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ANNETOCIN: AN OXYTOCIN-RELATED PEPTIDE ISOLATED FROM THE EARTHWORM, EISENIA FOETIDA

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An oxytocin-vasopressin-related peptide, Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH_2, was isolated from the lumbricid earthworm, Eisenia foetida and termed annetocin. Annetocin potentiated not only spontaneous contractions of the gut but also pulsatory contractions and bladder-shaking movement of the nephridia. Annetocin may be involved in osmoregulation of the animal through nephridial function. © 1994 Academic Press, Inc.

Oxytocin and vasopressin are well-known neuropeptides or hormones, whose involvement in osmoregulation and male or female reproductive events in mammals has been well documented [1, 2]. Peptides belonging to the oxytocin-vasopressin superfamily have been identified mainly in vertebrates. Recently, invertebrate peptides of this superfamily have been found in the insect, Locusta migratoria [3], the gastropod molluscs, Conus geographus, Conus striatus [4], the cephalopod mollusc, Octopus vulgaris [5], and the tunicate, Styela plicata [6]. McMaster et al. [7] showed an evidence strongly suggesting that the gastropod mollusc, Aplysia kurodai, has the oxytocin-vasopressin-related peptide, Lys-conopressin, which was firstly identified in C. geographus by Cruz et al. [4]. van Kesteren et al. [8] have cloned a cDNA encoding a vasopressin-related peptide, Lys-conopressin, from the mollusc Lymnaea stagnalis and demonstrated that the Lymnaea conopressin precursor was organized similar to the precursors of the vertebrate oxytocin-vasopressin superfamily peptides. In some animals of the Annelida, which is one of the major

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Abbreviations: DTT, dithiothreitol; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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invertebrate phyla, oxytocin-vasopressin-like substances have been immunologically detected [9, 10]. However, their structure has not been determined yet.

In the present study, we isolated a novel oxytocin-vasopressin-related peptide from an annelid. Here, we report isolation, structure determination and actions of the peptide. This is the first report on identification of a member of the oxytocin-vasopressin superfamily in annelids.

Materials and Methods

Purification

The lumbricid earthworms, Eisenia foetida (980 g), were purchased from a fishing-bait store and maintained for three days on wet filter papers which were changed daily. The the earthworms were then frozen in liquid nitrogen, pulverized and boiled for 15 min in water containing 4% acetic acid (4 l). An equal volume of ethanol was added to the extract and the mixture, including tissue debris, was homogenized with a Waring blender. The homogenate was centrifuged at 10,000 g for 30 min (4 ^{o}C). The precipitate was mixed with 50% ethanol-2% acetic acid, resuspended, and centrifuged again. The two supernatants were combined and evaporated almost to dryness. The residue was dissolved in 0.1% TFA, and centrifuged (10,000 g, 30 min, 4 ^{o}C). The supernatant was forced through five C-18 cartridge columns in series (Sep-Pak Vac, Waters). The columns were washed with 10% methanol in 0.1% TFA, and the retained material was eluted with 50% methanol in 0.1% TFA. The eluate was evaporated to 0.5 ml in a vacuum centrifuge and filtered through a membrane filter (Ultrafree C3, Millipore; 0.22 μm). The filtrate was then subjected to HPLC using a C-18 reversed-phase column (CAPCELL-PAK, Shiseido; 10 x 250 mm). The column was eluted witha 120-min linear gradient of 0-60% acetonitrile in 0.15 TFA at a flow rate of 1 ml/min. An aliquot (1/1000) of each 2 ml-fraction was evaporated to dryness, dissolved in earthworm physiological saline (102 mM NaCl, 1.6 mM KCl, 1.8 mM $CaCl_2$, 5 mM Tris-HCl; pH 7.5) and used for bioassay. The fractions with bioactivity, eluted with 12-15% acetonitrile, were combined and subjected to cation-exchange HPLC (SP-5PW, Tosoh; 7.5 x 75 mm) with a 70-min linear gradient of 0-0.7 M NaCl in 10 mM phosphate buffer (pH 7.0). The active fractions eluted with around 0.15 M NaCl were applied onto C-18 reversed-phase column (ODS-80TM, Tosoh, 4.6 x 150 mm) and eluted with a 50-min linear gradient of 10-20% ACN in 0.1 % TFA. By an additional five steps of reversed-phase (ODS-80TM) and two steps of cation-exchange (SP-5PW) HPLC, an active substance was finally purified.

Structure determination

The purified substance was subjected to amino acid sequence analysis by the automated Edman degradation method with a gas-phase sequencer (Shimadzu PSQ-1), amino acid analysis of phenylthiocarbamyl derivatives by pre-column method (WAKOPAK WS-PTC column 4.0 x 200 mm) and molecular weight determination with a fast atom bombardment mass spectrometry (FAB-MS; JEOL JMS-HX 110/110A). A peptide having the sequenced structure was

synthesized by a solid-phase peptide synthesizer (Applied Biosystems 430A) using the FastMoc method. The intramolecular disulfide bond formation was achieved by the oxidation with $K_3[Fe(CN)_6]$ according to the method of Minamitake et al. [11].

Bioassay

The bioactivity of each HPLC fraction was examined on two systems of the earthworm, the nephridia and the gut (crop-gizzard complex). For the bioassay using the nephridia, the dorsal body wall of the earthworms was cut open, and the gut was removed. The body wall with the nephridia was cut off to a length of 5-6 mm and pinned on a rubber plate in a small petri dish filled with the saline (1.5 ml), which was constantly aerated. The test substance was dissolved in 0.1 ml saline and introduced into the dish and examined for bioactivity of eliciting spontaneous pulsatory and bladder-shaking movements of the nephridia under a dissection microscope. The nephridia isolated from the body wall were used for recording of bioactivity of synthetic peptide. The response of an isolated nephridium to the peptide was recorded photoelectrically on an electronic polyrecorder (TOA, EPR-221A) using a photoconductive cell attached to a microscope. For bioactivity on the gut, the effects of test substances were examined for spontaneous contractions of the crop-gizzard complex isolated from the earthworms. Both ends of the isolated gut were tied with two cotton threads, one being connected to the bottom of a trough and the other to a force-displacement transducer (SB-1T, Nihon Kohden) for tension recording. The trough was filled with physiological saline (1.5 ml), which was constantly aerated. The test substance dissolved in 0.1 ml saline was injected into the trough. All the bioassays were performed at room temperature (23-27 ^{o}C).

Results and Discussion

The active substance was finally purified on the C-18 reversed-phase column (Fig. 1). An aliquot (1/100) of the purified substance elicited pulsatory and bladder-shaking movements of the nephridia (data not shown), and potentiated spontaneous rhythmic contractions of the isolated gut (Fig. 1).

Quantitative amino acid analysis of the substance showed the following amino acid composition normalizing on Phe=1.0: $Asp_{1.1}$ $Gly_{1.1}$ $Arg_{0.9}$ $Thr_{1.1}$ $Pro_{1.0}$ $Val_{0.8}$ $Phe_{1.0}$. The determined sequence and detected amount (pmoles) of each amino acid except for the first and sixth residues were as follows: X-Phe (168)-Val (130)-Arg (25)-Asn (107)-X-Pro (73)-Thr (31)-Gly (36). No PTH-amino acid was observed in the first sequence cycle, while the sixth cycle showed two peaks, DTT-adduct of PTH-dehydroalanine and PTH-Arg. Nokihara et al. [12] studied the sequence analysis of a cystine containing peptide with the present sequencer (PSQ-

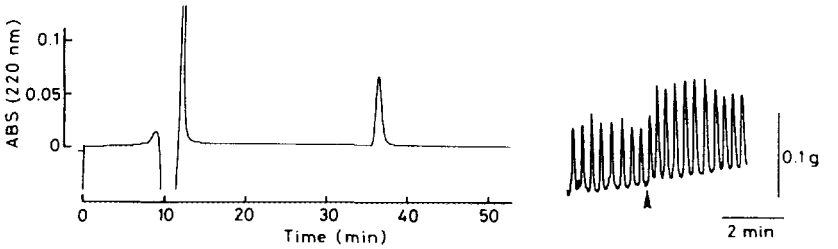

Fig. 1. The HPLC profile of the final purification of annetocin using a reversed-phase column (ODS-80TM) with an isocratic elution of 15% acetonitrile (0.1% TFA) at a flow rate of 0.3 ml/min (left) and bioactivity of the purified substance on an isolated gut (right). An aliquot (1/100) of the purified substance was introduced into trough at the time indicated by the arrow head.

1), and reported that the first half cystine was not appeared on the chromatogram, while the second half cystine was detected as two specific peaks, DTT-adduct of PTH-dehydroalanine and PTH-cysteine, the latter being eluted very close to PTH-Arg. Therefore, the unidentified residues at positions 1 and 6 were expected to be a Cys joined by a disulfide bond. Molecular ion, 993.4 m/z $(M+H)^+$, in the FAB-MS analysis of the substance further supported this hypothesis. The value was identical to the mass calculated for the above sequence including a cystine residue at positions 1 and 6 and an amide at C-terminus. We carried out reductive S-pyridylethylation before Edman degradation. Reasonable amount of PTH-S-(4-pyridylethyl)Cys was detected at the first and sixth cycles of the analysis. To confirm these sequence information, we synthesized a peptide having the proposed sequence and co-eluted the synthetic and native peptides on two HPLC systems (Fig. 2). As a result, structure of the purified substance was confirmed to be:

$$H\text{-}\overbrace{Cys\text{-}Phe\text{-}Val\text{-}Arg\text{-}Asn\text{-}Cys}\text{-}Pro\text{-}Thr\text{-}Gly\text{-}NH_2$$

and this peptide was termed annetocin.

Annetocin potentiated pulsatory contractions of the isolated nephridium of the earthworm at concentrations of 10^{-8} M or higher (Fig. 3A). In the quiescent nephridium, the peptide elicited pulsatory contractions (Fig. 3B). Annetocin also potentiated spontaneous contractions of the isolated gut (Fig. 4). The threshold concentration was between 10^{-9} M and 10^{-8} M.

Annetocin is clearly a member of the oxytocin-vasopressin superfamily (Table 1). Amino acid residues at position 1, 5, 6,

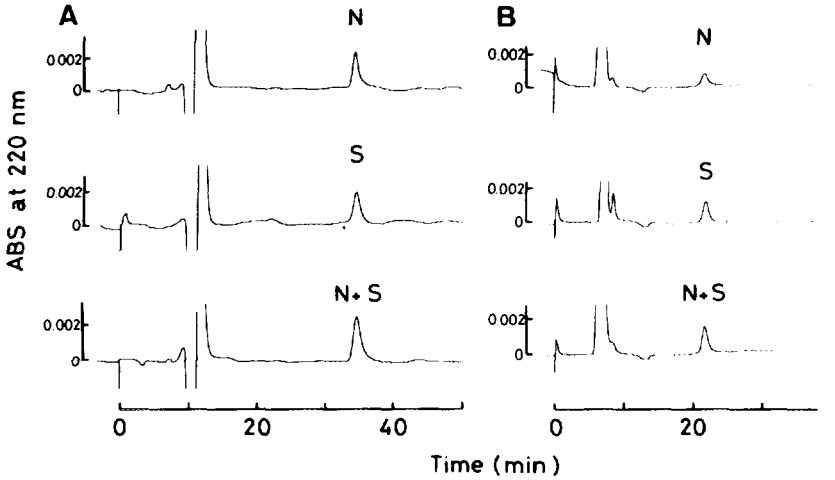

Fig. 2. Comparison of HPLC behavior between native (N) and synthetic (S) peptides. A: The reversed-phase column (ODS-80TM) was eluted isocratically with 15% acetonitrile in 0.1% TFA at a flow rate of 0.3 ml/min. B: The cation-exchange column (SP-5PW) was eluted with a 70-min linear gradient of 0.07-0.14 M NaCl in 10 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. In each HPLC, approximately 1/15 of the purified native peptide and 150 pmol of the synthetic peptide were injected into the HPLC system.

7 and 9 are common to all the superfamily members except for the tunicate peptide, indicating that the oxytocin-vasopressin superfamily is a highly conservative peptide group. Among the oxytocin-vasopressin superfamily peptides, annetocin is more closely related to the three molluscan peptides, cephalotocin, Lys-conopressin and Arg-conopressin than the other peptides. Notably, annetocin differs from Lys-conopressin only in position 3 and 8 residues.

Physiological role of the invertebrate peptides of oxytocin-vasopressin superfamily is not clear. The antiparallel

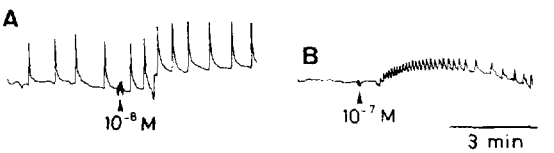

Fig. 3. Bioactivity of the synthetic peptide on two different preparations (A and B) of the isolated nephridia. The pulsatory contractions of the bladder of each nephridium were recorded photoelectrically. The peptide was applied at the time indicated by the arrow heads. 10^{-8} M annetocin elicited a slight increase in contraction frequency (A). In the quiescent nephridium, 10^{-7} M annetocin induced spontaneous pulsatory contractions (B).

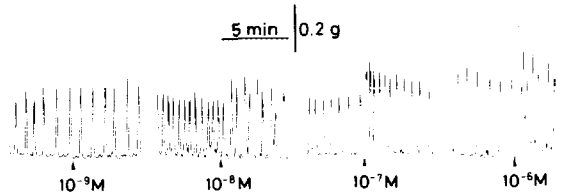

Fig. 4. Bioactivity of different concentrations of the synthetic peptide on an isolated gut (crop-gizzard). The peptide was applied at the time indicated by arrow heads.

dimer of the Locusta peptide has been reported to have diuretic action, but not the corresponding monomer [3]. Annetocin elicited pulsatory contraction of the nephridia of the earthworms, suggesting that it plays some role in osmoregulation through its action on nephridial functions. However, further study is needed to confirm this hypothesis.

Table 1. Members of the oxytocin-vasopressin superfamily

Vertebrate oxytocin-related peptides	
* * * * *	
Cys Tyr Ile Gln Asn Cys Pro Leu Gly-NH_2	Oxytocin
Cys Tyr Ile Gln Asn Cys Pro Ile Gly-NH_2	Mesotocin
Cys Tyr Ile Ser Asn Cys Pro Ile Gly-NH_2	Isotocin
Cys Tyr Ile Ser Asn Cys Pro Gln Gly-NH_2	Glumitocin
Cys Tyr Ile Gln Asn Cys Pro Val Gly-NH_2	Valitocin
Cys Tyr Ile Asn Asn Cys Pro Leu Gly-NH_2	Aspargtocin
Vertebrate vasopressin-related peptides	
Cys Tyr Phe Gln Asn Cys Pro Arg Gly-NH_2	Arg-vasopressin
Cys Tyr Phe Gln Asn Cys Pro Lys Gly-NH_2	Lys-vasopressin
Cys Tyr Ile Gln Asn Cys Pro Arg Gly-NH_2	Vasotocin
Cys Phe Phe Gln Asn Cys Pro Arg Gly-NH_2	Phenypressin
Invertebrate oxytocin-vasopressin-related peptides	
Cys Tyr Ile Ser Asp Cys Pro Asn Ser-	*Styela* oxytocin-
-Arg Phe Trp Ser Thr-NH_2	like peptide
Cys Leu Ile Thr Asn Cys Pro Arg Gly-NH_2	Insect DH
Cys Phe Ile Arg Asn Cys Pro Lys Gly-NH_2	Lys-conopressin
Cys Ile Ile Arg Asn Cys Pro Arg Gly-NH_2	Arg-conopressin
Cys Tyr Phe Arg Asn Cys Pro Ile Gly-NH_2	Cephalotocin
Cys Phe Val Arg Asn Cys Pro Thr Gly-NH_2	Annetocin

In each peptide, the cysteine residues form an internal disulfide bond. Residues at positions 1, 5, 6, 7, and 9 that are marked with asterisks are common to all members except for *Styela* oxytocin-like peptide whose C-terminus is extended. Adapted from Reich [5]. DH, diuretic hormone.

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