FEBS Letters 352 (1994) 105–108

FEBS 14541

Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma

Junichiro Sakata[a,*], Toru Shimokubo[a], Kazuo Kitamura[a], Masatoshi Nishizono[a], Yoshinari Iehiki[a], Kenji Kangawa[b], Hisayuki Matsuo[b], Tanenao Eto[a]

[a]*First Department of Internal Medicine, Miyazaki Medical College, Kihara Kiyotake, Miyazaki 889-16, Japan*
[b]*National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565, Japan*

Received 10 June 1994

Abstract Adrenomedullin is a new bioactive peptide recently isolated from pheochromocytoma. We report on the rat adrenomedullin distribution and molecular forms in various tissues and plasma. Using a sensitive radioimmunoassay system for rat adrenomedullin, high concentrations of immunoreactive rat adrenomedullin were detected in adrenal gland, lung and cardiac atrium. In lung and atrium, the immunoreactivity concentration in rat was about 6–10 times higher than that in human. The mean plasma concentration of immunoreactive rat adrenomedullin was 3.60 ± 0.34 fmol/ml (mean ± S.D.). Analysis in adrenal gland, lung and atrium with reverse-phase and gel-filtration high-performance liquid chromatography showed that most immunoreactive rat adrenomedullin emerged as a single peak at a position exactly identical to that of the authentic rat adrenomedullin peptide, synthesized according to the sequence predicted from the cDNA.

Key words: Adrenomedullin (rat); Radioimmunoassay; Distribution; Characterization; Plasma; High-performance liquid chromatography

1. Introduction

A potent vasorelaxing and hypotensive peptide, designated adrenomedullin (AM), has been recently isolated from human pheochromocytoma. It is a likely candidate for a new hormone because of its considerable amount of immunoreactivity in healthy human plasma [1,2]. In our previous study, the primary structure of rat AM and its precursor were determined by cDNA cloning [3]. The precursor was 185 amino acids in length, including a 21-residue putative signal peptide at the N-terminus. Rat AM consists of 50 amino acids, similar to but distinct from human AM; 2 residues are deleted and 6 residues are substituted when compared to the human peptide. Rat AM peptide, synthesized according to the sequence encoded in the cDNA, was shown to possess a potent hypotensive activity as well as an elevating activity on rat platelet cAMP [3]. RNA blot analysis showed that rat AM mRNA was expressed in various tissues including adrenal gland, lung, kidney, heart, spleen, duodenum and submandibular glands [3]. However, we lack any quantitative and qualitative information about the biosynthesized AM in rat tissue.

In the present study, we measured the concentration of rat AM in various tissues and plasma by using a radioimmunoassay (RIA) system for rat AM, and then characterized immunoreactive rat AM (ir-rAM) in tissue and plasma with chromatography.

2. Materials and methods

2.1. Peptide

According to the primary structure predicted from the nucleotide sequence [2], rat AM was synthesized by the solid phase method and purified by reverse-phase HPLC in the Peptide Institute, Inc. (Osaka, Japan).

2.2. RIA for rat AM

RIA for rat AM was carried out by the previously described method [6] using an antiserum (#172CI-7) against human AM[40–52]NH_2 [4], since rat AM was found to have 100% crossreactivity with it. The incubation buffer for RIA was 50 mM sodium phosphate buffer (pH 7.4), containing bovine serum albumin (BSA) treated with 0.5% *N*-ethylmaleimide, 0.5% Triton X-100, 80 mM sodium chloride, 25 mM EDTA·2Na, 0.05% sodium azide. One hundred μl of standard of rat AM or unknown sample was incubated for 2 h with 50 μl of the antiserum diluent, followed by addition of 50 μl of the tracer (18,000 cpm) iodinated by the Bolton and Hunter method [5]. After additional incubation for 24 h, free and bound tracers were separated by centrifugation (2,000 × *g*, 30 min) in the presence of polyethyleneglycol (MW 6,000) and bovine γ-globulin. The supernatant was aspirated, then radioactivity in the pellet was counted with a gamma counter (Aloka ARC-600). Assays were performed in duplicate at 4°C.

2.3. Preparation of tissue samples

Tissue samples were prepared by a method similar to that previously described [7,8]. After decapitation, various tissues of Sprague–Dawley rats were resected, quickly frozen with liquid nitrogen and stored at −80°C until use. Each tissue was weighed, diced and boiled in 5 volumes of 1 M acetic acid for 7 min to inactivate intrinsic proteases. Boiled tissue was extracted with a Polytron mixer for 2 min. The homogenate was centrifuged at 18,000 × *g* for 30 min and the supernatant was applied to a Sep-Pak C-18 cartridge (0.9 ml, Waters, MA, USA). Adsorbed materials were eluted with a solution of 60% CH_3CN containing 0.1% trifluoroacetic acid (TFA) after washing the cartridge with 0.5 M acetic acid. The eluate was then evaporated in vacuum to dryness and stored before use for measurement of ir-rAM.

2.4. Preparation of plasma sample

Whole blood was collected in ice-cooled tubes containing 1/10 volume of 10 mg/ml EDTA·2Na and 700 μg/ml of Trasylol. The plasma sample, obtained by centrifugation at 3,000 × *g* for 20 min at 4°C, was diluted with an equal volume of saline. The sample was then loaded on a Sep-Pak C-18 cartridge preequilibrated with saline. The cartridge was washed with saline and then 0.1% TFA. Adsorbed materials were eluted and evaporated as described above.

2.5. Characterization of ir-rAM in tissue

To identify and characterize ir-rAM, adrenal gland, lung and atrium were extracted by the method described above. The C-18 resin treated fractions (10 mg wet tissue eq.) were subjected to reverse-phase HPLC on a TSK ODS 120A column (4.6 × 150 mm, Tosoh, Japan). Reverse-phase HPLC was performed with a linear gradient elution of CH_3CN from 10% to 60% in a solution of 0.1% TFA. Gel-filtration HPLC on

*Corresponding author. Fax: (81) (985) 85 6596.

SSDI 0014-5793(94)00928-7

a TSK-Gel 2000SW column (8 × 500 mm, Tosoh, Japan) was carried out with a solvent containing 30% CH_3CN, 0.1% TFA and 0.2 M sodium chloride. An aliquot of each fraction obtained by reverse-phase and gel-filtration HPLC was submitted to RIA for rat AM.

2.6. Characterization of ir-rAM in plasma

The C-18 column cartridge adsorbed fraction of plasma samples (60 ml eq.) was separated by Sephadex G-50 fine column (1.5 × 100 cm) using 1 M acetic acid as a solvent. An aliquot of each fraction was submitted to RIA for rat AM. The fractions containing ir-rAM were further analyzed by reverse-phase HPLC on a column of TSK ODS 120A (4.6 × 150 mm) with a linear gradient elution of CH_3CN from 10% to 60% in a solution of 0.1% TFA.

3. Results and discussion

3.1. RIA for rat AM

An antiserum (#172CI-7) was raised against the carboxy terminal region of human AM by the described method [4,9]. It showed 100% crossreactivity with synthetic rat AM, whose 7 amino acid residues in the carboxy terminus were identical to those in human AM. The antiserum was used in the RIA for rat AM, proven to be usable at a final dilution of 1:220,000. As shown in Fig. 1, half maximal inhibition of tracer binding by rat AM was observed at 10 fmol/tube and the peptide was measurable in a range of 1 to 256 fmol/tube. The intra and inter-assay coefficients of variance were less than 6% and 9%, respectively. The dilution curve of rat tissue extracts was parallel to the standard curve. In addition, rat calcitonin gene-related peptide (CGRP) and amylin showed no crossreactivity with the antiserum, although they have a slight homology in amino acid residues with rat AM. These results indicate that this assay system is sufficiently sensitive and specific for detection of rat AM.

3.2. Distribution of ir-rAM in tissue and plasma

Using the RIA for rat AM, distribution of ir-rAM in tissue and plasma was investigated. As can be clearly seen in Table 1, concentrations of ir-rAM were high in adrenal gland (12.2 ± 0.9 fmoVmg wet tissue), lung (13.5 ± 2.8 fmol/mg wet tissue) and cardiac atrium (9.21 ± 1.48 fmol/mg wet tissue). Previously, we reported high level of rat AM gene expression in heart as well as in lung and adrenal gland [3]. Rat AM gene

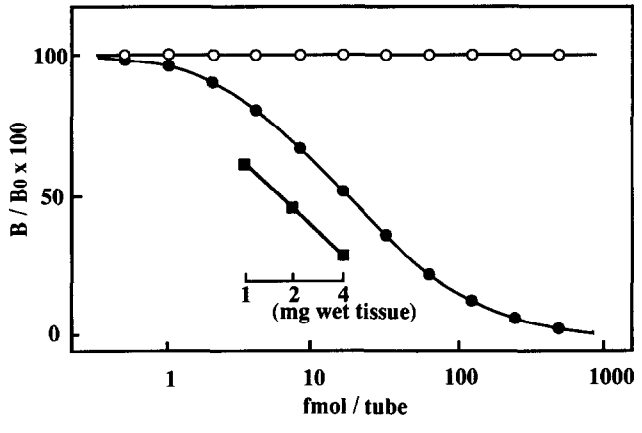

Fig. 1. Standard curve of RIA for rat AM and crossreactivity of antiserum #172CI-7. Inhibition of ^{125}I-labeled tracer binding to the antiserum by serial dilutions of rat AM (●), CGRP and amylin (○), extract from adrenal gland (■). The dilution curve of adrenal gland extract is roughly parallel to the standard culve.

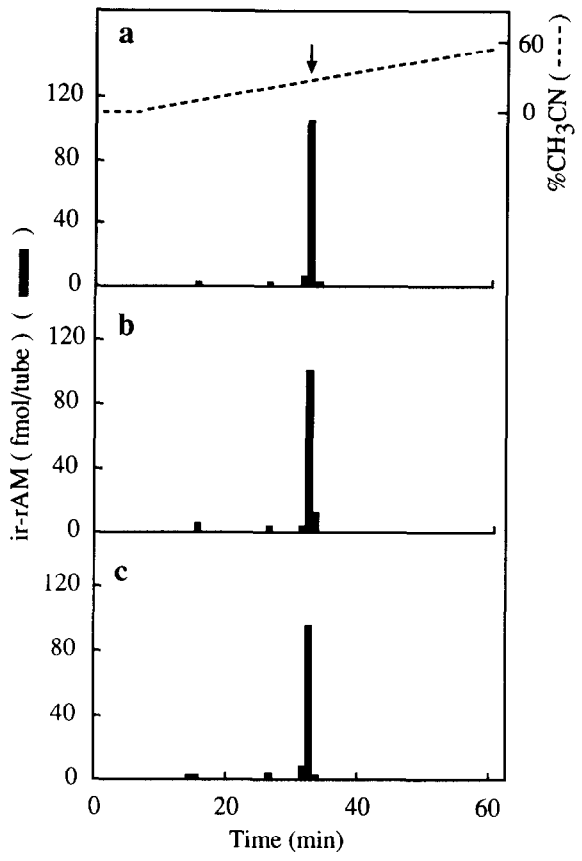

Fig. 2. Reverse-phase HPLC of rat tissue extract monitored by RIA for rat AM. Sample: (a) adrenal gland (10 mg eq.), (b) lung (10 mg eq.), (c) atrium (10 mg eq.). Column: TSK ODS 120A (4.6 × 150 mm). Flow rate: 1.0 ml/min. Solvent system: a linear gradientelution of CH_3CN from 10% to 60% in 0.1% TFA over 60 min. The arrow indicates an elution position of rat AM.

Table 1
Regional distribution of ir-rAM in tissue and plasma

Region	ir-rAM (fmol/mg wet tissue)
Brain	0.11 ± 0.01
Cerebellum	0.08 ± 0.01
Pituitary gland	1.12 ± 0.25
Submandibular gland	0.58 ± 0.1
Thyroid gland	0.96 ± 0.12
Cardiac atrium	9.21 ± 1.48
Cardiac ventricle	0.29 ± 0.05
Aorta	0.12 ± 0.03
Lung	13.5 ± 2.8
Adrenal gland	12.2 ± 0.9
Kidney	0.32 ± 0.05
Spleen	0.22 ± 0.02
Duodenum	0.25 ± 0.02
Pancreas	0.14 ± 0.02
Liver	0.14 ± 0.01
Testis	0.15 ± 0.03
Muscle	0.07 ± 0.01
Plasma*	3.60 ± 0.34

Data are mean ± S.D. (n = 4).
*Plasma concentration of ir-rAM is expressed as fmol/ml.

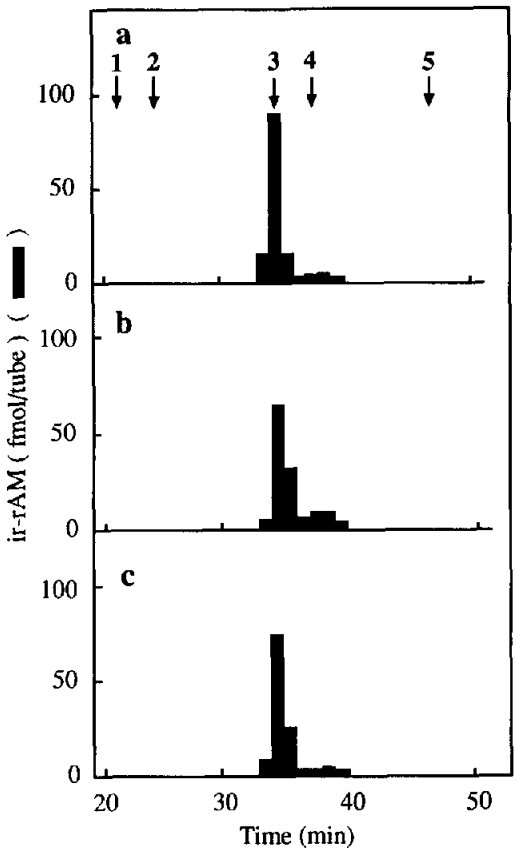

Fig. 3. Gel-filtration HPLC of rat tissue extract monitored by RIA for rat AM. Sample: (a) adrenal gland (10 mg eq), (b) lung (10 mg eq.), (c) atrium (10 mg eq.). Column: TSK-Gel 2000SW (8 × 500 mm). Flow rate: 0.5 ml/min. Solvent: 30% CH_3CN, 0.1% TFA and 0.2 M sodium chloride. Arrows indicate elution positions of (1) Vo, (2) BSA, (3) rat AM, (4) insulin chain B and (5) Vt.

expression in atrium and ventricle was further examined by RNA blot analysis, which indicated that the signal from atrium was stronger than that of ventricle (approximately 2:1) (data not shown). The immunoreactivity concentration ratio of atrium to ventricle was higher than the mRNA signal intensity ratio. In lung and atrium, the immunoreactivity concentration of rat AM was found to be about 6–10 times higher than that of human AM [4]. Thyroid gland also contained rat AM immunoreactivity in a relatively high concentration; about four times higher than that of human AM. AM immunoreactivity was detected for the first time in pituitary gland and submandibular gland in a considerable concentration. Only low level of ir-rAM was seen in the central nervous system, liver, pancreas, testis and muscle. Ir-rAM of kidney and spleen was found to be low, although gene expression of rat AM in these tissues was as high as in adrenal gland [3]. This discrepancy was also observed in the case of human [4], suggesting that AM in these tissues may be released into circulation or metabolized quickly after biosynthesis.

Prior to the measurement of plasma level of ir-rAM, plasma was applied to a Sep-Pak C-18 cartridge to remove protein which interfere with the RIA system. ir-rAM in plasma could be detected stably, with the mean concentration of plasma ir-rAM being 3.60 ± 0.34 fmol/ml (mean ± S.D.).

3.3. Characterization of ir-rAM in adrenal gland, lung, atrium and plasma

To characterize the molecular form of rat AM, the immunoreactivities found in rat adrenal gland, lung and atrium were analyzed by reverse-phase and gel-filtration HPLC. As shown in Fig. 2, reverse-phase HPLC patterns of these tissues indicate that more than 90% of ir-rAM was eluted as a single peak at a position exactly identical to that of authentic rat AM synthesized according to the sequence predicted from the cDNA [3]. In Fig. 3, gel-filtration HPLC of the tissue extracts on a column of TSK-Gel 2000SW shows that the major peak of ir-rAM in each tissue also emerged at an identical position with authentic rat AM consisting of 50 amino acids with a predicted molecular weight of 5,732 Da. These results indicate that rat AM exist in vivo and confirm the identity of the cDNA encoding rat AM as determined in our previous study.

The molecular forms of rat AM in plasma were also characterized by gel filtration on a Sephadex G-50 column coupled with RIA for rat AM. As seen in Fig. 4a, several peaks of ir-rAM were observed. Peak I emerged at an identical position with authentic rat AM. Peak II, the highest one, was found in a position corresponding to lower molecular weight. A smaller peak of ir-rAM was also seen in the void volume position, suggesting the possibility that some of rat AM binds to high molecular weight protein in plasma. Peak I and II of ir-rAM in the gel filtration of plasma were further analyzed by reverse-phase HPLC coupled with RIA for rat AM. Fig. 4b shows that peak I of ir-rAM emerged at the position where synthetic rat AM peptide was eluted. On the other hand, Fig. 4c shows that peak II of ir-rAM separated into several peaks located in earlier positions. Lower molecular weight forms of rat AM in plasma are thought to include the carboxy terminal region which antiserum #172CI-7 recognizes. Further structural study of these forms of rat AM is in progress.

In this study, using an antiserum which recognizes the carboxy terminal region of rat AM, a specific and sensitive RIA

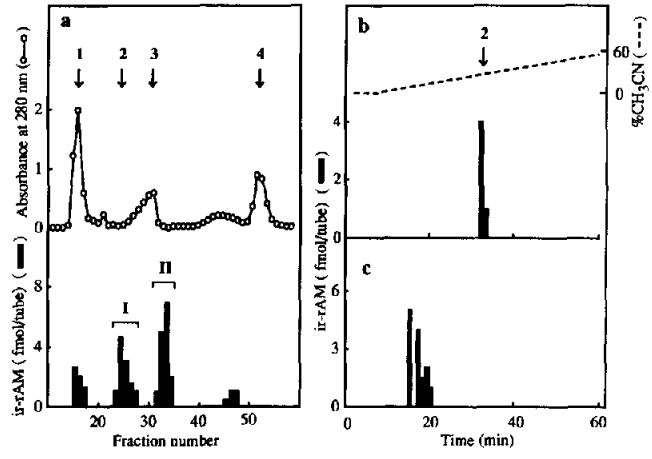

Fig. 4. Gel-filtration chromatography (a) and reverse-phase HPLC (b,c) of plasma sample monitored by RIA for rat AM. Samples: (a) 60 ml of rat plasma treated with Sep-Pak C-18 cartridge as described in the text, (b) half of peak I (fraction 24–28) in Fig. a, (c) half of peak II (fraction 32–35) in Fig. a. Condition of gel filtration (a); Column: Sephadex G-50 (1.5 × 100 cm). Solvent: 1 M acetic acid. Fraction size: 4 ml/tube. Flow rate: 12 ml/h. Condition of reverse-phase HPLC (b, c) was identical to that in Fig. 2. Arrows indicate elution positions of (1) Vo, (2) rat AM, (3) aprotinin and (4) Vt.

for rat AM has been established. High concentrations of ir-rAM were found in adrenal gland, lung and atrium. The immunoreactivity of AM was identified in pituitary gland and submandibular gland for the first time. We also confirmed that the behavior of ir-rAM in chromatography of rat tissue was completely identical with that of synthetic rat AM peptide predicted from the cDNA. Ir-rAM in plasma was found to consist of plural molecular forms including rat AM itself. Based on these data, we are continuing studies using rat models under various conditions in order to elucidate the role of AM.

Acknowledgements: This work was supported in part by research grants from Miyazaki Medical College and the Ministry of Education, Science and Culture of Japan.

References

[1] Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 192, 553–560.
[2] Kitamura, K., Sakata, J., Kangawa, K., Kojima, M., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 194, 720–725.
[3] Sakata, J., Shimokubo, T., Kitarnura, K., Nakamura, S., Kangawa, K., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 195, 921–927.
[4] Ichiki, Y, Kitamura, K., Kangawa, K., Kawamoto, M., Matsuo, H. and Eto, T. (1994) FEBS Lett. 338, 6–10.
[5] Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539.
[6] Ueda, S., Minamino, N., Aburaya, M., Kangawa, K., Matsukura, S. and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. 175, 759–767.
[7] Kitamura, K., Yukawa, T., Morita, S., Ichiki, Y., Eto, T. and Tanaka, K. (1990) Biochem. Biophys. Res. Commun. 170, 497–503.
[8] Minamino, N., Masuda, H., Kangawa, K. and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 124, 731–738.
[9] Miyata, A., Kangawa, K., Toshimori, T., Hatoh, T. and Matsuo, H. (1985) Biochem. Biophys. Res. Commun. 129, 248–255.