

Teleost isotocin receptor: structure, functional expression, mRNA distribution and phylogeny

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Abstract A cDNA encoding a receptor for the oxytocin-related peptide isotocin has been identified by screening a lambda gt11 library constructed from poly(A)⁺ RNA of the hypothalamic region of the teleost *Catostomus commersoni*. The probe used was obtained by PCR amplification of white sucker genomic DNA using degenerate primers based on conserved sequences in the mammalian receptor counterparts. The full-length cDNA specifies a polypeptide of 390 amino acid residues that displays the typical hydrophobicity profile of a seven transmembrane domain receptor and which exhibits greatest similarity to mammalian oxytocin receptors. Oocytes that express the cloned receptor respond to the application of isotocin by an induction of membrane chloride currents indicating that it is coupled to the inositol phosphate/calcium pathway. The isotocin receptor (ITR) can also be activated by vasotocin, mesotocin, oxytocin and Arg-vasopressin, although these have lower potencies than isotocin. ITR-encoding mRNA has been detected in brain, intestine, bladder, skeletal muscle, lateral line, gills and kidney indicating that this receptor may mediate a variety of physiological functions.

Key words: Neuropeptide; Oxytocin receptor; Vasopressin receptor; White sucker; *Catostomus commersoni*; Hypothalamus

1. Introduction

The mammalian neurohypophyseal nonapeptide hormones vasopressin (VP) and oxytocin (OT), and their cognate receptors, are involved in a variety of functions such as the regulation of osmotic balance and blood pressure (VP), and the contraction of uterine and mammary smooth muscle during birth and lactation (OT). The functional roles of their non-mammalian counterparts vasotocin and isotocin/mesotocin are less well defined. However, recent binding, in situ hybridization, and physiological studies suggest that these peptides mediate a number of diverse activities in lower vertebrates including reproductive, renal, cardiovascular, metabolic, and hydroosmotic functions; in addition, they may play a role in urine excretion and in the modulation of neurotransmission [1–10]. This broad range of physiological effects and the observation of diverse signal transduction pathways point to the existence of a number of nonapeptide receptor subtypes in non-mammalian species. Re-

cently an Arg-vasotocin receptor has been cloned from the teleost fish white sucker, *Catostomus commersoni* [11] by PCR amplification using degenerate oligonucleotide primers based on conserved regions of the mammalian vasopressin V1a, and V2 and oxytocin receptors [12–18]. We report here on the identification of a teleostean isotocin receptor¹, its primary structure and functional properties.

2. Materials and methods

2.1. Materials

Nonapeptides were obtained from Saxon Biochemicals (Hannover, Germany). Taq DNA polymerase, a T7 RNA polymerase transcription kit from Promega Biotec (Madison, WI, USA), RNazol B from Biotex Lab. Inc. (Houston, TX, USA), and RNasin and DNase I from Boehringer (Mannheim, Germany).

2.2. Cloning of the isotocin receptor

Degenerate PCR primers based on conserved sequences of mammalian nonapeptide receptors were used to amplify *C. commersoni* genomic DNA [11]. Fragments of about 0.35 kb in size were cloned into the *Pst*I restriction site of pBluescript and sequenced. One clone that displayed greatest sequence similarity to mammalian oxytocin receptor sequences was labelled with [³²P]CTP and used for screening (3 × 10⁸ dpm/μg DNA, 1 × 10⁶ dpm/ml of hybridization buffer) a *C. commersoni* hypothalamic lambda gt11 cDNA library (3 × 10⁶ recombinant phages, 5 × 10⁴ plaque forming units per plate). One positive clone (ITR) was isolated, its 3.1 kb insert subcloned into pBluescript, and subjected to automated DNA sequencing (Applied Biosystems Model 373 A) with dye-labelled primers and dye terminators.

2.3. Functional expression in oocytes

Synthesis of in vitro transcribed RNA was carried out using, as template, pBluescript containing the ITR cDNA linearized at the *Sal*I restriction site present in the vector polylinker and a T7 RNA polymerase transcription kit (using the protocol of the manufacture). RNA was dissolved in H₂O (40 ng/μl) and injected (50 nl) into stage V *Xenopus laevis* oocytes. Oocytes were kept at 20°C for two to three days. Voltage clamp measurements were performed with a conventional two-electrode voltage clamp (CA100, VF1800, VF180, Biologic, Claix, France) at room temperature. Membrane currents were elicited by a 1 min application of peptide agonists to the injected oocytes.

2.4. mRNA distribution

Total RNA was extracted from various white sucker tissues using RNazol B. 100 μl containing 2 μg of total RNA and 64 units of RNasin were incubated with 10 units of DNase I at 37°C for 30 min. RNA was extracted with phenol and chloroform, ethanol precipitated, dissolved in H₂O and converted into single-stranded cDNA using oligo(dT) by reverse transcription [19]. Forward (positions 941–961) and reverse (positions 1346–1366) primers (1 μM each) corresponding to the ITR cDNA sequence (Fig. 1) were employed in the PCR (2.5 units Taq DNA polymerase/100 μl; 35 cycles at 94°C for 1 min, 55°C for 1 min, and

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Abbreviations: ITR, isotocin receptor; VTR, Arg-vasotocin receptor; VP, Arg-vasopressin; OT, oxytocin; IT, isotocin; VT, Arg-vasotocin.

¹The sequence reported in this paper has been deposited in the GenBank database (accession no. X87783).

72°C for 1 min) with 1/20th of the single-stranded cDNA. Following agarose gel electrophoresis, fragments were blotted onto nylon membranes and hybridized under stringent conditions (final wash, 65°C, for 1 h, in 0.1 × SSC, 0.1% SDS; ref. [19]) with a ³²P-labelled ITR cDNA probe.

3. Results and discussion

3.1. Cloning and structure of the isotocin receptor

Using degenerate primers based on DNA sequences encoding the second extracellular loop and transmembrane domain VI of mammalian nonapeptide receptors [12–18], a cDNA fragment has been amplified the specified product of which exhibits greatest similarity to mammalian oxytocin receptors. Using this fragment as a probe a cDNA clone of 3152 bp has been isolated from a *C. commersoni* hypothalamic cDNA library (Fig. 1). This includes 213 bp of 5'-untranslated and 1769 bp of 3'-untranslated sequence. The presence of a short stretch of 9 adenine residues that are 21 nucleotides downstream of a canonical polyadenylation signal suggests that the complete 3' end has been cloned. An open reading frame of 1170 bp predicts a protein of 390 amino acid residues with a molecular weight of 44487 Da.

Hydrophobicity analysis of the deduced amino acid sequence indicates the presence of seven putative transmembrane regions that are characteristic of G-protein coupled receptors. The sequence displays a high degree of similarity to mammalian oxytocin receptors and shares several features with these receptors that are not found in vasotocin and vasopressin receptors such as an Asp-Arg-Cys variation of the conserved Asp-Arg-Tyr motif at the end of transmembrane region III. As shown in Fig. 1 the ITR displays several other characteristics of G-protein coupled receptors such as (i) N-linked glycosylation sites in its N-terminal extracellular domain (Asn-14, -19, -24, -30), (ii) consensus sequences for phosphorylation by protein kinase C (Thr/Ser-X-Arg/Lys) in the first (positions 70–72) and third (positions 239–241, 262–264, 273–275) intracellular loops and in the C-terminal intracellular tail (positions 365–367, 380–382), (iii) for protein kinase A (Arg-X-Ser/Thr) in the third intracellular loop (positions 259–261) and in the cytoplasmic tail (positions 366–368), and (iv) for casein kinase II (Thr/Ser-X-X-Asp/Glu) in the third intracellular loop (positions 239–242). A cysteine doublet 14 residues after transmembrane domain VII may be modified by palmitoylation and may thereby anchor the cytoplasmic tail in the lipid bilayer [20]. Sequence

ACGGACAGCAAGTGAAGGACCTGCTAAAAATGGATAAATATCGTTGAAAGCAACTGAGGGATCACCACCAACAGTAAAGGTCACAGTGT	- 124
ATCCAAATCTCTCGGTGACACCTGTTAGGCTATTTAAATTAAGTGGCTGAATATGCGCGAGGAAAGCTTGTCGGTCTATTGATACATT	- 34
TATGGAAGTAGACGTGCTCTTTTCTGGCATCATGGAGGAGATGTTCAAGGAGCAAGACTTTTGGTCTTCAACGAATCATCTAGAAAC	57
M E E M F K E Q D F W S F N E S S R N	19
TCCACCGTCGGCAATGAGACCTTTGGTGGGAACACAGACGTAATCCCTTGAGAGAAATGAAGAGGTGGCCAAAGTAGAAGTTACTGTG	147
S T V G N E T F G G N Q T V N P L K R N E E V A K V E V T V	49
CTTGGCTTGGTCTGTTTGGTCTTGTCTGTAACCTATGCGTCTTATTCGATCTACACAGCCCAAGCACTCAATCGCGCATGTAT	237
L A L V L F L A L A G N L C V L I A I Y T A K H T Q S R M Y	79
TACCTCATGAAGCACCTACGATTGCGATCTGGTGGTGGCTGTCTTCCAGGTTCTGCGCTCAACTCATTTGGGACATCACCTTCGCTTC	327
Y L M K A H S L A D L V A V F O V L P O L I W D I T F R F	109
TATGGGCGAGACTTCCTTTGCGAGTGGTAAATATCTCCAGACGTCGGGATGTTGCGCTCCACTTACATGCTCGTGTGATGTCATA	417
Y G P D F L C R L V K Y L Q T V G M F A S T Y M L V L M S I	139
GACAGATGTTATCTATCTGTCAGCCCTCTGTTCTTTACACAAGCGAAAGGACCGCTGTATGTGATTTCTTGGGCACTGAGCTTA	507
D R C I A Q P L R S L C H K R K D R C Y V I V S W A L S L	169
GTTTTTCAGTGTCCACAAAGTTTATATATCTCTTACGTGAGATGGTAAATGGAGTCTACGATTGCTGGGAGATTTCTGTCAGCCTTGG	597
V F S V P O V Y I F S L R E I G N G V Y D C W G D F V Q P W	199
GGAGCAAAAGCTTACATTTACATGGATAAGTCTGACATAATACATCATACCAGTGGCCACTATGGGTGGTGTCTATGACTGATAGCTTT	687
G A K A Y I T A W I S L T I Y I I P V A I L G G C Y G L I S F	229
AAAATATGGCAAACTTTAAAGGAAAACGAAGAGGACCACTGTATCTCACTCTCACTGCGGCAATCGAAAGCCAAACGCGTCGCGG	777
K I W Q N F K R K T K K D Q C I T L T T A A S K A N A L A R	259
GTGACGAGCTCAAACTGTTTCCAGGCGCAAAATCACCCCGTTAAATGACTTTTGTATTTGCTGGCTTATATAGTTTGTGAGCT	867
V S V K L V S K A K I T T V K M T F V I V L A Y I V C W T	289
CCGTTTCTTCTGTCAGATGTTGTCAGCTTGGGATCCAGACCAAGGAGCCATGCCCTTTATCATCTCCATGTTTACTGATGAC	957
P F F F V Q M W S A W D P E A P R E A M P F I I S M L L A S	319
CTGAACAGCTGCTTAACCCCTGGATCTACATGTTTTCGCGGGCATCTATTCACGACCTGAAGCAGAGCTGCTGTGTTGTTCCACA	1047
L N S C C N P W I Y M F F A G H L F H D L K Q S L L C C S T	349
CTGTATCTAAAGCTTCTTCAGTGTGTTGTGTCAGGAACACGACTCACGCAAGAGCAACTGCCCTTATGTCATTAAAGACCCAGC	1137
L Y L K S S Q C R C D Q E H D S R K S N C S T Y V I K S T S	379
AGCCAGAGGAGATCACCCAGCTCTCCATCATATAATATATATTTGAGCTTTTGAAGGCTGGGAGATTCAGATGAAGGAA	1227
S Q R S I T Q S S I T	390
GGACAATAAAAACAGTAACCCAAAGTCTCCAAATCTCACTATGAAGTTAAACATCAGTATGTTTCTTTTGGAAATATCCATTCCTCAA	1317
TGCAATTTCAATCACTCACTCTTTCGACTCTTTTCGTAAGTCTTATCTTATCTTAAAGATCTTGAAGGACCAACAA	1407
ACGAAGACAGAGTAAATGTTCTTACCTTGTGACTATGTTTATGAGATGTAACATGGGAAAGATCTGAATTTCTTAAAGTTGTAGC	1497
AAGAAGATGCTGTTGAAATTTGAAATGTAATACACAGCTTTGTTTAAATTCGAGAGCTCAAGTGGAGGAAATTTGATCCGCTCTGCG	1587
GTTTTGAGATACATACAGAAGAAATATGCTTTTATCTTTAATGCTGCGTTACAGTACACACCGCGCAAGTCAATGCAAGATG	1677
CGAATAGACGCCAAAATCCGATGACGCTTTTTCGCGAAGTCTTTCAGCATTCGAGATTTGAGGATGTCAGTCAATGACATGAT	1767
GATGTAGTTGCAAGTGAATGTTGTAACAAATACGGAATGAGCGATTTTCAGCTTCTAGTTTACGTTTCAATTCATTCACGAC	1857
ATCCGCGCGAAATTCGCGTCTATTTCGATCTTTTCGATTTGATGTAATCGCGCTGCGCAAGCAGCTGTTTCGCGTTGATGTG	1947
AACGCCACAGATGAAAGAGAGTGTGAAGACACATAAGTGTGAATCCCTTATTTTCAATTCGCAACCAATGACAAACATGTCGAAG	2037
GTTTGACATTTCTGAAGTGAACATCAGGATCATGCCATTTTATACATCAAAAATGTTTGTCAAGATGCAAAACATCTCATGATTTGA	2127
GAACCCACAGATGAAAGAGAGTGTGAAGACACATAAGTGTGAATCCCTTATTTTCAATTCGCAACCAATGACAAACATGTCGAAG	2217
TTGCTCTTTTAAAGTGTCTTACAAGAGACACATGCAAAATTTTATTTTAAAAAAATCATAAAGACACAAAGCATTGTCATTTT	2307
GTACATAGACATTCATATTTTTCAGTTCACCTTCTTGTGATTTTCATGTAACCAACAAACATGTCATGCCCGAGGCGCTATATACATTTGCA	2397
TAATGTTTCTGCAATGATATTTTAAACAAATTTTGTGTTTGTGTTTAAAGTTAAAGATTTGCTGTTCTCTTTCATTTTATTTGTTG	2487
TTAGTCTTTGATGTTTATTCAGTTCATATTTTACTTATTTGCTTAAACAAATGCTTTAAGTCTATTTTGTGCTGATTCATTCATA	2577
AAATTTTCTTAAAGTAAACACTCAAGAGGACAAAGCATTAAACACAGTTTACTAGTGTGCAAGAAAGAAATCTGCTTTTACCT	2667
TTTAAAGGAAATGAGGAGCTTTTATCAGCAGAAGATTTATTTTACAATGTTTAAATACATATTCAGTGAAGCTCTGTTAGTCTCT	2757
CATTTTAAAGCTACTGATTTTATCTCTGAAAAATATATGTCATATTTACATTTAAGTGTGCTAGATGTAGTCTTATTTATTTAAAT	2847
AAATGTAATGTGTTGATACGTTCAAAAAA	2879

Fig. 1. Nucleotide and deduced amino acid sequence (single letter code) of the *C. commersoni* isotocin receptor. Transmembrane regions are underlined, N-linked glycosylation sites are indicated by *, potential phosphorylation sites for protein kinase C by ●, for protein kinase A by #, for casein kinase II by ◇, and a putative palmitoylation site by +.

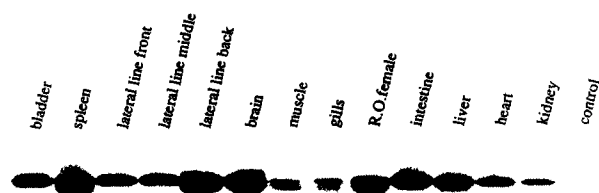


Fig. 2. Tissue distribution of ITR transcripts (for details see section 2). R.O., reproductive organs; control, reverse transcription and amplification in the absence of RNA. Not shown are negative controls where VTR cDNA run on an agarose gel and blotted onto a nylon membrane failed to give hybridization signals with a ^{32}P -labelled ITR cDNA probe; no amplified fragment was obtained when using the ITR forward and reverse primers and the VTR cDNA as template.

analyses also reveal the presence of residues that are conserved among nonapeptide receptors but which are absent from other G-protein coupled receptors. Residues Phe-Gln-Val-Leu-Pro-Gln-Leu at the C-terminal end of transmembrane region II, Gly-Pro-Asp in the first and Asp-Cys-Trp and Pro-Trp-Gly in the second extracellular loops have been suggested to contribute to the ligand binding domain [21].

3.2. Functional expression in *Xenopus laevis* oocytes

Oocytes previously injected with ITR-RNA respond to bath application of isotocin by exhibiting large chloride currents indicating the coupling of the ITR to the inositol phosphate/ Ca^{2+} signalling pathway (Table 1). The threshold concentration of IT that induced a significant membrane current of 1 to 5 nA was 2 ± 1.5 nM and the EC_{50} value for this agonist was 80 ± 30 nM. The teleost ITR can also be activated by Arg-vasotocin, mesotocin, oxytocin and Arg-vasopressin, although these have lower potencies than IT. It is interesting to note that the ITR differs in several respects from the previously cloned vasotocin receptor (VTR). Firstly, the VTR has a high peptide selectivity; the threshold for its activation by IT and corresponding EC_{50}

value are about 10000-fold higher than those for VT [11]. In contrast, the ITR displays only a three-fold preference for IT compared to VT. Secondly, the rank order of potencies being oxytocin > mesotocin > Arg-vasopressin for the VTR and mesotocin > oxytocin > Arg-vasopressin for the ITR. Thirdly, the concentrations of agonists needed to induce membrane currents in RNA injected oocytes is about 100-fold higher for the ITR than for the VTR.

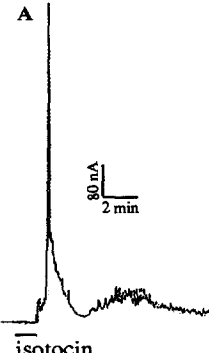
3.3. ITR mRNA distribution

RNA blot analyses with either total RNA or poly(A)⁺ RNA from different *C. commersoni* tissues and ^{32}P -labelled ITR-cDNA probe failed to reveal bands indicating that the ITR mRNA is of low abundance in the tissues examined. Therefore reverse transcribed RNA was amplified using primers specific for the white sucker ITR nucleotide sequence. Fig. 2 shows that ITR transcripts are present in brain, spleen, lateral line, ovary, bladder, intestine, heart and liver; products are also detectable in skeletal muscle, gills and kidney. In some lanes double bands can be seen suggesting the existence of ITR isoforms.

3.4. Phylogeny of the nonapeptide receptors

Fig. 3 shows that, based on sequences, the known nonapeptide receptors fall into four major groups. The ITR is most closely related to mammalian oxytocin receptors; it is also related to the human V_{1b} receptor. The fish VTR and the mammalian V_{1a} receptors form a second group being related to the third, the V_2 receptor group. A fourth group having only a low degree of sequence similarity with the IT/OT receptor and the VT/VP receptor subfamilies consists of the conopressin receptors recently cloned from the pond snail *Lymnaea stagnalis* [24] suggesting that the molluscan genes separated early in evolution probably before the separation of the other members of the nonapeptide receptor family. Based on the divergence of amino acid sequences the VT/ V_{1a} receptors and the V_2 receptors may have emerged by duplication of an ancestral neurohypophyseal hormone receptor gene about 640 million years ago, before the

Table 1
Pharmacological characterization of the white sucker isotocin receptor (ITR) expressed in *Xenopus laevis* oocytes

A	B		ITR EC ₅₀ values [nM]	
	<u>active peptides</u>			
	isotocin	CYISNCPIG*	80 ± 30	
	Arg-vasotocin	CYIQNCPRG*	300 ± 90	
	oxytocin	CYIQNCPLG*	950 ± 420	
	mesotocin	CYIQNCPIG*	440 ± 110	
	Arg-vasopressin	CYFQNCPRG*	3500 ± 2100	
	<u>inactive peptides</u>			
	conopressin S	CIIRNCPRG*	---	
	conopressin G	CFIRNCPKG*	---	
	tocinoic acid	CYIQNC	---	

(A) Typical membrane current trace of an oocyte injected with 2 ng of ITR-RNA, incubated as described in section 2, and then exposed for 1 min (bar) to 300 nM isotocin. Membrane currents were recorded at a holding potential of -50 mV. (B) The indicated ligands were applied at concentrations ranging from 1 nM to $10 \mu\text{M}$ for 1 min to the voltage-clamped oocytes. For the calculation of EC_{50} values dose-response curves were constructed (data not shown). Membrane currents were integrated over time; these values were then expressed relative to the maximal transported charge and plotted semilogarithmically versus ligand concentration. Each EC_{50} value derives from three to six experiments. Maximal amplitudes of the induced currents were 710 ± 290 nA. For those peptides for which no EC_{50} values are given, the ITR was not activated at concentrations of $10 \mu\text{M}$ (---). G*, amidated glycine residue.

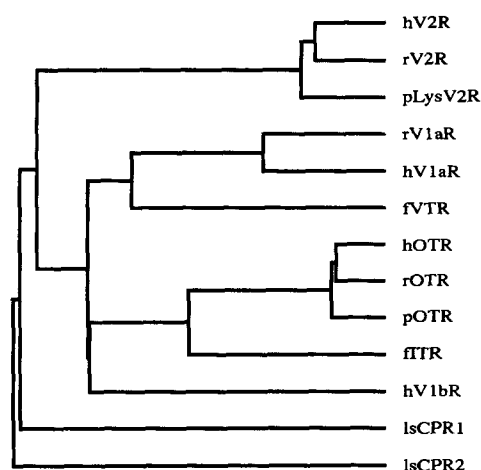


Fig. 3. Sequence relationships of the nonapeptide receptor family depicted in the form of a dendrogram. The amino acid sequences of the various receptors were aligned using the PILEUP algorithm (Genetics Computer Group, Madison, WI, USA). The lengths of the horizontal lines indicate reciprocally the sequence similarities. hV2R, human Arg-vasopressin V2 receptor [15]; rV2R, rat Arg-vasopressin V2 receptor [14]; pLysV2R, pig Lys-vasopressin V2 receptor [17]; rV1aR, rat Arg-vasopressin V1a receptor [12]; hV1aR, human Arg-vasopressin V1a receptor [13]; fVTR, fish (*C. commersoni*) Arg-vasotocin receptor [11]; hOTR, human oxytocin receptor [18]; rOTR, rat oxytocin receptor [22]; pOTR, pig oxytocin receptor [17]; fITR, fish (*C. commersoni*) isotocin receptor; hV1bR, human Arg-vasopressin V1b receptor [23]; LSCPR1, *Lymnaea stagnalis* conopressin receptor 1 [24]; LSCPR2, *Lymnaea stagnalis* conopressin receptor 2 (van Kesteren et al., manuscript submitted).

separation of the cyclostomes from other vertebrates. The IT/OT receptors evolved later, about 460 million years ago before cartilaginous fish originated. Thus, OT-like receptors should be present in all non-mammalian vertebrates except for cyclostomes; this is indeed consistent with the presence of OT-like peptides in non-mammalian vertebrates [25].

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