Purification and characterization of PAMP-12 (PAMP[9–20]) in porcine adrenal medulla as a major endogenous biologically active peptide

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Abstract Proadrenomedullin N-terminal 20 peptide (PAMP-20) is a potent hypotensive peptide processed from the adrenomedullin (AM) precursor. We developed a specific radioimmunoassay which recognizes the C-terminal region of PAMP-20. Using this radioimmunoassay, the distribution of immunoreactive (ir-) PAMP was determined in porcine tissues. High concentrations of ir-PAMP were observed in the adrenal medulla and in the atrium, and these values were comparable to the corresponding concentrations of ir-AM. The concentration of ir-PAMP was almost the same as that of ir-AM in the kidney, while ir-PAMP was significantly lower than ir-AM in the ventricle, lung, and aorta. Reversed-phase high performance liquid chromatography in each porcine tissue sample revealed that two major peaks of ir-PAMP existed: one emerged at a position identical to that of authentic porcine PAMP-20; the other unknown peak was eluted earlier. The unknown peptide was purified to homogeneity from porcine adrenal medulla, and its complete amino acid sequence was determined. This peptide was found to be PAMP[9–20] with a C-terminal amide structure, and was named PAMP-12. Intravenous injections of PAMP-12 in anesthetized rats showed a significant hypotensive effect in a dose-dependent fashion, and the effect was comparable to that of PAMP-20. These data indicate that PAMP-12, a major component of ir-PAMP, is processed from the AM precursor, as is PAMP-20, and may participate in cardiovascular control.

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Key words: Proadrenomedullin N-terminal 20 peptide; Radioimmunoassay; Distribution; Characterization; Peptide purification; Hypotensive peptide

1. Introduction

Adrenomedullin (AM), originally isolated from human pheochromocytoma tissue [1], has been shown to possess a potent hypotensive action [1,2]. A cDNA cloning of an AM precursor (preproadrenomedullin) was carried out in humans, pigs, and rats [3–5]. The precursor of porcine AM consists of 188 amino acids, including a 21-residue signal peptide at its N-terminus. The N-terminal portion of proadrenomedullin contains a unique 20-residue sequence, which is followed by the typical amidation signal of Gly-Lys-Arg. The peptide thus predicted was designated as proadrenomedullin N-terminal 20 peptide (PAMP-20), whose C-terminus is amidated.

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Abbreviations: PAMP, proadrenomedullin N-terminal 20 peptide; AM, adrenomedullin; ir-, immunoreactive; RIA, radioimmunoassay; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid

We previously established a radioimmunoassay (RIA) which recognizes the entire PAMP-20 molecule, but has little cross-reactivity with fragment peptides of PAMP-20 [6]. With the use of this RIA, the existence of PAMP-20 in vivo was demonstrated by the isolation and characterization of the peptide from both human pheochromocytoma and porcine adrenal medulla [7,8]. PAMP-20 elicited a potent hypotensive effect when injected intravenously in anesthetized rats [8]. PAMP-20 also inhibited carbachol-induced catecholamine synthesis and secretion in cultured bovine adrenal medullary cells [9,10]. It has been reported that the vasodilating activity of PAMP-20 is due mainly to an inhibition of catecholamine release from peripheral sympathetic nerve endings in rat mesenteric artery [11]. These data indicate that PAMP may play a key role in cardiovascular control through a mechanism different from that of AM.

Although both PAMP-20 and AM are produced from the same precursor, the immunoreactive (ir-) PAMP-20 concentrations in tissue and plasma, when assayed with the RIA recognizing the entire PAMP-20 molecule, were lower than those of ir-AM [6,12]. These data suggest that the biosynthesis or metabolism of PAMP may be different from that of AM.

In the present study, to elucidate the biosynthesis and metabolism of PAMP, we developed a new RIA which recognizes the C-terminal region of PAMP. Using this RIA, we examined the distribution of ir-PAMP in porcine tissues. We also demonstrated that PAMP[9–20] (PAMP-12) is a major product of the AM precursor, as is PAMP-20, and that PAMP-12 possesses hypotensive activity comparable to that of PAMP-20.

2. Materials and methods

2.1. Synthetic peptides
N-Tyr-PAMP[10–20], human PAMP[1–20] (PAMP-20), PAMP[9–20] (PAMP-12), [10–20], [13–20], and [16–20] were synthesized by the solid phase method with a peptide synthesizer (Model 431A, Applied Biosystems, Foster, CA, USA), and purified by reversed-phase high performance liquid chromatography (RP-HPLC).

2.2. Preparation of antiserum against PAMP
PAMP[10–20] (8 mg) and bovine thyroglobulin (10 mg) were conjugated by the carbodimide method as described [13]. The reaction mixture was dialyzed four times against one liter of 0.9% NaCl and twice against one liter of 0.05 M sodium phosphate buffer (pH 7.4) containing 0.08 M NaCl. The dialyzed solution was emulsified with an equal volume of Freund's complete adjuvant, then used for immunizing New Zealand white male rabbits as described [14].

2.3. Radioiodination of ligand
N-Tyr-PAMP[10–20] was dissolved in 25 μl of 0.4 M sodium acetate buffer (pH 5.6) in a reaction tube. Lactoperoxidase (Calbiochem,

San Diego, CA, USA: 600 ng/10 µl of 0.1 M sodium acetate buffer, pH 5.6) and H_2O_2 (70 ng/5 µl of water) were added to the reaction tube. After the tube was left standing for 10 min at 30°C, H_2O_2 (70 ng/5 µl of water) was added, and the tube was again left for 10 min at 30°C. ^{125}I-labeled peptide was purified by RP-HPLC on a TSK ODS 120A column (4.6×150 mm, TOSOH, Tokyo, Japan), then stored at −80°C after adding 1% bacitracin and 1% Triton X-100.

2.4. RIA for PAMP and AM

The RIA for PAMP[10–20] was carried out as follows. The RIA incubation buffer for PAMP was 0.05 M sodium phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin, 0.5% Triton X-100, 0.025 M EDTA·2Na, 0.05% NaN₃, and 500 KIU/ml of aprotinin. The RIA incubation mixture was composed of 100 µl of the standard PAMP[10–20] or the unknown sample with 200 µl of an antiserum which contained 0.5% normal rabbit serum, which was designated PAMP Ab-M#6-3, at the dilution of 1:6650. After incubation for 12 h, 100 µl of ^{125}I-N-Tyr-PAMP[10–20]NH₂ (18 000 cpm) was added and incubated for 24 h. One hundred µl of anti-rabbit IgG goat serum diluent was then added, and incubation was continued for 24 h. The tubes were centrifuged at 2000×g at 4°C for 30 min, and the radioactivity of the precipitate was measured with a gamma-counter (Aloka ARC-600, Tokyo).

Ir-AM in the tissue sample was measured by an RIA for human AM using the previously described method [12]. This RIA has shown 100% cross-reactivity with porcine AM.

All assay procedures were done in duplicate at 4°C.

2.5. Preparation of tissue samples

The peptide extract was prepared by the method described previously [1]. Porcine adrenal medulla, atrium, ventricle, lung, kidney, and aorta were collected at a local slaughterhouse soon after killing. After weighing, each tissue sample ($n = 4$, each type of tissue) was boiled for 10 min in 1 M acetic acid containing 20 mM HCl to inactivate intrinsic proteases. After cooling, extractions were homogenized with a Polytron mixer at 4°C, and the mixture was centrifuged at 24 000×g at 4°C for 30 min. The supernatant solution was applied to a Sep-Pak C-18 cartridge column (1 ml, Waters, Milford, MA, USA) pre-equilibrated with 0.5 M acetic acid, and the absorbed material was eluted with 4 ml of 60% CH_3CN containing 0.1% trifluoroacetic acid (TFA). After lyophilization of the sample, the residue was dissolved in the RIA buffer for use in the RIAs for both PAMP and AM.

2.6. Purification and structural analysis of PAMP-12

Porcine adrenal medulla (12 g) was boiled for 10 min in 60 ml of 1 M acetic acid containing 20 mM HCl. After cooling, the extractions were homogenized with a Polytron mixer at 4°C. The extract supernatant obtained after a 30-min centrifugation at 17 000×g was loaded onto a Sep-Pak C-18 cartridge column (20 ml, Waters). After washing the column with 0.5 M acetic acid, the materials absorbed on the column were eluted with 60% CH_3CN containing 0.1% TFA, and the eluent was concentrated. In the subsequent purification procedure, an aliquot of each fraction was examined by the RIA for the C-terminal region of PAMP-20. The concentrated solution was loaded onto a gel-filtration chromatography system (Sephadex G-50, fine, 3×150 cm) which was equilibrated with 1 M acetic acid. The fractions containing ir-PAMP were then separated by RP-HPLC on a column of Chemcosorb 7-ODS-H (10×250 mm, Chemco, Osaka, Japan) (Fig. 3A). The highest unknown peak of ir-PAMP, which emerged earlier than that of porcine PAMP-20, was further applied to ion-exchange HPLC on a column of TSK CM-2SW (4.0×300 mm, TOSOH) (Fig. 3B). The fraction containing the major ir-PAMP was finally purified by RP-HPLC on a column of Chemcosorb 3-ODS-H (2.1×75 mm, Chemco) (Fig. 3C). The purified peptide was sequenced with a gas-phase sequencer (Model 494, Applied Biosystems). The column effluents on the RP-HPLC were monitored by simultaneously measuring the absorbance at 210 and 280 nm.

2.7. Hypotensive activity

The depressor effect of PAMP-12 was examined by methods similar to those reported for human AM [2]. Seven-week-old male Wistar rats (ca. 300 g) were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Blood pressure was monitored continuously from a right carotid artery catheter (PE-50) connected to a Statham pressor transducer (Model P231D, Gould, Saddle Brook, NJ, USA).

A PE-10 catheter was inserted into the right jugular vein for the administration of both maintenance solution and peptides. After equilibration for at least 60 min, PAMP-12 or human PAMP-20 was injected intravenously.

2.8. Statistical analysis

All data are presented as means ± S.E.M. For the comparison of statistical significance between two groups, Student's t-test for unpaired data was used. A value of $P < 0.05$ was accepted as significant.

3. Results

3.1. RIA for PAMP

The antiserum to PAMP[10–20], PAMP Ab-M#6-3, recognized synthetic PAMP[10–20] with high affinity at a final dilution of 1:13 300. As shown in Fig. 1, the half-maximal inhibition of radio-iodinated ligand binding by PAMP[10–20] occurred at 30 fmol/tube. The measurable range of this RIA was from 2 to 256 fmol/tube. The intra- and inter-assay coefficients of variance were 6% and 9%, respectively. The dilution curve of the extracts of porcine adrenal medulla was roughly parallel to the standard curve of PAMP[10–20]. The RIA had 100% and 50% cross-reactivity with PAMP-12 and human PAMP-20, respectively, but had less than 0.01% cross-reactivity with PAMP[13–20] and [16–20].

3.2. Distribution of ir-PAMP and ir-AM in porcine tissues

High concentrations of ir-PAMP in the adrenal medulla and in the atrium were comparable to the corresponding concentrations of ir-AM (Table 1). The concentration of ir-PAMP was almost the same as that of ir-AM in the kidney, while ir-PAMP was significantly ($p < 0.05$) lower than ir-AM in the ventricle, lung, and aorta. In the lung, the ir-PAMP concentration was only 8% of that of ir-AM.

3.3. Characterization of ir-PAMP

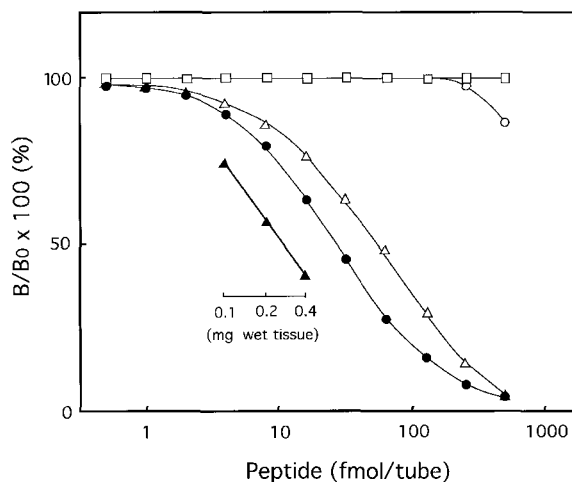The characterization of ir-PAMP in the porcine adrenal

Fig. 1. Standard curve of RIA for PAMP and cross-reactivity of antiserum. Inhibition of ^{125}I-labeled human PAMP[10–20] binding to antiserum by serial dilutions of human PAMP[1–20] (−△−), PAMP[9–20] and [10–20] (−●−), [13–20] (−○−), and [16–20] (−□−). PAMP[9–20] (PAMP-12) and human PAMP[1–20] (PAMP-20) showed 100% and 50% cross-reactivity with antiserum. PAMP[13–20] and [16–20] exhibited less than 0.01% cross-reactivity with antiserum. The dilution curve of the porcine adrenal medulla sample (−▲−) was parallel to the standard curve.

medulla and atrium, whose PAMP concentrations were abundant, was performed by RP-HPLC, coupled with the present RIA for PAMP. As shown in Fig. 2, an unknown major peak emerged at 22 min in both tissues. The elution time was 8 min earlier than that of synthetic porcine PAMP-20. The unknown major immunoreactivity was approximately twice as high as that of ir-PAMP-20. The unknown peak was therefore purified to determine its complete amino acid sequence, using porcine adrenal medulla which contained a high PAMP concentration.

3.4. Isolation and structural analysis

The unknown major peak of ir-PAMP in the porcine adrenal medulla extract was purified to homogeneity using the present RIA for PAMP. In gel-filtration chromatography analysis, one ir-PAMP peak was observed at M_r 1500–2500. These fractions containing ir-PAMP were further separated by RP-HPLC. As shown in Fig. 3A, two major peaks of ir-PAMP were found: One (peak II) emerged at a position identical to that of synthetic porcine PAMP-20; the other unknown peak (peak I) emerged earlier and contained a 1.8-fold greater amount of ir-PAMP-20. The unknown major peak was applied to ion-exchange HPLC. A major ir-PAMP peak was eluted later than that of synthetic porcine PAMP-20 (Fig. 3B). The fraction containing the unknown major ir-
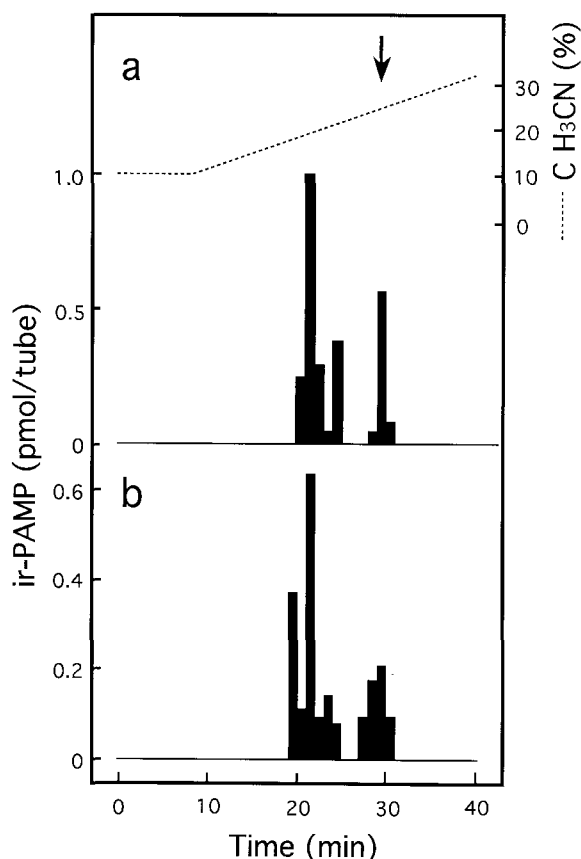

Fig. 2. Characterization of ir-PAMP by RP-HPLC. Sample: (a) adrenal medulla (8 mg eq.), (b) atrium (1.6 g eq.). Column: TSK ODS 120A, 4.6×150 mm. Flow rate: 1.0 ml/min. Solvent system: (a) H₂O:CH₃CN:10% TFA=90:10:1; (b) H₂O:CH₃CN:10% TFA=40:60:1. Linear gradient from (a) to (b) for 60 min. The arrow shows the elution position of porcine PAMP-20.

Table 1
Regional distributions of ir-PAMP and ir-AM in porcine tissue

Region	ir-PAMP	ir-AM
Adrenal medulla	222.3 ± 27.2	205.4 ± 15.4
Heart atrium	1.50 ± 0.36	1.56 ± 0.37
Heart ventricle	0.14 ± 0.02	0.24 ± 0.02*
Lung	0.13 ± 0.02	4.28 ± 0.17*
Kidney	0.19 ± 0.01	0.29 ± 0.03
Aorta	0.11 ± 0.01	0.22 ± 0.04*

Results are expressed as fmol/mg wet tissue. All values are means ± S.E.M. of four samples. *$P < 0.05$ vs. ir-PAMP.

PAMP was finally purified by RP-HPLC. The HPLC yielded a homogeneous peptide peak (Fig. 3C). The recovery of this peptide was 90 pmol, starting from 12 g of porcine adrenal medulla.

Half of the purified peptide was subjected to a gas-phase automated sequencer. Its complete amino acid sequence was identified as Phe-Arg-Lys-Lys-Trp-Asn-Lys-Trp-Ala-Leu-Ser-Arg (Table 2). The yield of Trp, Ser, and Arg was too low to give accurate values, although they appeared in detectable amounts. Thus, the major immunoreactive peptide was identified as PAMP[9–20], which lacked eight amino acids at the N-terminal site of PAMP. This peptide was named PAMP-12.

The C-terminus of Arg was found to be amidated; the elution position of this peptide in RP-HPLC and ion-exchange HPLC was found to be identical to that of synthetic PAMP-12.

3.5. Hypotensive effect

The hypotensive effects of the PAMPs in anesthetized rats are demonstrated in Fig. 4. The intravenous bolus injection of PAMP-12 in doses of 10 to 50 nmol/kg caused a significant hypotensive effect in a dose-dependent manner. When PAMP-12 at 50 nmol/kg was injected intravenously, the maximum decrease of mean blood pressure was 39.7 ± 3.8 mmHg ($n = 6$). The hypotensive activity of PAMP-12 did not differ significantly from that of human PAMP-20.

4. Discussion

In this study, we established an RIA for the C-terminal region of PAMP, and demonstrated the regional distribution of ir-PAMP in porcine tissues. We also identified PAMP-12 in porcine adrenal medulla as a major endogenous hypotensive peptide from the AM precursor.

The distribution of PAMP was similar to that of AM, implying that PAMP is biosynthesized from the AM precursor. The concentration of ir-PAMP was comparable to that of ir-AM in the adrenal medulla, atrium, and kidney. Unexpectedly, however, its value was less than half of that of ir-AM in other tissues. These data suggest that the mechanism of biosynthesis or metabolism of PAMP may be different among these tissues.

It is noteworthy that the ir-PAMP concentration in the lungs was very low, compared to ir-AM. Owji et al. reported that rat lung has the most abundant binding sites of AM, about seven-fold that of the adrenal gland [15]. Additionally, in patients with ischemic heart disease, the plasma concentration of AM in the aorta was significantly lower than that in the pulmonary artery, suggesting that pulmonary circulation may be one of the important sites for endogenous AM clear-
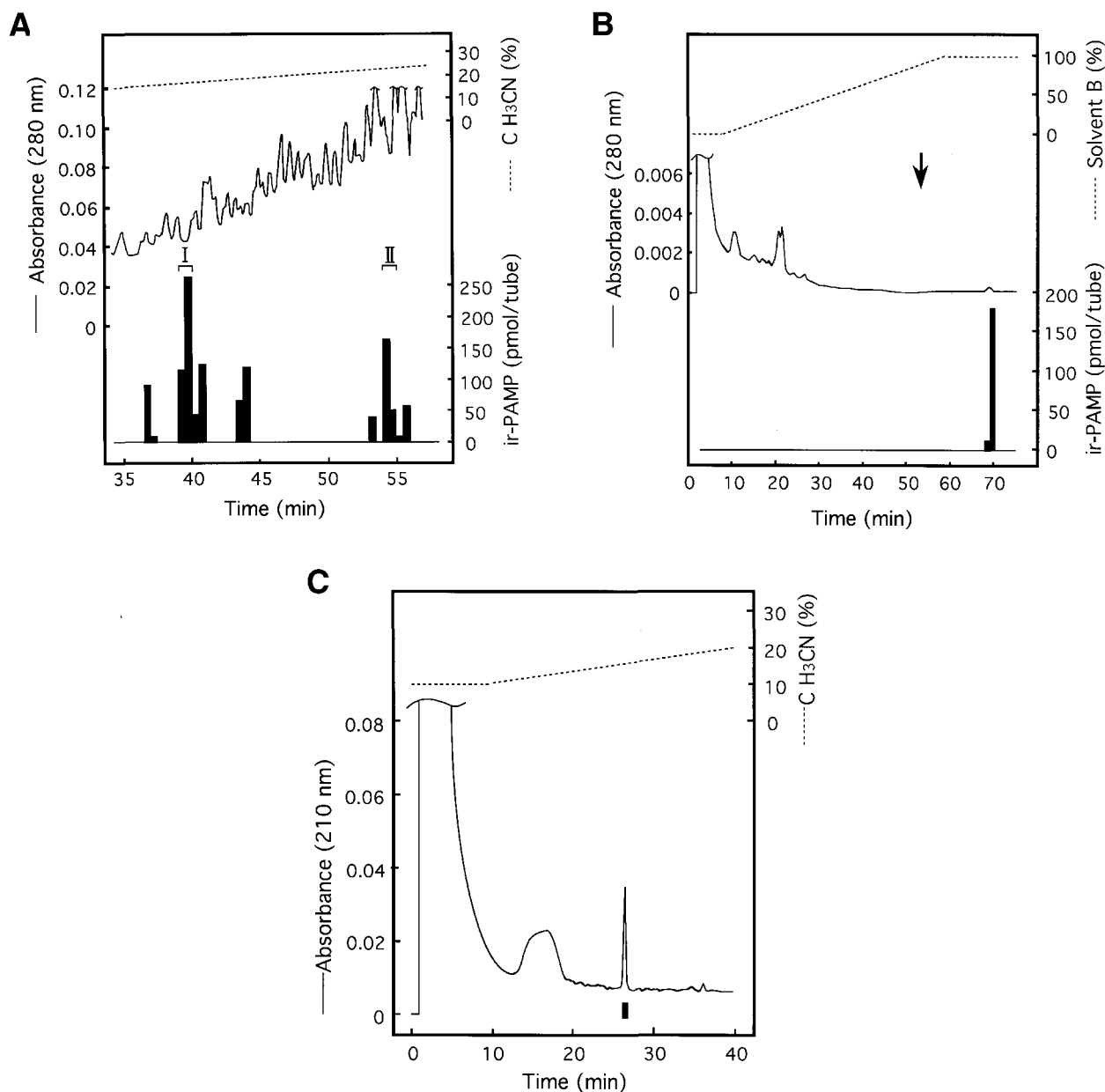
Fig. 3. Purification of porcine adrenal medulla monitored by RIA for PAMP. (A) Reversed-phase HPLC. Sample: Immunoreactive (ir-) PAMP obtained from 12 g porcine adrenal medulla tissue by gel-filtration (Sephadex G-50). Column: Chemcosorb 7-ODS-H, 10×250 mm. Flow rate: 2.0 ml/min. Solvent system: Linear gradient from (A) to (B) for 120 min. (A) and (B): the same as Fig. 2. Peak I:380 pmol or ir-PAMP; Peak II:210 pmol of PAMP-20. (B) CM ion-exchange HPLC. Sample: peak I in A. Column: TSK CM-2SW, 4.0×300 mm. Flow rate: 1.0 ml/min. Solvent system: (A) 10 mM HCOONH$_4$ (pH 6.5): CH$_3$CN=90:10 (V/V); (B) 1 M HCOONH$_4$ (pH 6.5):CH$_3$CN=90:10 (V/V). Linear gradient from (A) to (B) for 60 min. The arrow shows the elution position of porcine PAMP-20. (C) Final purification of ir-PAMP by RP-HPLC. Sample: major ir-PAMP in B. Column: Chemcosorb 3-ODS-H, 2.1×75 mm. Flow rate: 0.2 ml/min. Solvent system: Linear gradient from (A) to (B) for 80 min. (A) and (B): the same as Fig. 2. Absorbance at 210 nm (—) was monitored. Black bar indicates 90 pmol of ir-PAMP.

ance [16]. In contrast, PAMP receptors in the rat lung is one-fifth of that in the adrenal gland [17]. The difference of receptor binding sites for AM and PAMP may be the main contributor to the discrepancy of immunoreactive concentrations between these peptides.

Here we purified 90 pmol of PAMP-12, starting from 12 g of porcine adrenal medulla which contained 1850 pmol of total ir-PAMP. The low recovery of purified PAMP-12 is probably due to its strong basic and hydrophobic characteristics. However, a major component of ir-PAMP in adrenal medulla must be PAMP-12, because the purified ir-PAMP

was eluted at the same position where the major ir-PAMP in the crude adrenal medulla extract was eluted. In addition, the recovery rate of ir-PAMP-12 in each purification step after the initial RP-HPLC analysis was more than 46%. We thus purified the major ir-PAMP peaks from porcine adrenal medulla.

The structure of PAMP-12, determined by sequence analyses (Table 2), was further confirmed by HPLC analyses of purified PAMP and synthetic PAMP. Since the elution profiles of the two peptides were identical on RP-HPLC and ion-exchange HPLC, the purified peptide in porcine adrenal me-
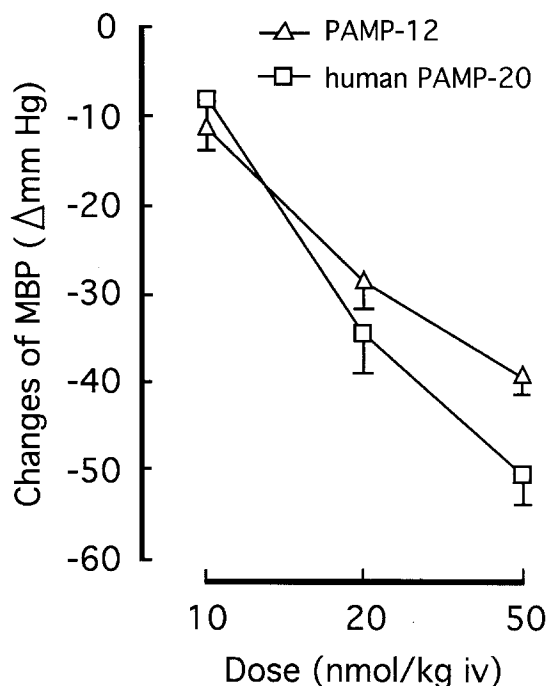
Fig. 4. Hypotensive responses of anesthetized rats to intravenous bolus injections of PAMPs. A bolus injection of PAMP-12 caused a significant hypotensive effect in a dose-dependent fashion. The maximum decrease of mean blood pressure (MBP) ($n=6$) was 39.7 ± 3.8 mmHg for 50 nmol/kg. The hypotensive activity of PAMP-12 did not differ significantly from that of human PAMP-20.

dulla is identical to synthetic PAMP-12 with an amidated carboxy terminus.

Since the previous RIA for the entire PAMP-20 molecule showed only 19% cross-reactivity with PAMP-12 [6], the existence of PAMP-12 in vivo was not identified in previous studies [7,8]. In the present study, the immunoreactive peak of PAMP-12 was demonstrated to be reproducibly higher than that of PAMP-20 by the RP-HPLC analyses, indicating that this peptide exists in vivo as an endogenous peptide. In the adrenal medulla and atrium, in particular, the ir-PAMP-12 concentration was approximately twice as high as that of ir-PAMP-20 (Fig. 2). The new RIA for the C-terminal region of PAMP-20 showed 100% and 50% cross-reactivity with PAMP-12 and PAMP-20, respectively. These findings indicate

Table 2
Amino acid sequence determination of isolated peptide

Cycle number	Peptide	(pmol)
1	Phe	3.30
2	Arg	1.05
3	Lys	1.86
4	Lys	1.99
5	Trp	
6	Asn	0.97
7	Lys	2.16
8	Trp	
9	Ala	0.83
10	Leu	0.70
11	Ser	
12	Arg	

that the presence of PAMP-12 and that of PAMP-20 are comparable in both tissues. Further studies are required to elucidate the mechanism by which PAMP-12 is biosynthesized in vivo.

We previously demonstrated that PAMP-20 shows a potent hypotensive activity [8], and that its plasma concentration is increased in spontaneously hypertensive rats and in patients with congestive heart failure or renal failure [18–20]. In the present study, it was found that PAMP-12 also had a significant hypotensive action, comparable to that of PAMP-20. PAMP[10–20] also showed considerable hypotensive activity, but PAMP[16–20] and PAMP without the C-terminal amide structure showed little hypotensive activity (unpublished observations). The sequence between the 9th and 20th amino acid of PAMP-20 is well conserved among species [3–5]. Taken together, these findings suggest that the C-terminal region including the amide structure of PAMP plays an important role in the biological activity of PAMP.

In conclusion, we have identified PAMP-12 in porcine adrenal medulla as a major product of the AM precursor, and demonstrated that it exhibits a potent hypotensive action. Although the existence of a specific receptor for PAMP-12 and PAMP-20 remains obscure, PAMP-12 might share a biological function with PAMP-20.

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