Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 131-139

PURIFICATION AND COMPLETE AMINO ACID SEQUENCE OF α-HUMAN ATRIAL NATRIURETIC POLYPEPTIDE (α-hANP)

Kenji KANGAWA and Hisayuki MATSUO

Department of Biochemistry, Miyazaki Medical College,
Kiyotake, Miyazaki 889-16, JAPAN

Received November 28, 1983

SUMMARY: The present survey for natriuretic factors in human atrial extract was performed by using in vitro assay for the relaxant effect on the contractility of chick rectum. Three distinct components (α, β and γ) of a potent relaxant activity were found in the chromatographic regions of the crude extract. As α-component of Mr 3,000 daltons, a 28-amino acid peptide has been isolated in a pure state and found to elicit potent diuretic and natriuretic activities as well as vasorelaxant activity, when injected into the assay rats. Accordingly, we proposed a name "α-human atrial natriuretic polypeptide (α-hANP)" for the peptide. The complete amino acid sequence of the peptide has been established by microsequencing as well as synthesis.

Mammalian atrial cardiocytes contain storage granules, referred to as the specific atrial granules, which morphologically resemble ones found in peptide hormone-producing cells (1). The observations that mammalian atrial extract causes natriuresis and diuresis, when injected into rats, imply a possible role of atrial granules in the regulation of extracellular fluid volume and water-electrolyte balance (2,3). Multimodal natriuretic factors of peptide nature have been suggested to exist in mammalian atrial extract (4,5). However, nothing has been identified as yet. Cardionatrin I named for one of natriuretic factors from rat atria is the only peptide, whose amino acid composition has been reported (6). On the other hand, it has been known that the natriuretic activity in mammalian atrial extract comigrates in gel filtration together with the chick rectum relaxant activity (7). This fact indicates a possibility that in vitro smooth muscle assay would enable rapid assessment of atrial natriuretic activity to facilitate its purification. As will be reported in the present paper, a 28-amino acid peptide with a potent relaxant activity has been purified from human atrial extract. The peptide thus isolated elicits potent natriuretic and diuretic activities as well as vasorelaxant activity, when injected into the assay rats. Accordingly, the peptide will be henceforth designated as α-human atrial natriuretic polypeptide (α-hANP). We will report here isolation and complete amino acid sequence of α-hANP. Bioactivities of the peptide will also be discussed.

0006-291X/84 $1.50

METHODS AND MATERIALS

Isolation: Human atrial tissue (40 g) was resected within 10 hr postmortem. Diced tissue was boiled for 5 min in 7x volumes of 1M AcOH containing 20 mM HCl to inactivate intrinsic proteases. After cooling, extractions were performed at 4°C by homogenizing with a polytron mixer. The supernatant of the extracts, obtained after 30-min centrifugation at 12,000 x g, was desalted through an Amicon UM-2 membrane to afford crude concentrate. In the preliminary experiment, an aliquot of the crude concentrate was subjected to reverse phase HPLC. Chick rectum relaxant activity in effluents was assayed (Fig.1). The residual concentrate (50 ml) obtained above was subjected to acetone-precipitation at a concentration of 66%. After removal of the precipitates, the supernatant was evaporated in vacuo to dryness. The residual materials were dissolved again in 1M AcOH and then adsorbed on a column of SP-Sephadex C-25 (H^+-form) (1.5 x 14 cm), pre-equilibrated with 1M AcOH. Successive elutions with 1M AcOH, 2M pyridine and 2M pyridine-AcOH (pH 5.0) afforded three respective fractions of SP-I, SP-II and SP-III. Lyophilization of SP-III yielded 69 mg of the dry SP-III concentrate, which was used for further purification. Gel-filtration of SP-III was performed on a column of Sephadex G-50 (fine). Column effluents were monitored by measuring absorbance at 280 nm. An aliquot of each fraction was subjected to bioassay for chick rectum relaxant activity (Fig.2). Fractions (#46-50) (3.8 mg) eliciting the rectum activity were subjected to preparative HPLC on a reverse phase column and α-hANP was isolated as a single peptide peak (Fig.3). Purity of α-hANP was examined by another reverse phase HPLC (Fig.4A). Column effluents on HPLC were monitored by measuring absorbance at 210 and 280 nm, simultaneously.

Bioassay: 1) Chick rectum relaxant activity was measured according to the method reported by Currie, et al. (7) by using strips of freshly isolated chick rectum, bathed in Krebs-Henseleit solution at 37°C. Muscle tone was induced in the rectum strips by carbachol (2×10^{-8} M).

2) Diuretic and natriuretic activities were assayed by the method described by DeBold, et al. (3). Male Sprague-Dawley rats (weight range 300-400 g) were anesthetized by i.p injection of pentobarbital (60 mg/kg) and prepared for bioassay. Quantitative collection of urine was performed through a bladder catheter. After an equilibration of 1 hr, 30-min collection of urine was taken at the control period. After 50 µl of saline solution containing the test material and bacitracin (5 µg) was injected into jugular vein in one shot, urine was consecutively collected every 5 min for 30 min. Sodium and potassium concentrations in urine were measured with a flame-photometer (Shimadzu CIM-101A). Chloride ion concentrations were electrometrically titrated with a chloride analyzer #925 (Corning). Urine volumes were measured by weighing. Increase in excretion of sodium, potassium, chloride ions and urine is expressed as the percent change (mean ± standard errors) from 30-min urine samples before and after injection of the test material. Three observations were made for each dose (Table 2).

Sequence analyses: Amino acid analyses were carried out with an amino acid analyzer Hitachi-835, after hydrolysis of the peptide (ca. 0.5 nmol) in 3N mercapto-ethanesulfonic acid or 6 N HCl containing 0.1% phenol at 110°C for 20 hr. Analyses of amino acids released after enzymatic digestion were carried out on a picomole level with a pre-fluorescence-labeling analysis system (Waters) (8). Reductive S-carboxymethylation of α-hANP was performed by reduction with 50 mM dithiothreitol (DTT) in 0.5 M Tris·HCl buffer (pH 8.0) for 4 hr at 37°C, followed by treatment with 100 mM iodoacetate for 5 min. Resulted RCM-α-hANP was purified by reverse phase HPLC. Cyanogen bromide cleavage of RCM-α-hANP (5.0 nmol) was performed by the described method (9). After converting the resulting C-terminal homoserinelactone to the corresponding homoserine as previously described (8), the BrCN fragments were separated by HPLC (Fig.4C). Tryptic digestion of the peptide (2 nmole) was carried out with 2 µg of trypsin (TPCK-treated: Worthington) in 25 µl of 1% ammonium bicarbonate (pH 8.0) at 37°C for 3 hr. Carboxy-terminal analyses of the native α-hANP (100-200 pmol) were carried out by digesting

with carboxypeptidase A and B (Sigma : 100 ng each) in a buffer of 0.2M N-ethyl-morpholine-acetate (pH 8.0) at 37°C, followed by analyzing the residues released at appropriate time intervals. Sequence analyses of RCM-α-hANP and BrCN fragment (CB-2) were performed by stepwise Edman degradation, using a gas-phase automated sequenator (Applied Biosystems, model 470A), coupled with HPLC identification of resulted PTH-amino acids.

Synthesis: Total synthesis of α-hANP was performed by solid phase procedures, conducted on a chloromethylated polystyrene resin. O-2,6-dichlorobenzyl-BOC-Tyr, Tosyl-BOC-Arg, O-Benzyl-BOC-Ser, S-4-methylbenzyl-BOC-Cys and p-nitrophenylesters of BOC-Gln and BOC-Asn were used for coupling. Otherwise, BOC-amino acids were coupled. After HF-treatment, the deblocked peptide was reduced with DTT at pH 8.5 to afford crude α-hANP (reduced form). After removal of excess DTT at pH 3.0, the resulting peptide was subjected to air-oxidation in a buffer of ammonium acetate (pH 7.5) at a peptide concentration of 10^{-5} M for 2 days at room temperature to result in the formation of disulfide linkage. The major product of the synthesis was purified by gel-filtration, ion exchange HPLC and reverse phase HPLC. Amino acid analysis and microsequencing confirmed the correct synthesis of α-hANP.

RESULTS AND DISCUSSION

Isolation of α-hANP: The present survey for natriuretic factors in human atrial extract was performed by utilizing a rapid and simple in vitro assay for relaxant effect on chick rectum in place of bioassay for diuresis or natriuresis. In our preliminary experiment, reverse phase HPLC of crude extract of human atrial tissue indicated the presence of at least three distinct components (α, β and γ) with a potent rectum relaxant activity (Fig.1). Approximate molecular weights of these three components were estimated by gel-filtration to be; α, ca. 3,000 daltons; β, ca. 5,000 and γ,

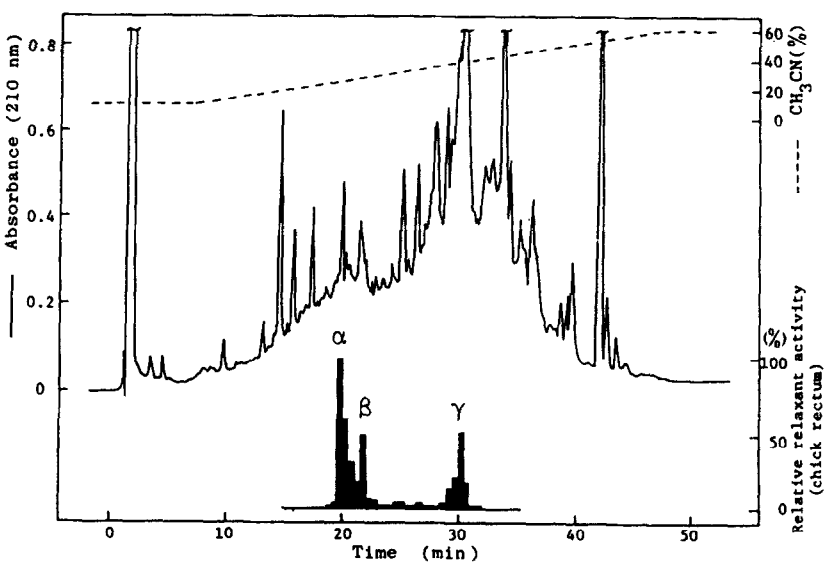

Fig.1. Reverse phase HPLC of crude extract of human atrial tissue. Sample : 1/100 portion of acid extract. Flow rate : 2.0 ml/min. Column : TSK LS-410 ODS SIL (4.0 x 250 mm, ToyoSoda) Solvent system : H_2O : CH_3CN : 10% TFA = (a) 90 : 10 : 1, (b) 40 : 60 : 1 (v/v). Linear gradient from (a) to (b) (40 min). Three major peaks (α-, β- and γ-) of a potent rectum relaxant activity were observed.

ca. 13,000 daltons. The present purification is concerned with α-component of Mr 3,000 daltons and was performed in a manner similar to our previous isolations of neuropeptides from brain extracts(8). After heat-treatment to abolish intrinsic protease activity, acid extract of human atrial tissue (40 g) was desalted by ultrafiltration, and then subjected to acetone-precipitation. Peptides in the supernatant were adsorbed on SP-Sephadex C-25 and then eluted with 1M AcOH (SP-I), 2M pyridine (SP-II) and 2M pyridine-AcOH (pH 5.0) (SP-III), successively. Rectum activity was eluted in SP-II (γ-component) and SP-III (α- and β- components). Gel-filtration of SP-III containing basic peptides on a column of Sephadex G-50 afforded two major peaks (α- and β-components) of chick rectum relaxant activity (Fig.2). Fractions #46-50 corresponding to α-component were further purified by reverse phase HPLC, where a bulk of rectum activity was eluted in a single peptide peak at 20-21 min (Fig.3). Another HPLC of the peptide revealed that α-component of rectum relaxant activity was isolated in a pure state (Fig.4A). Amino acid composition of the peptide thus purified indicates its 28-amino acid peptide structure with a disulfide linkage in the molecule (Table 1). Based on these data, it was estimated that 92 nmoles of the peptide were isolated from human atrial tissue (40 g). Intravenous injection into rat of the pure peptide (0.4 nmol; 1.2 μg) induced rapid and potent diuretic and natriuretic responses (Fig.6 and Table 2). Accordingly, the peptide will be referred to as α-hANP for α-human atrial natriuretic polypeptide. Purification of β- and γ-components is now going on in our laboratory.

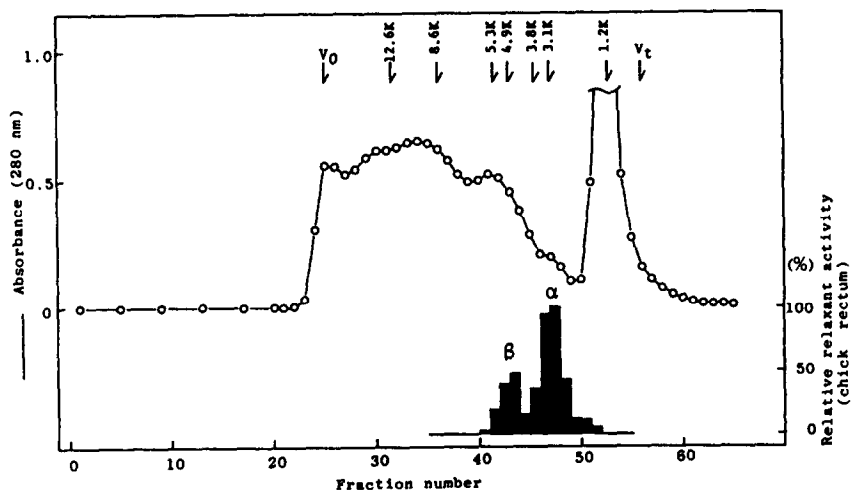

Fig.2. Gel-filtration of the basic peptide fraction (SP-III).
Sample : SP-III concentrate (69 mg) obtained after SP-Sephadex C-25.
Column : Sephadex G-50 (fine) (1.2 x 103 cm). Eluent : 1M AcOH
Flow rate : 5.4 ml/hr. Fraction size : 2 ml/tube. Two components (α- and β-) of a potent rectum activity were separated.

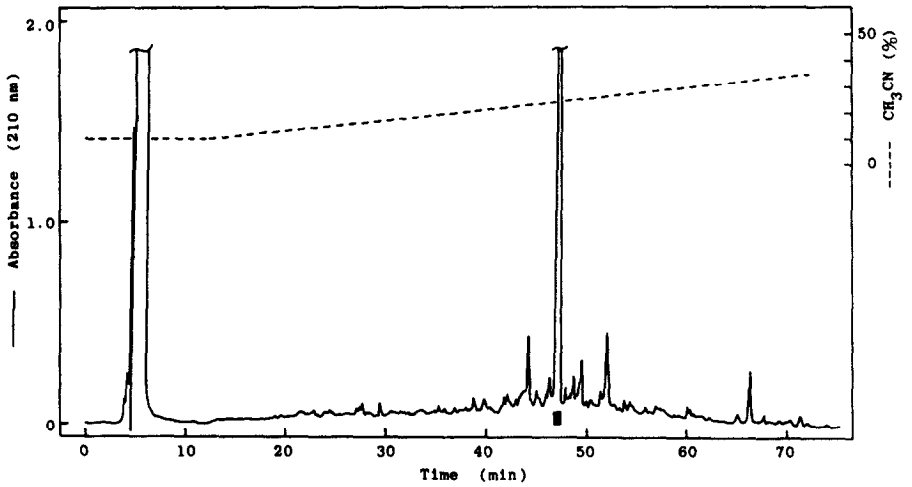

Fig.3. Reverse phase HPLC of rectum relaxant α-component.
Sample : Fr.#46-50 (3.8 mg) obtained in Fig.2. Flow rate : 2.0 ml/min.
Column : Chemcosorb 5 ODS-H (8.0 x 250 mm : Chemco). Solvent system : Linear gradient from (a) to (b) (120 min). (a), (b): the same as in Fig.1.
Black bar indicates a peak of a potent relaxant activity (α-hANP)

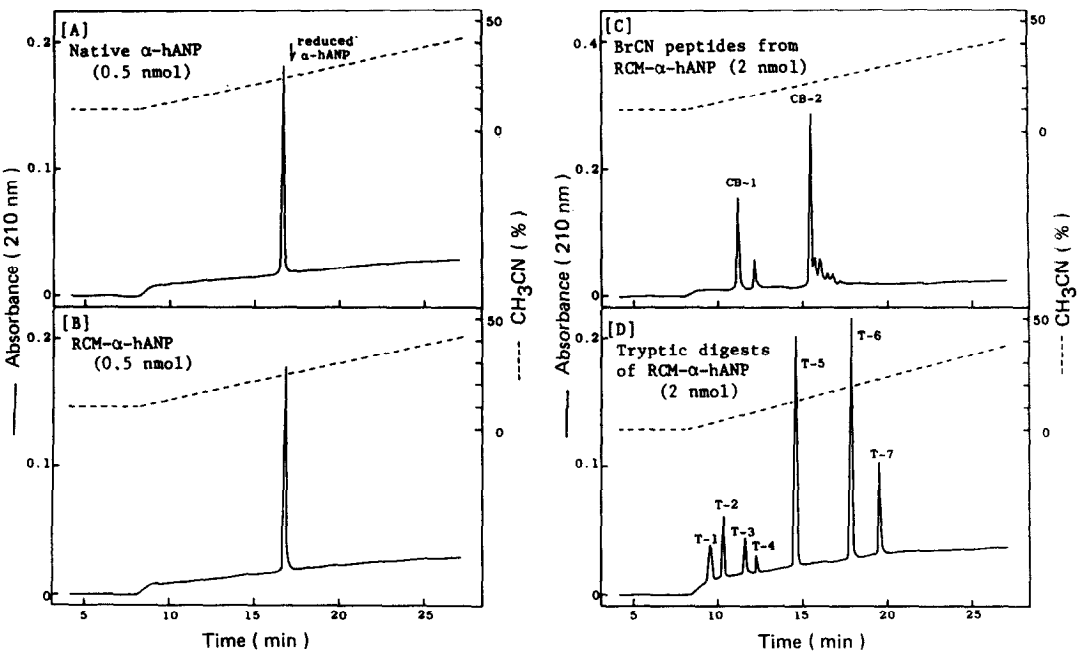

Fig.4. Reverse phase HPLC of α-hANP and related peptides (native and synthetic).
Column : Chemcorsorb 3 ODS-H (4.6 x 75 mm, Chemco) Flow rate : 1 ml/min.
Solvent system : H_2O : CH_3CN : 10% TFA = (a) 100 : 0 : 1, (b) 90 : 10 : 1, (c) 40 : 60 : 1 (v/v). Linear gradient from (b) to (c) (30 min) in the cases of (A), (B), (C) and from (a) to (c) (30 min) in the case of (D).
Sequences of BrCN peptides (CB-1, -2) and tryptic peptides (T-1∿ T-7) are shown in Fig.5B.

Table 1 Amino acid compositions (residues per mole) of α-hANP, RCM-α-hANP and BrCN peptides

	α-hANP	RCM-α-hANP	CB-1	CB-2
CmCys	----	1.95 (2)	1.07 (1)	1.03 (1)
Asp	2.09 (2)	1.95 (2)	----	2.10 (2)
Ser	4.94 (5)	4.90 (5)	3.02 (3)	2.10 (2)
Glu	1.10 (1)	1.06 (1)	----	1.05 (1)
Gly	4.99 (5)	5.03 (5)	2.07 (2)	2.95 (3)
Ala	1.12 (1)	1.11 (1)	----	1.02 (1)
$(Cys)_2$ *	0.80 (1)	----	----	----
Met	0.95 (1)	0.90 (1)	** (1)	----
Ile	1.01 (1)	1.00 (1)	----	0.93 (1)
Leu	2.00 (2)	2.00 (2)	1.00 (1)	1.00 (1)
Tyr	0.99 (1)	1.02 (1)	----	0.93 (1)
Phe	1.99 (2)	2.05 (2)	1.12 (1)	1.02 (1)
Arg	4.92 (5)	5.19 (5)	3.02 (3)	1.93 (2)
total	(28)	(28)	(12)	(16)

*) $(Cys)_2$: cystine **) Determined as homoserine and its lactone
Numbers in parentheses represent the nearest integers.

Structural Analyses: Since amino acid analysis indicated that α-hANP has a disulfide linkage in the molecule (Table 1), sequence analyses of the peptide was performed after reductive carboxymethylation to convert to the corresponding RCM-α-hANP, whose amino acid composition and HPLC pattern are shown in Table I and Fig.4B, respectively. Stepwise Edman degradation of RCM-α-hANP (1.0 nmol) was performed by an automated gas-phase sequenator. PTH-amino acids liberated were successfully identified up to the 22nd step, as shown in Fig.5. C-Terminal sequence of RCM-α-hANP was determined by carboxypeptidase digestion. The first release of Tyr from RCM-α-hANP took place by carboxypeptidase A digestion. The second release of Arg was achieved after further addition of carboxypeptidase B in the reaction solution and then spontaneously followed by successive release of Phe and Ser. Thus, C-terminal sequence of RCM-α-hANP was identified to be: -Ser-Phe-Arg-Tyr (Fig.5). Cyanogen bromide cleavage of RCM-α-hANP afforded two fragment peptides, CB-1 and CB-2, which were separated by reverse phase HPLC (Fig.4C). Amino acid compositions of CB-1 and CB-2 were found to correspond to N-terminal half [1-12] and

Table 2 Diuretic and natriuretic responses induced by native α-hANP (expressed by % change(mean ± S.E.) from 30-min urine samples collected before and after injection)

	native α-hANP injected(i.v.)		
	0.1 nmole	0.2 nmole	0.4 nmole
Urine output	222 ± 44(%)	375 ± 48(%)	557 ± 104(%)
Na^+excretion	299 ± 98	541 ± 149	911 ± 154
K^+ excretion	152 ± 27	274 ± 75	138 ± 7
Cl^-excretion	243 ± 62	567 ± 163	872 ± 112

Three assay rats were used for each dose.

C-terminal half [13-28] of RCM-α-hANP, respectively. Automated sequence analysis of CB-2 (0.2 nmol)proceeded up to the 14th step, as shown in Fig.5. From these data, the complete amino acid sequence of RCM-α-hANP was unambiguously determined (Fig.5). The presence of a disulfide linkage in native α-hANP was suggested from the amino acid analysis data showing that one cystine residue was recovered in a fair yield (0.8 mole equiv.) after acid hydrolysis of α-hANP (Table 1). Furthermore, reduction of α-hANP with DTT afforded the corresponding reduced form which was distinguishable from α-hANP by HPLC (Fig.4A). The possibility that the peptide is a dimer or higher molecular weights polymer, was excluded by its behavior on gel-filtration. These data strongly indicate that α-hANP is a monomeric peptide with a disulfide bridge. Positive confirmation of the structure for α-hANP was provided by synthesis. The linear 28-amino acid peptide (reduced form), corresponding to the sequence determined above, was synthesized by solid-phase techniques and then converted to the oxidized form by air-oxidation under high dilution conditions. Synthetic α-hANP (oxidized form) comigrates with the native peptide on reverse phase HPLC (Fig.4A), confirming that native α-hANP had been isolated as the oxidized form. Furthermore, the reduced form and RCM-peptide prepared from synthetic α-hANP were found to be chromatographically identical with those derived from native α-hANP, respectively. It was also evidenced that both RCM-peptides derived from synthetic and native α-hANP underwent cyanogen bromide cleavage as well as tryptic digestion in exactly the same manner (Fig.4C and 4D). Finally, synthetic α-hANP was found to elicit the full activities of native α-hANP in our preliminary in vitro and in vivo assays. Thus, the complete structure of α-hANP was unambiguously established as shown in Fig.5.

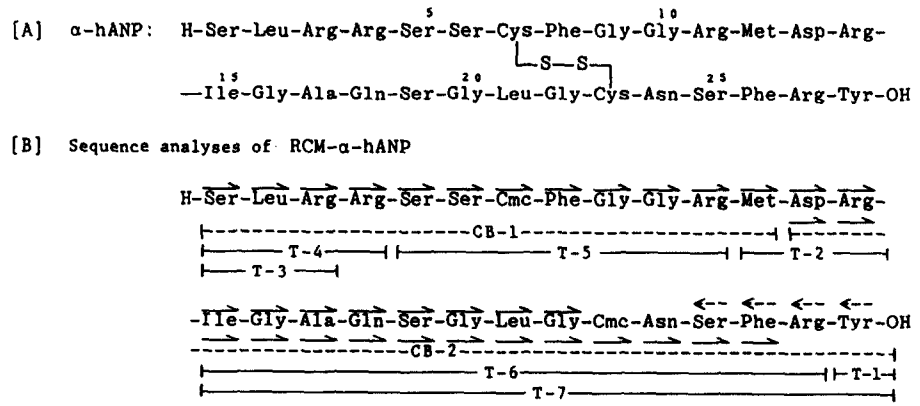

Fig.5. Sequence determination : [A] shows the complete amino aicd sequence of α-hANP. Analyses were made with RCM-α-hANP in the manners shown in [B]. (—→) : by automated sequenator, (←--) : by carboxypeptidase method. Cmc : S-carboxymethylcysteine

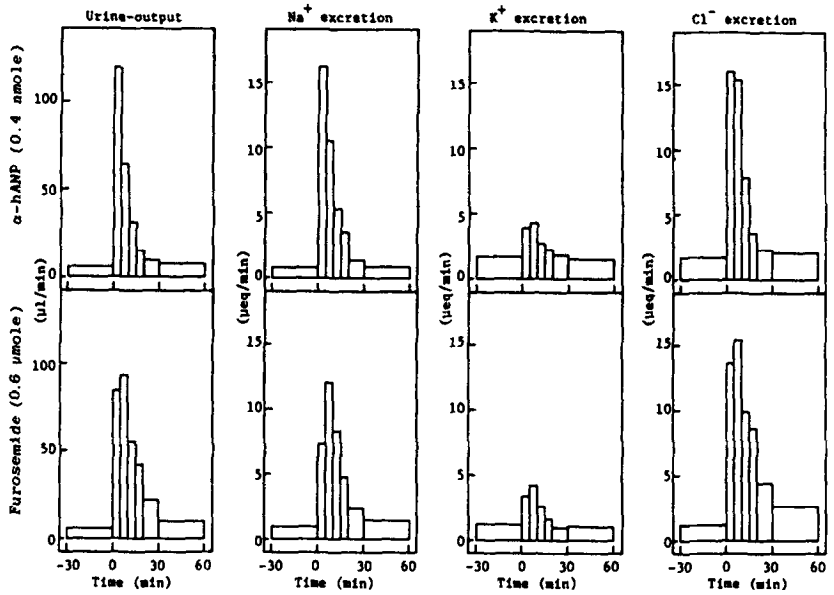

Fig.6. Time course of urine output and Na^+, K^+, Cl^- excretions in urine induced by native α-hANP (0.4 nmol), comparing with furosemide (0.6 μmol). 30-Min urine before injection was collected as a control. After injection at 0 min, urine was collected every 5 min for 30 min. Analyses of each urine sample were made as described in the text.

Biological Activities: Native α-hANP isolated as one of rectum relaxant components elicits potent diuretic and natriuretic activities (Fig.6 and Table 2), when injected intravenously into the assay rats. Fig.6 represents time course of diuretic and natriuretic responses induced by native α-hANP (0.4 nmol), comparing with those induced by furosemide (0.6 μmol). Injection of α-hANP resulted in a rapid increase of NaCl and fluid excretion, which reached maximum within 5 min. The response was essentially complete in 30 min. At the maximum response, α-hANP (0.4 nmol) caused a 20 x fold increase in urine output and a 30 x fold increase in excretions of Na^+ and Cl^-, comparable to the responses induced by ca. 1.0 μmol of furosemide. Our preliminary data listed in Table 2 show that α-hANP caused a marked and dose-dependent increase in the excretion of NaCl as well as in urine output. However, the excretion of K^+, only doubled at each dose, was not dose-dependent. A small but reproducible decrease (15-20 mmHg) in blood pressure was observed during 45 min after injection, likely due to fluid loss by urinary excretion. These data indicate that α-hANP is an extremely potent inhibitor of renal tubular reabsorption of NaCl. It is also obvious that α-hANP is one of the substances responsible for the natriuretic effect of atrial extract.

We have observed the presence in crude atrial extract of two other components (β- and γ-) of higher molecular weights, both of which are

expected to have natriuretic activity. DeBold and Flynn also reported a similar observation that there are four distinct species of natriuretic activity, named cardionatrin I-IV in chromatographic regions of rat atrial extracts(6). Such multimodal distributions of the activity may indicate the presence of precursor-intermediate-product relationships between them, as has often been observed in peptide hormones.

ACKNOWLEDGMENT

We are grateful to Dr.I. Kubota and Mr.Y.Hayashi, Suntory Institute for Biomedical Research for their help in conducting of automated sequence analyses and bioassays, respectively. We also thank Misses A.Fukuda, T.Hatou and R. Nishi for the skilled technical assistance.

REFERENCES

1)Jamieson,J.D. and Palade,G.E. (1964) J. Cell. Biol., 23, 151-172.
2)DeBold,A.J. (1979) Proc. Soc. Exp. Biol. Med., 161, 508-511.
3)DeBold,A.J., Borenstein,H.B., Veress,A.T. and Sonnenberg,H. (1981) Life Sci., 28, 89-94.
4)Keller,R. (1982) Can. J. Physiol. Pharmacol., 60, 1078-1082.
5)Trippodo, N.C., MacPhee,A.A., Cole,F.E. and Blakesley,H.L. (1982) Proc. Soc. Exp. Biol. Med., 170, 502-508.
6)DeBold,A.J. and Flynn,T.G. (1983) Life Sci., 33, 297-302.
7)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.
8)Kangawa,K., Minamino,N., Fukuda,A. and Matsuo,H. (1983) Biochem. Biophys Res. Commun., 114, 533-540.
9)Gross,E. (1967) Methods in Enzymol., 11, 238-255.