biosoft© 1994–1997). The final sequence 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.; web site: http://www.ncbi.nlm.nih.gov/BLAST/). The hydrophobic regions indicating putative transmembrane domains and sequence homology with known 5-HT$_{1D}$ receptors from other species were established.

Inverse PCR

Since the forward and reverse oligonucleotide primers used in RT–PCR were designed from the guinea-pig 5-HT$_{1D}$ receptor sequence, we identified the 5′ and 3′ ends of our cloned porcine cDNA by inverse PCR (Ochman *et al*., 1988). Porcine genomic DNA was digested with *Eco*RI restriction enzyme, as the cloned 5-HT$_{1D}$ receptor cDNA did not show any restriction site for *Eco*RI. 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. Using porcine specific inverse primers, the ligated DNA fragments, as circles of different sizes in various dilutions, were subjected to PCR amplification. The internal oligonucleotide inverse primers, designed on the basis of porcine 5-HT$_{1D}$ receptor cDNA sequence, were 5′-GCATTGGAAAGGACAGTGGC-3′ (for 5′ end) and 5′-TCATCTGCTGGTTGCCCTTC-3′ (for 3′ end). The PCR products were separated on a 1% agarose gel, purified, cloned and sequenced as described above.

Transient transfection and ligand receptor binding assay

The purified full-length pig 5-HT$_{1D}$ receptor cDNA insert was subcloned into dephosphorylated eukaryotic expression vector, pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) and transformed into TOP10 competent cells. The clones containing the insert were selected and screened with restriction enzyme (*Hind*III and *Eco*RI) for appropriate orientation. 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 bovine 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 50 nM Tris-HCl 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 *et al*., 1996). The membrane protein concentration was measured by dye binding assay (Bradford, 1976) using a Bio-Rad Kit and bovine serum albumin as a standard.

Binding assays to membranes obtained from transfected Cos-7 cells were performed using either 3.0 nM [^{3}H]GR125743 ([^{3}H]N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl) benzamide) or 1.0 nM [^{3}H]5-carboxamidotryptamine ([^{3}H]5-CT) as radioligands. Incubation mixtures consisted of 0.40 ml of cell membrane preparation (30–50 μg of protein), 0.05 ml of one of the radioligands 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). In the case of [^{3}H]-GR125743, 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 did not display specific [^{3}H]-5-CT or [^{3}H]-GR125743 binding.

[^{35}S]GTPγS binding

CHO-K1 cells co-expressing the porcine 5-HT$_{1D}$ receptor and mutant $G_{\alpha i1}Cys^{351}Ile$ 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 30 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ascorbic acid. 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), ketanserin (10 μM) or zolmitriptan (0.1–10 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 Whatman 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. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. 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.

Materials

All oligonucleotide primers were commercially procured from Life Technologies b.v. (Breda, The Netherlands). pGEMT-Easy vector system, Wizard® PCR prep and mini-prep DNA purification systems were purchased from Promega Benelux b.v. (Leiden, The Netherlands). AmpliTaqGold and dye terminator/cycle sequencing ready reaction kit were procured from Perkin Elmer Applied Biosystem Benelux (Nieuwerkerk a/d Ijssel, The Netherlands). Oligotex mRNA purification kit was purchased from Qiagen GmbH (Hilden, Germany). Guanidinium thiocyanate was purchased from U.S. Biochemicals (Cleveland, OH, U.S.A.). AMV-Reverse transcriptase enzyme was obtained from Pharmacia-LKB (Uppsala, Sweden). All other chemicals used in this study were of molecular biology and/or tissue culture grade.

The compounds used in pharmacological assays were: 5-HT creatinine sulphate (Sigma Chemicals, St. Louis, MO, U.S.A.), [^{3}H]-5-CT (56.5 Ci mmol^{-1}, New England Nuclear, Les Ulis, France), BRL15572, CP122638 (N-methyl-3-[pyrrolidin-(R)-yl-methyl]-1H-indol-5-ylmethyl sulphanomide), [^{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, 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), methiothepin, ritanserin, sumatriptan, SB224289 and zolmitriptan. Except BRL15572 (gift: Dr A.A. Parsons, SmithKline Beecham Pharmaceuticals, Harlow, Essex, U.K.) and L694247 (Tocris Cookson, Bristol, U.K.), all compounds were synthesized at Centre de Recherche Pierre Fabre (Castres, France).

Results

Cloning of 5-HT$_{1D}$ receptor cDNA

Figure 1 shows the gel electrophoresis of RT–PCR products of cDNA prepared from the poly(A)$^+$ mRNA of porcine brain cortex used for the amplification of 5-HT$_{1D}$ receptor. The quality of cDNA was established by detecting a DNA fragment of expected size (625 bp) encoding β-actin cDNA. Employing this cDNA as a template and oligonucleotide primers designed from the known guinea-pig 5-HT$_{1D}$ receptor cDNA sequence (Wurch *et al*., 1997; Zgombick *et al*., 1997), a DNA fragment of expected size ($\sim$1140 bp) was amplified by PCR (left panel). The PCR amplified product was cloned and the presence of the insert checked with *Eco*RI (right panel).

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

The full-length band disclosed a nucleotide sequence of 1134 bp encoding a 377 amino acid long protein. A BLAST search at the GenBank revealed that the identified sequence most closely matched with the sequence of 5-HT$_{1D}$ receptor and the software DNAMAN analysis predicted seven hydrophobic transmembrane domains (Figure 2). Since the porcine 5-HT$_{1D}$ receptor cDNA was amplified using primers based on 5′ and 3′ ends of guinea-pig 5-HT$_{1D}$ receptor sequence (Wurch *et al*., 1997; Zgombick *et al*., 1997), the 5′ and 3′ terminals of the porcine cDNA were confirmed by inverse PCR. Using a set of porcine specific internal inverse primers (based on the derived sequence) and the porcine ligated genomic DNA fragments, a PCR product of approximately 400 bp was amplified, cloned and sequenced. Sequence analysis revealed that, compared to the derived porcine 5-HT$_{1D}$ receptor cDNA, there was a 100% identity with 170 nucleotides from the 5′ end (Figure 3, upper panel) and 203 nucleotides from the 3′ end (Figure 3, lower panel). The 5′ (N) and 3′ (C) terminals were identical to those obtained by cDNA sequencing (Figure 3, shaded boxes). In the intervening portion (not shown in the figure), there were only 20 nucleotides derived from the porcine genomic DNA, where the *Eco*RI site was present 5 nucleotides away from 3′ end and 15 nucleotides before 5′ end.

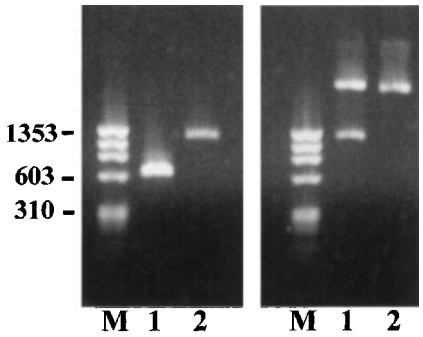

Figure 1 Left panel: agarose gel electrophoresis of RT–PCR products of cDNA synthesized from porcine brain cortex (see Methods). M, $\phi \times 174$ DNA/*Hae*III marker; Lanes 1, RT–PCR product of 625 bp using β-actin primers; Lane 2, RT–PCR product of approximately 1200 bp obtained using forward and reverse primers of 5-HT$_{1D}$ receptors. A control without template cDNA (in absence of reverse transcriptase) did not show any signal (not included in the figure). Right panel: agarose gel electrophoresis of recombinant plasmid with 5-HT$_{1D}$ receptor cDNA. M, $\phi \times 174$ DNA/*Hae*III marker; Lane 1, recombinant plasmid DNA restricted with *Eco*RI enzyme and showing a DNA insert of approximately 1200 bp; Lane 2, non-digested recombinant plasmid DNA. The size (bp) of three marker bands is indicated in the left margin.

Figure 4 compares the amino acid sequence of porcine 5-HT$_{1D}$ receptor with those of other mammalian species. It should be noted that towards the N-terminal of porcine 5-HT$_{1D}$ receptor a number of amino acids (Val^{8,36}, Asp^{10}, Gly^{14}, Thr^{15}, Lys^{27}, Pro^{97} and Glu^{102}) were different from those in the other species. However, there was a high homology in the overall sequence of the receptor across the species (from 88% in dog to 92% in humans). Between the pig and human, the transmembrane-3 and transmembrane-7 regions showed an identical sequence while the remaining transmembrane regions showed a 92–96% homology.

Binding properties of recombinant porcine 5-HT$_{1D}$ receptor

Membrane preparations from COS-7 cells transfected with the porcine 5-HT$_{1D}$ receptor showed high affinity for the agonist [^{3}H]-5-CT as well as the antagonist [^{3}H]-GR125743. The equilibrium dissociation constant (K_D) of [^{3}H]-5-CT and [^{3}H]-GR125743 for the porcine 5-HT$_{1D}$ receptor was, respectively, 1.08 ± 0.04 nM and 1.47 ± 0.13 nM (both $n=3$). The B_{max} of [^{3}H]-GR125743 binding (2.70 ± 0.59 pmol mg^{-1} protein; $n=3$) was about one and a half times as high as that of [^{3}H]-5-CT binding (1.74 ± 0.04 pmol mg^{-1} protein; $n=3$), suggesting that part of the 5-HT$_{1D}$ receptor population was in the high affinity state.

Table 1 shows pK$_i$ values of a number of 5-HT receptor agonists and antagonists for the displacement of [^{3}H]-5-CT and [^{3}H]-GR125743 from membranes obtained from COS-7 cells expressing the cloned porcine 5-HT$_{1D}$ receptor. There was an excellent correlation ($r_S=0.984$; $P \leqslant 0.05$) between values obtained with the two radioligands. The rank order of affinity of agonists was L694247 > 5-CT > zolmitriptan > 5-HT > sumatriptan = CP122638 = ergotamine > dihydroergotamine, while that of the antagonists was methiothepin > GR127935 > ketanserin > ritanserin > > SB224289 $\geqslant$ BRL 15572.

In Figure 5, the affinity constants of the 14 compounds (eight agonists and six antagonists; see Table 1) investigated in the present experiments with the cloned pig 5-HT$_{1D}$ receptor have been plotted against values obtained earlier with the same compounds using membranes from cells expressing the human 5-HT$_{1B}$ or 5-HT$_{1D}$ receptor (Pauwels *et al*., 1996). The correlation between the affinity constants at the cloned porcine 5-HT$_{1D}$ receptor, particularly using [^{3}H]-GR125743 as ligand, was clearly higher with the cloned human 5-HT$_{1D}$ than with 5-

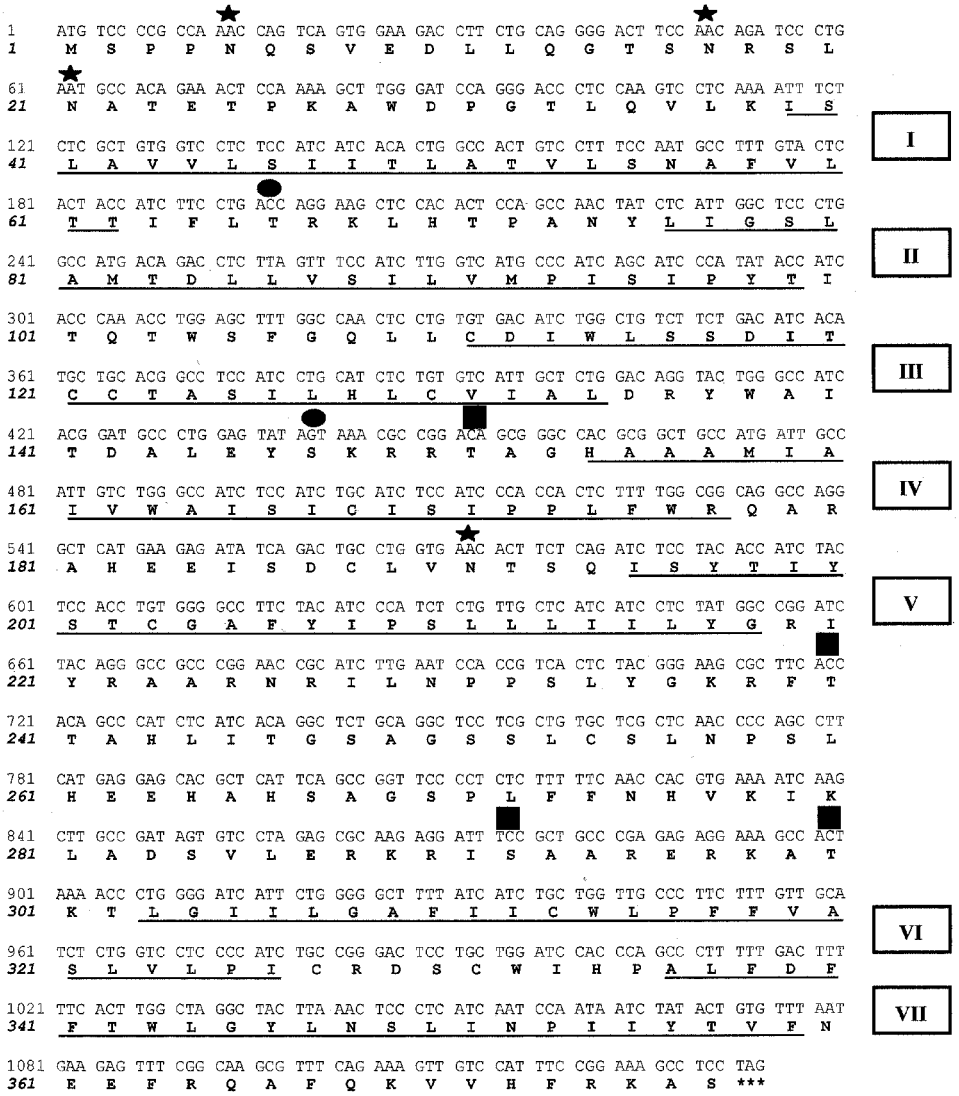

Figure 2 Nucleotide and deduced amino acid (in bold) sequences of the pig 5-HT$_{1D}$ receptor (GenBank accession number: AF 117655). The computer predicted (software DNAMAN, version 3.2, Lynnon Biosoft©) transmembrane domains I–VII (underlined) and putative N-glycosylation (★), protein kinase A phoshorylation (■) and protein kinase C phosphorylation (●) sites are indicated in the sequence.

HT$_{1B}$ receptor (r_s=0.903 and 0.608, respectively; Figure 5, right panels). Admittedly, this difference was not as great using [^{3}H]-5-CT as ligand (r_s=0.724 and 0.716, respectively; Figure 5, left panels). However, it is interesting to point out that the selective antagonist at the human 5-HT$_{1D}$ receptor, BRL15572 (Price *et al*., 1997; Schlicker *et al*., 1997), displayed much less affinity at the pig 5-HT$_{1D}$ receptor (Figure 5, upper panels). As can be expected, the selective antagonist at the human 5-HT$_{1B}$ receptor, SB224289 (Gaster *et al*., 1998; Selkirk *et al*., 1998), did not show a high affinity at the pig 5-HT$_{1D}$ receptor (Figure 5, lower panels).

When BRL15572 was excluded from analysis, the correlation between the pK$_i$ values at pig and human 5-HT$_{1D}$ receptors was increased (r_s=0.798 and 0.957 using [^{3}H]-5-CT and [^{3}H]-GR125743, respectively), but that between pig 5-HT$_{1D}$ and human 5-HT$_{1B}$ receptors was decreased (r_s=0.602 and 0.386 using [^{3}H]-5-CT and [^{3}H]-GR125743, respectively).

[^{35}S]GTPγS binding

The basal [^{35}S]-GTPγS binding in membranes obtained from CHO-K1 cells co-expressing the cloned porcine 5-HT$_{1D}$ receptor and the rat G_{zil}Cys^{351}Ile protein was 277±37 fmol [^{35}S]-GTPγS mg^{-1} protein. 5-HT (10 μM) elicited an increase of 44% over the basal level. Zolmitriptan caused a concentration-dependent increase in [^{35}S]-GTPγS binding and elicited a near full agonist response at the receptor (83±6% increase as compared to 10 μM 5-HT; Figure 6). The potency of zolmitriptan (pEC$_{50}$: 8.46±0.08) was found to be close to the binding affinity at the porcine 5-HT$_{1D}$ receptor (pK$_i$ 8.38 and 8.67, see Table 1). The putative 5-HT$_{1D}$ receptor antagonist, ketanserin (10 μM), decreased basal [^{35}S]-GTPγS binding by about 25%, thus exhibiting a negative efficacy.

Discussion

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

Using primers based on the nucleotide sequence of the guinea-pig 5-HT$_{1D}$ receptor gene (Wurch *et al*., 1997; Zgombick *et al*., 1997) and cDNA derived from porcine cerebral cortex, a band of expected fragment size (~1140 bp) was amplified, cloned and sequenced. The BLAST search of the cloned porcine

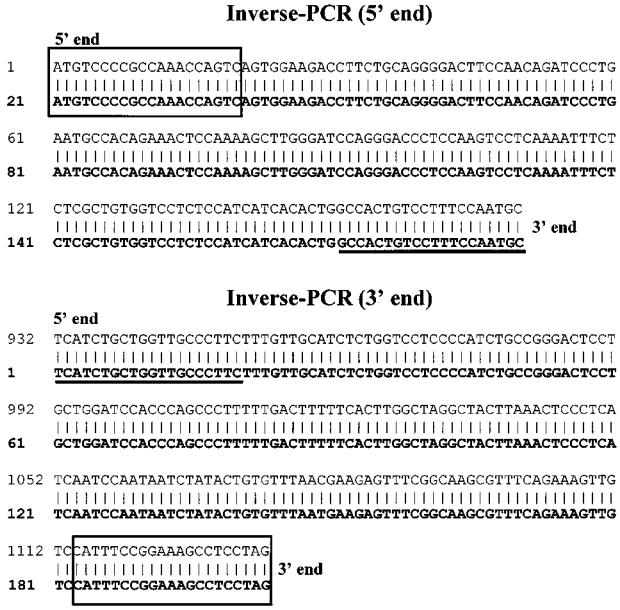

Figure 3 Sequence of inverse-PCR amplified products (in bold letters) showing a 100% homology at both 5′ and 3′ ends with the sequence of the cDNA identified from the pig brain cortex (in normal letters). The 5′ and 3′ terminal sequences are identified in boxes, while the inverse PCR primers are underlined.

Table 1 pK_i values for a series of serotonergic compounds for the inhibition of [^{3}H]-5-CT and [^{3}H]-GR125743 binding to cloned pig 5-HT$_{1D}$ receptor expressed in Cos-7 cells.

Compound	*[^{3}H]-5-CT*	*[^{3}H]-GR125743*
Agonists		
L694247	9.29±0.01	9.50±0.03
5-CT	8.79±0.06	9.00±0.05
Zolmitriptan	8.38±0.15	8.67±0.08
5-HT	8.22±0.12	8.40±0.09
Sumatriptan	7.97±0.04	8.06±0.04
CP122638	7.84±0.11	8.67±0.08
Ergotamine	8.00±0.04	7.89±0.03
Dihydroergotamine	7.87±0.12	7.73±0.24
Antagonists		
Methiothepin	8.74±0.06	8.81±0.14
GR127935	7.92±0.18	7.98±0.17
Ketanserin	7.17±0.06	7.42±0.04
Ritanserin	6.80±0.13	7.26±0.18
SB224289	6.15±0.22	6.39±0.08
BRL15572	5.94±0.09	5.93±0.09

Data are mean±s.e.mean (n=3–7). The pK_i values obtained with the two ligands correlated significantly (Spearman correlation coefficient r_s=0.984; $P \leqslant 0.05$, Slide-Write plus for Windows, Advanced Graphics Software, Encinitas, CA, U.S.A.).

cDNA fragment revealed high resemblance with previously cloned 5-HT$_{1D}$ receptors. The full-length nucleotide sequence containing 1137 bp encoded a 377 amino acid peptide, which showed a close (88–92%) similarity with the human (Hamblin & Metcalf, 1991; Weinshank *et al*., 1992), rabbit (Harwood *et al*., 1995), guinea-pig (Wurch *et al*., 1997; Zgombick *et al*., 1997), mouse (Wurch *et al*., 1997), rat (Hamblin *et al*., 1992; Wurch *et al*., 1997) and dog (Libert *et al*., 1990; Zgombick *et al*., 1991) 5-HT$_{1D}$ receptor. The similarity with the human 5-HT$_{1D}$ receptor in the predicted seven transmembrane regions was even higher (92–100%). Moreover, the full-length amino acid sequence completely matched the partial sequence of the porcine 5-HT$_{1D}$ receptor, previously submitted to the GenBank by T. Wurch and colleagues (accession number

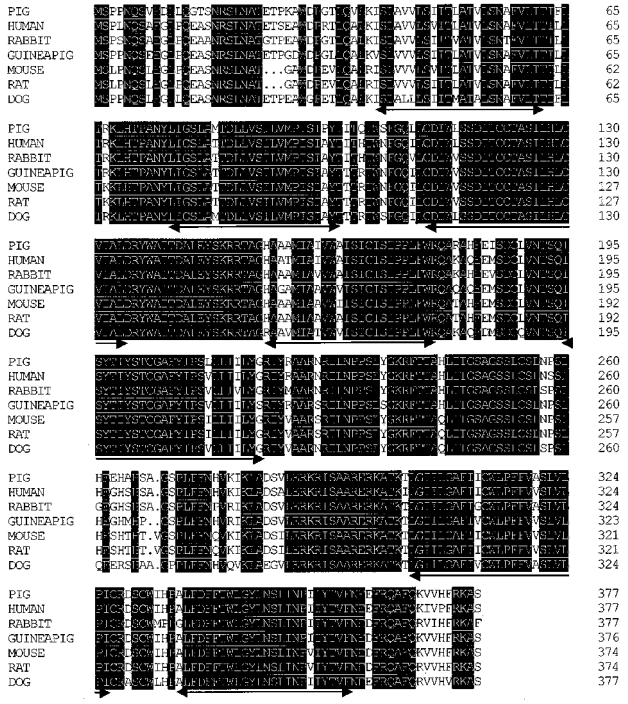

Figure 4 Comparison of amino acid sequences of the pig 5-HT$_{1D}$ receptor with human (GenBank accession number M89955), rabbit (Z50162), guinea-pig (X94436), mouse (X94908), rat (M89953) and dog (X14049) 5-HT$_{1D}$ receptors (software DNAMAN, version 3.2, Lynnon Biosoft©). The arrows drawn across the amino acid sequence indicate the seven transmembrane regions. Shaded boxes show identity across the different species.

P79400). Thus, the nucleotide sequence of the mRNA obtained from the porcine cerebral cortex encodes the 5-HT$_{1D}$ receptor.

The computer-predicted hydrophobicity showed seven transmembrane spanning regions as well as putative N-linked glycosylation sites also observed in 5-HT$_{1D}$ receptors from the other species (Wurch *et al*., 1997). However, the N-terminal of porcine 5-HT$_{1D}$ receptor (see Figure 3) showed several unique amino acids not present in other species (Harwood *et al*., 1995; Wurch *et al*., 1997). The divergent nucleotides within the 5′ (N) and 3′ (C) ends were confirmed by inverse PCR using primers based on the derived sequence of the porcine 5-HT$_{1D}$ receptor. It may, however, be noted that the threonine residue, being the seventh amino acid in the transmembrane-7 region of thus far cloned 5-HT$_{1D}$ (Thr^{342}) as well as non-rodent 5-HT$_{1B}$ (Thr^{355}) receptors (see Zgombick *et al*., 1991; Harwood *et al*., 1995; Pregenzer *et al*., 1997; Wurch *et al*., 1997; Zgombick *et al*., 1997), was conserved. The presence of threonine at this position seems to be important for the largely similar ligand binding properties of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Interestingly, a single amino acid change at the seventh residue (from Thr$^{354/355}$ in non-rodent species to Asp^{351} in the rat or mouse) in the 5-HT$_{1B}$ receptor is known to confer major pharmacological differences between the two groups of 5-HT$_{1B}$ receptors (Oksenberg *et al*., 1992; Zgombick *et al*., 1997).

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

The cloned porcine 5-HT$_{1D}$ receptor expressed in COS-7 cells showed high affinity for the two 5-HT$_{1B/D}$ receptor radioligands ([^{3}H]-5-CT and [^{3}H]-GR125743) and the rank order of agonists (L694247 > 5-CT > zolmitriptan > 5-HT > sumatriptan = CP122638 = ergotamine > dihydroergotamine) as well as antagonists (methiothepin > GR127935 > ketanserin > ritan-

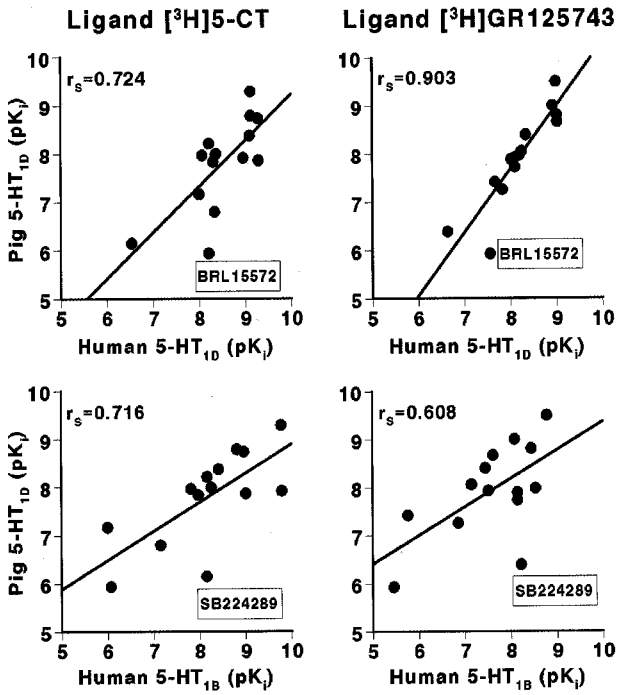

Figure 5 Regression analysis of pK_i values (affinity constants) of 5-HT receptor ligands for the cloned pig 5-HT_{1D} receptor (see Table 1 for ligands and data) against the cloned human 5-HT_{1D} (upper panels) and 5-HT_{1B} (lower panels) receptors (data from Pauwels *et al*., 1996; Wurch *et al*., 1998), using either [^{3}H]-5-CT (left panels) or [^{3}H]-GR125743 (right panels). The Spearman correlation coefficient r_s, calculated by using SlideWrite plus for Windows (Advanced Graphics Software, Encinitas, CA, U.S.A.), is listed in each panel. The compounds clearly falling outside the regression line (BRL15572 and SB224289) are identified in the graphs.

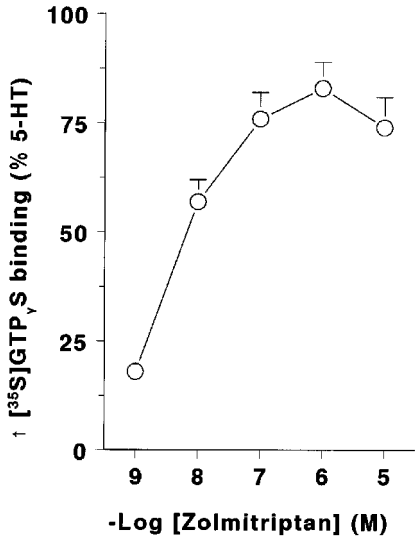

Figure 6 Increase in [^{35}S]-GTPγS binding, as percentage of the response to 10 μM 5-HT, by zolmitriptan in CHO-K_1 cells co-transfected with the cloned pig 5-HT_{1D} receptor and rat $G_{\alpha i1}Cys^{351}Ile$ protein. The basal [^{35}S]-GTPγS binding (277 ± 37 fmol mg^{-1} protein) was increased by 5-HT (10 μM) to 399 ± 77 fmol mg^{-1} protein (44%) and decreased by ketanserin (10 μM) to 220 ± 23 fmol mg^{-1} protein (-25%).

serin $>>$SB224289$\geqslant$BRL 15572) affinity was found to be the same using either radioligand. The affinity constants of these 14 compounds obtained in the present experiments with the cloned porcine 5-HT_{1D} receptor showed a high correlation with those previously reported with the cloned human 5-HT_{1D} receptor (Figure 5, Pauwels *et al*., 1996). However, the most salient finding in the present investigation was that BRL15572, which behaves as a selective antagonist at the human 5-HT_{1D} receptor (Price *et al*., 1997; Schlicker *et al*., 1997), did not show high affinity at the porcine 5-HT_{1D} receptor. The profound implications of this finding are obvious; a ligand selectively recognizing a particular receptor may fail at other species' homologues. Indeed, this is also the case with ketanserin, which has a moderate selectivity for the 5-HT_{1D} over the 5-HT_{1B} receptors in the human (Zgombick *et al*., 1995; Pauwels & Colpaert, 1996; Pauwels *et al*., 1996), rabbit (Harwood *et al*., 1995; Bard *et al*., 1996), rat (Bach *et al*., 1993; Wurch *et al*., 1997) and guinea-pig (Wurch *et al*., 1997), but not in the dog (Zgombick *et al*., 1991). Similarly, certain isochroman derivatives show a differential pharmacology at the guinea-pig and gorilla 5-HT_{1D} receptors (Pregenzer *et al*., 1999). Such species differences in the pharmacology of homologue receptors can be used to explore the role of divergent amino acid residues in the receptor-ligand interaction as well as the validation of animal models with respect to drug discovery for human diseases.

In view of the poor affinity of BRL15572 at the cloned porcine 5-HT_{1D} receptor, we have to admit that the use of this compound by us to rule out the involvement of 5-HT_{1D} receptor in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses (De Vries *et al*., 1999b) was not adequate. However, the conclusions that the response to sumatriptan is mediated by 5-HT_{1B} receptor still seems valid, since both selective (SB224289, Gaster *et al*., 1998; Selkirk *et al*., 1998) as well as non-selective (GR127935, Pauwels *et al*., 1996; Skingle *et al*., 1996) 5-HT_{1B} receptor antagonists potently antagonized the carotid arteriovenous anastomotic constriction by sumatriptan (De Vries *et al*., 1996; 1999b).

Functional characterization of porcine 5-HT_{1D} receptor

Functional properties of recombinant 5-HT_{1D} receptors have been established using predominantly cellular responses employing cyclic AMP and [^{35}S]-GTPγS binding assays (Thomas *et al*., 1995; Pauwels *et al*., 1996; 1997). Using membranes from cells co-expressing the porcine 5-HT_{1D} receptor and rat $G_{\alpha i1}Cys^{351}Ile$ protein, we showed that 5-HT (10 μM) increased basal [^{35}S]-GTPγS binding by 44% and that zolmitriptan behaved as a near full agonist in this respect. This result is in agreement with earlier observations on recombinant human 5-HT_{1D} receptor (Pauwels *et al*., 1997). Moreover, as can be expected, the potency of zolmitriptan in the [^{35}S]-GTPγS binding assay (pEC_{50}: 8.46 ± 0.08, see Figure 6) closely agreed with its affinity in the ligand binding assay (pK_i 8.38 and 8.67, see Table 1). Finally, the putative 5-HT_{1D} receptor antagonist ketanserin (10 μM) inhibited basal [^{35}S]-GTPγS binding, thus exhibiting negative efficacy (inverse agonism) as noticed earlier using the recombinant human 5-HT_{1D} receptor (Thomas *et al*., 1995; Pauwels *et al*., 1997).

In conclusion, we have established the cDNA sequence of cloned porcine 5-HT_{1D} receptor, which shows a similar ligand binding profile as the cloned human 5-HT_{1D} receptor, except that BRL15572, a selective antagonist at the human 5-HT_{1D} receptor, is not recognized by the porcine homologue. As shown by the increase in [^{35}S]-GTPγS binding, the cloned porcine 5-HT_{1D} receptor is also functionally active.

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