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Isolation of the opioid heptapeptide Met-enkephalin[Arg^6, Phe^7] from bovine adrenal medullary granules and striatum

(chromaffin granules/high-performance liquid chromatography)

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ABSTRACT **Bovine adrenal chromaffin granules have been shown to contain, in addition to Met-enkephalin and Leu-enkephalin, at least three small peptides with opiate receptor activity. One of these adrenal peptides has been purified to homogeneity and its sequence was shown to be Met-enkephalin-[Arg^6, Phe^7]. This heptapeptide was also found in beef striatal extracts in amounts comparable to those of Leu-enkephalin.**

The opioid pentapeptides Met-enkephalin and Leu-enkephalin were originally isolated from whole brain (1). When the Met-enkephalin sequence was found at the NH_2 terminus of the pituitary hormone β-endorphin, it was assumed that Met-enkephalin is derived from β-endorphin. However, this relationship has never been established. Indeed, there have been studies that suggest a biosynthetic pathway for the brain enkephalins that does not include β-endorphin as an intermediate (2–4).

Recently, immunologic procedures have revealed the presence of opioid peptides in adrenal medulla, localized in chromaffin cells (5–9). We have not only corroborated these findings but also have found that the adrenal medulla contains more enkephalin-like material than does the brain. Furthermore, the medulla is also rich in several proteins and large peptides that yield opioid activity after treatment with trypsin (10). None of the proteins is related to β-endorphin or its pituitary precursors.

In the course of these studies we undertook the chemical characterization of those peptides in the medulla that are in the molecular weight range of the enkephalins and that show direct opioid activity. We found not only Met- and Leu-enkephalin, as expected, but also, at least three other small peptides with opioid activity. One of these unknown peptides has been purified to homogeneity and its sequence was shown to be Met-enkephalin[Arg^6, Phe^7]. This peptide is found not only in the adrenal gland but also in the striatum in amounts comparable to those of Leu-enkephalin. Details of the isolation and characterization of this opioid heptapeptide are presented here.

MATERIALS AND METHODS

Bovine adrenal glands were obtained from a local slaughterhouse and stored on ice until used (1–2 hr). The medullas were dissected out, and chromaffin granules were prepared by the procedure of Smith and Winkler (11). The isolated chromaffin granules were lysed in a solution (1:10, wt/vol) containing 1 M acetic acid, 20 mM HCl, 1 μg each of phenylmethylsulfonyl fluoride and pepstatin per ml, and 0.1% 2-mercaptoethanol. Membranes and cell debris were removed by centrifugation at 100,000 × g for 30 min, and proteins in the supernatant solution were precipitated by the addition of 50% (wt/vol) trichloroacetic acid to achieve a 10% final concentration. The precipitate was then removed by centrifugation at 26,000 × g for 15 min. After removal of trichloroacetic acid and lipids from the supernatant solution by ether extraction (three times with equal volumes), the sample was lyophilized. The residue was then dissolved in buffer and subjected to high-performance liquid chromatography (HPLC) on a Lichrosorb RP-18 (reverse-phase) column (4.6 × 250 mm, either 5- or 10-μm particle size, Brownlee Labs, Santa Clara, CA), as described below.

Striata were removed from beef brains and homogenized as described (2). After centrifugation, proteins in the supernatant solution were precipitated with trichloroacetic acid and extracted with ether as described above. The sample was then applied to the reverse-phase column.

Peptides in column effluents were monitored with fluorescamine (12). The peptide concentration in individual fractions was determined with fluorescamine, with Leu-enkephalin as the calibration standard. Amino acid analyses were performed with fluorescamine detection (13) utilizing 50–200 pmol of peptide. Sequence determination was carried out by the dansyl-Edman method, essentially as described (14) and the dansyl amino acids were identified by HPLC (15). Met-Enkephalin[Arg^6, Phe^7] was synthesized by Peninsula Laboratories (San Carlos, CA) and purified by HPLC.

Aliquots of the collected fractions were tested for opioid activity by a radioreceptor binding assay using neuroblastoma–glioma hybrid cells (16) with [^{3}H]tyrosine-labeled Leu-enkephalin as the competing ligand. Radioimmunoassays were performed by using a COOH-terminal-directed Leu-enkephalin antiserum (RB-92) and an NH_2-terminal-directed Met-enkephalin antibody (JR-235). The characteristics of these antisera have been described (17, 18). The COOH-terminal-directed Leu-enkephalin antiserum has a crossreactivity of 3% with Met-enkephalin. The NH_2-terminal-directed antiserum crossreacts 41% with Leu-enkephalin. Further experimental details are given in the figure legends.

RESULTS

Isolation of Opioid Peptides from Adrenal Medullary Granules. Chromatography of extracts of bovine adrenal medulla or purified chromaffin granules on Sephadex G-100 yields five peaks of radioreceptor active material (17) corresponding in molecular weights to 20,000–24,000 (peak I), 10,000–15,000 (peak II), 7000–10,000 (peak III), 3000–5000 (peak IV), and <1000 (peak V). The material in an acid extract of chromaffin granules, after deproteinization with trichloroacetic acid, corresponds to peak V (17). The deproteinized acid extract obtained from the granules of bovine adrenal medullas was

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Abbreviation: HPLC, high-performance liquid chromatography.

chromatographed on the RP-18 column and assayed for opioid activity. Opioid activity was present not only in the calibrated Met-enkephalin and Leu-enkephalin regions, but also in two other regions: one preceding Met-enkephalin (fractions 6 and 7) and the other following Leu-enkephalin (fraction 16) (Fig. 1). To determine whether fractions 6, 7, and 16 were indeed entities separate from the enkephalins, these fractions were lyophilized, dissolved in buffer, and rechromatographed. Each opioid peptide eluted in its original position (Fig. 2). Note that fraction 7 contained some Met-enkephalin.

Fractions from the first chromatography were further characterized by radioimmunoassay using the two different antisera. All of the fractions interacted well with the NH_2-terminal-specific antiserum but not at all with the COOH-terminal-specific antiserum (Table 1). Thus, these fractions appear to represent homologs of the enkephalins with additional residues at the COOH terminus.

Purification and Analysis of an Opioid Peptide from Fraction 16. Material corresponding to fraction 16 (see Fig. 1) was prepared from 15 g of chromaffin granules and chromatographed under conditions giving highest resolution (i.e., isocratic elution) on an RP-18 column (Fig. 3). Two peaks of opioid activity were observed, indicating that fraction 16 contains two distinct opioid peptides. The region of highest specific activity (141–147 min) from the major peak was rechromatographed with the same system and a symmetrical peak of fluorescence was obtained, indicative of homogeneity (Fig. 4*A*). This purified peptide was subjected to structural analysis.

Amino acid assay on three preparations indicated the composition Tyr_1, Gly_2, Phe_2, Met_1, Arg_1. Sequence analysis, carried out with 5 nmol, revealed the primary structure Tyr-Gly-Gly-Phe-Met-Arg-Phe. In addition, chromatography of the purified heptapeptide from fraction 16 yielded a peak at exactly the same position with the same peak height and the same specific activity as was obtained with the same quantity of synthetic Met-enkephalin[Arg^6, Phe^7] (Fig. 4 *A* and *B*).

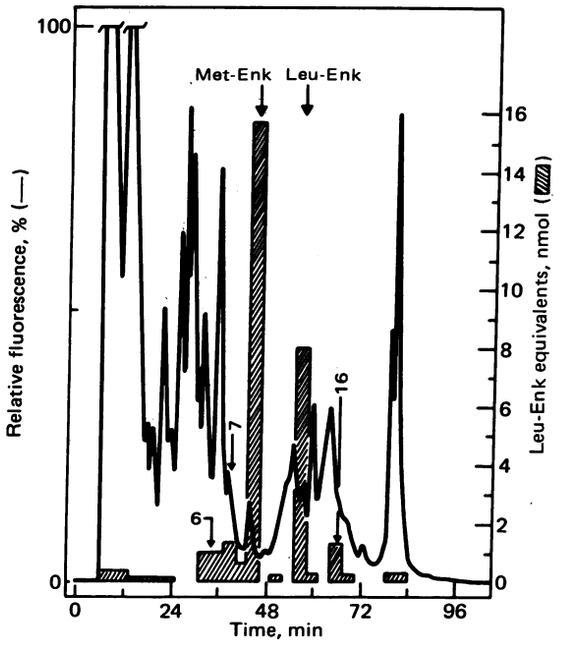

FIG. 1. Chromatography of peptides from chromaffin granules. A deproteinized extract obtained from five adrenal glands was applied to an RP-18 column (10 μm, 4.6 × 250 mm) and eluted at 35 ml/hr with 0.5 M formic acid/0.4 M pyridine, pH 4.0, and a stepwise gradient of *n*-propanol: 0% (5 min), 5% (5 min), 10% (25 min), 15% (30 min), and 40% (17 min). A portion (5%) of the column effluent was diverted to the fluorescamine monitoring system. Fractions (1.75 ml) were collected and aliquots (20 μl) were lyophilized and assayed for opioid activity. Met-Enk, Met-enkephalin; Leu-Enk, Leu-enkephalin; arrows, fractions 6, 7, and 16.

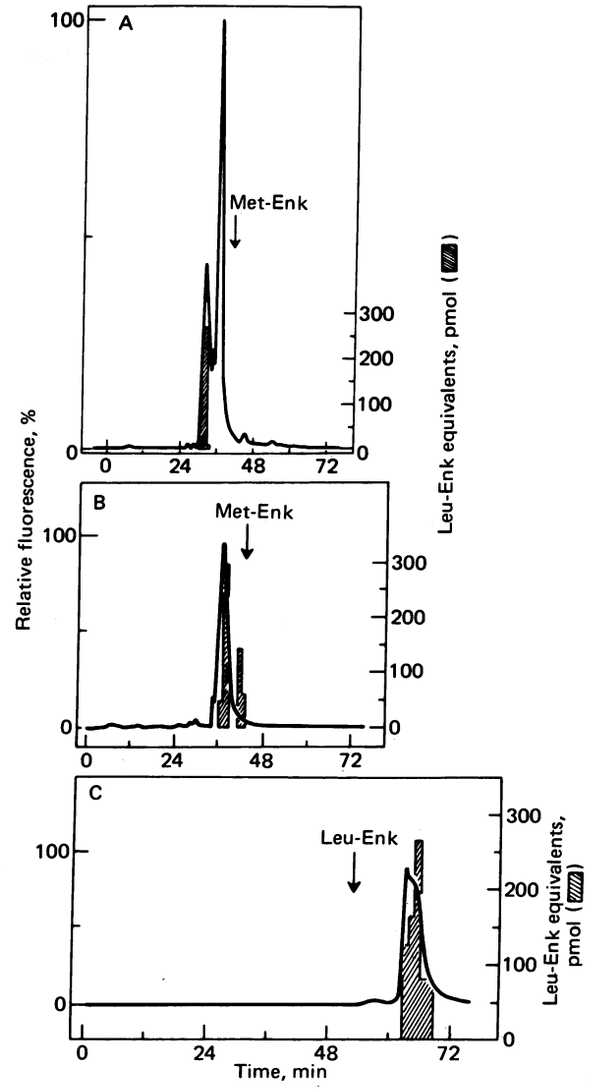

FIG. 2. Rechromatography of fractions obtained from RP-18 chromatography of deproteinized lysed granules (Fig. 1). Fractions 6 (30–36 min), 7 (36–39 min), and 16 (66–69 min) were separately lyophilized, redissolved in buffer, and applied to the column, which was developed as in Fig. 1. Fractions (0.6 ml) were collected and aliquots (30 μl) were lyophilized and assayed for opioid activity. (*A*) Eluate from fraction 6; (*B*) eluate from fraction 7; (*C*) eluate from fraction 16.

Opioid Peptides in Beef Striatum. When a deproteinized beef striatal extract was subjected to HPLC, opioid activity was found not only in the calibrated Met- and Leu-enkephalin regions but also in a region (24–33 min) preceding Met-enkephalin and in another (60–63 min) following Leu-enkephalin (Fig. 5). The NH_2-terminal-specific antiserum interacted well with the material in both regions whereas the COOH-terminal-specific antibody interacted only with material in the Leu-enkephalin region.

The chromatographic pattern of striatal peptides (Fig. 5) corresponds qualitatively with that obtained from chromaffin granule extracts (Fig. 1). Although elution times differed somewhat in the two studies because slightly different gradients were used, the positions of these unknown opioid peptides

Table 1. Radioreceptor assay and radioimmunoassay of adrenal opioid-containing fractions

Fraction	Radioreceptor, pmol	Radioimmunoassay, pmol	
		NH_2 terminal	COOH terminal
6	13	10	<0.1
7	17	13	0.1
16	17	5	0.2
Synthetic Met-enkephalin (100 pmol)	100	100	3.0
Synthetic Leu-enkephalin (100 pmol)	100	40	100
Synthetic Met-enkephalin [Arg^6, Phe^7] (100 pmol)	40	10	1.2

Tissue levels are presented as pmol/g of adrenal medulla, and fractions were obtained as described in the legend to Fig. 1. Results with synthetic compounds (100 pmol) are shown for comparison.

relative to Met-enkephalin and Leu-enkephalin are identical to those obtained in adrenal fractions 6, 7, and 16.

When the striatal material, which eluted in the region following Leu-enkephalin (Fig. 5), was rechromatographed under isocratic conditions, opioid activity appeared at exactly the same elution position (Fig. 4*C*) as the synthetic (Fig. 4*B*) and adrenal-derived (Fig. 4*A*) heptapeptide Met-enkephalin[Arg^6, Phe^7]. Rechromatography of the area preceding the Met-enkephalin marker revealed opioid activity eluting in regions corresponding to fractions 6 and 7 of adrenal extracts (results not shown).

Quantitation. In the radioreceptor assay the specific activity of the heptapeptide, both synthetic and adrenal-derived, was about 40% that of Leu-enkephalin. If one corrects for this difference in biological activity, adrenal chromaffin granules contain typically, per gram of adrenal medulla, 200 pmol of Met-enkephalin, 65 pmol of Leu-enkephalin, and 45 pmol of Met-enkephalin[Arg^6, Phe^7]. The activities in fractions 6 and 7 are approximately 13 and 17 pmol, respectively. These last two values are uncorrected and are probably higher with respect to peptide concentration. The amounts of opioid peptide extracted per gram of striatal tissue are typically 290 pmol of Met-enkephalin, 65 pmol of Leu-enkephalin, and 50 pmol of Met-enkephalin[Arg^6, Phe^7]. These values are not corrected for recovery.

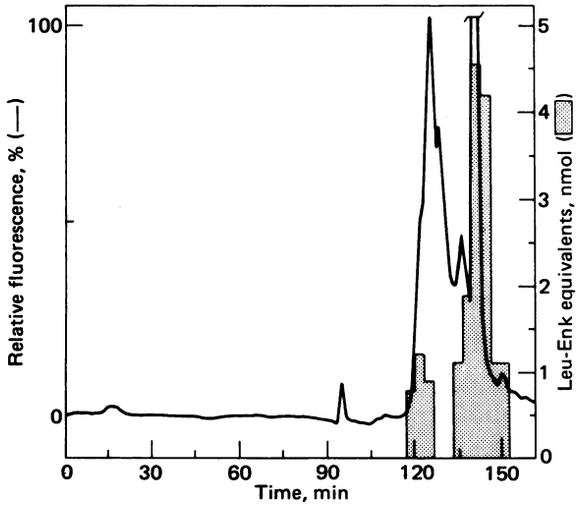

FIG. 3. Rechromatography of fraction 16 under isocratic conditions. Fraction 16 obtained from an extract of 15 g of chromaffin granules was applied to an RP-18 column (5 μm, 4.6 × 250 mm) in buffer and washed for a total of 12 min with the same buffer. Elution was carried out at 14 ml/hr with 17.3% *n*-propanol in the buffer. A portion (9%) of the column effluent was diverted to the fluorescamine monitoring system. Fractions (700 μl) were collected and aliquots (5 μl) were lyophilized and assayed for opioid activity.

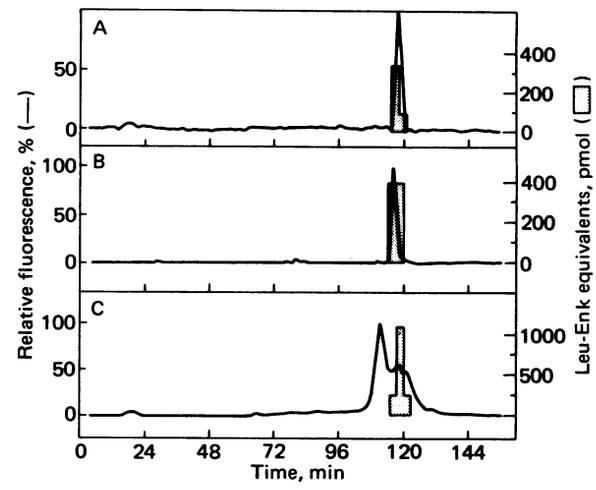

FIG. 4. Chromatographic comparison of adrenal (*A*) and striatal (*C*) heptapeptides with synthetic Met-enkephalin[Arg^6, Phe^7] (*B*). Synthetic and adrenal peptides (1 nmol by amino acid assay) were applied to the column, eluted, and assayed as described in Fig. 3. The striatal peptide was prepared as in Fig. 5, lyophilized, redissolved in buffer, applied to the column, and eluted as in Fig. 3.

DISCUSSION

The identification of the heptapeptide Met-enkephalin[Arg^6, Phe^7] introduces a new sequence in the opioid field, one that is not found in β-endorphin or α-neo-endorphin (19) (Fig. 6).

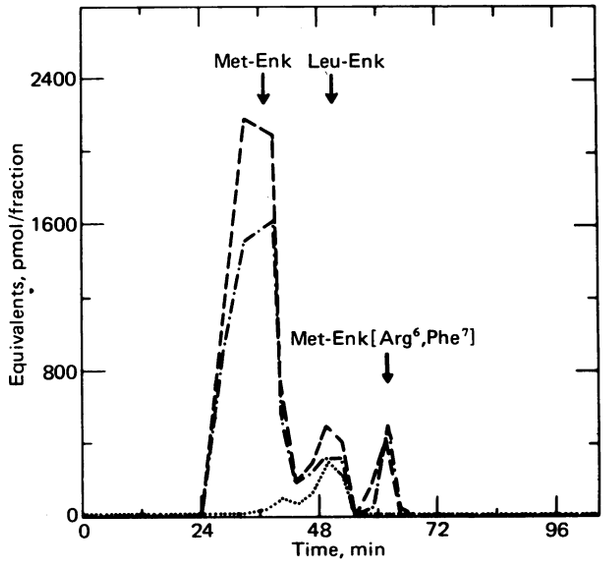

FIG. 5. Chromatography of striatal opioid peptides. A deproteinized extract obtained from 7.6 g of striatum was adjusted to pH 4.0 with pyridine and applied to the column (10 μm, 4.6 × 250 mm). Elution was carried out at 35 ml/hr with 0.5 M formic acid/0.4 M pyridine, pH 4.0, using a stepwise gradient of *n*-propanol: 0% (5 min), 5.3% (5 min), 10.7% (25 min), 14.7% (30 min), and 40% (17 min). A portion (5%) of the effluent was diverted to the fluorescamine monitoring system. Fractions (1.75 ml) were collected and aliquots (10 μl) were subjected to radioimmunoassay and radioreceptor assay. ---, Leu-Enkephalin radioreceptors; -·-, Met-enkephalin NH_2 terminus antibody; ·····, Leu-enkephalin COOH terminus antibody.

β-Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-
α-Neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-
Adrenal heptapeptide	Tyr-Gly-Gly-Phe-Met-Arg-Phe
Adrenal tryptic peptide	Tyr-Gly-Gly-Phe-Met-Lys

FIG. 6. Recognized structures that contain an enkephalin sequence.

This heptapeptide and the other small peptides (fractions 6 and 7) may represent products derived from the larger opioid-containing proteins in the adrenal medulla (17). Several of these proteins have now been purified to homogeneity, and sequence determinations will establish their relationship to the heptapeptide and the enkephalins.

The discovery of Met-enkephalin[Arg^6, Phe^7] along with other, as yet uncharacterized, peptides in adrenals as well as brain (fractions 6 and 7) may explain why radioreceptor assay and COOH-terminal-specific enkephalin immunoassay have not yielded the same values when applied to various tissues (20). Thus, the presence of all these related opioid peptides opens to question any results obtained exclusively by immunologic methods.

An important conclusion of these studies is that, in addition to Met-enkephalin and Leu-enkephalin, there are other peptides that have opioid activity. This raises the question as to whether the two recognized enkephalins are the only opioid peptides of physiologic significance. There is the possibility that Met-enkephalin[Arg^6, Phe^7] possesses a unique physiologic role of its own. Its presence in chromaffin granules (in amounts comparable to the enkephalins) indicates that it, too, is released into the blood along with catecholamines. The extension at the COOH terminus may make the peptide more resistant to degradation in blood. In this respect, it is of interest that the heptapeptide produces analgesia when injected intracerebrally into mice (C. Inturrisi, personal communication).

Apparently, the heptapeptide Met-enkephalin[Arg^6, Phe^7] is not the only enkephalin-containing sequence in the opioid-active adrenal proteins. We have recently isolated the tryptic peptide Met-enkephalin[Lys^6] from some of the adrenal opioid-containing proteins (17). Thus, as shown in Fig. 6, there are now four known, distinct enkephalin sequences. It should be noted that all of them, with the exception of β-endorphin, possess lysine or arginine at position 6, making it possible to generate enkephalin by a combination of trypsin and carboxypeptidase B activities. In the light of the present findings, it will be necessary to reevaluate the simplistic scheme of opioid neurobiology based solely on Met- and Leu-enkephalinergic innervation.

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