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Probable precursors of [Leu]enkephalin and [Met]enkephalin in adrenal medulla: Peptides of 3–5 kilodaltons

(trypsin/high-performance liquid chromatography)

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ABSTRACT **Adrenal chromaffin granules contain at least 10 peptides, ranging in size from 3 to 5 kilodaltons, that yield, upon digestion with trypsin, peptides that show specific binding to opiate receptors. All are distinctly different from β-endorphin. Two of these peptides have been purified to homogeneity and subjected to chemical analysis. One is apparently a [Met]enkephalin precursor containing two copies of the [Met]enkephalin sequence. The other peptide contains both [Leu]enkephalin and [Met]enkephalin sequences and is presumably a common precursor of the two forms of enkephalin.**

Although the [Met]enkephalin sequence is contained within the pituitary peptide β-endorphin, there is now evidence to indicate that the latter is not the precursor of this opioid pentapeptide (1–4). Because [Met]enkephalin and [Leu]enkephalin are found in many tissues (brain as well as peripheral nervous system), their precursors may also be found in these tissues. The recent discovery of the enkephalins in the adrenal medulla (5, 6) led us to examine this tissue. We found (7, 8) that bovine adrenal chromaffin granules contain many peptides that either bind directly to opiate receptors or do so after treatment with trypsin. These peptides range in size from 500 to 25,000 daltons. The larger ones, although similar in size, are distinct from the known opioid-containing peptides in the pituitary, β-endorphin, β-lipotropin, and pro-opiocortin (9). In an earlier report, several opioid peptides in the size range of the enkephalins were shown to be present in chromaffin granule extracts; one of these was purified to homogeneity and its sequence was shown to be [Met]enkephalin-Arg^6-Phe^7 (10). The identification of this heptapeptide introduced a new sequence in the opioid field and provided further evidence that [Met]enkephalin is formed via a biosynthetic pathway that does not include β-endorphin, even though the latter also contains a [Met]enkephalin sequence (11).

In the present study we undertook the characterization of the peptides of intermediate size [3–5 kilodaltons (kDal)] present in adrenal chromaffin granule extracts (7, 8). At least 10 peptides in this range, with opiate receptor binding activity after trypsin digestion, were found, all of which are distinctly different from β-endorphin. Two of these peptides have been purified to homogeneity. One contains two copies of the [Met]enkephalin sequence; the other contains both a [Leu]-enkephalin sequence and a [Met]enkephalin sequence. Details of the purification and chemical characterization of these peptides and their tryptic products are presented.

MATERIALS AND METHODS

Bovine adrenal glands were obtained from a local slaughterhouse and stored (2 hr) on ice until used. The medullas were dissected out and chromaffin granules were prepared by the procedure of Smith and Winkler (12). The isolated chromaffin granules were homogenized (10:1 vol/wt) in 1 M acetic acid, 20 mM HCl, and 0.1% 2-mercaptoethanol containing 1 μg/ml each of phenylmethanesulfonyl fluoride and pepstatin. The procedures for extraction, elution from Sephadex columns, and digestion with trypsin have been reported (1, 7). The radioreceptor assay used neuroblastoma–glioma hybrid cells with tritiated [Leu]enkephalin as the competing ligand (13). Radioimmunoassays were performed with two enkephalin antisera or β-endorphin antiserum, as described (14).

Reverse-phase high-performance liquid chromatography was carried out with Lichrosorb RP-18 (Ace Scientific, Edison, NJ), Ultrasphere Octyl (Altex, Berkeley, CA), or Spherisorb CN (Lab Data Control, Riviera Beach, FL). Formic acid (0.5 M) /pyridine (0.4 M) buffer, pH 4.0, was used with gradients of 1-propanol to elute the peptides and proteins (15). An automated fluorescence detection system using fluorescamine (Hoffmann-La Roche) was used for monitoring peptides in column effluents (16). Amino acid analysis was performed at the picomole level with a fluorescamine amino acid analyzer (17). Sequence analyses were carried out by dansyl and Edman methods at the nanomole level, essentially as described (18, 19), and the dansyl and phenylthiohydantoin amino acids were identified by high-performance liquid chromatography (20, 21). All solvents (Fisher) were distilled over ninhydrin prior to use.

[Met]Enkephalin-Arg^6-Phe^7 and [Met]enkephalin-Arg^6 were obtained from Peninsula Laboratories (San Carlos, CA). We also wish to thank J. Meienhofer (Hoffmann-La Roche) for preparing [Met]enkephalin-Lys^6 and additional quantities of [Met]enkephalin-Arg^6. Radioimmunoassays for corticotropin were generously performed by T. Mowles (Hoffmann-La Roche) and by the Nichols Institute (San Pedro, CA). Radioimmunoassays for β-endorphin were performed by C. Inturrisi (Cornell University Medical College).

RESULTS

The separation of peptides in extracts of bovine adrenal chromaffin granules on a Sephadex G-100 column was described in a previous report (7). In that study, five peaks containing peptides active in the opiate radioreceptor assay or yielding

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Abbreviation: kDal, kilodaltons.

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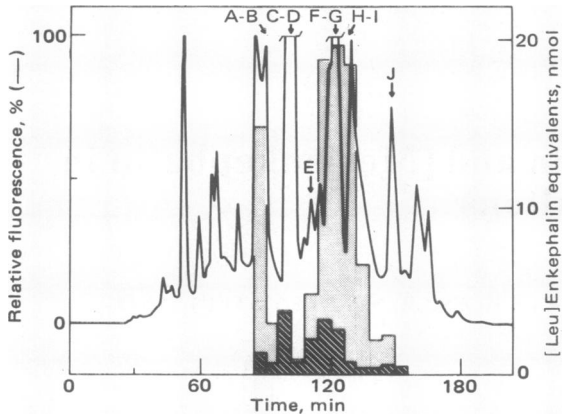

FIG. 1. High-performance liquid chromatography of peak IV on Lichrosorb RP-18. The column (10 μm, 4.6 × 250 mm) was eluted at 30 ml/hr with 0.5 M formic acid/0.4 M pyridine, pH 4.0, with a linear 0–20% gradient of 1-propanol for 120 min. A portion of the column effluent (2%) was directed to the fluorescamine monitoring system. Aliquots of each fraction (1.5 ml) were analyzed by the radioreceptor binding assay with (gray area) and without (hatched area) prior digestion with trypsin. The active fractions are labeled A–J.

active tryptic peptides were observed. These peaks corresponded to the following size ranges (in Dal): 20,000–24,000 (peak I), 10,000–15,000 (peak II), 7,000–10,000 (peak III), 3000–5000 (peak IV), and <1000 (peak V). The present studies were undertaken to characterize the peptides in peak IV. The fractions corresponding to peak IV from the Sephadex G-100 column were pooled and pumped directly onto a Lichrosorb RP-18 column (Fig. 1). A number of fractions were obtained that had opiate-receptor-binding activity or yielded tryptic peptides with such activity. All of the fractions, except fraction C-D, showed much greater activity after trypsin digestion. Further chromatography of each of these fractions with Lichrosorb RP-18, Ultrasphere Octyl, or Spherisorb CN columns resolved at least 10 distinct active peptides (Fig. 2). The relative proportions of the peptides varied from preparation to preparation, but peptides B, E, F, and I were always the major peptides present.

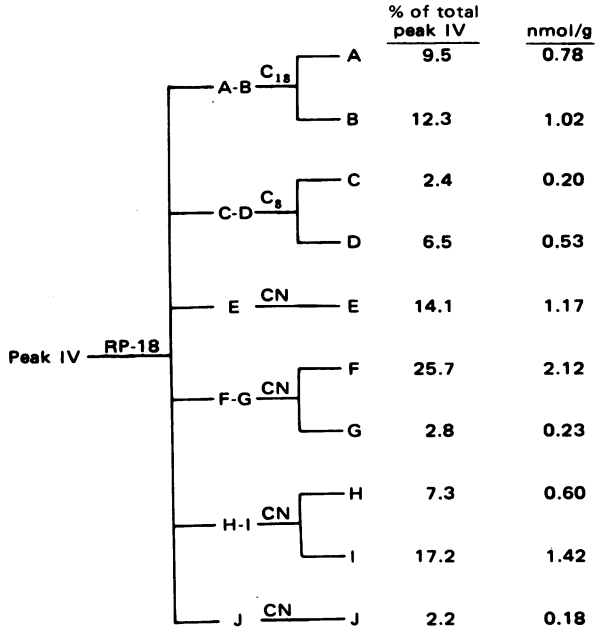

FIG. 2. Summary of the amounts of the 10 peptides in peak IV (3–5 kDal). Lichrosorb RP-18 (C_{18}), Ultrasphere Octyl (C_8), and Spherisorb CN (CN) columns were used for separations. The amount of each of the 10 peptides represents the average of 10 isolations using 6 g of chromaffin granules in each case. Also shown is the percentage of each peptide recovered, based on the total radioreceptor binding activity in the original peak IV mixture.

Purification and Analysis of Opioid Peptides F and I. Fractions F-G and H-I obtained by RP-18 chromatography (Fig. 1) were further purified on a Spherisorb column (Fig. 3). Each fraction yielded two peaks of activity. These were digested with trypsin prior to radioreceptor assay. The major active peptides, F (Fig. 3*A*) and I (Fig. 3*B*) were purified to homogeneity by rechromatography on the same CN column.

Amino acid analyses of the two peptides are shown in Table 1. Molecular weights of 3800 and 4700 for peptides F and I, respectively, were calculated from these analyses. These molecular weights are in accord with their elution positions on gel chromatography. Their compositions indicate that peptides F and I are distinct entities and that neither has any relationship to β-endorphin (11) or to α-neoendorphin (22) (Table 1). End-group analysis by the dansyl method revealed a single amino acid for each of the peptides, confirming their homogeneity. The amino terminus of peptide F is Tyr and that of peptide I is Ser.

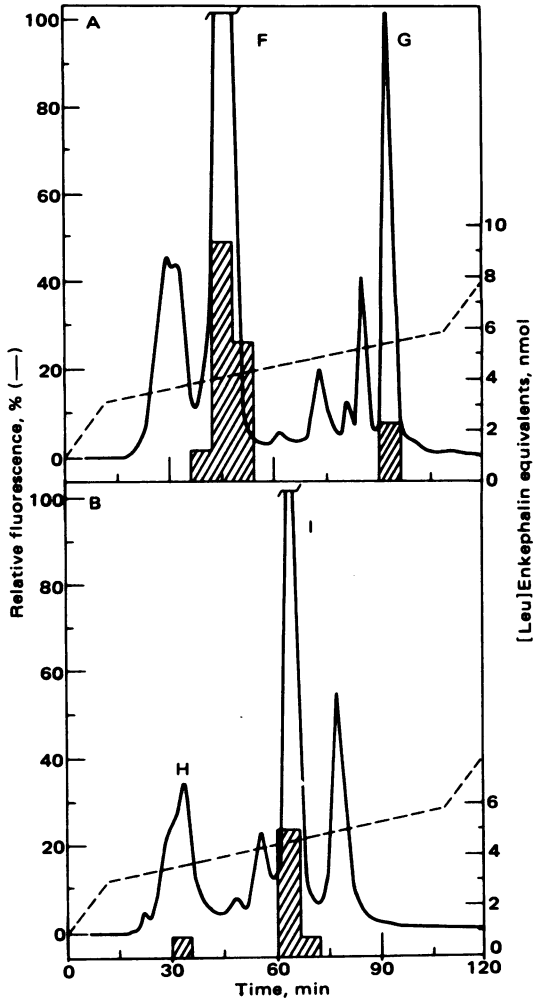

FIG. 3. Chromatography of fractions F-G and H-I on Spherisorb CN columns. Fractions F-G and H-I obtained from RP-18 chromatography of peak IV (Fig. 1) were each applied to a Spherisorb CN column (5 μm, 4.6 × 250 mm). The column was eluted at 30 ml/hr with 0.5 M formic acid/0.4 M pyridine, pH 4.0, with a linear 12–28% (- - -) gradient of 1-propanol. Aliquots were subjected to radioreceptor assay (hatched area) after treatment with trypsin. (*A*) F-G; (*B*) H-I.

Table 1. Amino acid composition of adrenal peptide F and peptide I

Amino acid	F	I	β-Endorphin*	α-Neoendorphin†
Asx	2.11 (2)	2.03 (2)	2	0
Thr	0.07 (0)	0.95 (1)	3	0
Ser	0.09 (0)	0.90 (1)	2	0
Glx	5.68 (6)	5.63 (6)	3	0
Pro	1.16 (1)	3.53 (4)	1	1
Gly	6.19 (6)	4.58 (5)	3	3
Ala	1.09 (1)	0.05 (0)	2	0
Cys	0.00 (0)	0.00 (0)	0	0
Val	2.00 (2)	1.00 (1)	1	0
Met	3.11 (3)	2.13 (2)	1	0
Ile	0.04 (0)	0.00 (0)	2	0
Leu	3.16 (3)	3.20 (3)	2	1
Tyr	2.86 (3)	3.04 (3)	1	3
Phe	1.90 (2)	1.93 (2)	2	1
His	0.04 (0)	0.95 (1)	1	0
Lys	2.81 (3)	2.79 (3)	5	2
Arg	0.98 (1)	4.85 (5)	0	4
Trp	0.00 (0)	0.00 (0)	0	0
Total	33	39	31	15

Samples of 100 pmol were hydrolyzed at 110°C for 22 hr in 200 μl of constant boiling HCl containing 0.1% thioglycolic acid. Tryptophan was determined by hydrolysis in the presence of 4% (vol/vol) thioglycolic acid. All values represent the averages of three (F) and five (I) analyses. The nearest integer values are indicated in parentheses. Val was arbitrarily set equal to 2.0 and 1.0 in F and I, respectively.
* See ref. 11.
† See ref. 22.

Tryptic Peptides Derived from Peptides F and I. For further characterization, peptides F and I were each treated with trypsin and the tryptic peptides were separated by chromatography on an Ultrasphere Octyl column (Fig. 4). Two peaks with opiate-receptor-binding activity were obtained in the digest of peptide F, designated F-T-1 and F-T-2 according to their order of elution. The most active tryptic peptide (F-T-2) was chromatographically identical to authentic [Met]enkephalin (Fig. 4, *A* and *B*). The other tryptic peptide (F-T-1) eluted at a position preceding synthetic [Met]enkephalin-Arg^6 and coinciding with synthetic [Met]enkephalin-Lys^6.

As shown in Table 2, the amino acid composition of F-T-2 was identical to that of [Met]enkephalin. F-T-1 had the same amino acid composition as [Met]enkephalin-Lys^6. Based on amino acid analyses, equimolar amounts of both tryptic peptides, [Met]enkephalin and [Met]enkephalin-Lys^6, were derived from peptide F. Partial sequence analysis of peptide F yielded Tyr^1-Gly^2-Gly^3-Phe^4. On the basis of all the above data, the 33-residue peptide F must contain two copies of the [Met]-enkephalin sequence. One is located at the amino terminus, followed by Lys and yielding [Met]enkephalin-Lys^6 on digestion with trypsin. The other is at the carboxyl terminus, preceded by Lys or Arg and yielding free [Met]enkephalin on digestion with trypsin (Fig. 5).

For peptide I, only one tryptic peptide with opiate-receptor-binding activity, designated I-T-2, was found. This peptide was chromatographically identical to authentic [Leu]enkephalin (Fig. 4, *A* and *C*). The amino acid composition of I-T-2 was also the same as synthetic [Leu]enkephalin (Table 2). Although no additional active peptides were detected in the tryptic digests, a peak (I-T-1), eluting in the same position as [Met]enkephalin-Arg^6, was observed. The amino acid analysis of I-T-1 was also the same as [Met]enkephalin-Arg^6 (Table 2). Both the tryptic peptide I-T-1 and synthetic [Met]enkephalin-Arg^6 were

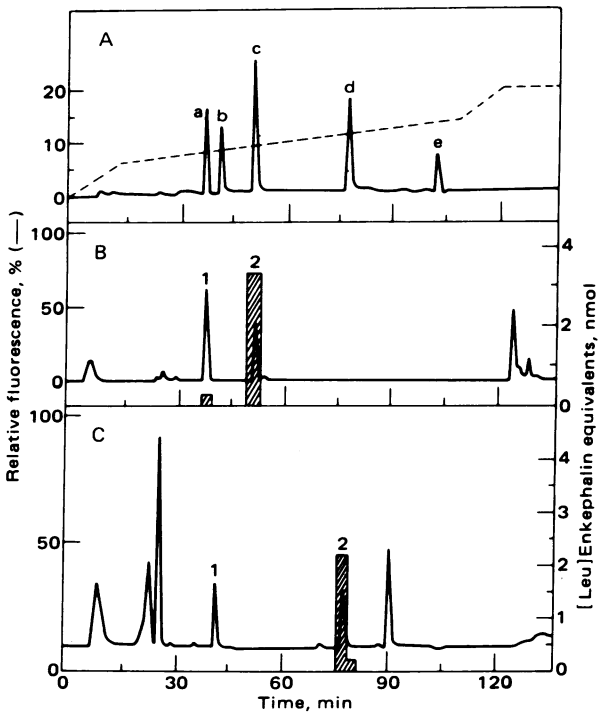

FIG. 4. Tryptic peptides derived from peptide F and peptide I. Each peptide was treated with trypsin and the digests were applied to an Ultrasphere Octyl column (5 μm, 4.6 × 250 mm). The column was eluted at 30 ml/hr with 0.5 M formic acid/0.4 M pyridine, pH 4.0, by using a linear 6–14% (- - -) gradient of 1-propanol. Aliquots of the column effluents were lyophilized and assayed for opiate-receptor-binding activity (hatched area). (*A*) Standard: a, [Met]enkephalin-Lys^6; b, [Met]enkephalin-Arg^6; c, [Met]enkephalin; d, [Leu]enkephalin; e, [Met]enkephalin-Arg^6-Phe^7. (*B*) Tryptic digest of peptide F. (*C*) Tryptic digest of peptide I.

subsequently shown to be active in the radioreceptor assay, but at far higher concentrations than were originally used. All the above data indicate that the 39-residue peptide I contains a

Table 2. Amino acid composition of opioid-active tryptic peptides from peptide F and peptide I

Amino acid	F-T-1	F-T-2	I-T-1	I-T-2
Asx	0	0	0	0
Thr	0	0	0	0
Ser	0	0	0	0
Glx	0	0	0	0
Pro	0	0	0	0
Gly	2	2	2	2
Ala	0	0	0	0
Cys	0	0	0	0
Val	0	0	0	0
Met	1	1	1	0
Ile	0	0	0	0
Leu	0	0	0	1
Tyr	1	1	1	1
Phe	1	1	1	1
His	0	0	0	0
Lys	1	0	0	0
Arg	0	0	1	0
NH_2-terminal	Tyr	Tyr	Tyr	Tyr

Samples (100 pmol) were hydrolyzed as in the legend for Table 1. Tryptophan was not determined because peptide F and peptide I contain no tryptophan residue. NH_2-terminal amino acids were determined by the dansyl method.

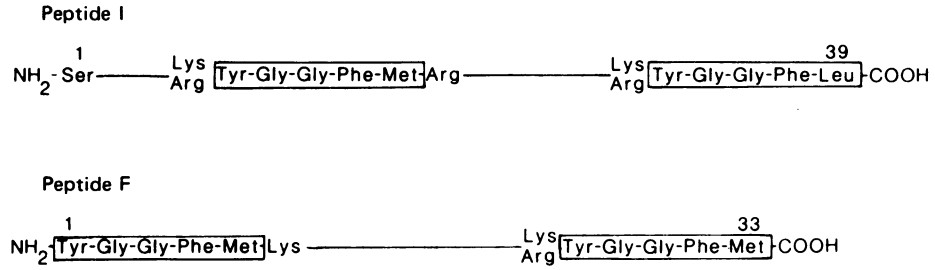

FIG. 5. Schematic structures of peptide I and peptide F.

[Leu]enkephalin sequence that is located at the carboxyl terminus and is preceded by Lys or Arg and a [Met]enkephalin sequence in the internal portion of the peptide preceded by Lys or Arg and followed by Arg (Fig. 5).

Radioreceptor Binding Activity and Immunoreactivity of Peptides F and I. Although amino acid analyses showed equal amounts of the tryptic peptides [Met]enkephalin and [Met]enkephalin-Lys^6 in peptide F, the radioreceptor binding activity of the two tryptic fragments were clearly different (Fig. 4*B*). The binding curves of the two tryptic peptides showed that [Met]enkephalin-Lys^6 binds only 5% as well to the receptor as does [Met]enkephalin (data not shown). Intact peptide F, which has a [Met]enkephalin-Lys^6 sequence at its amino terminus, showed a binding activity similar to that of synthetic [Met]-enkephalin-Lys^6. The binding curve for the unfractionated tryptic digest of peptide I was almost identical to that of synthetic [Leu]enkephalin (data not shown). As noted above, [Met]enkephalin-Arg^6 binds very poorly to the receptor and thus contributes very little to the activity of the tryptic digest of peptide I. This hexapeptide, as well as intact peptide I, exhibited less than 3% of the binding activity of [Leu]enkephalin.

Radioimmunoassay with an amino-terminal-directed [Met]enkephalin antiserum showed that intact peptide F does crossreact and that there is a significant increase (1.4-fold) in crossreactivity after trypsin digestion. Intact peptide I showed crossreactivity with the above antiserum only after trypsin digestion. Both intact peptides F and I showed about 0.1% crossreactivity towards a carboxyl-terminal-directed [Leu]-enkephalin antiserum. However, after trypsin digestion, the crossreactivity of peptide I increased to 100%, whereas the crossreactivity of peptide F was unchanged. None of the 10 peptides in peak IV exhibited crossreactivity with an antiserum to β-endorphin. Unfractionated chromaffin granule extracts were also negative to a radioimmunoassay for corticotropin.

Both the radioreceptor binding assays and the radioimmunoassays are in accord with the structures shown in Fig. 5—i.e., the assignment of a [Met]enkephalin sequence at the amino terminus and another at the carboxyl terminus of peptide F. The presence of the [Leu]enkephalin sequence at the carboxyl terminus and an internal [Met]enkephalin sequence in peptide I was also confirmed by the data from these two types of assays.

DISCUSSION

The peptides [Met]enkephalin, [Leu]enkephalin, [Met]enkephalin-Lys^6, and [Met]enkephalin-Arg^6, which were isolated from tryptic digests of peptides F and I, were identified by high-performance liquid chromatography, amino acid analysis, binding to opiate receptors, and interaction with the corresponding antisera. The markedly diminished opiate-receptor-binding activities of both [Met]enkephalin-Lys^6 and [Met]enkephalin-Arg^6 and of peptide F indicate that the presence of a positively charged group added to the carboxyl terminus of an enkephalin sequence hinders its binding to the receptor. β-Endorphin, which does not contain either Arg or Lys in position 6, is very active in the opiate-receptor-binding assay (13).

Although the presence of the [Met]enkephalin and [Leu]-enkephalin sequences in these two peptides does not establish them as precursors of the enkephalins, their cleavage by trypsin to generate the pentapeptides does strongly suggest a precursor relationship. In a previous paper (10) we identified [Met]enkephalin and [Leu]enkephalin as naturally occurring peptides in chromaffin granules. Recently, we have found that free [Met]enkephalin-Arg^6 and [Met]enkephalin-Lys^6 are also present in chromaffin granules (unpublished observations). These findings add further evidence to the probability that peptide F and peptide I are enkephalin precursors. Although peptide F seems to qualify as a [Met]enkephalin precursor, the presence of the two [Met]enkephalin sequences, one at the amino terminus and the other at the carboxyl terminus, suggests a more complex relationship than simply that of precursor and product.

Peptide I represents the largest peptide thus far isolated that contains the [Leu]enkephalin sequence. Of even greater interest is the presence of both [Met]enkephalin and [Leu]enkephalin sequences in the same peptide. The amino acid composition of peptide I (Table 1) is quite different from that of α-neoendorphin isolated by Kangawa *et al.* (22) from hypothalamic extracts. It is conceivable that α-neoendorphin and peptide I may represent cleavage products of an even larger precursor (see below) because [Leu]enkephalin is located at the amino terminus of α-neoendorphin and at the carboxyl terminus of peptide I.

Peptides F and I are unique because of the presence of more than one copy of a biologically active peptide in these apparent precursor molecules. The situation may be more complicated than that because we have also isolated from adrenal extracts larger peptides (15–25 kDal) that contain even more than two copies of opioid peptides per mole (unpublished observations). The presence of multiple enkephalin sequences in these peptides suggests that the ratio of [Leu]enkephalin to [Met]enkephalin may be determined by their relative proportions in a single large precursor protein (7, 8). If this is true, then variations in the relative proportions of the two enkephalins from tissue to tissue would depend on differences in the metabolism of the precursor and the pentapeptides. In view of our finding that a single peptide, an apparent precursor, contains both [Met]enkephalin and [Leu]enkephalin sequences, reports that [Met]enkephalin and [Leu]enkephalin occur in separate neurons are surprising (23).

These intermediate-sized peptides (3–5 kDal), which themselves possess very low opiate-receptor-binding activity, may be the forms secreted from the adrenal gland and thus serve as a means of transporting the more labile enkephalins to the appropriate receptors. It remains to be determined whether these same peptides also serve as precursors of the enkephalins in the brain and in other peripheral tissues.

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