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Cloning and functional characterization of the rat stomach fundus serotonin receptor

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A DNA segment homologous to the third exons of the serotonin 1C and 2 receptor genes was isolated from a mouse genomic library. The positions of the introns flanking these exons were conserved in the three genes. To examine whether the new fragment was part of an active gene, we used a quantitative PCR protocol to analyse rat RNAs from different tissues and ages. The gene was expressed in stomach fundus at an abundance of 1×10^5 mRNA molecules. This tissue contracts in response to serotonin via a receptor that has previously resisted classification. We constructed a cDNA library from rat stomach fundus and isolated clones containing 2020 bp inserts with open reading frames of 465 amino acids comprising seven putative membrane-spanning regions. The protein was transiently expressed in COS cells and binding of serotonergic ligands to the membranes was analysed. The pharmacological profile resembled that described for the serotonin-stimulated contraction of the stomach fundus. After expression of this receptor in *Xenopus* oocytes, the application of serotonin triggered the typical chloride current which presumably results from the activation of phospholipase C. The coupling to this response system was less efficient than that of the 5-HT_{1C} or 5-HT_2 receptors.

Key words: COS cell expression/5-HT receptor/voltage clamp/*Xenopus* oocytes

Introduction

Serotonergic neurons of the central nervous system are located mainly in the raphe nuclei in the brain stem and the pons. Most regions of the brain are densely innervated by these neurons. The receptors are located primarily in the brain, the spinal cord and in smooth muscle. By pharmacological and physiological criteria several 5-HT receptor types can be distinguished: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_4 receptors regulate cAMP formation, 5-HT_{1C} and 5-HT_2 receptors stimulate phosphatidylinositol hydrolysis while 5-HT_3 receptors are ligand-gated ion channels (Hoyer and Schoeffter, 1991; Julius, 1991). Several reports describe 5-HT receptors with pharmacological profiles that distinguish them from the above types, one of them occurring in the stomach fundus (Cohen and Wittenauer, 1985; Clineschmidt *et al.*, 1985). Serotonin causes contraction of this tissue via a receptor that is similar but not identical to the 5-HT_{1C} receptor (Baez *et al.* 1990; Kalkman and Fozard, 1991).

The isolation of mouse and rat 5-HT_{1C} (Lübbert *et al.*, 1987a; Julius *et al.*, 1988), rat 5-HT_2 (Pritchett *et al.*, 1988; Julius *et al.*, 1990; Foguet *et al.*, 1992), human and rat 5-HT_{1A} (Fargin *et al.*, 1988; Albert *et al.*, 1990), rat 5-HT_{1B} (Voigt *et al.*, 1991) and human 5-HT_{1D} receptor clones (Hamblin and Metcalf, 1991) has been reported. The amino acid sequences in the membrane-spanning regions of the 5-HT_2 and 5-HT_{1C} receptors are highly conserved (~81%) while those of the 5-HT_{1A} receptor are only 40% homologous to the other two 5-HT receptors and more closely related to the hydrophobic regions of the β-adrenergic receptor (48%).

Analysis of the mouse 5-HT_{1C} and 5-HT_2 receptor genes has revealed that, in contrast to the 5-HT_{1A} and 5-HT_{1D} receptor genes, they contain two introns in conserved positions within the coding region (Foguet *et al.*, 1992). In an attempt to identify related genes we have previously isolated a genomic clone (Foguet *et al.*, 1992), called *SRL* (*s*erotonin *r*eceptor-*l*ike), containing a putative exon which had the same conserved exon–intron boundaries and displayed strong sequence similarity to the corresponding exons of the other two receptors (62 and 65%, respectively).

Here, we describe the tissue distribution, cDNA cloning, expression and functional characterization of this receptor. Using a quantitative PCR approach, expression of this receptor was detected primarily in the stomach fundus. A cDNA clone encoding this receptor was then isolated from a rat stomach fundus cDNA library. The receptor was expressed in COS cells for the analysis of its binding properties. The pharmacological profile resembled most closely that of the serotonin-stimulated contraction of the stomach fundus. After expression in *Xenopus* oocytes, the activated receptor triggered the oscillating chloride current thought to be a consequence of phosphatidylinositol breakdown.

Results

Expression of the SRL gene

The spatial and temporal expression of the *SRL* gene in the rat was analysed by PCR. The RNA used in these experiments was treated with an excess of RNase-free DNase prior to the reverse transcription. After reverse transcription two oligonucleotides flanking the ends of the putative exon were used to analyse *SRL* cDNA (Figure 1). This exon was previously isolated by low stringency screening of a mouse genomic library with a probe containing sequences of the 5-HT_{1C} receptor (Foguet *et al.*, 1992). PCR was employed since we were unable to detect any transcripts on RNA blots.

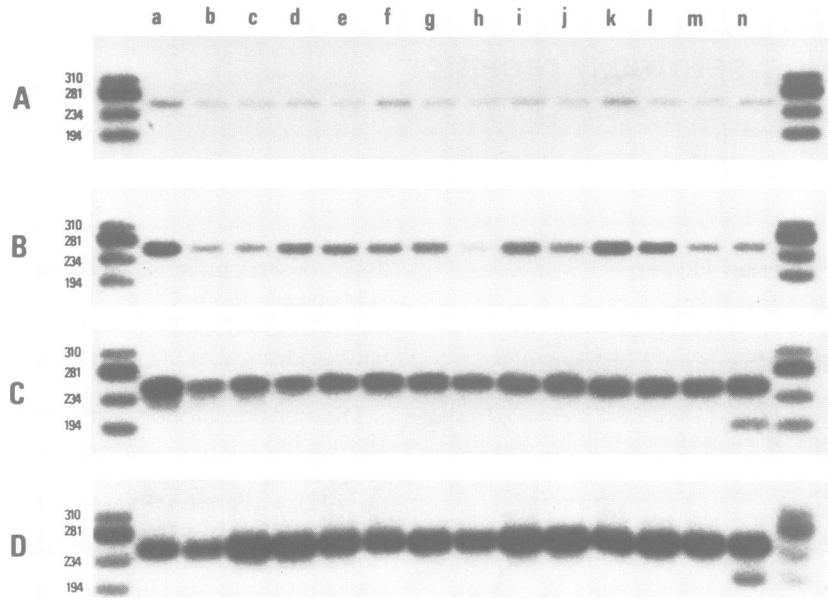

Fig. 1. Distribution and quantification of *SRL* gene expression. A cRNA (360 fg, corresponding to 4×10^5 molecules) containing the sequences of the two PCR primers was mixed with 1 μg of total rat RNA isolated from (a) day E9 whole embryo, (b) E14 brain, (c) E18 brain, (d) newborn brain, (e) P7 brain, (f) P14 brain, (g) adult brain, (h) muscle, (i) lung, (j) liver, (k) gut, (l) heart, (m) kidney and (n) stomach fundus. The RNAs in lanes g–n were isolated from adult rats. After reverse transcription of the RNAs, 26 (**A**), 28 (**B**), 30 (**C**) and 32 (**D**) cycles of PCR were performed. PCR products were separated by gel electrophoresis. The sizes of the standards are indicated in base-pairs. The 252 bp band corresponds to the internal control (cRNA), the 196 bp band to the mRNA.

As an internal standard for the RNA quantifications, we constructed a clone containing the sequence of the exon III of the *SRL* gene extended by 56 bases. This construct was transcribed *in vitro* and 4×10^5 molecules of cRNA (360 fg) were mixed with 1 μg of total RNAs prepared from various rat tissues prior to the reverse transcription (see legend to Figure 1). The PCR products were separated by agarose gel electrophoresis. The 252 bp band represented the internal standard, the 196 bp band the mRNA present in the respective tissue.

After 28 cycles of PCR the 196 bp band became clearly visible in stomach fundus RNA. When the reverse transcription step was omitted the 196 bp band did not appear, indicating that the bands shown in Figure 1 were not caused by contamination of the RNA preparations with DNA (data not shown).

Two different approaches were employed for the quantification of the *SRL* RNA present in stomach fundus. First, samples were analysed after 26, 28, 30 or 32 PCR cycles, the bands were cut from a gel and the incorporated radioactivity was determined by scintillation counting. Alternatively, reverse transcription and PCR were performed in the presence of increasing amounts of the cRNA standard to determine the amount needed to obtain equally strong bands in a competitive PCR (Gilliland *et al.*, 1990). With both methods, it was found that $\sim 3 \times 10^5$ *SRL* mRNA molecules were present in 1 μg of total RNA from stomach fundus. This corresponds to a ratio of *SRL* mRNA to poly(A^+) RNA of ~1:100 000 assuming that 2% of the total RNA is polyadenylated.

Cloning and sequence analysis of the SRL cDNA

We constructed a cDNA library from rat stomach fundus. Several clones were isolated by pre-screening pools of clones with PCR and subsequent standard filter hybridizations of positive pools. The nucleotide and deduced amino acid sequences are shown in Figure 2. The longest open reading frame encoded a protein of 479 amino acids.

The hydrophobicity plot displayed seven hydrophobic, putative membrane-spanning regions and looked very similar to those of the 5-HT_{1C} and 5-HT_2 receptors (data not shown). The additional amino-terminal hydrophobic region characteristic for the 5-HT_{1C} receptor (Yu *et al.*, 1991) was absent in the *SRL* gene product. The latter contained five consensus sequences for N-linked glycosylation (Hubbard and Ivatt, 1981), two of them located in the third and fourth extracellular loops (Figure 2). There was no potential N-glycosylation site within the amino-terminal extracellular domain. A potential site for palmitoylation was present in the carboxy-terminal part of the molecule (Figure 2). Depalmitoylation at an analogous site of the β_2-adrenergic receptor has resulted in receptor uncoupling (O'Dowd *et al.*, 1989). Although the sequence contained recognition motifs for various protein kinases (Kemp and Pearson, 1990), including cAMP- and cGMP-dependent protein kinases, casein kinase II and S6 kinase, there was no consensus sequence for phosphorylation by protein kinase C. Of particular interest may be the recognition sequences for cAMP-dependent protein kinases in the third intracelluar loop and the carboxy-terminus.

The SRL amino acid sequence revealed strong similarities to the 5-HT_{1C} and 5-HT_2 receptors (51 and 45%, respectively) and significantly lower homology (30%) to the 5-HT_{1A} receptor. The usual amino acids are conserved in this new member of the family of G-protein coupled receptors. An alignment of the SRL, 5-HT_{1C}, and 5-HT_2 receptor sequences are depicted in Figure 3.

Pharmacological profile of the receptor expressed in COS cells

Membranes prepared from transfected COS cells displayed high affinity [^{3}H]5-HT binding: B_{max} = 1122 ± 54

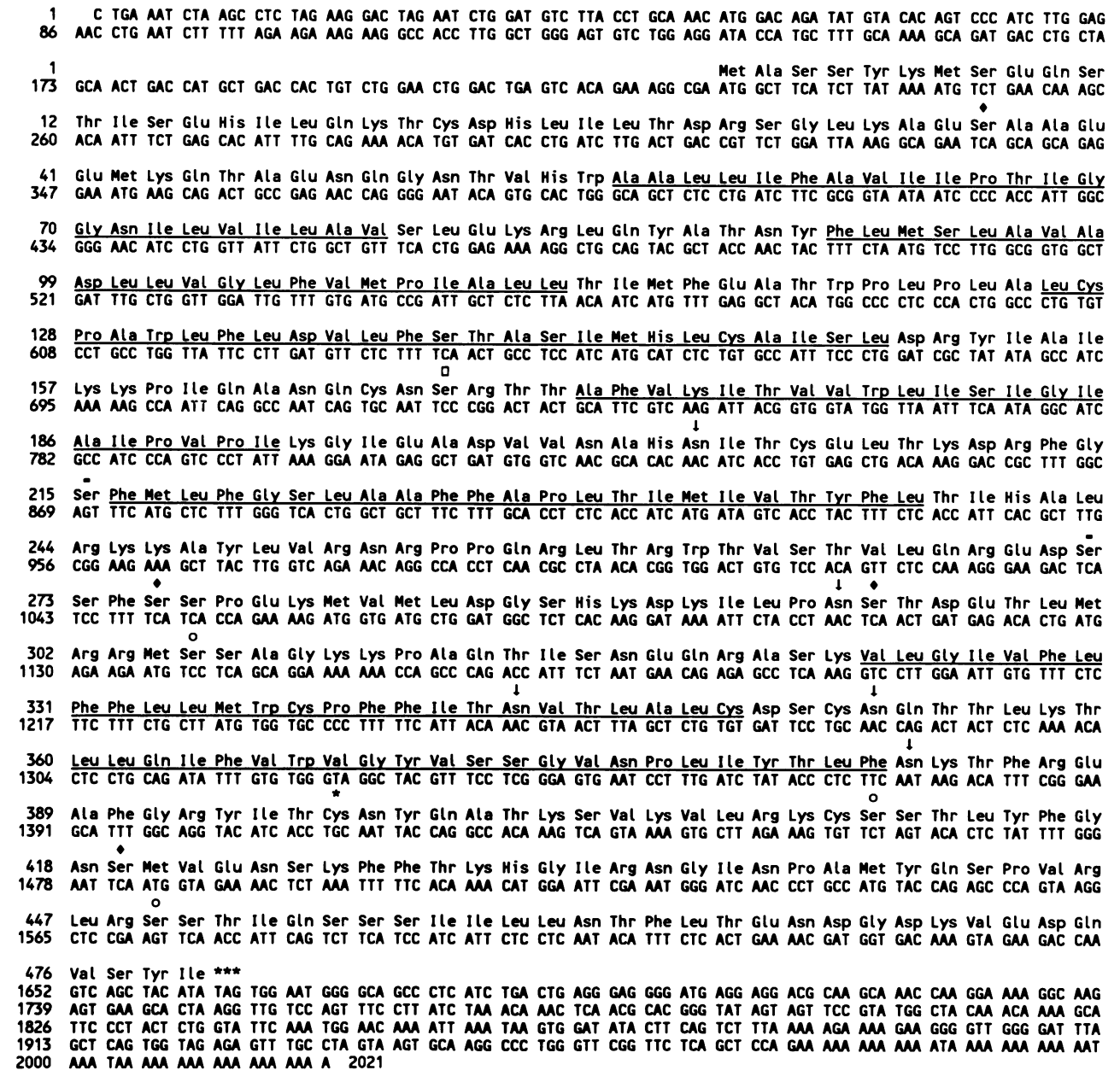

Fig. 2. Nucleic acid and deduced amino acid sequence of the *SRL* cDNA (EMBL database accession number X66842). The putative membrane spanning regions are underlined. Consensus sequences for N-linked glycosylation are marked by an arrow; an asterisk marks the cysteine residue that may attach the C-terminal region to the membrane via a palmitoyl anchor. Several recognition motifs for protein kinases are indicated (●, cAMP-dependent protein kinase; □, cGMP-dependent protein kinase; ♦, casein kinase II; =, S6 kinase).

fmol/mg, $pK_D = 7.39 \pm 0.01$ (mean values ± SD, $n = 2$). Non-transfected cells did not have binding sites. The affinity of 5-HT in the saturation binding experiments agreed well with that obtained in competition experiments. Examples for typical ligand displacement curves are shown in Figure 4 and the results listed in Table I. The pharmacological profile of the binding sites was different from that of other known 5-HT recognition sites (e.g. subtypes of 5-HT_1, 5-HT_2, 5-HT_3 or 5-HT_4). It was characterized by high affinity for pizotifen, ergolines, yohimbine and rauwolscine. There was a good correlation ($r = 0.88$) with results obtained in functional analyses of rat fundic strip preparations (Buchheit *et al.*, 1986; Clineschmidt *et al.*, 1985; Cohen and Wittenauer, 1986; Kalkman and Fozard, 1991).

Expression in Xenopus oocytes and electrophysiology

cDNA clones of the mouse 5-HT_{1C} (Yu *et al.*, 1991), rat 5-HT_2 (Foguet *et al.*, 1992) and SRL receptors were transcribed *in vitro* and RNA was injected into *Xenopus* oocytes. Application of serotonin (200 nM and 10 μM, respectively) triggered the opening of a chloride channel in the membranes of oocytes injected with 5-HT_{1C} and 5-HT_2 receptor RNA (Figure 5A and B). Generally, the current amplitude was higher for the 5-HT_{1C} than for the 5-HT_2 receptor as expected based on previous reports (Lübbert *et al.*, 1987b). Generally, oocytes injected with the *SRL* RNA did not respond to 2−20 nM 5-HT, whereas clear currents were observed with 200 nM. Higher concentrations (1−10 μM) resulted in very slow recovery. Hence, 200 nM

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SRL                          MASSYKMSE QSTISEHILQ KTCDHLILTD RSGLKAESAA EEMKQTAENQ GNTVHWAALL IFAVIIPTIG GNILVILAVS
5-HT1C                       MVNLGNAVRS LLMHLIGL*V WQF*IS*SPV AGIVTDTFNS SDGGRLFQFP DGVQN*P**S *VVI**M*** ******M***
5-HT2  MEILCEDNIS LSSIPNSLMQ LGDGPRLYHN DFNSRDANTS EASNWT*DAE NRTNLSCEGY LPPTCLSILH LQEKN*S*** TTV***L**A ******M***

SRL    LEKRLQYATN YFLMSLAVAD LLVGLFVMPI ALLTIMFEAT WPLPLALCPA WLFLDVLFST ASIMHLCAIS LDRYIAIKKP IQANQCNSRT TAFVKITVVW
5HT1C  M**K*HN*** *******I** M****L***L S**A*LYDYV ****RY***V *IS******* ********** ****V**RN* *EHSRF**** K*IM**AI**
5HT2   ***K**N*** *******I** M*L*FL***V SM***LYGYR ****SK**AI *IY******* ********** ****V**QN* *HHSRF**** K**L**IA**

SRL    LISIGIA--I PVPIKGIEAD V-VNAHNITC ELTKDRFGSF MLFGSLAAFF APLTIMIVTY FLTIHALRKK AYL-VRNRPP QRL-TRWTVS TVLQREDSSF
5HT1C  A****VSVP* **IGLRD*SK *F**--*T** V*N-*PN--* V*I**FV*** I*****VI** ****YV**RQ TLMLL*---- --------GH *EEELANM*L
5HT2   T**V**SMP* **FGLQDDSK *FKE--GS-* L*A-*DN--* V*I**FV*** I*****VI** ****KS*Q*E *T*C*SD--- --*S**AKLA SFSFLPQ**L

SRL    SSPEKMVMLD GSHKDKILPN STDETLMRR- -MSSAGKKPA QTISNEQRAS KVLGIVFLFF LLMWCPFFIT NVTLALCD-S CNQTTLKTLL QIFVWVGYVS
5HT1C  NFLNCCCKKN *-GEEENA** PNPDQKP**K KKEKRPRGTM *A*N**KK** *******FV* *I******** *ILSV**GKA ***KLMEK** NV***I***C
5HT2   **-**----- ---------- -LFQRSIH*E PG*Y**RRTM *S*****K*C *******FL* VV********* *IMAVI*KE* **ENVIGA** NV***I**L*

SRL    SGVNPLIYTL FNKTFREAFG RYITCNYQAT KSVKVLRKCS STLYFGNSMV ENSKFFTKHG IRNGINPAMY QSPVRLRSST IQSSSIILLN TFLTENDGDK
5HT1C  **I***V*** ***IY*R**S K*LR*D*KPD *KPP*RQIPR VAATALSGRE L*VNIYRHTN E*VARKANDP EPGIEMQVEN LELPVNPSNV VSERISSV
5HT2   *A****V*** ****Y*S**S ***Q*Q*KEN RKPLQ*ILVN TIPALAYKSS QLQVGQK*NS QEDAEQTVDD C*M*T*GKQQ SEENCTDNIE *VNEKVSCV

SRL    VEDQVSYI
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Fig. 3. Alignment of the SRL, rat 5-HT_{1C} (Julius *et al.*, 1988) and rat 5-HT_2 (Julius *et al.*, 1990) receptor sequences. Bars represent the putative membrane spanning regions. Amino acid identities are illustrated by asterisks.

5-HT was used throughout. The average amplitude under these conditions was 73 ± 23 nA (mean ± SEM, n = 7), compared with an average amplitude of 555 ± 78 nA (n = 11) for the activation of the 5-HT_{1C} receptor under identical conditions.

A typical response to 200 nM 5-HT is shown in Figure 5C. The latency in current onset as well as the time to peak current were long. Current oscillations were also observed, superimposed on the elevated baseline. Similar results were observed in nine out of 12 oocytes, although the size of both the peak current and oscillations was variable (13−260 nA and <10−40 nA, respectively). Such oscillations in current are typical for Ca^{2+}-mobilizing agonists (Berridge, 1988). Caffeine, by depleting the internal Ca^{2+}-induced Ca^{2+} release sensitive store, can abolish oscillations induced by several agonists. A 4 min pre-exposure to 10 mM caffeine abolished the oscillations induced by 5-HT and greatly reduced the non-oscillatory current component (data not shown). Generally, the latency of onset was slower for the responses mediated by SRL than by the 5-HT_{1C} receptor. For the 5-HT_{1C} response it was 39 ± 3 s (n = 11) after the initiation of agonist perfusion, whereas for SRL it was 108 ± 10 s (n = 9). These long latencies were seen irrespective of the size of the current in a particular oocyte and therefore most probably do not simply reflect weaker expression of SRL.

The effects of yohimbine on the 5-HT-induced current were studied and a typical result is also shown in Figure 5C. After obtaining the control response, oocytes were exposed to 1 μM yohimbine for 2 min and then challenged with 5-HT. Yohimbine pretreatment abolished the response to 5-HT (five out of five oocytes) in a reversible manner.

Discussion

We have previously isolated a putative exon of a gene, called the *SRL* gene, with strong homologies to corresponding exons in the genes of the closely related 5-HT_{1C} and 5-HT_2 receptors (Foguet *et al.*, 1992). The most significant expression of this gene was found in the stomach fundus. This tissue displays a contractile response to serotonin. Although known for a long time, the pharmacological nature of the fundus 5-HT receptor has remained unclear (Kalkman and Fozard, 1991). The receptor is not of the 5-HT_2, 5-HT_{1A} or 5-HT_{1B} type (Clineschmidt *et al.*, 1985; Cohen and Wittenauer, 1987). Based on the order of potency of a large series of agonists, it was initially proposed that the fundus receptor is of the 5-HT_{1C} type (Buchheit *et al.*, 1986), but this could not be confirmed with antagonists (Baez *et al.*, 1990; Kalkman and Fozard, 1991). Although some pharmacological similarities also exist between the fundus and the 5-HT_{1D} receptor, there is certainly no identity between these two receptors (Kalkman and Fozard, 1991).

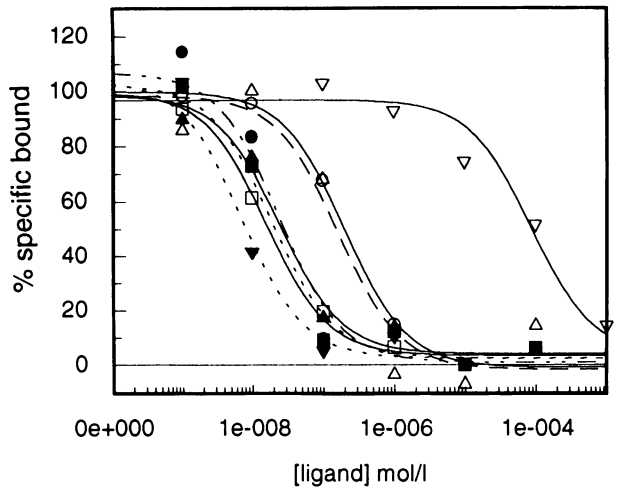

Fig. 4. Displacement of [^{3}H]5-HT bound to transfected COS cells by a variety of ligands. The results are from a typical experiment performed in triplicate. The figure depicts specific binding at the indicated concentrations of competing drugs. ●- — -, 5-MeOT; ○—, tryptamine; □—, rauwolscine; ▼---, methylsergide; △— — —, 5-HT; ■----, pizotifen; ▽—, 8-OH-DPAT; ▲—, 1-NP.

The high abundance of the *SRL* gene expression in stomach fundus (1:100 000) and the low or absent expression elsewhere imply that this gene encodes the stomach fundus 5-HT receptor.

The pharmacological profile of the expressed receptor resembled that of the serotonin stimulated contraction of the fundus more closely than that of any other serotonin receptor type (correlation coefficient r = 0.88). Several compounds do, however, display significant differences in their affinities

Table I. Pharmacology of the rat stomach fundus 5-HT receptor expressed in COS cells

Drug	COS cell binding	Function	5-HT_{1C} binding	5-HT_2 binding
Agonists				
5-HT	7.55 ± 0.30 (4)	7.97	7.5	5.5
5-MeOT	8.15 ± 0.38 (4)	7.79	7.6	5.5
Tryptamine	6.70 ± 0.22 (4)	7.20	7.2	5.0
2-Me-5-HT	6.50 ± 0.20 (3)	6.22	5.8	5.0
8-OH-DPAT	5.05 ± 0.55 (4)	4.58	5.2	5.0
Antagonists				
Pizotifen	7.41 ± 0.34 (5)	7.66	8.1	8.8
Rauwolscine	7.23 ± 0.26 (6)	8.42	5.8	6.1
1-Naphtylpiperazine	7.17 ± 0.35 (5)	8.86	8.2	7.2
Yohimbine	6.97 ± 0.48 (4)	7.89	4.4	6.0
Methysergide	7.06 ± 0.84 (3)	8.48	8.6	8.6
Dihydroergotamine	6.84 ± 0.45 (4)	7.12	7.5	8.6
Mianserin	6.70 ± 0.10 (4)	6.60	8.0	8.1
Cinanserin	5.78 ± 0.41 (4)	4.83−5.29	6.7	8.0
Pirenperone	5.67 ± 0.83 (3)	5.50	7.3	8.8
Quipazine	<5.20 (3)	6.00	6.7	6.2
MK 212	<4.00 (3)	5.91	6.2	5.3
Spiperone	<4.00 (2)	<5.20	5.9	8.8

Radioligand binding studies were performed with [^{3}H]5-HT in membranes from transfected COS cells (pK_D values, −log mol/l); the number of experiments is indicated in parenthesis. Results from functional experiments performed in rat fundus strips are reported as pEC_{50} (agonists) or pA_2, pK_B or pD'_2 values (antagonists). Functional data are from Buchheit *et al.* (1986), Clineschmidt *et al.* (1985), Kalkman and Fozard (1991) or from H.O.Kalkman, J.R.Fozard and D.Hoyer (unpublished). 5-HT_{1C} and 5-HT_2 binding data, shown for comparison, were derived from pig choroid plexus and rat cortex (Hoyer, 1991). A correlation coefficient of 0.879 ($P < 0.01$) was obtained when binding and function were compared. Correlations with 5-HT_{1C} and 5-HT_2 binding were much lower.

in both systems. This may be attributed to differences in post-translational modifications or G-protein coupling. The characterization is complicated by the fact that the fundus may not have a homogeneous population of 5-HT receptors. Indeed, concentration response curves to 5-HT and a variety of other agonists are unusually shallow, suggesting the coexistence of at least two receptors (see Clineschmidt *et al.*, 1985; Buchheit *et al.*, 1986). Thus, values obtained in functional tests with both agonists and antagonists may represent approximations. Actually, many antagonists tested in this model do not display true competitive antagonism; among other explanations, receptor heterogeneity can certainly lead to this type of complex behaviour. Thus, it may not be too surprising that a perfect match was not observed between functional data and results obtained in the radioligand binding experiments reported here. Nevertheless, the pharmacological characteristics of the expressed receptor were unique and compared best with those observed in the rat fundus preparation.

Smooth muscle preparations almost always contract in response to 5-HT via a stimulation of either a pure 5-HT_2 population or a mixed 5-HT_{1D}-like/5-HT_2 population (Frenken and Kaumann, 1985; Borton *et al.*, 1990; Hamel and Bouchard, 1991). The rat fundus preparation has previously resisted definition and the present data offer an explanation, in that a novel 5-HT receptor mediates contraction in this tissue.

The stomach fundus 5-HT receptor is a typical G-protein coupled receptor with seven putatively membrane spanning hydrophobic regions and several other conserved features. Based on its sequence and the location of introns in its gene (Foguet *et al.*, 1992), it is closely related to the 5-HT_{1C} and 5-HT_2 receptors. Interestingly, like the 5-HT_2 receptor, it contains neither the additional characteristic hydrophobic region present at the amino-terminus of the 5-HT_{1C} receptor

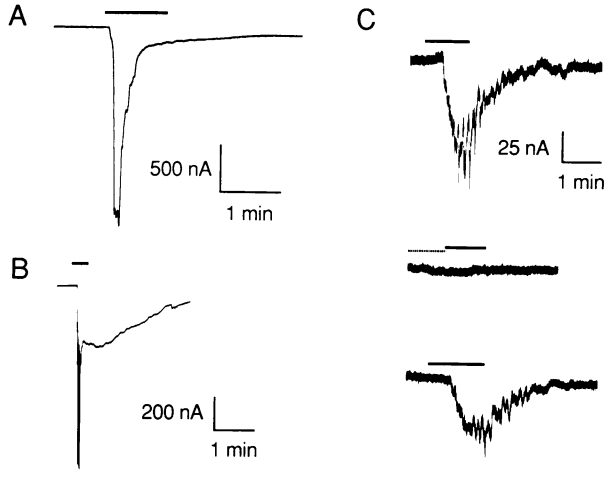

Fig. 5. Chloride currents recorded from an oocyte under perfusion with frog Ringer's. **A**: Response to 200 nM 5-HT of an oocyte injected with 5-HT_{1C} RNA. **B**: Response of a 5-HT_2 RNA injected oocyte to 10 μM 5-HT. **C**: The first line shows the control response of a *SRL* RNA-injected oocyte to 200 nM 5-HT. The response is blocked by pretreatment with 1 μM of yohimbine (second line) in a reversible manner (third line). The bars illustrate the times of serotonin application, the stippled bar that of the pretreatment with yohimbine.

(Yu *et al.*, 1991) nor the intron that is present in this part of the 5-HT_{1C} receptor gene (Foguet *et al.*, 1992).

It is well established that agonist binding to the 5-HT_{1C} and 5-HT_2 receptors expressed in *Xenopus* oocytes mediates the activation of phospholipase C followed by Ca^{2+} mobilization and the opening of Ca^{2+}-activated Cl^- channels in the oocyte membrane (Dascal *et al.*, 1986; Lübbert *et al.*, 1987b; Pritchett *et al.*, 1988). The typical oscillating Cl^- conductance has been triggered by the stomach fundus serotonin receptor. The affinities of 5-HT

and yohimbine in this assay correlated perfectly with those observed in the COS cells. We concluded that in oocytes the stomach fundus receptor is also coupled to phosphatidylinositol (PI) hydrolysis. Previous authors have not been able, however, to detect a significant serotonin stimulated PI increase in the rat fundus (Cohen and Wittenauer, 1987). Receptors expressed in *Xenopus* oocytes couple to intrinsic G-proteins. This coupling is significantly more efficient for 5-HT_{1C} than for 5-HT_2 receptors (Lübbert *et al.*, 1987b). Based on the amplitudes of the membrane depolarizations, the coupling of the stomach fundus 5-HT receptor to the oocyte second messenger system is even weaker. Therefore, one has to be careful in extrapolating the oocyte results to the situation in the fundus. However, in all experiments that we are aware of, the second messenger coupling of a receptor expressed in the oocyte system has reflected the coupling in its natural cellular environment.

As determined by PCR amplification of genomic DNA, the *SRL* gene is present in the mouse, rat and human genomes (data not shown). This raises the question if and where this receptor is expressed in the human body. It should be noted that a tissue like the extremely muscular and distendable rat fundus is not present in the human stomach. Isolated tissues with receptors displaying a similar pharmacological profile have been described. For instance, endothelium-dependent vasorelaxation to 5-HT in the jugular vein, and possibly also other blood vessels takes place via a 5-HT receptor like that in the rat fundus (Leff *et al.*, 1987; Mylecharane, 1990). This renders the analysis of the expression of this receptor in the human body highly interesting.

Materials and methods

Tissue distribution of SRL mRNA

RNA was prepared according to the procedure of Aguzzi (1990). In addition, since DNA contamination would interfere with reverse transcription/PCR analysis (only the sequence from one exon was known), 20 μg of RNA was treated twice for 1 h with 10 units of RQ1 RNase-free DNase (Promega) in the presence of 40 units of RNAsin (Promega) according to the supplier's instructions, followed by phenol extraction and ethanol precipitation. To ensure that the RNA was free of contaminating DNA, PCR reactions were also performed without prior reverse transcription.

To serve as internal standards for PCR reactions (Wang *et al.* 1989) the segment of the *SRL* gene between the PCR primers was extended by 56 nucleotides. An oligonucleotide (GGGCTACTGCATTCAGATTCCAG-TCTGGAATTCGGTACCGGATCCATCGTAGGCTCCTGATTCGATA-ACAGTCAAGATTACAG) was synthesized containing nucleotides 175–189 of the *SRL* gene sequence (Foguet *et al.*, 1992), an insertion of 56 randomly chosen nucleotides, and the complement of the first 11 nucleotides of the 3′-oligonucleotide used for the detection of the *SRL* gene (see below). These two oligonucleotides were allowed to form primer dimers in a standard PCR reaction, the resulting product was eluted from a gel and inserted by blunt-end ligation between the *Sma*I restriction site at position 172 (Foguet *et al.*, 1992) of an *SRL* gene subclone in pBluescript (Stratagene) and the *Sma*I site in the vector. A clone containing the insert in the correct orientation was isolated and cRNA was prepared by *in vitro* transcription using slightly modified standard procedures. Briefly, 2 μg of plasmid DNA previously linearized with *Sca*I was transcribed with 70 units of T3 RNA polymerase (Boehringer) under standard conditions. After a 1 h incubation at 37°C, the same amount of T3 RNA polymerase was added and the incubation was continued for another hour. To remove the DNA template, the cRNA was incubated twice for 1 h with 20 units of RQ1 DNase (Promega, RNase-free) followed by phenol extraction and ethanol precipitation.

For the RT-PCR 1 μg of each RNA was mixed with 360 fg of the internal standard RNA. The reverse transcription was performed in a final volume of 20 μl using MMLV reverse transcriptase (BRL) in PCR buffer. 10 μl of this reaction were then used in a PCR with Ampli-Taq (Perkin–Elmer Cetus) according to the supplier's instructions. The reactions contained 5 μCi of [α-^{32}P]dCTP and 0.2 μM of each primer (AGGCTATATGGCC-CCTCCCACT and GAAATTAACCATACCACTGTAATCTTG) in a final volume of 50 μl. Following the amplification, the ^{32}P-labelled PCR products were separated on 4% agarose gels. The gels were dried and exposed to X-ray film. For quantification, the bands of interest were cut from the gel and the radioactivity determined by scintillation counting.

Isolation of SRL cDNA clones

To prevent self-ligation of the vector we inserted an oligonucleotide (TCGACCATTGTGCTGGATCCGATCTACCAGCACAATGGAGCT) containing two *Bst*XI restriction sites into pBluescript M13+ (Stratagene) between the *Sal*I and the *Sac*I restriction sites. Digestion of the vector with *Bst*XI then created two overhanging ends (5′...TGTG 3′, similar to the vector constructed by Aruffo and Seed, 1987). The fragment between the two *Bst*XI sites was removed by agarose gel electrophoresis. Blunt-ended cDNA was synthesized from 5 μg of rat stomach fundus poly(A)$^+$ RNA using the Amersham cDNA synthesis system. Double-stranded adapters (5 μg) containing a *Sal*I restriction site and creating a 5′...CACA-3′ overhang (TGTCGACTGCACA) were ligated to the ends in a final volume of 300 μl according to standard procedures. Subsequently the cDNA was separated by gel electrophoresis and molecules >1.5 kb were recovered from the gel by adherence to glass milk (Quiaex, Diagen). After ligation of the cDNA to 2 μg vector in a final volume of 400 μl, the constructs were extracted with phenol three times and precipitated with ethanol. The library was transformed into *Escherichia coli* WM1100 by electroporation (Gene Pulser, Bio-Rad). It contained 2×10^7 independent transformants with an average insert size of 2 kb. 23 pools of 5×10^4 clones each were grown over night and plasmid DNA was isolated. From each pool, 0.5 μg of plasmid DNA was linearized and PCR was performed using the *SRL* primers as described above. 13 pools gave rise to the expected PCR product. Three of them were plated on agar and screened by standard filter hybridizations (Sambrook *et al.*, 1989) using an oligonucleotide probe (AGGCTATATGGCCCCT-CCCACTGGCCCTG). DNA sequencing was performed with the T7 sequencing kit from Pharmacia. The DNASIS and PROSIS programs (Hitachi) were used for the sequence analysis.

COS cell expression and radioligand binding studies

COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum. For transfection, the cells were plated at 1.5×10^6 cells/13.5 cm plate and used for transfection 24 h later. The cDNA was subcloned into *Sal*I-cut pXMD1 and transfected with the DEAE–dextran method as described (Kluxen *et al.*, 1992). Two days later, membranes were prepared by hypotonic lysis as described (Hoyer and Neijt, 1988) and stored at −80°C or used immediately. Membranes were suspended in buffer (50 mM Tris–HCl, 4 mM $CaCl_2$, pH 7.7). The assays consisted of 50 μl of radioligand, 200 μl of competing drug and 750 μl of membrane suspension (protein concentration = 60–80 μg/ml). Saturation experiments were performed with eight concentrations of radioligands in triplicate determinations. Competition experiments were performed with six to eight concentrations of competing ligands with [^{3}H]5-HT at a final concentration of 4–5 nmol/l. The mixture was incubated at 37°C for 30 min and the assay terminated by rapid filtration (twice with 5 ml of cold 20 mM Tris–HCl, 154 mM NaCl) over Whatman GFB filters. Filters were dried rapidly and counted by liquid scintillation. Non-specific binding was determined in the presence of an excess of 5-HT (10 μM). Bound radioligand represented <2% of free radioligand. In competition experiments, specific binding represented ~40% of total binding. Results are expressed as pK_D values (−log mol/l) SEM. Data were analysed by computerized iterations as described earlier (Hoyer and Neijt, 1988).

Expression in Xenopus oocytes and electrophysiology

In vitro transcription, oocyte injection and current recording were performed essentially as described (Stühmer *et al.*, 1989; Yu *et al.*, 1991). The follicular layer of the oocytes was removed enzymatically before injection while the vitellin membrane was left intact. Oocytes were injected with 20 ng of RNA synthesized *in vitro*. Electrophysiological measurements were performed 3 days after injection of the oocytes. All experiments were conducted under two electrode voltage clamp with a holding potential of −80 mV. Oocytes were perfused at a high flow rate (~5 ml/min) in order to achieve a rapid and uniform exposure to serotonin. 15 min recovery time was allowed between successive applications of serotonin.

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