Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 338-344

SYNTHESIS OF ATRIAL NATRIURETIC PEPTIDES AND STUDIES ON STRUCTURAL FACTORS IN TISSUE SPECIFICITY

M. Sugiyama*, H. Fukumi, R.T. Grammer, K.S. Misono, Y. Yabe*, Y. Morisawa*, T. Inagami

Department of Biochemistry, Vanderbilt University, Nashville, TN 37232
*Chemical Research Laboratories, Sankyo Co. Ltd., Shinagawa, Tokyo 140, Japan

Received July 20, 1984

Two atrial natriuretic peptides, containing 25 amino acid residues, ANF IV, and 21 amino acid residues, ANF V, were synthesized by a solid phase method and oxidized with $K_3Fe(CN)_6$ to form a disulfide bridge. Synthetic ANF IV exhibited a natriuretic activity with an ED_{50} 70 times higher than that of synthetic ANF V, whereas the longer peptide was only 2.5 times more potent in chick rectal smooth muscle relaxant activity. Both peptides inhibited norepinephrine-induced contraction of rabbit aorta. The shorter peptide, ANF V, was 300 times less efficient than the longer peptide, ANF IV. It is proposed that the carboxy-terminal of ANF IV seems to have a modulating effect on receptor affinity in kidney and vascular tissue.

Atrial extract contains peptides which cause marked natriuresis and diuresis (1) and relaxes norepinephrine- (2) or angiotensin II-(3) induced vascular smooth muscle tone or carbacol-induced contraction of chick rectal smooth muscle (2). We have shown that purified peptides, designated atrial natriuretic factor (ANF), possess both the relaxant and natriuretic activity (4). The amino acid sequences of rat ANF have been determined (5,6,7,8,9,10). ANF peptides with widely varying molecular weights were shown to be a family of related peptides, derived by limited proteolytic degradation. Currie et al. (7) have reported that atriopeptin I (ANF V) (H_2N•S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S•COOH) with 21 amino acids exhibits natriuretic activity and relaxes chick rectal smooth muscle but not aortic smooth muscle strips. On the other hand, ANF IV (H_2N•R-S-S-C-F-G-G-R-I-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y•COOH) with 25 amino acids purified by us (5) exhibits the aortic smooth muscle relaxant activity as well as the natriuretic, diuretic and intestinal smooth muscle relaxant activities. In this report a synthetic approach was initiated to identify the structural determinants for the various biological activities of ANF and to establish reference standards for the synthesis of ANF. We have synthesized two natriuretic pep-

0006-291X/84 $1.50

tides, one with a complete carboxyl terminal sequence found in most of the reported ANF peptides (5,11) and the other with a minimum of the carboxyl terminal structure, and compared their biological activities to determine the role of these portions of the peptides. Synthetic peptide ANF IV was compared with native ANF IV of identical structure to validate the synthetic methodology. Since no detail (7,8,9) on the synthesis of rat ANF peptides with a macro ring structure has been published, the present work is intended to serve as an initial step of and validation for the synthetic approach to the study of ANF.

METHODS AND PROCEDURES

Peptide synthesis was performed essentially by the solid phase method of Merrifield (12), according to Stewart and Young (13) with the following modifications in a Beckman Model 990B peptide synthesizer. The functional groups of amino acids used in the synthesis were protected by the following groups: N^{α}-amino groups by t-Boc- except Aoc- for arginine, O-benzyl- for the the β-hydroxyl group of serine, 4-methoxybenzyl- for the sulfhydryl group of cysteine, benzyl ester for the β-carboxyl group of aspartic acid, 2,6-dichlorobenzyl- for the 4-hydroxyl group of tyrosine, and tosyl- for the guanidino group of arginine. After the reaction of t-Boc amino acid residue with resin (14), the coupling of subsequent residues was done using dicyclohexylcarbodiimide (0.3M) in methylene-cholride for 60 min followed by an additional 60 min reaction in the presence of 0.05 M dimethylaminopyridine. Boc-Asn, Boc-Leu, Boc-Gln, Boc-Ile, and Aoc-Arg(Tos) were first dissolved in 1 to 10 ml of dimethylformamide, then made up to 10 ml with methylene chloride. If the coupling was incomplete by the ninhydrin test of Kaiser (15), additional reaction was carried out using hydroxybenzotriazole (2.5 equivalents) in 2 ml of dimethylformamide and the mixture was allowed to stand for 3 to 6 hrs. Small amounts of free amino groups still remaining after the additional reaction were blocked by treatment with fluorescamine or the symmetric anhydrides of the α-N-protected amino acids (16) used for a given coupling stage.

The protected peptide-resin (2.0 g) was treated with HF in the presence of 10% (v/v) anisol at 0°C for 1 hr to release free peptide. After evaporating HF under vacuum and washing with ethyl acetate, the peptide was extracted in 2N acetic acid (200 ml) and the extract was diluted to 2 l (5×10^{-5} M peptide) with distilled water. The mixed solution was adjusted to pH 7 with dilute ammonium hydroxide, and 0.01 N $K_3Fe(CN)_6$ solution was added slowly at room temperature to form an intramolecular disulfide linkage. The solution was allowed to stand for 1 hr at room temperature, the pH was adjusted to 5.0, and 8 grams of BioRad AG3-X4A resin (Cl-form) was added. The mixture was allowed to stand for 30 min and filtered. The filtrate was concentrated under vacuum to a small volume. Peptides in the concentrate were separated from salt by gel filtration on a column (2.5 x 96 cm) of Sephadex G-25 in 10% acetic acid. The peptide-containing fractions were pooled, freeze-dried and further purified by ion-exchange chromatography on an SP-Sephadex C-25 column (2 x 18.5 cm) with a concentration gradient generated between 300 ml each of 0.01 M ammonium acetate and 0.15 ammonium acetate, pH 6.1 (Fig. 1). Fractions under the third peak was pooled, freeze-dried, chromatographed on a column (2.5 x 96 cm) of Sephadex G-25 in 10% acetic acid and purified further by HPLC on an octadecyl column with acetonitrile gradient (10-40%). A single major peak was separated by repeating the chromatography under the same condition (Fig. 2). The homogeneity of the substance was confirmed by additional HPLC on a diphenyl (Vydac) and CN (Zorbax, DuPont) column.

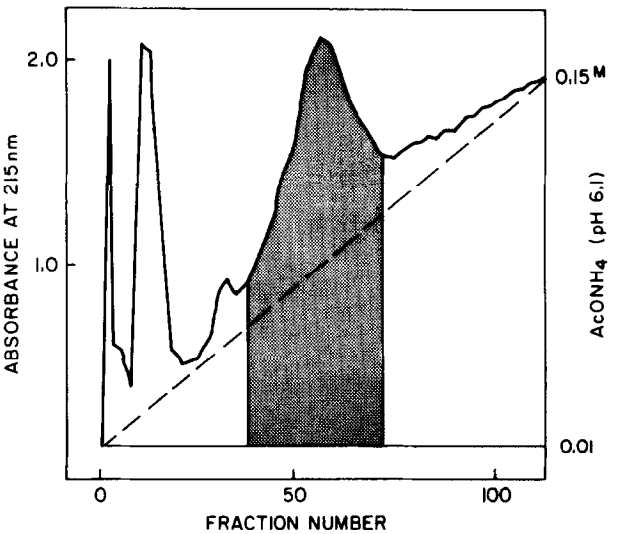

Figure 1: Ion exchange chromatography of synthetic ANF V on SP-Sephadex. Synthetic ANF IV showed the same pattern.

Biological Activity Natriuretic activity was determined by the method described previously (4,5) using urethane anesthesized female rats cannulated in the jugular vein and bladder. To reduce inter-animal variation the following method was used. All assay rats were injected with a standard dose of 1.0 mg furosemide per kg body weight prior to ANF injection. For dose-response studies the total natriuretic response (μEq Na^+) of each rat to a given dose of the ANF was divided by the total response to the standard dose of furosemide. The smooth muscle relaxant activity was determined by the inhibition of norepinephrine induced contraction of aortic strips and carbacol elicited contraction of chick rectum in tissue both containing Krebs-Henseleit buffer with 95% O_2 5% CO_2 as described previously (4,5).

Amino acid composition was determined with reduced and carboxymethylated ANF peptide after 24 hr hydrolysis in 6 N HCl. Amino acid sequence was determined by Edman degradation of the performic acid oxidized ANF peptides. PTH-amino acids were determined by HPLC as described previously (5).

RESULTS AND DISCUSSION

Two ANF peptides were synthesized. The 25-residue peptide corresponds to ANF IV which we had isolated as the smallest of the four major ANF peptide species from acid extract of rat atrium (11). The 21-residue ANF V (atriopeptin I) corresponds to the smaller peptide isolated by Currie et al. (7). Each synthetic peptide was purified by gel filtration, ion-exchange chromatography and reverse phase HPLC (Fig. 2A-B) to homogeneity. The overall yields of synthesis, calculated from the amino acid analyses of the initial resin coupled with the first residues and the final purified peptides, were 13.7% and 15.5% for ANF IV and ANF V, respectively. Amino acid analysis (Table I) showed correct compo-

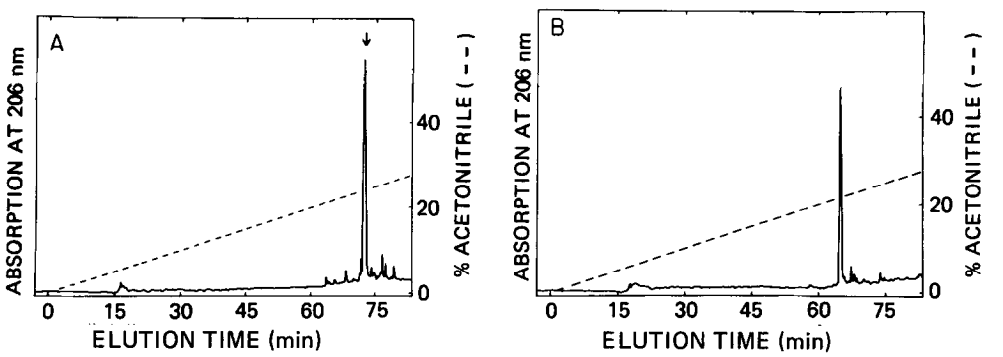

Figure 2: HPLC chromatography of synthetic ANF peptides on a C_{18} reverse phase column.
(A) Synthetic ANF IV. Arrow shows the elution position of native ANF IV.
(B) Synthetic ANF V.

sitions of these peptides. The amino acid sequences were confirmed by automated Edman degradation of performic acid-oxidized peptides.

The comparison of the dose response curves of aortic relaxant activities of the native (5) and synthetic ANF IV indicated that the two curves are essentially superimposable (Fig. 3A). Similarly, inhibition of carbacol-induced chick rectum contraction (Fig. 3B) and natriuretic responses by the synthetic and native ANF IV showed practically identical responses.

The availability of two synthetic ANF differing in the carboxyl terminal structure and the amino terminal arginyl residue allowed us to evaluate the role of these terminal structures of ANF. The 21 residue ANF V is less efficient than

Table I

Amino acid composition of synthetic natriuretic peptides

Amino acid	ANF IV Residues/mol	ANF V Residues/mol	Amino acid	ANF IV Residues/mol	ANF V Residues/mol
Cys/2[a]	2.0 (2)	1.8 (2)	Met[b]	0	0
Asp	2.1 (2)	1.9 (2)	Ile	1.4 (2)	1.9(2)
Thr	0	0	Leu	1.0 (1)	1.0 (1)
Ser	3.7 (4)	3.6 (4)	Tyr	0.6 (1)	
Glu	1.0 (1)	1.0 (1)	Phe	1.7 (2)	1.0 (1)
Pro	0	0	His	0	0
Gly	4.6 (5)	4.9 (5)	Lys	0	0
Ala	1.0 (1)	1.0 (1)	Arg	3.4 (4)	1.8 (2)
Val	0	0	Trp	N.D.[c]	N.D.[c]

[a] Half-cystine was determined as cysteic acid. [b] Methionine was determined as methionine sulfone. [c] Not determined.

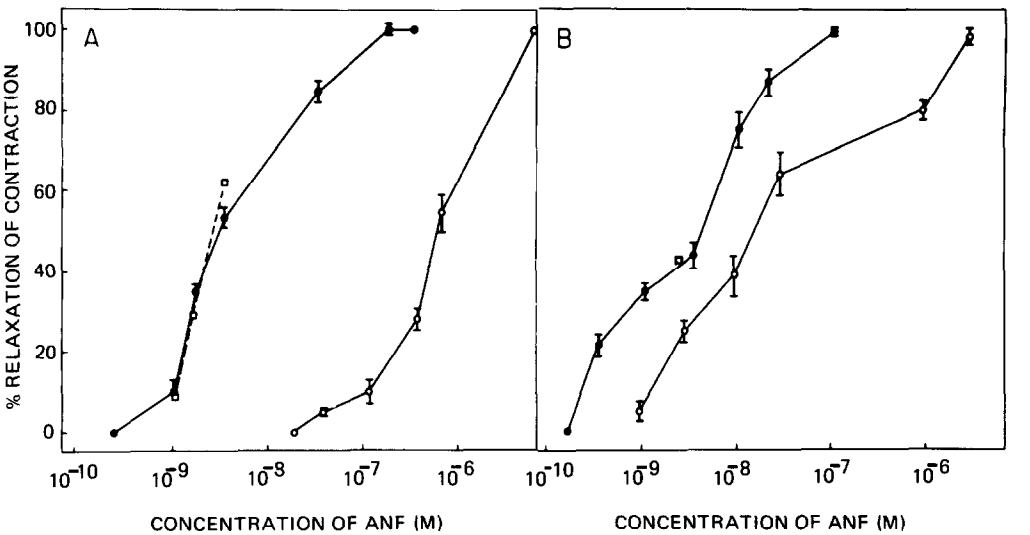

Figure 3: Dose response curve for synthetic ANF peptides.
(A) Rabbit strip relaxant activity of synthetic ANF IV (●—●), synthetic ANF V (o—o), and native ANF IV (□--□).
(B) Chick rectum relaxant activity of synthetic ANF IV (●—●), synthetic ANF V (o—o), and native ANF (□).
Responses are plotted as means ± SE.

the 25 residue ANF IV in inhibiting the carbacol-induced chick rectal contraction (11 nM vs 4.4 nM) with a 2.5-fold difference in half maximum concentration in the dose-inhibition curve (Fig. 3B). The half-maximum inhibition of norepinephrine-induced aortic contraction ANF V (600 nM) is approximately 300 times higher than the longer ANF (2.1 nM) (Fig. 3A). Also, recovery from inhibition of ANF V is much faster than the 25 residue peptide. Currie et al. reported that atriopeptin I, had no ability to inhibit norepinephrine induced aortic contraction (7). The explanation for such a discrepancy may be found in the different assay methods. Currie et al. used tissue strips in a cascade of bathing medium whereas our studies kept aortic strips bathed in the medium in an aerated flask.

The half-maximal natriuretic response for ANF IV was elicited by 0.9 μg (320 pmol) while that for ANF V was ca. 49 μg (23.5 nmol), a 70-fold difference (Fig. 4). This difference again indicates that the structural differences between ANF IV and ANF V have significant biological consequences.

Thus, in these studies we have developed methods for the synthesis of ANF peptides with the biologically essential ring structure (5) and shown that the

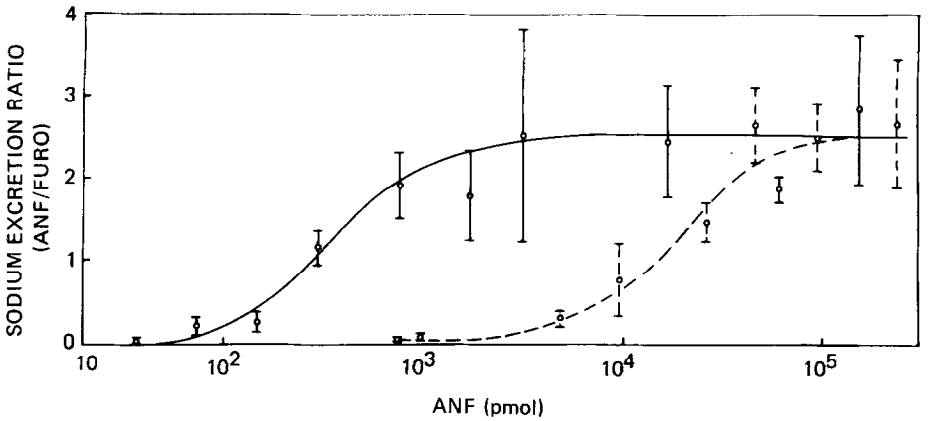

Figure 4: Natriuresis dose-response curves for synthetic ANF peptides. Responses are plotted as means ± SE. ANF IV, (———), ANF V, (— — —).

synthetic ANF IV is indistinguishable from the corresponding native peptide in its chemical and biological properties. Moreover, we have found significant differences in natriuresis and vasorelaxation between ANF IV and ANF V, indicating that the carboxyl-terminal tripeptide sequence of ANF IV, or less likely the amino-terminal arginine, modulates the binding of the peptide to its receptor and/or its potency in physiological actions.

ACKNOWLEDGMENTS

We acknowledge the skillful technical assistance of William Burkhart, Edward Price, Jr., and Rita Smith. We thank Drs. H. Nakao and H. Koike of Sankyo Co. Ltd. for valuable discussion. This work was supported by Research Grants HL-14192, HL-22288 from NIH, 84-1291 and from American Heart Association. R.T.G. and K.S.M. are Investigators of Tennessee Affiliate of the American Heart Association.

REFERENCES

1. deBold, A.J., Borenstein, H.B., Veress, A.T., and Sonnenberg, H. (1981) Life Sci. 28, 89-94.
2. Currie, M.G., Geller, D.M., Cole, B.R., Boylan, J.G., Yu Sheng, W., Holmberg, S.W., and Needleman, P. (1983) Science 221, 71-73.
3. Kleinert, H.D., Maack, T., Atlas, S.A., Januszewica, A., Sealey, J.E., and Laragh, J.H. (1984) Hypertension 6 (Supple 1), I-143-I-147.
4. Grammer, R.T., Fukumi, H., Inagami, T. and Misono, K.S. (1983) Biochem. Biophys. Res. Commun. 116, 696-703.
5. Misono, K.S., Fukumi H., Grammer, R.T., and Inagami T. (1984) Biochem. Biophys. Res. Commun. 119, 524-529.
6. Flynn, T.G., deBold, M.L., and deBold, A.J. (1983) Biochem. Biophys. Res. Commun. 117, 859-865.
7. Currie, M.G., Geller, D.M., Cole, B.R., Siegel, N.R., Fok, K.F., Adams, S.P., Galluppi, G.R., and Needleman, P. (1984) Science 223, 67-69.
8. Seidah, N.G., Lazure, C., Chretien, M., Thibault, G., Garcia, R., Cantin, M., Genest, J., Nutt, R.F., Brady, S.F., Lyle, T.A., Paleveda, W.J., Colton,

C.D., Ciccarone, T.M., and Veber, D.F. (1984) Proc. Natl. Acad. Sci. USA 81, 2640-2644.
9. Atlas, S.A., Kleinert, H.D., Camargo, M.J., Januszewicz, A., Sealey, J.E., Laragh, J.H., Schilling, J.W., Lewick, J.A., Johnson, L.K., and Maack, T. (1984) Nature 309, 717-719.
10. Kangawa, K., Fukuda, A., Kubota, I., Hayashi, Y., and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 121, 585-591.
11. Inagami, T., Misono, K.S., Maki, M., Fukumi, H., Takayanagi, R., Grammer, R.T., Tibbetts, C., Pandey, K.N. (1984) J. Hypertension (in press).
12. Merrifield, R.B. (1964) Biochemistry 3, 1385-1390.
13. Stewart, J.M., and Young, J.D., (1969) Solid Phase Peptide Synthesis, Freeman, W.H., & Co., San Francisco.
14. Yamashiro, D., and Li, C.H. (1973) J. Am. Chem. Soc. 95, 1310-1315.
15. Kaiser, E., Colescott, R.L., Bossinger, C.D., and Cook, P.I. (1970) Anal. Biochem. 34, 595-598.
16. Blake, J., and Li, C.H. (1975) Int. J. Pept. Protein Res. 7, 495-501.