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Secretion and biosynthesis of atrial natriuretic factor by cultured adrenal chromaffin cells

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In our previous work, the existence of the precursor and mature forms of atrial natriuretic factor (ANF) within the bovine chromaffin granules has been reported. To confirm the endogenous character of these peptides, we demonstrate that nicotinic activation and depolarization by KCl increase their co-secretion from cultured chromaffin cells. The increase of intracellular levels of these atrial peptides by phorbol ester is potentiated by addition of forskolin. The release of ANF and their de novo synthesis within the cultured chromaffin cells emphasize the usefulness of this model in the study of the mechanisms of release and storage of these peptides in the neuronal tissues.

Atrial natriuretic peptide; Adrenal medulla; Neuropeptide; Chromaffin system; Neurosecretory system; Phorbol ester; (Chromaffin cell)

1. INTRODUCTION

It is now well established that myoendocrine cells in the mammalian heart synthesize and secrete a hormone called atrial natriuretic factor (ANF) which modifies renal and cardiovascular functions. ProANF, the 126-amino acid precursor, is the main constituent of the intracellular pool of atrial ANF as well as the unique form secreted by cultured cardiocytes [1,2]. It seems that no maturation occurred within the atrial secretory granules, suggesting that proANF would be processed upon its release to the circulating form ANF^{99-126} [1–3]. On the one hand, in vivo and in vitro studies on isolated perfused heart suggested that the common physiological stimulus for ANF secretion is atrial distension [4]. On the other hand, glucocorticoids [5] and thyroid hormone [6] induced in vivo and in vitro cardiac tissue mRNA accumulation, suggesting an increase of ANF synthesis. However, a specific secretagogue or inducer of ANF biosynthesis for in vitro cultured cardiocytes has not yet been reported.

We have recently demonstrated the co-existence of ANF^{99-126} and its precursor ANF^{1-126} within the bovine chromaffin granules, confirming the maturation process of atrial peptides in the adrenal medulla [7,8]. We now report the modulation of the biosynthesis of ANF and its precursor as well as their secretion from cultured chromaffin cells.

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2. MATERIALS AND METHODS

2.1. *Chromaffin cell culture*

Chromaffin cells were isolated from bovine adrenal medulla by the method of Livett [9] and were seeded in 75 cm^2 flasks (Miles Laboratories, Rexdale, Ont.) at a density of 2×10^7 cells in F-12 medium supplemented with 10% (v/v) horse serum, 2% fetal calf serum and 1% penicillin-streptomycin-fungizone (Gibco, Burlington, Ont.) for 5 days. For biosynthesis experiments, chromaffin cells were treated for the last 3 days with phorbol ester (12-*O*-tetradecanoyl phorbol-13-acetate, TPA) (Sigma, St. Louis, MO), forskolin (FKL) (Calbiochem, San Diego, CA) or both. On day 6, the monolayer was washed twice with Locke's buffer consisting of (in mM): 145 NaCl, 5.6 KCl, 1.2 $MgSO_4$, 2.2 $CaCl_2$, 5.6 glucose, 0.01% (w/v) heat-inactivated BSA and 10 Hepes, pH 7.4, and cells were harvested in 2 M acetic acid containing 20 mM HCl, 0.1 mM EDTA and

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0.1 mM phenylmethylsulfonyl fluoride, sonicated, and centrifuged at 30000 × *g* for 30 min. For secretion experiments, the monolayer was exposed for 15 min at 37°C to stimulating agents in the presence of 1 μM each of aprotinin, leupeptin, pepstatin A and 10 μM 1,10-phenanthroline (Sigma) and the secretion medium was then collected in 1% trifluoroacetic acid (Pierce, Rockford, IL).

2.2. *Reversed-phase chromatography*

Secretion media and cellular extracts were first applied on a precolumn (RP-300, 4.6 mm × 3 cm) (Brownlee Labs, Santa Clara) equilibrated with 15% acetonitrile (ACN) in 0.25 N triethylamine-formate buffer (TEAF), pH 4.0, at a flow rate of 2 ml/min. After 4 min of washing with the same buffer, the pre-column was then connected to a Vydac C_{18} (4.6 mm × 25 cm) (Separation Group, Hesperia, CA) via a Rheodyne 7000 switching valve (Rheodyne, Berkeley, CA) and ANF was separated using a linear gradient of 15–55% ACN in 0.25 N TEAF, pH 4.0, for 40 min at a flow rate of 1 ml/min.

2.3. *AFN detection and data analysis*

Aliquots of the HPLC profile were dried under reduced pressure prior to estimation both by radioimmunoassay [10] (RIA) using anti-human ANF antibody from Peninsula (Belmont, CA) and by radioreceptor assay [11] (RRA) using bovine adrenal cortex membranes. Determination of bound ^{125}I-labeled ANF^{99-126} was used for computerized data analysis using a four parameter logistic equation [12]. Statistical differences between treatment groups were evaluated by one-way analysis of variance followed by a Dunnett's *t*-test according to a bilateral null hypothesis. Results are expressed as mean ± SE.

3. RESULTS

In order to eliminate the interferences in the ANF assays and to minimize peptide losses, the use of an on-line system with a pre-column was found necessary for the chromatography of the secretion media as well as for chromaffin cell extracts. The mature form AFN^{99-126} eluting at 26% acetonitrile was equally well detected by RIA and by RRA (fig.1). In contrast, the proANF form eluting at 39% acetonitrile was selectively detected by RIA because of its low cross-reactivity in RRA. The characteristic elution position and differential detectability of the two forms of ANF unequivocally identifies them in HPLC eluates of chromaffin cell extracts.

One of the characteristics of an endogenous neuropeptide is its secretion from target cells or tissues following appropriate stimuli. Fig.2 illustrates the co-secretion of ANF^{99-126} and its precursor$^{1-126}$ following exposure of chromaffin cells to various secretagogues. Nicotine (10 μM) induced respectively a 7- and 27-fold increase over the basal secretory level of immunoreactive ANF

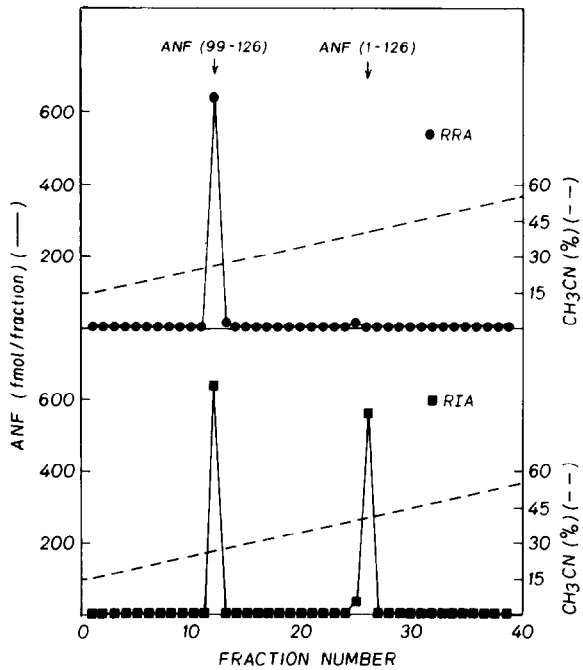

Fig.1. Reversed-phase HPLC separation profile of purified ANF^{99-126} and its precursor$^{1-126}$ from bovine chromaffin granules on the bidimensional system (RP-300-Vydac C_{18}) with a linear gradient of acetonitrile of 15–55% in 0.25 N triethylamine formate, pH 4, over a 40 min period. The ANF activity in the eluted fractions were determined by both radioreceptor assay (RRA, upper panel) and radioimmunoassay (RIA, lower panel).

(ir-ANF) (from 27 ±12 up to 186 ± 52 fmol/2 × 10^7 cells) and of immunoreactive proANF (ir-proANF) (from 10 ± 1 up to 269 ± 40 fmol/2 × 10^7 cells). KCl (56 mM) also produced a concomitant 7- and 34-fold enhancement of the release of ir-ANF (from 27 ± 12 up to 180 ± 53 fmol/2 × 10^7 cells) and of ir-proANF (from 10 ± 1 up to 339 ± 48 fmol/2 × 10^7 cells), respectively. The corresponding intracellular contents of ir-ANF and ir-proANF were respectively 1030 ± 146 and 2058 ± 213 fmol/2 × 10^7 cells (n = 7). Basal ir-ANF and ir-proANF secretions were 2.5 and 0.5% of their respective contents. Nicotine induced the release of 16% and 14% of the intracellular contents of ir-ANF and ir-proANF, respectively. KCl-induced depolarisation released 10 and 12% of the ir-ANF and its precursor intracellular contents, respectively.

Another characteristic of an endogenous neuro-

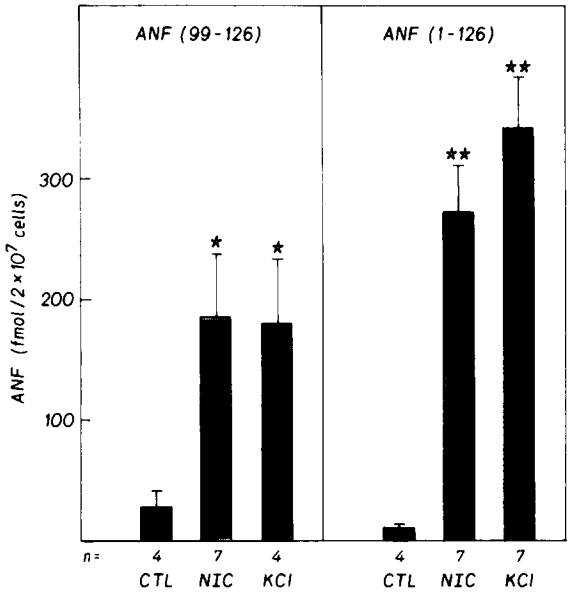

Fig.2. Co-secretion of ANF^{99-126} (left panel) and its $precursor^{1-126}$ (right panel) from cultured chromaffin cells stimulated by nicotine or KCl for 15 min at 37°C. Secretion medium was submitted to reversed-phase HPLC prior to ANF activity determination by radioimmunoassay. CTL, control; NIC, 10 μM nicotine; KCl, 56 mM KCl; n, number of cultures. * $p < 0.05$, ** $p < 0.01$ compared to control by Dunnett' t-test.

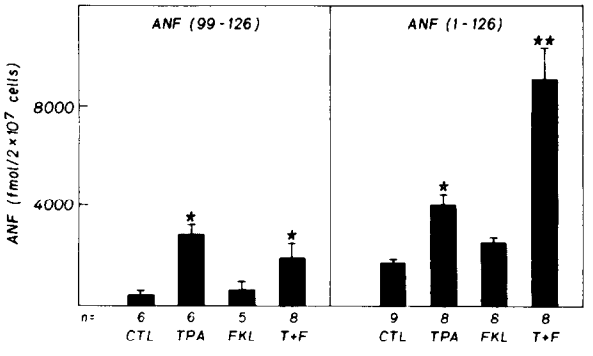

Fig.3. Biosynthesis of ANF^{99-126} (left panel) and its $precursor^{1-126}$ (right panel) by cultured chromaffin cells upon exposure to phorbol ester (12-*O*-tetradecanoyl phorbol-13-acetate), forskolin or the combined treatment for 72 h at 37°C. Intracellular acid extracts were subjected to reversed-phase HPLC prior to activity determination by radioimmunoassay. CTL, control; TPA, 0.1 μM phorbol ester; FKL, 1 μM forskolin; T+F, 0.1 μM phorbol ester and 1 μM forskolin; n, number of cultures. * $p < 0.05$, ** $p < 0.01$ compared to control by Dunnett's t-test.

peptide is its in situ biosynthesis by target cells or tissues. Fig.3 illustrates the effect of 0.1 μM TPA, 1 μM FKL, or the combined treatment on chromaffin cells intracellular ANF contents. TPA induced a concomitant 5- and 2-fold enhancement of intracellular levels of ir-ANF (from 449 ± 171 up to 2371 ± 544 fmol/2 × 10^7 cells) and of ir-proANF (from 1784 ± 103 up to 4013 ± 426 fmol/2 × 10^7 cells), respectively. Although FKL did not produce a significant increase in either forms of ANF, the combined treatment (T+F) induced respectively a 4- and 5-fold enhancement of ir-ANF (from 449 ± 171 up to 1912 ± 498 fmol/2 × 10^7 cells) and of ir-proANF (from 1784 ± 103 up to 8952 ± 1337 fmol/2 × 10^7 cells).

4. DISCUSSION

Immunohistochemical studies have revealed the presence of ANF-like material in sympathetic ganglia and adrenal medulla [13]. We have further reported the identification by amino acid sequencing of both proANF and its mature form within chromaffin granules, suggesting a possible modulation of the biosynthesis, maturation and secretion of these atrial peptides by cultured chromaffin cells [7]. We provide here the first biochemical evidence that ANF^{99-126} and its $precursor^{1-126}$ are co-secreted by chromaffin cells. This observation is reminiscent of previous studies on the co-release of enkephalin and its enkephalin-containing polypeptides [9,14]. Hence, in contrast to cardiomyocytes [1,2], chromaffin cells secreted both proANF and its mature product, suggesting that the maturation process might occur in chromaffin granules [8].

It has been shown that cAMP, phorbol ester and KCl-induced depolarization increased neuropeptide synthesis in chromaffin cells [15]. cAMP and KCl depolarisation induced the accumulation of proenkephalin messenger RNA prior to the appearance of the enkephalins pentapeptide [15]. We have shown in this report that TPA alone or in combination with forskolin enhanced production of both forms of ANF, indicative of de novo synthesis. The potentiation by forskolin of the phorbol ester effect has been also observed for the release of ANF by perfused heart [4] and would suggest the convergence of cAMP and phosphati-

dylinositol pathways on the regulation of ANF biosynthesis in chromaffin cells. The results indicate that ANF behaves as an endogenous neuropeptide within the adrenal gland.

Since adrenomedullary ANF represents 7% of those of cardiac origin [7], the relative contribution of medullary ANF to the circulating level is presumably minor. However, previous reports have demonstrated that atrial peptides inhibit aldosterone production by a direct action on the adrenal cortex both in vivo [16] and in vitro [17]. Morphological studies indicated the presence in mammalian adrenal of rays of chromaffin cells across the cortex [18,19], again compatible with a paracrine role of medullary ANF. Intermixing of cortical and medullary adrenal cells is even more pronounced in other species e.g. amphibians [20].

In summary, bovine chromaffin cells thus provide an interesting model alternative to cultured cardiocytes for investigating biosynthesis, maturation and secretion of atrial peptides. Since chromaffin cells are considered as typical paraneurons and since most neuropeptides are also present in sympathetic ganglia [9], this in vitro model would be suitable for investigating the biosynthesis of ANF as well as the physiological role of the cosecretion of neuropeptides and catecholamines in the nervous system.

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