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A highly potent 3200-dalton adrenal opioid peptide that contains both a [Met]- and [Leu]enkephalin sequence

(adrenal medulla/guinea pig ileum/hormone/sequence microanalysis)

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ABSTRACT **A 3200-dalton adrenal enkephalin-containing peptide, designated peptide E, that exhibits high opiate activity in the guinea pig ileum longitudinal muscle preparation was purified, and its structure was determined. It contains an amino-terminal [Met]enkephalin sequence and a [Leu]enkephalin sequence at the carboxyl terminus. Sequence analysis revealed that peptide E arises from a previously characterized 4900-dalton adrenal enkephalin-containing peptide. Peptide E was shown to be 30 times more potent than [Met]enkephalin in the guinea pig ileum assay, which suggests that the adrenal enkephalin-containing peptide may perform a unique biological function *in vivo*.**

A large number of enkephalin-containing polypeptides are present in the adrenal medulla (1–3). We have purified most of them to homogeneity, characterized them chemically, and determined the total sequence of a few of them (4–9). Although all of the hitherto reported peptides contain one or more enkephalin sequences, none possesses significant intrinsic opiate receptor binding activity, and all require treatment with trypsin and carboxypeptidase B to yield the free enkephalins for biological activity to be demonstrated. We now have isolated and totally sequenced an adrenal peptide that is a far more potent opiate agonist than [Met]enkephalin in the isolated guinea pig ileum and is almost as potent as dynorphin (10). This peptide is 3200 daltons in mass and contains within it both a [Met]enkephalin and a [Leu]enkephalin sequence.

MATERIALS AND METHODS

Peptide Isolation. Partial purification of the 3200-dalton peptide from purified chromaffin granules of bovine adrenal medullas has been described (5). Final purification of the peptide (shown as peptide E in Fig. 1) was achieved by reverse-phase high-performance liquid chromatography (HPLC) with an Altex Ultrasphere ODS column (5 μm; 10-nm pore size) and a diphenyl reverse-phase HPLC column (10 μm, 10 nm pore size) (11, 12).

Peptide Characterization. Tryptic peptides derived from purified peptide E were separated by HPLC as reported (5). Amino acid analyses were performed at the picomole level with a fluorescamine amino acid analyzer (13). Carboxypeptidase Y time-course analyses were carried out by the procedure of Jones *et al.* (14). Automated sequence analyses were performed as described (6).

Measurement of Opiate Activity. Opiate activity was determined in the guinea pig ileum longitudinal muscle preparation (15). All chambers used in the ileum experiments were first treated with Prosil-28 (PCR Research Chemicals, Gainsville, FL) to minimize surface adsorption of the peptides. Opiate receptor binding activity was measured by using NG-108 neuroblastoma × glioma cell membranes and [^{3}H]naloxone as the competing ligand (16).

RESULTS AND DISCUSSION

Acid extracts of isolated chromaffin granules were chromatographed on Sephadex G-75, and the fractions corresponding to the 3000- to 5000-dalton region were pooled and pumped directly onto a Lichrosorb RP-18 column. When aliquots of column fractions were assayed prior to digestion for opiate activity in the guinea pig ileum preparation, a major peak of activity was observed corresponding to peptide E. This peak was only a minor component of the total receptor binding activity obtained after digestion of fraction aliquots with trypsin and carboxypeptidase B (5). Fractions containing the major peak of ileum activity were pooled and further purified on an Ultrasphere ODS column (Fig. 1*A*). Final purification was achieved by chromatography on a diphenyl reverse-phase column (Fig. 1*B*). End group analysis revealed a single amino acid (tyrosine) consistent with homogeneity. Amino acid analysis indicated that the purified polypeptide contained 25 amino acid residues. From these data the molecular mass was calculated to be 3200 daltons.

Peptide E was treated with trypsin, and the resulting tryptic peptides were separated by reverse-phase HPLC on a Lichrosorb RP-18 column. Two of the tryptic peptides were found to possess opiate receptor binding activity. The amino acid composition of one corresponded to [Met]enkephalin-Arg^6, whereas the other corresponded to [Leu]enkephalin. This demonstrated that peptide E contains a [Leu]enkephalin sequence at its carboxyl terminus and a [Met]enkephalin sequence either at the amino terminus or internally.

The amino-terminal amino acid sequence of peptide E was determined by automated Edman degradation. Unambiguous results were obtained for the first 23 cycles. The remainder of the primary structure was established by carboxypeptidase Y time-course hydrolysis. The complete sequence is given in Fig. 2, which also shows the amino acid sequences of a number of other enkephalin-containing peptides that have been characterized. It is quite clear that peptide E represents residues 15 to 39 of the 4900-dalton adrenal peptide (peptide I) that has been reported (9). Both are presumably cleavage products of a much larger multivalent enkephalin-containing polypeptide, proenkephalin, that may be the product of translation of the enkephalin gene (9). It would appear that the enkephalin-containing polypeptides represent intermediates in the processing of the multivalent proenkephalin to free [Met]- and [Leu]enkephalin. Another possibility that was raised in an earlier report (20) is

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Abbreviation: HPLC, high-performance liquid chromatography.

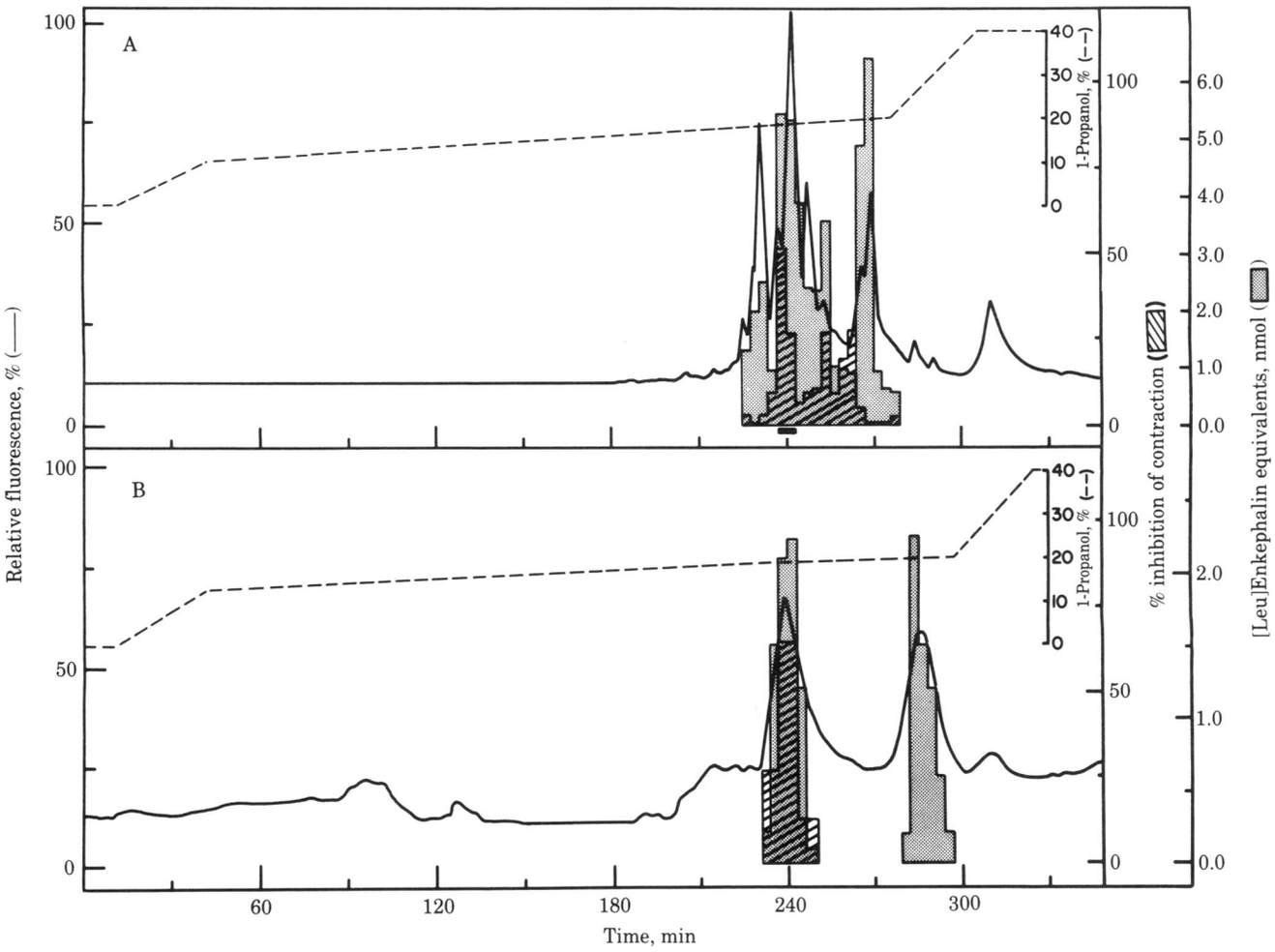

FIG. 1. Purification of the 3200-dalton enkephalin-containing peptide (peptide E). (*A*) Reverse-phase HPLC of partially purified material from Lichrosorb RP-18 column (ref. 5, peak E) on an Ultrasphere ODS column (5 μm; 10-nm pore size). Starting buffer was 0.5 M formic acid/0.4 M pyridine, pH 4.0, and elution of peptides was accomplished with a 1-propanol gradient (----). Flow rate was 20 ml/hr. Opiate receptor binding activity was determined after digestion with trypsin (100 μg/ml) and carboxypeptidase B (0.5 μg/ml), whereas opiate activity in the guinea pig ileum preparation was determined without prior enzymatic digestion. (*B*) Reverse-phase HPLC of guinea pig ileum-active material from ODS column (marked by black bar in *A*) on a diphenyl column (10 μm; 10-nm pore size).

that one (or more) of the adrenal enkephalin-containing polypeptides may possess unique activity of its own and may serve as an adrenal hormone(s). The heptapeptide [Met]enkephalin-Arg^6-Phe^7, which was first isolated from adrenal extracts, is indeed more active than enkephalin when injected intracerebroventricularly (21). However, none of the larger adrenal peptides isolated thus far possesses appreciable intrinsic opiate activity. By contrast, peptide E is a highly potent opiate agonist (Fig. 1).

A summary of the studies that were carried out on the guinea pig ileum longitudinal muscle preparation is shown in Table 1. Of all the adrenal peptides we have found, peptide E is unique. It is about 30 times more potent than [Met]enkephalin and β-endorphin and about half as active as $dynorphin_{1-13}$ (10), and its effects are completely reversed by naloxone (100 nM). Like $dynorphin_{1-13}$, the twitch inhibition produced by peptide E is reversed very slowly on washing the preparation ($t_{1/2} \approx 4$ min). The affinity of peptide E for the opiate receptor of NG-108 cells was found to be slightly lower than that of [Met]enkephalin or $dynorphin_{1-13}$ (Table 1).

Removal of the carboxyl-terminal residue Leu^{25} from peptide E did not alter its potency in the guinea pig ileum assay (Table 1). Similar treatment of free [Leu]enkephalin is known to reduce its opiate receptor affinity by more than 90% (22). Thus, the carboxyl-terminal [Leu]enkephalin moiety of peptide E does not contribute significantly to its opiate activity. Therefore, the amino-terminal [Met]enkephalin must be the major determinant of the peptide's potency. Similarly, both $dynorphin_{1-13}$ and α-neoendorphin (19), two peptides with potent opiate activity in the guinea pig ileum assay, contain an amino-terminal [Leu]enkephalin sequence (Fig. 2). The lower potency of peptide I, the precursor of peptide E, undoubtedly is due to the lack of such an exposed enkephalin sequence at the amino terminus (Table 1; Fig. 2). However, the presence of an amino-terminal enkephalin sequence does not ensure high opiate activity in the ileum or receptor assay, as can be seen for peptide F. This peptide also contains an amino-terminal [Met]enkephalin sequence (Fig. 2) but is a much weaker opiate agonist than even free [Met]enkephalin (Table 1).

The length of peptide E and the sequence of the residues subsequent to the [Met]enkephalin sequence must play important roles in determining biological activity. We have used carboxypeptidase Y to prepare peptide E_{1-12}, equivalent to BAM-12P (18). As was observed by Mizuno *et al.* (18), the activity of this peptide fragment in the guinea pig ileum assay was quite low (Table 1). As stated above, removal of the carboxyl-

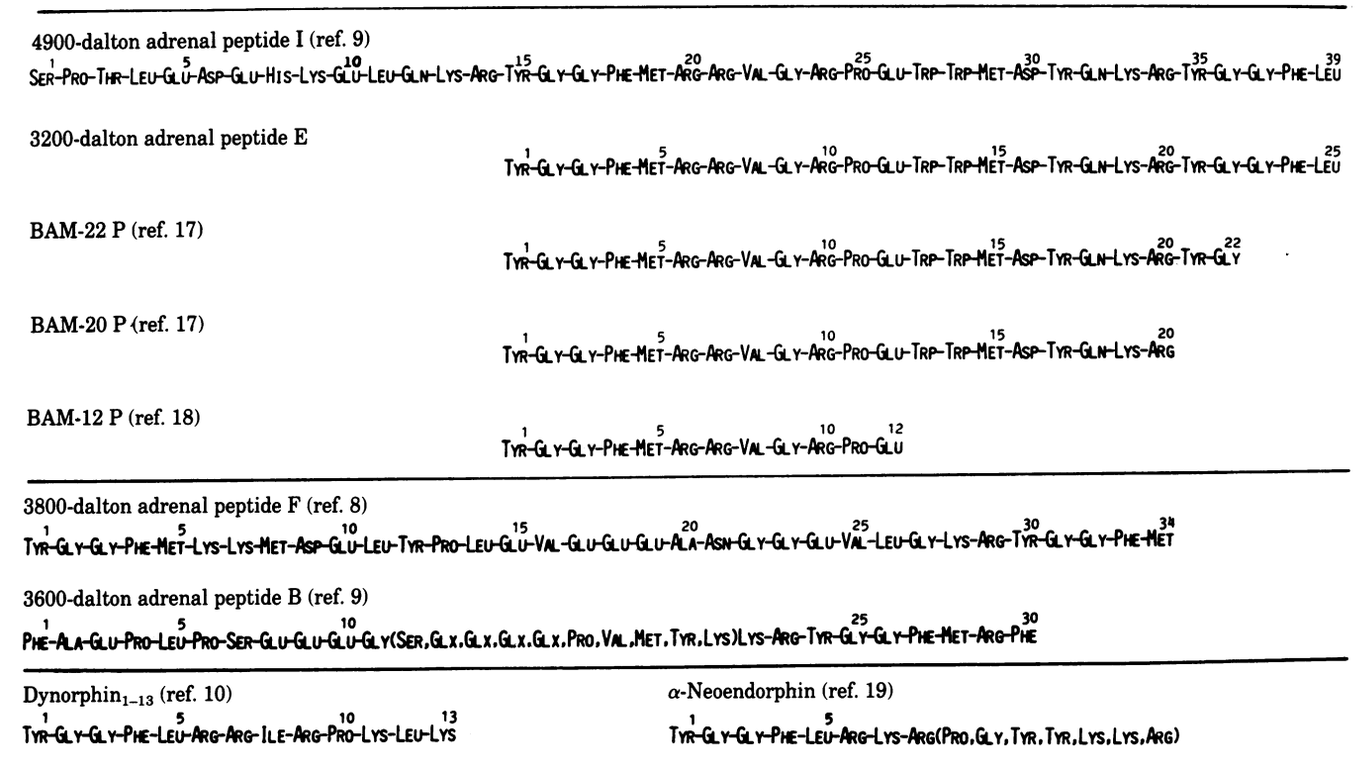

FIG. 2. Structures of some naturally occurring enkephalin-containing peptides.

terminal Leu from peptide E did not lower its activity appreciably. From the studies of Mizuno *et al.* (17), at least three residues can be removed from the carboxyl terminus (BAM-22P, Fig. 2) without much loss of opiate activity in the guinea pig ileum assay (Table 1). However, removal of five residues from the carboxyl terminus (BAM-20P, Fig. 2) reduces the activity of peptide E substantially (Table 1).

It is of interest that Mizuno *et al.* (17, 18) have not isolated peptides I and E, which we find in abundance. In turn, we have not observed BAM-12P, -20P, or -22P in our extracts of beef adrenal medulla. All of the peptide fragments that we have isolated can be produced by the enzymes normally operative in precursor processing in animal tissues [i.e., tryptic- and carboxypeptidase B-like activities (23)]. Two of the peptides isolated by Mizuno *et al.* (17, 18) were most likely generated from peptide E or peptide I by enzymes not generally involved in precursor processing (cleaving at a Gly-Gly bond and a Glu-Trp bond). Enzymes that cleave at such sites are probably not of physiologic import (23). In this instance, they actually destroyed the [Leu]enkephalin sequence at the carboxyl terminus of peptide E (Fig. 2). Differences in the pattern of peptides isolated by the two groups may be due to differences in the procedures for extracting the opioid peptides from beef adrenals. In our studies, we first purify chromaffin granules and free them from lysosomal contamination (6). The granules are then homogenized in a mixture of pepstatin and phenylmethylsulfonyl fluoride to inhibit proteolysis. Mizuno *et al.* (18) extract from homogenates of whole adrenal medullas and use only phenylmethylsulfonyl fluoride. Acidic lysosomal carboxypeptidase acting on peptide E could explain some of the BAM peptides reported by Mizuno *et al.* (17, 18).

Table 1. Biological activities of enkephalins and naturally occurring enkephalin-containing peptides

Peptide	IC_{50}, nM*	
	Guinea pig ileum assay	Receptor binding assay
[Met]Enkephalin	36	20
[Leu]Enkephalin	504	—
β-Endorphin	31	—
Dynorphin1–13	0.52	15
Adrenal opioid peptides		
Peptide I	>100	—
Peptide F	306	100
Peptide B	>200	—
Peptide E	0.96	40
Des-Leu^{25}-peptide E†	1.5	—
BAM-12P (ref. 18)	15.5	—
BAM-20P (ref. 17)	2.2	—
BAM-22P (ref. 17)	1.3	—

Values reported for both guinea pig ileum and receptor binding activities are from individual experiments. Similar results were found in three other experiments.

* Concentration that inhibits maximal response by 50%.

† The carboxyl-terminal residue of peptide E, Leu^{25}, was removed by digestion with carboxypeptidase Y as described (14). Analysis of the digest mixture demonstrated greater than 95% removal of Leu^{25}.

It is apparent that the processing of proenkephalin can lead to intermediates, such as peptide E and perhaps BAM-20P, that are far more active on isolated test systems than are the presumed end products [Met]enkephalin and [Leu]enkephalin. It should be noted that both dynorphin1–13 (10) and α-neoendorphin (19) must also arise from larger precursors. It is possible that they too are derived from the same proenkephalin (9) that gives rise to peptide E and all the other enkephalin-containing peptides, large and small, found in the adrenal medulla (1), brain (24, 25), and intestine (24).

Thus, free enkephalins may not be the only physiologically important end-products in the processing of proenkephalin. Peptide E, dynorphin1–13, α-neoendorphin, and BAM-20P merit further evaluation as unique physiologic regulatory substances.

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