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IDENTIFICATION OF THE OCTAPEPTIDE [MET]ENKEPHALIN -Arg^6-Gly^7-Leu^8 IN EXTRACTS OF BOVINE ADRENAL MEDULLA

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SUMMARY

The primary structure of the 5300 dalton adrenal enkephalin-containing polypeptide was shown to contain at its carboxyl terminus the sequence -Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu-COOH (Jones et al., (1981) Proc. Natl. Acad. Sci. USA, in press). From knowledge of the type of processing that occurs at paired basic amino acid residues such as -Lys-Arg-, it was predicted that the octapeptide Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu should be produced and exist in free form in the adrenal gland. This octapeptide has now been purified from bovine adrenal chromaffin granules. Its structure was determined by amino acid analysis, carboxypeptidase Y time course hydrolysis and sequential digestion with trypsin and carboxypeptidase B. The octapeptide has 35% the opiate receptor binding activity of [Met]enkephalin.

INTRODUCTION

Adrenal medullary chromaffin granules are a rich source of enkephalin-containing polypeptides (ECPs)[1] (1-8). A common feature of nearly all these ECPs is the bracketing of internal enkephalin sequences by paired basic residues (arginine and/or lysine). These are typical cleavage sites for processing enzymes and have been observed in many other prohormones (9). The only previous exception to such a bracketing among the ECPs was in a 3600 dalton polypeptide in which a [Met]enkephalin is preceded by paired basic residues (-Lys-Arg-) but is followed by the sequence -Arg-Phe-COOH (7). Since this sequence forms the carboxyl terminus of the polypeptide,

[1]ECP, enkephalin-containing polypeptide; HPLC, high performance liquid chromatography; ODS, octadecylsilane.

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processing should give rise to [Met]enkephalin-Arg^6-Phe^7, which is indeed present in free form in adrenal extracts (2,4). We have recently characterized a 5300 dalton ECP which has at its carboxyl terminus a [Met]enkephalin sequence preceded by a -Lys-Arg- linkage and followed by the sequence -Arg-Gly-Leu-COOH (10). By analogy, processing of this ECP should lead to the formation of a previously uncharacterized octapeptide, [Met]enkephalin-Arg^6-Gly^7-Leu^8. We now report the isolation of this octapeptide from bovine adrenal extracts and its characterization.

MATERIALS AND METHODS

Bovine adrenal chromaffin granules were isolated, extracted in acid, and the extracts chromatographed on Sephadex G-75, as previously described (1). Fractions from the low molecular weight region (<1000 daltons) were pooled and chromatographed on a Lichrosorb RP-18 HPLC column (4.6 x 250 mm, 5 μM particle size; Ace Scientific, Edison, NJ). Final purification was carried out on an Ultrasphere ODS column (4.6 x 250 mm, 5 μM particle size; Rainin Instrument, Ridgefield, NJ). A flow rate of 20 ml/hr was used. Gradients of 1-propanol in 0.5 M formic acid/0.4 M pyridine (pH 4.0) were used for column elution and column effluent was monitored for peptides using a fluorescamine detection system (11). Enkephalin-containing peptides were detected after digestion of fraction aliquots with trypsin and carboxypeptidase B (1,12) using a [Met]enkephalin radioimmunoassay with [^{125}I]-[Met]enkephalin as the competing ligand (Immunonuclear, MN).

Amino acid analyses were done at the picomole level using a fluorescamine amino acid analyzer (13). Carboxypeptidase Y time course hydrolysis was carried out according to Jones *et al.* (14). The opiate receptor binding activity of the purified octapeptide was determined using NG-108 cells and [^{3}H]-[Leu]enkephalin as the competing ligand (15).

RESULTS AND DISCUSSION

From previous experience, it seemed likely that a peptide with the structure [Met]enkephalin-Arg^6-Gly^7-Leu^8 would have a retention time on a reverse-phase column similar to that of [Met]enkephalin -Arg^6-Phe^7. A peak of opiate activity eluting just before the heptapeptide on HPLC had in fact been observed previously (4). At that time it was thought to be the methionine-sulfoxide form of [Met]enkephalin-Arg^6-Phe^7, although this was not verified. This peak of opiate activity that preceded the heptapeptide was therefore re-examined.

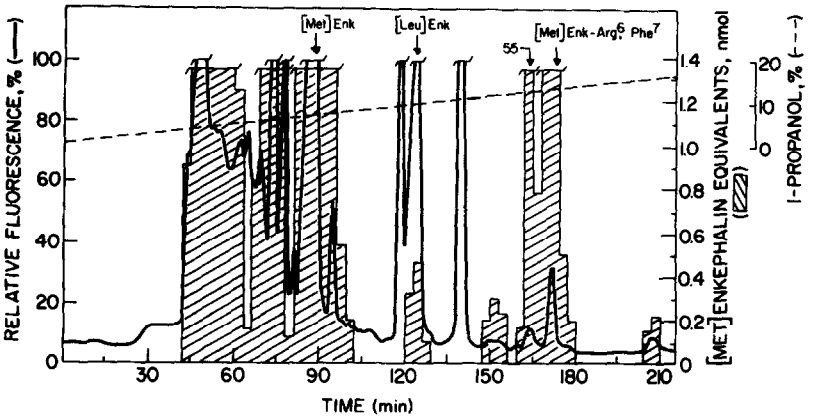

Fig. 1. HPLC of acid extracted low molecular weight material from bovine adrenal chromaffin granules on a Lichrosorb RP-18 column. Aliquots (5 µl) were removed from 1 ml fractions and digested with trypsin and carboxypeptidase B prior to being assayed for [Met]enkephalin. The elution positions of peptide standards and the enkephalin peak eluting in front of [Met]enkephalin-Arg^6-Phe^7 (fraction 55) are indicated by arrows.

Acid extracts of bovine chromaffin granules were separated according to size on a Sephadex G-75 column and the region containing the enkephalins and small ECPs was chromatographed on a Lichrosorb RP-18 column (Fig. 1). A peak of enkephalin activity was observed just before the elution position of [Met]enkephalin-Arg^6-Phe^7 (fraction 55). The material from this fraction was rechromatographed under isocratic conditions on an Ultrasphere ODS column as shown in Fig. 2. A sharp symmetrical peak of

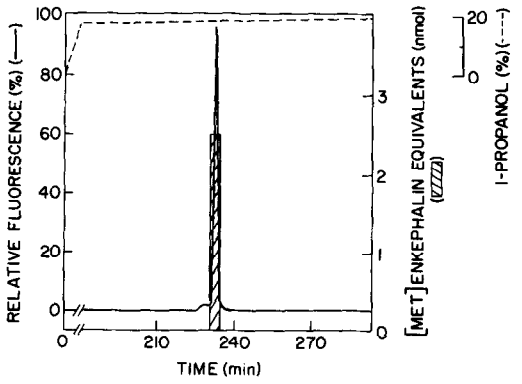

Fig. 2. Rechromatography of fraction 55 on an Ultrasphere ODS column. Fractions were assayed for [Met]enkephalin as before.

TABLE I

AMINO ACID COMPOSITIONS OF THE PURIFIED ECPs

Amino Acid	Ultrasphere ODS Column Fraction 78	Lichrosorb RP-18 Column Fraction 58
ASX	0.0	0.0
THR	0.0	0.0
SER	0.1	0.1
GLX	0.0	0.2
PRO	0.0	0.0
GLY	3.1	2.0
ALA	0.0	0.0
CYS	0.0	0.0
VAL	0.0	0.0
MET	0.9	1.0
ILE	0.0	0.0
LEU	1.1	0.0
TYR	0.9	1.0
PHE	1.0	1.9
HIS	0.0	0.0
LYS	0.0	0.0
ARG	1.0	1.0
TRP	0.0	0.0

100-200 pmol of peptide were hydrolyzed at 110°C for 22 hr in 150µl of constant boiling HCl containing 0.1% thioglycolic acid. Each value is the average of 2 analyses.

fluorescence was obtained, indicative of homogeneity. This peptide peak yielded free enkephalin after treatment with trypsin and carboxypeptidase B. Amino acid analysis of this purified material gave the composition Tyr_1, Gly_3, Phe_1,Met_1, Arg_1,Leu_1 (Table I). This is the expected composition of the octapeptide [Met]enkephalin-Arg^6-Gly^7-Leu^8. The near integral values found for each residue also suggest homogeneity. The purified peptide clearly is not oxidized [Met]enkephalin-Arg^6-Phe^7. The elution position of the reduced heptapeptide on Lichrosorb RP-18 chromatography (Fig. 1) was also verified by amino acid analysis (Table I).

To establish the sequence of amino acids at the carboxyl terminus of the purified peptide, enzymatic sequence analysis using carboxypeptidase Y was carried out (Fig. 3). Leucine was released rapidly followed by glycine, arginine, methionine and phenylalanine. These results indicate a carboxyl terminal sequence of -Phe-Met-Arg-Gly-Leu-COOH. To more firmly establish the presence of [Met]enkephalin within the structure of

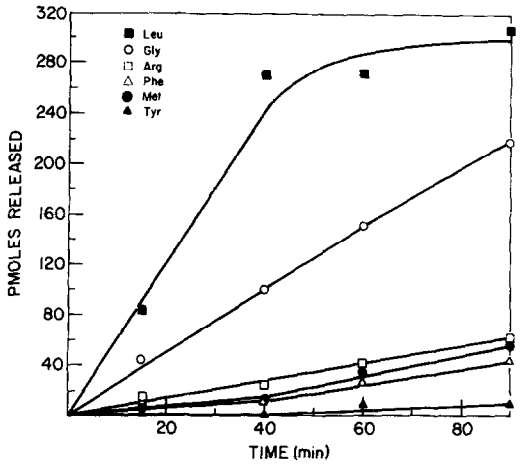

Fig. 3. Carboxypeptidase Y time course hydrolysis of putative octapeptide. The sample, 300 pmol, was incubated with 1 µg of enzyme in 50 µl of 100 mM NaOAc (pH 5.5). At the indicated time intervals, 5 µl aliquots were removed for amino acid analysis.

the peptide, 700 pmol were digested with trypsin and the digest chromatographed on a Lichrosorb RP-18 column (Fig. 4B). Two tryptic peptides were observed. One was shown by amino acid analysis to contain equimolar amounts (640 pmol each) of glycine and leucine, while the other comigrated with [Met]enkephalin-Arg^6. This is the expected tryptic cleavage pattern of the putative octapeptide. Further digestion of the peak corresponding to [Met]enkephalin-Arg^6 with carboxypeptidase B (Fig. 4C) converted it to [Met]enkephalin (590 pmol) as determined by its elution position, radioimmunoassay, and amino acid analysis (data not shown). The structure of the purified peptide is thus established as [Met]enkephalin-Arg^6-Gly^7-Leu^8.

The opioid activity of the intact octapeptide was determined by measuring its binding to the opiate receptor of cultured NG-108 cells. As shown in Table II, [Met]enkephalin-Arg^6-Gly^7-Leu^8 exhibits significant binding activity, approximately 35% as much as [Met]enkephalin. This should not be interpreted to mean that the octapeptide is less active than [Met]enkephalin *in vivo*. [Met]enkephalin-Arg^6-Phe^7 has 40% the binding activity of the free enkephalins to the same cells, yet possesses

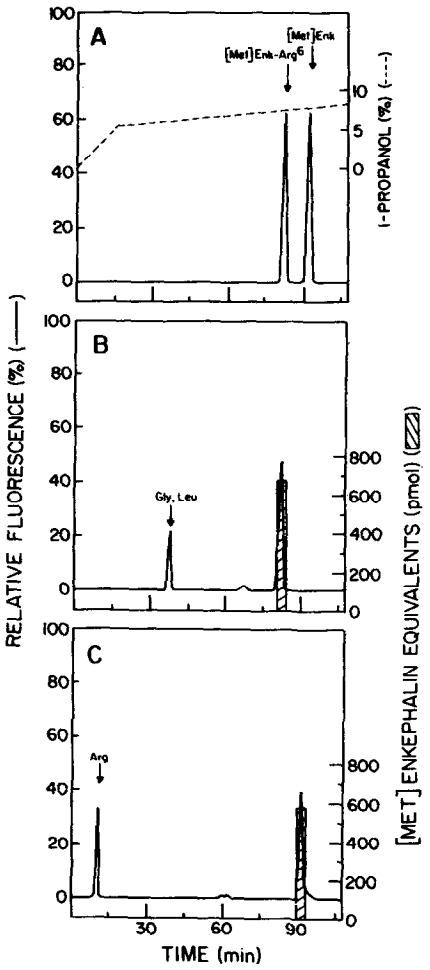

Fig. 4. Sequential digestion of the putative octapeptide with trypsin and carboxypeptidase B. Digests were chromatographed on a Lichrosorb RP-18 column. (A) The elution positions of [Met]enkephalin-Arg^6 and [Met]enkephalin standards are indicated. (B) The elution profile after digestion of the putative octapeptide (700 pmol) with trypsin (1 µg). (C) Digestion of the ECP produced by trypsin with carboxypeptidase B (0.1 µg).

significantly greater analgesic activity than the enkephalins when injected intraventricularly (4,16).

The amounts of the octapeptide in bovine chromaffin granules were found to be approximately half that of [Met]enkephalin-Arg^6-Phe^7 based on [Met]enkephalin content. This is not very different from the ratio found between what was originally thought to be oxidized [Met]-enkephalin-Arg^6-Phe^7 and the reduced heptapeptide by Stern et al. in

TABLE II

OPIATE RECEPTOR ACTIVITY OF [MET]ENKEPHALIN-ARG^6-GLY^7-LEU^8

Peptide	IC_{50}(nM)*
[Met]enkephalin-Arg^6-Gly^7-Leu^8	17.0
[Met]enkephalin	6.2

*Concentration of peptide that displaces 50% of receptor-bound [^{3}H]-[Leu]enkephalin. Peptide concentrations were determined by amino acid analysis as described in Methods.

bovine adrenal medulla (188/243 pmoles/gm) (4). An opioid peptide with a retention time on HPLC identical to [Met]enkephalin-Arg^6-Gly^7-Leu^8 has also been found in bovine striatum (4) and in guinea pig adrenal, myenteric plexus, and striatum (unpublished observations). The presence of this unique octapeptide in species as divergent as cow and guinea pig is further evidence of the ubiquitous nature of proenkephalin and the ECPs produced from it (12). Furthermore, since the octapeptide is apparently produced by recognized processing at the -Lys-Arg- linkage which preceeds it, it may have been designed for some specialized physiological function.

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