Volume 282, number 2, 317–320 FEBS 09681 May 1991

ADONIS 001457939100390O

A novel natriuretic peptide isolated from eel cardiac ventricles

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Received 8 February 1991; revised version received 1 March 1991

A new natriuretic peptide, which exhibits the entire spectrum of actions known to be characteristic of atrial and brain natriuretic peptides (ANP and BNP), was isolated from eel cardiac ventricles and has been named ventricular natriuretic peptide (VNP). The primary structure of eel VNP is characterized by its uniquely long C-terminal 'tail' that extends from the second half-cystine. Thus, eel VNP appears to be a novel natriuretic peptide of a type not found in mammals. With respect to natriuretic (rat) and vasodepressor (rat and eel) activities, eel VNP is much more potent than human ANP in eels and almost equipotent in rats. Strong tachyphylaxis is observed for the vasodepressor effect in both rats and eels, whereas it is not observed for the natriuretic effect in rats.

Atrial natriuretic peptide; Cardiac ventricle; Natriuresis; Hypotension; Tachyphylaxis; Eel; *Anguilla japonica*

1. INTRODUCTION

Euryhaline fish are capable of osmoregulation by changing their drinking rate, rate of intestinal absorption, gill permeability, and rate of renal excretion, of water and electrolytes [1]. Among the euryhaline species, eels are one of the few that can survive a direct transfer from fresh water to seawater or vice versa. The ways that they cope with the drastic changes in environmental osmotic pressure have been studied in some detail, and data are accumulating that implicate ANP in fish osmoregulation [2–5]. However, mammalian ANP has been used in such studies in fishes, and immunoreactive fish ANP has been measured only by heterologous radioimmunoassays (RIA) for mammalian ANP [6].

We previously reported that mammalian ANP had little biological effect in eels, and that an RIA for human ANP did not measure levels of eel ANP correctly [7]. Therefore, we isolated eel ANP from atria, determined its amino acid sequence [8], and established a specific RIA for eel ANP [9]. The homologous RIA measured 700-fold higher values for ANP levels in eel atria than did the heterologous RIA for human ANP. Unexpectedly, however, levels of ANP in eel cardiac ventricles were only a few-fold different between the 2 measurements (unpublished data). Thus, it is likely that a different type of ANP is present in the eel ventricle, as was the case in the brain [10].

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2. MATERIALS AND METHODS

The hearts of cultured Japanese eels, *Anguilla japonica*, were isolated immediately after decapitation and placed on ice. The ventricles were isolated from the still-beating hearts, and frozen immediately on dry ice. They were stored at −25°C until use.

2.1. *Extraction and purification*

The ANP-like peptide, later named eel ventricular natriuretic peptide (VNP), was extracted from 2500 eel cardiac ventricles (225 g) as reported previously [8]. In brief, the pulverized frozen tissues were boiled in water, acidified with AcOH, homogenized, and centrifuged. The supernatant was treated with 67% and 98.5% acetone to remove high-molecular-weight proteins and lipids, respectively. The crude extract was subjected to gel-filtration chromatography on a column of Sephadex G-25 (Fig. 1a), cation-exchange chromatography on a column of SP-Sephadex C-25 (1.6 × 15 cm), cation-exchange HPLC on an IEC-CM column (Fig. 1b), and reverse-phase HPLC on an ODS-120T column (Fig. 1c). The activity of VNP was assayed at each step by measuring relaxant activity in the chick rectum [11]. Semi-purified material was reduced and *S*-carboxymethylated, as described previously [8], and was finally purified by reverse-phase HPCL (Fig. 1d).

2.2. *Analysis of amino acid sequence*

The amino acid sequence of the purified and carboxymethylated eel VNP was determined with an automated gas-phase protein sequencer (Applied Biosystems, Model 470A/120A). The correct sequence was verified by amino acid analysis with a PICO TAG Work Station (Waters), and by co-chromatography of the purified and synthetic peptide by reverse-phase HPLC with 2 different solvent systems [10]. The eel VNP was synthesized with a peptide synthesizer (Applied Biosystems, Model 430A), and its amino acid sequence was confirmed by the amino acid analyzer, sequencer and mass spectrometer [10].

2.3. *Relative bioactivity of eel VNP*

Procedures for the measurement of arterial pressure in eels, and arterial pressure and renal sodium excretion in rats have been described previously [8]. Eels (199 ± 7 g, $n=8$) were anesthetized and the ventral and dorsal aortae were cannulated for injections and measurements of blood pressure, respectively. Since eels became

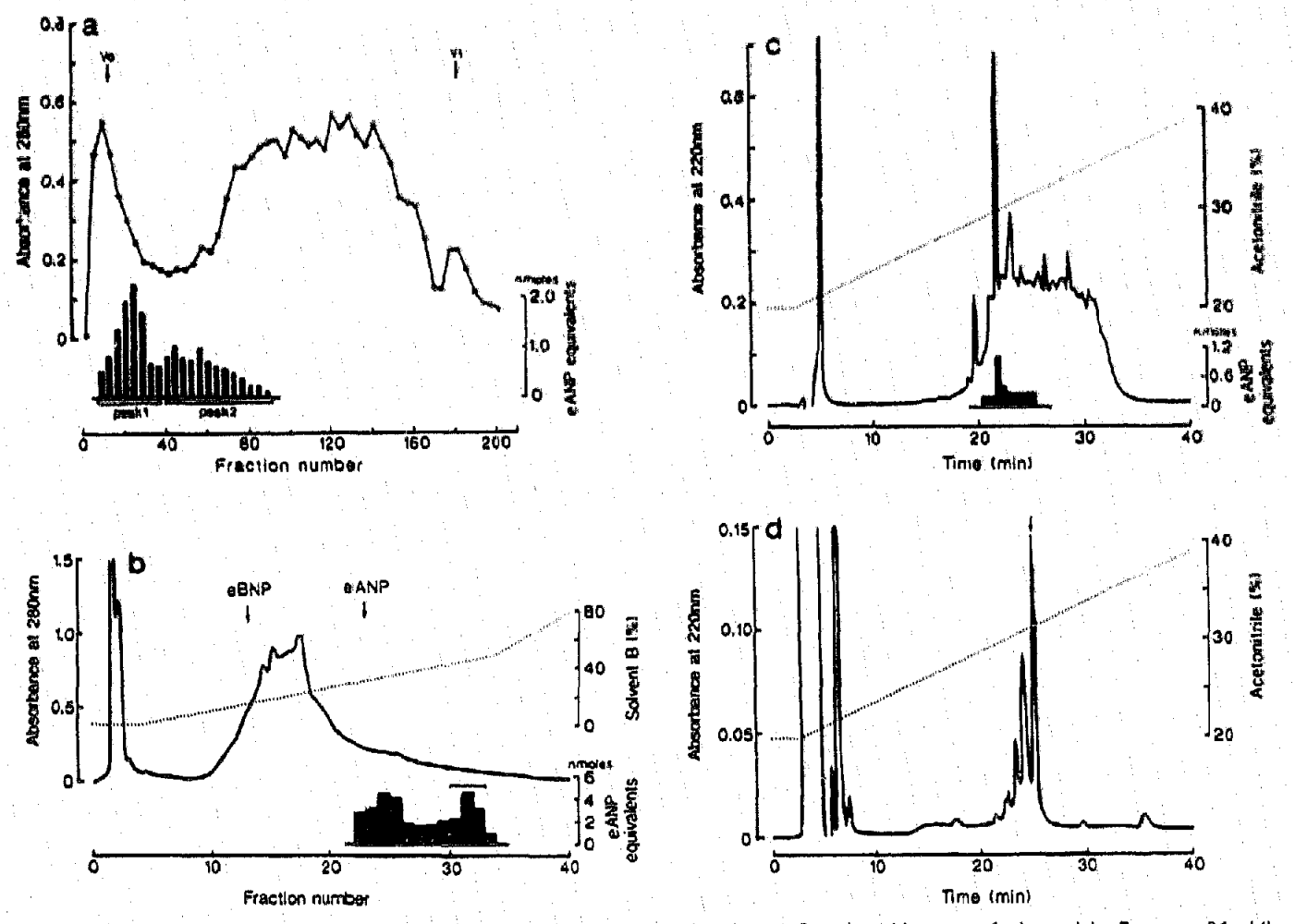

Fig. 1. (a) Gel-filtration chromatography on a column of Sephadex G-25 fine (5 × 82 cm). Sample, acid extract of eel ventricle; flow rate, 96 ml/h; eluent, 1 M AcOH; fraction size, 6.5 ml/tube; V_0, void volume; V_t, total bed volume. Black bars represent activities equivalent to eel ANP in the chick-rectum relaxant assay. Eel VNP was recovered from the peak 1. (b) Cation-exchange high-performance liquid chromatography (HPLC) on an IEC-CM column (7.5 × 75 mm, Jasco, Tokyo). Sample, a fraction eluted with pyridine-AcOH from a column of SP-Sephadex C-25; flow rate, 1 ml/min; solvent system, linear-gradient elution from solvent A (10 mM NH_4OAc, pH 6.8, plus CH_3CN at a ratio of 9:1) to solvent B (1 M NH_4OAc, pH 6.8, plus CH_3CN at a ratio of 9:1); fraction size, 2 ml/tube. Each bioactive fraction was subjected to reverse-phase HPLC on a ODS-120T column (4.6 × 250 mm, Tosoh, Tokyo). Eel VNP was recovered from the marked fractions. (c) Reverse-phase HPLC on an ODS-120T column. Sample, bioactive fractions obtained from the first reverse-phase HPLC of the marked fractions in (b); flow rate, 1 ml/min; solvent system, linear-gradient elution from CH_3CN at a concentration of 20% to 40% in 0.1% trifluoroacetic acid for 40 min. (d) Reverse-phase HPLC on an ODS-120T column. Sample, carboxymethylated materials in a bioactive fraction in (c). Other conditions are identical to those described in (c). Fractions are collected by each peak. An arrow shows the peak of carboxymethylated VNP.

unresponsive after the injection of high doses of eel VNP because of tachyphylaxis, the injections were started from the lowest dose, and intervals between injections of more than 1 h were introduced at high doses. Sprague-Dawley rats (286 ± 3 g, $n = 7$) were anesthetized and the femoral vein was cannulated for injections and continuous infusions of a Ringer solution; the femoral artery was cannulated for measurement of blood pressure; and the urinary bladder was cannulated for collection of urine. Urine volume, and urinary concentrations of sodium, potassium and chloride were measured.

3. RESULTS AND DISCUSSION

We have isolated, from eel cardiac ventricles, a highly basic peptide that exhibits a relaxant activity in the chick rectum after reduction and *S*-carboxymethylation of the semi-purified peptide (Fig. 1). The yield was 8 μg equivalent to eel ANP by absorbance at 220 nm. One half and one fifth of the carboxymethylated peptide were used, respectively, for the sequence analysis and amino acid analysis. The remainder was used for the purity check and comparison of the sequence with the synthetic peptide. The complete amino acid sequence of the carboxymethylated peptide was determined by the sequencer (Fig. 2). The result of amino acid analysis supports the sequence: Asp, 4.7 (5); CM-Cys, 2.4 (2); Ser, 4.6 (5); Gly, 6.9 (6); Arg, 2.1 (2); Thr, 2.0 (2); Met, 0.9 (1); Ile, 1.9 (2); Leu, 2.2 (2); Phe, 2.5 (3); Lys, 5.0 (5); Trp, undetectable (1). The values in perentheses are those obtained by the sequence analysis. Thus, this peptide consists of 36 amino acid

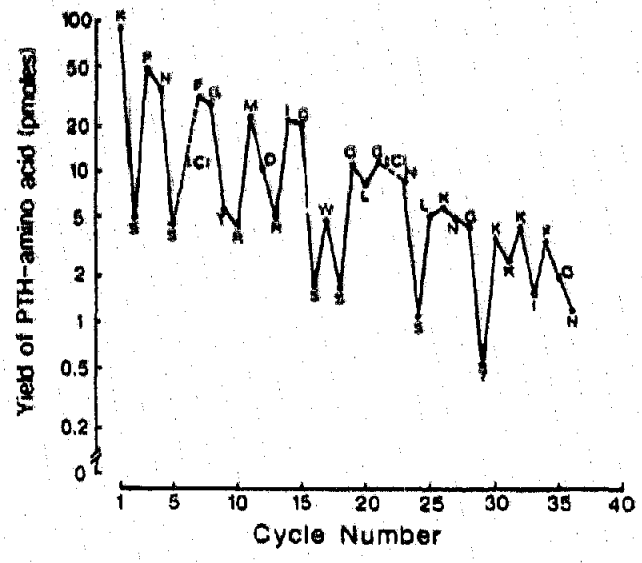

Fig. 2. Yield of phenylthiohydantoin-derivatized (PTH) amino acid at each cycle of Edman degradation of the *S*-carboxymethylated peptide isolated from eel cardiac ventricles. Amino acids are denoted by the single-letter code.

residues, including a tryptophane residue. The sequence thus deduced was finally confirmed by co-chromatography of the isolated and synthetic peptides in reverse-phase HPLC with different solvent systems. Since this peptide is a new type of natriuretic peptide which has long C-terminal 'tail' sequence (Fig. 3), and since this peptide was isolated from eel ventricles, we have named it venticular natriuretic peptide (VNP).

Eel VNP caused hypotension in conscious eels and natriuresis and hypotension in anesthetized rats (Fig. 4). In the homologous eel, eel VNP is, like eel ANP and BNP, 100-fold more potent than human ANP. Unlike the case with eel ANP and BNP, however, strong tachyphylaxis was observed after injection of VNP, and this reaction lasted for several hours at high doses. Since the dose given at each injection was increased gradually, the effect became smaller at higher doses (Fig. 4a). Tachyphylaxis was also noted in association with the vasodepressor effect in rats after injection of VNP (data not shown). The natriuretic effect of eel VNP in rats was much stronger than the effects of eel ANP and BNP (Fig. 4b). Eel VNP also caused diuresis and chloridouresis, as well as antikaliuresis at high doses. It is noteworthy that tachyphylaxis was not observed in association with the natriuretic effect.

The natriuretic peptides identified to date have been classified into 3 groups based on structural similarities and sites of production (Fig. 3). All members of the first group are isolated from atria and each is called ANP [8,12]. The members of the second group are mostly called BNP because the first such peptide was isolated from the porcine brain [3], even though all the other peptides and their messenger RNAs of this group were isolated from the heart [14–17]. Fowl ANP isolated from the heart [18] and bovine aldosterone secretion inhibitory factor (ASIF), isolated from cultural adrenal chromaffin cells [19], also belong to this group. The members of the third group are all isolated from the brain and are called by various names. However, we shall call the members of this group C-type natriuretic peptide (CNP), because this name is used most frequently (Fig. 3).

Three species of natriuretic peptides are found in

Eel VNP **KSFNS-CFGTRMDRIGSWSGLGCNSL-KNGTKKKIFGN**

A-type Natriuretic Peptide

Eel ANP	SKS**SS**P**CFG**GK**LDRIG**SY**SG**L**GC**NS-**R**K
Bullfrog ANP	**SS**D**CFG**SR**IDRIG**AQ**SG**M**GC**-G-**R**RF
Human ANP	SLRR**SS**-**CFG**GRM**DRIG**AQ**SG**L**GC**NSF**R**-Y
Rat ANP	SLRR**SS**-**CFG**GRI**DRIG**AQ**SG**L**GC**NSF**R**-Y

B-type Natriuretic Peptide

Fowl ANP	**M**MRDSG-**CFG**RRI**DRI**GSL**SG**M**GC**NGS**R**KN
Porcine BNP	SPKT**M**-RDSG-**CFG**RRL**DRI**GSL**SG**L**GC**NVL**R**RY
Canine BNP	SPK-**M**MHKSG-**CFG**RRL**DRI**GSL**SG**L**GC**NVL**R**KY
Bovine ASIF*	PK-**M**MRDSG-**CFG**RRL**DRI**GSL**SG**L**GC**NVL**R**RY
Human BNP	SPK-**M**VQGSG-**CFG**RKM**DRI**SSS**SG**L**GC**KVL**R**RH
Rat BNP*	S-K-**M**AHSSS-**CFG**QKI**DRI**GAV**S**R**L**GC**DGL**R**LF

C-type Natriuretic Peptide

Eel BNP	**GWNRG-CFGLKLDRIGSL SGLGC**
Killifish brain ANP	**GWNRG-CFGLKLDRIGSM SGLGC**
Porcine CNP	**GLSKG-CFGLKLDRIGSM SGLGC**

Fig. 3. Comparison of amino acid (single-letter code) sequences of eel VNP and other natriuretic peptides sequenced to date. Identical amino acid residues within the same group are printed in bold face. *These peptides have longer N-terminal amino-acid regions [19,22]. Gaps have been introduced to optimize matching.

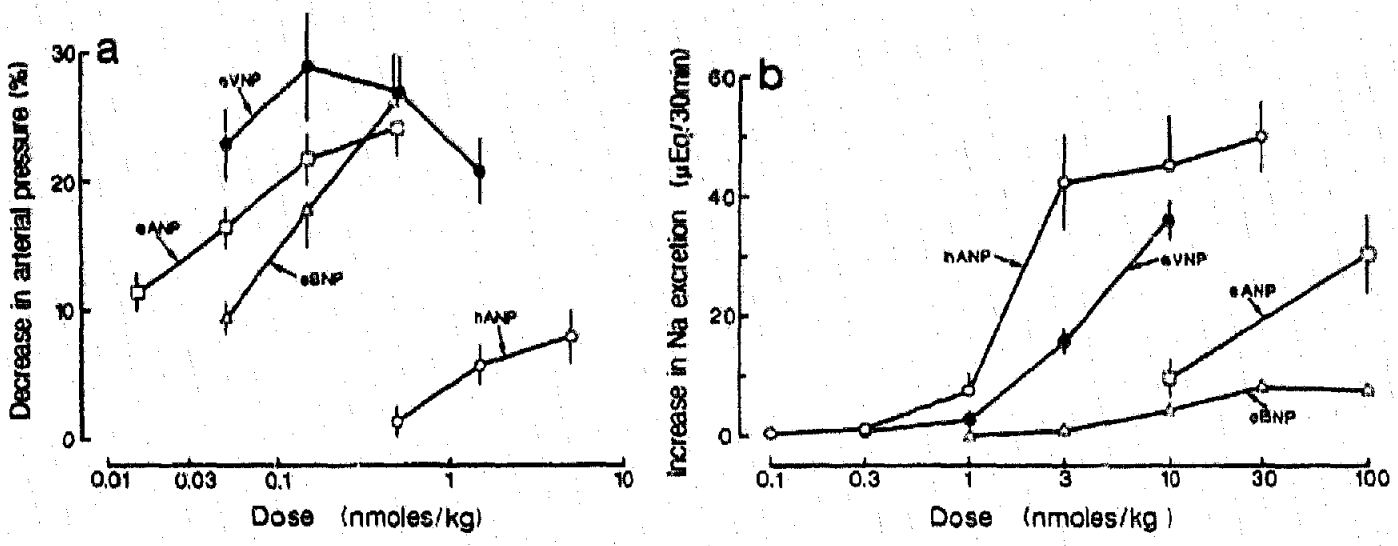

Fig. 4. Dose-response relationships for the vasodepressor effect in the eel (a) and for the natriuretic effect in the rat (b) of eel VNP (●), eel ANP (□), eel BNP (△), and human ANP (○). Eight eels and 6–8 rats were used in each assay. The data for eel ANP and BNP were obtained from the previous studies [8,10] after correction for the response to human ANP. Values are means ± SEM.

eels, and 2 of them (eel ANP, and eel BNP or CNP) are classified as belonging to the A-type and C-type natriuretic peptides (Fig. 3). Thus, it is possible that eel VNP belongs to the B-type natriuretic peptide. Comparing the amino acid sequences, however, no B-type natriuretic peptides have more than 6 amino acids at the C-terminus which extend from the second half-cystine. Furthermore, similarities in terms of amino acid sequences (present results) and of cDNA sequences (unpublished results) between eel VNP and the B-type natriuretic peptides are no greater than such similarities to the A-type and C-type natriuretic peptides. Thus, eel VNP may be a new type of natriuretic peptide. However, we have to identify B-type natriuretic peptide in the eel, and the fourth peptide of a VNP type in mammals, to verify this assumption.

Acknowledgements: We thank Dr. T. Takao and Dr. Y. Shimonishi for performing mass spectrometry. We also thank Miss S. Nishida, and Miss Y. Itahara for their excellent technical assistance. This investigation was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (02640584), and by the Terumo Foundation, the Nakatomi Foundation, and the Kitasato Research Foundation.

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