

Possible Functions of Oxytocin/Vasopressin-Superfamily Peptides in Annelids With Special Reference to Reproduction and Osmoregulation

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ABSTRACT Annetocin is an earthworm oxytocin-related peptide that we previously isolated from the whole body of a lumbricid earthworm *Eisenia foetida*. We have reported that annetocin induces egg-laying-like behaviors in *E. foetida* and a gnathobdellid leech, *Whitmania pigra*, when it is injected into the respective animals. The present study was undertaken to probe physiological functions of invertebrate oxytocin-vasopressin-superfamily peptides with special reference to reproductive and osmoregulatory events in which vertebrate peptides of this superfamily are involved. Annetocin, Lys-conopressin (a leech vasopressin-related peptide) and two analog peptides, [Tyr³]-annetocin (³Y-annetocin) and [Phe³]-annetocin (³F-annetocin), were compared for their activities to induce egg-laying-like behavior and to change body weight as a measure of water balance in the leech *W. pigra*. Injection of annetocin, Lys-conopressin, and ³F-annetocin caused both egg-laying-like behavior and reduction of body weight in the animals, but ³Y-annetocin induced neither. Furthermore, leeches in the non-breeding season responded to peptides less conspicuously than those in the breeding season. Such a concomitant induction of egg-laying-like behavior and body-weight reduction suggests that these two phenomena are unitary and might be accounted for by the fact that egg-laying in leeches and earthworms is accompanied by secretion of a large quantity of mucus, which should significantly contribute to body-weight loss. *J. Exp. Zool.* 284:401–406, 1999. © 1999 Wiley-Liss, Inc.

The neurohypophysis of mammals releases two nonapeptides: oxytocin and vasopressin. Oxytocin is best known for its stimulation of uterine smooth muscle contraction during parturition and of milk ejection from the mammary gland. The principal function of vasopressin is to promote water retention by stimulating reabsorption of water in the renal tubules. Each vertebrate species except for cyclostomes has two neurohypophysial peptides: oxytocin-related and vasopressin-related ones. It has been believed that oxytocin-related and vasopressin-related peptides have arisen from the common ancestral form, vasotocin, by gene duplication that occurred after vertebrates and invertebrates diverged from a stem group, the Archaemetazoa, 600 to 700 million years ago (Acher, '80, '93; Hyodo et al., '91). In fact, no in-

vertebrate species that possesses both oxytocin-related and vasopressin-related peptides have been known so far. Van Kesteren et al. ('95) have reported that the pulmonate mollusc *Lymnaea stagnalis* has only the vasopressin-related peptide, Lys-conopressin but not the oxytocin-related peptide and that cDNA or the gene for an oxytocin-like peptide is likely to be absent. Thus, the occurrence of oxytocin-related or vasopressin-related peptide in a certain invertebrate species has been suggested to depend on occasional mutation

Grant sponsor: Suntory Institute for Bioorganic Research, Osaka, Japan, SUNBOR GRANT.

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Received 6 March 1998; Accepted 30 June 1998.

of a single gene during evolution rather than gene duplication (Van Kesteren et al., '95).

As for invertebrate oxytocin/vasopressin (OT/VP) superfamily peptides, the following peptides have been isolated; antiparallel homodimer of Arg-vasopressin-like peptide from the insect *Locusta migratoria* (Proux et al., '87); Arg-conopressin from *Conus striatus* (Cruz et al., '87); Lys-conopressin from the gastropod mollusc *Conus geographus* (Cruz et al., '87), the pharyngobdellid leech *Erpobdella octoculata* (Salzet et al., '93a), *L. stagnalis* (Van Kesteren et al., '92) and the sea slug *Aplysia kurodai* (McMaster et al., '92); cephalotocin from the cephalopod mollusc *Octopus vulgaris* (Reich, '92); and annetocin from the earthworm *Eisenia foetida* (Oumi et al., '94). Among these invertebrate peptides, the *Locusta* peptide and Lys-conopressin have been reported to play an osmoregulatory role in *L. migratoria* (Proux et al., '88; Picquot and Proux, '90) and the leech *E. octoculata* (Salzet et al., '93a,b), respectively. On the other hand, the involvement in reproductive events has been suggested for Lys-conopressin in *L. stagnalis* (Van Kesteren et al., '95) and for annetocin in the earthworm *F. foetida* and the leech *Whitmania pigra* (Oumi et al., '96).

In the present study, we examined the effects of annetocin, Lys-conopressin, and two annetocin-related analog peptides on egg-laying induction and body-weight change as measures for reproduction and osmoregulation, respectively, at the organismal level in leeches. Possible physiological functions of invertebrate OT/VP superfamily peptides are discussed.

MATERIALS AND METHODS

Animals

Several hundred gnathobdellid leeches, *Whitmania pigra*, were collected in a brook and paddy field (Higashi Hiroshima, Japan) in June to August and maintained in dechlorinated tap water with constant aeration in the dark at room temperature. Experiments for breeding-season leeches were performed in this season (June–August). Since it is difficult to collect the animals from the field in winter, those collected in summer and kept for several months under laboratory conditions without food were used as the non-breeding-season leeches, and experiments were done in winter (January–February).

Physiological conditions of these leeches might not exactly reflect those of the animals in the natural field. Furthermore, sexuality of the ex-

perimental animals was not checked, although all leeches are protandric hermaphrodites (Barnes, '87). Despite the lack of clarity about physiological conditions and sexuality, we still obtained some information on the correlation between egg-laying-related behaviors and hydromineral regulation with respect to the effect of annetocin.

Experimental procedures

In the previous study, we reported that annetocin induced egg-laying-like behaviors not only in the earthworm *E. foetida*, from which annetocin was isolated, but also in the leech *W. pigra* (Oumi et al., '96). In the present study, leeches were used as experimental animals, because injection into the earthworms was more difficult than that into the leeches mainly due to the size and because visible changes during egg-laying behaviors were more conspicuous in leeches than in earthworms. One nmole of peptides (1 μ l of 10^{-3} M solution in distilled water) or the same volume of distilled water for controls was injected dorsally into the coelom of the leeches. The peptides tested were annetocin, 3 Y-annetocin, 3 F-annetocin, and Lys-conopressin; 3 Y- and 3 F-annetocin were the analog peptides in which the amino acid residue (Val) at the third position of annetocin was replaced with Tyr or Phe, respectively (Table 1).

As mentioned above, the injection experiments were performed in both the breeding (June–July) and non-breeding (January–February) seasons; the leeches in respective seasons weighed 0.5–1.0 g and 0.3–0.4 g.

The injected leeches were placed on filter papers (no. 2; 90 mm in diameter) in polystyrene petri dishes (90 \times 15 mm), which were moistened with 1 ml of dechlorinated tap water, and observed with respect to the egg-laying-like behaviors such as characteristic changes in the body shape and mucus secretion from the clitellum (Oumi et al., '96). Eight animals were examined for each test peptide or for the control. Further-

TABLE 1. Primary structures of annetocin and its related peptides

Annetocin	Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH ₂
3 Y-Annetocin	Cys-Phe-Tyr-Arg-Asn-Cys-Pro-Thr-Gly-NH ₂
3 F-Annetocin	Cys-Phe-Phe-Arg-Asn-Cys-Pro-Thr-Gly-NH ₂
Lys-conopressin	Cys-Phe-Ile-Arg-Asn-Cys-Pro-Lys-Gly-NH ₂
Arg-vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH ₂
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH ₂
Vasotocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH ₂

more, at specified times (0, 1, 2, 4, and 20 hr) after injection, the animals were weighed to the nearest 1 mg as a measure of water balance.

Peptides

Annetocin, ^3Y - and ^3F -annetocin were synthesized with a solid-phase peptide synthesizer (Applied Biosystems 430A, Foster City, CA) and purified by reverse-phase HPLC (Oumi et al., '94; Ukena et al., '95). Lys-conopressin was purchased from Peninsula Lab. Inc. (Belmont, CA).

Statistical analyses

Significantly different means of body weights were identified by the multiple Student's *t*-test. Significant difference in induction of egg-laying behaviors between the control and peptide-injected groups was analysed by the χ^2 -test. The significance level accepted was $P < 0.05$.

RESULTS

In the breeding season (June–July), most leeches injected with 1 nmol of annetocin, ^3F -annetocin, and Lys-conopressin expressed a series of stereotyped behaviors involved in egg laying, which included constriction of both ends of the clitellum, accordion-like movement of the clitellum, secretion of mucous substance (cocoon membrane and nutritious substance) from the clitellum, and slenderizing of the body anterior to the clitellum ($P < 0.001$ as compared with control, Table 2). But, ^3Y -annetocin did not induce any such behaviors. Correspondingly, the body weights of leeches injected with ^3Y -annetocin were not significantly different from the control animals ($P > 0.5$ at 4 hr, Fig. 1A). In contrast, the leeches injected with Lys-conopressin ($P < 0.001$ at 4 hr), annetocin ($P < 0.001$ at 4 hr) and ^3F -annetocin ($P < 0.05$ at 4 hr) showed a significant decrease in body weights; the potency was in this order. Upon injection with Lys-conopressin, for example, the mean body weight decreased 75%

in 4 hr and returned toward the initial level increasing to 91% in 20 hr, though the value was still significantly different from the control level ($P < 0.001$ at 20 hr).

In non-breeding season (January–February), the same amounts of peptides except for Lys-conopressin were injected, though leeches in this season were somewhat smaller in size than those in breeding season. The leeches injected with annetocin showed only slightly slenderizing of the anterior body, but no mucous secretion (data not shown). No visible changes in body shape or mucous secretion was observed in leeches injected with ^3F -annetocin, ^3Y -annetocin, or distilled water only. Correspondingly, body-weight changes in leeches injected with peptides were much smaller than those in breeding season (Fig. 1B). But, the decreases in body weight were still statistically significant in annetocin- ($P < 0.001$ at 4 hr) and ^3F -annetocin-injected leeches ($P < 0.05$ at 4 hr), as compared with the control animals.

DISCUSSION

The functions of OT/VP superfamily peptides have been only partially delineated in invertebrates. The locust antiparallel homodimer of vasopressin-like peptide has been reported to show a diuretic activity through its direct action on the Malpighian tubules (Proux et al., '88; Picquot and Proux, '90). Salzet et al. ('93a) have isolated Lys-conopressin, a vasopressin-related peptide, from the leech *E. octoculata* and have reported that the peptide has a diuretic effect since the leech injected with the peptide showed a significant loss of the body mass. In the current experiments, annetocin and Lys-conopressin induced both egg-laying-like behavior and loss of body weight in the leech *W. pigra*. Furthermore, the effects of the four peptides, including two analog peptides on induction of egg-laying behavior, corresponded to those on body-weight change. These results suggest that the two phenomena are closely linked. Leeches and earthworms exhibiting egg-laying behaviors secrete a large quantity of mucous substance, which afterwards forms cocoon membrane and a nutritious substance (Barnes, '87; Oumi et al., '96). Some part of the body-weight loss observed in the present study should be due to the secretion of the mucus. In the non-breeding season, leeches injected with annetocin did not secrete mucous substance, but showed only a slight change of body shape with respect to egg laying. They also lost weight to a lesser extent than leeches in breeding season. Therefore, the body-weight loss observed in the present experiments

TABLE 2. Induction of egg-laying-like behavior in leeches¹

Peptides (1 nmole)	No. of leeches (responded/tested)
Control	0/8
Annetocin	8/8
Lys-Conopressin	8/8
^3Y -Annetocin	0/8
^3F -Annetocin	7/8

¹Each leech was injected with 1 μl of saline or 10^{-3} M peptide solution. Leeches that showed typical body-shape changes were judged as "responded."

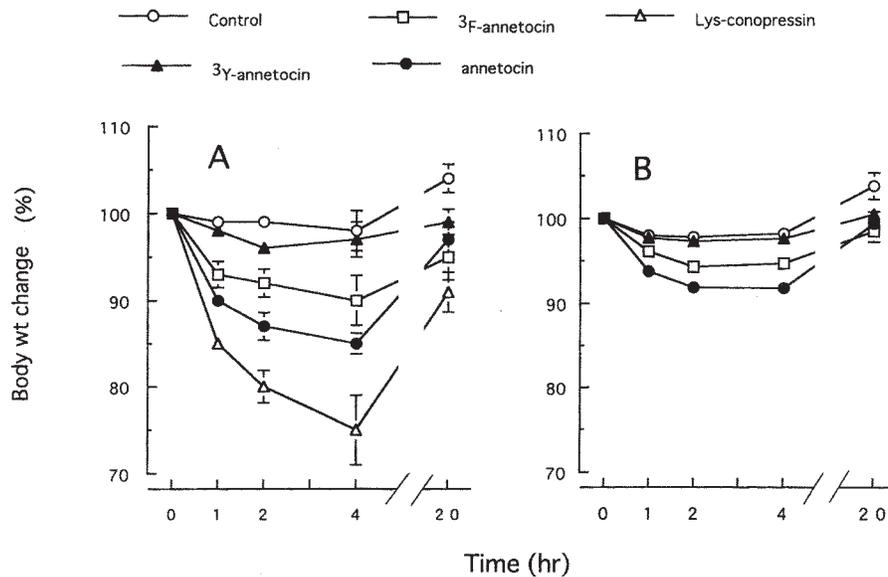


Fig. 1. Time course of changes in body weight of the leeches injected with saline or peptide solutions ($1 \mu\text{l}$ of 10^{-3} M solution in saline for each leech). For each of the control and peptide series of experiments, the mean percent change

in body weight \pm SE is plotted ($n = 8$). Experiments were done in the breeding (A) and non-breeding seasons (B). The effect of Lys-conopressin could not be tested in the non-breeding season, because no peptide was available.

seems to be an egg-laying-related phenomenon. In fact, serosal application of annetocin at as high as 10^{-5} M showed no effect on transepithelial potential difference, short-circuit current, and transepithelial resistance across the skin of *W. pigra* (unpublished data), indicating that annetocin did not influence ionic transport across the skin. The presence of amiloride-sensitive Na^+ channels has been reported in the skin of the leech, *Hirudo medicinalis* (Weber et al., '95). In vertebrate epithelia, OT/VP superfamily peptides have been known to activate Na^+ influx (Omachi et al., '74; Helman et al., '83; Lester et al., '88; Bevevino et al., '96).

It has been suggested that the difference in orientation of ^2Y side chain between mammalian oxytocin and vasopressin accounts for their functional difference, namely oxytocic or antidiuretic activity, respectively (Langs et al., '86). In vasopressin, two aromatic residues at the second and third positions are in perpendicular orientation with each other, which is important for exertion of antidiuretic action. In oxytocin, such an interaction no longer exists because ^3F is replaced with an aliphatic residue ^3I . In the present study, therefore, we prepared two annetocin-analog peptides with two aromatic residues at the second and third positions, ^3Y - and ^3F -annetocin, and tested for their effects on body weight and egg-laying-like behaviors. However, the two synthetic analogs did

not show any functional shift from egg laying to weight loss in leeches. Thus, OT/VP superfamily peptides do not appear to be functionally differentiated between reproduction and osmoregulation in leeches (Acher, '80, '93; Hyodo et al., '91; Van Kesteren et al., '92, '95). In this connection, we should not call the egg-laying-related body-weight loss osmoregulation or hydromineral regulation and might conclude that annetocin is primarily involved in reproduction rather than osmoregulation. Furthermore, the quite different activities between ^3Y - and ^3F -annetocin suggest that hydroxy group of ^3Y interferes with exertion of the effects, because it is the only structural difference between the two amino acids, tyrosine and phenylalanine.

Lys-conopressin has also been identified in the freshwater pulmonate snail *L. stagnalis* as the precursor protein deduced from a cloned cDNA (Van Kesteren et al., '92) and is expressed in neurons innervating the penis complex and the vas deferens, showing excitatory effects on the vas deferens muscular contractions; Lys-conopressin appears to be involved in male sexual behavior in *L. stagnalis* (Van Kesteren et al., '95). In the present study, Lys-conopressin and annetocin induced female sexual behavior, a series of egg-laying-like behaviors. These peptides of the OT/VP superfamily may cause specific sexual behaviors at the organismal level by regulating activities of

either male or female reproductive organs especially in hermaphroditic animals such as earthworms, leeches and pulmonate molluscs like *L. stagnalis*. In mammals, oxytocin regulates both sexual behaviors, that is, milk ejection from the mammary gland and uterine muscular contraction in females and ejaculation of semen in males. In vertebrates other than mammals, however, the function of oxytocin-related peptides is not clear. On the other hand, vertebrate vasopressin-related peptides, vasopressin and vasotocin, are involved in hydromineral regulation. In mammals and birds, the principal role of vasopressin and vasotocin in hydromineral regulation is activation of water reabsorption in renal tubules, causing antidiuresis. In other poikilothermal vertebrates, however, constriction of preglomerular vessel is a major strategy for antidiuresis, which reduces glomerular filtration rate (GFR); this is clearly a result of the action of vasotocin as a vasopressor. Especially in bony fishes, vasotocin has potent systemic vasopressor effect causing hypertension. This effect is more than preglomerular vessel effect, resulting in diuresis due to increased GFR. Thus, the function of vasopressin and vasotocin seems to be originally vasopressive and to evolve along with advance onto the terrestrial environment (Takei, '92).

We have demonstrated immunohistochemically that annetocin is a neuropeptide in the earthworm *E. foetida*, showing annetocin-immunoreactivity in the central nervous system of the earthworm (Takahama et al., '98). Recently, Salzet et al. ('97) isolated a peptide with homology to *Aplysia* egg-laying hormone and *Lymnaea* caudo-dorsal cell hormone from the central nervous system of the rhynchobdellid leech *Theromyzon tessulatum*, the function of which has not been determined. Annetocin appears to play a key role in releasing egg-laying behaviors, though the detailed mechanism is still unclear.

ACKNOWLEDGMENTS

We thank Professor Y. Muneoka, Hiroshima University, for his constant encouragement and valuable advice throughout the study.

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