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Ca^{2+}-dependent cosecretion of adrenomedullin and catecholamines mediated by nicotinic receptors in bovine cultured adrenal medullary cells

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Abstract

Bovine cultured adrenal medullary cells (4×10^6) contained 4266.5 ± 370.0 fmol of immunoreactive adrenomedullin and 373.4 ± 32.6 nmol of catecholamines. Nicotinic (but not muscarinic) receptors mediated the Ca^{2+}-dependent co-secretion of adrenomedullin and catecholamines, with the molar ratio of adrenomedullin/catecholamines secreted into the medium being equal to the ratio stored in the cells. The concentration–response curve of carbachol for adrenomedullin secretion (EC_{50} 42 μM) was similar to that for catecholamine secretion (EC_{50} 63 μM). Reverse phase HPLC analysis showed that immunoreactive adrenomedullins in the cells and secreted into the medium were both eluted exclusively at the position almost identical to synthetic human adrenomedullin[1–52]NH_2.

Key words: Adrenomedullin; Catecholamine; Secretion; Ca^{2+}; Nicotinic receptor; Adrenal medulla

1. Introduction

Adrenomedullin (AM) is a novel hypotensive peptide recently discovered in human pheochromocytoma [1], by using an assay system which monitored its activity to elevate cAMP in rat platelets [1]. AM, consisting of 52 amino acids, has one intramolecular disulfide bond and shows a 17% of amino acid sequence homology with calcitonin gene-related peptides (CGRP-I, II) that also raise cAMP levels to cause vasodilatation. In anesthetized rats, the intravenous bolus administration of AM produced a rapid, marked and long-lasting decrease in blood pressure [1,2]. In the perfused rat mesenteric vasculature, AM produced a concentration-dependent vasodilatation, and it was inhibited by $CGRP^{8-37}$, an antagonist for CGRP receptor, but not by antagonists for muscarinic and β-adrenergic receptors [3].

Adrenal medullary cells (embryologically derived from neural crest) share common properties with adrenergic neurons. The functions of adrenal medulla are under the control of acetylcholine released from preganglionic splanchnic nerves. Tissue concentrations of ir-AM in human pheochromocytoma and adrenal medulla are three to four orders of magnitude higher than those in kidney and lung [1]. The ir-AM was also detected in plasma, suggesting that an endogenous AM in circulation may be involved in the regulation of blood pressure. However, it has not been documented that the secretion of AM is modulated by neuro/humoral-transmitter(s). In addition, the origin(s) of plasma ir-AM and the molecular form(s) of ir-AM secreted into circulation are unknown. We now provide the first evidence that AM[1–52]NH_2 is secreted along with catecholamines by a Ca^{2+}-dependent exocytosis following the stimulation of nicotinic receptors in bovine cultured adrenal medullary cells.

2. Materials and methods

2.1. Primary culture

Bovine adrenal medullary cells were isolated and cultured (4×10^6 cells/dish, Falcon 35 mm) in Eagle's minimum essential medium containing 10% calf serum and antibiotics [4]. Oxygenated Krebs–Ringer phosphate (KRP) buffer (mM) (NaCl 154, KCl 5.6, $MgSO_4$ 1.1, $CaCl_2$ 2.2, NaH_2PO_4 0.85, Na_2HPO_4 2.15, glucose 10; and 0.5% bovine serum albumin, pH 7.4) was used throughout.

2.2. Secretion of AM and catecholamines

Secretion of AM and catecholamines was measured by incubating the cells at 37°C in 1.0 ml of KRP buffer with or without test compounds [4]. The reaction was terminated by transferring the medium to an assay tube that contained EDTA (3 mM) and aprotinin (500 KIU/ml) to inactivate proteases.

2.3. Measurement of AM

AM secreted into the medium was measured in duplicate by radioimmunoassay (RIA) [5]. The incubation buffer for RIA 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 and 0.05% NaN_3. An aliquot (100 μl) of the secretion medium was reacted at 4°C for 24 h with 50 μl of rabbit antiserum (dilution 1:182,250) raised against human AM[40–52]NH_2 and 50 μl of ^{125}I-labelled human AM[40–52]NH_2 (18,000 cpm) prepared by Bolton–Hunter method. The antiserum specifically recognized the C-terminal region of AM, and did not exhibit cross-reactivity with CGRP-I, II and neuropeptide Y [5]. The RIA has 100% cross-reactivity with bovine AM[45–52]NH_2 de-

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Abbreviations: ir-AM, immunoreactive adrenomedullin; CGRP, calcitonin gene-related peptide; ANP, atrial natriuretic peptide; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; KRP, Krebs–Ringer phosphate; EC_{50}, half-maximal effective concentration.

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duced from cDNA analysis of bovine AM (manuscript in preparation), whose amino acid sequence is identical to human AM[45–52]NH_2. Free and bound tracers were separated by polyethyleneglycol method, and the radioactivity in the pellet was counted in a γ-counter (ARC-600, Aloka).

To measure AM in cells, cells were detached, boiled in 1 M acetic acid (to inactivate proteases), homogenized and centrifuged at 24,000 × *g* for 30 min. The resultant supernatant was applied to a Sep-Pak C-18 cartridge (Waters, Inc., MA, USA) pre-equilibrated with 0.5 M acetic acid. After washing the column with 0.5 M acetic acid, the adsorbed materials were eluted with 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA), evaporated and finally dissolved in RIA buffer to submit RIA.

The ir-AMs in cell extract and secretion medium were analyzed by reverse phase HPLC, using a column of TSK ODS 120A (4.6 × 150 mm, Tosoh) [1,5].

2.4. Measurement of catecholamines (epinephrine plus norepinephrine)

Catecholamines were adsorbed to alumina, eluted with 0.2 N perchloric acid and measured by HPLC [6]. Recovery of the internal standard 3,4-dihydroxybenzylamine was 87.5 ± 1.7% (n = 10).

2.5. Chemicals

Eagle's minimum essential medium, Nissui Seiyaku; calf serum and EDTA, Nacalai Tesque; atropine, carbachol, hexamethonium and muscarine, Sigma; nicotine, Tokyo Kasei; aprotinin, Bayer AG. Human synthetic AM was prepared by solid phase methods in Peptide Institute, Inc., Osaka, Japan.

2.6. Statistical analysis

All values were expressed as mean ± S.E.M.

3. Results

3.1. Time courses of carbachol-induced secretion of AM and catecholamines

Carbachol evoked a rapid and transient secretion of AM and catecholamines, both of which occurred within 1 min and then leveled off, whereas the basal secretion of AM and catecholamines remaind at low levels (Fig. 1). Over a period of 5 min, carbachol (300 μM)-induced secretion of AM and catecholamines were 710.8 ± 30.4 fmol (n = 5) and 62.28 ± 9.80 nmol (n = 6) from 4 × 10^6 cells, corresponding to 16.7 and 16.7% of the total cellular AM and catecholamines, respectively.

3.2. Concentration–response curves of carbachol for secretion of AM and catecholamines

Carbachol, at the same concentration range, increased the secretion of both AM and catecholamines in a concentration-dependent manner, and they attained their maximal level at 300 μM carbachol (Fig. 2). The EC_{50} values of carbachol for AM and catecholamine secretion were 42 and 63 μM, respectively.

3.3. Molar ratio of ir-AM and catecholamines in secretion medium and adrenal medullary cells

Bovine cultured adrenal medullary cells (4 × 10^6) contained 4266.5 ± 370.0 fmol of ir-AM and 373.4 ± 32.6 nmol of catecholamines, the molar ratio of ir-AM/catecholamines being 11.41 ± 1.14 × 10^{-6} (n = 5). The molar ratios of ir-AM/catecholamines secreted into the medium during a 5 min incubation period were 11.90 ± 0.40 × 10^{-6}, 11.87 ± 0.27 × 10^{-6}, 11.77 ± 0.74 × 10^{-6} and 10.33 ± 0.34 × 10^{-6} (n = 4) at 50, 100, 300 and 1000 μM of carbachol, respectively.

3.4. Effect of extracellular Ca^{2+} removal

In adrenal medulla, an influx of Ca^{2+} via voltage-dependent Ca^{2+} channels is indispensable for the exocytotic secretion of catecholamines [4]. Over a 5 min incubation period, the secretion of AM and catecholamines induced by carbachol (300 μM) were abolished in the Ca^{2+}-free medium, although the basal secretion of AM and catecholamines were not significantly altered (data not given).

3.5. Effects of nicotinic and muscarinic agents

Carbachol-induced secretion of AM was inhibited by hexamethonium (1 mM), an antagonist for nicotinic re-

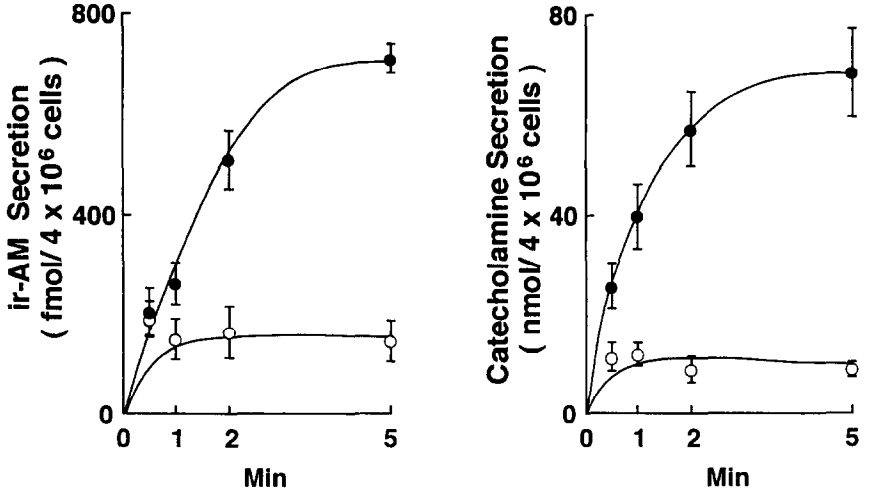

Fig. 1. Time courses of carbachol-induced secretion of ir-AM (A) and catecholamines (B) from cultured bovine adrenal medullary cells. The cells were incubated in 1.0 ml of KRP buffer at 37°C in the absence (○) or presence (●) of carbachol (300 μM).

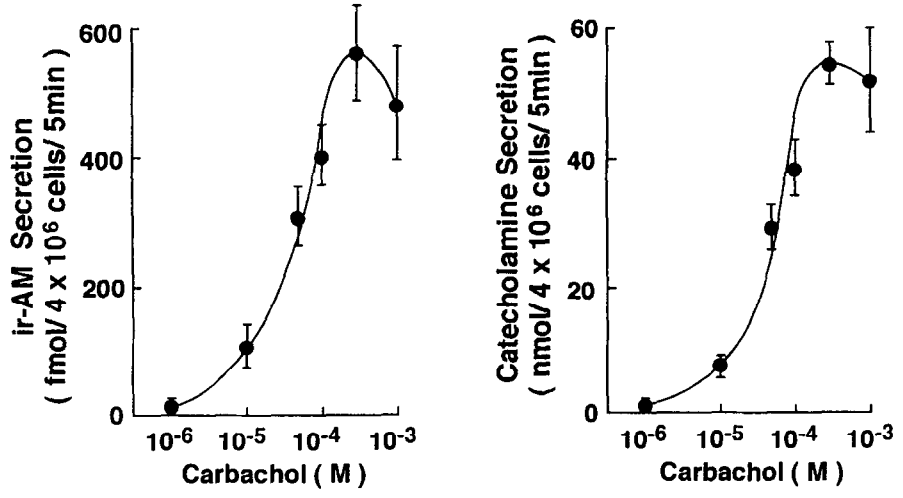

Fig. 2. Concentration–response curves of carbachol for the secretion of ir-AM (A) and catecholamines (B). Cultured cells were stimulated at 37°C for 5 min with various concentrations of carbachol (CCh). Values obtained at 37°C without carbachol were subtracted. Each point represents the mean ± S.E.M. ($n = 4$).

ceptors, but not by atropine (1 μM), an antagonist for muscarinic receptors (Fig. 3). Nicotine (100 μM) induced AM secretion to the same extent as that caused by carbachol (300 μM), whereas muscarine (100 μM) did not increase AM secretion.

3.6. *Analysis of ir-AM in the secretion medium and cell extract of adrenal medulla*

Molecular forms of ir-AM in the secretion medium and acid extract of adrenal medullary cells were characterized by reverse phase HPLC, using a column of TSK ODS 120A coupled with RIA for AM. Molecular forms of ir-AM in both preparations were composed of the major peak and several minor peaks, as shown in Fig. 4. The major peak in the secretion medium appeared at an elution position almost identical to that of cell extract, and they corresponded to the elution position of synthetic human AM[1–52]NH_2.

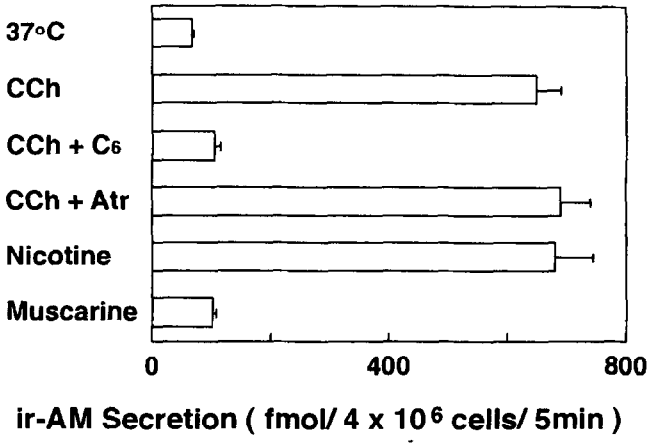

Fig. 3. Effects of cholinergic agents on ir-AM secretion. The cells were incubated at 37°C for 5 min with or without the indicated drugs. Carbachol (CCh), 300 μM; hexamethonium (C_6), 1 mM; atropine (Atr), 1 μM; nicotine, 100 μM and muscarine, 100 μM. Means ± S.E.M. ($n = 4$).

4. Discussion

In the present study, carbachol caused a rapid and transient secretion of ir-AM in a time course similar to that of catecholamine secretion. The concentration–response curve of carbachol for ir-AM secretion was also indistinguishable from that for catecholamine secretion. Carbachol-induced secretion of ir-AM was abolished by hexamethonium, but not by atropine. Nicotine evoked ir-AM secretion, while muscarine had no effect. These results indicate that nicotinic (but not muscarinic) receptors mediate the secretion of ir-AM, in accordance with the secretion of catecholamines [4].

In adrenal medulla, catecholamines are stored exclusively in chromaffin granules and secreted by a Ca^{2+}-dependent exocytosis [7]. Chromaffin granules have been documented to contain a number of biologically active peptides [8,9], such as enkephalins [10–12], neuropeptide Y [13,14], and atrial natriuretic peptide (ANP)-related peptides [15,16], which are cosecreted along with catecholamines by a Ca^{2+}-dependent exocytosis.

In the present study, carbachol failed to stimulate the secretion of ir-AM as well as catecholamines in a Ca^{2+}-free medium. The molar ration of ir-AM/catecholamines secreted into the medium was quite similar to the ratio stored in adrenal medullary cells. Our previous experiments documented that the mature form of AM in adrenal medulla is produced by the processing from prepro AM via pro AM, and the major ir-AM in human adrenal medulla is mature AM which consists of 52 amino acids

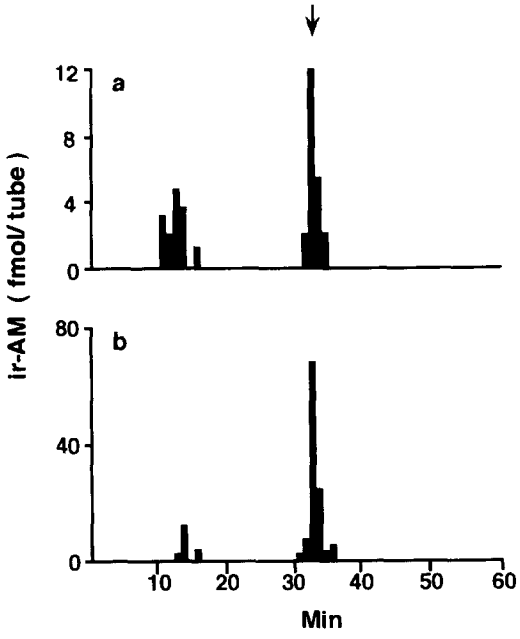

Fig. 4. Reverse phase HPLC analysis of ir-AM in the secretion medium and in cell extract of adrenal medulla. Sample: (a) secretion medium, (b) cell extract. Column: TSK ODS 120A (4.6 × 150 mm, Tosoh). Flow rate: 1.0 ml/min. Solvent system: (A) H_2O/CH_3CN/10% TFA, 90:10:1 (v/v); (B) H_2O/CH_3CN/10% TFA, 40:60:1 (v/v). Linear gradient from (A) to (B) for 60 min. The arrow indicates the elution position of synthetic human AM[1–52]NH_2. Although CGRPs were also eluted at a similar position, they did not cross-react with the antiserum raised against human AM[40–52]NH_2 [5].

[5]. In the present study, reverse-phase HPLC analysis showed that ir-AM stored in the cells and secreted into the medium were both eluted at the position almost identical to human AM[1–52]NH_2. These results suggest that the processing pathway of bovine AM is quite similar to that of human AM, and AM is cosecreted with catecholamines in the same molecular form as it exists in chromaffin granules.

Although it is still obscure to what extent AM secreted from adrenal medulla contributes to its plasma level, and the physiological significance of endogenous AM is not precisely known, a large amount of AM secreted from adrenal medulla seems to be involved in the modulation of vascular tone. In addition, enkephalin [17] and neuropeptide Y [18] secreted from adrenal medulla were reported to inhibit nicotinic receptor-mediated catecholamine secretion. Our previous study in bovine cultured adrenal medullary cells also documented that ANP-related peptides phosphorylated and activated tyrosine hydroxylase, the enzyme catalyzing the rate-limiting step in the biosynthesis of catecholamines, in a cGMP-dependent process(es) [19]. It is intriguing to conjecture that AM, the potent vasorelaxing peptide, cosecreted with catecholamines, would modulate the secretion and/or synthesis of catecholamines, although the existence of the AM receptor in adrenal medulla, by itself, is still putative.

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