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Synthesis and biological properties of two dimeric forms of human α-atrial natriuretic peptide

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Two dimeric forms of human α-atrial natriuretic peptide (α-ANP) were synthesized by solution methods and compared with monomeric α-ANP in terms of some biological and immunochemical properties. The parallel form (β′-ANP) and the antiparallel form (β-ANP) were equipotent in smooth muscle relaxant activity in isolated rat aorta and their ED_{50} values were estimated to be 1.7×10^{-8} M and 1.6×10^{-8} M, respectively. Diuretic and natriuretic responses induced by β-ANP and β′-ANP in anesthetized rats were equally less potent but exhibited a significantly longer duration than those induced by α-ANP. β-ANP and β′-ANP possessed immunoreactivities of 60–100% and 50–90% (α-ANP, 100% on a molar basis), respectively, with three different antisera raised against α-ANP-related peptides.

Peptide synthesis *Atrial natriuretic peptide* *Muscle relaxation* *Diuresis* *Natriuresis* *Immunoreactivity*

1. INTRODUCTION

Since a potent diuretic-natriuretic activity was observed in extracts derived from rat atria [1], multiple forms of ANP have been isolated from rat atria [2–8] and three distinct forms of ANP have been found in the human atrium [9,10]. Of the three forms of human peptide, α-ANP is a 28-amino acid peptide with an intramolecular disulfide linkage [9]. γ-ANP is a large peptide comprising 126 amino acid residues and contains the α-ANP sequence at the carboxyl end of the molecule [10]. Both α-ANP and γ-ANP molecules seem to be derived from a common 151-residue precursor [11], while β-ANP has been reported to be an antiparallel dimer of α-ANP [10]. The origin and mechanism of the formation of β-ANP are still unknown. Such a dimeric form of ANP has not been found in other mammalian species. Kangawa et al. [10] reported that diuretic-natriuretic responses to β-ANP showed a slower onset and longer duration than that to α-ANP or γ-ANP in the rat. These interesting features of β-ANP prompted us to study the two dimeric forms of α-ANP. We report here the synthesis of the human α-ANP dimers and their biological and immunochemical properties. The parallel and antiparallel dimers of α-ANP are tentatively designated as β′-ANP and β-ANP, respectively, in this communication.

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Abbreviations: Acm, acetamidomethyl; ANP, atrial natriuretic peptide; Bzl, benzyl; CM, carboxymethyl; Dcb, 2,6-dichlorobenzyl; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; HPLC, high-performance liquid chromatography; Mob, 4-methoxybenzyl; Pac, phenacyl; Tce, 2,2,2,-trichloroethyl; TFA, trifluoroacetic acid; Tos, tosyl; TPCK, L-1-tosylamido-2-phenylethyl chloromethylketone

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2. MATERIALS AND METHODS

2.1. *Materials*

Human α-ANP, α-ANP-(7-28), and α-ANP-(17-28) were synthesized in these laboratories. Human α-ANP was also obtained from the Peptide Institute, Osaka, Japan. Aminopeptidase M (EC 3.4.11.2) was purchased from Pierce and TPCK-treated trypsin (EC 3.4.21.4) from Worthington.

2.2. *Peptide synthesis*

Protected octacosapeptides (**5** and **6**) corresponding to the total amino acid sequence of α-ANP were synthesized by the conventional solution methods as shown in fig.1A. An equimolar mixture of **5** and **6** was treated with HF at 0°C for 60 min in the presence of anisole and methionine, followed by air-oxidation at pH 6.5 and 25°C. The resulting material was purified on a CM-cellulose (Whatman CM-52) and a reversed-phase (Wako RQ-2) column in the usual manner. Finally reversed-phase HPLC was carried out repeatedly on a Nucleosil $5C_{18}$ column (150 mm × 4.6 mm ID) using 26% acetonitrile in 0.1% TFA to separate three components (fig.1B), [Cys(Acm)^{7}, Cys(Acm)$^{7'}$]-β'-ANP (**7**), [Cys(Acm)^{23}, Cys(Acm)$^{23'}$]-β'-ANP (**8**), and [Cys(Acm)^{7}, Cys(Acm)$^{23'}$]-β-ANP (**9**), in their pure forms. For removal of the Acm groups [12] and concomitant oxidation, compound **9** was treated with iodine (45 mol/mol peptide) in 90% acetic acid at 23°C for 3 h in the presence of methionine (20 mol/mol peptide). After addition of ascorbic acid to quench the excess iodine, the mixture was evaporated in vacuo and the residue was subjected to HPLC with 26% acetonitrile in 0.1% TFA to give β-ANP in sufficient purity. In the same manner, β'-ANP was obtained from **8**.

2.3. *Bioassays*

2.3.1. In vitro smooth muscle relaxant activity

The assay was performed using spiral strips of isolated rat thoracic aorta suspended in a Krebs bicarbonate solution (95% O_2 and 5% CO_2) at 37°C. Contraction of the strips was induced with 50 mM KCl, and the percent relaxation was measured for various peptide concentrations, in which the relaxation obtained by 0.1 mM papaverine hydrochloride was taken as 100. Logarithmic dose-response curves obtained with 10^{-10}–10^{-6} M peptide solutions permitted the determination of ED_{50} values.

2.3.2. Diuretic and natriuretic activities

Male Sprague-Dawley rats (~350 g) were anesthetized with sodium pentobarbital (30 mg/kg i.p.), and then an intrafemoral vein catheter for infusion and a bladder catheter were installed. The animals received a constant infusion of 0.9% NaCl at a rate of 0.15 ml/10 min per rat from 30 min before a single injection of peptide (0.1 ml/rat through the femoral vein catheter) and during the evaluation period. Urine was collected through the bladder catheter and simultaneous measurements of urinary volume [13] and electric conductivity were performed automatically. Urinary sodium and potassium were measured by flame photometry. The data were treated with the paired Student's t-test and given as mean ± SE.

2.4. *Radioimmunoassay*

Antisera were produced by immunizing Japanese white rabbits in the usual manner with α-ANP-(17-28) [14] or human α-ANP-(7-28) conjugated to bovine thyroglobulin (Sigma) by the carbodiimide method [15]. ^{125}I-labelled human α-ANP was prepared by the chloramine T method [16] and purified by HPLC. A 0.1 mM phosphate buffer (pH 7.0) containing 0.1% bovine serum albumin, 1 mM Na_2EDTA, and 0.01% sodium azide was used as the assay buffer. A mixture of standard human α-ANP or sample (0.1 ml), human ^{125}Iα-ANP (0.1 ml), a properly diluted antiserum (0.1 ml), and the assay buffer (0.2 ml) in polystyrene tubes was incubated at 4°C for 20 h. To this were added non-immune rabbit serum (1:20, 0.05 ml) and then second antiserum (goat anti-rabbit IgG antiserum, MBL, Nagoya, Japan; 1:60, 1 ml) containing 6% polyethyleneglycol 6000. The mixture was incubated at 4°C for 60 min to separate bound and free ligands. Centrifugation at 2000 × g for 20 min was followed by counting the radioactivity of the precipitates.

3. RESULTS AND DISCUSSION

In the present synthesis of two dimeric forms of human α-ANP, protected peptides **5** and **6** cor-

responding to the 28-amino acid sequence of the monomer were built up from four fragments **1**–**4** (fig.1A). These fragments were synthesized basically following a step-by-step strategy, in which the two selectively removable sulfhydryl protecting groups, Mob and Acm, were used alternatively to protect the two cysteine residues (positions 7 and 23 in α-ANP) as shown in fig.1B. This made it possible to form two disulfide crosslinks between two molecules of the monomer as desired. The resulting dimers β-ANP and β'-ANP were found to be sufficiently pure by amino acid analysis (table 1) and analytical HPLC. HPLC separation of α-ANP, β-ANP and β'-ANP is shown in fig.2.

To confirm the structure of the synthetic peptides, tryptic digests of β-ANP and β'-ANP were compared with that of human α-ANP in HPLC. As shown in fig.3, β-ANP but not β'-ANP gave a peptide map identical with that derived from α-ANP, clearly indicating that β-ANP is the antiparallel dimer and β'-ANP is the parallel dimer of α-ANP, as expected.

Fig.4 shows the time course of the smooth muscle relaxant activity in rat aorta at a peptide concentration of 3×10^{-8} M. The slower response to the dimeric peptides than to the monomer is noteworthy. From the data obtained for various peptide concentrations (not shown), ED_{50} values of α-ANP, β-ANP and β'-ANP were estimated to be 8.4×10^{-9} M, 1.6×10^{-8} M and 1.7×10^{-8} M, respectively ($N = 4$).

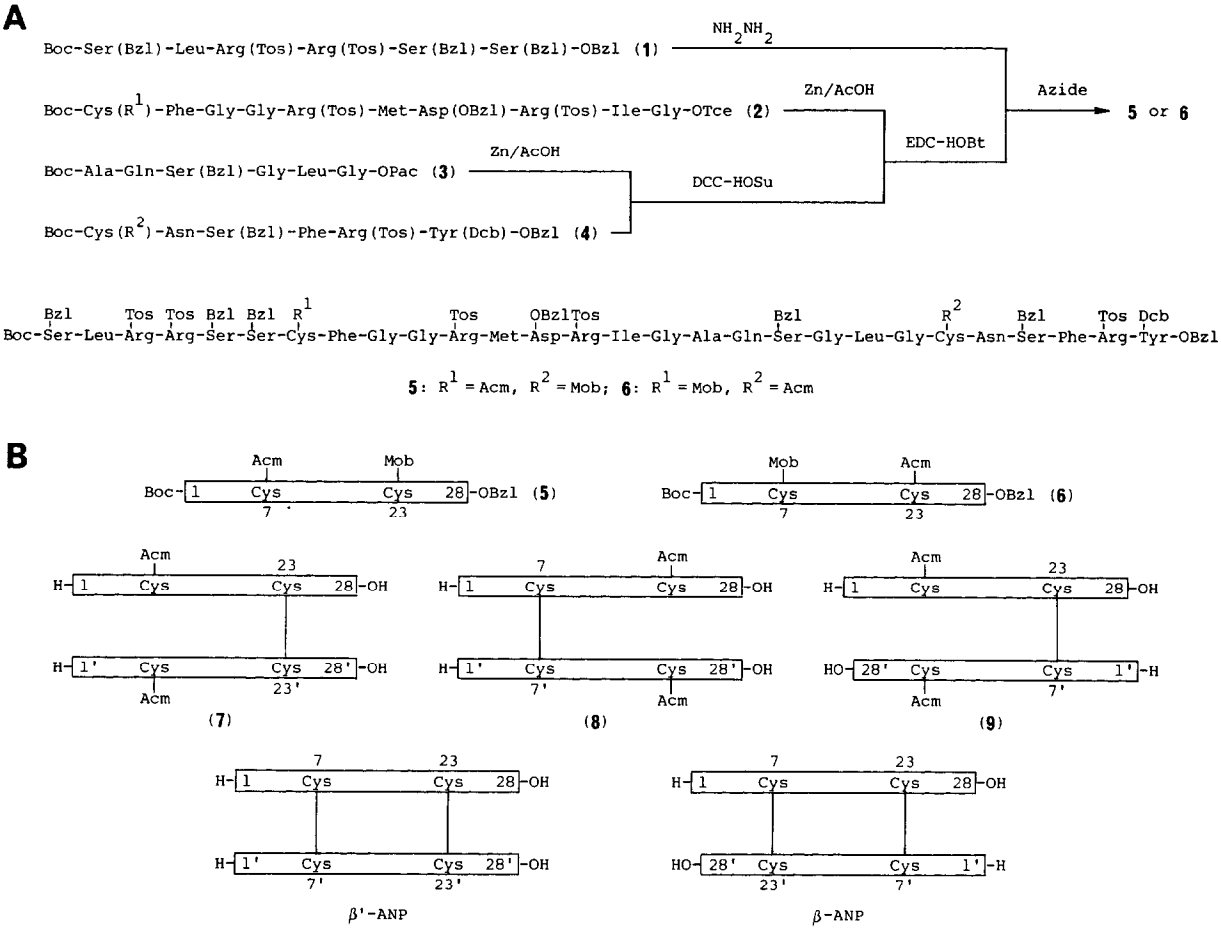

Fig.1. (A) Synthesis of octacosapeptide derivatives (**5** and **6**) as intermediates to the parallel dimer (β'-ANP) and antiparallel dimer (β-ANP) of α-ANP. (B) Schematic illustration of β-ANP, β'-ANP, and their synthetic intermediates.

Table 1

Amino acid ratios in aminopeptidase M digests (APM)[a] and acid hydrolysates (Acid)[b] of β-ANP and β'-ANP

Amino acid	β-ANP		β'-ANP	
	APM	Acid	APM	Acid
Asp	2.06(2)[c]	4.01(4)	2.04(2)	4.01(4)
Ser	9.89(14)	7.84(10)	9.92(14)	7.75(10)
Glu	–	2.21(2)	–	2.31(2)
Cit	9.42(10)	–	9.00(10)	–
Gly	9.63(10)	10.00(10)	9.46(10)	10.01(10)
Ala	2.18(2)	2.17(2)	2.23(2)	2.23(2)
$(Cys)_2$	1.74(2)	nd	1.57(2)	nd
Met	2.00(2)	2.03(2)	1.96(2)	2.04(2)
Ile	2.30(2)	2.05(2)	2.36(2)	2.07(2)
Leu	4.00(4)	4.00(4)	4.00(4)	4.00(4)
Tyr	2.11(2)	2.01(2)	2.16(2)	2.02(2)
Phe	3.75(4)	3.99(4)	3.70(4)	4.00(4)
Arg	nd[d]	9.76(10)	0.72	9.69(10)

[a] For enzymatic hydrolysis the peptide (2 nmol) in 25 mM phosphate buffer (pH 7.0, 40 μl) was treated with trypsin (2 μg) at 37°C for 3 h and aminopeptidase M (2 μg) at 37°C for 21 h, successively. Glutamine and asparagine were coeluted with serine. These three amino acids were therefore estimated as serine. Arginine was mostly converted into citrulline (Cit) owing to the presence of an arginine oxidase in the enzyme preparation used

[b] Acid hydrolysis was performed with 6 M HCl at 110°C for 20 h in the presence of phenol

[c] Theoretical values are given in parentheses

[d] nd, not determined

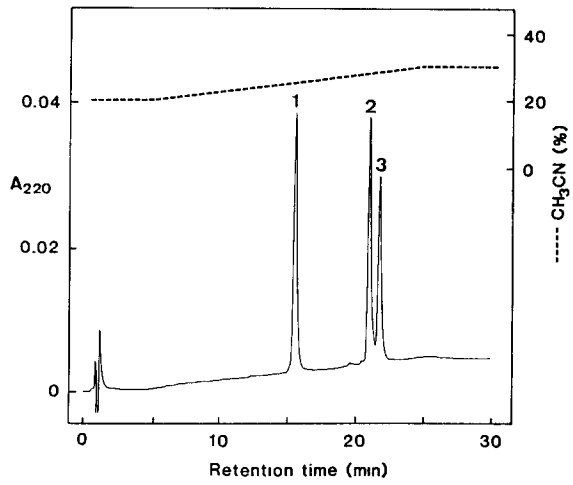

Fig.2. HPLC separation of α-ANP (1), β-ANP (2), and β'-ANP (3). Column: Nucleosil $5C_{18}$, 150 mm × 4.6 mm ID; mobile phase: generated by mixing solution A (0.1% TFA in water) and solution B (0.1% TFA and 50% acetonitrile in water), 1.8 ml/min; detection: 220 nm (range 0.08). The column was eluted with an acetonitrile concentration of 20% for 5 min and then eluted with a linear gradient of 0.5%/min to a final concentration of 30%.

The time courses of diuretic and natriuretic activities of α-ANP and β-ANP in rats are shown in fig.5. In response to a single injection of α-ANP (1 μg/rat), urinary volume and Na^+ excretion immediately increased to a maximum and then decreased to a control level in 20 min. When β-ANP (3 μg/rat) was injected, however, the diuretic and natriuretic responses appeared more slowly and lasted for longer periods of time (fig.5). A separate experiment also showed that β-ANP (3 μg/rat) and β'-ANP (3 μg/rat) had identical time courses (not shown). Independent administration of α-ANP (1 μg or 0.3 nmol/rat) or β-ANP (3 μg or 0.5 nmol/rat) increased urinary volume by 1.67 ± 0.31 ml and 2.47 ± 0.39 ml, respectively, and Na^+ excretion by 263 ± 43 μEq and 225 ± 83 μEq, respectively, in 60 min after injection (N = 7). In another experiment, β-ANP (3 μg/rat) and β'-ANP (3 μg/rat) increased urinary output by 1.59 ± 0.37 ml and 1.36 ± 0.52 ml, respectively, and Na^+ excretion by 185 ± 53 μEq and 206 ± 55 μEq, respectively, in 60 min after injection (N = 8). These results indicate that there is no significant difference between β-ANP and β'-ANP with respect to their diuretic-natriuretic activities. β-ANP and β'-ANP seem to be somewhat less active than α-ANP when compared on a molar basis, but the diuretic and natriuretic responses induced by these dimeric forms exhibited a slower onset and longer duration than those induced by monomeric α-ANP. Kangawa et al. [10] have reported that a second component of ANP in human atrium named β-hANP [9] is an antiparallel dimer of human α-ANP and induces protracted diuretic-natriuretic responses in rats. The general similarity of our present observations with those reported by Kangawa et al. [10] strongly suggest that the synthetic β-ANP is identical with natural β-hANP.

The immunoreactivity of β-ANP and β'-ANP with ANP-specific antibodies was compared on a

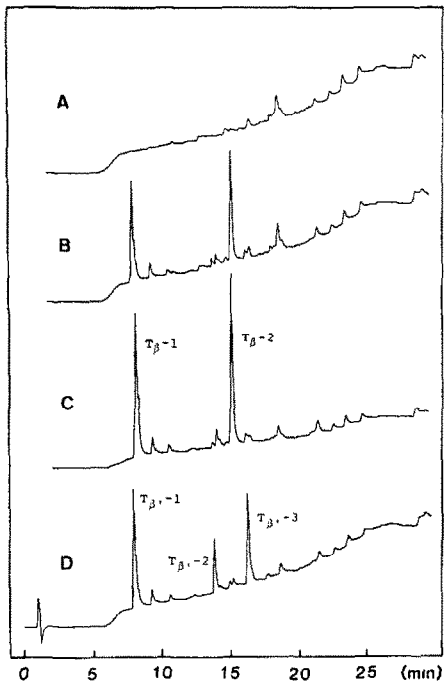

Fig.3. HPLC profiles of tryptic hydrolysates of α-ANP and its dimers. (A) Blank (no peptide), (B) hydrolysate of α-ANP, (C) hydrolysate of β-ANP, and (D) hydrolysate of β'-ANP. For HPLC conditions, see the legend of fig.2. The column was eluted with a linear gradient of acetonitrile concentration from 0 to 50% at a rate of 2.5%/min followed by isocratic elution for 10 min. Tryptic peptides isolated from peaks T_β-2, $T_{\beta'}$-2 and $T_{\beta'}$-3, were found to contain the Cys^7-$Cys^{23'}$, Cys^7-$Cys^{7'}$, and Cys^{23}-$Cys^{23'}$ linkages, respectively. Both T_β-1 and $T_{\beta'}$-1 contained tyrosine only.

molar basis with that of α-ANP using three different antisera F045, F054, and F069 (table 2). Although β-ANP and β'-ANP may have two equivalent antigenic sites within their molecules, they do not seem to bind more than one antibody molecule. This and some conformational differences at or near the antigenic sites may explain the different immunoreactivities among α-ANP, β-ANP, and β'-ANP.

In the present work we were able to synthesize the two dimeric forms of α-ANP and to show for the first time that these dimers were equipotent and significantly longer-acting than the monomer in their diuretic-natriuretic responses, although the mechanism of prolongation of action remains to be elucidated.

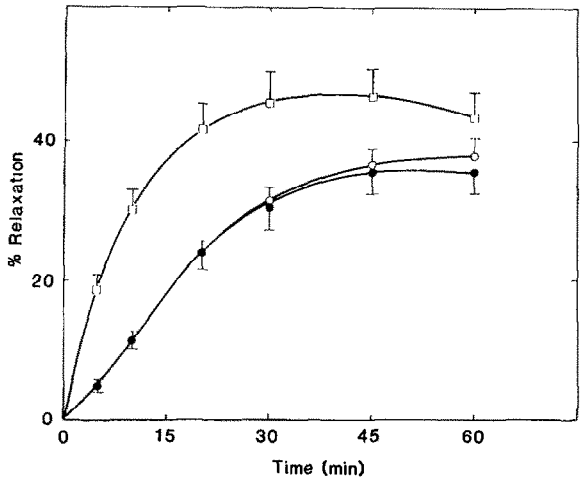

Fig.4. Time course of smooth muscle relaxant activity of α-ANP (□), β-ANP (●), and β'-ANP (○) in isolated rat aorta (N = 4). Peptide concentration: 3×10^{-8} M.

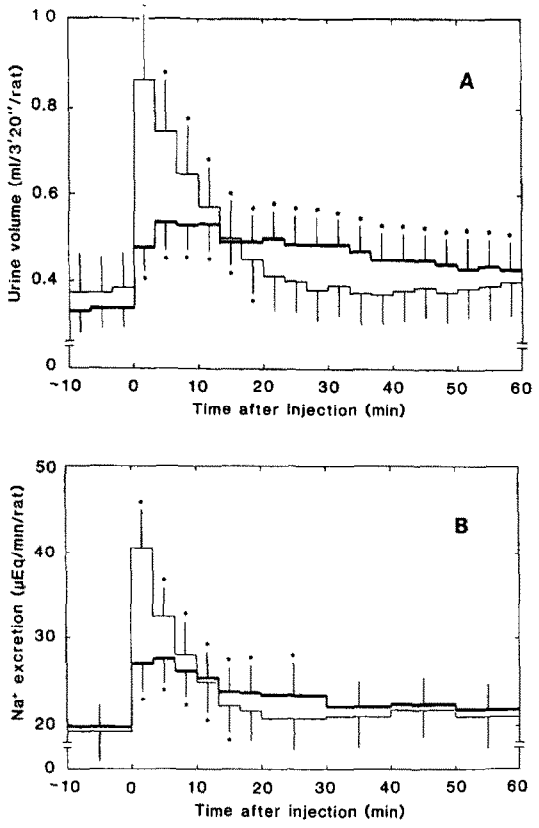

Fig.5. Time course of diuretic and natriuretic activities of α-ANP and β-ANP in rats (N = 7). Urine volume (A) and Na^+ excretion (B) were measured for α-ANP (1 μg/rat, light lines) and β-ANP (3 μg/rat, bold lines). Asterisks represent $p < 0.05$.

Table 2

Crossreactivity of peptides related to human α-ANP as estimated with antisera F045, F054 and F069

Peptide	% crossreactivity (α-ANP = 100)[a]		
	F054	F045	F069
α-ANP	100	100	100
β-ANP	60	93	59
β'-ANP	49	88	50
α-ANP-(7-28)	100	100	130
α-ANP-(17-28)	98	<0.3	41

[a] Calculated from peptide concentrations yielding 50% displacement of ^{125}I-α-ANP on a molar basis

F054 is an antiserum raised against an α-ANP-(17-28)-bovine thyroglobulin conjugate and recognizes the C-terminal portion of the α-ANP molecule. F045 and F069 are antisera raised against a human α-ANP-(7-28) conjugate. F045 mainly recognizes the ring structure, while F069 recognizes almost the whole molecule of α-ANP-(7-28)

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