Differential Secretion of Enkephalin-Like Peptides from Bovine Adrenal Chromaffin Cells

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Stimulation of chromaffin cells in culture with 1,1-dimethyl-4-phenylpiperazinium (DMPP) or depolarizing concentrations of K^+ results in a significant secretion of high and low molecular weight enkephalin-like peptides (ELPs) into the culture medium. Bio-Gel P-10 chromatography was used to characterized the ELPs in the extract of chromaffin cells and in culture media before and after stimulation. DMPP (50μM) stimulation produced a significant secretion of primarily low molelcular weitht (3-kDa) ELPs whereas 56 mM K^+ caused a secretion of both the high and low molecular weights ELPs. Subsequent to the secretion of these ELPs the expected decrease in the content of cellular low molecular weight peptides was not observed. This suggests that stimulation of chromaffin cells by either an action on nicotinic receptors or by membrane depolarization enhances processing of ELPs. Our results support the hypothesis that specific stimuli bring about the secretion of ELPs of differing molelcular weight.

Key words : 1,1-dimethyl-4-phenylpiperazinium, high K^+, chromaffin cells, enkephalin-like peptide secretion, proenkephalin processing

(Met^5)-enkephalin and its various precursors are present in adrenomedullary chromaffin cells [1-3]. The concentration of enkephalin-like peptides (ELPs) in chromaffin cells is species dependent being highest in dog and cattle adrenal medullae, and lowest in rat adrenal medulla[1]. The ELPs are co-stored with the catecholamines, norepinephrine and epinephrine, in chromaffin granules[2-3] Studies with retrogradely perfused canine adrenal glands[3], retrogradely perfused beef adrenal glands[4], canine[5] and feline[6-7] adrenal glands *in situ*, and bovine chromaffin cells in culture[8] reveal that ELPs are released, in a calcium-dependent manner, along with catecholamines. The release is believed to occur primarily through nicotinic receptor activation[2-5, 9-10]. Furthermore, the secretion of various sizes of ELPs has been shown to be influenced by the type of stimuli[4-7]. Hexum et al.[11] reported that splanchnic nerve stimulation in the anesthetized dog resulted in increased secretion of primarily low molecular weight ELPs. Studies with perfused beef adrenal glands showed that a portion of each of ELPs present in the glands is released by 50 μM nicotine or 5 mM Ba^{+2} [4]. Chaminade et al.[6-7] using perfused cat adrenal glands *in situ*, reported a release of (Met^5)-enkephalin immunoreactive material and precursors of different proportions, depending upon whether stimulation was due to the splanchnic nerve, 50mM K^+ or 100 μM acetylcholine.

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The present study provides evidence of differential release of the ELPs from bovine chromaffin cells in culture in response to stimulation by either DMPP, a potent nicotinic receptor agonist, or high K^+ concentration. DMPP-stimulation preferentially causes a secretion of low molelcular weight ELPs which constitute up to 85% of the total ELPs released while high K^+ induces marked secretion of both the high and low molecular weight peptides. As a consequence of this immediate secretion of the peptides there is no change in the cellular content of the small ELPs following both DMPP- or high K^+ -stimulations. This suggests that the repletion of the cellular small ELPs has occurred through the processing of large ELPs.

Materials And Methods

Primary culture of chromaffin cells

Chromaffin cells were isolated essentially as described by Greenberg and Zinder[12] Briefly, adrenal glands were retrogradely infused with Ca^{+2}-, Mg^{+2}-free balanced salt solution containing 2.5 mg/ml collagenase (Type V, Sigma) and 13.3 mg/L DNase I(Type II,Sigma) and incubated twice, 15 min each at 37°C. The cortices were removed and the medullae were finely chopped and subjected to another 30 min incubation with fresh mixture of collagenase/DNase solution. The minced tissue was filtered on a nylon mesh (74 mesh, Spectra). The filtrate was centrifuged at 100 x g for 10 min and the pellet was washed twice with Locke's containing 5 mg/ml bovine serum albumin and filtered again prior to dispensing into the culture medium. Cell viability, determined with Trypan blue, was usually greater than 95% of which 90% was shown to be cells containing catecholamines as assessed by neutral red dye. A yield of approximately 1 x 10^8 viable cells per medulla was normally obtained. Cells were resuspended in 25mM HEPES DMEM, pH 7.4, supplemented with 10% fetal calf serum (Gibco), 584 µg/ml glutamine (Gibco), 50 units/ml nystatin (Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin, 40µg/ml gentamicin, and 10 µM 5-fluorodeoxyuridine. All antibiotics and antimitotics agents were purchased from Sigma. Cells were cultured in rat-tail collagen-coated 100 x 15 mm petri dishes (Optilux Dish, Falcon 1001) at a density of 2 x 10^7 cells/dish in 10 ml DMEM and kept at 37°C in water-saturated 5% CO_2/95% air. After 2 days in cultlure, cells showed confluency, and half of the medium was replaced with fresh medium. Cells were used after 3 days in culture.

Stimulation of ELP-release from chromaffin cells

Culture media were aspirated and cells washed briefly with fresh Locke's. Cells were preincubated in this buffer at 37°C for 10 min. Aliquots of preincubation medium were removed to iced tubes to determine basal release of ELPs. 50 µM DMPP in Locke's or Locke's with 56 mM K^+ (sodium corrected) was added into the culture dish. Cells were stimulated with the same concentrations of these secretagogues three times, 5min each at 37°C. At the end of each 5 min stimulation period, all culture media were removed to tubes on ice and pooled. Following the last stimulation period cells were rapidly washed once with ice-cold Locke's and this washing was combined with the previously pooled medium. Cell suspensions were prepared by scraping the cells from the bottom of the culture dish with a rubber policeman and ice-cold Locke's. Both pooled culture media and cell suspensions were boiled at 95°C for 10 min, then cooled. ELPs were extracted from cell suspensions by brief sonication at 0°C. Aliquots from culture media and cell extracts were taken for radioimmunoassay[9]

Bio-Gel P-10 chromatography

Culture media and cell extracts were centrifuged, 12,100 x g for 10 min, desalted and concentrated on a Sep-Pak C-18 cartridge (Waters Associates) Samples eluted from Sep-Pak cartridges (100% CH_3CN) were

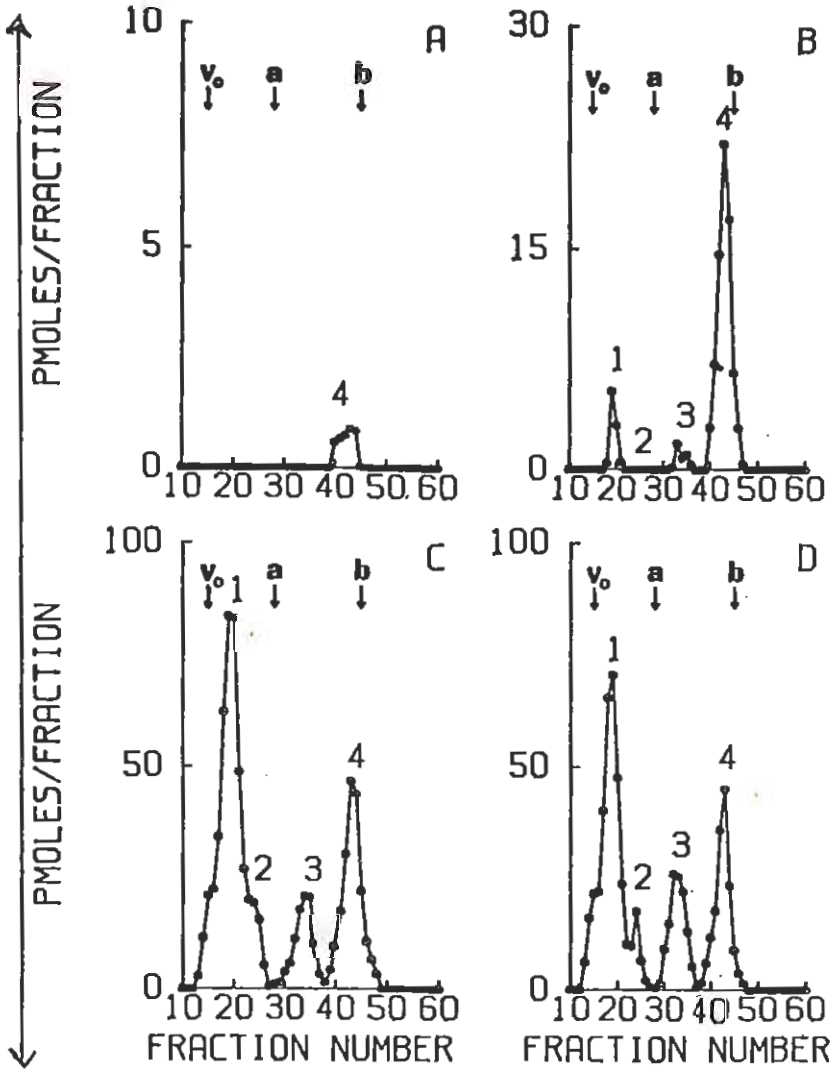

Figure 1. Enkephalin-like peptide profiles after gel permeation chromatography on a Bio-Gel P-10 column equilibrated with 0.1 M acetic acid.

Aliquots from column fractions were digested with trypsin and carboxypeptidase B and total (Met^5)-enkephalin was determined by radioimmunoassay (see METHODS). A and B represent the ELPs released into the culture medium under basal or 50 μM DMPP, respectively. C and D represent cellular peptides before and after 50 μM DMPP, respectively. Markers are: Vo, blue dextran; a, cytochrome C oxidase; d, peptide E.

lyophilized, reconstituted in 0.1 M acetic acid and applied onto a Bio-Gel P-10 column (0.9 x 58 cm) that had been equilibrated with 0.1 M acetic acid. Aliquots of column fractions were freeze-dried, reconstituted in 0.05 M Tris-HCl, pH 8.0, containing 2 mM $CaCl_2$ and subjected to sequential digestion with trypsin and carboxypeptidase B (Cooper Biomedical, Calbiochem)[13] before determination of ELPs by radioimmunoassay.

Peptide radioimmunoassay

ELP content was determined using a highly specific C-terminal directed antiserum to (Met^5)-enkephalin as described elswhere[9]. All radioimmunoassays were carried out in a total volume of 550 μl using ^3H(Met^5)-enkephalin 52 Ci/mmol (Amersham) as a tracer. The buffer used was 0.5 MTris-HCl, pH 7.4, containing 0.2% bovine serum albumin (RIA grade, Sigma) and 0.15% dextran (Sigma). After

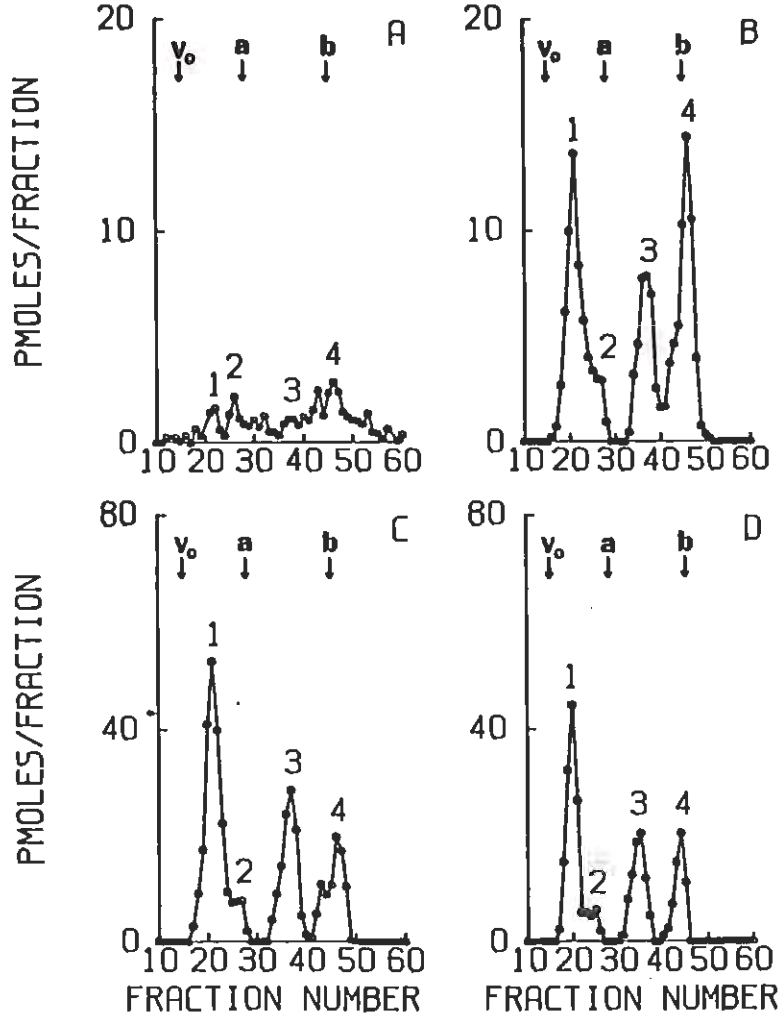

Figure 2. Effect of high K^+ on the secretion and content of enkephalin-like peptides.
A and B represent the peptides released into the culture medium before and after high K^+. C and D represent the cellular peptides before and after high K^+ Other descriptions are as provided in Figure 1.

incubation overnight at 4°C, the antibody bound tracer was separated from unbound tracer by adsorption to charcoal followed by centrifugation[14] Radioactivity in the supernatant was determined using a Beckman liquid scintillation spectrometer.

Results

Figure 1 (A and B) shows the profiles of ELPs released into the culture medium in response to nicotinic receptor stimulation. DMPP (50 μM) significantly increased the secretion of ELPs found in Peak 4 which have a molecular weight of approximately 3-kDa since they co-elute with peptide E (3.2-kDa). Peak 4 contains upto 85% of the total immunoreactive material recovered in the culture medium after stimulation of the cultured cells. When cells were stimulated with 56 mM K^+ the molecular forms of the released ELPs changed. As shown in Figure 2B, elevated K^+ caused a significant increase in the secretion of immunoreactive material found in Peaks 1 and 3 as well as Peak 4. Peaks 1 and 3 contain ELPs with corresponding molecular weight of approximately 20- and 10-kDa since they co-elute with blue dextran (20-kDa) and cytochrome C oxidase (12-kDa), respectively.

Comparision of the Bio-Gel P-10

profile of culture medium shown in Figure 1B with that in Figure 2B reveals that elevated K^+ causes a significant rise in the percentage of immunoreactive material recovered in Peaks 1 and 3 (Figure 2B) when compared to the effect of DMPP (Figure 1B). Whereas DMPP causes a secretion of about 85% of immunoreactivity as Peak 4, depolarizing concentration of K^+ results in only 37% of immunoreactive material secreted being found in Peak 4.

Discussion

The results reported here are consistent with the results obtained from perfused feline[6-7] adrenal glands *in situ* which showed that mild stimulation of splanchnic nerve (15 Hz, 15 V, 1 ms square pulse, 10 min) produced secretion of small peptides predominantly whereas drastic stimulation (50 mM K^+ or 100 µM acetylcholine) brought about the additional secretion of ELPs in the form of larger fragment of the precursors.

The release of ELPs from chromaffin cells would be expected to produce a corresponding alteration in the cellular content of the peptides if there is no replacement of the lost peptides. However, while stimulation of chromaffin cells with 50 µM DMPP or 56 mM K+ results in a decrease in the cellular immunoreactive material found in Peak 1 that in Peak 4 remains relatively constant (Figures 1D, 2D). Moreover, it is apparent that stimulation by these secretagogues leads to a significant secretion of immunoreactive material as Peak 4 (Figures 1B, 2B) when compared with their corresponding controls (Figures 1A, 2A). Despite the significant rise of immunoreactive material seen in Peak 4 of the culture medium, the corresponding cellular immunoreactivity in Peak 4 does not simultaneously decrease (Figures 1D, 2D). Thus, the results suggest that cellular repletion of ELPs found in Peak 4 occurs concurrently with the accelerated secretion induced by these two secretagogues.

The significance of these findings is two-fold. First, as the extension of the in vivo demonstrations[6-7, 11] this study suggests that there is selective secretion of the ELPs. Mild stimulation might represent the physiological response through splanchnic nerve stimulation resulting in substantial secretion of low molecular weight ELPs probably from the mature granules that contain the small biologically active enkephalins. Alternatively, drastic stimuli leads to the secretion of partially processed precursors which are probably from the immature granules. The difference in release patterns of the ELPs in response to specific stimuli further implies a different role for the various ELPs released from the adrenal gland. The small ELPs may produce an immediate physiological effect by acting on peripheral tissues such as the heart or the vasculatures. The larger ELPs may cause a subsequent delayed effect due to their degradation to small peptides by blood borne proteases. Another possibility is that the large ELPs may be gradually processed to small peptides by blood borne proteases during transport to the brain. The resultant small peptides then may interact with the opiate receptors at or near the blood brain barrier or be transported across the barrier into the brain[15] and exert their effects. The latter was shown to be the case as exemplified by the behavioural effects of pharmacological doses of opioid peptides given systemically[16-17]. Further elaboration regarding physiological significance of this differential secretion remains to be explored. Secondly, the observed cellular repletion induced by DMPP or elevated K^+ suggests that there is a stimulus-secretion-synthesis coupling of the opioid peptides in the adrenal chromaffin cells. Stimuli that cause depletion of cellular ELPs by secretion may also activate the processing of pre-existing precursor peptides to replete the secreted ELPs. Therefore, the homeostasis of opioid peptides in the chromaffin cells is regulated and the ELPs can be released in response to stimulation.

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