A new natriuretic peptide isolated from cardiac atria of trout, *Oncorhynchus mykiss*

Y. Takei[a,*], A. Fukuzawa[a], Y. Itahara[b], T.X. Watanabe[b], K. Yoshizawa Kumagaye[b], K. Nakajima[b], A. Yasuda[c], M.P. Smith[d], D.W. Duff[e], K.R. Olson[a,d]

[a] *Laboratory of Physiology, Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164, Japan*
[b] *Peptide Institute, Inc., Protein Research Foundation, Minoh, Osaka 562, Japan*
[c] *Suntory Institute for Bioorganic Research, Mishima, Osaka 618, Japan*
[d] *Indiana University School of Medicine, South Bend Center, University of Notre Dame, Notre Dame, IN 46556, USA*
[e] *Indiana University at South Bend, South Bend, IN 46565, USA*

Received 23 July 1997

Abstract Atrial and brain natriuretic peptides (ANP and BNP, respectively) are two cardiac natriuretic peptides (NPs) found in tetrapods from amphibians to mammals, whereas ANP and ventricular NP (VNP) have been identified in eel hearts. Because VNP has also been found in the rainbow trout ventricle, we attempted to isolate NP from trout cardiac atria in order to determine whether ANP and VNP are common cardiac NPs in teleosts. In the present experiments, we isolated VNP and a novel atrial NP consisting of 29 amino acid residues from the atria. This new trout NP exhibited similar sequence identity to mammalian ANP and BNP (50–60%). Its homology to eel ANP was low (52%) compared with high homology of trout and eel VNP (78%). Based on yield, the content of this new NP in trout atria may be even smaller than that of VNP. The new trout atrial NP exhibited low relaxant activity in the chick rectum (only 1/10 of that of trout VNP), and extremely low vasorelaxant activity in the rat aortic strip (only 1/400 of that of human ANP). However, the new trout NP was equipotent with trout VNP and human ANP in relaxing trout epibranchial artery. Based on the sequence similarity with other NPs and on atrial content, the new NP isolated from trout atria cannot yet be assigned to a known member of the NP family.

Key words: Atrial natriuretic peptide; Brain natriuretic peptide; Ventricular natriuretic peptide; Molecular evolution; Vasorelaxant activity; Rainbow trout (*Oncorhynchus mykiss*)

1. Introduction

Atrial, brain, and C-type natriuretic peptides (ANP, BNP and CNP) form a peptide family which displays a spectrum of actions involved in volume and pressure homeostasis [1]. ANP and BNP are true hormones secreted by the heart, while CNP is a paracrine factor in the brain and other peripheral tissues [2]. All three peptides (ANP, BNP and CNP) appear to be present in tetrapods [3]. In the eel, however, ANP and ventricular NP (VNP) are the only known cardiac hormones, CNP may originate in the brain, and all three can be found in the circulation [4]. CNP is the only hormone in the heart and brain of the more primitive cartilaginous fish and it is believed to be an ancestral molecule of the NP family [5].

In view of the evolution of NP molecules, it is of interest to determine if BNP and VNP are unique to tetrapods and teleosts, respectively, or if they are on the same line of molecular evolution in the NP family. Since both the amino acid and nucleotide sequences of VNP are more similar to ANP than to BNP [6], VNP may have diverged from CNP before ANP. Alternatively, both VNP and BNP may have branched from ANP early in the course of NP evolution.

VNP has previously been isolated from the cardiac ventricle of rainbow trout [7]; however, several previous attempts to isolate ANP from atria, including efforts from our laboratory, have not been successful. This may be due to the low immunoreactivity to antisera against mammalian ANP and/or low biological activity in the assay system. Because ANP is a rather conservative molecule in the NP family, it is possible that a new type of NP could be present in trout atria. In the present study, we isolated a new NP from trout atria which is equally homologous to ANP and to BNP. This trout NP has very low biological activity in the chick rectum and rat aorta compared with trout VNP, but exhibited similar vasorelaxant activity in trout arteries.

2. Materials and methods

2.1. Isolation of NP from atria

Cardiac atria were isolated from 250–400 g rainbow trout, *Oncorhynchus mykiss*, of both sexes just after decapitation, and frozen immediately on dry ice. The frozen tissues were shipped on dry ice from Indiana University to University of Tokyo and stored at −80°C until use.

Frozen atria (300 g) were pulverized and heated in a boiling water bath with 5 volumes of distilled water for 10 min. After cooling, acetic acid (AcOH) was added to a final concentration of 1 M. The mixture was homogenized with a Polytron (Kinematika, Switzerland) for 3 min at maximum speed. The homogenate was centrifuged at 17700×*g* for 30 min at 4°C. The supernatant was treated with 66.7% and 98.5% (final) of cold acetone to remove high-molecular protein and lipids, respectively. The sample was applied to a column of Sephadex G-25 fine (5×80 cm, Pharmacia, Sweden) for desalting. The NP activity in each fraction was assayed by relaxant activity in the chick rectum as described below. Bioactive fractions were subjected to cation-exchange chromatography in an SP-Sephadex C-25 column (100 ml, Pharmacia), and adsorbed materials were eluted successively with 400 ml each of 1 M AcOH, 2 M pyridine, and 2 M pyridine-AcOH, pH 5.0. Since bioactivity was evident in the pyridine-AcOH fraction, it was subjected to cation-exchange high performance liquid chromatography (HPLC) in an SP-2SW column (4.6×250 mm, Tosoh, Japan). Each bioactive fraction was then subjected to gel-permeation HPLC in a column of Superdex Peptide HR 10/30 (10×300 mm, Pharmacia). Each bioactive fraction was further purified by sequential reverse-phase HPLC, first on an ODS-120T (4.6×250 mm, Tosoh) column and then on a 219TP54 diphenyl (4.6×250 mm, Vydac, USA) column. Elution conditions for HPLC are described in the legend of each figure. The column effluents were monitored by absorbance at 220, 230 or 280 nm.

*Corresponding author. Fax: (81) (3) 5351-6464.
E-mail: takei@ori.u-tokyo.ac.jp

PII S0014-5793(97)01050-8

2.2. Mass spectrometry and sequence analysis

Mass spectrometry was carried out with 1/100 of the purified peptide in a KOMPACT MALDI III mass spectrometer (Shimadzu, Japan). Amino acid sequence was determined with half of the purified peptide in an automated gas-phase protein sequencer (Model 476A, Applied Biosystems Inc., USA). The remaining peptide was used for confirmation of identity with the synthetic peptide (see below).

2.3. C-terminal analysis

Since the molecular mass calculated from the sequenced data did not coincide with the data of mass spectrometry, C-terminal analysis of the purified peptide was performed by carboxypeptidase digestion coupled with mass spectrometry [8]. The accuracy of this method resolves one mass difference, thereby enabling differentiation between an amidated and non-amidated C-terminus. The peptide was digested with carboxypeptidase Y (Wako Pure Chemicals, Japan) at 2 pmol/5 mU in 100 mM *N*-ethylmorpholine (Wako), pH 6.0, at 22°C for 15 min, and analyzed in a mass spectrometer (Voyager Elite, Applied Biosystems Inc.). An amino acid residue at the C-terminus was determined by the difference of molecular mass between undigested and digested peptide. Since a non-amidated Ser residue was detected at the C-terminus, it was synthesized in the protein synthesizer (Model 430A, Applied Biosystems Inc.). The remaining purified peptide was finally co-chromatographed with synthetic peptide to confirm the identity in ODS-120T and 219TP54 diphenyl columns at a gradient of 10–30% CH_3CN concentration for 40 min.

2.4. Relative potency of new NP

Biological activity of the new atrial NP relative to trout VNP and human ANP was examined in three in vitro systems: relaxant activity in the chick rectum [9], vasorelaxant activity in the rat thoracic aorta [10], and vasorelaxant activity in the trout epibranchial artery [11]. Isotonic displacement was measured for the chick rectum and rat vessel, while isometric tension was measured for the trout vessel. The synthetic new trout NP, trout VNP, and human ANP (Peptide Institute Inc.) were dissolved in isotonic (0.9%) NaCl solution, and administered in random order of drugs and doses. The relative potency of the trout NP was calculated by 2×3 point analysis after parallelism of two dose-response curves was confirmed [12]. The difference of the effects of two drugs was statistically examined by ANOVA. The ratio was expressed with 95% confidence interval. Other data were expressed as means ± S.E.M.

3. Results

3.1. Isolation and sequence determination

After desalting with Sephadex G-25 column, SP-Sephadex C-25 chromatography yielded 40 nmol of rectum relaxant activity equivalent to eel ANP in the pyridine-AcOH fraction. After cation-exchange HPLC of the fraction, the bioactivity was scattered over many fractions (Fig. 1a). The bioactive fractions were divided into three groups according to the peaks, and each group with same relaxant activity (7 nmol) was pooled and further purified by gel-permeation HPLC and several steps of reverse-phase HPLC. A new peptide was iso-

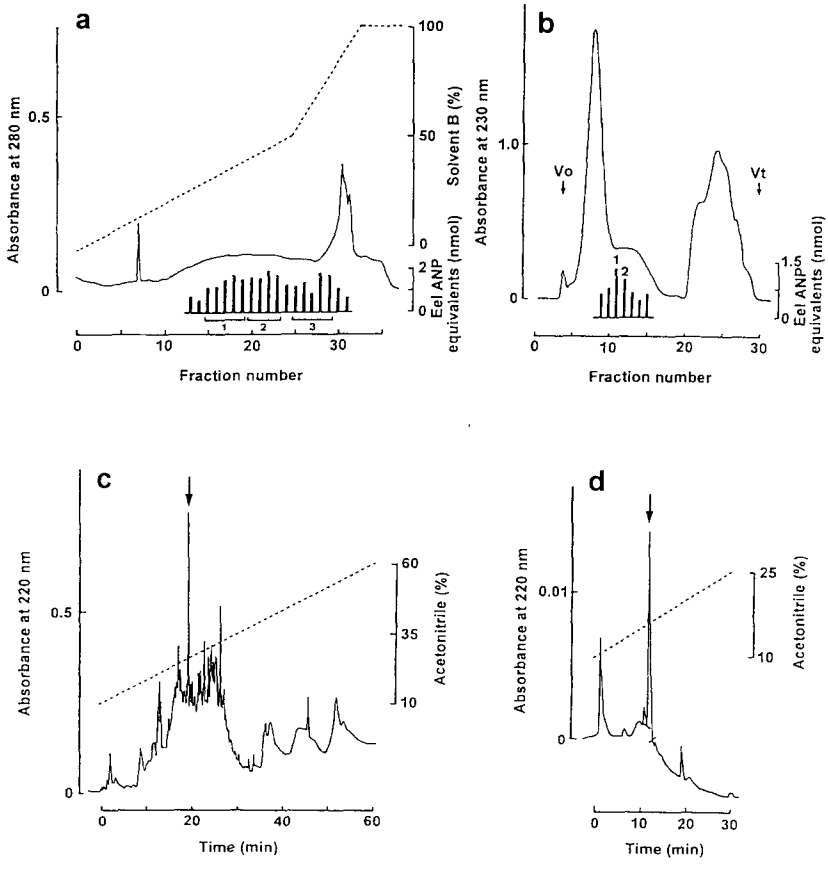

Fig. 1. a: Cation-exchange HPLC on an SP-2SW column. Sample: fraction eluted with 2 M pyridine-AcOH from SP-Sephadex C-25. Elution: from solvent A (10 mM $AcONH_4$ in 10% CH_3CN) to 50% solvent B (1 M $AcONH_4$ in 10% CH_3CN) for 60 min, and to 100% solvent B for 20 min. Flow rate: 1 ml/min. Fraction size: 2.5 ml/tube. Black columns represent relaxant activities in the chick rectum expressed as equivalents of eel ANP. b: Gel-permeation HPLC on a Superdex Peptide column. Sample: peak 1 in (a). Flow rate: 0.25 ml/min. Fraction size: 0.625 ml/tube. Eluant: 30% CH_3CN in 0.1% trifluoroacetic acid (TFA). c: Reverse-phase HPLC on an ODS-120T column. Sample: peak 1 in (b). Elution: 10% to 60% CH_3CN in 0.1% TFA for 60 min. Flow rate: 1 ml/min. Fraction was collected by each peak. d: Reverse-phase HPLC on a 219TP54 diphenyl column. Sample: peak marked in (c). Elution: 10% to 25% CH_3CN in 0.1% TFA for 30 min. Other conditions were identical to (c).

lated from peak 1 (see below), whereas trout VNP-(1–36) was isolated from peak 2 and trout VNP-(1–32) was isolated from peak 3 (data not shown). The final recovery of VNP-(1–36) and VNP-(1–32) was 470 and 350 pmol, respectively, equivalent to eel ANP as determined by absorbance at 220 nm.

After gel-permeation HPLC of peak 1, two fractions exhibited high rectum relaxant activity (Fig. 1b). The fraction displaying the highest activity was subjected to reverse-phase HPLC with an ODS column, but the assay using 1/5 of the fraction collected by each peak reflection did not show any significant activity (Fig. 1c). However, a possible fraction marked by an arrow was further purified by another reverse-phase HPLC with a diphenyl column, and the material in the major peak reflection was analyzed by mass spectrometry (Fig. 1d). Mass analysis of the final product revealed a single peak with a molecular mass of 2972 (data not shown). Sequence analysis of half of the purified peptide resulted in Ser-Lys-Ala-Val-Ser-Gly-Cys-Phe-Gly-Ala-Arg-Met-Asp-Arg-Ile-Gly-Thr-Ser-Ser-Gly-Leu-Gly-Cys-Ser-Pro-Lys-Arg-Arg. The fraction showing the second highest activity after gel-permeation HPLC was also purified as above, and the same peptide was recovered (data not shown). The final yield from both fractions was 400 pmol equivalent to eel ANP by absorbance at 220 nm.

The molecular mass calculated from the above sequence, however, was ca. 87 smaller than the value measured by mass spectrometry. Mass spectrometry after digestion of the peptide with carboxypeptidase Y displayed two distinct peaks, one at 2972.48 (major) and a second at 2885.38 (minor) (data not shown). Since the difference (87.1) exactly coincided with a Ser residue, we determined that a non-amidated Ser residue is attached to the C-terminal Arg residue of the deduced sequence (Fig. 2). The theoretical molecular mass calculated from this sequence was 2972.4. The sequence was finally confirmed by co-chromatography of the purified peptide with synthetic peptide in two different HPLC systems.

3.2. Biological activity

The trout NP induced a dose-dependent relaxation in the precontracted chick rectum, but it was significantly less potent than either trout VNP or human ANP (Fig. 3a). The potency ratio for trout NP was 0.09 (0.08–0.10) compared to trout VNP and 0.25 (0.18–0.34) compared to human ANP. Trout

Fig. 2. Comparison of amino acid sequence of new trout atrial NP with other natriuretic peptides sequenced to date. Amino acid residues identical to trout atrial NP are reversed.

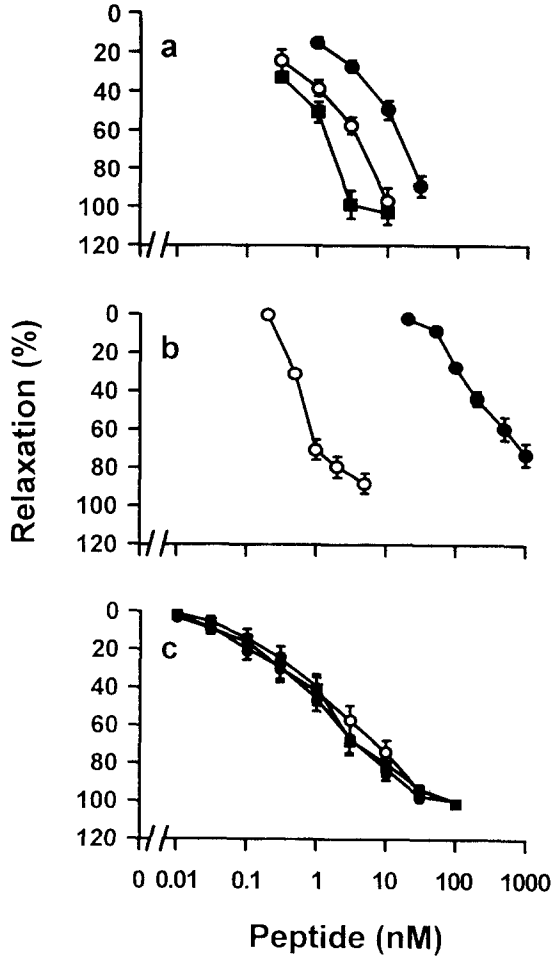

Fig. 3. a: Relaxant activity in the chick rectum ($n=8$). b: Vasorelaxant activity in the rat aortic strip ($n=6$). c: Vasorelaxant activity in the trout efferent epibranchial artery ($n=8$) of trout atrial NP (●), trout VNP (■) and human ANP (○). Values are means ±S.E.M.

NP was even less efficacious in the precontracted rat thoracic aorta (Fig. 3b) where the potency ratio was only 0.0027 (0.0019–0.0033) compared to human ANP. However, trout NP was equipotent with both trout VNP and human ANP in its ability to relax the trout efferent epibranchial artery (Fig. 3c). Neither homologous trout peptide was more potent than human peptide in the trout artery.

4. Discussion

Rainbow trout are the most frequently used fish species for basic fish research and aquaculture [13]. They are particularly good research models for osmoregulatory studies because of their tolerance to a wide range of environmental salinities [14]. Thus a detailed examination of osmoregulatory hormones, such as NPs, in these fish has both general and practical value. Surprisingly, however, only VNP has been isolated from rainbow trout [7] or from a related species, the chum salmon, *Oncorhynchus keta* (A. Fukuzawa and Y. Takei, unpublished observation). Even though these and other studies attempted to identify a salmonid ANP using the classical chick rectum bioassay, they were not successful. In addition, a number of attempts have been made to isolate ANP from trout or salmon atria using immunoreactivity to mammalian ANP as an

assay system; however, to our knowledge these have likewise been unsuccessful. This suggests that either there is quantitatively little trout atrial NP or it is functionally and/or structurally quite different from other ANP molecules. In the present study, a new NP was isolated from trout atria even though its biological activity, as determined with standard bioassay techniques, was extremely low. Our success may be partially due to our use of gel-permeation HPLC which permitted us to decrease the frequency of subsequent reverse-phase HPLC. In fact, isolation of VNP from atria in this study was also due to this improved technique.

The new trout atrial NP is equally similar to both ANP and BNP and it is thus far impossible to determine whether it is ANP or BNP. The ANP content in atria was several orders of magnitude larger than that of BNP or VNP in any mammalian and teleostean species thus far examined [15,16]. However, atrial content of the new NP appears to be smaller than that of VNP judging from the recovery of each peptide from trout atria. The sequence of the C-terminal end of the present NP consists of 6 amino acid residues, which has more similarity to BNP (6 residues) than to ANP (5 residues), but there are some exceptions [3] (Fig. 2). If the new NP belongs to the ANP group, it is evident that ANP is structurally more variable than VNP; sequence identity of ANP between trout and eel (52%) is much less than that of VNP (78%) [7]. In mammals, ANP is more highly conserved than BNP [4]. If the new NP is BNP, on the other hand, it will provide a new insight into the molecular evolution of the NP family. In order to reach a conclusive decision, it is necessary to isolate the peptide's cDNA and examine whether it possesses seven repetitions of the ATTTA sequence in the 3′ noncoding region because this characteristic is common to all BNP cDNAs thus far cloned [17].

It is perhaps surprising that human ANP was as effective as homologous trout NPs for the vasorelaxant activity in the trout artery. In the eel, for instance, the vasodepressor activity of eel NPs are 100-fold more potent than that of human ANP, and the opposite is true in the rat [18]. Since homology between human ANP and trout NPs is low, it is possible that these peptides may not share the same receptors. Therefore, another NP that is more closely related to human ANP and shares a receptor with human ANP might be present in the trout. In mammals, two guanylyl cyclase (GC) coupled receptors responsible for biological actions of NP peptides are identified: GC-A for ANP and, to a lesser extent, BNP and GC-B for CNP [19]. In teleost fishes, GC-B has been cloned in the eel [20]. Eel GC-B expressed in COS cells exhibited a potency order for cGMP production of CNP > VNP > ANP. The presence of GC-A type receptor is also demonstrated in the kidney and intestine of the eel [21], and eel GC-A cDNA has recently been cloned (M. Kashiwagi and S. Hirose, personal communication). Since VNP has a unique structure with a long C-terminal sequence, it is possible that new receptors specific to VNP are also present in teleost fishes. In this way, studies on the fish NP system will give us a new insight into the evolution of this system.

Acknowledgements: We express cordial thanks to Dr. K. Kangawa and Dr. H. Matsuo of the National Cardiovascular Center Research Institute for inviting K.R.O. through Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan (Encourage System of C.O.E.). The authors also express their gratitude to Dr. Scott LaPatra and J. Winkle and the staff at Clear Springs Foods Inc., Buhl, ID, USA for their assistance in obtaining trout tissues. This work was supported in part by Grant 08640843 from the Ministry of Education, Science and Culture of Japan to Y.T. and National Science Foundation Grants IBN-9105247 and IBN-9723306 to K.R.O.

References

[1] Brenner, B.M., Ballermann, B.J., Gunning, M.E. and Zeidel, M.L. (1990) Physiol. Rev. 70, 665–699.
[2] Barr, C.S., Rhodes, P. and Struthers, A.D. (1996) Peptides 17, 1243–1251.
[3] Fukuzawa, A., Watanabe, T.X., Itahara, Y., Nakajima, K., Yoshizawa-Kumagaye, K. and Takei, Y. (1996) Biochem. Biophys. Res. Commun. 222, 323–329.
[4] Takei, Y. and Balment, R.J. (1993) Fish Physiol. Biochem. 11, 183–188.
[5] Suzuki, R., Togashi, K., Ando, K. and Takei, Y. (1994) Gen. Comp. Endocrinol. 96, 378–384.
[6] Takei, Y., Ueki, M. and Nishizawa, T. (1994) J. Mol. Endocrinol. 13, 339–345.
[7] Takei, Y., Takano, M., Itahara, Y., Watanabe, T.X., Nakajima, K., Conklin, D.J., Duff, D.W. and Olson, K.R. (1994) Gen. Comp. Endocrinol. 96, 420–426.
[8] Biemann, K. (1992) Annu. Rev. Biochem. 61, 977–1010.
[9] Currie, M.G., Geller, D.M., Cole, B.R., Boylan, J.G., Yusheng, W., Holmberg, S.W. and Needleman, P. (1983) Science 221, 71–73.
[10] Watanabe, T.X., Noda, Y., Chino, N., Nishiuchi, Y., Kimura, T., Sakakibara, S. and Imai, M. (1988) Eur. J. Pharmacol. 147, 49–58.
[11] Conklin, D.J. and Olson, K.R. (1994) Am. J. Physiol. 266, R1856–R1860.
[12] Warner, B.T. (1963) J. Pharm. Pharmacol. 16, 220–233.
[13] Gall, G.A.E. (1992) The Rainbow Trout, Elsevier, Amsterdam.
[14] Woods, C.M. and Shuttleworth, T.J. (1995) Cellular and Molecular Approaches to Fish Ionic Regulation. Academic Press, San Diego, CA.
[15] Mukouyama, M., Nakao, K., Hosoda, K., Suga, S., Saito, Y., Ogawa, Y., Shirakami, G., Jougasaki, M., Obata, K., Yasue, H., Kambayashi, Y., Inoue, K. and Imura, H. (1991) J. Clin. Invest. 87, 1402–1412.
[16] Takei, Y., Takahashi, A., Watanabe, T.X. and Ando, K. (1994) J. Endocrinol. 141, 81–89.
[17] Akizuki, N., Kangawa, K., Minamino, N. and Matsuo, H. (1991) FEBS Lett. 280, 357–362.
[18] Takei, Y., Takahashi, A., Watanabe, T.X., Nakajima, K. and Sakakibara, S. (1991) FEBS Lett. 282, 317–320.
[19] Gerbers, D.L. (1992) Cell 71, 1–4.
[20] Katafuchi, T., Takashima, A., Kashiwagi, M., Hagiwara, H., Takei, Y. and Hirose, S. (1994) Eur. J. Biochem. 222, 835–842.
[21] Loretz, C.A. and Takei, Y. (1997) Fish Physiol. Biochem. (in press).