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Identification and hypotensive activity of proadrenomedullin N-terminal 20 peptide (PAMP)

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Abstract

Proadrenomedullin N-terminal 20 peptide (PAMP) is a candidate for a novel biologically active peptide processed from an adrenomedullin precursor. Using a radioimmunoassay for human PAMP, major and minor immunoreactive PAMPs were purified from porcine adrenal medulla and complete amino acid sequences were determined. The major immunoreactive peptide was PAMP itself with an amidated carboxy terminus. The minor one was determined to be PAMP[5–20]. An intravenous bolus injection of human PAMP in anesthetized rats caused a rapid and strong hypotensive effect in a dose dependent manner. The present data indicate that PAMP is an endogenous biologically active peptide which is processed from adrenomedullin precursor.

Key words: Adrenomedullin; Proadrenomedullin N-terminal 20 peptide (PAMP); Peptide purification; Amino acid sequence; Hypotensive peptide

1. Introduction

Adrenomedullin is a newly identified hypotensive peptide isolated from human pheochromocytoma. This novel peptide was discovered while monitoring the elevating activity of rat platelet cAMP [1]. Subsequently, cDNA clones encoding a human and a porcine adrenomedullin precursor were isolated from human pheochromocytoma and from porcine adrenal medulla cDNA library and their nucleotide sequences were determined [2,3]. The precursor for porcine adrenomedullin (preproadrenomedullin) consists of 188 amino acids in length, including the porcine adrenomedullin sequence. In addition to adrenomedullin, proadrenomedullin contains a unique 20 residue sequence followed by Gly-Lys-Arg in the N-terminal region. This peptide was termed proadrenomedullin N-terminal 20 peptide (PAMP) whose carboxy terminus may be amidated, although the biological activity of PAMP in vivo has not yet been explored. Very recently, we have established a radioimmunoassay (RIA) for human PAMP and examined immunoreacive PAMP in human tissue [4]. Immunoreactive PAMP was abundant in the adrenal medulla and the cardiac atrium as well as in pheochromocytoma tissue.

In the present study, using the RIA for human PAMP, we purified immunoreactive PAMPs from porcine adrenal medulla and determined their complete amino acid sequences. In the second part of the study, human PAMP was found to be a biologically active peptide which exerts a potent hypotensive activity in anesthetized rats.

2. Materials and methods

2.1. Peptides

N-Tyr-human PAMP[1–20]NH_2 and human PAMP[1–20]NH_2, -[16–20]NH_2, and -[16–20]C00H were synthesized by the solid phase method with a peptide synthesizer (431A, Applied Biosystems), and purified by reverse-phase high performance liquid chromatography (HPLC).

2.2. RIA for PAMP

The RIA for human PAMP was performed by the method as described recently [4]. 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, 0.08 M NaCl, 0.025 M EDTA-2Na, 0.05% NaN_3 and 500 kIU/ml of aprotinin. The RIA incubation mixture was composed of 100 μl of standard PAMP or the unknown sample, and 200 μl of antiserum at a dilution of 1:66,500 which contained 0.5% normal rabbit serum. After incubation for 12 h, 100 μl of ^{125}I-labelled ligand (18,000 cpm) was added. After incubation for 24 h, 100 μl of anti-rabbit IgG goat serum diluent was added and incubated for 24 h. The tubes were centrifuged at 2,000 × g for 30 min, and radioactivity of the precipitate was measured by a gamma-counter (Aloka ARC-600, Tokyo). All assay procedures were performed in duplicate at 4°C.

2.3. Preparation of peptide extract

Preparation of peptide extract was performed by a method similar to that described [1,3]. Diced pieces of porcine adrenal medulla (5 g) were boiled for 10 min in 10 vols. of 1 M acetic acid containing 20 mM HCl to inactivate intrinsic proteases. After cooling, the mixture was homogenized at 4°C with a polytron homogenizer. The extract supernatant, obtained after 30 min centrifugation at 22,000 × *g*, was applied to a Sep-Pak C-18 cartridge column (20 ml, Waters). After washing the column with 0.1% trifluoroacetic acid (TFA), the materials adsorbed onto the column were eluted with 60% CH_3CN containing 0.1% TFA.

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Abbreviations: PAMP, proadrenomedullin N-terminal 20 peptide; RIA, radioimmunoassay; ir-, immunoreactive; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid.

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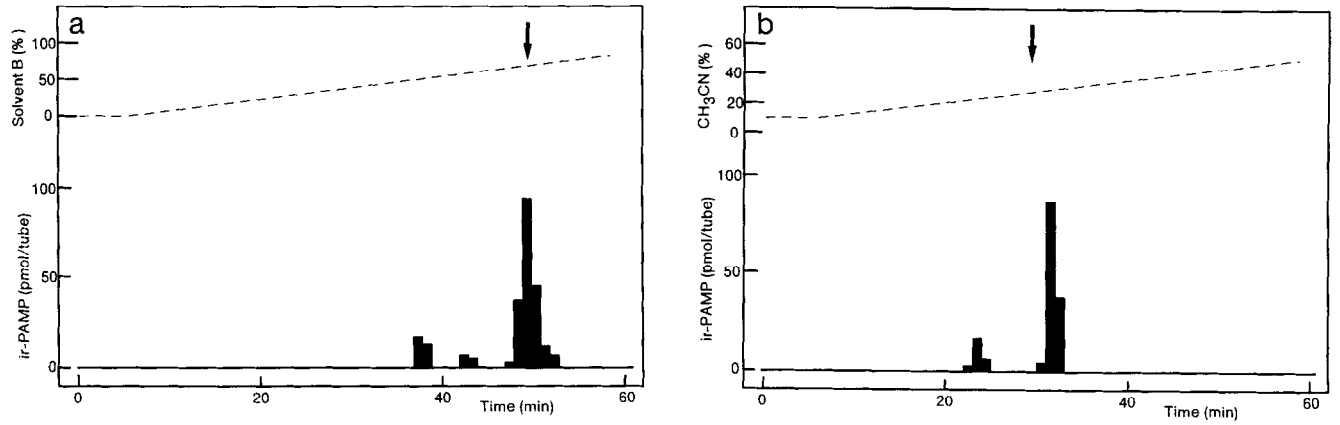

Fig. 1. (a) Ion-exchange HPLC of porcine adrenal medulla monitored by RIA for human PAMP. Sample: Immunoreactive (ir-) PAMP obtained from 5 g porcine adrenal medulla tissue by gel filtration (Sephadex G-50). Column: TSK CM-2SW, 4.0 × 300 mm. Flow rate: 1 ml/min. Solvent system: (A) 10 mM $HCOONH_4$ (pH 6.5): CH_3CN = 90:10 (by volume), (B) 1 M $HCOONH_4$ (pH 6.5): CH_3CN = 90:10 (by volume). Linear gradient elution from (A) to (B) for 60 min. Arrow indicates the elution position of synthetic human PAMP. Fig. 1b: Reverse phase HPLC of porcine adrenal medulla monitored by RIA for human PAMP. Sample, The major immunoreactive fractions shown in Fig. 1a. Column: ODS 120A column (4.6 × 250 mm, Tosoh). Flow rate: 1.0 ml/min. Solvent system: linear gradient elution from A:B = 100:0 to A:B = 0:100 (60 min). (A) H_2O:CH_3CN:10% TFA = 90:10:1 (by volume); (b)H_2O:CH_3CN: 10% TFA = 40:60:1 (by volume). Arrow indicates the elution position of synthetic human PAMP.

The eluate was concentrated, then was used as the starting material in the present study.

2.4. Structural analyses

Amino acid sequence analyses of the peptides were performed by a gas-phase sequencer equipped with phenylthiohydantoin amino acid analysing HPLC system (Model 470A/120A, Applied Biosystems). Phenylthiohydantoin amino acids were detectable as low as 0.1 pmol [5].

2.5. Hypotensive effect

Depressor effect of PAMP was examined by methods similar to those reported for human adrenomedullin [6]. Seven-week-old male Wistar rats (300 g) were anesthetized by an intraperitoneal injection of pentobarbital sodium (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). A PE-10 catheter was inserted into the right jugular vein for administration of both maintenance solution and peptides. After equilibration for at least 60 min, human PAMP was injected intravenously.

3. Results and discussion

We have already established a sensitive RIA system which can detect human PAMP [4]. Using this RIA system, we started purification of porcine PAMP from adrenal medulla. Porcine adrenal medulla peptide extract, prepared as described in section 2, was separated by gel filtration chromatography (Sephadex G-50, fine, 1.6 × 150 cm). One major ir-PAMP was observed in M_r 2000–2500. The peptides in the fraction were further separated by CM ion exchange HPLC on a column of TSK CM-2SW (4.0 × 150 mm, Tosoh). As shown in Fig. 1a, one major immunoreactive PAMP is observed at the same position as synthetic human PAMP, and two minor peaks emerge earlier. The major immunoreactive peak which contained more than 90% of total PAMP immunoreactivity was further purified by reverse phase

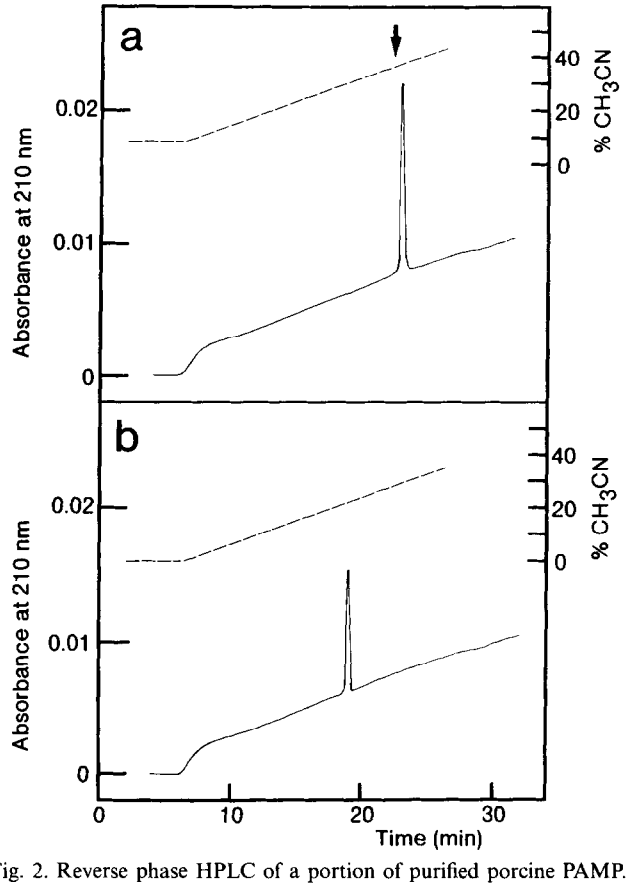

Fig. 2. Reverse phase HPLC of a portion of purified porcine PAMP. Sample (a): 15 pmol of purified major immunoreactive PAMP as described in the text. Sample (b): 10 pmol of purified minor immunoreactive PAMP as described in the text. Column: μ-Bondasphere ODS 300A (2.1 × 150 mm, Waters). Flow rate: 0.3 ml/min. Solvent system: linear gradient elution from A:B = 100:0 to A:B = 0:100 (40 min). (A) H_2O:CH_3CN:10%TFA = 90:10:1 (by volume); (b) H_2O:CH_3CN: 10%TFA = 40:60:1 (by volume). Absorbance at 210 nm (——) was monitored. Arrow indicates the elution position of synthetic human PAMP.

HPLC using an ODS 120A column (4.6 × 250 mm, Tosoh). As shown in Fig. 1b, one major and one minor immunoreactive peaks were observed and they were finally purified by reverse phase HPLC on Chemcosorb 3ODS-H column (4.0 × 150 mm, Chemco, Osaka). The recovery of major and minor peptides was 150 pmol and 20 pmol, respectively, starting from 5 g of porcine adrenal medulla. As shown in Fig. 2, reverse phase HPLC on a semimicro column of a portion of purified PAMPs gives a single peptide peak, confirming the homogeneity of these peptides. The major peak was eluted a little later than the synthetic human PAMP (Fig. 2a), while the minor immunoreactive peptide emerged 3.5 min earlier than that of human PAMP (Fig. 2b).

Each purified peptide was subjected to a gas phase sequencer and complete amino acid sequences of major and minor immunoreactive peptides were determined as shown in Fig. 3. The major immunoreactive peptide was 20-residue porcine PAMP itself, whose amino acid sequence is identical to that of deduced amino acid sequence by cDNA analysis. The carboxy-terminus of Arg was found to be amidated. The amino acid sequence of the minor immunoreactive peptide was found to be PAMP[5–20] which lacked four amino acids at the amino-terminal site of PAMP. Because the recovered amount of PAMP[5–20] is low compared to that of PAMP, PAMP[5–20] may be a mere metabolite of PAMP by proteolyses before extraction. But it cannot be denied at present that PAMP[5–20] as well as PAMP has some biological function in vivo as an endogenous peptide.

As a first step in investigating the biological activity of PAMP, we examined the vasodepressor effect of human PAMP, whose amino acid sequence was identical with that of porcine PAMP except for one amino acid residue. Human PAMP was found to elicit a potent hypotensive effect in anesthetized rats. Typical hypotensive profiles for anesthetized rat are shown in Fig. 4. An intravenous bolus injection of PAMP caused a rapid and strong hypotensive effect in a dose dependent manner. When PAMP at 50 nmol/kg was injected intravenously, the maximum decrease of mean blood pressure was 49 ± 4.3 mmHg (mean ± S.E.M., $n = 6$). This value is comparable to that of a 3 nmol/kg adrenomedullin injection [1]. The significant hypotensive effect lasted for 5–10 min which was much shorter than that of adrenomedullin [1]. Consequently, it was concluded that human PAMP has a potent hypotensive effect. Very recently, it has been reported that human PAMP had no hypotensive activity in anesthetized rats [7]. We cannot explain reasons for this discrepancy with our data, but our data have reproducibly demonstrated that human PAMP exerted hypotensive activity following their intravenous injections.

Major Immunoreactive PAMP

Ala-Arg-Leu-Asp-Val-Ala-Ala-Glu-Phe-Arg-Lys-Lys-Trp-Asn-Lys-Trp-Ala-Leu-Ser-Arg-NH_2

(5 10 15 20)

Minor Immunoreactive PAMP

Val-Ala-Ala-Glu-Phe-Arg-Lys-Lys-Trp-Asn-Lys-Trp-Ala-Leu-Ser-Arg-NH_2

Fig. 3. Complete amino acid sequence of porcine PAMP. Sequence analysis was performed with major and minor immumoreactive PAMPs isolated from porcine adrenal medulla.

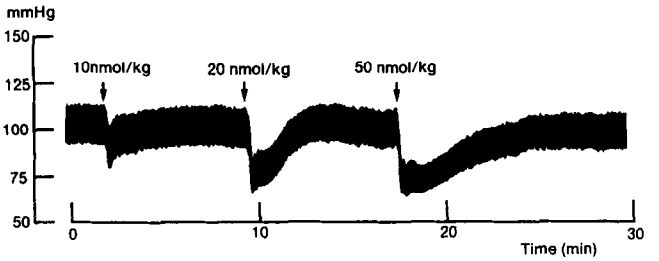

Fig. 4. Typical recordings for hypotensive responses in anesthetized rats to intravenous bolus injection of human PAMP. The maximum decrease of mean blood pressure (mean ± S.E.M., $n = 6$) was 9.0 ± 2.9 mmHg for 10 nmol/kg of PAMP, 33 ± 5.3 mmHg for 20 nmol/kg of PAMP and 49 ± 4.3 mmHg for 50 nmol/kg of PAMP.

In conclusion, we identified the porcine PAMP as an endogenous peptide and demonstrated for the first time that human PAMP had potent hypotensive activity. The present data indicate that PAMP as well as adrenomedullin is biosynthesized from adrenomedullin precursor and may participate in circulation control.

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