Selective Regulation of Carboxypeptidase Peptide Hormone-processing Enzyme during Enkephalin Biosynthesis in Cultured Bovine Adrenomedullary Chromaffin Cells

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Bovine adrenomedullary chromaffin cells in culture were incubated with reserpine or forskolin, two agents acting through different mechanisms, which increase cellular [Met]enkephalin levels by 2-fold after 72 h. Cells were harvested and chromaffin granules were purified on a linear sucrose gradient. After reserpine treatment, carboxypeptidase-processing enzyme specific activity in chromaffin granule fractions was stimulated 1.9-fold, and COOH-terminal carboxypeptidase specific activity was stimulated 3-fold. The increase in enzyme activity was dependent on the time of reserpine treatment. Forskolin, on the other hand, had no significant effect on carboxypeptidase activity. The differential effects of reserpine and forskolin suggest that the carboxypeptidase-processing enzyme may be selectively regulated during periods of elevated enkephalin formation.

Kinetic studies revealed that in cells exposed to reserpine, the $K_m$ value for [Met]enkephalin-Arg$^2$ for the CO$_2^+$-stimulated carboxypeptidase activity was lowered to 0.136 from 0.447 mM, but there was no change in the $K_m$ values of the non-CO$_2^+$-stimulated carboxypeptidase activity from reserpine and control groups. Cellular levels of immune-reactive carboxypeptidase-processing enzyme, measured by a radioimmunoassay method, were not altered after reserpine treatment. These data suggest that while the total number of carboxypeptidase enzyme molecules remained constant, there may be a conversion of existing enzyme molecules to a more active form which displays a higher affinity for [Met]enkephalin-Arg$^2$ in the presence of CO$_2^+$.

Regulation of peptide hormone biosynthesis can occur at several cellular levels: transcription of the peptide hormone gene, RNA processing, translation of the mature mRNA, and post-translational processing of the precursor form of the peptide. Among these steps, much has yet to be learned about the enzymes involved in post-translational processing. These enzymes represent an important potential point of regulation, since it is at this step where the biologically inactive peptide hormone precursor is converted to its final smaller biologically active form.

Within the preproenkephalin precursor (1–3) and many other peptide hormone precursors (4–7) the peptide hormone sequences are characteristically flanked at their NH$_2$ and COOH termini by pairs of basic amino acid residues (lysine and arginine). Several different proteases are thought to be involved in prohormone processing. A trypsin-like endopeptidase that cleaves at the pairs of basic amino acid residues could liberate peptide hormone(s) extended at the carboxyl terminus with lysine or arginine. The COOH-terminal basic residue can be clipped off by a carboxypeptidase-processing enzyme resulting in the formation of the small active peptide. If the endopeptidase cleaves between the pairs of basic residues, an aminopeptidase may be required to remove the NH$_2$-terminal basic residue extension on the peptide.

Prohormone converting activity involved in processing proopiomelanocortin in rat pituitary anterior and intermediate lobes (8–10) and provasopressin in pituitary neural lobe (11) has been identified and appears to be a unique acid thiol “trypsin-like” secretory granule peptidase. Several studies (12–15) have described trypsin-like activities in adrenomedullary chromaffin granules which may be involved in processing proenkephalin; however, the identity of these activities is not yet clear.

Carboxypeptidase-processing enzyme activities have been identified in purified secretory granules from bovine adrenal medulla (16–21), rat anterior and intermediate lobes (22), rat neural lobe (22), and rat pancreatic tumor cells (23), which are thought to be involved in the processing of proenkephalin, proopiomelanocortin, provasopressin, and proinsulin, respectively. These carboxypeptidase activities display similar properties. Such activity has also been identified in bovine pituitary and brain (18, 19, 24, 25) and has previously been referred to as “enkephalin convertase” (17–19, 24, 25). Characterization of the purified carboxypeptidase (enkephalin convertase) has revealed that the bovine pituitary, brain, and adrenal medulla forms are identical with respect to their kinetics, substrate specificity, inhibitor profile, and size (24, 25). This suggests that a common carboxypeptidase enzyme may be involved in the processing of many peptide precursors.

The processing carboxypeptidase’s acidic pH optimum, thiol dependence, molecular weight, and potency of Zn-metalloprotease inhibitors differentiate it (16–25) from pancreatic carboxypeptidase B and plasma carboxypeptidase N (26, 27), but the processing carboxypeptidase resembles carboxypeptidase B in its specificity for basic amino acid residues. Specific rabbit polyclonal antibodies against the purified bovine pituitary processing carboxypeptidase do not cross-react with carboxypeptidases B, N, Y, P, or A, suggesting that this enzyme may be a structurally distinct protein from other known carboxypeptidases.\(^1\) Thus, a unique carboxypeptidase

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appears to be involved in peptide hormone precursor processing. An important question to investigate is whether or not the processing enzyme activities can be altered when cellular peptide levels are elevated or lowered and, if so, what is the mechanism of this regulation. In this report we have studied carboxypeptidase-processing enzyme activity in cultured bovine adrenal medulla chromaffin cells after reserpine and forskolin treatment. Reserpine is an inhibitor of vesicular catecholamine reuptake (28), and forskolin is a stimulator of adenylyl cyclase (29) which results in elevated cellular cyclic adenosine 3':5'-monophosphate levels. These two agents both increase enkephalin levels by 2-fold in the chromaffin cells (30–32), yet they have differential effects on carboxypeptidase-processing enzyme activity.

**MATERIALS AND METHODS**

**Bovine Chromaffin Cell Culture**—Fresh bovine adrenal glands were obtained from a local slaughter house (Mt. Airy, MD) and the adrenal medulla tissue was dissected. Chromaffin cells were dispersed from adrenal medulla as described previously (22). 25 × 10⁶ cells were plated in 175 cm² flasks (Falcon) in 50 ml of Dulbecco’s modified Eagle’s medium containing 100 units/ml of penicillin, 100 µg/ml of streptomycin, 10% fetal calf serum and 10-4 M tyrosine arabinoside, and cells were kept at 37 °C in an air/5% CO₂ incubator. After 1–4 days in culture, reserpine (10-5 M in dimethyl sulfoxide) or forskolin (50 µM in dimethyl sulfoxide) was added to the culture medium to give final concentrations of 10-5 M reserpine or 50 µM forskolin. Control groups had the drug vehicle (0.1% dimethyl sulfoxide) in the culture medium. After 24 or 72 h of treatment with reserpine or forskolin, cells were harvested and chromaffin granules isolated as indicated below.

**Purification of Granules from Chromaffin Cells in Culture**—Chromaffin cells were harvested by removing the culture medium and rinsing the cells 5 times with 5 ml of phosphate-buffered saline, pH 7.4, without Ca²⁺ and Mg²⁺. The cells were then incubated at 37 °C with 5 ml of 0.02% EDTA in phosphate-buffered saline. The detached cells were collected, and the flask was rinsed 3 times with 5 ml of phosphate-buffered saline to collect any remaining cells. Cells were centrifuged at 400 × g for 10 min, and the cell pellets were homogenized in 20 ml of 0.32 M sucrose with 20 strokes of a Dounce B homogenizer. The homogenate was centrifuged at 10,000 × g for 5 min, and the resultant supernatant was centrifuged at 30,000 × g for 35 min. The pellet was resuspended in 1.0 ml of 0.32 M sucrose and applied to a linear sucrose gradient for 45 min in a Beckman ultracentrifuge at 32,000 rpm for 30 min in an SW 65 rotor. A puncture was made at the bottom of the centrifuge tube and fractions of 0.25 ml (15 fractions) were collected. Carboxypeptidase-processing enzyme activity and immunoreactivity, [Met]enkephalin immunoreactivity, catecholamine content, acid phosphatase (lysosomal enzyme marker), and protein content were measured in each fraction.

**Purification of Chromaffin Granules from Adrenal Medulla Tissue**—Chromaffin granules were purified from fresh bovine adrenal medulla tissue as described previously (16, 21). Granules were lysed by 0.015 M KCl and kept at 4 °C for 2 h. After centrifugation for 45 min at 10,000 rpm in an SS 34 rotor, the supernatant and pellet were taken as the soluble and membrane granule fractions, respectively.

**Carboxypeptidase Assay for the Conversion of [125I]-Met-[Enkephalin-Arg⁶ to [125I]-Met-[Enkephalin-Arg⁶ to [125I]-Met-[Enkephalin—[Met]Enkephalin-[Met]Enkephalin-[Met]Enkephalin—[Met]Enkephalin-[Met]Enkephalin—[Met]Enkephalin-[Met]Enkephalin—[Met]Enkephalin in the reaction mixture were separated by thin layer chromatography on Silica Gel G plates (Analtech, Newark, DE) in a solvent system of petroleum ether/acetone/28% acetic acid in water (3:5:2.5:4.5:1.4:5:4.8:3.7. Ethyl acetate was first washed with 10 nm ferric sulfate (ethyl acetate/ ferric sulfate was 10:1, v/v). Radioactivity was scanned along each lane with the use of a BID/System 100 radiographic imaging system (Biocis, Washington, D. C.). [125I]-[Met]Enkephalin-Arg⁶ and [125I]-[Met]enkephalin migrated on the TLC plates with Rₜ values of 0.21 and 0.47, respectively. Total cpm of [125I]-[Met]enkephalin was determined by computing the integral of each peak with an HP-86 computer.

The carboxypeptidase assay was linear in the range of 5-35% conversion of radiolabeled substrate to product. Therefore, several different volumes (1–10 µl) of the gradient fractions were assayed for carboxypeptidase activity in order to make measurements in the linear range of the assay. The carboxypeptidase-processing enzyme present in chromaffin granule fractions has been found to be stimulated by Co²⁺ (17). When carboxypeptidase was assayed in the presence of CoCl₂, reaction tubes were first preincubated with a final concentration of 5 mM CoCl₂ at 4 °C for 30 min. To provide enough sample for the determination of Kₘ, constant in chromaffin granule fractions from the linear sucrose gradient, every 2 fractions were pooled (fractions 5–6, 7–8, 9–10, and 11–12 were combined). Endogenous substrate present in these fractions was removed by dialysis and concentration with an Amicon Centrifloc 10 microconcentrator which had a Mₘ = 10,000 cut-off membrane. Enzyme activity in these dialyzed samples was measured at substrate concentrations of 23, 51, 80, 118, 232, and 346 µM ([Met]enkephalin-Arg⁶).

**Carboxypeptidase-processing Enzyme Radioimmunoassay—Specific rabbit polyclonal antibodies against purified bovine pituitary carboxypeptidase-processing enzyme have been prepared and characterized.**

[125I]-Carboxypeptidase (approximate specific activity 120 µCi/0.1 pmol) was prepared by a lactoperoxidase method using Enzymobeads (Bio-Rad), a solid phase support for lactoperoxidase and glucose oxidase. 5 µg of [125I]-carboxypeptidase (400,000 cpm) in 150 µl of 200 mM sodium phosphate buffer, pH 7.2, 2 µl of Na⁺I (1 mCi, Amersham), and 25 µl of 1.0 M glucose at room temperature for 45 min. After centrifugation for 5 min in a Beckman Microfuge, the supernatant was applied to a G-25 PD10 (Pharmacia) gel filtration column equilibrated with 0.1% sodium dodecyl sulfate, 1% bovine serum albumin, 150 mM NaCl, 100 mM sodium phosphate, pH 7.4, to remove free I⁻.

Because carboxypeptidase enzyme activity has been found in both soluble and membrane (16–21) components of bovine adrenomedullary chromaffin granules, gradient fractions were first solubilized by dilution (1:2) with a Triton-containing buffer (100 mM sodium phosphate, pH 7.4, 0.5 of trypsin inhibitor units/ml of Trasylol (Sigma), 0.1% bovine serum albumin, 0.01% Merthiolate, 0.1% Triton X-100, and 10 mM EDTA) to solubilize membrane-bound enzyme. After remaining on ice for 2 h, the solubilized gradient fractions were centrifuged in a Beckman Microfuge for 5 min and the supernatants were assayed for carboxypeptidase enzyme immunoreactivity. Radioimmunoassay was performed as described previously by using purified soluble form of bovine pituitary carboxypeptidase as standard (a gift from Dr. Lloyd Fricker, University of Oregon, Eugene, and Dr. Solomon H. Snyder, Johns Hopkins University, Baltimore, MD).

Each tube contained in a total volume of 400 µl of radioimmunoassay buffer (100 mM Tris-HCl, pH 8.5, 0.5% bovine serum albumin, 0.01% Merthiolate, 0.5 mg/ml of soybean trypsin inhibitor), 8,000–10,000 cpm of [125I]-carboxypeptidase, standard or experimental samples of carboxypeptidase, and rabbit antiserum (A2) at a final dilution of 1:15,000. After overnight incubation at 4 °C, 100 µl of goat anti-rabbit immunoglobulins (Calbiochem-Behring) (diluted 1:10 in radioimmunoassay buffer) was added and incubated again overnight at 4 °C. Samples were centrifuged for 20 min at 2000 rpm in an International centrifuge and the pellet was counted in a Micromed γ counter. Nonspecific [125I]-carboxypeptidase binding was 4–5% of total counts in each tube, and antibody at 1:15,000 dilution bound 19–22% of total [125I]-carboxypeptidase.

[Met]Enkephalin—Radioimmunoassay—[Met]Enkephalin immunoreactivity was determined in sucrose gradient fractions after dilution and boiling in 0.1 N HCl, as previously described (34) using an enkephalin antiserum (RB-4) kindly provided by Dr. Steven Sabol (National Heart, Lung and Blood Institute, Bethesda, MD), carboxyamidomethylation—Catecholamines were measured by the method of von Euler et al. (35) using epinephrine (Sigma) as standard.

**Acid Phosphatase**—Acid phosphatase activity, an enzyme marker for lysosomes, was determined according to the method of Barrett (36). Protein was determined according to the method of Lowry et al. (37).

**RESULTS**

**Carboxypeptidase-processing Enzyme Activity in Granules of Cultured Bovine Chromaffin Cells**—Chromaffin granules were...
Alterations in Carboxypeptidase-processing Enzyme Activity

purified from cultured bovine adenomedullary chromaffin cells on a linear sucrose gradient (Fig. 1). In the control group (Fig. 1, panel I), two peaks of granules containing [Met] enkephalin and catecholamines (fractions 3-6 and 6-9) were separated from the majority of acid phosphatase activity, a marker enzyme for lysosomes. Carboxypeptidase-processing enzyme activity which converted $^{35}$S-[Met]enkephalin-Arg$^6$ to a single product, $^{35}$S-[Met]enkephalin, was present in both populations of granules. This granular carboxypeptidase activity was stimulated by cobalt (Co$^{2+}$), as Fricker and Snyder have reported (17).

After the chromaffin cells were treated for 72 h with reserpine or forskolin, [Met]enkephalin content in the granular fractions was increased over controls (Fig. 1). Catecholamine content in the granular fractions was depleted by reserpine but not by forskolin. Co$^{2+}$-stimulated carboxypeptidase specific activity in the [Met]enkephalin-containing granule fractions was dramatically stimulated in reserpine-treated cells, and there appeared to be a small increase in the forskolin-treated cells. It is interesting to note that the two peaks of granules from the reserpine group were less dense (peak fractions were 7 and 9) than those from the control group (peak fractions were 4-5 and 7-8) (38). In reserpine-treated cells, the greatest increase in Co$^{2+}$-stimulated carboxypeptidase activity was present in the less dense population of granules.

The increase in granular [Met]enkephalin content was dependent on the length of reserpine treatment (Table I). When total chromaffin granule [Met]enkephalin content was calculated, 24- and 72-h reserpine treatments increased granule [Met]enkephalin to 151 and 190%, respectively, of control values. After 72 h of forskolin treatment, [Met]enkephalin content was 212% of control values. Other studies (37) have shown that 24 h of forskolin treatment does not alter chromaffin cell [Met]enkephalin content.

The reserpine-induced increase in carboxypeptidase-processing enzyme specific activity was also dependent on the time of drug treatment (Table II). After 24 and 72 h of reserpine treatment carboxypeptidase specific activity (assayed without Co$^{2+}$) increased 1.5- and 1.9-fold over control, respectively, and the Co$^{2+}$-stimulated specific activity increased 1.8- and 2.9-fold over control. Carboxypeptidase activity from reserpine-treated cells was more sensitive to stimulation by Co$^{2+}$ than the activity from control cells. Total granule carboxypeptidase activity was also increased by reserpine. 72 h of reserpine increased total activity by approximately 2-fold.

Fig. 1. Sucrose density gradient fractionation of granules from reserpine- or forskolin-treated bovine chromaffin cells. Bovine adenomedullary chromaffin cells were treated with reserpine (1 X 10$^{-6}$ M) or forskolin (50 μM) for 72 h as described under "Materials and Methods." Cells were harvested and chromaffin granules were purified on a linear sucrose gradient. Sucrose gradient fractions from control (I) and reserpine (II)- and forskolin (III)-treated cells were assayed for carboxypeptidase-processing enzyme activity and immunoreactivity (panel A), [Met]enkephalin and catecholamine content (panel B), and protein and acid phosphatase content (panel C).
Alterations in Carboxypeptidase-processing Enzyme Activity

Table I

| [Met]Enkephalin content in granules purified from chromaffin cells in culture after reserpine and forskolin treatment |
|---------------------------------------------------------------|
| Chromaffin cells were treated with 1 x 10^{-4} M reserpine or 50 \mu M forskolin. Cells were harvested and chromaffin granules were purified on a linear sucrose gradient as described under "Materials and Methods" and as shown in Fig. 1. [Met]Enkephalin immunoreactivity was determined in each gradient fraction and the sum of [Met]enkephalin content in the enkephalin-containing fractions (Nos. 5-11) was taken as total [Met]enkephalin in the chromaffin granule fractions. These values are expressed below as per cent control to normalize for the slight variations from experiment to experiment in number of cells plated for each treatment group. |
| | % control (mean ± S.E.) |
| Control | 100 |
| Reserpine, 24 h | 159 ± 43* |
| Reserpine, 72 h | 190 ± 45* |
| Forskolin, 72 h | 212 ± 19* |
| * Statistically significant, p < 0.01. |

Table II

Carboxypeptidase-processing enzyme specific activity and total activity in granules purified from chromaffin cells in culture after reserpine and forskolin treatment

| Specific activity (mean ± S.E.) | Total activity |
|---------------------------------|---------------|
| -Co^{2+} | +Co^{2+} |
| pmol [^{3}H]-[Met]enkephalin/µg protein | % control |
| Control | 8.81 ± 0.74 | 11.27 ± 1.32 | 100 |
| Reserpine, 24 h | 13.40 ± 5.16 | 19.98 ± 3.54 | 126 ± 34 | 125 ± 27 |
| Reserpine, 72 h | 16.56 ± 2.56 | 32.40 ± 2.70 | 185 ± 22 | 262 ± 39 |
| Forskolin, 72 h | 7.32 ± 1.47 | 15.69 ± 3.61 | 90 ± 7 | 134 ± 16 |
| * Statistically significant, p < 0.05, compared to control using Student's two-tailed t test (n = 3). |

Although forskolin, like reserpine, increased [Met]enkephalin content 2-fold after 72 h of treatment (Table I), forskolin elicited a small but insignificant elevation in Co^{2+}-stimulated carboxypeptidase specific activity and total activity (Table II).

To determine whether reserpine had a direct or indirect effect on the processing enzyme, carboxypeptidase activity in chromaffin granules purified from whole bovine adrenal medullary tissue was assayed in vitro in the absence or presence of reserpine (Table III). Reserpine, at final concentrations of 1 x 10^{-8} to 1 x 10^{-4} M, had no effect on carboxypeptidase activity in vitro. Therefore, the increase in enzyme activity seen in reserpine-treated chromaffin cells probably involves indirect effect(s) of reserpine.

Small enkephalin peptides, including the carboxypeptidase substrates [Met]enkephalin-Arg^{6} and [Met]enkephalin-Lys^{6}, are present in large amounts in adrenomedullary chromaffin granules (39). Because the carboxypeptidase-processing enzyme was assayed in whole chromaffin granules, it is possible that alterations in granular carboxypeptidase activity might be due to changes in granule substrate concentrations induced by reserpine. To determine if the reserpine-induced increase in carboxypeptidase activity was independent of endogenous substrates in the chromaffin granules, enzyme activity was measured after removal of low molecular weight peptides and proteins (M, less than 10,000) (Table IV). Total carboxypeptidase activity in the granule fractions was not altered in control or reserpine groups after removal of the low molecular weight proteins. Therefore, the increase in enzyme activity was probably independent of endogenous substrate concentration, and may be due to a more active form of the enzyme or an increase in the number of active enzyme molecules.

Kinetic Studies of Carboxypeptidase Activity with [Met]Enkephalin-Arg^{6}—After incubation of the chromaffin cells with reserpine for 72 h, the K_{m} for carboxypeptidase was determined in the absence and presence of Co^{2+} (Fig. 2). Granule fractions 5-6, 7-8, 9-10, and 11-12 from the sucrose gradient were combined to form the pooled fractions A, B, C, and D, respectively. After removal of low molecular weight proteins (M, less than 10,000), the K_{m} values were measured in each pooled fraction (A-D) from control and reserpine-treated cells and Lineweaver-Burk plots are shown in Fig. 2. The 1/S axis intercept plotted for each granule fraction was similar within each group: control ± Co^{2+} (Fig. 2, panels I and II) and reserpine ± Co^{2+} (Fig. 2, panels III and IV). In the presence of Co^{2+}, the K_{m} value determined for carboxypeptidase from the reserpine-treated group (0.136 ± 0.007 mM) was markedly lowered to one-fourth the K_{m} determined for control Co^{2+}-stimulated carboxypeptidase (0.447 ± 0.054 mM) (Figs. 2 and 3). When assayed in the absence of Co^{2+}, there was no significant difference in K_{m} values between control (0.576 ± 0.112 mM) and reserpine carboxypeptidase (0.727 ± 0.067). Thus, after exposure of chromaffin cells to reserpine, the carboxypeptidase-processing enzyme appears to display a greater affinity for substrate in the presence of Co^{2+}.
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FIG. 2. Lineweaver-Burk plots for carboxypeptidase-processing enzyme. Chromaffin granules from 72-h reserpine (1 × 10^{-6} M)-treated cells were purified on a linear sucrose gradient (see "Materials and Methods"). Granule fractions 5-6, 7-8, 9-10, and 11-12 were combined and pooled as fractions A, B, C, and D, respectively. Low M_r proteins (less than 10,000) were removed by dialysis by a factor of 40 times with an Amicon Centricon 10 microconcentrator. Carboxypeptidase activity in fractions A-D was measured by following the conversion of Ord-[Met]enkephalin-Arg to Ord-[Met]enkephalin in the presence of increasing concentrations of nonradioactive [Met]enkephalin-Arg. Lineweaver-Burk plots are shown for (I) control carboxypeptidase without Co^{2+}, fractions B-D; (II) control carboxypeptidase with 3 mM Co^{2+}, fractions B-D; (III) reserpine carboxypeptidase without Co^{2+}, fractions A-D; and (IV) reserpine carboxypeptidase with 3 mM Co^{2+}, fractions B-D.

![Graph showing Lineweaver-Burk plots](image-url)

FIG. 3. K_m values for carboxypeptidase-processing enzyme in chromaffin granules from control and reserpine-treated cells. The K_m values are illustrated as the mean ± S.E. of fractions A-D or B-D (as indicated in Fig. 2) from control and reserpine groups, measured in the absence (■) or presence (●) of 3 mM CoCl_2. * Statistically significant, p < 0.005, compared to control using Student's two-tailed t test.

![Graph showing K_m values](image-url)

Carboxypeptidase enzyme molecules in chromaffin cells after the drug treatments. Carboxypeptidase immunoreactivity was present in two subpopulations of granule fractions containing [Met]enkephalin and catecholamines (Fig. 1, panel I). Carboxypeptidase activity and immunoreactivity were found in the same region of the sucrose gradient. In the reserpine and forskolin group sucrose gradients (Fig. 1, panels II and III) carboxypeptidase immunoreactivity was present in fractions containing [Met]enkephalin and smaller amounts of immunoreactivity were also present in other fractions. Total carboxypeptidase immunoreactivity in granule fractions and in the entire sucrose gradient was not altered after 72 h of reserpine or forskolin treatment (Table V).

Immunoreactivity was also measured in whole chromaffin cells after 72 h of reserpine treatment (Table VI), because, in addition to the granules, the carboxypeptidase enzyme may be present in other organelles, such as rough endoplasmic reticulum and Golgi apparatus where it is synthesized and transported to new granules. Reserpine at final concentrations of 10^{-7} to 10^{-6} M had no effect on total chromaffin cell carboxypeptidase immunoreactivity.

DISCUSSION

Reserpine and forskolin are two agents which both increase chromaffin cell [Met]enkephalin levels 2-fold, but presumably, by different mechanisms. Reserpine is known to inhibit catecholamine reuptake into the chromaffin granules, resulting in a depletion of granular catecholamine stores (28).

![Table V](image-url)

Table V: Carboxypeptidase-processing enzyme immunoreactivity in granules isolated from reserpine- and forskolin-treated chromaffin cells

| CARBOXYPEPTIDASE IMMUNOREACTIVITY | GRANULE FRACTIONS (Nos. 5-12) | TOTAL IN SUCROSE GRADIENT |
|-----------------------------------|-----------------------------|---------------------------|
| CONTROL                           | 48.9                        | 98.1                      |
| Reserpine, 72 h                   | 56.1                        | 91.3                      |
| Forskolin, 72 h                   | 48.0                        | 95.8                      |
Forskolin is a diterpene (29) which elevates cellular cAMP levels through activation of adenylate cyclase. Such agents which act through different mechanisms to elevate cellular enkephalin peptide levels were used in this study as tools for understanding how the respective peptide precursor processing enzymes might be regulated during enkephalin biosynthesis.

After bovine adrenal medullary chromaffin cells were incubated with forskolin for 72 h, carboxypeptidase-processing enzyme specific activity and total activity in purified granules was increased significantly by 2 to 3 times over control. Forskolin appeared to induce a small increase in carboxypeptidase specific and total activities, but these alterations were not statistically significant. This suggests that cAMP is probably not an important molecule involved in the regulation of carboxypeptidase-processing enzyme activity in chromaffin cells. Thus, while both agents elevated [Met]enkephalin levels, only reserpine, and not forskolin, increased carboxypeptidase-processing enzyme activity. These experiments demonstrate that the carboxypeptidase-processing enzyme may or may not be increased during periods of elevated enkephalin biosynthesis.

The mechanism for the reserpine-elicted increase in carboxypeptidase activity was investigated. Reserpine had no direct effect on carboxypeptidase activity (in vitro) and the increase in enzyme activity was not due to changes in endogenous chromaffin granule substrate concentration, since the increased enzyme activity was retained after removal of low molecular weight peptides and proteins (M, less than 10,000). However, reserpine dramatically lowered the K for CG24-stimulated carboxypeptidase to one-fourth the value for CG24-stimulated carboxypeptidase from control cells. Furthermore, carboxypeptidase immunoactivity in the chromaffin cells was not altered by reserpine, indicating that the number of carboxypeptidase enzyme molecules remained constant. The increased carboxypeptidase enzyme activity was, therefore, not due to an elevation in the number of enzyme molecules. These data suggest that during reserpine treatment, inactive enzyme molecules may be converted to active molecules or that less active enzyme molecules have been converted to more active molecules which display a higher affinity for [Met]enkephalin-Arg-G substrate.

While reserpine appears to rapidly elevate cellular [Met] enkephalin levels, at least in part through selective stimulation of carboxypeptidase enzyme activity, forskolin must induce [Met]enkephalin levels by other means since it had no significant effect on carboxypeptidase activity. Other studies in this laboratory (31) have found that forskolin elicits a 3- to 5-fold increase in chromaffin cell preproenkephalin messenger RNA (mRNA) levels while exposure to reserpine reduced cellular mRNA levels. Further, forskolin was found to increase levels of high molecular weight enkephalin-containing peptides, but reserpine decreased their levels. These data suggest a scheme where forskolin increases cellular preproenkephalin mRNA to result in greater levels of enkephalin precursors, and reserpine enhances the processing of existing high molecular weight precursors.

Reserpine has been found to induce a more rapid increase in chromaffin cell enkephalin levels than forskolin (31). It is possible that the storage of high molecular weight enkephalin precursors and activation of the processing enzymes may allow the cell to rapidly synthesize enkephalins, whereas, more long term induction of peptide levels may involve elevated mRNA and its translation to proenkephalin. The carboxypeptidase-processing enzyme is one of several enzymes thought to be required for the complete conversion of proenkephalin to the small biologically active peptides. It will be interesting to determine if the "trypsin-like" endopeptidase and perhaps aminopeptidase-processing enzymes may be involved in the rapid reserpine induction of enkephalin levels. Such studies can also suggest whether the processing enzymes may be regulated together in a coordinate fashion.

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