Cloning, expression and pharmacological characterization of a vasopressin-related receptor in an annelid, the leech *Theromyzon tessulatum*

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Abstract

In annelids, it has been established that arginine-vasopressin (AVP)/oxytocin (OT) superfamily peptides are involved in the maintenance of water and electrolyte homeostasis as well as reproduction. At present, there is little information on their receptors. In this study, we report the characterization of a 1.7 kb cDNA for an AVP-related receptor from the leech *Theromyzon tessulatum*. The open reading frame encodes a 435-amino-acid transmembrane protein that displays seven segments of hydrophobic amino acids, typical of G-protein-coupled receptors. The overall predicted protein exhibits about 30% amino-acid identities to other invertebrate, as well as vertebrate, AVP/OT receptor family members, and displays conserved characteristic features belonging to the AVP/OT receptor superfamily. RT-PCR expression experiments showed that mRNA is expressed in the genital tract, the ovary and the brain. The receptor expression is stage specific, showing a weak expression after the two first blood meals, increasing dramatically after the last blood meal during the period of sexual maturation and disappearing after egg laying. Thus, the leech AVP-related receptor may mediate reproductive functions. When expressed in COS-7 cells, the receptor binds ligands with the following rank order of potency: AVP=Arg-vasotocin > Arg-conopressin > mesotocin = OT = Lys-conopressin = isotocin > anetocin. This shows an AVP-like pharmacological profile. The transfected receptor mediates AVP-induced accumulation of inositol phosphates, indicating that the leech AVP-related receptor is functional. This study describes the characterization of a novel AVP/OT superfamily receptor in annelids, which are considered the most distant group of coelomate metazoa possessing a functional AVP/OT-related endocrine system.

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Introduction

The arginine-vasopressin (AVP)/oxytocin (OT) neuroendocrine system is widely distributed in the animal kingdom. In vertebrates, more than 10 related peptides and over 30 related receptors belonging to the G-protein-coupled receptor (GPCR) family have been described from cyclostomes to mammals (Acher 1993, Acher et al. 1999). Neurohypophyseal peptides and their receptors can be regarded as having two distinct lineages with divergent physiological activity: the vasotocin-vasopressin line concerned with water and electrolyte balance, and the isotocin-mesotocin-oxytocin line concerned with reproduction (Darlison & Richter 1999).

In invertebrates, data are scarce. Seven AVP/OT-related nonapeptides have been isolated in different phyla. By the relationships of the precursor primary structure and physiological actions, these peptides may be associated with either AVP or OT lineages, whereas they often control both osmoregulation and reproductive functions (Hoyle 1999). In insects, one brain AVP-like diuretic hormone has been isolated in *Locusta migratoria*. This nonapeptide acts as a neurohormone on Malpighian tubules to enhance water excretion (Proux et al. 1987). Although several putative
AVP/OT-related receptors are detected in silico in Drosophila melanogaster genome databases, no insect receptor of this superfamily has been fully characterized.

In molluscs, a structurally AVP-related peptide termed ‘Lys-conopressin’ was purified and cloned from the pond snail Lymnaea stagnalis. This nonapeptide functions as a neurotransmitter/neuromodulator in both the brain and the male copulatory organs, acting as a vertebrate OT-like peptide in reproduction (Van Kesteren et al. 1995a). In the cephalopod Octopus vulgaris, two structurally OT-related peptides termed ‘cephalotocin’ and ‘octopressin’ have been purified; they act as multifunctional neurohormones that control reproduction, homeostasis and feeding (Takuwa-Kuroda et al. 2003). At present, only three functional mollusc AVP/OT receptors have been reported from the L. stagnalis genital tract, the so-called conopressin receptors (LSCP1 and LSCP2) (Van Kesteren et al. 1995b, 2003) and from O. vulgaris (CTR) (Kanda et al. 2003). In the nematode Caenorhabditis elegans, several putative AVP/OT-related receptors are also detected in silico in the genome database but without any pharmacological evidence of AVP/OT receptor lineages.

In annelids, which are considered the most distant group of coelomate metazoans possessing a functional AVP/OT-related endocrine system, immunoreactivities with antibodies raised against vertebrate peptides were first detected in the brain of several species of leech, including Theromyzon tessulatum (Malecha 1983). Then, an AVP-related peptide that exhibits identical sequence with mollusc Lys-conopressin was purified from Eupodella octoculata. In leeches, this peptide mainly exerts a potent diuretic effect via pulsatory contractions and bladder-shaking movements of the nephridia as well as regulation of reproduction (Fujino et al. 1999). In the lumbricid earthworm Eisenia fetida, an OT-related peptide termed ‘annetocin’ has also been characterized (Oumi et al. 1994, Satake et al. 1999). In annelids, annetocin induces egg laying, which is accompanied by secretion of mucus from the clitellum (Ukena et al. 1995). However, little information is currently available on OT/AVP-related receptors in this phylum. This study describes the cloning and pharmacological characterization of an AVP-related receptor in an annelid, the leech Theromyzon tessulatum.

Materials and Methods

Animals, dissections and surgical procedures

Rynchobdellid leeches of the species T. tessulatum were reared under laboratory conditions, as previously described (Malecha 1983). Their life cycle is subdivided into stages defined by taking as indicators the three blood meals (stage 0, from hatching until the first blood meal, and stages 1–3, after respectively the first, second and third blood meals). Despite high blood volume absorption, after the first and the second meals, total body water content is low and undergoes little change. These stages are characterized by important diuretic mechanisms. Stage 3 is subdivided into substages (A–F) defined by the degree of sexual maturation and reproductive functions. In short, soon after the third and last meal corresponding to stage 3A, spermatogenesis and ovogenesis occur during stages B and C–D respectively. These stages are characterized by male and female genital tract maturation as well as intake of water at stage 3B, followed by water retention until the end of the life cycle. After egg laying and hatching maternal care that correspond to stage 3E, the animals die, representing stage F. After anaesthesia with chlorexone, the animals were pinned flat, ventral side up, in leech Ringer’s solution. For RNA extraction, excised tissues were immediately frozen in liquid nitrogen and stored at −70 °C before use.

RNA preparation, RT-PCR and rapid amplification of cDNA ends (RACE) cloning

Based on leech physiological data, receptor cloning was performed with enriched central nervous system and genital tract tissues isolated from 3A–D stage animals. Total cellular RNA was extracted with Trizol reagent (Gibco BRL, Strasbourg, France), according to the manufacturer’s instructions. An amount of 3 μg RNA was converted into single-stranded cDNA with 200 U MMLV reverse transcriptase, primed with 3 μg random primers (Life Technologies, Cergy Pontoise, France) in a 50 μl solution containing 50 mM KCl, 50 mM Tris–HCl, 10 mM MgCl₂ (pH 8·3) and 2 mM each of dNTP and 20 U RNasin. The reaction mix was incubated for 1 h at 37 °C. Alignment of AVP/OT superfamily receptors allowed us to design two degenerate oligonucleotides, forward TGCTGG(GT)CGCCITTCCTTT(GC)TI(GT) (CT)ICAGATGTGG and reverse (GT)A(GT)CCA(GC) GGTTTC(AG)TGCAGCTGTTCAG, directed to transmembrane domain (TM) 6 and TM 7 conserved regions respectively. The first-strand synthesis reaction (1:6) was amplified for 35 cycles with 1 U Taq Polymerase (Eurogentec, Liège, Belgium) and 100 pmol of each forward and reverse primer, as previously described (Breton et al. 1995). The cycling parameters were: 94 °C for 90 s, 42 °C for 90 s and 72 °C for 120 s. Negative control RT-PCR reactions were performed by omitting reverse transcriptase or RNA from the reaction mixture. After amplification, the samples were separated on 2% agarose gel and revealed by ethidium bromide staining. PCR products of the expected size were subcloned into pGEM-T easy vector (Promega) and sequenced with an automated DNA sequencer (ABI PRISM 377 DNA sequencer; Applied Biosystems, Courtaboeuf, France) and the ABI PRISM Original Drhodamine Terminator Cycle Sequencing Kit (Applied Biosystems). Among the mixture of amplified cDNAs, one fragment displaying greatest sequence homologies with AVP/OT receptor was used to design forward- and reverse-specific primers. In order to
obtain a complete sequence, 3′- and 5′-RACE were performed with the SMART RACE cDNA amplification kit (Clontech), as described by the manufacturer.

**RT-PCR expression analysis**

Male and female stage-specific animal tissue total RNA was extracted and used as a template for semiquantitative RT-PCR assay. The method, indicated above, has been previously described and validated (Breton et al. 1995). To exclude the possibility that a positive signal was due to contamination with genomic DNA during RNA preparation, the PCR was also performed without preincubation with reverse transcriptase or with ribonuclease-free deoxyribonuclease I-treated total RNA, showing similar results. Primers specific for leech AVP-related receptor and actin were used to amplify tissue cDNA. For the receptor amplification, a sense oligonucleotide 5′-GGT GCTATGTA CGGTCGTCGTCTTACA-3′ and an antisense oligonucleotide 5′-CCTTCACCGCTTCTGTAT TCTCGCA-3′ were designed to amplify a 359 bp cDNA fragment (corresponding to residues 133–254). As control for amplification, a sense oligonucleotide 5′-CCCTGGAAG AACATCCAGTCC-3′ and an antisense oligonucleotide 5′-CGAGAGATGATGAAGCAGCA-3′ were designed to amplify a 419 bp mRNA (residues 59–199). The T. tessulatum actin cDNA sequence was kindly provided by Dr Christophe Lefebvre. It was cloned in our laboratory and served as control of amplification (the sequence is available at the GenBank Database, accession No. p8 CK640381). The cycling parameters were as follows: 94°C for 90 s, 60°C for 90 s and 72°C for 120 s. Amplification of the receptor and actin cDNA was carried out for 35 and 22 cycles respectively. PCR amplification products were sequenced to verify the specificity of the amplification. All experiments were performed in triplicate with similar results.

**Preparation of expression plasmid and transient transfection of COS7 cells**

A sense oligonucleotide 5′-CGGGATCCCGGTATA TCAGCACATCAGATTTACGACGTGA-3′ located 72 bp upstream of the ATG codon and an antisense oligonucleotide 5′-CCCAGCTTTGCAAACAAAAAGTAGTATTTAGTGACAGAAT-3′ centred 30 bp downstream of the stop codon were designed to amplify the full-length cDNA encoding the leech AVP-related receptor. At the 5′-end, the primers contained a recognition sequence for the restriction endonucleases EcoRI and BamHI respectively. Oligo(dT)-primed cDNA was synthesized with SuperScript Plus transcriptase and amplified for 30 cycles, using 2 U of proofreading pfu Taq, as described by the manufacturer (Promega). The cDNA fragment was digested with restriction enzymes and ligated into a eukaryotic expression vector already used to subclone rat and human AVP and OT receptors (Mouillac et al. 1995). The entire PCR product was sequenced twice in both strands to verify absence of amplification errors, thus confirming the sequence obtained by RACE procedures. Expression plasmid DNA was prepared for transfections by the Plasmid Maxi Kit (Qiagen) procedure. The receptor was transiently expressed in COS-7 cells by electroporation, as previously described (Mouillac et al. 1995, Breton et al. 2001). Briefly, cells were suspended in electroporation buffer (107 cells per 0·3 ml) and incubated with plasmid DNA (20 µg carrier DNA and 2 µg expression vector containing the cDNA insert) for 1 min at room temperature before being pulsed (280 V, 950 µF, GeneZapper system, Kodak Scientific Imaging). After electroporation, cells were plated in Petri dishes and grown in Dulbecco’s modified Eagle’s medium (DMEM, BioWhittaker, Cambrex Bio Science, Emerainville, France) supplemented with 10% fetal calf serum, 1 MEM non-essential amino acids (Invitrogen), 500 units/ml penicillin and streptomycin each, and 0·25 µg/ml amphotericin B in an environment containing 95% air and 5% CO2 at 37°C. Transfected cells were harvested 48 h after electroporation, and membranes were prepared. Cells were washed twice in PBS without Ca2+ and Mg2+. Polytron-homogenized in lysis buffer (15 mM Tris–HCl (pH 7·4), 2 mM MgCl2 and 0·3 mM EDTA) and centrifuged at 800 g for 5 min at 4°C. Supernatants were recovered and centrifuged at 44 000 g for 20 min at 4°C. Pellets were washed in Buffer A (50 mM Tris–HCl (pH 7·4) and 5 mM MgCl2) and centrifuged at 44 000 g for 20 min at 4°C. Membranes were suspended in a small volume of Buffer A, and protein content was determined by the Bradford method (BioRad), using bovine serum albumin (BSA) as the standard. Aliquots of membranes were used immediately for binding assays or stored at −80°C.

**Radioligand binding assays**

The peptides Arg-vasopressin, Arg-vasotocin, Arg-conopressin (conopressin G), Lys-conopressin (conopressin S), OT, mesotocin and isotocin were obtained from Bachem, Voisins-le-Bretonneux, France. Annetocin was synthesized by Drs Maurice Manning and Ling L Cheng (Medical College of Ohio, Toledo, OH, USA) and characterized by HPLC and MALDI mass spectrometry. [3H]AVP (60–80 Ci/mmol) was purchased from NEN-PerkinElmer, Courtabeuf, France. The leech AVP-related receptor binding assays were performed at 30°C, using [3H]-AVP as the radioligand and 15–20 µg membrane proteins in standard radioligand saturation and competition binding assays, as previously described (Mouillac et al. 1995, Breton et al. 2001). Briefly, membranes were incubated in Buffer A supplemented with 1 mg/ml BSA (binding buffer) and with radiolabelled and displacing ligands for 30 min. Affinities (Kd) for
\[^3\text{H}\] \text{AVP} \text{ (concentrations 0.1–20 nM) were directly determined in saturation experiments. Affinities (K) for the unlabelled ligands were determined by competition experiments using } [^3\text{H}] \text{AVP (1–2 nM) as the radioligand. The concentration of the unlabelled ligands varied from 1 \mu\text{M to 10 \mu\text{M. In saturation and competition experiments, non-specific binding was determined by adding 10 \mu\text{M unlabelled AVP. Bound and free radioactivities were separated by passing over Whatman GF/C filters presoaked in 10 mg/ml BSA solution for 3–4 h. The ligand binding data were analysed by non-linear least-squares regression by the computer program Ligand (Elsevier-Biosoft, Cambridge, UK). Unless otherwise indicated, all assays were performed in triplicate on at least three separate batches of electroporated cells.}

\textit{Inositol phosphate assays}

The accumulation of inositol phosphates was determined as described earlier (Mouillac et al. 1995, Breton et al. 2001). Briefly, COS-7 cells transfected with the leech AVP-related receptor were plated and grown in 12-well clusters for 24 h in Dulbecco’s modified Eagle’s supplemented medium and then labelled with \textit{L-myo-[1,2-\textsuperscript{3}H]}inositol (65 Ci/mmol; PerkinElmer) at a final concentration of 1 \textmu Ci/ml in a serum-free, inositol-free medium (Invitrogen). Cells were washed twice in PBS medium, equilibrated at 37 °C in PBS medium for 1 h, and then incubated for 20 min in PBS medium supplemented with 10 mM LiCl. COS-7 cells were then stimulated for 20 min with increasing concentrations of AVP (from } 10^{-12} \text{ to } 10^{-5} \text{ M) or with a maximal concentration of other natural peptide hormones (10^{-5} \text{ M). The reaction was stopped by adding ice-cold perchloric acid 5%. After neutralizing of the samples, total inositol phosphates were extracted and purified on anion-exchange chromatography columns (Dowex AG 1–X8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total inositol phosphates was extracted and counted. An EC\textsubscript{50} constant corresponding to concentrations of AVP leading to half-maximum accumulation of inositol phosphates was calculated by non-linear regression analysis with a sigmoidal dose–response equation (Prism4, GraphPad Software, Macintosh, GraphPad Software Inc., San Diego, USA). All assays were performed at least in triplicate unless otherwise indicated from three separate batches of electroporated COS-7 cells.

\textbf{Results}

\textit{Cloning of the leech AVP-related receptor}

With degenerate primers, a 126-bp cDNA fragment has been amplified from } T. \textit{tessulatum} \text{ brain- and genital tract-enriched RNA. This sequence exhibited a maximum 68% identity with the human V1b receptor amino-acid sequence as well as characteristic features belonging to the AVP/OT receptor superfamily. Using } 5' \text{ - and 3' -specific primers and RACE strategy, we were able to clone about 1700 bp of the leech AVP-related receptor. This includes 216 bp of 5'–untranslated and 123 bp of 3’–untranslated sequence. Taken together, the unique 3' poly(dT) primer used for RACE technique and the presence of a canonical polyadenylation signal suggest that the complete 3’-end has been cloned. The cDNA contains an open reading frame (ORF) of 1305 bp encoding a 435-amino-acid protein with a predicted molecular mass of 50.3 kDa (Fig. 1). The ORF is preceded by an in-frame stop codon at position –186, indicating that the coding region is complete at the 5’-end. Although an in-frame ATG sequence is also present at position –126, the ATG at position 1 is suggested to be the translation initiation site since it best fits both the GCC(A/G)/CCATGG Kozak consensus sequence and the predicted peptide signal position. Hydrophobicity analysis of the deduced amino-acid sequence indicates the presence of seven putative transmembrane-spanning regions that are characteristic of GPCR (Fig. 2). As indicated in Fig. 1, this sequence displays several other characteristics of GPCR. Although no potential N-linked glycosylation (Asn-X-Ser/Thr) sites were present, several putative phosphorylation sites were predicted. These sites include 10 serine, 4 threonine and 3 tyrosine phosphorylation sites, such as consensus sequences (i) for phosphorylation by protein kinase C (Thr/Ser-X-Arg/Lys) in the second (positions 159–161) and third (positions 286–288, 321–323 and 348–350) intracellular loops, (ii) for protein kinase A (Arg-X-Ser/Thr) (positions 354–356), (iii) for casein kinase II (Thr/Ser-X-X-Asp/Glu) (positions 349–352) and (iv) for cAMP-dependent protein kinase (Arg/Lys-Arg/Lys-X-Ser/Thr) (positions 259–262) in the third intracellular loop.

\textit{Structure of the leech AVP-related receptor}

At the amino-acid level, the entire ORF exhibited about 30% identities with other invertebrate and vertebrate AVP/OT receptors. The N-terminal extracellular domain shows hardly any sequence homology. However, surprisingly, sequences including TM and extracellular loops exhibit the closest similarity with vertebrate AVP/OT receptors. Among these regions known to be crucial for ligand binding, sequences comparison analysis reveals over 65% amino-acid identities with vertebrate AVP as well as vasotocin receptors, but it displays only 40% identities with the three mollusc receptors. As shown in Fig. 3, the third intracellular loop is considerably longer than the vertebrate as well as invertebrate receptors described so far, containing 55 amino-acid residues more than mollusc receptors (Van Kesteren et al. 1995b, 1996, Kanda et al. 2003). In addition, a cysteine doublet after TM 7 that may be modified by palmitoylation and may thereby anchor the
The primary structure of the leech AVP-related receptor. Nucleotide sequence and deduced amino-acid sequence of the leech AVP-related receptor cDNA have been submitted to the GenBank database with accession no. AY560590. Nucleotides and amino acids are numbered on the right, starting at the first ATG corresponding to the methionine initiation residue. Nucleotides upstream from the first ATG corresponding to the 5′ untranslated sequence are indicated by negative numbers. An in-frame stop codon at position –93 is in boldface. Following ATG, the predicted peptide signal position is underlined. The stop codon is in boldface. Within the 3′ untranslated sequence, the AATAAA potential polyadenylation signal is in boldface and underlined. Putative transmembrane domains in the predicted protein sequence are shaded. Putative sites for phosphorylation are indicated by sublineal black dots.

cytoplasmic tail in the lipid bilayer is absent as well as the C-terminal domain corresponding to the cytoplasmic tail. Further analyses also reveal the presence of the most characteristic sequences common to this family as well as residues that are conserved only among nonapeptide receptors but which are absent from other GPCR.
Expression of the leech AVP-related receptor

To investigate the developmental expression of the leech AVP-related receptor, total RNA was extracted from leeches at different developmental stages defined on the basis of feeding pattern and reproductive activity (Malecha et al. 1986). Because leech AVP-related receptor mRNA is hardly detectable by Northern blot analysis, due to the low number of animals available, we performed semi-quantitative RT-PCR analysis. As shown in Fig. 4A, a faint signal was observed after the first (stage 1) and second (stage 2) blood meals. However, soon after the third and last blood meal (stage 3A), receptor mRNA level increased dramatically and remained high during most of the time occupied by spermatogenesis (stage 3B) and ovogenesis (stage 3C). A high expression level was still maintained thereafter during the final stage of ovogenesis (stage 3D). In contrast, the transcript gradually disappeared after egg laying (stage 3E) and returned to a barely detectable level when the animal died (stage 3F). These findings correlate well with the maturation of reproductive organs as well as reproduction processes, with an important increase of receptor expression. We have thus performed RT-PCR experiments using total RNA (stage 3C) from anterior, medium and posterior parts of animals and from ovary, testis (female and male genital tract) and brain. As shown in Fig. 4B, our analysis revealed that receptor transcripts were present in all tissues in different abundance. The medium part containing most of the mature female reproductive tract (ovary and oviduct), the posterior part and the dissected female genital tract showed the more intense signal, suggesting that this receptor may mediate reproductive functions. A weak PCR signal was also identified in the anterior part that does not contain genital organs and in the brain, suggesting that this receptor may have additional functions. This receptor is probably involved in the central regulation of reproduction.

Pharmacological properties of the leech AVP-related receptor expressed in COS-7 cells

Because the leech AVP-related receptor possesses some of the hallmark residues of the AVP/OT family of GPCRs as well as some conserved residues involved in the binding of the neurohypophyseal hormones to their respective receptors, we investigated its pharmacological properties by transient expression in the heterologous COS-7 cell system. Thus, the receptor cDNA was subcloned into a...
eukaryotic expression vector, and cells were transfected by electroporation. The natural peptide ligand for the leech AVP-related receptor being not known in the *T. tessulatum* species, the affinity of various peptide hormones of the AVP/OT family was measured in standard binding assays. AVP, which is available as a tritiated compound, [3H]AVP, was first tested in a saturation-binding experiment. As shown in Fig. 5, the mammalian peptide hormone bound to the COS-7 cell membranes expressing the receptor with a very high affinity. Indeed, as illustrated in a representative Scatchard plot experiment, an apparent dissociation constant (*K*~d~) for [3H]AVP of 0·36 nM and a maximal binding capacity of 303 fmol/mg of membrane proteins were calculated respectively. Surprisingly, the invertebrate leech AVP-related receptor binds AVP with an affinity equivalent to the one described for human or rat receptors. In addition, using [3H]AVP as the radioligand, competition experiments were carried out to determine the pharmacological activities of the leech AVP-related receptor toward other peptides belonging to the superfamily. A series of natural peptide hormones isolated from other vertebrates and invertebrates was tested. As shown in Fig. 6, most of the ligands used in the study were able to displace [3H]AVP binding. Calculated inhibition constants (*K*~i~) are summarized in Table 1. Peptides containing an arginine residue at position 8, such as Arg-vasotocin or Arg-conopressin, displayed a relatively high affinity, like AVP, in the 1–10 nM range. Peptides, such as OT, mesotocin, isotocin or Lys-conopressin were at least 100 times less efficient than AVP, having a *K*~i~ in the 200–400 nM range. Finally, annetocin, isolated from the earthworm *Eisenia foetida*, was almost inactive, having a *K*~i~ above µM concentrations. In conclusion, the rank order of potency was AVP = Arg-vasotocin > Arg-conopressin > mesotocin = OT = Lys-conopressin = isotocin > annetocin.

**Functionality of the leech AVP-related receptor expressed in COS-7 cells**

To determine whether the leech AVP-related receptor expressed in COS-7 cells is able to couple to G protein and phospholipase C/calcium signalling pathways, as most AVP/OT members of this receptor family do, we
stimulated cells with different agonist ligands and measured the consecutive accumulation of inositol phosphates. As illustrated in Fig. 7A, the mammalian hormone AVP induced a concentration-dependent accumulation of inositol phosphates (eightfold above the basal value with a maximal $10^{-5}$ M concentration). The EC_{50} for AVP, calculated from the dose–response curve, was $3.13 \pm 0.9$ nM ($n=3$), a value in agreement with the affinity of the ligand calculated in saturation-binding assays ($0.44$ nM). This result demonstrated that the leech AVP-related receptor, transfected in a mammalian cell system, is able to couple to mammalian Gq protein and inositol phosphate/calcium signalling and thus is a functional receptor. In addition, as depicted in Fig. 7B, all natural vertebrate and invertebrate peptide hormones of the AVP/OT superfamily for which affinities have been determined were able to stimulate the leech AVP-related receptor. Indeed, Arg-vasotocin (AVT) and Arg-conopressin, when used at $10^{-5}$ M, were almost as potent as AVP. Mesotocin, oxytocin, Lys-conopressin, isotocin or annetocin, although less potent than AVP, AVT or Arg-conopressin, all stimulated a significant accumulation of inositol phosphates. In terms of efficiency, the rank order of the different peptides seems to be in perfect agreement with the one defined in binding assays, except for isotocin. In conclusion, the leech AVP-related receptor is functional and can be activated by vertebrate and invertebrate AVP-related hormones.

**Discussion**

In the present paper, we reported, for the first time, the molecular cloning, expression and pharmacological profile of an AVP-related receptor in an annelid, the leech *T. tessulatum*.

A large body of evidence strongly suggests that this primary sequence, so-called leech AVP-related receptor, belongs to the AVP/OT receptor superfamily. First, following database searches, this sequence exclusively
exhibits the highest sequence homologies with the AVP/OT receptor family. Secondly, the leech receptor sequence possesses some of the hallmark residues of the AVP/OT receptor family (Peter et al. 1995, Howl & Wheatley 1996, Barberis et al. 1998). In addition, RT-PCR genomic analysis revealed the presence of a unique 82 bp intron located between TM 6 and 7 (data not shown). The position of the intron is conserved in all members of the AVP/OT superfamily characterized so far (Hoyle 1999). Finally, the longer third intracellular loop, compared with the vertebrate receptor counterpart, appears to be a characteristic of invertebrate receptor within this AVP/OT superfamily (Van Kesteren et al. 1995b, 1996, Kanda et al. 2003).

Analysis of the transcript expression also shows that this receptor belongs to the AVP/OT superfamily. We showed that the receptor mRNA level is low during the first and second stages, whereas the expression increases dramatically during the third stage corresponding to the reproductive organs maturation of the adult animal. Although the exact function of the leech AVP-related receptor is unclear, these findings indicate that this receptor plays an important role in the animal’s physiology with special reference to peripheral and central reproductive events in which AVP/OT-related peptides are involved (Fujino et al. 1999). Our results are in good agreement with the fact that Lys-conopressin and annetocin, when injected in leeches and earthworms, induce rapid egg-laying-like behaviour accompanied by much mucus secretion. Furthermore, leeches in the non-breeding season responded to peptides less conspicuously than those in the breeding season, suggesting that the involvement of peptides in the control of reproductive events is periodic (Ukena et al. 1995, Satake et al. 1999). These findings also correlate well with the pond snail LSCPR functions that induce muscle contractions of the vas deferens, thus stimulating the transport of both eggs and semen during copulation and inhibiting the central neuroendocrine cells that coordinate female reproductive behaviour (Van Kesteren et al. 1995b, 1996). Moreover, in line with known AVP/OT-related peptide functions, we cannot rule out that the leech AVP-related receptor is expressed in other organs involved in osmoregulation (Salzet et al. 1993, Fujino et al. 1999).

Finally, the pharmacological characterization convincingly demonstrated that the leech receptor belongs to the AVP/OT receptor family. Interestingly, among various nonapeptides of the AVP/OT superfamily, the leech AVP-related receptor can distinguish ligands having an Arg residue at position 8 from those having another residue at this position. Indeed, ligands with an Arg residue (AVP, Arg-vasotocin or Arg-conopressin) displayed a nanomolar affinity equivalent to that determined for these agonists to vertebrate receptors, whereas the affinity for OT-related ligands (OT, mesotocin or isotocin), Lys-conopressin and annetocin is much lower.

As far as AVP is concerned, the leech AVP-related receptor presents a mammalian AVP-like pharmacological profile (Thibonnier et al. 1998). Strikingly, both TM and extracellular loop sequences of the leech AVP-related receptor found to play an important role in the binding of these ligands are more closely related to those of vertebrates than of invertebrates. Among these regions, conserved Gln residues in TM 2, 3, 4 and 6, as well as a Lys residue in TM 3, are crucial (Mouillac et al. 1995, Thibonnier et al. 2000). These residues are present in the leech AVP-related receptor (Gln108, -131, -185 and -395) except for the Lys in TM 3. A Gln residue in the first extracellular loop (Glu115) is in a position equivalent to that of the Asp/Tyr/Phe residues in the V2, V1a and

<table>
<thead>
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<th>Table 1 Affinity of natural peptide hormones of the leech AVP-related receptor. All values are expressed as the means ± s.e. calculated from at least three independent experiments done on three different batches of electroporated cells. The numbers of independent determinations are given in parentheses.</th>
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<tr>
<td><strong>Peptide</strong></td>
<td><strong>Sequence</strong></td>
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<tr>
<td>Arg-vasopressin*</td>
<td>CYFQNCPRG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Arg-vasotocin</td>
<td>CYIQCPRG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>Arg-conopressin</td>
<td>CIIRNCPRG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>CYIQCPIG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>Oxytocin</td>
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<td>Isotocin</td>
<td>CYISNCPKG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Annetocin</td>
<td>CFVRNCPPTG-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

*Affinity (K<sub>i</sub>) for [3H]AVP was directly determined in saturation experiments. Affinity (K<sub>i</sub>) for other ligands was determined in competition binding by displacement of [3H]AVP. Binding of the natural peptide hormones was performed as described in the Materials and Methods. Affinities (K<sub>i</sub>) were determined with the equation K<sub>i</sub> = IC<sub>50</sub> /[L]. K<sub>i</sub> is the affinity of [3H]AVP for the leech AVP-related receptor, [L] is the concentration of labelled AVP and IC<sub>50</sub> is the concentration of peptide able to displace 50% of the maximal [3H]AVP binding capacity.
OT receptors respectively, which have been defined as crucial determinants for binding selective analogues (Chini et al. 1995, Ufer et al. 1995). The Arg residue at position 8 in the AVP molecule has been demonstrated to interact directly with these residues in the first extracellular loop.

AVP in the leech AVP-related receptor could interact with the Glu115 in a similar way (ionic bond between Glu and Arg8) as in the V2 receptor (presence of a negative charge; Asp residue), explaining such a high affinity for this hormone. An aromatic cluster in TM 6 (Trp388, Phe391, Phe392), involved in discriminating between agonists and antagonists, is also present (Phalipou et al. 1999, Cotte et al. 2000). In the N-terminal part of the AVP/OT receptors, a conserved Arg residue (for example, Arg46 in the human V1a receptor) has been demonstrated to be necessary for agonist binding (Hawtin et al. 2002). Such an Arg residue (Arg44) is also present in the leech receptor. Altogether, these residues could define a binding pocket adapted to AVP.

It is interesting to note that Arg-conopressin and Lys-conopressin, which both display a positively charged residue at position 8 (Arg and Lys respectively), do not bind equivalently the leech AVP-related receptor (10·7 vs 331 nM). Presumably, the guanidinium group of Arg is better for interacting with Glu115. Moreover, one could also explain this significant difference by the presence in these two peptides of a variable residue at position 2. Indeed, Ile is found in the Arg-conopressin and Phe in the Lys-conopressin. According to the three-dimensional models developed for AVP receptors (Mouillac et al. 1995, Fanelli et al. 1999), this part of the peptide ligand should be embedded deeply in the receptor TM cleft. It would have to be demonstrated that Ile is better docked into this binding pocket than Phe. Thus, further structure/function analysis of leech functional domains should provide valuable information for understanding the molecular determinants responsible for the ligand-binding pocket.

Expression of the cDNA in COS-7 cells and measurement of AVP-induced accumulation of inositol phosphates confirmed that the AVP-related receptor is fully functional. The increase of AVP as well as other AVP/OT-related peptide hormones in inostitol phosphate turnover suggests the coupling of the leech AVP-related receptor to the phospholipase C/IP3/intracellular calcium signalling pathway, as is the case for most mammalian receptors of this family.

To date, the leech AVP-related receptor is the only invertebrate receptor of the AVP/OT family able to bind AVP. It is noteworthy that there are only a few peptide-binding GPCR that have been shown to have high pharmacological conservation over such a long evolutionary distance (Darlison & Richter 1999, Hoyle 1999). Thus, why the leech AVP-related receptor displays such a high affinity for AVP or vasotocin, and whether this property is related to the parasitism and/or aquatic physiology of this particular annelid, are still open questions. However, the leech receptor’s resemblance to vertebrate AVP and vasotocin receptors does not seem to be solely related to the parasitic existence of T. tessulatum, because AVP-related receptors with similar
features are present in other non-parasitic annelids (data not shown).

As described in molluscs (Van Kesteren et al. 1995b, 1996, Kanda et al. 2003), we speculate that there are at least two different receptors also present in annelids that might fulfill more than one physiological function. This hypothesis is supported by several arguments. First, annetocin, F3-annetocin and Lys-conopressin elicited equivalent potency for both egg-laying behaviour and reduction of body weight in other leeches (Fujino et al. 1999, Satake et al. 1999). Secondly, injection of Lys-conopressin exerts a potent diuretic effect in stage 2 T. tessulatum, leading to a severe loss of mass, while the expression of leech receptor is barely detectable (Malecha et al. 1986, Salzet et al. 1993). Thirdly, at least two different PCR products encoding AVP-related receptor isoforms have been characterized in several annelids, including T. tessulatum (data not shown).

In conclusion, we have cloned a novel receptor of the AVP/OT receptor superfamily from T. tessulatum that might mediate reproductive as well as mucus excretion functions during the period of sexual maturation. Pharmacological and functional experiments showed that this receptor displays a strikingly mammalian AVP-like profile. Taken together, our data provide additional evidence for the conservation of the AVP/OT neuroendocrine system during the course of evolution.

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Leech AVP-related receptor · A LEVOYE, B MOUILLAC and others

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