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ISOLATION AND IDENTIFICATION OF RAT BRAIN NATRIURETIC PEPTIDES IN CARDIAC ATRIUM

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SUMMARY: The amino acid sequence of a precursor for rat brain natriuretic peptide (BNP) has recently been deduced by the cDNA cloning method. By using a radioimmunoassay (RIA) system newly established for rat BNP, a high concentration of ir-BNP was found to exist in rat cardiac atrium. Two ir-BNPs of different molecular weights (11K and 5K) were isolated from rat cardiac atria by anti-rat BNP IgG immunoaffinity chromatography and reverse phase high performance liquid chromatography (HPLC). By microsequencing, the high molecular weight (MW) BNP was deduced to be a pro-BNP of 95 residues (γ-BNP). The low MW BNP was demonstrated to be a C-terminal 45-amino acid peptide (BNP-45) of pro-BNP. Based on these results, BNP-45 and γ-BNP are shown to be two major forms in rat cardiac atrium, indicating a unique processing pathway of rat BNP precursor.

BNP, originally isolated from porcine brain, is a novel type of natriuretic peptide, having an amino acid sequence and pharmacological activity remarkably similar to that of atrial natriuretic peptide (ANP) (1). By utilizing cDNA of porcine BNP precursor as a probe (2), we have recently cloned and sequenced cDNA encoding rat BNP precursor (3) along with cDNA encoding human BNP precursor (4). Structural elucidation of porcine, rat and human BNP precursors have demonstrated that structural species differences observed among mammalian BNPs are much larger than those among mammalian ANPs even in the C-terminal amino acid sequences essential for exerting the biological activity. Thus, for further study on physiologic functions of BNP in rats, it is necessary to identify endogenous molecular forms of rat BNP.

As a first step, an antiserum against a synthetic peptide corresponding to a C-terminal 26-amino acid peptide (BNP-26) of rat pro-BNP was prepared. By using an RIA specific to rat BNP, we found the highest concentration of

Abbreviations: BNP, brain natriuretic peptide; ANP, atrial natriuretic peptide; RIA, radioimmunoassay; ir, immunoreactive; MW, molecular weight; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; TFA, trifluoroacetic acid; RCM, reduced and S-carboxymethylated; PTH, phenylthiohydantoin.

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ir-BNP in rat cardiac atrium. Two BNP-immunoreactive peptides of different MWs were isolated from the extracts of rat atria and determined their amino acid sequences, as reported in the present paper.

MATERIALS AND METHODS

Isolation: Hearts were collected from 55 male Wistar rats (about 10-14 weeks old, body weight 300-400 g) after decapitation. Cardiac atria (wet tissue weight, 6.35 g) were dissected from whole hearts and boiled for 10 min in 10 volumes of water to inactivate intrinsic proteases. After cooling, glacial acetic acid was added (final concentration = 1M), and the resulting mixture was homogenized with a Polytron mixer for 4 min. The supernatants, obtained after centrifugation at 32,000 x g for 30 min, were divided into 2 portions and were each loaded onto a reverse phase C-18 column (15 ml, LC-SORB SPW-C-ODS, Chemco). After washing with 0.5M acetic acid, adsorbed materials were eluted with 60% CH_3CN containing 0.1% trifluoroacetic acid (TFA). Each eluate was mixed, evaporated in vacuum, and then lyophilized. The lyophilizate (dry weight, 48.7 mg) was dissolved in 5 ml of 1M acetic acid and subjected to gel filtration on a Sephadex G-75 column (1.8 x 137 cm, Pharmacia). An aliquot of each fraction was submitted to RIAs for rat BNP and ANP. Pooled fractions #45-49 and #52-58 exhibiting ir-BNP were lyophilized, and then subjected to immunoaffinity chromatography on an anti-rat BNP IgG-AFFI-GEL HZ column (see below). The peptide fraction adsorbed on the immunoaffinity column was purified by successive reverse phase HPLCs first on a μ-Bondasphere C-18 column (3.9 x 150 mm, 300A, Waters), and then on phenyl columns (219TP54 4.6 x 250 mm, and 219TP5215 2.1 x 150 mm, Vydac) with a linear gradient elution of CH_3CN from 10% to 60% in 0.1% TFA. The column effluents of HPLC were monitored by measuring absorbance at 210 and 280 nm. Aliquots of all the fractions from immunoaffinity chromatography and reverse phase HPLCs were submitted to RIAs for rat BNP and ANP.

RIAs for rat BNP and rat ANP: Details on RIA for rat BNP will be reported in a separate paper. Antiserum #179-3, which was raised in rabbit by immunizing rat BNP-26-thyroglobulin conjugate, recognizes rat BNP-26 and BNP-32 at the same affinity. RIA was performed as reported (5), using rat BNP-32 as a standard. When the antiserum was used at a final dilution of 1:480,000, peptides were measurable in a range of 0.5-50 fmol/tube, and porcine BNP-26, human BNP-32 and rat α-ANP did not show significant crossreactivity. RIA for rat α-ANP was performed as reported previously (6). This RIA system had 0.01% crossreactivity with rat BNP-32.

Immunoaffinity chromatography: Anti-BNP immunoaffinity column was prepared mainly as reported for the isolation of porcine BNP-32 (7). In brief, IgG fraction of antiserum #179-3, prepared with Protein A-Sepharose CL-4B (Pharmacia), was coupled with AFFI-GEL HZ according to the manual of Bio-Rad. Samples dissolved in 0.1M sodium phosphate buffer (pH 7.4) were loaded onto the immunoaffinity column (total bed volume: 1 ml). After washing the column, the adsorbed materials were eluted with 1M acetic acid containing 10% CH_3CN.

Sequence analyses: Amino acid sequence analyses were performed using a gas-phase sequencer equipped with a phenylthiohydantoin (PTH)-amino acid analyzing HPLC system (Model 470A/120A, Applied Biosystems). The purified γ-BNP, BNP-45 and lysyl endopeptidase digests of reduced and S-carboxymethylated (RCM-) BNP-45 prepared as described below, were each submitted to the sequencer. The native BNP-45 (ca. 1.0 μg) was reduced with 20mM dithiothreitol in 0.5M Tris-HCl (pH 8.5) at 37°C for 4 hr, and then was carboxymethylated with sodium monoiodoacetic acid. The RCM-BNP-45 was purified by reverse phase HPLC on a μ-Bondasphere C-18 column and was lyophilized. Lysyl endopeptidase digestion of RCM-BNP-45 was performed with 200 ng of the enzyme (Wako Pure Chemicals) in 100 μl of 50mM Tris-HCl buffer (pH 8.5) at 37°C for 2 hr, and the digestion mixture was subjected to reverse phase HPLC on a μ-Bondasphere C-18 column (3.9 x 150 mm) with a linear gradient elution of CH_3CN in 0.1% TFA. Half of

LEP-3 obtained by the first digestion was further digested with 1 μg of lysyl endopeptidase at 37°C for 7 hr and was then subjected to reverse phase HPLC as described above.

RESULTS AND DISCUSSION

Based on the structure of rat BNP precursor elucidated by the cDNA analysis, rat BNP-26, corresponding to a C-terminal 26-residue peptide of the precursor, was synthesized and an antiserum against this peptide was prepared. By utilizing the antiserum against rat BNP (#179-3), we preliminarily studied on distribution of ir-BNP in rat and found the highest concentration of ir-BNP in heart, especially in the cardiac atrium, as is the case with porcine BNP (8,9). These results led us to isolate rat BNP from heart tissue and identify it at the peptide level.

Since tissue concentration of ir-BNP in rat cardiac atrium was 5-10 times higher (ca. 1-2 μg/g) than that of porcine ir-BNP in porcine atrium (9), the present isolation was performed with 6.35 g of cardiac atria. After extraction, the peptide fraction of rat atria was condensed and desalted with a reverse phase C-18 column, and then subjected to Sephadex G-75 gel filtration. As shown in Fig. 1, two peaks of ir-BNP were observed on the chromatogram, although the majority of ir-ANP was eluted as a single peak at the position indicated by arrow 2 (data not shown). The minor peak emerged at fractions #45-49 (fraction A) corresponding to 10-12K daltons, which was a little later than rat γ-ANP, and the major ir-BNP peak was eluted at fractions

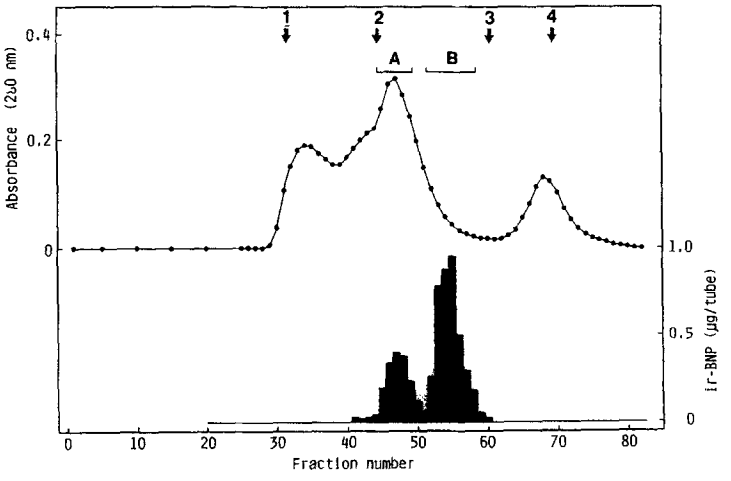

Figure 1. Sephadex G-75 gel filtration of acid extracts of rat cardiac atria.
Sample: Adsorbed fraction to C-18 silica gel column of the acid extracts of rat cardiac atria (6.35 g). Solvent: 1M acetic acid.
Column: Sephadex G-75 (1.8 x 137 cm, Pharmacia).
Fraction size: 5 ml/tube. Flow rate: 8 ml/hr.
Arrows indicate elution positions of 1) bovine serum albumin, 2) rat γ-ANP, 3) rat α-ANP and 4) NaCl, respectively.

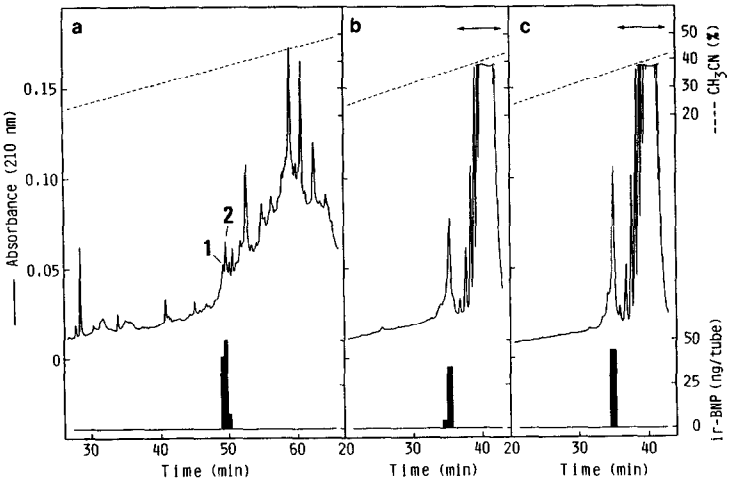

Figure 2. Successive purification of γ-BNP by reverse phase HPLC.
Sample: a) Anti-BNP IgG immunoaffinity chromatography purified fraction obtained from fraction A (#45-49) in Fig. 1.
b) Peak 1, exhibiting BNP immunoreactivity in (a).
c) Peak 2, exhibiting BNP immunoreactivity in (a).
Column: a) μ-Bondasphere C-18 (3.9 x 150 mm, 300A, Waters).
b,c) 219TP5215 phenyl (2.1 x 150 mm, Vydac).
Solvent: H_2O:CH_3CN:10% TFA = (I) 90:10:1 (v/v), (II) 40:60:1 (v/v). Linear gradient elution from (I) to (II) for 70 min in (a), and for 60 min in (b) and (c).
Flow rate: (a) 1 ml/min and (b) 0.3 ml/min. Temperature: ambient.
Peaks marked with arrows in (b) and (c) were derived from Triton X-100.

#52-58 (fraction B) corresponding to 5K daltons. After lyophilization, fractions A and B were each subjected to anti-BNP IgG immunoaffinity chromatography. For the high MW ir-BNP (fraction A), the lyophilized samples were solubilized with a low concentration of Triton X-100. The adsorbed peptides of fractions A and B on the affinity columns were eluted, and then each was separated first by reverse phase HPLC on a μ-Bondasphere C-18 column. As shown in Fig. 2a, the high MW ir-BNP (fraction A) was eluted as two peaks, peak 1 and peak 2. Peak 1 and peak 2 gave finally purified peptides by reverse phase HPLC on a Vydac phenyl column (Figs, 2b and 2c), in yields of about 100 ng and 150 ng, respectively. The immunoaffinity-adsorbed peptides of fraction B were also purified by reverse phase HPLCs on a C-18 column and then on a phenyl column to a homogeneous state, as shown in Figs. 3a and 3b. The yield of the peptide purified from low MW ir-BNP was estimated to be about 1.5 μg.

First, an amino acid sequence of low MW BNP with major immunoreactivity in the atrium was determined. One-fourth of the finally purified peptide was submitted to N-terminal sequence analysis, and the amino acid sequence was determined up to the 38th residue except for the 23rd residue (Fig. 5b). Two-thirds of the purified peptide was reduced, carboxymethylated, and then digested with lysyl endopeptidase. Three peaks of LEP-1 to LEP-3 were isolated (Fig. 4a), and the amino acid sequence of each peptide was completely

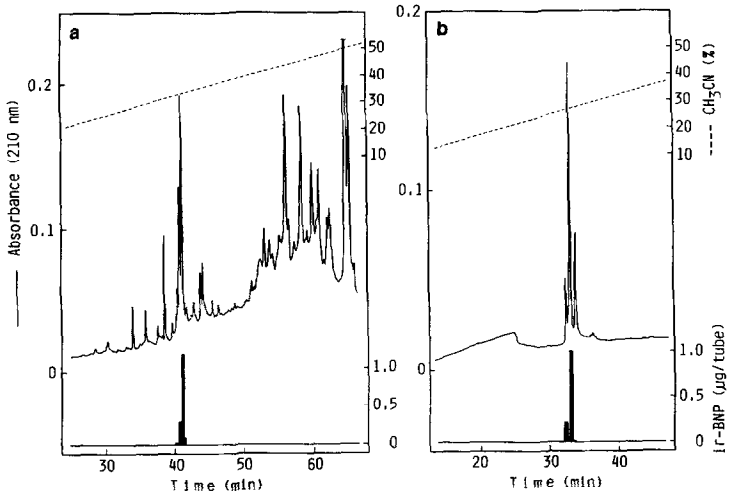

Figure 3. Successive purification of BNP-45 by reverse phase HPLC.
Sample: a) Anti-BNP IgG immunoaffinity chromatography purified fraction obtained from fraction B (#52-58) in Fig. 1.
b) BNP immunoreactive peak in (a).
Column: a) μ-Bondasphere C-18 (3.9 x 150 mm, 300A, Waters).
b) 219TP54 phenyl (4.6 x 250 mm, Vydac).
Solvent: H_2O:CH_3CN:10% TFA = (I) 90:10:1 (v/v), (II) 40:60:1 (v/v).
Linear gradient elution from (I) to (II) for 70 min.
Flow rate: 1 ml/min. Temperature: ambient.

determined, covering the whole sequence of the original peptide. Based on these results, the amino acid sequence of low MW BNP was determined as shown in Fig. 5b and found to be identical to a C-terminal 45-amino acid peptide of

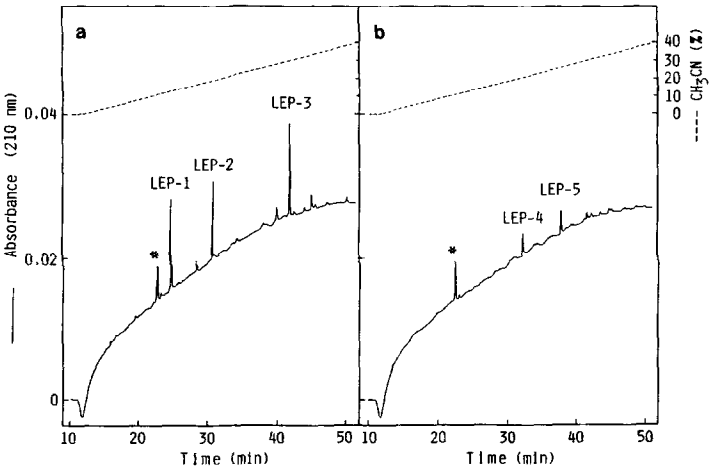

Figure 4. Reverse phase HPLC of lysyl endopeptidase digests of RCM-BNP-45.
Sample: a) Lysyl endopeptidase digests of purified RCM-BNP-45 (ca. 1 μg).
b) Lysyl endopeptidase digests of half of peak LEP-3 in (a).
Column: μ-Bondasphere C-18 (3.9 x 150 mm, 300A, Waters).
Solvent: H_2O:CH_3CN:10% TFA = (I) 100:0:1 (v/v), (II) 40:60:1 (v/v).
Linear gradient elution from (A) to (B) for 60 min.
Flow rate: 1 ml/min. Temperature: ambient.
Peaks marked with asterisks were subjected to sequence analyses, but no PTH-amino acid was detected in 10 cycles of Edman degradation.

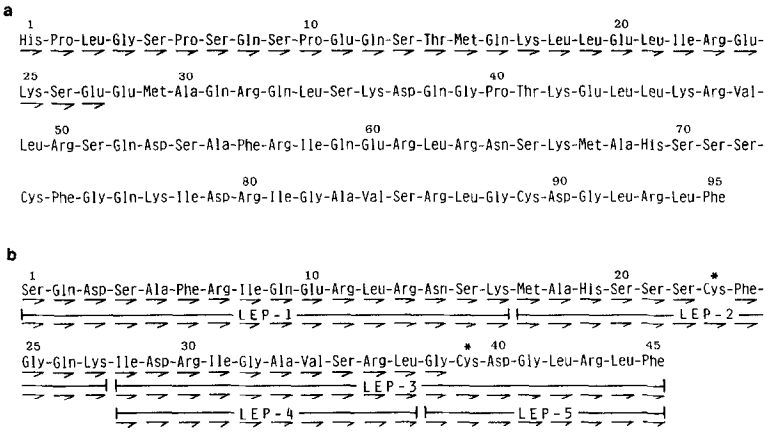

Figure 5. Amino acid sequences of rat (a) γ-BNP and (b) BNP-45. Amino acid sequence analyses were performed directly with purified γ-BNPs (BNP-immunoreactive peaks in Figs. 2b and 2c), and purified BNP-45 (BNP-immunoreactive peak in Fig. 3b). Fragmented peptides prepared with lysyl endopeptidase digestion of RCM-BNP-45 (designated LEP) were also sequenced. LEP-1~LEP-5 correspond to peptides indicated in Figs. 4a and 4b. Arrows indicate amino acid residues identified by stepwise Edman degradation. Two cysteines in each molecule form a disulfide linkage. *) identified as a carboxymethylcysteine.

rat pro-BNP, which was designated BNP-45. To confirm the C-terminal sequence of BNP-45, the fragmented peptide (LEP-3) was intensively digested with lysyl endopeptidase, and amino acid sequences of two peptides (LEP-4 and LEP-5) were determined (Figs. 4b and 5b). Thus, the amino acid sequence of low MW BNP, comprising about 70% of total ir-BNP in the acid extracts of cardiac atria, was finally established and identified as BNP-45.

All the peptides isolated from peak 1 and peak 2 of high MW BNP were directly submitted to sequence analyses, since limited amounts of peptides were purified (Figs. 3a and 3b). PTH-amino acids were identifiable up to the 24th step and the 27th step (Fig. 5a) from the N-termini of the peptides purified from peak 1 and peak 2, respectively. Among the residues identified as PTH-amino acids, no difference in the amino acid sequence was observed between the two peptides. Furthermore, the determined amino acid sequences were completely identical to the N-terminal sequence of rat pro-BNP deduced from the cDNA analysis (3), and these two peptides showed BNP immunoreactivity comparable to BNP-45 on a molar basis, indicating the presence of BNP-45 units in their C-termini. Thus, the high MW form of BNP was identified to be the pro-BNP of 95 residues (Fig. 5a), which was directly generated from pre-BNP by removal of the signal peptide, as predicted in our previuos paper (2). Differences in elution times of the two peptides on reverse phase HPLC may be due to conformational differences or oxidation of methionine residues of the peptides, since similar facts have also been observed during previous purification of human and rat γ-ANPs (unpublished observations).

By identification of BNP-45 and γ-BNP in cardiac atrium, rat pro-BNP deduced from the cDNA analysis was verified to exist as a peptide in heart. The deduced amino acid sequence of pro-BNP was thus confirmed by peptide sequencing. These results demonstrated that the BNP gene is expressed not only in pig but also in other mammals including rat.

In mammalian ANPs and porcine BNP, a high MW γ-form is known to be a major storage form in heart (10,11). In rat cardiac atrium, however, the present data indicate that BNP-45 is the major molecular form, with γ-BNP occupying only 1/3 - 1/4 of the total ir-BNP. Thus, biosynthetic and processing pathway of rat BNP in cardiac atrium was found to be rather different from those of rat ANP and porcine BNP. Since no peptide longer or shorter than BNP-45 (except for γ-BNP) could be isolated even as a minor component in the present study, BNP-45 in rat atrium should be programmed to be generated from γ-BNP and stored to exert the physiologic actions of rat BNP after secretion into the blood stream. In order to characterize rat BNP as a circulating hormone, however, its molecular form and concentration in rat plasma must still be elucidated.

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