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PUTATIVE ENKEPHALIN PRECURSORS IN BOVINE ADRENAL MEDULLA

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SUMMARY

Extracts from bovine adrenal medulla and adrenal medullary chromaffin granules were found to contain three proteins, 20,000, 10,000 and 5,000 approximate molecular weights which yield tryptic peptides with opioid activity. The opioid activity of these peptides was demonstrated with a radioreceptor assay and two radioimmunoassays. The three proteins yield the same active peptides all of which are chromatographically distinct from the tryptic opioid nonapeptide β-LPH 61-69, generated by trypsin digestion of pituitary endorphins and their precursors. Furthermore, these endorphins and their precursors do not appear to be present in the adrenal medulla. These findings further support the hypothesis that the enkephalin biosynthetic pathway is distinct from that leading to β-endorphin.

The enkephalins from the brain were the first opioid peptides to be isolated and characterized (1). The Met-enkephalin sequence was shown to be the same as the 61-65 sequence of the pituitary peptide β-lipotropin. It was therefore considered that β-lipotropin was a precursor of enkephalins. However, it has not been possible to show that Met-enkephalin is biosynthetically derived from β-endorphin. In fact, several lines of evidence argue against such a pathway. Hypophysectomy has been found to have no effect on the enkephalin content of the brain (2, 3). In the striatum of several species, where the enkephalin levels are the highest, no β-endorphin or its precursors could be detected (2, 3). Immunofluorescence studies have also shown individual neurons containing either enkephalin or β-endorphin, but not both (4). In addition, a possible Leu-enkephalin precursor termed α-neo endorphin has been isolated from porcine hypothalamus and, except for the enkephalin sequence, shown to be unrelated to β-endorphin (5).

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These findings support a separate biosynthetic pathway for the enkephalins not involving β-endorphin.

Immunocytochemical observations (6) had shown the presence of relatively large amounts of enkephalin immunoreactive material in the adrenal medulla from several species. We, therefore, selected to study the enkephalin biosynthetic pathway in this organ. In this communication we will show that high molecular weight proteins containing opioid peptides are present in high concentration in the chromaffin granules of the bovine adrenal medulla. Furthermore, these opioid peptides differ structurally from those found in the pituitary gland.

MATERIALS AND METHODS

Bovine adrenal glands were obtained from a local slaughterhouse within 30 min after death and stored on ice until used. The medullas were dissected out and chromaffin granules prepared by a modification (7) of the procedure of Smith and Winkler (8). The isolated chromaffin granules or whole adrenal medulla were homogenized in an acid solution with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The procedures for extraction (see Fig. 1 legend), Sephadex column elution, and trypsin digestion have been described previously (3). The radioreceptor assay employed neuroblastoma-glioma cells (9). Radioimmunoassays were performed using a C-terminal directed Leu-enkephalin antibody and an N-terminal directed Met-enkephalin antibody. The characteristics of the C-terminal Leu^5-enkephalin antiserum (RB 92) have been described (2). This antiserum has a cross-reactivity of 3% with Met^5-enkephalin. The N-terminus Met^5-enkephalin antiserum (JR235) cross-reacts 41% with Leu^5-enkephalin, 100% with β-LPH 61-69 and 1.4% with β-endorphin. It does not cross react with any des-Tyr peptide.

High performance liquid chromatography was carried out using Lichrosorb RP-18 columns (10 μ resin, 4.6 x 250 mm, EM Hibar II, Ace Scientific, Edison, NJ). Gradients of 1-propanol in 0.5 formic acid-0.4 M pyridine pH 4.0 were used to elute peptides (10). All solvents were distilled over ninhydrin. An automated fluorescent peptide analyzer was used to monitor column effluents (11). Further experimental details are provided in the text and figure legends.

RESULTS AND DISCUSSION

Chromatography of extracts of bovine adrenal medulla on Sephadex G-100 gave five peaks of radioreceptor active material (Fig. 1). These correspond in molecular weight to approximately 20,000 (I); 10,000 (II); 5,000 (III); 2,000 (IV) and < 1,000 (V). The receptor activity eluting

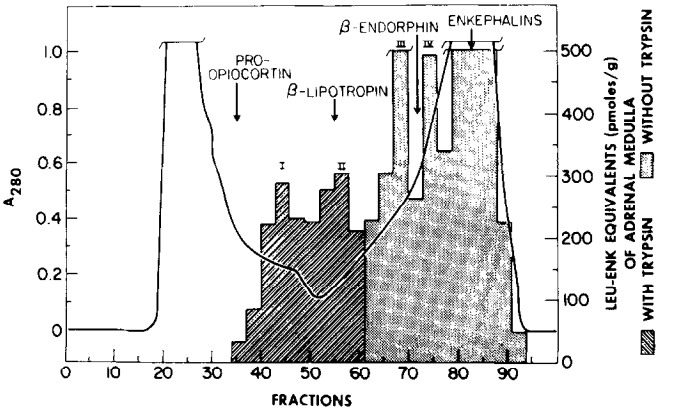

Fig. 1 Gel filtration of an acid extract (1 M acetic acid, 20 mM HCl, 10:1 v/w) of adrenal medulla (6.35 g) on a Sephadex G-100 column (5 x 100 cm). Aliquots (0.5 ml) of fractions (6 ml) 10 - 61 were treated with trypsin and assayed for opioid activity. The aliquots of fractions 62-99 were assayed directly for opioid activity. The solid line is the UV trace and the cross-hatched areas indicate radioreceptor active material.

in the regions of peaks I and II was detectable only when the material was digested with trypsin prior to assay.

To determine if peaks I and II are structurally related to β-endorphin or its precursors, trypsin digests of these peaks were chromatographed on a Lichrosorb RP-18 column. This method has been used previously (12) to show that the endorphin precursors all yield the same opioid active tryptic nonapeptide, β-LPH 61-69. The chromatograms of digests of peaks I and II showed the same pattern of active fragments. However, none of these active tryptic peptides corresponded to the elution position of β-LPH 61-69 (Fig. 2). Although an opioid active fragment elutes near the β-LPH 61-69 position in this chromatography, other chromatographic systems (data not shown) clearly separate this fragment from β-LPH 61-69. These findings imply that peaks I and II are structurally related to each other in the opioid peptide region and are different from the precursor peptides found in the pituitary.

Although the molecular weight of the peptide in Peak III was comparable to that of β-endorphin, when peak III (native, undigested material)

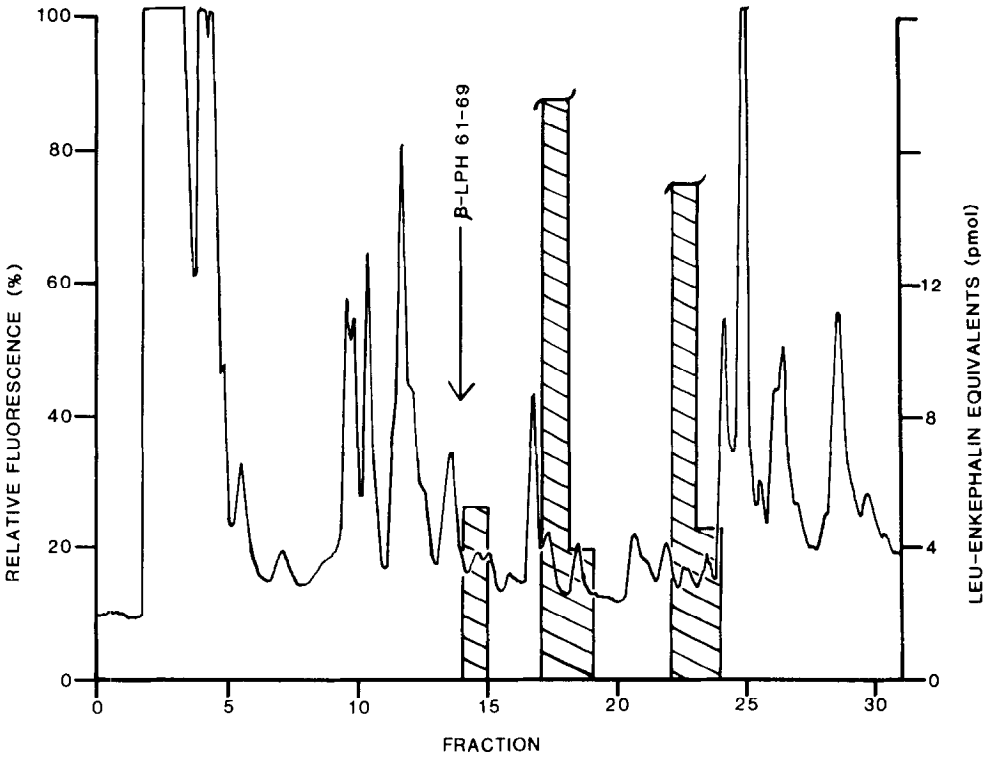

Fig. 2 Trypsin treated peak I (from G-100) was applied to a Lichrosorb RP-18 column (4.6 x 250 mm). The column was eluted at 16 ml/hr with 0.5 M formic acid - 0.4 M pyridine (pH 4.0) using a linear gradient of 1-propanol from 6% to 14%. A portion (5%) of the column effluent was diverted to the fluorescamine monitoring system. Fractions were collected, aliquots were lyophilized and assayed for opioid activity. The solid line is the fluorescence detection system trace and the crosshatched areas indicate radioreceptor active material.

was chromatographed on the RP-18 column, opioid active material eluted after the calibrated position of β-endorphin indicating that peak III is not β-endorphin (results not shown). In addition, a tryptic digest of Peak III, when chromatographed on the RP-18 column, gave active peptides which eluted in the same positions as the active tryptic peptides from peaks I and II.

Chromaffin granules from the bovine adrenal medulla were lysed and subjected to the same chromatography on Sephadex G-100 as described above. The same five peaks of activity were observed (results not shown). Opioid activity was quantitatively determined by the radioreceptor assay with

TABLE I

RADIORECEPTOR ASSAY OF PEAKS FROM SEPHADEX G-100 (FIG. 1)

Peak	Trypsin Digested	Native
Adrenal Medulla [1,3]		
I	6.9	0.6
II	12.5	N.D.
III	10.9	1.9
Chromaffin Granules [2,3]		
I	0.8	0.2
II	4.7	0.7
III	4.4	2.4

[1]These values are for 10 g wet weight of bovine adrenal medulla.

[2]These values are for 10 g wet weight of bovine adrenal medulla with a yield of 35 mg of chromaffin granules per gram of medulla.

[3]The values are expressed as nmoles/peak and N.D. indicates values below 0.05 nmole per 10 g of tissue.

and without trypsin digestion for each of the three pooled regions above 3,000 daltons from both whole medulla and granules. The results (Table I) show that the relative amounts of activity in peaks II and III, are the same from both sources. Although Smith and Winkler (8) obtained a 70% recovery of granules as determined by catecholamine assay with their procedure, our modified procedure gave a 40% recovery of peaks II and III in the granules. This lower recovery may be due to the homogenization conditions which we employed. Assuming this 40% recovery reflects the recovery of chromaffin granules, it suggests that most of the activity in peaks II and III is localized in the granules. The amount of peak I relative to the other peaks was found to be more than 3-fold higher in the whole medulla than in the granules. This may indicate uptake of the largest peptide by the granules followed by proteolytic processing within the granule. Since peaks I, II, and III all showed considerably greater activity in the radioreceptor assay when predigested with trypsin, it would appear that the active peptide sequence is contained within a larger peptide. From studies with the synthetic opioid peptides it is known that the N-terminal tyrosine cannot be blocked with another amino acid without

TABLE II

RADIOIMMUNOASSAYS OF PEAKS FROM SEPHADEX G-100 (FIG. 1)

Peak	N-Terminal [1,2] Trypsin Digested	N-Terminal [1,2] Native	C-Terminal [2,3] Trypsin Digested	C-Terminal [2,3] Native
Adrenal Medulla				
I	27.0	N.D.	0.5	N.D.
II	25.1	N.D.	0.5	N.D.
III	19.2	0.6	0.3	N.D.
Chromaffin Granules				
I	4.12	0.5	0.1	N.D.
II	10.3	0.6	0.3	N.D.
III	10.1	0.2	0.6	N.D.

[1]N-terminal directed Met-enkephalin antiserum has a 41% cross-reactivity with Leu-enkephalin.

[2]These values are expressed as nmoles/peak with tissue amounts identical to Table I and the values denoted by N.D. were not detectable being below 0.05 nmole per 10 g of tissue.

[3]C-terminal directed Leu-enkephalin antiserum has a 3% cross-reactivity with Met-enkephalin.

loss of activity. The opioid peptide prior to trypsin digestion, therefore, must have an extension at its amino terminus.

The three peaks were also characterized by radioimmunoassay using two different antisera, one directed toward the N-terminus of Met-enkephalin and one directed toward the C-terminus of Leu-enkephalin. Aliquots of peaks I, II, and III from granules and medulla were assayed before and after digestion with trypsin. As shown in Table II the tryptic peptides from peaks I, II, and III, interacted well with the N-terminal specific antiserum and gave values of the same order of magnitude as the radioreceptor assay. Digestion with trypsin also yielded peptides with immunoreactivity using the C-terminus specific antibody. These findings, that an immunoreactive peptide is present only after trypsin digestion of peaks I, II, and III, are in accord with those obtained with the radioreceptor assay. They indicate that an active peptide is released from a larger peptide by the trypsin digestion and that the larger peptides are not themselves active. The active tryptic fragment must have

peptide extensions at both the N- and C-terminuses since both antibodies detect immunoreactive material only after trypsin digestion.

As in the striatum (3) no β-endorphin, β-lipotropin or pro-opiocortin could be detected in the adrenal medulla, indicating that these are not the enkephalin precursors. In addition, the putative precursors found in the adrenal did not yield the same active tryptic peptide, β-LPH 61-69, obtained with β-endorphin or its precursors. The biosynthetic pathway leading to the enkephalins may be even more intricate as we have recently found several active opioid peptides of molecular weight less than 1,500 both in the adrenal medulla and in the striatum (7). In a recent abstract Yang et al. (13) reported the presence of enkephalin and enkephalin precursors unrelated to β-endorphin in the adrenal medulla.

The demonstration by chemical methods that the putative enkephalin precursors are localized in the chromaffin granules of the adrenal medulla is in accord with the immunocytochemical findings of Shultzberg, et al. (6). It may indicate that the enkephalins and their precursors are released along with catecholamines during adrenal medullary stimulation. The physiologic role of these opioid peptides in the adrenal gland is at present not known.

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