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RAT ATRIAL NATRIURETIC FACTOR. PURIFICATION AND VASORELAXANT ACTIVITY

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The atrial natriuretic activity of rat heart has been found to exist in multiple forms. One of these factors has been purified to apparent homogeneity by a combination of gel filtration and high pressure liquid chromatography in two different systems and its amino acid composition determined. The purified active peptide is shown to have a molecular weight of approximately 3800. In addition, the vasorelaxant activity of rat atrium has been purified and found to co-chromatograph with the natriuretic activity in all chromatographic systems employed. Thus, the vasorelaxant activity resides in the natriuretic factor. The existence of this new multifunctional peptide implies a higher level of complexity for cardiovascular control of blood volume and pressure.

A wide range of physiological experiments has indicated the existence of humoral factors which stimulate natriuresis and diuresis (1-3). Extracts of heart atrial, but not ventricular, tissue have been shown to exhibit natriuretic and diuretic activity (4-8), and studies of these crude extracts have indicated their site of action to be the kidney (7-10). Partial purification and relative dose-response relationships of the substance exhibiting the natriuretic activity, termed atrial natriuretic factor (ANF)[1], have been reported (11-13).

In addition, atrial tissue has also been found to be the source of vasorelaxant substances (14) which, taken together with the existence of ANF, suggests an endogenous system for the control of fluid volume and vascular function.

It has not been clear whether these different physiological responses are elicited by separate, chemically distinct substances or by a single multifunctional factor. In the work reported here, we have sought to clarify our understanding of the role of the atrium in regulating fluid volume and vascular function by the purification of its natriuretic and vasorelaxant factors.

Abbreviations: ANF, atrial natriuretic factor; PBS, 0.02 M potassium phosphate, pH 7.2, 0.9% NaCl; NE, norepinephrine.

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METHODS

Purification of rat atrial natriuretic factor Atria excised from 400 adult Sprague-Dawley rats were extracted twice with 150 ml of 1.0 M acetic acid containing 50 mM triethylamine and 10 μM pepstatin by homogenization in a Polytron homogenizer with the setting at 7 for 1 min followed by centrifugation at 100,000 x g for 30 min. The supernatants were combined and lyophilized. The freeze-dried material was suspended in 50 ml of the same buffer. Boiling water (four volumes) was added to the suspension which was then heated in a boiling water bath for 15 min. After cooling in an ice-bath, the precipitate was removed by centrifugation and the supernatant lyophilized. The lyophilized material was dissolved in 50 ml of 0.1 M acetic acid and, after removing insoluble material by centrifugation at 100,000 x g for 30 min, was chromatographed on a column of Sephadex G-50 (5 x 90 cm) in 0.5 M acetic acid containing 10 mM triethylamine and 1 μM pepstatin (Fig. 1). Fractions 110-139, containing both natriuretic activity and smooth muscle relaxant activity, were pooled and lyophilized. The material was dissolved in 10 ml of 20 mM sodium phosphate, pH 8.0, and applied to a column of DEAE-cellulose (1 x 8 cm) equilibrated with the same phosphate buffer. The pass-through fraction and wash fraction (two column volumes of the same buffer) were combined and lyophilized.

The material was dissolved in a small volume of water and chromatographed on a SynChropak ODS column (0.46 x 25 cm, SynChrom) using a linear gradient of acetonitrile (0-40%) in 0.1% trifluoroacetic acid over 2 h. Both natriuretic and smooth muscle relaxant activities were concentrated in the fractions eluted with retention time from 39 to 48 min, which were pooled and lyophilized. The material was dissolved in 0.1 M acetic acid and rechromatographed on a SynChropak ODS column (0.46 x 25 cm) under the conditions shown in the legend to Fig. 2. The activities coeluted under two peaks with retention times of 39 min and 44 min, respectively. The material under the latter peak was chromatographed on a Zorbax CN column (0.46 x 25 cm, DuPont) as shown in Fig. 3. It was resolved into several UV-absorbance peaks among which two major peaks contained both natriuretic and vasorelaxant activity. The material under the first peak was re-chromatographed on the same column to remove minor contaminating material from the preceding peak (Fig. 4).

Natriuresis Assay Bioassay of natriuretic activity was performed on female Sprague-Dawley rats (175-225 g). Bladder and intrajugular vein catheters were installed under urethane anesthesia (1 g/kg i.p.). After equilibration for at least 15 minutes, urine was collected for three 10-minute control periods before sample injection (i.v., 0.2 ml in 2 min) and for successive 10-minute intervals after sample injection until the urine flow rate returned to control levels. During each collection interval PBS, 0.02 M potassium phosphate, pH 7.2, 0.9% NaCl, was injected (0.1 ml/min) in volumes calculated to maintain fluid balance. Sodium concentration was measured with a flame photometer. All values are reported as urinary sodium flow rate during the peak response period.

Aortic Strip Relaxation Assay Female New Zealand White rabbits weighing 1.9 to 3.2 kg were sacrified by injection of air into an ear vein. The thoracic aorta (2.5-5.0 mm in diameter) was quickly excised and helically cut at an angle of approximately 80° to the longitudinal axis into strips (15). One end of the strip was connected to a force-displacement transducer (Grass Instrument, FT .03), then immersed in a bathing solution (25 ml) consisting of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.68 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM dextrose (Krebs-Henseleit solution), which was maintained at 37±0.5°C and bubbled with a mixture of 95% O_2 and 5% CO_2. An initial resting tension of 1 g was applied to the strip. An appropriate amount of ANF was added to the bathing solution and 10 minutes later norepinephrine (NE) was added to 5×10^{-8} M. Percent suppression of NE-induced contraction was estimated in comparison with the contraction in the absence of ANF and was used as an index of vasorelaxant activity.

Chick Rectum Relaxation Assay White Leghorn chicks up to 7 days old were starved overnight, then sacrificed with ether. The rectum was quickly removed and washed

in Krebs-Henseleit solution. One end of the rectum was attached to the lever of a force-displacement transducer and a tension of 2 g applied. After carbachol (5 x 10^{-8}M) was added to the solution, relaxation of contraction was induced by addition of ANF.

Amino Acid Analysis was performed on the peptide oxidized with performic acid according to Hirs (16) and hydrolyzed in 6 N HCl containing 0.2% phenol at 110°C for 24 h.

RESULTS AND DISCUSSION

The purification of ANF was carried out by a combination of gel filtration, ion-exchange chromatography and HPLC in which both the natriuretic and vasorelaxant activities were monitored. The gel filtration profile (Fig. 1) was essentially in agreement with the results of others (11, 13). The active mate-

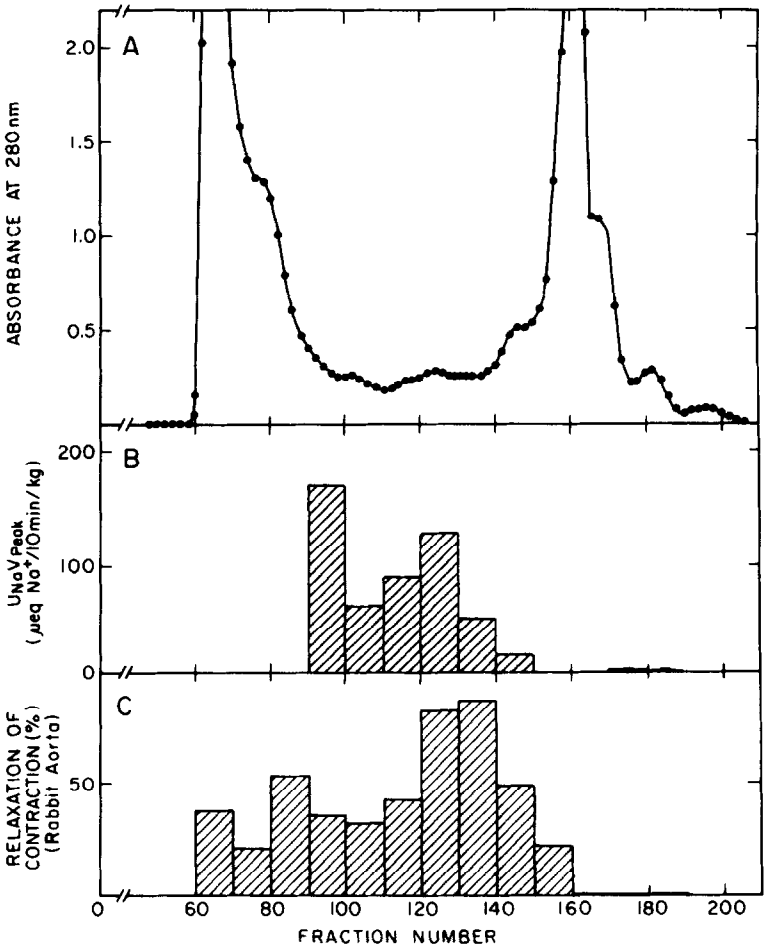

Figure 1. Gel filtration of rat atrial extract on Sephadex G-50 in 0.5 M acetic acid. Flow rate was 5 ml/cm^2/hr. A, Fractions of 11 ml were collected and A_{280} measured. Five ml aliquots from pools of 10 fractions were lyophilized, resuspended in 0.5 ml of PBS and centrifuged to remove insoluble material. B, Urinary sodium excretion was measured following injection of 0.2 ml aliquots. C, 20 μl was used for the assay of vasorelaxant activity toward contraction of rabbit aorta.

rial from the DEAE column was separated into two peaks exhibiting natriuretic and smooth muscle relaxant activities upon reverse-phase chromatography on the Syn-Chropak ODS column (Fig. 2). The latter of the two peaks was further separated into two active peaks by HPLC on a Zorbax CN column (Fig. 3). Thus, a number of different active peptides are present in rat atria. The first active peak on the cyanopropyl column induced a urinary sodium flow rate of 226 μEq Na^+/10 min-kg upon injection of 1.8 μg of peptide. This peak was re-chromatographed on the same column (Fig. 4) which revealed a single active peak with separation of a

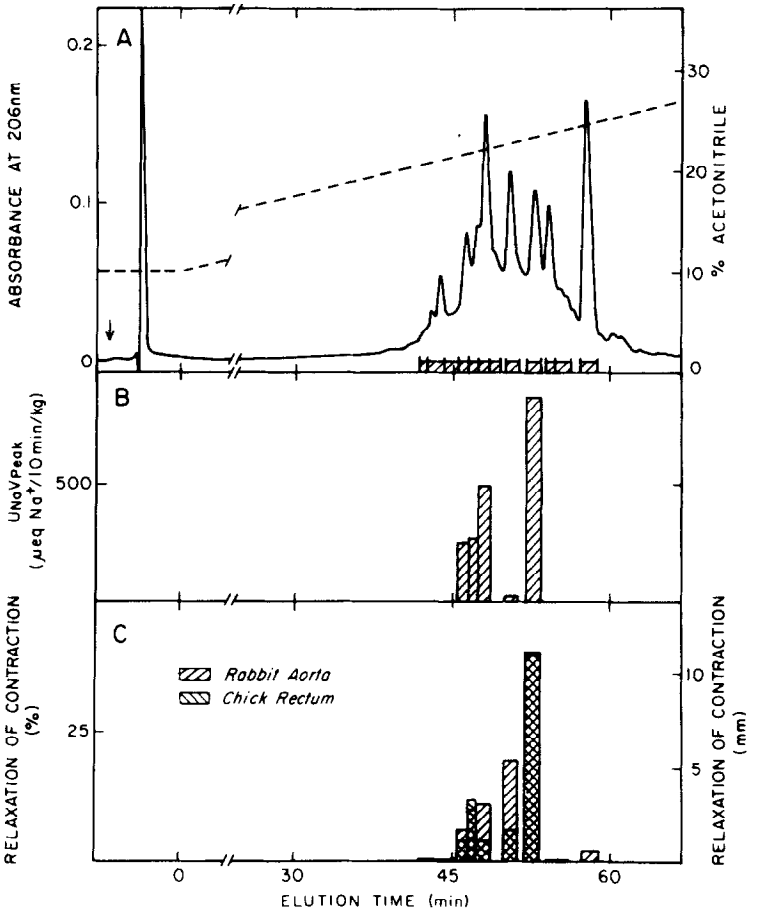

Figure 2. HPLC separation of ANF fraction on a Synchropak RP-P column. A, Elution was done by a linear gradient of acetonitrile (---) from 0 to 40% over 2 h in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Arrow indicates the time of injection. Fractions were collected manually as indicated by hatched bars. Fractions obtained from three similar HPLC separations were combined, lyophilized and dissolved in 550 μl of PBS. B, 400 μl was used for assay of natriuretic activity, and C, 1.1 μl aliquots were used for assay of relaxant activity toward contraction of rabbit aorta and 2.2 μl aliquots were used for relaxant activity toward contraction of chick rectum.

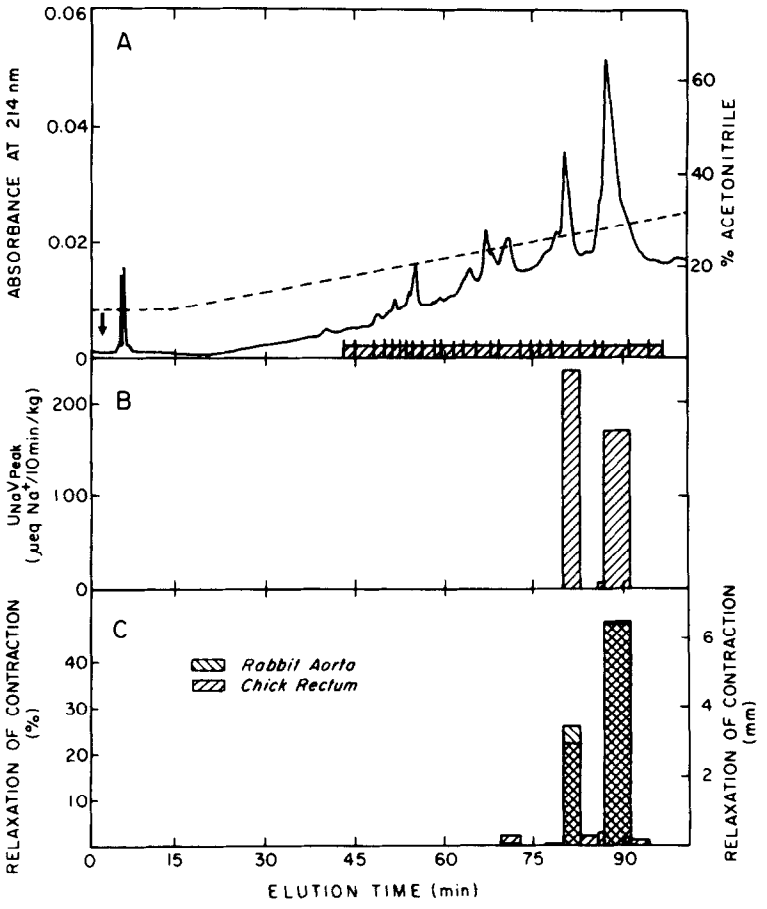

Figure 3. HPLC separation of the fraction from Synchropak RP-P column (Fig. 2) on a Zorbax CN column. A, Elution was done by a linear gradient of acetonitrile (---) from 10 to 40% over 2h in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Arrow indicates time of injection. Fractions were collected manually, as indicated by hatched horizontal bars, lyophilized and dissolved in 500 μl of PBS. B, 340 μl aliquots of the fractions were used for assay of natriuretic activity, and C, 40 μl aliquots was used for assays of relaxant activities toward contractions of rabbit aorta and chick rectum.

minor, inactive component. Thus, this active fraction exhibits apparent homogeneity upon Zorbax CN chromatography. A 20% inhibition of aortic strip contraction was induced by 0.14 μg of the final material. This material was subjected to amino acid analysis.

Based on the amino acid analysis and an approximate M_r estimated from the elution position in the Sephadex G-50, the amino acid composition of the peptide was calculated as shown in Table I. Using this composition, its molecular weight was calculated to be approximately 3800. The peptide contains a large number of

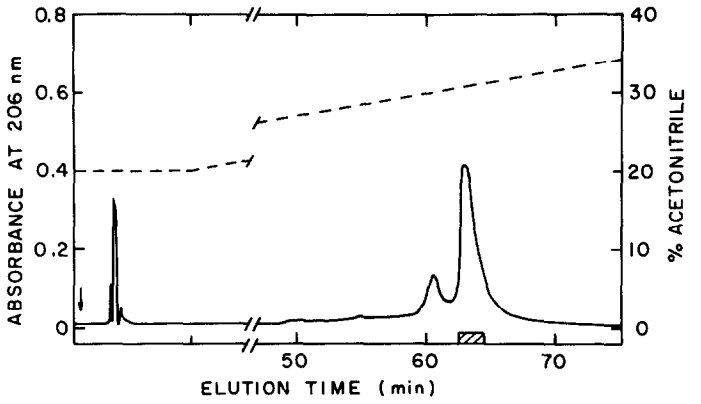

Figure 4. Rechromatography of material under the first activity peak from Zorbax CN column (Fig. 3). Chromatography was carried out on the same column, using a gradient of acetonitrile from 20% to 40% over 2h in 0.1% trifluoroacetic acid. Active peptide was collected as shown by the hatched bar. The preceding small peak did not contain activity.

arginine residues, consistent with its basic nature as revealed by its behavior at pH 8 on the DEAE-cellulose column. It contains 2 half-cystine residues. No methionine was found. Recently, the purification of a natriuretic peptide from rat atria was reported (17). That peptide has a larger molecular weight than that reported here, and its amino acid composition is substantially different. Its relationship to the peptide reported here cannot yet be determined.

Throughout these chromatographic separations, the natriuretic activity and smooth muscle relaxant activity toward norepinephrine-induced contraction of

Table I

Amino acid composition of atrial natriuretic factor from rat

Amino acid	Residues/ mole	Nearest Integer	Amino acid	Residues/ mole	Nearest Integer
Cys/2[a]	2.02	2	Met[b]	0	0
Asp	2.97	3	Ile	1.90	2
Thr	0.69	1	Leu	1.93	2
Ser	4.82	5	Tyr	0.86	1
Glu	1.94	2	Phe	1.84	2
Pro	0.93	1	His	0.63	1
Gly	6.03	6	Lys	1.41	1
Ala	2.26	2	Arg	3.85	4
Val	0.88	1	Trp	N.D.[c]	-

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

aortic strip and carbachol-induced contraction of chick rectum co-chromatographed, indicating that both activities are contained in the same set of peptides. Earlier studies provide evidence that the natriuretic activity of ANF is due to a direct effect on tubular sodium reabsorption rather than an effect on renal blood flow (4, 7-10). Thus, the multiple physiological responses elicited by ANF include suppression of the contraction of adrenergic vascular smooth muscle, suppression of the cholinergic contraction of chick rectum and inhibition of renal tubular sodium reabsorption. Whether these actions of ANF are mediated by a single mechanism common to all of the three or by different mechanisms is not clear. It is possible that ANF may affect calcium ion movement or cyclic nucleotide metabolism which may be common to these responses.

The demonstration of natriuretic and vasorelaxant activities within this new peptide and the location of its storage site near receptors sensitive to blood volume and salt concentration (18) strongly suggest a pivotal role for ANF in control of blood volume and pressure.

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