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C-type natriuretic peptide in bovine chromaffin cells

The regulation of its biosynthesis and secretion

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We report here the regulation of the biosynthesis and the secretion of C-type natriuretic peptide (CNP) in cultured bovine chromaffin cells. The combined treatment with protein kinase A and -C activators induced a 6-fold increase of intracellular levels of CNP-(1–103). The biosynthesized CNP-(1–103) was co-released with its mature forms, typically CNP-(51–103), upon stimulation by nicotine or depolarizing agents. This confirms the neuropeptidic character of this third member of the natriuretic peptide family, which might act as a neuromodulator or neurotransmitter.

Natriuretic peptide; Chromaffin cell

1. INTRODUCTION

Cardiac natriuretic peptides are a family of factors isolated from cardiac and brain extracts, sharing common regulatory properties of vascular tone, kidney function and blood pressure. They include atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). This third hormone, a 22 amino acid peptide, CNP-(82–103), and its elongated form of 53 residues, CNP-(51–103), first isolated from porcine brain [1,2], appears to be exclusively localized in the central nervous system according to Northern analysis of CNP mRNA distribution, and immunoreactivity detection in various porcine and rat tissues [3,4]. Moreover, high immunoreactivity levels are not only detected in specific regions of human brain, such as the hypothalamus and the cerebellum, but also in the pituitary gland, suggesting its role as a neuromodulator [5]. In fact, CNP appears to bind and activate the ANP-B receptors which are found in the brain and pituitary gland as well as in the adrenal medulla [6]. However, the sites and regulation of the biosynthesis for this peptide have not yet been documented. In our preliminary experiments, we have detected CNP immunoreactive-like activity within bovine chromaffin granules. This prompted us to select bovine chromaffin cells as a model to document the biosynthesis and secretion of this peptide.

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2. MATERIALS AND METHODS

2.1. *Materials*

Rat CNP-(82–103) and CNP-(51–103) and anti-rat CNP antiserum were obtained from Peninsula Laboratories (Belmont, CA). Carrier-free $Na^{125}I$ was from Amersham Corp. (Arlington Heights, IL). Enzymobeads were purchased from Bio-Rad (Richmond, CA). Bovine serum albumin, collagenase, DNAse, aprotinin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), thiorphan, phorbol ester (12-*O*-tetradecanoyl phorbol-13-acetate, TPA), forskolin (FKL), norepinephrine bitartrate, epinephrine bitartrate, and dihydroxybenzylamine-HBr were all purchased from Sigma (St. Louis, MO). All tissue culture media, sera and antibiotics were obtained from Gibco (Grand Island, NY). All chromatographic solvents were obtained from Burdick and Jackson (Muskegon, MI).

2.2. *Chromaffin cell culture and peptide extraction*

Chromaffin cells were isolated from bovine adrenal medullae according to Livett's method [4]. Briefly, adrenal glands were perfused for 30 min at 37°C in a retrograde manner with Ca^{2+}- and Mg^{2+}-free Locke's buffer containing 0.25% (w/v) collagenase and 0.025% DNAse. The Ca^{2+}, Mg^{2+}-free Locke's buffer consisted of (in mM): NaCl 145, KCl 5.6, glucose 5.6 and HEPES 10, pH 7.4. The medullary tissue was then isolated, finely minced and incubated with fresh collagenase for another 30 min. Chromaffin cells were then washed twice in Locke's solution for 7 min at 250 × *g*. Plating was accomplished in 75 cm^2 flasks (Miles Scientific, Napierville, IL) at a density of 2×10^7 cells in F12 medium supplemented with 10% (v/v) horse serum, 2% fetal bovine serum, 100 U/ml penicillin G sulfate, 100 μg/ml streptomycin sulfate and 2.5 μg/ml amphotericin B. CNP biosynthesis studies were carried out 48 h after plating. Chromaffin cells were exposed to FKL (1 μM), TPA (0.1 μM) or to both agents simultaneously for 72 h. After this treatment, the monolayers were washed twice with Locke's buffer and cells were harvested in 2 M acetic acid containing 20 mM HCl, 0.1 mM EDTA, 0.1 mM PMSF and 0.01 mM pepstatin A. The cell suspensions were then sonicated, centrifuged at 30,000 × *g* for 30 min and the supernatants were subjected to HPLC analysis. CNP secretion studies were performed 4 days after initial plating with cells previously treated with both FKL and TPA. After washing with Locke's buffer, monolayers were exposed for 20 min at 37°C to nico-

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tine (10 μM) or KCl (56 mM) in Locke's solution containing 2.2 mM $CaCl_2$ and protease inhibitors such as PMSF (1 mM), aprotinin (2 μg/ml), leupeptin (5 μg/ml) and pepstatin (5 μg/ml). Secretion media from 6×10^7 cells were pooled and acidified to a final concentration of 1% trifluoroacetic before HPLC analysis.

2.3. *Natriuretic peptide assay and data analysis*

HPLC analysis of both cell extracts and secretion media was performed according to the method of Nguyen et al. [8]. Briefly, samples were first loaded onto a reverse-phase precolumn (RP-300, 4.6 × 30 mm) (Brownlee Labs., Santa Clara, CA) at a flow rate of 2 ml/min using a mobile phase consisting of 15% acetonitrile (CH_3CN) in 25 mM triethylamine-formate buffer, pH 4. After washing with the same buffer, the precolumn was connected online with a reverse-phase analytical column (Vydac C_{18}, 0.46 × 25 cm) (Separation group, Hesperia, CA) through a switching valve. Mature and precursor forms of CNP were then separated with a 40 min linear gradient from 15 to 55% of CH_3CN in 25 mM triethylamine formate, pH 4, at a flow rate of 1 ml/min. Fractions of 1 ml were collected and dried under reduced pressure prior to estimation by radioimmunoassay for CNP. CNP-(82–103) was radioiodinated by solid phase using Enzymobeads (Bio-Rad, Richmond, CA) according to the manufacturer's directives. The radiolabelled peptide was then purified by reverse-phase HPLC on an Exsil C_{18} column (0.46 × 15 cm) (CSC, Montréal, Qué.) using a linear gradient of CH_3CN (0.5%/min) in 0.1% trifluoroacetic acid. The specific activity of the monoiodinated peptide was 1,500 Ci/mmol, as determined by self-displacement using a specific radioimmunoassay for CNP. The radioimmunoassay conditions for CNP consisted of standards or reconstituted HPLC fractions in 50 mM phosphate assay buffer, pH 7.4 (100 μl), [^{125}I]CNP-(82–103) (100 μl, 15,000 cpm), antiserum (50 μl). The anti-rat CNP antiserum displays full crossreactivity between both forms of CNP, namely CNP-(82–103) and CNP-(51-103). Standard curves for CNP were constructed from 1 to 10^5 fmol per tube in the assay buffer. The incubation was set for 24 h at 4°C. Bound and free fractions of [^{125}I]CNP-(82–103) were separated by the polyethylene glycol method and data were computer analyzed using the four parameter logistic equation [9]. Statistical differences between treatment groups were evaluated by one way analysis of variance followed by Dunnett's test for post hoc multiple comparisons. The contrast between the combined treatment with FKL and TPA as compared to the individual treatments with FKL or TPA was used to evaluate the synergistic effect of the combined treatment. The minimal level of significance was $P < 0.05$. Results are expressed as means ± S.E.M.

3. RESULTS

Fig. 1 displays the chromatographic profile of CNP-(51–103) and CNP-(1–103) found in the chromaffin cell acid extracts and eluting, respectively, at 34% and 47% of acetonitrile. In our chromatographic conditions, the immunoreactive signal corresponding to the shorter mature form, CNP-(82–103), and eluting at 37% of acetonitrile, could not be detected in the bovine chromaffin cell extracts. Shown in Fig. 2 are the effects of 1 μM FKL, 0.1 μM TPA and the combined treatment with both protein kinase A and -C activators over a 72-h period on the CNP levels within cultured chromaffin cells. In contrast to FKL or TPA alone, which do not appear to induce any significant change in the levels of both the mature and precursor forms of CNP, the combined treatment with both FKL and TPA elicits a synergistic effect on CNP-(1–103) levels (from 46 ± 9 to 298 ± 66 from/4 × 10^7 ($P < 0.001$) which corresponds to

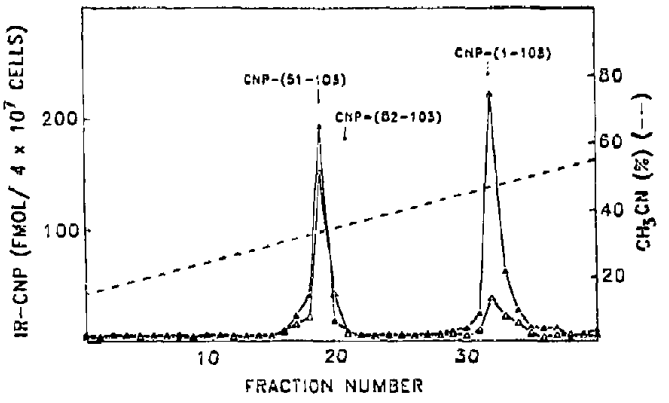

Fig. 1. Reverse-phase HPLC separation profile of unstimulated (△) or stimulated (▲) chromaffin cell extracts on the bidimensional system. Peptides were eluted with a 40 min linear gradient from 15 to 55% CH_3CN in 25 mM triethylamine formate, pH 4.0, at a flow rate of 1 ml/min. The HPLC eluates (1 ml) were evaporated to dryness and the immunoreactive peptides were determined by radioimmunoassay. CNP-(51–103), CNP-(82–103), and CNP-(1–103) eluted at 34, 37 and 47% of acetonitrile, respectively. Elution positions of standards CNP-(82–103) and CNP-(51–103) are indicated by arrows (↓). The elution position of CNP-(1–103) is inferred from its relative retention time to that of CNP-(82–103) and CNP-(51–103) ( ).

a 6-fold stimulation. This stimulatory effect appears not to be as potent on the mature form of CNP (from 140 ± 20 to 244 ± 51 fmol/4 × 10^7 cells). Fig. 3 illustrates the co-secretion of both mature and precursor forms of CNP from chromaffin cells in which the CNP biosynthesis was first stimulated by pretreatments with both FKL and TPA for 72 h before subjecting the cells to 10 μM nicotinic stimulation or to 56 mM KCl as

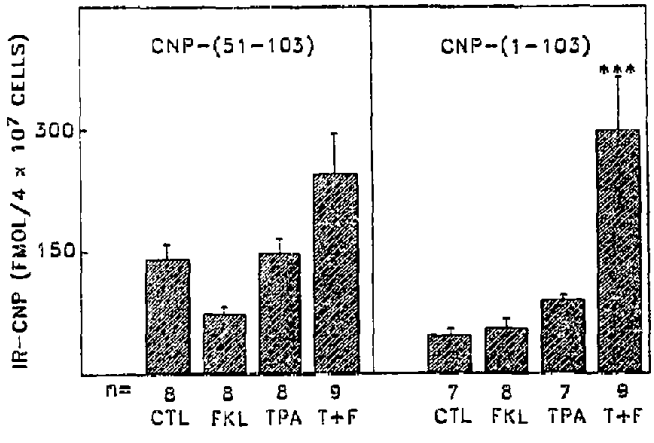

Fig. 2. Biosynthesis of CNP-(51–103) and its precursor CNP-(1–103) by cultured chromaffin cells. Cells were seeded in 75 cm^2 flasks and left to settle for 48 h after which they were stimulated by 1 μM forskolin (FKL), 0.1 μM phorbol ester (TPA) or the combined treatment (T + F) for 72 h. Cellular acid extracts were subjected to reverse-phase HPLC followed by RIA of CNP activity-containing fractions. Data are represented as mean ± S.E.M. ($n = 8$). Statistical significance was evaluated by ANOVA for CNP and its precursor levels followed by comparison to control (CTL) with Dunnett's test. ***$P < 0.001$.

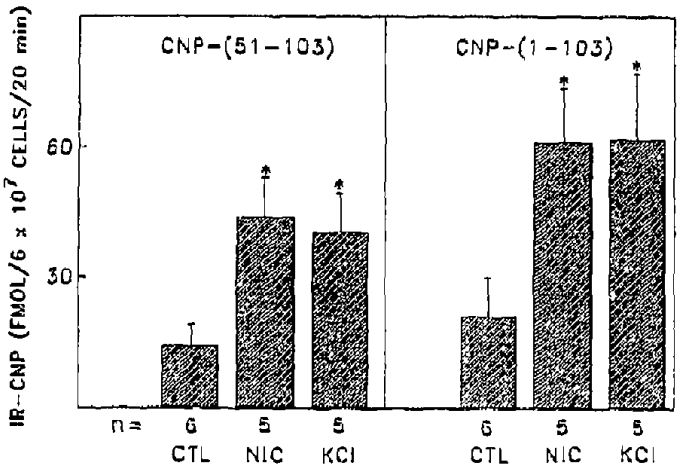

Fig. 3. Co-secretion CNP-(51–103) and its precursor CNP-(1–103) by cultured chromaffin cells. After 48 h settling and 72 h stimulation with FKL (1 μM) and TPA (0.1 μM), cells were exposed to 10 μM nicotine (NIC) or 56 mM KCl (KCl). Extracellular secretion media were subjected to HPLC followed by RIA for CNP immunoreactivity detection. Data are represented as mean ± S.E.M. Statistical significance was evaluated by ANOVA for CNP and its precursor levels followed by comparison to control (CTL) with Dunnett's test. $^*P < 0.05$.

depolarizing agent. When 10 μM nicotine was added to the secretion medium, the basal levels of CNP-(51–103) and its precursor form, CNP-(1–103), which were 14 ± 5.2 fmol and 20.8 ± 9 fmol/6 × 10^7 cells, respectively, increased up to 43.6 ± 9.4 fmol and 61 ± 12.4 fmol/6 × 10^7 cells, corresponding to a 3-fold increase above the control levels.

4. DISCUSSION

The neuropeptide character of CNP, the third member of the natriuretic peptide family recently isolated from porcine brain, has been demonstrated by its biosynthesis and secretion from cultured bovine chromaffin cells. Indeed, the net 6-fold increase of the intracellular levels of the precursor form of CNP, CNP-(1–103), observed under the combined treatment with FKL and TPA, is indicative of de novo synthesis of this peptide within the chromaffin cells. However, no further maturation process of CNP-(1–103) was observed under this stimulatory condition since the proportion of the mature and precursor forms of CNP relative to each other appears to decrease. This contrasts with both ANF and ASIF whose intracellular ratio of mature vs. precursor forms remained unchanged in the presence of FKL and TPA [8]. The biosynthesis profile of the mature form of CNP is reminiscent of that of ANF with a 3–4 fold increase in intracellular levels promoted by protein kinase A and -C activators. As observed for other neuropeptides found in the chromaffin granules, e.g. enkephalin-containing peptides [10] and other natriuretic peptides [8], the concomitant exocytosis of both precursor and mature forms of CNP could be equipotently induced by nicotinic activation or KCl depolarization, corresponding to an average release of 20% of their intracellular content. Interestingly, the ratio of CNP-(1–103) to its mature form CNP-(51–103) released in the incubation media, which averaged 1.4, was maintained upon the stimulation with both secretagogues and remained identical to the corresponding intracellular ratio, suggesting that no further processing seems to occur during exocytosis. Furthermore, the processing pattern of CNP in chromaffin cells is similar to that in the brain since CNP-(51–103) is the major mature form found in both tissues [2]. In contrast to other natriueretic peptides, it has been reported that CNP exhibits more potent vasoactive effects [11]. Therefore, its potential role on the regulation of the catecholamine secretion might be expected. Further work is needed to document the role of CNP in the modulation of catecholamines using the cultured chromaffin cells as model.

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