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RAT ATRIAL NATRIURETIC FACTOR: COMPLETE AMINO ACID SEQUENCE AND DISULFIDE LINKAGE ESSENTIAL FOR BIOLOGICAL ACTIVITY

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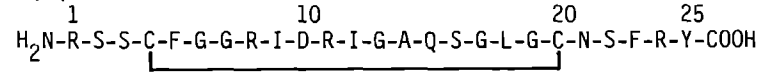
The complete amino acid sequence of an atrial natriuretic peptide from rat possessing both natriuretic and smooth muscle relaxant activity has been determined. The peptide has the structure



and a calculated molecular weight of 2,706. The ring structure formed by the disulfide linkage between the two half-cystine residues was found essential for both the natriuretic activity and smooth muscle relaxant activity. The purified peptide caused 50% relaxation of norepinephrine (5×10^{-8} M) induced contraction of rabbit thoracic aorta at the concentration of 2×10^{-9} M and complete relaxation at 6×10^{-9} M.

It has been known that stimulation of the baroreceptor of heart atrium by expanded thoracic blood volume results in increases in urinary flow and sodium excretion. The mechanism of this atrium-mediated fluid volume regulation has not been clear. Recently, de Bold has found that the atrium of mammalian heart contains a potent, rapid- and short-acting natriuretic factor stored in specific atrial granules (1,2). The factor, designated atrial natriuretic factor, was subsequently shown to inhibit sodium reabsorption in the medullary collecting duct of the kidney (3-5). In addition, the extract of the heart atrium has been found to cause relaxation of vascular smooth muscle contraction induced with norepinephrine (6). Partial purification and relative dose-response relationships of these substances have been reported (7-9). Recently, we have demonstrated by purifying atrial peptides that the natriuretic activity and smooth muscle relaxant activity reside inseparably in these peptides (10).

In this report, we present the complete amino acid sequence of one of the peptides isolated from rat atrium which exhibits both natriuretic and smooth

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muscle relaxant activity. The peptide was found to contain a disulfide linkage essential for the biological activities.

MATERIALS AND METHODS

Purification of the natriuretic and smooth muscle relaxant peptide from rat atrium - The atrial peptide was purified by the method described previously (10). The last step of the purification method involved separation by HPLC on a Zorbax CN column (DuPont) which yielded two peaks containing both natriuretic and smooth muscle relaxant activity. The peptide eluted under the early peak was used for the present study. Due to tailing of peaks eluted from the column, certain preparations of the peptide contained small amounts of contaminating material(s) as judged by amino acid analyses. The contaminants were removed by rechromatography of the peptide preparation on the same column in which only fractions at the peak portion were collected. The purified peptide had both the natriuretic activity and smooth muscle relaxant activity.

Natriuretic activity was determined by bioassay in anesthetized rats as described previously (10). The activity is expressed as net increase in sodium excretion (μEq) in the urine collected over 10 min per kg body weight of the bioassay rats.

Smooth muscle relaxant activity was determined *in vitro* using strips of rabbit thoracic aorta and chick rectum maintained in tone with norepinephrine (5×10^{-8} M) and carbacol (5×10^{-8} M), respectively. Relaxation by the atrial peptide of the drug-induced contraction was determined as described previously (10).

Reduction and carboxymethylation of ANF. The purified peptide was reduced with 25 mM dithiothreitol and carboxymethylated with [1-^{14}C]-iodoacetic acid (200 dpm/nmole, Amersham) in 6 M guanidine HCl according to Hirs (11). The modified peptide was isolated by HPLC of the reaction mixture on a Vydac 318TP octadecyl column (0.46 x 25 cm, Separation Group). Elution was carried out with a linear concentration gradient of acetonitrile from 0 to 40% over 2 h in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The carboxymethylated peptide was eluted at 75 min after the beginning of the gradient. The unmodified peptide was eluted at 73 min under the same conditions.

Amino acid analysis was performed after hydrolysis in 6 N HCl containing 0.2% phenol for 20 h at 110°C. Tryptophan was determined after hydrolysis in 3 N mercaptoethanesulfonic acid (12).

Edman degradations were performed in a Beckman 890B sequencer using polybrene (3 mg, Aldrich), 0.55 M Quadrol buffer and a combined benzene-ethyl acetate wash program essentially as described elsewhere (13).

RESULTS AND DISCUSSION

The complete amino acid sequence of the atrial natriuretic peptide from rat possessing both natriuretic and smooth muscle relaxant activity (10) was determined by Edman degradation of 3 nmoles of the reduced and [^{14}C]-carboxymethylated derivative (Table I). The structure deduced from this study is shown in Figure 1. The molecule consists of 25 amino acid residues with the molecular weight of 2,706 calculated from the structure shown. The amino acid sequence is in agreement with the amino acid composition shown in Table II. The presence of the disulfide linkage involving Cys-4 and Cys-20 in the native molecule was deduced from the observation that reduction and carboxymethylation of the pep-

Table I. Automated Edman Degradation of Natriuretic Peptide from Rat Atrium[a]

Cycle number	Amino Acid	Yield (nmole)	^{14}C radio-activity[c] (cpm)	Cycle number	Amino Acid	Yield (nmole)	^{14}C radio-activity (cpm)
1	Arg	0.58	37.3	14	Ala	0.87	25.8
2	Ser[b]	N.D.	39.3	15	Gln	0.51	22.2
3	Ser[b]	N.D.	39.5	16	Ser[b]	N.D.	23.9
4	Cys	0.38	170.0	17	Gly	0.61	25.2
5	Phe	2.7	39.2	18	Leu	0.81	23.8
6	Gly	1.5	33.0	19	Gly	0.44	24.4
7	Gly	1.5	29.9	20	Cys	0.09	38.9
8	Arg	0.70	31.5	21	Asn	0.19	28.8
9	Ile	1.6	30.4	22	Ser[b]	N.D.	22.5
10	Asp	0.24	30.6	23	Phe	0.37	
11	Arg	0.55	33.3	24	Arg	0.24	
12	Ile	1.50	24.8	25	Tyr	0.14	
13	Gly	0.84	24.2				

[a]Sequence analysis of 3 nmoles of the [^{14}C]-carboxymethylated atrial natriuretic peptide.

[b]PTH-serine was not quantitated due to low and variable recoveries. Serine residues were recovered mostly as the PTH-serine dehydro derivative.

[c]Sample aliquots of 60% were taken for counting ^{14}C radioactivity. The numbers shown are not corrected for background counts.

tide abolished both the natriuretic activity and smooth muscle relaxant activity (see below).

The purified peptide administered intravenously into anesthetized rat caused a rapid and marked increase in urine volume and sodium excretion for a short duration in a manner similar to responses elicited by crude atrial extracts. Due to relatively large animal-to-animal variations and the limited availability of the pure peptide, the specific natriuretic activity of the pep-

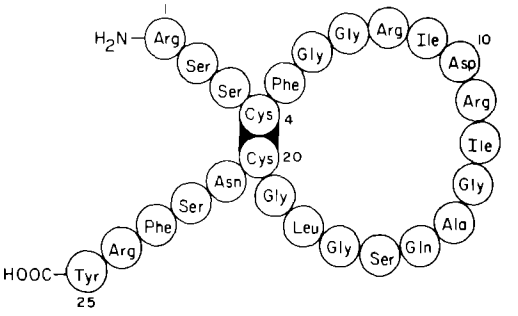

Figure 1: The structure of the atrial natriuretic peptide from rat. The complete amino acid sequence and the disulfide linkage between Cys-4 and Cys-20 found essential for the natriuretic activity and smooth muscle relaxant activity are shown schematically.

Table II. Amino Acid Composition of Natriuretic Peptide from Rat Atrium[a]

Amino Acid	Residues /mole	Amino Acid	Residues /mole
Cys/2[b]	2.01 (2)	Met	
Asx	2.05 (2)	Ile	1.80 (2)
Thr		Leu	1.07 (1)
Ser	3.70 (4)	Tyr	0.91 (1)
Glx	1.19 (1)	Phe	1.75 (2)
Pro		His	
Gly	5.38 (5)	Lys	
Ala	1.21 (1)	Arg	3.55 (4)
Val		Trp[c]	

[a]Values were calculated on the basis of the molecular weight of 2,706. Numbers in parentheses are those expected from the sequence.

[b]Determined as carboxymethylcysteine.

[c]Determined after hydrolysis in 3 N mercaptoethanesulfonic acid.

tide has not been exhaustively studied. Preliminary experiments indicated that 1.8 μg of the pure peptide administered intravenously in rat caused an increase in sodium excretion from 1.2 μEq/10 min/kg to 226 μEq/10 min/kg.

The contribution of the ring structure of the atrial natriuretic peptide to its natriuretic activity was examined by cleaving the disulfide linkage by reduction and carboxymethylation using partially purified peptide preparations as described in the legend to Figure 2. The reduced and carboxymethylated peptide failed to cause natriuretic and diuretic responses, while the same material treated in an identical manner in the absence of dithiothreitol retained the activity (Figure 2). The natriuretic and diuretic responses to the latter were comparable with those to the untreated material (not shown). These findings indicate that the ring structure in the molecule formed by the disulfide linkage is essential for the natriuretic and diuretic activities. The results also suggest that the peptide molecule readily forms its active structure even after it is exposed to denaturing conditions.

Similar results were obtained for the smooth muscle relaxant activity. The pure atrial peptide added to 2×10^{-9} M caused 50% relaxation of the contraction of rabbit aortic strips induced by 5×10^{-8} M norepinephrine. Complete relaxation was obtained at the peptide concentration of 6×10^{-9} M. On the other hand,

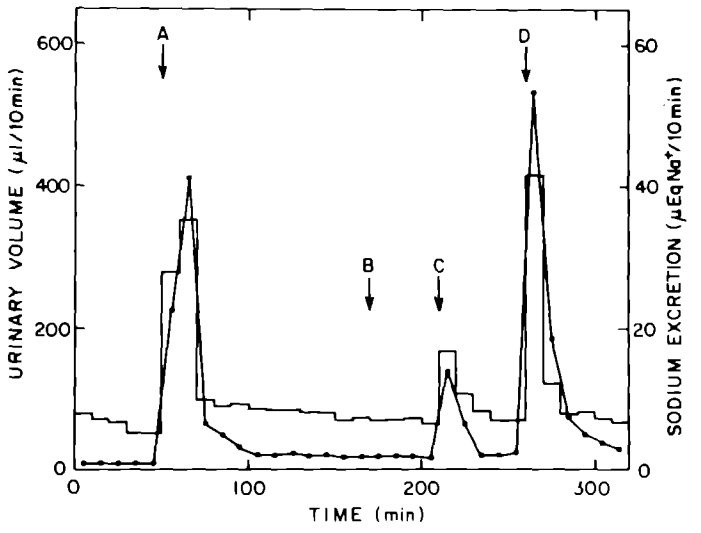

Figure 2: Natriuretic responses of rats to atrial peptide preparations and the effect of reduction and carboxymethylation of the peptide on the natriuretic activity. A typical result of assay using an urethane anesthetized rat (1g/kg body weight, i.p.) is shown. Injections of atrial peptide preparations were made though an intra-jugular vein catheter and urine was collected through a bladder catheter for every 10-min period. The urine volume was measured (bars) and sodium was determined by flame photometry (•—•). At time A, an aliquot (1/50) of a crude atrial extract prepared from 100 rats was injected to serve as a reference. After the urinary flow returned to the basal level, an aliquot of partially purified atrial natriuretic peptide which had been reduced and carboxymethylated in 6 M guanidine HCl (see below) was injected at time B. At time C, an identical amount of the same material treated in the same manner but without reduction was injected. At time D, the same aliquot of the crude extract as time A was injected.

Partially purified atrial natriuretic peptide was obtained after the first HPLC step on an ODS column in the purification procedure described previously (10). An aliquot of this material was dissolved in 6 M guanidine HCl containing 0.4 M Tris-HCl, pH 8.6, and 5 mM EDTA and dithiothreitol was added to 50 mM. After incubation for 2 hr at 37°C, sodium iodoacetate was added to 0.1 M and incubated in the dark at 37°C for 1 h. The reaction mixture was passed through a Sep-Pak C_{18} cartridge (Waters Assoc.) equilibrated with 0.1% trifluoroacetic acid. After washing with the same solvent, the bound material was eluted with 60% acetonitrile in 0.1% trifuluoroacetic acid and lyophilized. The lyophilized material was dissolved in water and injected into a rat at time B. Another aliquot was processed in the same manner but without reduction with ditniothreitol and was injected at time C.

reduced and carboxymethylated peptide at 6×10^{-9} M caused no relaxation. These results indicate that the ring structure is an important structural requirement also for the vascular smooth muscle relaxant activity.

The present finding that the atrial natriuretic factor is effective in counteracting the vasoconstricting action of norepinephrine at a concentration as low as 6×10^{-9} M and in causing marked natriuresis in microgram quantities administered in a whole rat suggests that this peptide is a potent substance with effects directed toward alleviating hypertensive effects of salt and norepine-

phrine and possibly other vasoconstrictors. Elucidation of the complete amino acid sequence of the atrial natriuretic peptide and its disulfide linkage essential for the biological activity provides a basis for investigating the molecular mechanism of its action.

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