Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 630-636

MET-ENKEPHALIN-ARG^6-PHE^7 METABOLISM : CONVERSION TO MET-ENKEPHALIN BY BRAIN AND KIDNEY DIPEPTIDYL CARBOXYPEPTIDASES

Myron Benuck, Martin J. Berg and Neville Marks

Center for Neurochemistry, Rockland Research Institute
Ward's Island, New York 10035

Received February 17, 1981

SUMMARY : Enzymes degrading Met-enkephalin-Arg^6-Phe^7, an endogenous brain peptide with enhanced opiate activity in vivo, were isolated from membrane preparations of rabbit kidney and brain, and their specificity compared. A preparation from kidney or brain containing the angiotensin converting enzyme (EC 3.4.15.1) released with time Arg-Phe, Met-enkephalin, Phe-Met and Tyr-Gly-Gly. Kinetic analysis revealed a product precursor relationship with conversion of hepta- to pentapeptide (Met-enkephalin) followed by release of Tyr-Gly-Gly and Phe-Met indicating sequential cleavage at the Met^5-Arg^6 and Gly^3-Phe^4 bonds. A second preparation devoid of angiotensin converting enzyme activity released the same products and in addition a tetrapeptide Phe-Met-Arg-Phe. Release of products with time indicated cleavage at Gly^3-Phe^4 by an endopeptidase and at the Met^5-Arg^6 and Gly^3-Phe^4 bonds by a dipeptidyl carboxypeptidase. The dipeptidyl carboxypeptidases thus provide a mechanism for the formation of Met-enkephalin from a potential precursor.

INTRODUCTION

The finding of enkephalins with C-terminal extensions such as Met-enkephalin-Arg^6-Phe^7 has focused interest on mechanisms available for their conversion and inactivation. This peptide is present in corpus striatum at levels exceeding those of Leu-enkephalin (1,2) is more potent than Met-enkephalin as an analgesic agent in vivo (1), and acts on a different subpopulation of opiate receptors (3). As a result its metabolism is of equal importance to that of the pentapeptide enkephalins. The possibility exists also that this peptide acts as a biosynthetic precursor of Met-enkephalin itself. Such a conversion may be mediated by dipeptidyl carboxypeptidases associated with particulates of brain or other tissue (4-7). Previously, we described a procedure for the separation of two distinct

Abbreviations used : ACE, Angiotensin converting enzyme ; HPLC, high performance liquid chromatography.

0006-291X/81/060630-07$01.00/0

dipeptidyl carboxypeptidases (4), but their role in the conversion or metabolism of larger enkephalins was not investigated. In this study we provide evidence that one of these enzymes, the angiotensin converting enzyme (ACE, EC 3.4.15.1) can convert the heptapeptide into the pentapeptide form of enkephalin. The second dipeptidyl carboxypeptidase preparation acts in a similar manner but in addition contains a membrane bound endopeptidase capable of inactivating the heptapeptide by cleavage at the Gly^3-Phe^4 bond with release of the C-terminal tetrapeptide Phe-Met-Arg-Phe.

METHODS

Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 were purchased from Bachem Fine Chemicals (Torrance, CA). A supply of antiserum to rabbit lung ACE was kindly provided by RL Soffer (Cornell Univ. Med. Ctr. N.Y., NY).

An extract from the crude mitochondrial fraction from rabbit brain or kidney was prepared and enzyme solubilized using Triton X-100. The extract was chromatographed on a DE 53 cellulose column followed by IgG-Sepharose using published procedures (8,9). Immunoaffinity chromatography was successful in separating ACE from other dicarboxypeptidase activity ; ACE was completely adsorbed to the IgG-Sepharose, and was assayed in its immobilized form while the unadsorbed material contained the dipeptidyl carboxypeptidase B activity previously described (4). Enzyme was stored at 4°C or frozen until use ; enzyme activity was retained for periods of 6 months or longer.

Enzyme assays. Reaction mixtures of 0.2 - 0.4 ml 20 mM Tris-HCl buffer, pH 7.6, contained 50-100 nmol of heptapeptide substrate, 60 - 120 µmol of NaCl, and 0.1 µg of purified ACE or 1-3 µg of the dipeptidyl carboxypeptidase preparation, and were incubated up to 120 min at 37°C. Aliquots of 50 µl were withdrawn at appropriate time intervals and used for analysis by HPLC. Cleavage products were eluted from a C-18 µBondapack column (Waters Assoc., Milford, MA) using a 0.1 M potassium phosphate buffer, pH 3.0, followed by a 0-60% acetonitrile gradient generated by the method of Lewis (10). Peaks were monitored at 210 nm and identified by their retention time compared to known standards or verified by amino acid analysis following hydrolysis for 18 hr with 6N HCl. The instrument set at its highest sensitivity was capable of detecting 0.05 nmol of product. The products Tyr-Gly-Gly (C_3) and Arg-Phe (C_2) were eluted isocratically ; Phe-Met (C_2), Phe-Met-Arg-Phe (C_4) Met-enkephalin (C_5) and Met-enkephalin-Arg^6-Phe^7 (C_7) were eluted after application of the acetonitrile gradient (Fig. 1 and 2), The heptapeptide was eluted last and was accompanied by a smaller peak. The latter peak was not altered during incubation with enzyme, and lacked a distinctive amino acid composition. Amino acid analysis of the peaks gave ratios agreeing with theoretical ; however values for methionine were low either from losses during hydrolysis or its presence as the sulfone or sulfoxide derivative.

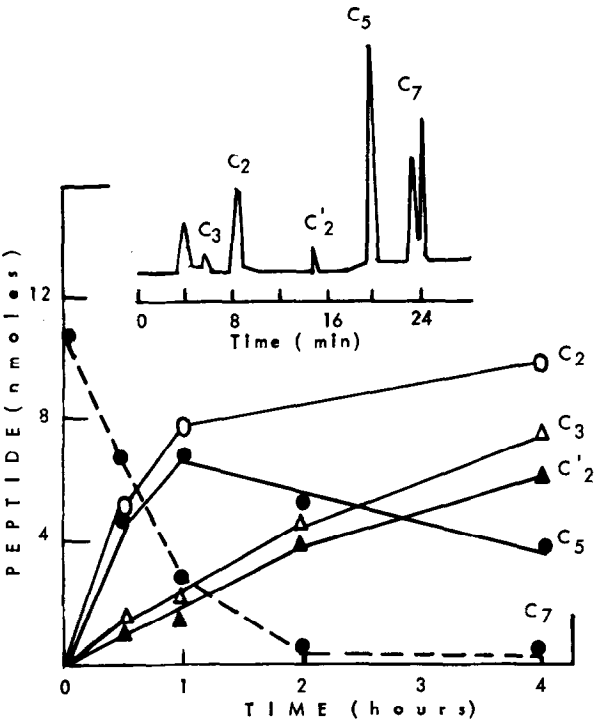

FIGURE 1. Time course of Met-enkephalin-Arg^6-Phe^7 (C_7) breakdown after incubation with purified kidney ACE and analysis by HPLC. The inset above represents the HPLC profile obtained after a 60 min incubation at 37°C. Values shown represent that present in 50 µl aliquots withdrawn from the incubation medium for analysis. In the figure, C_3 represents Tyr-Gly-Gly ; C_2, Arg-Phe; C'_2, Phe-Met; C_5 ; Met-enkephalin.

RESULTS AND DISCUSSION

Immobilized ACE purified from rabbit kidney degraded Met-enkephalin-Arg^6-Phe^7 with release of four products as detected by HPLC (Fig. 1). Their composition as determined from retention times compared to standards and by amino acid analysis were Tyr-Gly-Gly, Arg-Phe, Phe-Met and Met-enkephalin. The release of peptides with time followed a product-precursor relationship with evidence for the formation of Met-enkephalin as an intermediate followed by its cleavage to form Tyr-Gly-Gly and Phe-Met. Similarly, incubation of heptapeptide with purified ACE obtained from brain led also to the release sequentially of the C-terminal dipeptides with formation of Met-enkephalin as an intermediate. After a 30 min and 60 min incubation under the conditions

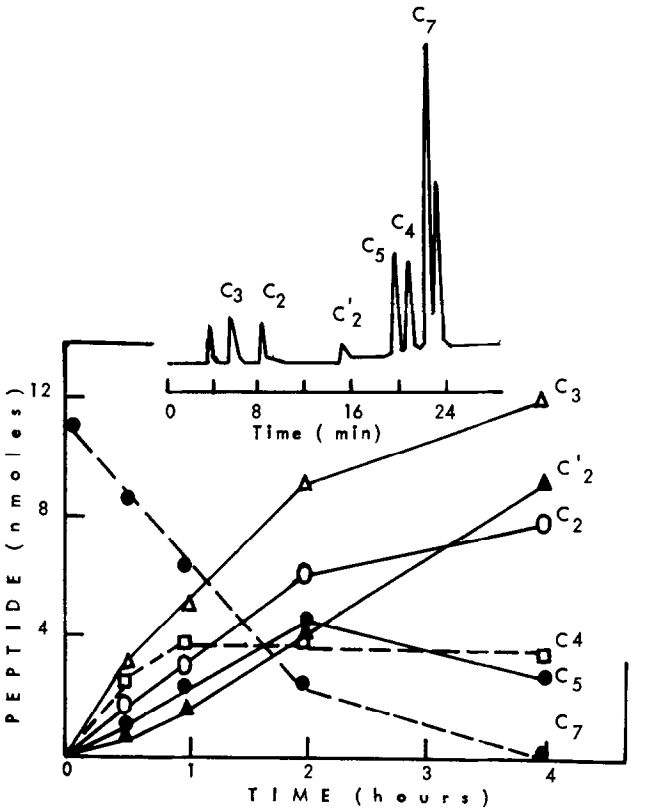

FIGURE 2. Time-course of Met-enkephalin-Arg^6-Phe^7 (C_7) breakdown after incubation with the dipeptidyl carboxypeptidase B preparation (see text). The inset above represents the HPLC profile obtained after a 60 min incubation at 37°C. In the figure, C_4 represents Phe-Met-Arg-Phe and for other details see Fig. 1.

selected, 12 and 26 nmol of Met-enkephalin were formed from 50 nmol of heptapeptide (Table 1). The larger quantities of Arg-Phe released (11 and 30 nmol) relative to Phe-Met (2.5 and 4.7 nmol) indicated that sequential release of dipeptides had occurred. Cleavage at the Met^5-Arg^6 bond would result in conversion of the heptapeptide into Met-enkephalin, also an active opiate peptide, but further action at the Gly^3-Phe^4 bond would result in inactivation since the products formed lack the enkephalinergic determinants (11).

The kidney enzymes remaining after removal of ACE by immunoaffinity chromatography and shown previously to contain a distinct dipeptidyl carboxypeptidase inactivating

Table 1. Release of peptides from Met-enkephalin-Arg^6-Phe^7 upon incubation with membrane bound ACE or unbound enzyme preparation from rabbit brain.

Product	Enzyme Activity			
	A		B	
	Products as per cent of substrate			
	30 min	60 min	30 min	60 min
Arg-Phe	22	59	4.3	8.8
Phe-Met	5.1	9.5	1.2	3.1
Tyr-Gly-Gly	6.0	9.5	7.1	18.4
Phe-Met-Arg-Phe	-	-	10.9	21.8
Met-enkephalin	25	53	8.0	11.0

The incubation mixture of 0.2 ml of 20 mM Tris-HCl buffer, pH 7.6, contained 50 nmol of heptapeptide, 60 µmol of NaCl, and 0.1 - 3 µg of enzyme protein. After a 30 and 60 min incubation, 50 µl aliquots were withdrawn and analyzed by the HPLC procedure described in the Methods section. In the Table, A refers to purified brain ACE, and B refers to the separate dipeptidyl carboxypeptidase preparation after removal of ACE by immunoadsorption (4). The values shown represent the products found by HPLC expressed as a per cent of substrate.

Met-enkephalin, released five rather than the four products when incubated with heptapeptide (Fig. 2). Four of the products were identical to those described above for ACE ; the composition of the fifth product was Phe-Met-Arg-Phe. The presence of the tetrapeptide suggested cleavage at the Gly^3-Phe^4 bond by an endopeptidase-like enzyme present in the preparation. This was a major site of cleavage as indicated by the amounts of tetrapeptide and Tyr-Gly-Gly found at the earlier incubation times relative to the other products. There was evidence also for action of a dipeptidyl carboxypeptidase since Met-enkephalin and Arg-Phe were formed concomitantly as products. Further cleavage of Met-enkephalin itself by the endopeptidase was discounted since earlier studies showed that modified enkephalins such as Met-enkephalinamide and Z-Met-enkephalinamide were not substrates (12). Failure to detect cleavage of an N-protected enkephalin

analog would exclude also the formation of Tyr-Gly-Gly by an aminopeptidase acting on the N-terminus of polypeptides with release of tripeptidyl units (13).

Incubation of Met-enkephalin-Arg^6-Phe^7 with a similar enzyme preparation from brain indicated that the same mechanisms were involved as that described for the kidney (Table 1). The release of Tyr-Gly-Gly and Phe-Met-Arg-Phe indicated that brain membrane fractions also contained an endopeptidase acting directly on the Gly-Phe bond. Also, the presence of Arg-Phe and Met-enkephalin indicated action of a dipeptidyl carboxypeptidase. The released tetrapeptide also served as a substrate since its levels did not continue to increase at later time points.

The non-ACE dipeptidyl carboxypeptidase is comparable to an 'enkephalinase' known to act on the C-terminus of enkephalins leading to their inactivation (6,7). We have proposed the alternative nomenclature of 'dipeptidyl carboxypeptidase B' for this enzyme since it is nonspecific and acts on other substrates. Cleavage of the Met^5-Arg^6 bond of the heptapeptide by this enzyme and by ACE can be viewed as a conversion processes rather than inactivation since release of the C-terminal dipeptide generated Met-enkephalin. In this respect the heptapeptide is seen as a biosynthetic precursor of the pentapeptide. The Met^5-Arg^6 bond may be accessible to cleavage also by the endopeptidase but additional purification is required to test this possibility. Cleavage at this site implies that enzymes involved in conversion recognize the C-terminal dipeptide Arg-Phe of the heptapeptide. It might be noted, however, that the presence of a charged group (Arg) in the penultimate position is contrary to the substrate requirements for the postulated active center for enkephalinase present on striatal membranes (14). This could suggest that alternate catalytic mechanisms are involved in conversion of the heptapeptide as compared to the pentapeptide.

Acknowledgements : This work was supported in part by grants from New York State Health Planning Commision, HRC, 9-013 and U.S.P.H.S. NS-12578.

REFERENCES

1. Rossier, J., Audigier, Y., Ling, N., Cros, J., and Udenfriend, S. (1980) Nature 288, 88-90.
2. Stem, A.S., Lewis, R.V., Kimura, S., Rossier, J., Gerber, L.D., Brink, L., Stein, S., and Udenfriend, S. (1979) Proc. Natl. Acad. Sci. (U.S.A.) 76, 6680-6683.
3. Audigier, Y., Mazarquil, H., Rossier, J. and Cros, J. (1980) Eur. J. Pharmacol. 68, 237-238.
4. Benuck, M. and Marks, N. (1980) Biochem. Biophys. Res. Commun. 95, 822-828.
5. Benuck, M. and Marks, N. (1979) Biochem. Biophys. Res. Commun. 88, 215-221.
6. Gorenstein, C. and Snyder, S.H. (1979) Life Sci. 25, 2065-2070.
7. Swerts, J.P., Perdrisot, R., Patey, G., De La Baume, S., and Schwartz, J.C. (1979) Eur. J. Pharmacol. 57, 279-281.
8. Marks, N., Datta, R.K. and Lajtha, A. (1968) J. Biol. Chem. 243, 2882-2889.
9. Das, M. and Soffer, R.L. (1976) Biochemistry 15, 5088-5094.
10. Lewis, R.V. (1979) Anal. Biochem. 93, 142-145.
11. Morgan, B.A., Smith, C.F.C., Waterfield, A.A., Hughes, J. and Kosterlitz, A.W. (1976) J. Pharm. Pharmacol. 28, 660-661.
12. Benuck, M., Berg, M.J. and Marks, N. (1981) Life Sci. In Press.
13. Aoyagi, T., Suda, H., Nagai, M., Tobe, H., Suzuki, J., Takeuchi, T. and Umezawa, H. (1972) Biochem. Biophys. Res. Commun. 80, 435-442.
14. Llorens, C., Gacel, G., Swerts, J.-P., Peridrisot, R., Fournie-Zaluski, M.-C., Schwartz, J.-C., Roques, B.P. (1980) Biochem. Biophys. Res. Commun. 96, 1710-1716.