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AMINO ACID SEQUENCE AND RELATIVE BIOLOGICAL ACTIVITY OF EEL ATRIAL NATRIURETIC PEPTIDE

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Summary: A peptide exhibiting vasodepressor and natriuretic activities in rats was isolated from eel atria, and its primary structure was determined as H-Ser-Lys-Ser-Ser-Ser-Pro-Cys-Phe-Gly-Gly-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Tyr-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Arg-Lys-OH. This peptide, termed eel atrial natriuretic peptide (ANP), has sequence homology of 59% to mammalian (human or rat) ANP, 52% to fowl ANP, and 46% to frog ANP. When the biological activity of synthetic eel ANP was compared with that of human ANP, the eel peptide was 110 times more potent for the vasodepressor activity in eels, nearly equipotent for the vasodepressor activity in quails, and 20 times less potent for the vasodepressor and natriuretic activity in rats.

The cardiac atria, which are thought to function as a sensor for volume homeostasis, have recently been shown to contain a hormone that may regulate water and electrolyte balance and blood pressure (1). This hormone, atrial natriuretic peptide (ANP), which is also known by other names, was soon sequenced in several species of mammals (2-7). The molecular structure of ANP from these animals are well-conserved; only one amino acid difference is noted in the circulating α-form. Histochemical studies also suggested the presence of immunoreactive ANP in several nonmammalian species (8, 9). So far, nonmammalian ANPs have been sequenced in fowl (10) and frogs (11, 12); their amino acid sequences are less homologous to mammalian peptides, in contrast to the high homologies among the mammalian ANPs. The biological activities of these nonmammalian ANPs in the homologous animals have not been examined yet.

Eels are euryhaline species that can survive in both fresh water and sea water. Thus, if ANP is actually involved in osmoregulation, it may play an important role in this species. Recently, we have demonstrated the presence of immunologically and biologically active ANP in eel heart and plasma (13). However, the molecular structure of eel ANP may be different from the human peptide because of its weak affinity to antibodies to human ANP (hANP). Thus,

Abbreviations: ANP, atrial natriuretic peptide; CM, S-carboxymethylated; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin-derivatized.

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it is essential to use native eel ANP for physiological studies on ANP from eels and to measure eel ANP by the homologous radioimmunoassay system. As a first step towards this goal, we report here the primary structure of eel ANP and its relative biological activity in homologous eels and heterologous animals, quails and rats.

MATERIALS AND METHODS

The hearts of cultured Japanese eels, Anguilla japonica, were isolated immediately after decapitation and placed on ice. The still-beating hearts were then divided into two parts, the atrium and ventricle, and frozen immediately on dry ice. The sinus venosus was attached to the atrium, and the bulbus arteriosus was discarded. The hearts were collected from 1690 eels.

Tissue Extraction and Purification: ANP was extracted only from atria (31.8 g) by a modified method based on that used by Kangawa and Matsuo (3). The frozen tissues were pulverized in a crusher and immediately boiled in 7 parts of distilled water for 5 min. After quick cooling, AcOH was added to a final concentration of 1 M. The mixture was then homogenized with a Polytron mixer for 3 min and then centrifuged at 25,000 x g for 30 min. The supernatant was mixed with cold acetone at a final concentration of 67%. The precipitate was removed after centrifugation at 16,000 x g for 30 min, and the supernatant was evaporated in vacuo to dryness. The dried material was dissolved in 30 ml of 1M AcOH, added to 2 liters of cold acetone, and left at 4°C overnight. After centrifugation at 16,000 x g for 30 min, the pellet was dried in vacuo and weighed (305 mg). The pellet was reconstituted in 39 ml of 1M AcOH, and applied onto a column of Sephadex G-25 fine (5 x 82 cm) and eluted with 1 M AcOH at a rate of 70 ml/hr. An aliquot of each fraction (6.5 ml) was assayed for the relaxant activity in the chick rectum (14). Bioactive fractions were pooled and applied onto a column of SP-Sephadex C-25 (1.6 x 17 cm) equilibrated with 1M AcOH. Successive elutions with 150 ml of 1M AcOH, 2M pyridine, and 2M pyridine-AcOH (pH 5.0), respectively, afforded 3 fractions: SP-I, SP-II and SP-III. After lyophilization, SP-III (60 mg), which showed the greatest bioactivity among the 3 fractions, was dissolved in 30 ml of 1M AcOH, applied onto a column of Sephadex G-75 fine (2.6 x 71 cm), and eluted with 1M AcOH at a rate of 36 ml/hr. An aliquot of each fraction (5 ml) was bioassayed, and active fractions were subjected to cation exchange high performance liquid chromatography (HPLC) on a column of Biofine IEC-CM (7.5 x 75 mm; Jasco). Each bioactive fraction was purified by reverse-phase HPLC on a column of Asahipak Gel ODP-50 (4.6 x 250 mm; Asahi Chemical Industry Co. Ltd.), and the bioactive peak was re-chromatographed on the same column. The column effluents of gel filtration chromatography and HPLC were monitored by absorbance at 280 or 220 nm.

Sequence Analysis: Purified eel ANP was subjected to reduction and S-carboxymethylation (15). The carboxymethylated eel ANP was purified by reverse-phase HPLC, and its amino acid composition was determined in an amino acid analyzer (Waters) after hydrolysis of the peptide (3). Sequence analysis of the ANP was performed by step-wise Edman degradation using a gas phase automated protein sequencer coupled with HPLC identification of the resulting PTH-amino acids (Applied Biosystems, Model 470A/120A).

Synthesis of Eel ANP: The eel ANP was synthesized by a peptide synthesizer (Applied Biosystems, Model 430A) with phenylacetamidomethyl-resin as a solid support (16). The correct sequence was confirmed by amino acid analysis and by co-eluting the synthetic and natural peptides in reverse-phase HPLC.

Relative Bioactivity of Synthetic Eel ANP: Cultured eels, Anguilla japonica, were purchased from a local dealer. After acclimation to laboratory conditions

for 1 wk, the eels were cannulated in the ventral aorta for injection of ANP and in the dorsal aorta for measurement of blood pressure as described previously (17). Eels weighed 205 ± 5 g (n=8) at the time of the experiment. Cultured male quails, Coturnix coturnix japonica, were purchased from a local dealer. After acclimation, the birds were cannulated in the external jugular vein for injections and in the common carotid artery for measurement of blood pressure (18). They weighed 107 ± 7 g (n=4) at the time of the experiment. Male Sprague-Dawley rats were purchased from a commercial source. After acclimation, they were cannulated in the urinary bladder for urine collection, in the femoral artery for measurement of blood pressure, and in the femoral vein for injection of ANP and continuous infusion of a Ringer solution (19). They weighed 271 ± 3 g (n=6) at the time of the experiment. Sodium and potassium concentrations were determined by an atomic absorption spectrometer (Hitachi), and chloride concentration was determined by a Digital Chloridometer (Haake-Buchler). The potency ratio (eel ANP/α-hANP) was estimated for each animal after parallelism of the dose-response curve was confirmed (18). All results were expressed as means ± SEM. Statistical significance was determined at $p<0.05$ by the paired t-test.

RESULTS

The recovery of ANP after Sephadex G-25 chromatography, as monitored by relaxant activity in the chick rectum, was equivalent to 311 μg of α-hANP. After ion-exchange chromatography on a SP-Sephadex C-25 column, the total α-hANP activity in SP-III was 152 μg. Sephadex G-75 chromatography of SP-III revealed that the bioactivity eluted near the position of α-hANP. Cation-exchange HPLC of these fractions (38 μg equivalent to α-hANP) resulted in bioactive peaks at fractions 21-23 (Fig. 1). These 3 fractions were separately subjected to reverse-phase HPLC. The elution pattern for the chromatography of fraction 23 is shown in Fig. 2A. The fraction that exhibited greatest

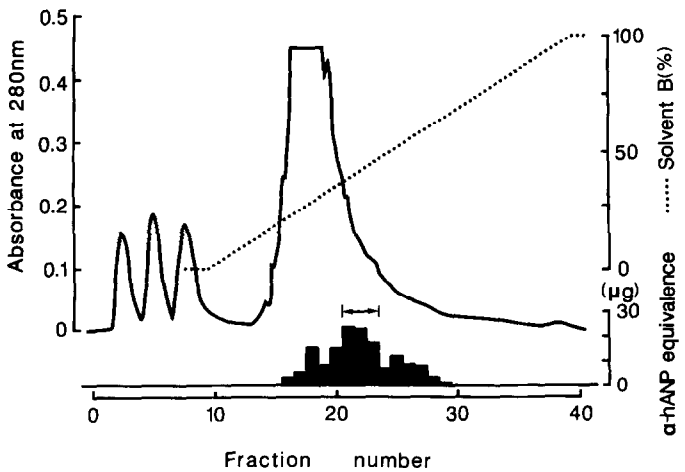


Figure 1 Cation-exchange HPLC on a Biofine IEC-CM column (7.5 x 75 mm, Jasco) of bioactive fractions obtained after Sephadex G-75 chromatography. Linear-gradient elution was performed from solution A (10 mM NH_4OAc, pH 6.8 : CH_3CN = 9 : 1) to B (1M NH_4OAc, pH 6.8 : CH_3CN = 9 : 1) for 60 min. Flow rate was 1 ml/min. Fraction size was 2 ml/tube. The ANP activity was expressed as μg equivalent to α-hANP as assayed by relaxant activity in the chick rectum. The respective fractions marked by an arrow were further subjected to reverse-phase HPLC.

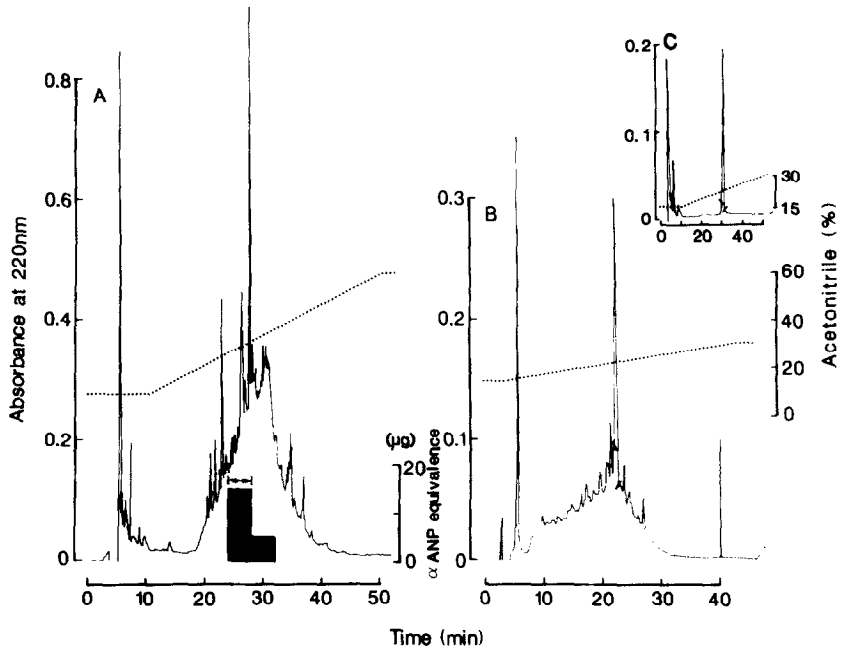
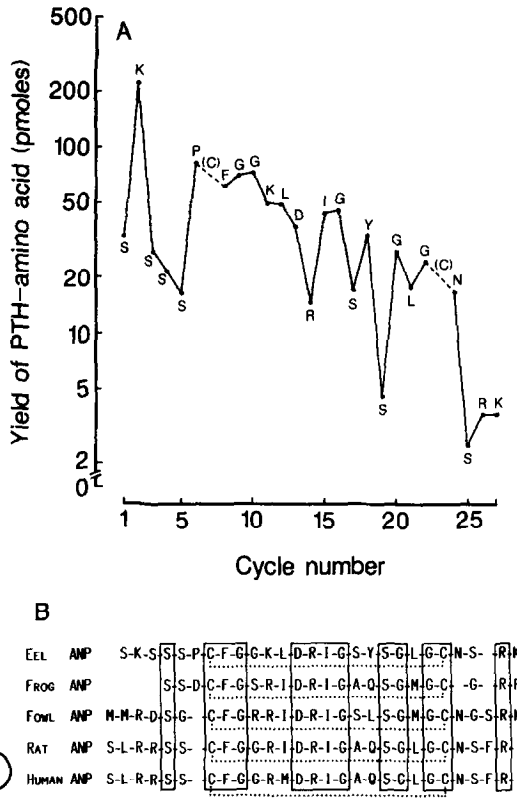


Figure 2 Reverse-phase HPLC on an Asahipak Gel ODP-50 column (4.6 x 250 mm, Asahi Chemical Co. Ltd.). Linear-gradient elution was performed from 10% to 60% or 15% to 30% of acetonitrile concentration in 0.1% trifluoroacetic acid for 40 min. Flow rate was 1 ml/min. A) Fraction 23 obtained after cation-exchange HPLC. B) Bioactive fraction marked in panel A by an arrow. C) Fraction marked in panel B by 2 nicks after reduction and S-carboxymethylation. The peak between 2 nicks in panel C was further processed by amino acid analyses and sequencing. The ANP activity was expressed as μg equivalent to α-hANP as assayed by relaxant activity in the chick rectum.

bioactivity was re-chromatographed (Fig. 2B), and the substance in the major peak was S-carboxymethylated (CM) and desalted (Fig. 2C). The amino acid analysis of this substance (final yield was 5.1 μg equivalent to α-hANP determined by the absorbance at 220 nm) indicated that it was composed of 27 amino acid residues: Asp, 2.0(2); Ser, 6.0(7); Gly, 5.4(5); CM-Cys, +(2); Arg, 2.0(2); Pro, 1.0(1); Tyr, 1.0(1); Ile, 1.0(1); Leu, 2.0 (2); Phe, 1.1(1); Lys, 2.5(3). The complete amino acid sequence of this peptide (eel ANP-(1-27)) was successfully determined as shown in Figure 3. Fractions 21 and 22 were processed in the same manner (data not shown), and eel ANP-(5-27) and (3-27) were finally isolated from the respective fractions. Final yields of eel ANP-(5-27) and (3-27) were 2.4 and 3.0 μg equivalent to α-hANP, respectively. No significant ANP-like peptides were recovered from the other fractions. Reverse-phase HPLC of the natural and synthetic peptides resulted in an identical retention time, which confirmed that the sequence was correct.

A bolus injection of synthetic eel ANP decreased the aortic pressure of conscious eels in a dose-dependent manner (Fig. 4). Eels responded to ANP concentrations as low as 0.05 nmole/kg or less. However, eels were much less sensitive to α-hANP (Fig. 4). The potency ratio was 110 ± 33 (n=8). A



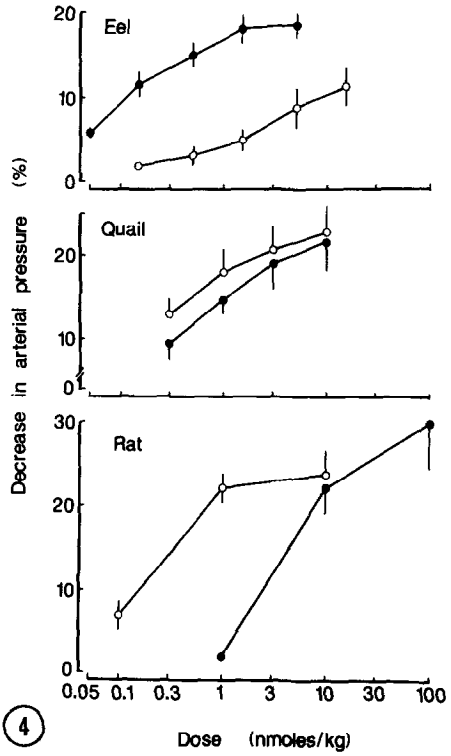


Figure 3 A) Yield of phenylthiohydantoin-derivatized (PTH) amino acid at each cycle of the Edman degradation of S-carboxymethylated eel ANP (Fig. 2C). B) Complete amino acid sequences (single letter code) of eel, frog (Rana catesbeiana) (11), fowl (10), rat (2), and human (3) ANP. Identical amino acid residues across all species are boxed. Broken lines indicate disulfide linkages of cystine residues.

Figure 4 Dose-response relationships for the vasodepressor effect of eel (●) and human (○) ANP in eels (n=8), quails (n=4), and rats (n=6). Numbers of rats examined at 0.1 nmole/kg of α-hANP and 1 nmoles/kg of eel ANP are 5 and 4, respectively. Decreases after injection of 0.9% NaCl were -1.0 ± 0.7% (n=8) for eels and -2.2 ± 0.8% (n=4) for quails. Injection of 0.9% NaCl was not performed in rats. All decreases in arterial pressure after injection of ANP were significant ($p<0.05$) in eels and quail when compared with the changes after injection of 0.9% NaCl.

comparable dose-dependent decrease in arterial pressure was observed in the quail after bolus injections of eel ANP and α-hANP (Fig. 4); the potency ratio was 0.53 ± 0.09 (n=4). In contrast to the results in eels, eel ANP was less effective than α-hANP in rats. Significant hypotension, natriuresis and diuresis were observed in rats at 1 nmole/kg of α-hANP, whereas the comparable effect was observed at 10 nmoles/kg or more of eel ANP (Fig. 4, Table 1). Thus, the potency ratio appears to be approximately 0.05, although the value could not be calculated by the method described above. Both α-hANP and eel ANP induced chloriduresis, but they were rather antikaliuretic at high doses (data not shown). Another interesting feature of eel ANP is that its effect continued much longer than that of α-hANP in all animals tested. In the eel,

Table 1. Changes in urine volume and Na excretion after intravenous injection of synthetic human and eel ANP in anesthetized rats (n=6)

ANP (nmole)	Time after injection of ANP (min)							
	Urine volume (μl/min)				Na excretion (μEq/min)			
	-10-0	0-10	10-20	20-30	-10-0	0-10	10-20	20-30
Human								
0.1	6.2 ± 0.3	6.9 ± 0.2	6.4 ± 0.3	5.8 ± 0.5	0.11 ± 0.06	0.13 ± 0.05	0.11 ± 0.05	0.10 ± 0.05
1	4.5 ± 0.4	8.5* ± 0.7	4.8 ± 0.4	4.7 ± 0.5	0.08 ± 0.04	0.29* ± 0.07	0.09 ± 0.04	0.07 ± 0.05
10	5.6 ± 0.6	15.9* ± 2.0	6.7 ± 0.8	6.1 ± 0.6	0.11 ± 0.06	1.77* ± 0.52	0.39* ± 0.13	0.22 ± 0.10
Eel								
10	6.3 ± 0.5	7.1 ± 0.7	6.3 ± 0.1	5.5 ± 0.4	0.08 ± 0.03	0.15* ± 0.04	0.11 ± 0.04	0.08 ± 0.02
100	6.1 ± 0.5	14.4* ± 1.5	6.9 ± 0.9	5.5 ± 0.3	0.09 ± 0.05	1.20* ± 0.37	0.37* ± 0.13	0.14 ± 0.06

*$p<0.05$
Values are means ± SEM.

e.g., the vasodepressor effect of 0.15 nmole/kg of eel ANP lasted for 10.5 ± 2.2 min, whereas the comparable effect produced by 15 nmoles/kg of α-hANP lasted for 6.7 ± 3.3 min (n=8 in each case).

DISCUSSION

We have purified and sequenced an eel atrial peptide with 27 amino acid residues, which causes natriuresis/diuresis in rats as do other mammalian and nonmammalian ANPs so far identified (2-7, 10-12). This is the first report on the amino acid sequence of fish ANP. This eel peptide is structurally similar to the other ANPs; it has an intramolecular ring structure with 17 amino acid residues formed by a disulfide bond, and the identical sequences conserved in all ANPs so far identified are also conserved in this peptide except for the replacement of the 11th Arg with Lys (Fig. 3). The COOH-terminus ends with Lys, which is also the case for the other two minor eel ANPs with truncated NH_2-termini. Thus, it is likely that eel ANP-(1-27) corresponds to an α-form, and the other 2 peptides may be produced during the purification processes. In mammals, α-ANP is a circulating form, but it has not been determined if this eel ANP-(1-27) is circulating in eel blood.

The biological activity of eel ANP relative to α-hANP differed greatly in different species. Eel ANP is 110 times more potent than α-hANP in eels, nearly equipotent in quails, and 20 times less potent in rats. For eels, α-hANP has a sequence homology of 59% to the native peptide. For rats, the homology of eel ANP to the native peptide is 59%, and that of α-hANP is 96%.

Thus, the activity of ANP in different animals appears to be closely related to the structural similarity to the native peptide. Quail ANP has not been sequenced yet, but if it is supposed to be identical to fowl ANP, both eel ANP and α-hANP have sequence homology of 52% to the quail peptide. Quail and fowl are taxonomically close (in the same order), and we recently found that the sequences of fowl and quail angiotensin I are identical (20).

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