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Enkephalin Biosynthesis in Adrenal Medulla

Modulation of Proenkephalin mRNA Content of Cultured Chromaffin Cells by 8-Bromo-Adenosine 3′,5′-Monophosphate

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SUMMARY

Incubation of primary cultures of chromaffin cells from bovine adrenal medulla with 8-bromo-adenosine 3′,5′-monophosphate (8-Br-cyclic AMP) resulted in an increase in proenkephalin mRNA content. The mRNA that increased was detected by hybridization analysis using a cDNA probe and migrated with an apparent size of approximately 1400 bases. The increase in proenkephalin mRNA following 8-Br-cyclic AMP treatment was apparent in 12 hr and continued over 2 days. Corresponding changes were detected in enkephalin-like immunoreactivity but with a 24-hr lag: the cellular content increased significantly after 2 days of treatment and continued to rise over the next 2 days, whereas changes in the amount released to the medium followed the same time course. Dose-response curves for the increase in the content of proenkephalin mRNA and of enkephalin-containing peptides were essentially identical. Chromatographic characterization of the enkephalin-like peptides demonstrated that 8-Br-cyclic AMP increased both the high molecular weight fraction and the low molecular weight fraction, which was shown by high-pressure liquid chromatography to contain Met^5-enkephalin, Leu^5-enkephalin, and Met^5-enkephalin-Arg^6-Phe^7. Previous results in chromaffin cells have demonstrated that the synthesis of tyrosine hydroxylase is also regulated by cyclic AMP, with a similar time course. These results therefore suggest the possibility of coordinate regulation by cyclic AMP of the expression of the cotransmitters, catecholamines and enkephalin peptides, in the adrenal medulla.

INTRODUCTION

The adrenal medulla contains not only catecholamines but also opioid peptides, most of which derive from proenkephalin (referred to as proenkephalin A by some authors) (1–5). Both high and low molecular weight forms of enkephalin-containing peptides have been found in adrenal medulla (2, 3, 6, 7) and in the blood effluent from the adrenal during splanchnic nerve stimulation (6, 8). Recent evidence suggests that the catecholamines and enkephalin peptides coexist in the same granules and can be released simultaneously either by splanchnic nerve stimulation (2, 8) or by stimulation of nicotinic receptors *in vivo* (9) and *in vitro* (6, 7). Transsynaptic induction of tyrosine hydroxylase in the adrenal medulla occurs in response to sustained splanchnic nerve activity (10, 11) via stimulation of nicotinic receptors and a rise in cyclic AMP (12). A rise in the number of enzyme molecules, maximal by 48 hr, can be elicited in cultured chromaffin cells by a 4-hr treatment with 8-Br-cyclic AMP[2] (13). In the present report, we have utilized a cDNA probe for proenkephalin (14) to measure the content of proenkephalin mRNA in cultured chromaffin cells in order to determine whether 8-Br-cyclic AMP can also increase the biosynthesis of the enkephalin peptides derived from proenkephalin (14–16).

MATERIALS AND METHODS

Chromaffin cell preparation and culture. Chromaffin cells were prepared from bovine adrenal medulla as previously described (17). Cells were plated in 24-well plates or in 60- or 100-mm Petri dishes at a density of 1 to 1.2 × 10^5 cells/cm^2 in Dulbecco's modified Eagle's medium (pH 7.4) containing 10% calf serum, 5 mM Hepes, penicillin (100 units/ml), streptomycin (100 μg/ml), gentamycin (40 μg/ml),

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[2] The abbreviations used are: 8-Br-cyclic AMP, 8-bromo-adenosine 3′,5′-monophosphate; 8-Br-cyclic GMP, 8-bromo-guanosine 3′,5′-monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; LMW, low molecular weight; HMW, high molecular weight; HPLC, high-pressure liquid chromatography.

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nystatin (50 units/ml), and 5–15 μM 5-fluorodeoxyuridine, and cultured for 3–10 days prior to use. Tissue culture reagents were obtained from Grand Island Biological Company (Grand Island, N.Y.); Hepes, 5-fluorodeoxyuridine, 8-Br-cyclic AMP, and 8-Br-cyclic GMP from Sigma Chemical Company (St. Louis, Mo.); Met^5-enkephalin from Peninsula Laboratories (Belmont, Calif.).

Preparation and measurement of RNA. Gel blot analysis of RNA was carried out with approximately 10^7 cells, which were scraped from the dishes in PBS, pelleted by centrifugation (500 × *g* for 5 min), rinsed twice with PBS, homogenized in 0.5 ml of 5 M guanidine thiocyanate/100 mM Tris (pH 7.6)/10 mM EDTA/5% (v/v) β-mercaptoethanol (18), and taken up to 8 ml total volume with the same buffer. The 8,000 × *g* supernatant was layered onto 4 ml of 5.7 M cesium chloride (Bethesda Research Laboratories, Bethesda, Md.)/25 mM sodium citrate-HCl (pH 7), and centrifuged for 14–16 hr at 150,000 × *g* (Beckman L2-65B; SW 40 rotor). The RNA pellet was analyzed by RNA gel blot as described previously (19), using the Hinc II 918 bp probe prepared from plasmid pHPE-9, a generous gift from Drs. Michael Comb and Edward Herbert (14).

For dot-blot hybridization analysis (20), 2 to 3 × 10^6 cells were scraped up, pelleted by centrifugation at 500 × *g* for 5 min, rinsed once with PBS, and homogenized in 200 μl of 10 mM Tris (pH 7.5)/5 mM EDTA/1% sodium dodecylsulfate/proteinase K (65 μg/ml). After incubation for 90 min at 42°, phenylmethylsulfonyl fluoride was added (0.03% w/v), and the samples were extracted twice with phenol/chloroform (1:1) and once with chloroform. The nucleic acids were precipitated with ethanol, dissolved in 50 μl of 10 mM Tris (pH 7.5)/5 mM EDTA, and 5 μl were used to measure total nucleic acids. The remainder of the sample was heated for 15 min at 60° with 30 μl of 3 M NaCl/0.34 M sodium citrate (pH 7) and 20 μl of formaldehyde (37%) prior to spotting on nitrocellulose paper using a Hybri-Dot manifold (Bethesda Research Laboratories). Hybridization and quantitative analysis by densitometric scanning were carried out as previously described (19). The dot-blot signal is linearly proportional to the amount of nucleic acids spotted over a range of approximately 0.5–5 μg. We spotted three dilutions of every sample chosen to contain 1–3 μg so that the signal would fall within the linear portion of the signal.

Quantitation of total nucleic acids was performed by spotting 5 μl of the redissolved ethanol precipitate onto a 2% agarose plate containing 150 mM NaCl-ethidium bromide (0.5 μg/ml). Quantities were determined by densitometric scan (19) of a photographic negative of the plate: tRNA was used as a standard. The density was linear over the range from 20 ng–1 μg of nucleic acids per spot. Recovery of total nucleic acids per sample was quite consistent both within an experiment and between experiments (variation within 20%). Average recovery was approximately 2 pg of nucleic acids per cell. Values for proenkephalin mRNA are presented in units which are defined as the peak area for proenkephalin probe hybridization per peak area for total nucleic acids per sample. These units are dependent on a number of factors, such as the amount of RNA per sample, the specific activity of the nick-translated probe, and the number of days of exposure of the autoradiogram. The dot-blot technique thus provides a relative quantitation within an experiment but one cannot directly compare the absolute value of units from one experiment to another. However, the relative increase in proenkephalin mRNA was consistent from one experiment to another (2 to 3-fold).

Radioimmunoassay of enkephalin-like peptides. Cells (2.5 × 10^5) were homogenized in 0.5 ml of 1 N acetic acid. An aliquot of the homogenate was assayed for protein (21): the rest was centrifuged at 15,000 × *g* for 2.5 min. The supernatant was lyophilized, taken up in 500 μl of 0.1 M $NaPO_4$ (pH 7.5), and 30–50 μl were used per assay. To assay medium enkephalin-like immunoreactivity, two 400-μl aliquots of the medium (total volume for incubation, 1 ml) were lyophilized, reconstituted in 100 μl of 0.1 M $NaPO_4$ (pH 7.5), and used directly for assay. Medium never exposed to cells was lyophilized, reconstituted, and assayed to provide a blank. The recovery of added standards ranged from 90% to 95%. The immunoassay was carried out in a total volume of 300 μl, with ^{3}H-Met^5-enkephalin (10,000 dpm) (ICN, Irvine, Calif.; 30 Ci/mmol) per tube. The assay was linear for Met-enkephalin standards of 0.1–1.6 ng/tube. The antibody used was raised against Met^5-enkephalin conjugated to succinylated hemocyanin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Relative to Met-enkephalin (100%), the cross-reactivities were as follows: Leu-enkephalin (40%); Arg^1-Met^5-enkephalin (39%); Met^5-enkephalin-Arg^6-Phe^7 (5%); des-Tyr-Met-enkephalin (<0.01%).

RESULTS

Exposure of primary cultures of bovine chromaffin cells to 1 mM 8-Br-cyclic AMP for 1 day resulted in a 70% increase of proenkephalin mRNA detectable by dot-blot hybridization (Fig. 1; Table 1). There was a corresponding increase in the cellular content of enkephalin-like immunoactivity during 3 days of treatment with 8-Br-cyclic AMP (Table 1). These changes were not replicated by 8-Br-cyclic GMP under similar conditions.

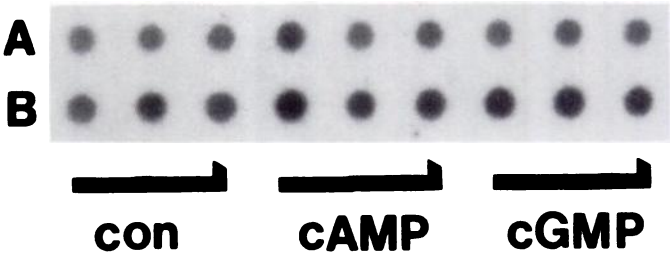

FIG. 1. *Dot-blot hybridization analysis of proenkephalin mRNA content of chromaffin cells*

Cells (3 × 10^6/60-mm dish) were cultured for 1 day without or with 1 mM 8-Br-cyclic AMP or 1 mM 8-Br-cyclic GMP. RNA samples were prepared as described under Materials and Methods. For each sample, 15 μl (A), 25 μl (B), and 40 μl (data not shown) were spotted on nitrocellulose (see Materials and Methods). Three dishes of cells were analyzed individually for control (*con*), for 8-Br-cyclic AMP (*cAMP*), and for 8-Br-cyclic GMP (*cGMP*). Shown is the autoradiogram obtained after hybridization of the dot-blot. Each spot was scanned, and the values from the three volumes (15 μl, 25 μl, and 40 μl) per sample were averaged and corrected for the content of total RNA. The results are presented as units of proenkephalin RNA in Table 1.

TABLE 1

Effect of 8-Br-cyclic AMP and 8-Br-cyclic GMP on proenkephalin mRNA content and enkephalin-like peptide content of chromaffin cells

Chromaffin cells were incubated for 1 day with or without 1 mM 8-Br-cyclic AMP or 8-Br-cyclic GMP for proenkephalin mRNA and for 3 days for enkephalin-like immunoreactivity. Proenkephalin mRNA (Proenk mRNA), presented as arbitrary units as described under Materials and Methods, was extracted from 3 × 10^6 cells/sample (60-mm Petri dish) and measured by dot-blot hybridization at three dilutions of each sample (mean ± standard error of the mean; $n = 3$ dishes). Cell enkephalin-like immunoactivity was extracted from 2.5 to 3.0 × 10^5 cells grown in 24-well Costar clusters (mean ± standard error of the mean; $n = 4$). The experiment was repeated three times. The percentage increases in Proenk mRNA due to 8-Br-cyclic AMP in the other experiments were 80%, 162%, and 190%. 8-Br-cyclic GMP never had a significant effect.

Treatment	Proenk mRNA	Enkephalin-like immunoactivity
	units	*ng/mg protein*
Control	1.59 ± 0.14	109 ± 2
8-Br-cyclic AMP	2.74 ± 0.14[a]	224 ± 13[a]
8-Br-cyclic GMP	1.77 ± 0.13	108 ± 4

[a] $p < 0.005$ versus control.

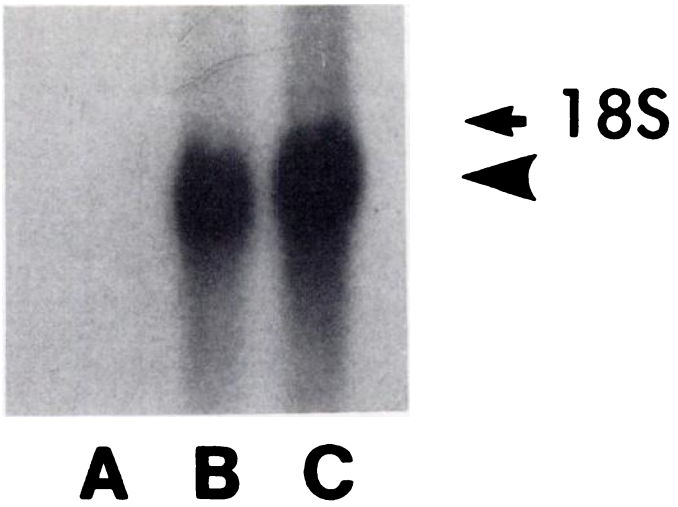

FIG. 2. *RNA gel blot analysis of chromaffin cell RNA*

Cells (2.4×10^7 cells/sample) were incubated for 1 day with (lane *C*) or without (Lane *B*) 1 mM 8-Br-cyclic AMP. Total RNA was prepared and fractionated on a 1.1% agarose-formaldehyde gel, blotted to nitrocellulose, and hybridized as described under Materials and Methods. Similar amounts of total RNA from control (48 μg) and 8-Br-cyclic AMP-treated (45 μg) cells were applied to the gel, and transfer to nitrocellulose was complete as judged by loss of ethidium bromide fluorescence from the gel. The position of proenkephalin-positive RNA is shown by the *arrowhead*. No hybridization was detected for 10 μg of poly(A)$^+$-RNA from rat liver (lane *A*), used as a negative control. Ribosomal RNAs (18 S and 28 S) were used as size markers.

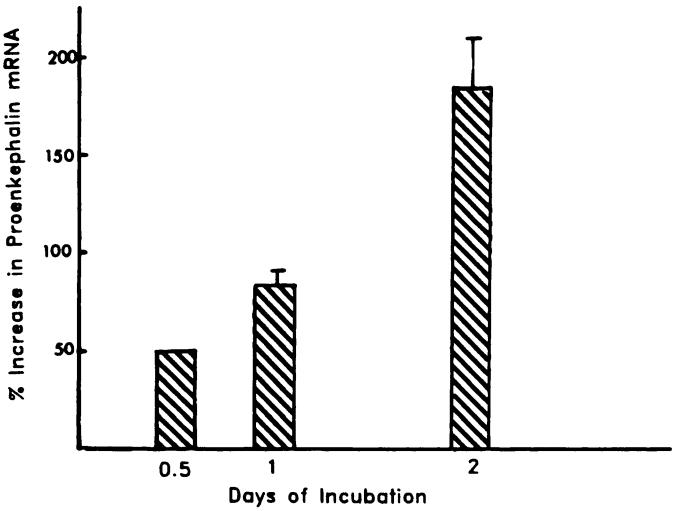

FIG. 3. *Time course of effect of 8-Br-cyclic AMP on proenkephalin mRNA*

Cells (3×10^6/60-mm dish) were incubated for 0.5–2 days with or without 1 mM 8-Br-cyclic AMP. Proenkephalin mRNA was measured by dot-blot hybridization. The results are presented as the percentage increase over control (no 8-Br-cyclic AMP) and represent the mean ± standard error of the mean for three experiments (the 0.5-day point was done only twice). The control content of proenkephalin mRNA did not change during the course of the incubation (zero time, 0.70 unit; 0.5 day, 0.78; 1 day, 0.99; 2 days, 0.88).

When the total RNA extracted from chromaffin cells was analyzed by formaldehyde-agarose gel and blot, a species of RNA (approximately 1400 bases) was detected that hybridized with the proenkephalin cDNA probe and was similar to those previously observed (14, 22) (Fig. 2).

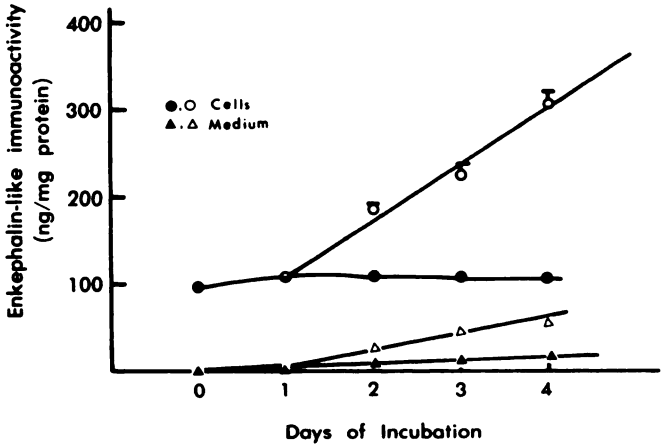

FIG. 4. *Time course of effect of 8-Br-cyclic AMP on enkephalin-like immunoactivity*

Cells (2.5×10^5/well) were incubated for 0–4 days with or without 1 mM 8-Br-cyclic AMP. Enkephalin-like immunoactivity was assayed in the cells (control, ●; 8-Br-cyclic AMP, ○) and in the medium (control ▲; 8-Br-cyclic AMP, △). Each point represents the mean from four wells. Points without bars indicating standard error of the mean had a standard error of the mean smaller than the symbol. The experiment was repeated twice with comparable increases in enkephalin content (at 2 days, 90%, 83%; at 3 days, 129%, 183%; at 4 days, 211%, 250%).

The size of the RNA was calculated relative to the migration of 18 S and 28 S ribosomal RNA markers. Treatment of the cells with 1 mM 8-Br-cyclic AMP for 1 day resulted in a 2-fold increase in the amount of this RNA species (as determined by densitometric scanning of the autoradiogram), with no change in total RNA or protein content or cell number elicited by 8-Br-cyclic AMP treatment.

The time course of the increase in proenkephalin mRNA caused by 8-Br-cyclic AMP is shown in Fig. 3. There was a 50% increase in proenkephalin mRNA within 12 hr of treatment: the increase continued essentially linearly over 2 days of treatment (Fig. 3), whereas the content of proenkephalin mRNA in control cells remained constant (data not shown). Both cell protein and total RNA remained constant in control as well as in treated cells over the 2 days of incubation. In contrast, the increase in enkephalin-like peptides could not be detected until 48 hr after addition of 8-Br-cyclic AMP; this increase continued in an essentially linear fashion for the next 48 hr (Fig. 4). The content of enkephalin-like peptides in control cells remained unchanged during 4 days of culture. Control cells released enkephalin-like peptides at a low but constant rate: 8-Br-cyclic AMP treatment accelerated this rate but only after 2 days of treatment, at the time when the cell content of enkephalin-like peptides was increased (Fig. 4).

There is a good correlation between the dose-response relationships for the increase in proenkephalin mRNA (Fig. 5A) and the increase in cellular enkephalin-like immunoactivity (Fig. 5B) as a function of the concentration of 8-Br-cyclic AMP. Chromatographic characterization of the enkephalin-like immunoreactivity by passage of cell extracts over a Bio-Gel P-2 column showed the presence of two immunoactive peaks (Fig. 6A). The LMW fraction was further separated by HPLC on a

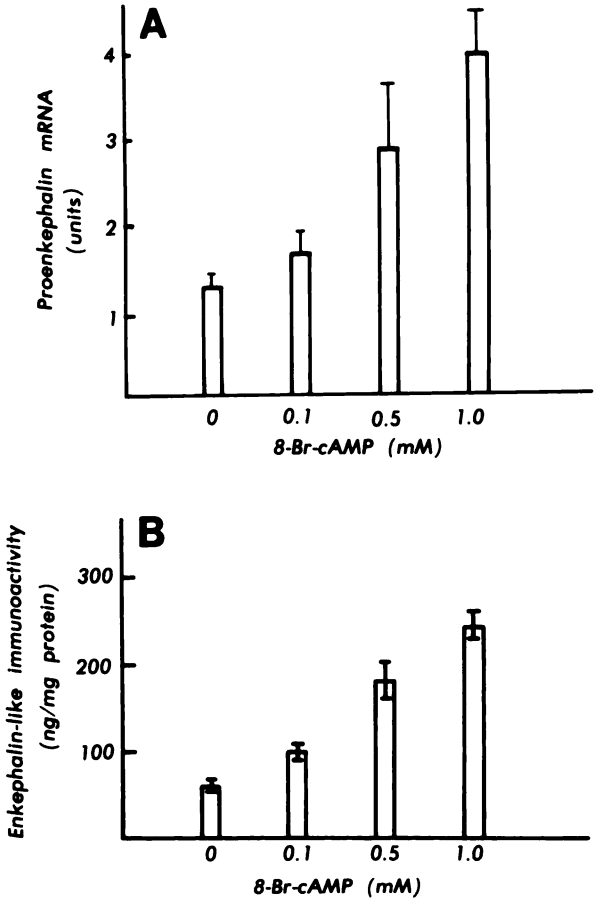

FIG. 5. *Dose response for effects of 8-Br-cyclic AMP on proenkephalin mRNA and enkephalin-like immunoactivity*

A. Cells (3×10^6/60-mm dish) were incubated for 1 day with the indicated concentrations of 8-Br-cyclic AMP. Proenkephalin mRNA was measured by dot-blot hybridization. Values are means ± standard error of the mean ($n = 3$ dishes). The experiment was repeated twice with comparable results (0.1 mM, 75% increase, 40%; 0.3 mM, 162%; 1 mM, 275%, 198%).

B. Cells (3×10^5/well) were incubated for 3 days with the indicated concentrations of 8-Br-cyclic AMP. Cell enkephalin-like immunoactivity was determined, and the data are presented as means ± standard error of the mean ($n = 4$ wells). The experiment was repeated twice with similar results.

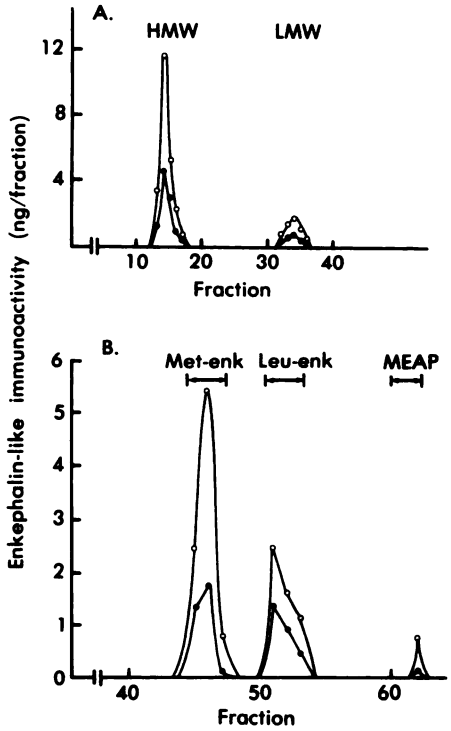

FIG. 6. *Chromatographic characterization of chromaffin cell enkephalin-like immunoactivity*

A. The acetic acid extract of 1.2×10^6 control cells (●) or 1.2×10^6 cells treated with 1 mM 8-Br-cyclic AMP for 3 days (○) was applied to a Bio-Gel P-2 column (0.9 × 60 cm) and eluted with 1 N acetic acid. Enkephalin-like immunoactivity was assayed in the lyophilized fractions (1 ml/fraction). The void volume represents fractions 13–16.

B. The LMW peak (fractions 32–36 from Bio-Gel P-2 column) of the control (●) or 8-Br-cyclic AMP-treated (○) sample was separated by HPLC on a Bio-Sil ODS-10 column. The mobile phase was 0.1% trifluoroacetic acid in water with a gradient of 0–60% acetonitrile for 2 hr. Fractions were 1 ml and the flow rate was 1.0 ml/min. Enkephalin-like immunoactivity was assayed in the lyophilized fractions. The bars indicate the elution positions of authentic Met^5-enkephalin (*Met-enk*), Leu^5-enkephalin (*Leu-enk*) and Met^5-enkephalin-Arg^6-Phe^7 (*MEAP*). The values were not corrected for differences in cross-reactivity to the Met-enk antiserum.

reverse-phase column using a 0–60% acetonitrile gradient (Fig. 6B). Immunoactivity was found to elute in three separate peaks, corresponding approximately to the elution positions of authentic Met^5-enkephalin, Leu^5-enkephalin, and Met^5-enkephalin-Arg^6-Phe^7. However, the broadness of the immunoreactivity peaks suggests that other peptides may be coeluting at these positions.

The HMW fraction from the Bio-Gel P-2 column (Fig. 6A) was further analyzed after digestion with trypsin and carboxypeptidase B (Table 2). The enzyme treatment increased the Met-enkephalin immunoactivity of the control HMW fraction about 6-fold, whereas the immunoreactivity of the HMW fraction from cells treated with 8-Br-cyclic AMP increased 9.5-fold. The cells thus contained much more HMW enkephalin-containing peptides than LMW, and treatment of the cells with 1 mM 8-Br-cyclic AMP for 3 days resulted in a somewhat larger increase in the HMW fraction (Table 2) than in the LMW fraction (Fig. 6).

DISCUSSION

In this paper we have demonstrated that 8-Br-cyclic AMP stimulates the expression of proenkephalin mRNA in primary cultures of bovine adrenal chromaffin cells and that this increase is followed by an increase in the cell content of enkephalin-containing peptides. Treatment of the cells with 8-Br-cyclic AMP causes a significant increase in proenkephalin mRNA within 12 hr: this mRNA continues to increase, essentially linearly, over a 2-day incubation, whereas the control mRNA remains constant over the 2 days. No change is detectable in the cell content of enkephalin-like immunoactivity after 24 hr of treatment, but by 48 hr there is a significant increase which also continues linearly for 3 days. The results thus suggest that 8-Br-cyclic AMP increases transcription of the gene for proenkephalin, resulting in the appearance of more mRNA, although we cannot at pres-

TABLE 2

Effect of treatment with trypsin and carboxypeptidase B on Met-enkephalin immunoactivity of Bio-Gel P-2 HMW fractions

Cells (1.2×10^6) were incubated for 3 days with or without 1 mM 8-Br-cyclic AMP. After homogenization in 1 N acetic acid, centrifugation, and passage of the supernatant over a Bio-Gel P-2 column (as in Fig. 6A), the HMW fractions were lyophilized, reconstituted in 0.1 M Tris (pH 7.5), and incubated alone or with TPCK-trypsin (10 μg/ml) (Sigma) for 2.5 hr at 37°, followed by carboxypeptidase B (1 μg/ml) (Worthington) for 30 min at 37°. The samples were then boiled for 5 min and assayed for Met-enkephalin immunoactivity. Recovery of protein and HMW activity from Bio-Gel P-2 is routinely 80–90% of the sample applied: the values shown have not been corrected for that factor, but have been corrected for the recovery (50%) of standard Met-enkephalin carried through the complete procedure.

Treatment	Met-enkephalin Equivalents	
	Control HMW	8-Br-cyclic AMP HMW
	ng	
None	11.0	19.9
Trypsin + carboxypeptidase B	65.3	189

ent rule out the possibility of a block in proenkephalin mRNA degradation. The mRNA which accumulates as a result of 8-Br-cyclic AMP treatment is translated into the precursor proenkephalin, which in turn is processed to the smaller enkephalin-containing peptides over the next 24 hr. The characterization of the enkephalin-like immunoactivity in control versus 8-Br-cyclic AMP-treated cells supports this interpretation, since 8-Br-cyclic AMP increased both the HMW and LMW enkephalin-like fractions. The HMW fraction was shown to consist of large enkephalin-containing peptides, since the enkephalin could be released by trypsin-carboxypeptidase B treatment (Table 2). The LMW fraction probably consists of authentic Met-enkephalin, Leu-enkephalin, and Met^5-enkephalin-Arg^6-Phe^7, as well as other small enkephalin-containing peptides. The results suggest that, as more precursor protein is synthesized, the processing enzyme(s) converts it to the smaller molecular weight peptides: the rate-limiting step appears to be synthesis of the mRNA. That more enkephalin is synthesized is confirmed by the increase of enkephalin-like immunoreactivity in the medium with time.

The presence of two (or more) transmitters/modulators in a single neuron has become a widespread observation. The presence of the catecholamines and the enkephalin peptides in the same vesicle of adrenal medullary cells is one example (1–4, 23). Little is known yet of how coexpression of two transmitters can be regulated, but the bovine adrenal chromaffin cells offer a model system in which to investigate such regulation. Previous work has shown that a long-term increase in cyclic AMP leads to an induction of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis, in the adrenal medulla both *in vivo* (12) and *in vitro* (13). In this paper we show that treatment with cyclic AMP also stimulates synthesis of the enkephalin-containing peptides through an increase of proenkephalin mRNA content. The results thus suggest the possibiity of coordinate regulation of expression of the cotransmitters of the adrenal medulla chromaffin cells, presumably through an activation of cyclic AMP-dependent protein kinase. Wilson *et al.* (24) have shown that reserpine, which also increases both opiate peptides and tyrosine hydroxylase, depletes catecholamine stores, but the effect of 8-Br-cyclic AMP on catecholamine content is unknown. Since cyclic AMP regulates the expression of both tyrosine hydroxylase, a soluble enzyme in the cytosol, and the polypeptide proenkephalin, which is stored in chromaffin granules, the regulation may occur at the level of transcription, independent of the storage site of the product. More definitive evidence as to whether the regulatory mechanisms for proenkephalin and tyrosine hydroxylase are similar requires comparable studies on the expression of tyrosine hydroxylase mRNA using a cDNA probe for the gene for this enzyme. Although we know that cyclic AMP increases the synthesis of tyrosine hydroxylase molecules (9), the ability to measure an increase of the specific mRNA is essential to studies on the mechanism whereby gene expression is regulated.

Cyclic AMP is known to regulate the expression of mRNA for many proteins, but a complete understanding of its mechanism of action is not yet in hand. In all cells, including chromaffin cells, a receptor-mediated increase of cyclic AMP leads to activation of the cytosolic cyclic AMP-dependent protein kinase(s), following which a translocation of catalytic subunits of the protein kinase into the nucleus of the cell has been observed in several tissues (25–28). Blockade of this translocation with colchicine or vinblastine prevents the subsequent responses to cyclic AMP (13, 28). The precise nature of the substrate or substrates for the translocated kinase and the exact mechanisms by which that phosphorylation can regulate specific gene expression remain to be established, but the chromaffin cells and the use of a cDNA probe to measure specific mRNAs directly offer an excellent model system in which to investigate these problems further.

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