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Identification and expression of two oxytocin/vasopressin-related peptides in the cuttlefish *Sepia officinalis*

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ABSTRACT

Two novel members of the oxytocin/vasopressin superfamily have been identified in the cephalopod *Sepia officinalis*. Oxytocin/vasopressin gene sequences were cloned by Race PCR. The two precursors we identified exhibit the classical organization of OT/VP superfamily precursors: a signal peptide followed by a nonapeptide and a neurophysin domain. The neurophysin domain is entirely conserved for the cuttlefish precursors, but the nonapeptides and the signal peptides differ. The first nonapeptide, called sepiatocin, is highly homologous to *Octopus vulgaris* octopressin. The second nonapeptide, called pro-sepiatocin, shows sequence homologies with a Crustacean oxytocin/vasopressin-like peptide identified in *Daphnia culex* and with a novel form of oxytocin described in New World monkeys. The expression of pro-sepiatocin is restricted to the supraesophageal and subesophageal masses of the brain whereas sepiatocin is expressed in the entire central nervous system. Sepiatocin, as described for octopressin, modulates the contractile activity of several muscles such as penis, oviduct and vena cava muscles; this suggests its involvement in reproduction and blood circulation. Pro-sepiatocin is released in the hemolymph; it is a neurohormone able to target numerous peripheral organs.

1. Introduction

Oxytocin and vasopressin (OT/VP) were discovered in the early XXth century from the mammalian posterior pituitary [1,13]. These regulatory peptides have a wide range of partially overlapping biological activities. OT is mainly known to stimulate uterus contractions during delivery and milk ejection [17], whereas VP regulates the osmotic balance and the contractions of smooth muscle cells in arteries [10]. Vasopressin and oxytocin are cyclic nonapeptides that contain two cysteines (positions 1 and 6) linked by a disulfide bond (Table 1). The main structural difference between vasopressin and oxytocin is the occurrence of a basic amino acid residue (mainly Arg) at position 8 in vasopressin as opposed to a neutral residue in oxytocin (Table 1). In the early 1980s, vasopressin and oxytocin preprohormones were cloned in rats [19,28]. Both precursor proteins contained, in addition to the OT/VP sequences, a larger polypeptide sequence named neurophysin, initially thought to be a vasopressin- or oxytocin-carrier protein but now believed to play a crucial role in the proper folding and sorting of vasopressin and oxytocin prohormones [11].

In cephalopods, the common octopus, *Octopus vulgaris*, possesses two peptides belonging to the oxytocin/vasopressin superfamily: cephalotocin (CT) and octopressin (OP) [24,30]. Takuwa-Kuroda and collaborators suggest that octopressin is a multifunctional neuropeptide that contributes to reproduction, cardiac circulation and feeding, and that cephalotocin plays important roles in metabolism as a neurohormone. In the cuttlefish *Sepia officinalis*, immunoreactivity was observed in several structures of the nervous system [4].

The present paper describes the identification of two novel members of the oxytocin/vasopressin family in *S. officinalis* through the cloning of the preprohormone using sequence homologies of the neurophysin domain.

2. Materials and methods

2.1. Animals and biological sample collection

All the cuttlefish were trapped in the Bay of Seine between January and June. They were maintained in 1000-L outflow tanks at 16 °C at the Marine Station of Luc sur Mer (University of Caen, France) under a natural photoperiod.

Mature cuttlefish were anesthetized using ethanol 3% [25]. Then hemolymph samples were taken, and several organs were dissected, *i.e.* the optic gland, the optic lobe, the sub-esophageal mass,

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Table 1
Structures of vasopressin, oxytocin, and some selected oxytocin-and vasopressin like peptides identified in vertebrates and invertebrates.

Name	Peptide structure	Source
OT family		
Oxytocin	CYIQNCPLGa	Mammals [13]
Pro-oxytocin	CYIQNCPPGa	*Callithrix jacchus* [20]
Isotocin	CYISNCPIGa	Fish [3]
VP family		
Vasopressin	CYFQNCPRGa	Mammals [1,14]
Lys-vasopressin	CYFQNCPKGa	Pig, some marsupials[6]
Phenypressin	CFFQNCPRGa	Some marsupials [7]
Vasotocin	CYIQNCPRGa	Non-mammalian vertebrates [2]
Invertebrate OT/VP-like peptides		
Inotocin	CLITNCPRGa	Various insects [18,23,29]
Arg-conopressin	CIIRNCPRGa	*Conus geographicus* [9]
Lys-conopressin	CFIRNCPKGa	Leech various mollusks [9,26,32]
Crustacean OT/VP-like peptide	CFITNCPPGa	*Daphnia pulex* [29]
Vasotocin	CFVRNCPPGa	*Platynereis dumerilii* [31]
Nematocin	CFLNSCPYRRYa	*Caenorhabditis elegans* [5]
Annetocin	CFVRNCPTGa	Various annelids [22]
Echinotocin	CFISNCPKGa	*Strongylocentrotus purpuratus* [15]
Cephalotocin	CYFRNCPIGa	*Octopus vulgaris* [24]
Octopressin	CFWTSCPIGa	*Octopus vulgaris* [30]
Sepiatocin	CFWTTCPIGa	*Sepia officinallis* (this paper)
Pro-sepiatocin	CFFRNCPPGa	*Sepia officinalis* (this paper)

the supra-esophageal mass, the vena cava, the oviduct, the male genital tract, the penis. These organs were frozen in liquid nitrogen and stored at −80 °C until RNA isolation or peptide extraction. Rectum, penis, oviduct, gill and vena cava were dissected for myotropic bioassays. These organs were maintained in sterile seawater supplemented with glucose.

No specific permits were required for the described field studies. The common cuttlefish is not an endangered or protected species.

2.2. Total RNA and mRNA preparation

Total RNA was isolated from the nervous system or genital organs of mature cuttlefish using Tri-Reagent (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. mRNAs were isolated using oligodT coupled to magnetic beads as described by the manufacturer (Dynal, Invitrogen, Carlsbad, CA, USA).

2.3. Oligonucleotide primers

Oligonucleotide primers (Table 2) were purchased from Eurobio (France) and Genecust (Luxembourg). RACE Primers were designed based on sequences of neurophysin domains, which are conserved among the OT/VP superfamily. NR and NR2 primers were synthesized for 5′-RACE from the neurophysin domain of the octopressin gene (GenBank accession no. AB108429). Spia1 and Spia2 primers, designed from OT/VP-like precursors, were used for 3′-RACE.

Table 2
Oligonucleotide sequences.

Name	Sequence
NR	5′-GCAAACACCGTCGGCAACACATCGTCCTTGT-3′
NR2	5′-GCGAGTGTGCCTATGATGCAACCGTCTTT-3′
Spia	5′-GGGGTTCCTGGGCTCTTTTGCTGCTTAT-3′
Spia2	5′-CGGGCCGATTCCTCTTTTCATCCACCAAG-3′
SepOT	5′-GCGAAGTGCTTCAGAATTCAGAG-3′
sepOTPP	5′-GAGAAGCGAAATGGGGTCGGG-3′
sepOT2Rev	5′-CTTTTTGTCTTGATGTTAGGGAACG-3′
Actin	5′-TCCATCATGAAGTGCGATGT-3′
ActinRev	5′-TGGACCGGACTCGTCATATT-3′
EFγ	5′-TACAGCGGGGCAAACGTGACTG-3′
EFγRev	5′-GGGTGATACGTTCACCCACCAGA-3′

For RT-PCR mapping, SepOT, SepOTTPP and SepOT2Rev were used (Fig. 1). Primers designed from Actin and Elongation factor γ were used to amplify reference genes.

2.4. 3′-RACE (rapid amplification of cDNA ends) and 5′-RACE

The transcriptional start site was determined by oligo-capping RACE methods using a GeneRacer kit (Invitrogen). First strand cDNA was synthesized from mRNA with the GeneRacer Oligo dT Primer supplied in the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. The first PCR was performed using the GeneRacer 5′ primer and primer NR with Platinum TaqDNA Polymerase High fidelity (Invitrogen), under the following conditions: 2 min at 94 °C, 5 cycles of 30 s at 94 °C and 2 min at 72 °C, 5 cycles of 30 s at 94 °C and 2 min at 68 °C, and 25 cycles of 30 s at 94 °C, 30 s at 62 °C, 2 min at 72 °C (7 min for the last cycle). The second PCR was performed using the GeneRacer 3′ nested primer and NR2 under the following conditions: 94 °C for 5 min, 35 cycles of 30 s at 94 °C, 30 s at 62 °C, 2 min at 72 °C (7 min for the last cycle). The second PCR products were subcloned and sequenced by genomexpress (Grenoble, France). The 3′-ends of the cDNAs were determined as follows: the first template was amplified using the GeneRacer 3′ primer and Spia or Spia2. Each of the first PCR products was re-amplified using the GeneRacer 3′ nested primer and Spia or Spia2. Both first and second PCR reactions were performed for 5 min at 94 °C, and then 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 2 min at 72 °C (7 min for the last cycle). The second PCR products were subcloned and sequenced by genomexpress (Grenoble, France).

2.5. Patterns of tissue-specific expression

We examined the patterns of tissue-specific expression for the sepiatocin transcripts. The variations in expression were assayed via RT-PCR from 5 tissues of the two sexes: the female genital tract (oviduct), the male genital tract, the sub-esophageal mass, the supra-esophageal mass, the optic lobes and the optic glands. After RNA quantification (Nanodrop, Thermoscientific), genomic DNA was digested for 30 min at 37 °C with 1U of RQ1 DNAse (Promega). Then, 1 μg of total RNA was reverse-transcribed 1 h at 37 °C, followed by 10 min at 70 °C with 200 U of MMLV-RT (Promega), 24 U of RNAsin and 250 ng of Random Primers. The resulting cDNAs were used to assay sepiatocin expression in different organs and actin gene expression was taken as a reference. Amplification reactions were performed in a Thermocycler (Biorad), at 95 °C for 3 min, and then 30 cycles of 95 °C for 30 s, 55 °C (or 60 °C with EFγ primers) for 30 s, 72 °C for 45 s, and final elongation for 10 min at 72 °C. The reaction mixes were composed of 1 μL of cDNA, 1 U of Go Taq (Promega) with the following primers: SepOTPP, SepOT and SepOT2Rev or Actin and ActinRev or EFγ and EFγRev.

For each set of primers, an equal amount of PCR amplicon (15 μl) from each template was electrophoresed at 100 V for 30 min in a 1.8% agarose gel, stained with ethidium bromide and visualized under UV light (alpha imager EP, alpha Innotech). The target-specific amplified products had expected sizes of 513 and 360 bp for Pro-Sep and Sep, respectively, 261 bp for actin and 406 bp for elongation factor γ.

2.6. Recovery of material from tissues

Tissues were dissected from mature cuttlefish anesthetized using ethanol 3% [24], frozen in liquid nitrogen and stored at −80 °C until peptide extraction.