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Secretion of proadrenomedullin N-terminal 20 peptide from cultured neonatal rat cardiac cells

Toshihiro Tsuruda[a], Johji Kato[a], Kazuo Kitamura[a], Kenji Kuwasako[a], Takuroh Imamura[a], Yasushi Koiwaya[a], Kenji Kangawa[b], Tanenao Eto[a,*]

[a]*First Department of Internal Medicine, Miyazaki Medical College Kihara, Kiyotake, Miyazaki 889-1692 Japan*
[b]*Department of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565-8565, Japan*

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Abstract

Proadrenomedullin N-terminal 20 peptide (PAMP) is generated from post-transcriptional enzymatic processing of a 185-amino acid precursor for adrenomedullin (AM), a potent vasodilator peptide. We have reported that AM is secreted from cultured neonatal rat cardiac myocytes and fibroblasts, and that secreted AM modulates the growth of these cells; however, it is unknown whether or not the cardiac cells produce PAMP. In this study, we examined the production of PAMP in cultured neonatal rat cardiac myocytes and fibroblasts. Both the cardiac myocytes and fibroblasts cultured with serum-free media secreted PAMP time-dependently at rates of 5.7 ± 0.9 fmol/10^5 cells/40 h and 8.4 ± 0.7 fmol/5×10^4 cells/48 h (mean$\pm$SD), respectively. Reverse-phase high performance liquid chromatography showed that immunoreactive PAMP secreted from these cells was identical to PAMP[1-20], a whole active molecule. PAMP and AM secretions were significantly ($P<0.01$) stimulated by 10^{-6} mol/L angiotensin II (Ang II) and 10% fetal bovine serum (FBS) in myocytes and fibroblasts, whereas the ratio of PAMP to AM secretion in the myocytes was smaller than that of the fibroblasts. These results suggest that PAMP is secreted along with AM from rat cardiac myocytes and fibroblasts, and the secretion is augmented by the growth-promoting stimuli of Ang II and FBS for these cells.

Keywords: Proadrenomedullin N-terminal 20 peptide; Adrenomedullin; Cardiac myocyte; Cardiac fibroblast; Angiotensin II

Introduction

Both adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are generated by the post-transcriptional enzymatic processing of the 185-amino acid preproAM [1].

* Corresponding author. Tel.: 81-985-85-0872; fax: 81-985-85-6596.
E-mail address: keto@post.miyazaki-med.ac.jp (T. Eto)

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These two peptides have been found to exert hypotensive effects through different mechanisms. AM dilates blood vessels via cAMP elevation in vascular smooth muscle cells [2], whereas PAMP lowers blood pressure by inhibiting the catecholamine release from the sympathetic nerve endings [3]. In addition, PAMP is shown to have inhibitory effects on the catecholamine release from adrenal medullary cells [4, 5] and on aldosterone secretion from cortex cells [6].

PreproAM mRNA is widely expressed in human and rat tissues including normal adrenal glands, lungs, kidneys, cardiac atria and ventricles [1, 7]. We previously reported that AM is produced from cultured neonatal rat cardiac myocytes and fibroblasts, and that AM modulates the growth of these cardiac cells in an autocrine or a paracrine manner [8, 9]. Compatible with the preproAM gene expression, PAMP is as widely distributed as AM in the tissues listed above [10, 11], and specific binding sites of PAMP are also observed in these tissues [12]. Plasma concentrations of PAMP are increased in patients with heart failure compared to control subjects [13], and PAMP content is augmented in a hypertrophied cardiac ventricle in hypertensive rats [14], though, it is unknown whether or not cardiac cells secrete PAMP. The aims of this study were to examine whether PAMP is secreted from cultured cardiac myocytes and fibroblasts and to compare secretion patterns of PAMP with those of AM.

Methods

Chemicals

Angiotensin II (Ang II) was purchased from Peptide Institute, Inc. (Osaka, Japan). Holotransferrin (human), collagenase (type IV), insulin (bovine pancreas), and trypsin were purchased from Sigma Chemical Co., and fetal bovine serum (FBS) from GIBCO BRL.

Cell culture

Cultures of cardiac myocytes and fibroblasts were prepared from cardiac ventricles of 1- to 3-day-old Wistar rats as previously described [8, 9]. In brief, after digestion of the minced ventricles with 0.12% trypsin and 0.03% collagenase, cells were placed on culture dishes for 30 min at 37 °C to allow selective attachment of nonmyocytes, which were mostly cardiac fibroblasts. Cardiomyocyte-enriched suspensions were removed from the culture dishes and plated at a density of 1×10^5 cells/cm^2 onto collagen type I-coated 24-well culture plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The myocytes were cultured for 48 h with Dulbecco's modified Eagle's medium (DMEM) containing 15 mmol/L of HEPES, 10% FBS, 10 μg/mL of insulin, 10 μg/mL of transferrin and 0.1 mmol/L bromodeoxyuridine (BrdU). The culture medium was then exchanged for serum-free DMEM containing the same additives except for BrdU. To prepare cardiac fibroblasts, the cells that adhered to the culture dishes during the pre-plating procedure were spilt into 24-well plain culture plates at a density of 2.5×10^4 cells/cm^2 and grown to confluence in DMEM supplemented with 10% FBS. After culturing the cardiac myocytes and fibroblasts for 24 h with the serum-free media, the cells were incubated in the presence or absence of 10^{-6} mol/L Ang II or 10% FBS in fresh serum-free media for the indicated time periods.

These experiments were performed according to the regulations of the Animal Research Committee of Miyazaki Medical College (1998-037-2). This investigation conformed with

the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Assays of PAMP and AM in conditioned medium

Conditioned media collected from the 24-well culture plates were acidified with acetic acid to a final concentration of 1.0 mol/L. After heating the media at 100 °C for 10 min, they were applied to a Sep-Pak C18 cartridge (Millipore-Waters, Milford, MA). The cartridge was washed with 10% CH_3CN in 0.1% trifluoroacetic acid, and then the adsorbed materials were eluted with 60% CH_3CN in 0.1% trifluoroacetic acid, lyophilized, and stored at −30 °C. The lyophilized samples were dissolved in a radioimmunoassay (RIA) buffer and subjected to RIA for rat PAMP and AM as previously described [10, 15]. The recoveries of PAMP and AM in those assay procedures were 90% and 82%, respectively.

Characterization of secreted PAMP

Molecular forms of immunoreactive PAMP (ir-PAMP) of the conditioned medium were analyzed by reverse phase high performance-liquid chromatography (HPLC) with a column of TSK ODS 120A (Tosoh, Tokyo, Japan) as previously described [10]. The extracts of the conditioned media were eluted from the column by a linear gradient of 10 to 60% acetonitrile in 0.1% trifluoroacetic acid, and ir-PAMP in each fraction was measured with RIA. The recovery of ir-PAMP in this HPLC was greater than 60%.

Statistical analysis

Multiple comparisons were made with one-way ANOVA followed by Scheffe's test. All data were expressed as means±SD of the samples examined, and P values less than 0.05 were considered significant.

Results

First, we examined whether the cultured neonatal rat cardiac myocytes and fibroblasts would secrete PAMP into the medium. As shown in Fig. 1, the cardiac myocytes and fibroblasts cultured in serum-free media secreted PAMP in a time-dependent manner at rates of 5.7±0.9 fmol/10^5 cells/40 h and 8.4±0.7 fmol/5×10^4 cells/48 h, respectively, which were smaller than those of AM secretion. We measured cellular contents of PAMP in these cardiac cells, but they were less than 0.5 fmol per 10^5 of myocytes or per 5×10^4 of fibroblasts, a level too low to be detected. Molecular forms of PAMP secreted into the medium were examined by reverse phase HPLC (Fig. 2). Immunoreactive PAMP secreted from the cardiac myocytes and fibroblasts consisted of a single peak that emerged at the elution position identical to that of authentic PAMP[1-20]-NH_2, a whole active molecule of rat PAMP [7].

To compare secretion patterns of PAMP and AM, we measured concentrations of both peptides in the conditioned media after incubating the cardiac myocytes and fibroblasts in the absence or presence of 10^{-6} mol/L Ang II or 10% FBS for 24 h. As shown in the table, both PAMP and AM secretions were significantly ($P<0.01$) increased, following the treatment with Ang II or FBS in both the cardiac cells. Interestingly, ratios of PAMP to AM secretion of

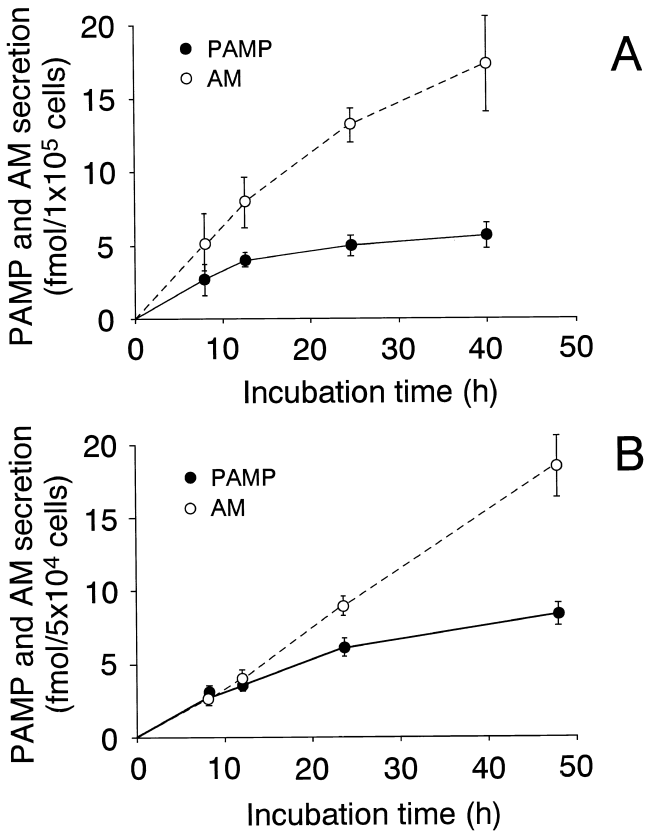

Fig. 1. Time course of PAMP and AM secretion from cardiac myocytes (**A**) and fibroblasts (**B**). The cells were incubated for the indicated periods in serum-free medium, and concentrations of PAMP and AM in the conditioned media were determined as described in Methods. Each value represents the mean±SD of six wells determined.

the fibroblasts were apparently higher than those of the myocytes, whereas the ratios were not so different within the treatment groups of the two types of cardiac cells.

Discussion

PAMP has been shown to be biosynthesized from preproAM, an AM precursor, by enzymatic cleavage [1]. Previous studies have revealed that PAMP is as widely distributed as AM in various tissues and organs including cardiac atria and ventricles, in rats and humans [10, 11, 14]. However, there has been no report showing secretion of PAMP from cardiac myocytes or fibroblasts. In this study, we demonstrated that both myocytes and fibroblasts actively secreted PAMP into the medium. HPLC analysis revealed that secreted immunoreactive PAMP was eluted at the position of PAMP[1-20], the whole active molecule of PAMP. When compared with concentrations of PAMP in the conditioned media, intracellular contents were at undetectable levels in the present study. This finding was in accord with the fact that tissue concentrations of PAMP in cardiac ventricles are much lower than those in adrenal glands

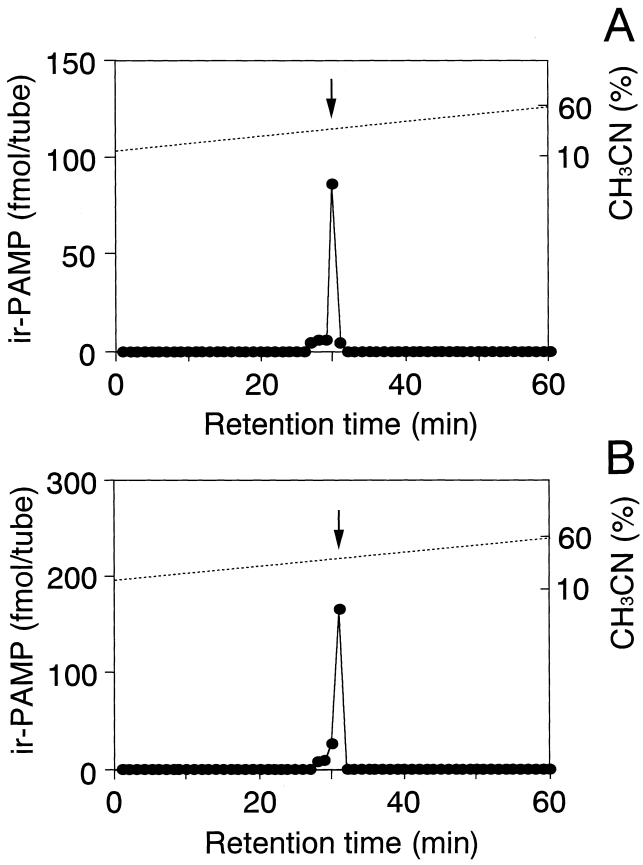

Fig. 2. Analysis of reverse-phase HPLC of immunoreactive PAMP (ir-PAMP) secreted from myocytes (**A**) and fibroblasts (**B**) into the media. A linear gradient of acetonitrile of 10% to 60% was made in 0.1% trifluoroacetic acid for 60 min at a flow rate of 1.0 mL/min. The arrow indicates the elution position of synthetic rat PAMP[1-20]-NH_2.

Table 1
Comparison of PAMP and AM secretion from cardiac myocytes and fibroblasts

Treatment	PAMP (fmol/10^5 cells)	AM (fmol/10^5 cells)	PAMP/AM
Cardiac myocytes			
Control	1.1 ± 0.3	9.5 ± 1.2	0.12
10^{-6} mol/L Ang II	2.3 ± 0.8*	20.4 ± 4.5*	0.11
10% FBS	5.0 ± 0.4*	38.3 ± 5.8*	0.13
Cardiac fibroblasts			
Control	8.4 ± 1.4	20.9 ± 2.6	0.40
10^{-6} mol/L Ang II	20.4 ± 2.8*	40.1 ± 6.0*	0.51
10% FBS	40.3 ± 10.1*	51.9 ± 9.6*	0.78

The cardiac myocytes and fibroblasts were incubated in serum-free media in the absence or presence of 10^{-6} mol/L of Ang II or 10% FBs. After incubation for 24 h, PAMP and AM secreted into the media were measured as described in Methods. Values are means±SD of the six wells examined. * $P<0.01$, compared with the controls.

despite the substantial amount of preproAM mRNA in the two tissues [1, 7, 10, 14]. Thus, PAMP and AM appear to be secreted in a constitutive fashion from the cardiac myocytes and fibroblasts with little intracellular storage. It has been shown that concentrations of PAMP are lower than those of AM in various tissues and organs [16]. Katoh et al. reported secretion of PAMP and AM from cultured bovine adrenal medullary cells at a ratio of 1:2 [4, 17]. In the present study, a similar ratio of secreted PAMP to AM from the cardiac fibroblasts was observed, whereas a lower ratio was noted from the myocytes. The ratios of PAMP to AM concentrations have been shown to vary from tissue to tissue in porcine [16]. Although a clearance of PAMP by the cardiac cells remains unclear, the processings or metabolisms of PAMP and AM also seem to be different depending upon the types of cells.

PAMP has been found to have inhibitory effects on catecholamine release from the adrenal medulla and sympathetic nerve endings [4, 5] and on aldosterone secretion from the adrenal cortex [6]. Ando et al. showed an inhibitory action of PAMP on the cellular growth of a neuroblastoma-derived cell line [18]. We previously reported that AM attenuates growth of the cardiac myocytes and fibroblasts, but synthetic PAMP failed to affect *de novo* protein synthesis of the cardiomyocytes and DNA synthesis of the cardiac fibroblasts, even at 10^{-6} mol/L of concentration (data not shown). In the meantime, specific binding sites for PAMP are present in the heart [12], and PAMP concentrations in hypertrophied cardiac ventricles of spontaneously hypertensive rats are shown to be higher than those of normotensive Wistar-Kyoto rats [14]. In the present study, PAMP secretion from the cardiac myocytes and fibroblasts was increased in the presence of Ang II or FBS, which induces the growth of the cardiac cells. These findings might imply a physiological or pathophysiological role of PAMP in the cardiac ventricle. However, further studies are necessary to answer this question.

In summary, cultured neonatal rat cardiac myocytes and fibroblasts actively secreted PAMP, and the PAMP secretion was increased by growth-promoting stimuli for these cells such as Ang II and FBS.

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