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ISOLATION AND SEQUENCE DETERMINATION OF RAT CARDIAC NATRIURETIC PEPTIDE

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SUMMARY: We have isolated a cardiac natriuretic peptide of 5K daltons from the rat atrium and determined its amino acid sequence. The 5K cardiac natriuretic peptide was elucidated to be a 45-amino acid peptide with the sequence of S-Q-D-S-A-F-R-I-Q-E-R-L-R-N-S-K-M-A-H-S-S-S-C-F-G-Q-K-I-D-R-I-G-A-V-S-R-L-G-C-D-G-L-R-L-F by sequencing the native peptide and its lysyl endopeptidase digests. The sequence of this peptide was identical to the amino acid sequence [51-95] of the rat brain natriuretic peptide (BNP) precursor deduced from the cDNA sequence. The 5K cardiac natriuretic peptide, or BNP[51-95], was identified as the major storage and secretory form derived from the BNP precursor in the rat heart.

Using radioimmunoassay (RIA) for the ring structure of iso-ANP, which is the highly conserved sequence of natriuretic peptides, and radioreceptor assay for atrial natriuretic peptide (ANP), we have already demonstrated the presence of a considerable amount of a novel cardiac natriuretic peptide with a molecular weight of 5K daltons in the rat atrium, which is distinct from iso-ANP and α-rat ANP isolated previously (1). We have also shown that the 5K cardiac natriuretic peptide is released from the heart in the Langendorff's perfusion experiment (1). However, we could not detect significant amounts of the cardiac natriuretic peptide in other rat tissues including the

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; RIA, radioimmunoassay; -LI, -like immunoreactivity; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid.

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brain (1). Thus, the biosynthesis and distribution of the 5K cardiac natriuretic peptide present a striking contrast to those of mammalian ANP and porcine brain natriuretic peptide (BNP) elucidated so far (2-12). Both ANP and porcine BNP are stored as precursor forms in the heart (2-5,11,12) and are secreted into the circulation from the heart as cleaved forms of about 3K daltons (4,12). They also distribute at the significant levels as low molecular forms in the brain (6-10).

In the present study, we have isolated the 5K cardiac natriuretic peptide from the rat atrium and determined the amino acid sequence. We have also clarified that the 5K cardiac natriuretic peptide is secreted from the rat heart.

MATERIALS AND METHODS

Peptides: Synthetic iso-ANP and α-rat ANP were purchased from Peninsula Laboratories Inc. The sequence of iso-ANP is S-Q-D-S-A-F-R-I-Q-E-R-L-R-N-S-K-M-A-H-S-S-S-C-F-G-Q-K-I-D-R-I-G-A-V-S-R-L-G-C-D-I-L-L-I-A-Q (1).

RIA: Detection of the cardiac natriuretic peptide was performed by the RIA for the ring structure of iso-ANP as we previously reported (1).

Preparation of immunoaffinity matrix: Antisera against iso-ANP[23-46] were prepared in Japanese white rabbits as we previously described (1). One of the antisera obtained (KY-RG) showed a specificity similar to the mouse antiserum (I-10) we previously reported (1). The RIA with KY-RG recognized the ring structure of iso-ANP. The cross-reactivity with α-rat ANP was less than 0.01 %. Three ml of the antiserum, KY-RG, was used for preparation of immunoglobulins by ammonium sulfate precipitation. The immunoglobulins were used for conjugation to cyanogen bromide-activated Sepharose 4B (3.9 g, Pharmacia).

Isolation of cardiac natriuretic peptide from rat atrium: Atrial tissues (65 g) obtained from 500 Wistar rats were boiled in 0.1 M acetic acid (650 ml) for 5 minutes. The boiled tissues were then homogenized in 1 M acetic acid (600 ml) for 10 minutes with a Polytron homogenizer. The supernatant obtained by centrifugation (20,000 x g, 30 minutes) was loaded on a SP-Sephadex C-25 column (25 mmID x 70 mm, Pharmacia) and eluted successively with 1 M acetic acid, 2 M pyridine and 2 M pyridine acetate according to the method reported elsewhere (9). The pyridine acetate solution was then treated with Sep-Pak C_{18} cartridges (Waters) and the eluate with 50 % acetonitrile-0.1 % trifluoroacetic acid (TFA) was lyophilized. The residual materials were dissolved in 0.2 % Triton X-100-0.1 M sodium phosphate (pH 7.2, 3.6 ml) and were applied on the affinity matrix described above. After the washing with 0.1 M sodium phosphate (pH 7.2), the adsorbed materials were eluted out with 10 % acetonitrile-1 M acetic acid. The eluate was treated with Sep-Pak C_{18} cartridges in the same way and then purified by reverse phase high performance liquid chromatography (reverse phase HPLC) on a Nucleosil $5C_{18}$ column (4.6 mmID x 150 mm, Nagel) with a linear gradient of acetonitrile from 25 % to 35 % in 0.1 % TFA. Fractions (0.5 ml) were collected and subjected to RIA. The main fraction detected was further purified by reverse phase HPLC on a 219TP54 diphenyl column (4.6 mmID x 250 mm, Vydac) with a linear gradient of acetonitrile from 10 % to 60 % in 0.1 % TFA.

Sequence analysis: Amino acid sequence analysis was performed by stepwise Edman degradation using a gas-phase sequencer equipped with a reverse phase HPLC system, Model 470A/120A (Applied Biosystems Inc.).

Reductive carboxymethylation and lysyl endopeptidase digestion: The isolated cardiac natriuretic peptide (700 ng) was reduced with 20 mM dithiothreitol in 0.5 M Tris-HCl (pH 8.5) at 37°C for 4 hours and then treated with 50 mM sodium monoiodoacetate for 5 minutes at 23°C. The reaction mixture was applied on a μ-Bondasphere C_{18} column (3.9 mmID x 150 mm, Waters) and eluted with a linear gradient of acetonitrile from 10 % to 60 % in 0.1 % TFA. A major peak emerging around 38 minutes was collected and lyophilized. The carboxymethylated peptide was then digested with lysyl endopeptidase (200 ng, Wako Pure Chemicals Industries, Ltd., Osaka, Japan) in 50 mM Tris-HCl (pH 8.5) at 37°C for 2 hours. The digests thus obtained were subjected to reverse phase HPLC on a μ-Boundasphere C_{18} column (3.9 mmID x 150 mm) with a linear gradient of acetonitrile from 0 % to 60 % in 0.1 % TFA. Each peptide fragment was subjected to sequence analysis.

Analysis of peptide in perfusate from isolated hearts: The perfusion of isolated beating rat hearts was performed as we described elsewhere (13). The perfusate (1 liter) was treated with a Sep-Pak C_{18} cartridge prior to application to reverse phase HPLC on a Nucleosil $5C_{18}$ column under the isocratical condition with 0.1 % TFA-28 % acetonitrile. Fractions (0.25 ml) were collected and subjected to RIA as described previously (1).

RESULTS

Isolation of novel cardiac natriuretic peptide

The total amount of the cardiac natriuretic peptide in 1 M acetic acid extract from rat atrial tissues (65 g) determined by the RIA for iso-ANP[23-46] was 18 μg. Figure 1 shows the reverse phase HPLC profile of the 2 M pyridine acetate fraction (SP-Sephadex C-25) of the 1 M acetic acid extract on a Nucleosil $5C_{18}$ column. The 5K cardiac natriuretic peptide was the major component of the iso-ANP[23-46]-like immunoreactivity (-LI), of which retention time was clearly different from that of iso-ANP. There was no peak of immunoreactivity at the

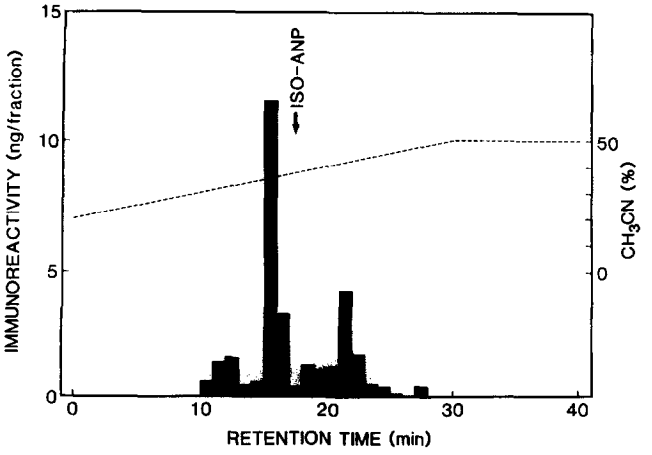

Figure 1. Reverse phase HPLC profile of the 2 M pyridine acetate fraction (SP-Sephadex C-25) of the 1 M acetic acid extract on a Nucleosil $5C_{18}$ (4.6 mmID x 150 mm). The scale on the left ordinate shows iso-ANP[23-46]-like immunoreactivity.

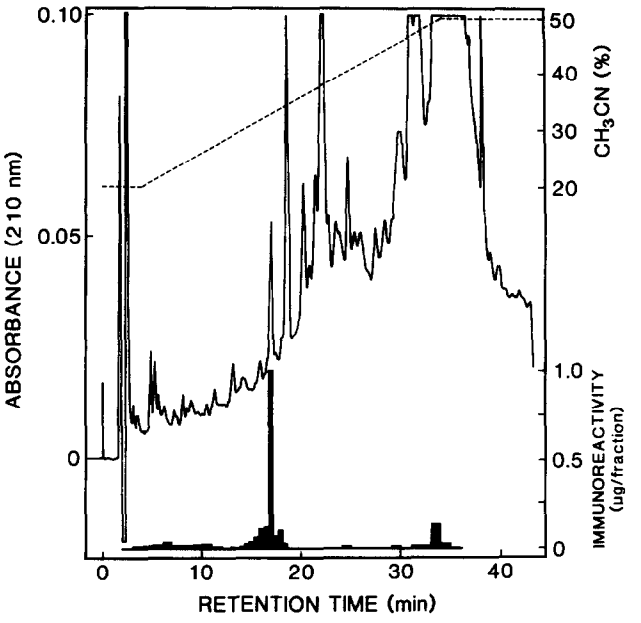

Figure 2. Reverse phase HPLC profile of the materials adsorbed on the immunoaffinity matrix on a Nucleosil $5C_{18}$ column (4.6 mmID x 150 mm). The bottom scale on the right ordinate shows iso-ANP[23-46]-like immunoreactivity.

retention time of iso-ANP. Figure 2 reveals the reverse phase HPLC profile of the materials adsorbed on the immunoaffinity matrix on a Nucleosil $5C_{18}$ column. As shown in Figure 2, the 5K cardiac natriuretic peptide was eluted at the retention time of 17 minutes. The peptide was further purified to complete homogeneity by the subsequent reverse phase HPLC on a diphenyl column as depicted in Figure 3.

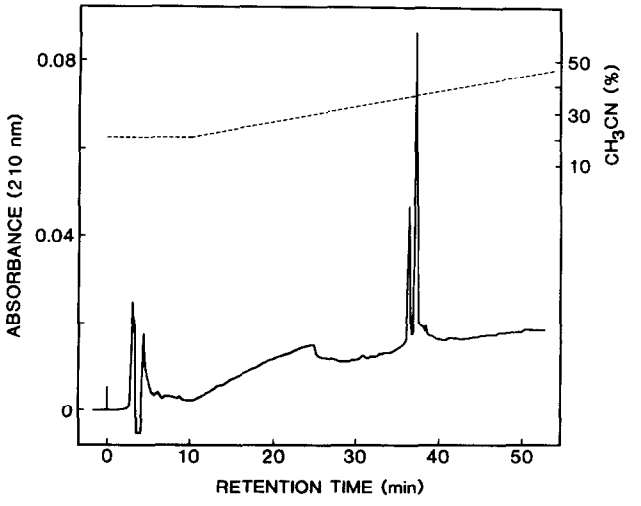

Figure 3. Reverse phase HPLC profile of the main fraction in the immunoaffinity chromatography on a 219TP54 diphenyl column (4.6 mmID x 250 mm).

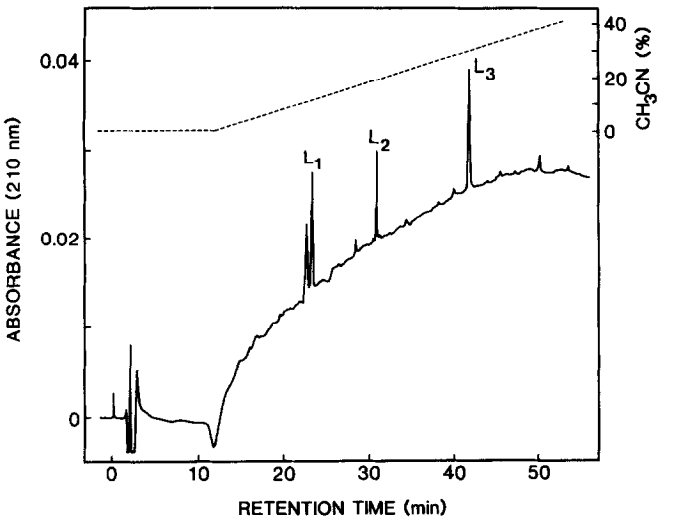

Figure 4. Reverse phase HPLC profile of lysyl endopeptidase digests on a μ-Boundasphere C_{18} column (3.9 mmID x 150 mm).

There was a minor peak preceding the major peak. This minor peak proved to have the same N-terminal sequence as that of the major peak in the sequencing. It is likely, therefore, that the minor component is the peptide with the same sequence, of which methionine residue is oxidized. Finally we obtained 1.6 μg of the 5K cardiac natriuretic peptide.

Amino acid sequence of cardiac natriuretic peptide

Sequence analysis of the 5K cardiac natriuretic peptide (about 300 ng) determined the structure of the N-terminal 37 amino acid residues. As shown in Figure 4, the enzymatic digestion of the native peptide with lysyl endopeptidase yielded four peaks. Among them three peaks designated L1, L2 and L3 proved to come from the

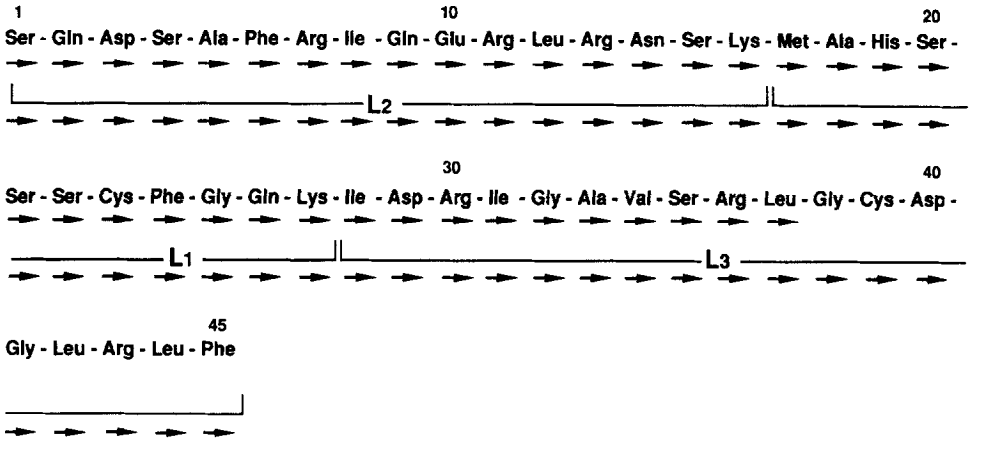

Figure 5. Amino acid sequences of native 5K cardiac natriuretic peptide and its lysyl endopeptidase digests.

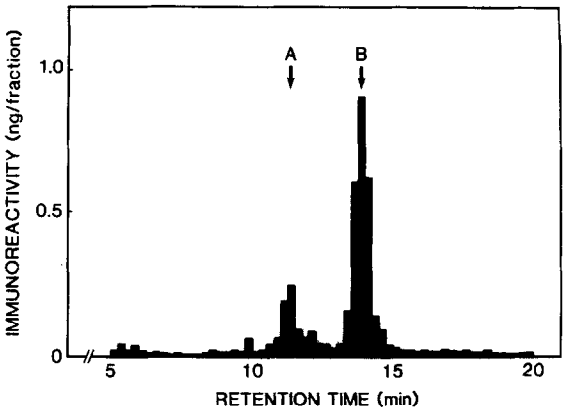

Figure 6. Reverse phase HPLC profile of extract of the perfusate from isolated rat hearts on a Nucleosil $5C_{18}$ column (4.6 mmID x 250 mm) under the isocratical condition with 0.1 % TFA-28 % acetonitrile. Immunoreactivity on the ordinate is based on the synthetic 5K cardiac natriuretic peptide. Arrows, A and B, indicate elution positions of the 5K cardiac natriuretic peptide with the oxidized methionine residue and the authentic 5K cardiac natriuretic peptide, respectively.

parent peptide. Another peak was not due to peptide fragment. Their detailed sequences are given in Figure 5. The 5K cardiac natriuretic peptide was clarified to be a 45-amino acid peptide with the sequence shown in Figure 5. The synthetic 5K cardiac natriuretic peptide was confirmed to be identical with the native peptide both in analytical HPLC and in peptide mapping using lysyl endopeptidase (data not shown).

Identification of secretory form

Figure 6 shows the elution profile of the cardiac natriuretic peptide in the perfusate from isolated rat hearts in reverse phase HPLC. Two peaks of immunoreactivity were detected in the perfusate. The major peak was co-migrated with the native 5K cardiac natriuretic peptide. The minor peak corresponded to the peptide with the methionine residue oxidized.

DISCUSSION

In the present study, we have isolated the 5K cardiac natriuretic peptide, which we previously identified in the rat heart (1), and determined its amino acid sequence. We have also elucidated that the 5K cardiac natriuretic peptide is secreted from the heart. The 5K cardiac natriuretic peptide was composed of 45 amino acids and had a ring structure with 17 amino acids looped by the disulfide linkage of two cysteine residues corresponding to the sequence [23-39], which was

thought to be essential for biological actions of a family of natriuretic peptides.

The N-terminal 40 amino acid sequence including the ring structure of the 5K cardiac natriuretic peptide was identical to the sequence of iso-ANP presented previously by Flynn et al., whereas the C-terminal sequence, G-L-R-L-F, of the 5K cardiac natriuretic peptide was markedly different from that of iso-ANP, I-L-L-I-A-Q.

In the course of the present study, Kojima et al. (14) reported the cloning and sequencing of cDNA encoding a precursor of rat BNP, though the natural form of rat BNP is not yet identified. The sequence of the 5K cardiac natriuretic peptide isolated in the present study is identical to the C-terminal 45-amino acid sequence [51-95] of the rat BNP precursor deduced from the cDNA sequence. Thus, the 5K cardiac natriuretic peptide is rat BNP[51-95]. Since the sequence of the 5K cardiac natriuretic peptide is preceded by the single arginine residue at the position 50 in the rat BNP precursor, it is conceivable that the proteolytic cleavage at this residue generates the 5K cardiac natriuretic peptide.

In the present study, we have also demonstrated that the 5K cardiac natriuretic peptide is not only the major storage form of BNP in the rat heart but also the sole secretory form from the heart. These results indicate that the 5K cardiac natriuretic peptide, or rat BNP[51-95] is a novel cardiac hormone secreted from the rat heart.

During the preparation of the present paper, Flynn et al. (15) reported the sequence of iso-ANP consisting of 45 amino acids, which was different from the original sequence with 46 amino acids shown in Materials and Methods. The C-terminal sequence of our cardiac natriuretic peptide, G-L-R-L-F, differs from the C-terminal sequence of the revised iso-ANP, G-L-R-Q-F.

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