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Arginine Vasotocin from the Pituitary Gland of the Lamprey (*Petromyzon marinus*): Isolation and Amino Acid Sequence

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Arginine vasotocin (AVT) was isolated from extracts of sea lamprey pituitary glands (*Petromyzon marinus*). The amino acid sequence Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Arg–Gly-NH_2 is identical to the molecule isolated from teleosts and tetrapods. The total yield was estimated to be 9.6 pmol per gland. No evidence could be found for the existence of a second neurohypophyseal nonapeptide in the lamprey pituitary.

The neurohypophyseal nonapeptide family is composed of at least 10 homologous peptide sequences. Of these, arginine vasotocin (AVT) appears to be phylogenetically the oldest (reviewed by Acher, 1974; Bentley, 1980). Whereas at least two different neurohypophyseal nonapeptides can be detected in gnathostome vertebrates, only AVT can be detected immunohistochemically in the neurosecretory tracts of cyclostomes (Goosens *et al.*, 1977). Neither the biochemical characteristics nor the amino acid sequence of a cyclostome pituitary hormone has been reported. Thus, we set out to isolate and characterize hormonal peptides from the pituitary of the sea lamprey (*Petromyzon marinus*).

The pituitary of the cyclostomes is similar to those of other vertebrates in that it contains anatomically identifiable adenohypophysis and neurohypophysis (Tsuneki and Gorbman, 1975a, b). However, there is some doubt about the role of the pituitary in cyclostome biology. The pars distalis in the lamprey and the entire adenohypophysis in the hagfish do not possess neural or vascular connections to the hypothalamus (reviewed by Gorbman, 1980). The significance of this problem lies in the antiquity of the cyclostome lineage, having diverged from the vertebrate line some 500 million years ago (see reviews: Jarvik, 1968; Bardack and Zangerl, 1971; Hardisty and Baker, 1982). Thus, the activities of the cyclostome pituitary are significant to studies of the evolution of all vertebrate pituitary functions.

The lamprey pituitary contains several peptides that are biologically active in other animals. The results suggest the presence of vasotocin-like (Sawyer *et al.*, 1961; Follett and Heller, 1964; Sawyer, 1965) and proopiomelanocortin-related peptides (Larson and Rothwell, 1972; Hardisty and Baker, 1982; Baker and Buckingham, 1983). Extensive immunohistochemical studies have tentatively identified several vertebrate peptide and protein hormones in specific cell types of the brain, hypothalamus, and pituitary (see Nozaki, 1985, for review). Among these substances, only hypothalamic GnRH has been isolated and characterized (from *P. marinus;* Sherwood *et al.*, 1986). While several pituitary hormones have been proposed for the cyclo-

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stomes, none has been isolated and chemically characterized.

Biochemical investigation of the cyclostome pituitary has been hindered by its extremely small size (0.7 mg in adult *P. marinus*) and the difficulty in obtaining quantities of tissue needed for purification studies. This paper describes the isolation and sequence of lamprey AVT, the first pituitary hormone to be chemically characterized from the lamprey.

MATERIALS AND METHODS

Isolation. Adult sea lamprey, *P. marinus,* were captured during their upstream migration and maintained at Hammond Bay Biological Station, Michigan. Pituitary glands were collected and were stored at −70° until used. Pituitaries (2220; 1.538 g wet wt.) were homogenized in ammonium bicarbonate buffer (0.1 *M* NH_4HCO_3, pH 9.0), 4°, in the presence of 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The crude extract was clarified by centrifugation (15,000*g*, 30 min, 4°) and fractionated by Sephadex G-100 (2.0 × 85 cm; Pharmacia) in 0.05 *M* NH_4HCO_3, pH 9.0. Peaks of absorbance (OD 280 nm) were lyophilized and the most retarded peak (containing peptides and salts) was fractionated as follows. Peptides were solubilized in 0.1% trifluoroacetic acid (TFA) and applied to a reverse-phase HPLC column (C-18 ODS-120T, 5-μm particle size, Toyo Soda, Japan; 4.6 × 250 mm, 1 ml · min^{-1}, 40°). Salts were eluted with buffer (0.1% TFA); peptides were eluted by a step gradient to 60% acetonitrile (CH_3CN) and lyophilized. The desalted peptides were fractionated first by carboxymethyl (CM)-HPLC. Peptides unabsorbed by CM-HPLC were lyophilized and rechromatographed on diethylamino ethyl (DEAE)-HPLC. All fractions were chromatographed to homogeneity on RP-HPLC. Fractionation on both ion exchangers involved solubilizing peptides in 10 m*M* ammonium formate (HCO_2NH_4) + 10% CH_3CN, pH 6.5, and applying them to columns equilibrated with the same buffer (TSK · CM-2SW and TSK · DEAE-2SW, Toyo Soda, Japan; 4.6 × 250 mm, 1 ml · min^{-1}, 40°). Peptides were eluted with a linear gradient to 1 *M* HCO_2NH_2 over 90 min. All fractions were lyophilized, solubilized in 0.1% TFA, and applied to RP-HPLC as above; peptides were eluted with a linear gradient from 10 to 50% CH_3CN over 40 min.

Native chum salmon AVT (*Oncorhynchus keta*) was the generous gift of Dr. I. Kawazoe and was isolated as described (Kawauchi *et al.*, 1984). The sample was repurified to homogeneity by RP-HPLC as described above.

Analysis. Absorbance peaks (OD 220 nm) recovered from RP-HPLC were analyzed for amino acid composition and N-terminal residue determination. Briefly, hydrolyzed amino acids were identified as their phenylthiocarbamyl (PTC) derivatives on a Jasco 800 series amino acid analyzer equiped with a TSK gel, ODS-80TM column. Half cystine content was determined by analysis of performic acid oxidized peptides followed by hydrolysis and PTC derivatization. The N-terminal residues were analyzed after labeling the intact peptide with dansyl-chloride (Gray, 1967). Fractions with apparent homology to vertebrate neuropeptides were subjected to amino acid sequencing. Briefly, sequencing involved sequential manual Edman cleavage of the phenylisothiocyanate (PITC)-labeled amino-terminal residues with anhydrous TFA. The released PITC conjugated amino acids were separated from the peptide and converted to the phenylthiohydantoin (PTH) derivative by acid hydrolysis. PTH-labeled amino acids were then determined using a Jasco 800 series amino acid analyzer equipped with a TSK gel, ODS-80TM column.

RESULTS

Pituitaries (2220) had a wet weight of 1.538 g. The material contained some blood and brain tissue, since this is practically unavoidable for this species. After chromatography on Sephadex G-100 and desalting, the peptide fraction contained 4.26 mg. Desalted peptides were chromatographed by CM-HPLC and 14 fractions were isolated (Fig. 1B). The unretarded material was then separated into 10 fractions by DEAE-HPLC (data not shown). All peaks from CM and DEAE were rechromatographed on RP-HPLC resulting in 54 major peaks (data not shown). Selection of major peaks was based on absorbance at 220 nm such that only fractions with OD greater than 0.45 were selected for further analysis. Seventy-four minor peaks were collected (OD 0.15 to 0.45) but were not analyzed in detail.

The pellet from the original extract was reextracted in acid acetone and chromatographed as above. No significant peptides were detected in this second extract (data not shown).

Amino acid composition analysis of peptides resulted in the identification of a vasotocin-like fraction. Figure 1 shows the

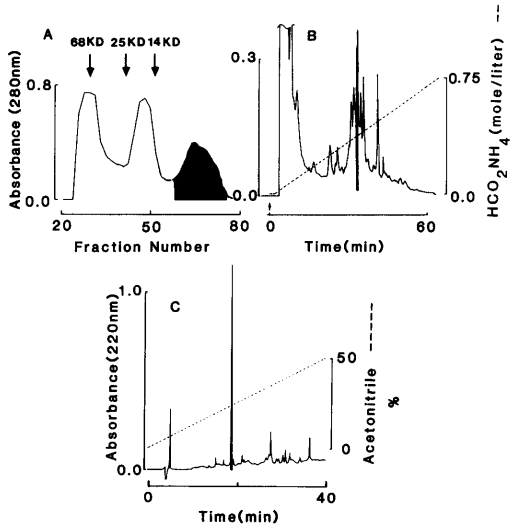

FIG. 1. Chromatographic isolation of lamprey AVT. (A) Sephadex G-100 fractionation of the crude extract. (B) CM-HPLC fractionation of the peptide peak. (C) RP-HPLC of the AVT fraction from CM-HPLC. Chromatographic methods as described in the text. Shaded areas represent AVT containing fractions as identified by source of the final isolate. Large arrows, elution position of molecular weight standards; small arrows, time of injection.

chromatographic isolation of lamprey AVT; including the G-100 chromatogram (Fig. 1A), CM chromatogram (Fig. 1B), and RP-HPLC of the AVT containing fraction (Fig. 1C). The amino acid composition of this fraction is presented in Table 1. Tyrosine residues are underrepresented because they are destroyed by acid hydrolysis.

Dansylation of the intact peptide resulted in no detectable amino terminus as expected for an N-terminal half cystine. A strong oxy-tyrosine signal was detected, confirming the amino acid composition assignment of tyrosine and assuring that a suitable amount of peptide was used.

Amino acid sequencing demonstrated a sequence: X–Tyr–Ile–Gln–Asn–X–Pro–Arg–Gly–NH_2 (data not shown). Half cystines were assigned to null (X) positions from the amino acid composition results. N-terminal cysteine is consistent with the results of N-terminal analysis of the intact

TABLE 1
AMINO ACID COMPOSITIONS OF LAMPREY AND CHUM SALMON ARGININE VASOTOCIN

	Lamprey AVT[a]	Salmon AVT[a]	AVT[d]
½ Cys[b]	1.6 (2)	1.6 (2)	2
Asp	1.2 (1)	1.2 (1)	1
Glu	1.2 (1)	1.0 (1)	1
Gly	1.2 (1)	1.2 (1)	1
Arg	1.1 (1)	0.6 (1)	1
Pro	1.4 (1)	0.8 (1)	1
Tyr	0.5 (1)[c]	0.1 (1)[c]	1
Ile	0.6 (1)	1.0 (1)	1
Total	8.8 (9)	7.5 (9)	9

[a] Means from two separate composition determinations using acid hydrolyzed peptides.
[b] Determined after performic acid oxidation.
[c] Verified by the recovery of *o*-Tyr after dansylation.
[d] Composition of AVT (Acher, 1974).

peptide and from the fact that no modification was required for sequencing, i.e., no pyro-glutamic acid, etc. RP-HPLC elution times of the native lamprey peptide was compared with AVT from chum salmon (*O. keta*) (Fig. 2). The purity and identity of the chum AVT were checked by RP-HPLC and amino acid composition (Table 1). No difference is seen in elution times, and a mixture of the two samples resulted in a single sharp peak and increased peak height (Fig. 2C).

Quantitative amino acid analysis of an aliquot of the original sample allowed the estimation of yield. Taking glycine as the standard, we estimated 21.06 nmol of AVT in the purified fraction. This estimate does not include losses during isolation and so may underestimate the actual gland content.

Neutral Nonapeptides

A study of amino acid compositions of peptides eluting in the unretarded peaks from both the CM- and DEAE-ion-exchange columns did not reveal any other homologous nonapeptide (data not shown).

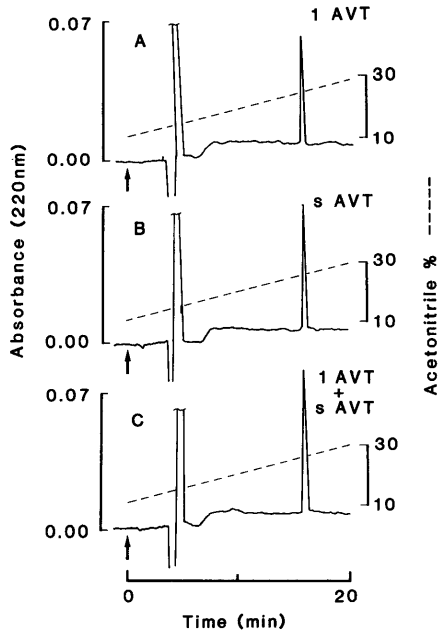

FIG. 2. Comparison of the RP-HPLC elution positions of lamprey AVT (A), Chum salmon AVT (B), and a 1:1 mix of lamprey and salmon preparations (C). RP-HPLC under identical conditions, as described in the text. Arrows, time of injection.

DISCUSSION

We have demonstrated the presence of AVT in the sea lamprey pituitary and thus confirmed previous bioassay and immunocytochemical localization in lampreys (Sawyer *et al.*, 1961; Follett and Heller, 1964; Sawyer, 1965; Rurak, and Perks, 1976, 1977; Goosens *et al.*, 1977). This work extends these earlier studies considerably by proving the structure of this peptide to be Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Arg–Gly-NH_2. Furthermore, we were able to estimate the pituitary content of AVT in adult sea lamprey during their spring upstream migration. We recovered roughly 21 nmol of AVT from 2220 pituitary glands. Since this calculation does not include loses during extraction, a mean of at least 9.6 pmol per gland (13.7 nmol/g wet wt) is calculated. This estimate can not be related to sex or body weight since the animals were both male and female and body weights ranged considerably.

Earlier attempts to isolate neurohypophyseal nonapeptide hormones from lamprey resulted in the tentative identification of arginine vasotocin based on the chromatographic behavior of bioactive substance (Sawyer *et al.*, 1961; Follett and Heller, 1964; Sawyer, 1965). Oxytocin-related peptides have generally not been identified in these extracts by bioassay or immunohistochemistry (Goosens *et al.*, 1977). A survey of major neutral peptides in our extract produced no evidence for peptides with homology to the neurohypophyseal nonapeptide family. This tends to support previous studies which have found no immunoreactivity with antisera generated against oxytocin, isotocin, or their homologs. While there are reports of oxytocic activity from lamprey pituitary extracts (Lanzing, 1954), it has so far been impossible to separate this activity from the AVT activity.

The common interpretation of this data is that cyclostomes represent a class of vertebrates that diverged before the gene duplication event which gave rise to multiple neurohypophyseal nonapeptides (reviewed by Acher, 1974). However, none of these studies can address the distinct possibility that another neurohypophyseal nonapeptide once existed, currently exists in very low concentrations, or exists in an unrecognized state, in the lamprey lineage. The gene for such a peptide may have been lost or undergone extensive modification over the 500 million years since the divergence of the cyclostome lineage.

In vivo actions of pituitary hormones in lampreys may be different from those in higher vertebrates. Removal of the pituitary gland does not markedly affect interrenal steroidogenesis, blood sugar levels, or thyroid function (reviewed by Hardisty 1979; Hardisty and Baker, 1982). Administration of exogenous AVT is reported to cause slight hyperglycemia and to elevate muscle glycogen of the lamprey *L. fluviatilis* (Bentley and Follett, 1965). AVT admin-

istration also acts as a vasoconstrictor in hagfish but the doses required may be pharmacological.

The neurohypophyseal nonapeptide family is classically referred to in discussions of water balance, blood pressure regulation, oviduct/uterine motility, and milk ejection of vertebrates (Bentley, 1980). The presence of at least two distinct peptides in gnathostome vertebrates can often be related to a partial separation of the hormonal pathways that regulate these reproductive and vasoactive functions. Sawyer has suggested that the vasopressor activity may be the original action of the family. In recent years the demonstration of several additional roles has forced a reinterpretation of the actions that these peptides play *in vivo*. In mammals, arginine vasopressin (AVP) also has thyrotropin-releasing activity equal to TRH (Lumpkin *et al.*, 1987) and behavioral effects (Koob and Bloom, 1982). In amphibians, AVT may be important in reproductive behavior (Moore and Miller, 1983) and, in teleost fish, AVT is reported to stimulate gonadotrophic cells in the pars distalis (Groves and Batten, 1986). Studies addressing these other effects have required sophisticated assay systems that are largely not applicable to species such as lamprey. Since appropriate information on other actions is not available in lower vertebrates, it is necessary to proceed cautiously with interpolation of the ancestral condition.

This paper confirms that AVT is present in all vertebrate classes. In light of the many bioactivities observed, it may be interesting to reexamine which of the activities is the ancestral function and to address the evolution of functions with a view toward the biology of particular groups. The work presented here proves the validity of applying AVT immunoassays to the study of this peptide in lampreys. Measurement of circulating levels and responses to experimental AVT administration are thus feasable and appropriate.

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