Rat Brain Natriuretic Peptide
Isolation From Rat Heart and Tissue Distribution

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We have isolated a cardiac natriuretic peptide of 5,000 d from atrial tissues from 500 rats and determined its amino acid sequence. The 5,000 d atrial natriuretic factor 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 precursor deduced from the complementary DNA (cDNA) sequence. The cardiac natriuretic peptide with a molecular weight of 5,000, or rat brain natriuretic peptide, was identified as the major storage form and as the sole secretory form derived from the brain natriuretic peptide precursor in the rat heart. The rat brain natriuretic peptide level in the atrium was 3.68±0.61 µg/g, which represents about 4% of that of atrial natriuretic factor. Rat brain natriuretic peptide was also detected in the ventricle. The ratio of brain natriuretic peptide to atrial natriuretic peptide in the ventricle was approximately 30% and much higher than that in the atrium. Rat brain natriuretic peptide, however, was not detectable in the brain. We conclude that the 5,000 d cardiac natriuretic peptide is rat brain natriuretic peptide with 45 amino acids derived from the brain natriuretic peptide precursor and is secreted from the rat heart as a novel cardiac hormone. (Hypertension 1990;15:774-778)

By using radioimmunoassay (RIA) for the ring structure of isoatrial natriuretic peptide (iso-ANP), which is the highly conserved sequence of natriuretic peptides, and radioreceptor assay for atrial natriuretic peptides (ANP), we have already demonstrated the presence of a considerable amount of a novel cardiac natriuretic peptide with a molecular weight of 5,000 in the rat atrium, which is distinct from iso-ANP and rat atrial natriuretic factor-(99-126) [rat ANP-(99-126)] isolated previously.1 We have also shown that the 5K cardiac natriuretic peptide (molecular weight 5,000) is released from the heart in the Langendorff perfusion experiment.1 We could not, however, detect significant amounts of the cardiac natriuretic peptide in other rat tissues including the brain.1 Thus, the biosynthesis and distribution of the 5,000 cardiac natriuretic peptide present a remarkable 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 heart2-5,11,12 and are secreted into the circulation from the heart as cleaved forms with a molecular weight of about 3,000.4,12 They also distribute at significant levels as low molecular forms in the brain.6-10

In the present study, we have isolated the 5,000 cardiac natriuretic peptide from the rat atrium and determined the amino acid sequence. We have also clarified the distribution of this natriuretic peptide.

Methods

Peptides

Synthetic iso-ANP and rat ANP-(99-126) were purchased from Peninsula Labs. Inc. (Belmont, California). 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-C-F-G-Q-

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K-I-D-R-I-G-A-V-S-R-L-G-C-D-I-L-L-I-A-Q. After the sequencing of 5,000 cardiac natriuretic peptide, the peptide was synthesized by the solid-phase method.

Radioimmunoassay
Detection of the cardiac natriuretic peptide was performed by RIA for the ring structure of iso-ANP as we previously reported. The ANP level was measured by RIA that recognizes the C-terminal sequence of immunoreactive ANP-(99-126).

Preparation of Immunoaffinity Matrix
Antisera against iso-ANP-(23-46) were prepared in Japanese White rabbits as we previously described. One of the antisera obtained (KY-RG) showed a specificity similar to the mouse antiserum (1-10). The RIA with KY-RG recognized the ring structure of iso-ANP. The cross-reactivity with rat ANP-(99-126) was less than 0.01%. Three milliliters antisera, 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, Uppsala, Sweden).

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 (Kinematika, Switzerland). The supernatant obtained by centrifugation (20,000g, 30 minutes) was loaded on a SP-Sephadex C-25 column (25 mm i.d.×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. The pyridine acetate solution was then treated with pyridine acetate fraction (SP-Sephadex C-25) of the materials adsorbed on the immunoaffinity matrix on a Nucleosil 5C18 cartridge (1 liter) was treated with a Sep-Pak C18 cartridge before application to reverse-phase HPLC on a Nucleosil 5C18 column under the isocratical condition with 0.1% TFA–20% acetonitrile.

Results
The isolated cardiac natriuretic peptide (700 ng) was reduced with 20 mM diithiothreitol in 0.5 M Tris-HCl (pH 8.5) at 37°C for 4 hours and then treated with 50 mM sodium monooiodoacetate for 5 minutes at 23°C. The reaction mixture was applied on a μ-Bondasphere C18 column (3.9 mm i.d.×150 mm, Waters) and eluted with a linear gradient of acetonitrile of 10-60% in 0.1% TFA. 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-Bondasphere C18 column (3.9 mm i.d.×150 mm) with a linear gradient of acetonitrile of 0-60% in 0.1% TFA.

Analysis of Peptide in Perfusate From Isolated Hearts
The perfusion of isolated beating rat hearts was performed as we described elsewhere. The perfusate (1 liter) was treated with a Sep-Pak C18 cartridge before application to reverse-phase HPLC on a Nucleosil 5C18 column under the isocratical condition with 0.1% TFA–28% acetonitrile.

Tissues and Extraction Procedure
Hearts, brains, and other tissues were obtained from male Slc: Wistar rats weighing 250–350 g (Shizuoka Animal Center, Shizuoka, Japan) immediately after decapitation. The apical half of the ventricular tissue was obtained for prevention of atrial contamination. Brains were dissected as previously reported. Tissues were extracted as previously described in detail.

Isolation of Novel Cardiac Natriuretic Peptide
The total amount of cardiac natriuretic peptide in 1 M acetic acid extract from rat atrial tissues (65 g) determined by RIA for iso-ANP-(23-46) was 18 μg. In 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 5C18 column, the 5,000 cardiac natriuretic peptide was the major component of the iso-ANP-(23-46)–like immunoreactivity of which retention time was clearly different from that of iso-ANP. There was no peak of immunoreactivity at the retention time of iso-ANP. In the reverse-phase HPLC profile of the materials adsorbed on the immunoaffinity matrix on a Nucleosil 5C18 column, the 5,000 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 1. There was a minor peak preceding the major peak. This minor
peak proved to be the peptide with the same sequence of which methionine residue is oxidized. Finally, we obtained 1.6 μg of the 5,000 cardiac natriuretic peptide.

**Amino Acid Sequence of Cardiac Natriuretic Peptide**

Sequence analysis of the 5,000 cardiac natriuretic peptide (about 300 ng) determined the structure of the N-terminal 37 amino acid residues. As shown in Figure 2, 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 parent peptide. Another peak was not caused by peptide fragmentation. Their detailed sequences are given in Figure 3. The 5,000 cardiac natriuretic peptide was clarified to be a 45 amino acid peptide with the sequence shown in Figure 3. The synthetic 5,000 cardiac natriuretic peptide was confirmed to be identical to the native peptide both in analytical HPLC and in peptide mapping with lysyl endopeptidase (data not shown).

**Identification of Secretory Form**

In reverse-phase HPLC analysis of the perfusate from isolated rat hearts, two peaks of immunoreactivity were detected in the perfusate. The major peak was comigrated with the native 5,000 cardiac natriuretic peptide or rat BNP. The minor peak corresponded to the peptide with the methionine residue oxidized. The level of rat BNP in the perfusate under nonstimulated conditions was shown together with that of ANP in Table 1.

**Tissue Distribution**

The rat BNP level in the atrium was 3.68±0.61 μg/g, which represents about 4% of that of ANP.
In the present study, we have isolated the 5,000 cardiac natriuretic peptide, which we previously identified in the rat heart, \(^1\) and determined its amino acid sequence. We have also elucidated that the 5,000 cardiac natriuretic peptide was distinctly different from that of iso-ANP, that is, I-L-L-I-A-Q. Recently, Flynn et al\(^{16}\) reported the sequence of iso-ANP consisting of 45 amino acids, which was different from the original sequence with 46 amino acids as shown in Methods. The C-terminal sequence of our cardiac natriuretic peptide, G-L-R-L-F, differs from the revised C-terminal sequence of iso-ANP, G-L-R-Q-F. We could not find iso-ANP (original and revised sequences) either in the rat heart or in other rat tissues.

In the course of the present study, Kojima et al\(^{17}\) reported the cloning and sequencing of complementary DNA (cDNA) encoding a precursor of rat BNP although the natural form of rat BNP is not yet identified. The sequence of the 5,000 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 5,000 cardiac natriuretic peptide is rat BNP. Because the sequence of the 5,000 cardiac natriuretic peptide is preceded by the single arginine residue at position 50 in the rat BNP precursor, it is possible that the proteolytic cleavage at this residue generates the 5,000 cardiac natriuretic peptide.

Regarding biological actions of rat BNP, we examined natriuretic and vasorelaxant actions of synthetic rat BNP in rats. Biological actions of rat BNP were almost equipotent with those of ANP (unpublished observation).

In the present study, we have also demonstrated that the 5,000 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 5,000 cardiac natriuretic peptide, or rat BNP, is a novel cardiac hormone secreted from the rat heart.

**TABLE 1. Concentrations of Brain Natriuretic Peptide (5,000 Cardiac Natriuretic Peptide) and Atrial Natriuretic Peptide in Rats**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>BNP</th>
<th>ANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrium</td>
<td>3.68±0.61 μg/g</td>
<td>56.67±16.9 μg/g</td>
</tr>
<tr>
<td>Ventricle</td>
<td>0.0109±0.0021 μg/g</td>
<td>0.0234±0.008 μg/g</td>
</tr>
<tr>
<td>Perfusate</td>
<td>0.061±0.009 ng/min</td>
<td>1.92±0.38 ng/min</td>
</tr>
<tr>
<td>Brain (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>&lt;0.5 ng/g</td>
<td>4.12±0.13 ng/g</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>&lt;0.05</td>
<td>3.51±0.24</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>&lt;0.05</td>
<td>1.27±0.19</td>
</tr>
<tr>
<td>Striatum</td>
<td>&lt;0.05</td>
<td>1.26±0.13</td>
</tr>
<tr>
<td>Thalamus</td>
<td>&lt;0.05</td>
<td>4.91±0.24</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>&lt;0.05</td>
<td>7.34±0.71</td>
</tr>
<tr>
<td>Septum</td>
<td>&lt;0.05</td>
<td>11.45±1.95</td>
</tr>
<tr>
<td>Midbrain</td>
<td>&lt;0.05</td>
<td>4.08±0.12</td>
</tr>
<tr>
<td>Pons</td>
<td>&lt;0.05</td>
<td>1.16±0.08</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>&lt;0.05</td>
<td>1.37±0.02</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>&lt;0.05</td>
<td>0.24±0.01</td>
</tr>
</tbody>
</table>

Values are the mean±SEM. BNP, brain natriuretic peptide; ANP, atrial natriuretic peptide.
Because BNP is originally isolated from the porcine brain as 26 and 32 amino acid peptides,\textsuperscript{8,9} BNP is expected to play a role or roles in the central regulation of body fluid and blood pressure alone or in concert with brain ANP.\textsuperscript{18-20} No significant amount of BNP, however, was detected in the rat brain in the present study, although our RIA against the ring structure of rat BNP could detect N-terminally and C-terminally modified (elongated or deleted) forms of rat BNP. Additionally, no BNP messenger RNA was detected in the rat brain. Recently, bovine BNP or aldosterone secretion inhibitory factor was isolated from chromaffin cells as a 35 amino acid peptide.\textsuperscript{21} More recently, we have isolated and sequenced human BNP with 32 amino acids in the human heart.\textsuperscript{19} These results indicate that the distribution and the processing of BNP show a pronounced species difference. It is likely, therefore, that the physiological and pathophysiological significance of the dual mechanism of ANP and BNP, especially BNP, must await further investigation based on these findings.

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References

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