Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 33, Issue of November 25, pp. 15821-15824, 1987

Invertebrate Vasopressin/ Oxytocin Homologs

CHARACTERIZATION OF PEPTIDES FROM *CONUS GEOGRAPHUS* AND *CONUS STRIATUS* VENOMS*

(Received for publication, July 30, 1987)

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The vasopressin-oxytocin family of peptides is of very ancient lineage, found in organisms as diverse as hydra and man. Although these peptides have been intensively studied in vertebrates, the presumably more extensive invertebrate series was defined primarily by immunological methods. In this report, we describe the purification and structures of two peptides of the vasopressin-oxytocin family from molluscs ("Conopressins"), which were found in the venom of fish-hunting marine snails of the genus *Conus*. The biological activity observed when the two snail peptides are injected intracerebrally into mice is very similar to that elicited by the vertebrate neurohypophyseal hormones and presumably reflects their actions upon a common receptor in the brain. The sequences of the purified peptides reveal unique features not found in the vertebrate peptide series, most notably an additional positive charge. These are the first members of the invertebrate series of the vasopressin-oxytocin family to be characterized biochemically.

The sequences of these peptides are: from *Conus geographus* venom, Lys-conopressin-G, Cys-Phe-Ile-Arg-Asn-Cys-Pro-Lys-Gly-NH_2; and from *Conus striatus* venom, Arg-conopressin-S, Cys-Ile-Ile-Arg-Asn-Cys-Pro-Arg-Gly-NH_2.

The vasopressin-oxytocin peptides were first characterized as neurohypophyseal hormones in mammals. Their biological activity was defined 30 years before (1) and their structures were elucidated in the 1950s by Du Vigneaud and his coworkers (2). In recent years, the proposed physiological role and phylogenetic distribution of this family of peptides has been greatly expanded. These peptides are released in tissues other than the pituitary in mammals, and vasopressin appears to play a role as a neurotransmitter or cotransmitter in mammalian brain. In addition, compelling evidence has accumulated for the presence of these peptides in a number of invertebrate systems, including insects (3–5) and molluscs (6, 7). This evidence is primarily immunological, most often immunocytochemical, staining with antipeptide antibodies.

Recently Grimmelikhuijzen *et al.* (8) reported oxytocin/vasopressin-like immunoreactivity in *Hydra*. This coelenterate has one of the simplest nervous systems known: although synapses and secretory vesicles are present, small molecule neurotransmitters such as acetylcholine and catecholamines appear to be absent (9, 10). The high concentration of peptides in the neurons of *Hydra* has led to the intriguing suggestion that in primordial nervous systems the first neurotransmitters may have been exclusively peptides (10). Thus, included in the set of putative *Hydra* neurotransmitters is a peptide of the vasopressin-oxytocin family (8).

These results suggest that these peptides have an evolutionary lineage that dates back ~10^9 years. To date, only the vertebrate members of this peptide family have been purified and fully characterized, leaving the relationship between them and the ancient line of invertebrate peptides undefined. In this report we describe the first purification and sequence analysis of invertebrate vasopressin-oxytocin peptides. These peptides have been purified from an unexpected source, the venom of the fish-hunting cone snails (*Conus*). We propose the generic term conopressin for all vasopressin-oxytocin homologs found in *Conus*. The *Conus* venoms have proven to be rich sources of biologically active peptides (11). Peptide toxins which inhibit calcium channels (12, 13), sodium channels (14, 15), and the acetylcholine receptor (16, 17), as well as a peptide which induces a sleep-like state in mice (18, 19), have already been described.

MATERIALS AND METHODS

Venoms—*Conus geographus* and *Conus striatus* were collected around the island of Marinduque, Philippines. Venom ducts were dissected from freshly killed snails, and the crude venom was subjected to preliminary purification as described previously (12).

Isolation and Bioassay of Peptides—Crude venom extract from *C. geographus* was fractionated on a Sephadex G-25 column as previously described (12) and the "scratcher peptide" was subsequently purified from the third major protein peak which was designated as Peak C by Olivera *et al.* (12). Reverse-phase HPLC[1] was used to separate different peptide fractions with the appropriate biological activity as shown in Fig. 1, and the peptides were assayed by intracerebral injection into mice as previously described (12). The scratcher peptide of *C. striatus* venom was purified essentially in the same manner.

Amino Acid Analysis—Peptide samples were hydrolyzed *in vacuo* in 6 N HCl, 1% phenol for 18 h at 105 °C. For *C. striatus*, amino acid analysis was done by reverse-phase HPLC of phenyl-thiocarbamyl derivatives (20).

Peptide Sequencing—Reduction and carboxymethylation was carried out as previously described (16) except that a volatile buffer (0.02 M *N*-ethylmorpholine) was used. Sequencing of reduced and carboxymethylated peptides was carried out by sequential Edman degradation in a Beckman 890D spinning cup sequenator, using 0.1 M Quadrol buffer and Polybrene carrier (21). Phenylthiohydantoin-derivatives were identified by HPLC on a Hewlett-Packard 1084B instrument using an Ultrasphere ODS column (0.46 × 15 cm, 5-μ particle size, endcapped) eluted with a gradient of acetonitrile in 0.05 M sodium acetate, pH 4.5 (16).

* This research was supported by National Institutes of Health Grants GM22737 (to B. M. O.) and GM34913 (to W. R. G.) and by a grant from the International Foundation for Science, Stockholm, Sweden (to L. J. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviation used is: HPLC, high performance liquid chromatography.

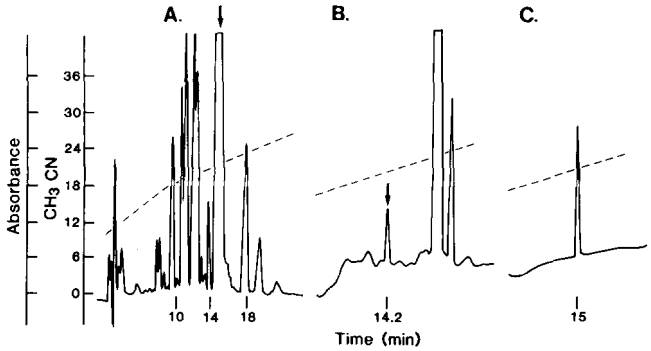

FIG. 1. **Purification of the scratcher peptide from *Conus geographus* venom.** One gram of crude lyophilized venom was extracted and fractionated on a Sephadex G-25 column as previously described (12). The first 6 of 21 successive fractions containing "shaker activity" (now known as the calcium channel blockers, ω-conotoxins) were pooled and chromatographed on a Vydac C_{18} semi-preparative column eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid (*A*). The peak indicated by an *arrow* elicited both shaking and scratching activities. Chromatography of material from this peak on a Vydac C_{18} analytical column eluted with a gradient of acetonitrile in 0.05% heptafluorobutyric acid (*B*) separated the "scratcher" from the "shaker" activity. The scratcher peak, when rechromatographed under the same conditions as in *B*, appears homogeneous (*C*). The absorbance plotted is at 214 nm. *Dashed lines* indicate the gradient of acetonitrile.

RESULTS

Assay and Purification of Conopressins—Intracerebral injection of these peptides at levels of >0.5 nmol causes mice to scratch themselves within a few minutes. The "scratcher" activity is masked in crude venom by the actions of other toxins, particularly the lethal ones. The first conopressin was detected in *Conus* venom fractions from Sephadex G-25 filtration, where its elution profile overlapped extensively with that of the Ca^{2+} channel toxins (ω-conotoxins). On reverse-phase HPLC in 0.1% trifluoroacetic acid/acetonitrile (Fig. 1*A*), the *C. geographus* activity was associated with the leading edge of the major peak of ω-conotoxin GVIA or the "shaker peptide". On rerunning in 0.05% heptafluorobutyric acid/acetonitrile (Fig. 1*B*) a clean separation was obtained, the scratching effects being elicited only by material from a minor peak. This material, which was apparently homogeneous, was then analyzed by sequencing. Purification of conopressin from *C. striatus* venom followed essentially the same steps.

Peptide Structural Analysis—The peptides were reduced and carboxymethylated as described under "Materials and Methods" (16) and then applied to the sequenator cup. In both cases the sequences were unambiguous, although there were heavy losses of the COOH-terminal Gly, a common experience with amidated peptides. Results of the analysis for both conopressins are given in Table I, and sequences of the two conopressins are shown in Table II.

Amino acid analysis of conopressin S was in agreement with the sequence, though the Ile·Ile dipeptide was only hydrolyzed to approximately 50%. Analysis was not carried out for conopressin G. The proposed structures, including the COOH-terminal amides, were also confirmed by fast atom bombardment mass spectrometry, with protonated molecular ions (MH^+) of 1034 and 1028 for conopressins G and S, respectively. The sequence assignments have been confirmed by peptide synthesis; details of the synthesis, comparisons of natural *versus* synthetic material, and a more detailed physiological characterization of these peptides will be described elsewhere.[2]

[2] W. R. Gray, R. Zeikus, and C. A. Ramilo, manuscript in preparation.

TABLE I
Sequence analysis of conopressins
PTH, phenylthiohydantoin-derivative.

Step	Conopressin G		Step	Conopressin S	
	Assigned residue	Yield		Assigned residue	Yield
		nmol of PTH			*nmol of PTH*
1	Cys	1.15	1	Cys	1.67
2	Phe	2.11	2	Ile	2.30
3	Ile	2.06	3	Ile	2.36
4	Arg	1.55	4	Arg	0.75
5	Asn	0.57	5	Asn	1.05
6	Cys	0.60	6	Cys	1.02
7	Pro	0.84	7	Pro	0.59
8	Lys	0.65	8	Arg	0.29
9	Gly	0.04	9	Gly	0.05

The structural similarity with vasopressin and other neurohypophyseal hormones is obvious from Table II. Thus, the peptide from *C. geographus* is designated Lys-conopressin-G and the peptide from *C. striatus* is designated Arg-conopressin-S. This nomenclature follows both the commonly used system for vasopressins, as well as for *Conus* peptides (22).

Biological Effects of Intracerebral Injection—The biological responses elicited by intracerebral injection of conopressin G were compared with those elicited by Arg-vasopressin and oxytocin (Table III). They were similar in all cases, with conopressin being intermediate in potency between the vertebrate hormones. The scratching response is dose-dependent with respect to time of onset and duration of symptoms. At very low doses, the mice have the appearance of grooming their faces. The symptomatology elicited by these peptides upon intracerebral injection into mice is highly characteristic (23–25), presumably diagnostic of a specific central nervous system receptor. This may be homologous to the receptor which controls the stereotypic "flanking marking" response characterized in Golden hamsters (26); scratching is one feature of this territorial behavior.

DISCUSSION

The peptides isolated from *C. geographus* and *C. striatus* are clearly members of the vasopressin-oxytocin family (Tables II and III), yet they have distinctive features. A basic residue, Lys or Arg, at position 8 is strongly correlated with pressor activity in vertebrate hormones and in hundreds of synthetic analogues (27). Since both of the *Conus* peptides share this feature, we propose the name "conopressin" for the group. The presence of Ile at position 3 could be used to argue in favor of "conotocin," but this is confusingly similar to the well-established "conotoxin". Both peptides have neutral hydrophobic residues at position 2, Phe in conopressin G and Ile in conopressin S. All the vertebrate peptides have Tyr at this position, with the sole exception of phenypressin from marsupials (see Table II). An aromatic residue at position 2 is believed to be specifically required for interaction with neurophysin in vertebrate systems (28). The presence of Ile in conopressin S implies either that there are no endogenous neurophysins for conopressin(s) or that *Conus* neurophysins are more tolerant of substitutions at position 2.

The unique feature shared by both snail peptides is the presence of a basic residue, Arg, at position 4. This gives them a net charge of +3, higher than that of the vasopressins (+2) or oxytocins (+1). Whether this contributes unique biological properties will be of great interest. An arginine vasopressin analogue with this substitution has been synthesized (29) and shown to be similar to vasopressin in its antidiuretic and pressor activities.

TABLE II

Amino acid sequences of the scratcher peptide and some vertebrate neurohypophyseal hormones

Peptide	Amino acid sequence	Charge	Animal group
A. Piscivorous *Conus* scratcher peptides			
Lys-conopressin-G[a]	Cys-Phe-Ile-Arg-Asn-Cys-Pro-Lys-Gly-(NH_2)	+3	Mollusc
Arg-conopressin-S	Cys-Ile-Ile-Arg-Asn-Cys-Pro-Arg-Gly-(NH_2)	+3	Mollusc
B. Vertebrate neurohypophyseal hormones[b]			
Lys-vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-(NH_2)	+2	Suiformes, peccaries, wart hogs, and hippopotami
Arg-vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-(NH_2)	+2	Mammals (except domestic pigs)
Phenypressin	Cys-Phe-Phe-Gln-Asn-Cys-Pro-Arg-Gly-(NH_2)	+2	Marsupials
Arg-vasotocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-(NH_2)	+2	Birds, reptiles, amphibians, bony fishes, and possibly cartilaginous fishes
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-(NH_2)	+1	Mammals
Glumitocin	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Gln-Gly-(NH_2)	+1	Cartilaginous fishes (ray)
Ancestral vertebrate peptide	Cys-Tyr-*X*-*X*-Asn-Cys-Pro-*X*-Gly-(NH_2)		

[a] We propose to call all peptides of this homologous peptide set "conopressins." The nomenclature is adapted from that used for conotoxins, *i.e.* G (for *C. geographus*), S (for *C. striatus*) (22). Thus, the first conopressin from *Conus magus* (if different in sequence) would be conopressin M.

[b] Adapted from Archer (31) and Hadley (32). The cysteine residues form internal disulfide bonds.

TABLE III

Biological activity

	Time of onset of scratching activity		Grooming behavior when injected with[a]
	5-nmol injection	0.5-nmol injection	
	s	*min*	*pmol*
Lys-conopressin-G	45	3.0	10
Arg-vasopressin	15	1.0	3
Oxytocin	60	3.2	20

[a] Different levels of each peptide starting at 5 nmol and using successive 2- to 5-fold dilution intervals were injected into a series of mice. Eleven mice were used for the Lys-conopressin-G series and 6 mice each for Arg-vasopressin and oxytocin. The lowest concentration of each peptide at which definite grooming behavior was observed is indicated. The experiments with conopressin were performed with two different batches of toxin.

Peptides of the vasopressin-oxytocin family are probably present in all nervous systems, including the highly primitive ones such as that of *Hydra* which lack the "classical" neurotransmitters. Their function in *Conus* is unknown, especially in the venom. In *Aplysia californica* Lukowiak *et al.* (30) demonstrated that arginine vasopressin enhances suppressive control over gill reflex behavior when it is applied to the abdominal ganglion at 10^{-12} M concentrations. They subsequently showed that the anterior ganglia of *Aplysia* contain an arginine vasotocin-like peptide, as judged by its immunological reactivity and HPLC behavior (7). In our HPLC systems the conopressins are resolved from arginine vasotocin.

It is noteworthy, given the conserved homology of the vertebrate vasopressin-oxytocin family, that the conopressins sequenced from two snails in the same genus differ in two out of nine residues. In contrast, arginine vasotocin from teleosts to birds exhibits no sequence changes at all (see Table II), and changes among other members are minor. Despite the lack of stringent sequence conservation in conopressins, the fact that the positive charge in position 4 is found in both *C. geographus* and *C. striatus* venoms suggest either that this structural feature is selected for and/or that both peptides evolved from an endogenous molluscan peptide with a positive charge at position 4. We expect that the occurrence of conopressins in *Conus* venoms is more likely due to evolution from pre-existing snail peptides, rather than to arriving at a functional analog by convergent evolution. Although conopressins may be secreted by neuronal tissue innervating the venom ducts, it is also possible that conopressins play a role in the envenomation process. We suggest that these peptides have been used in nervous systems continuously but have been used opportunistically for specialized functions, such as neurohypophyseal hormones in mammals (and possibly as venom peptides in *Conus*), and that in different taxa a variety of such specialized uses may be found.

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