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CHARACTERIZATION OF A DISTINCT MEMBRANE BOUND DIPEPTIDYL CARBOXYPEPTIDASE INACTIVATING ENKEPHALIN IN BRAIN

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SUMMARY: A dipeptidyl carboxypeptidase distinct from the angiotensin converting enzyme (EC 3.4.15.1) was isolated from membrane preparations of rabbit brain. The enzyme cleaved enkephalin at the Gly-Phe bond, releasing either Phe-Leu from Leu-enkephalin or Phe-Met from Met-enkephalin, and also acted on bradykinin, releasing the terminal dipeptide Phe-Arg. In contrast to the converting enzyme, however, this dipeptidyl carboxypeptidase did not act on angiotensin-I, and it did not degrade hippuryl-His-Leu. Chloride ions did not affect its activity, but the enzyme was inhibited by metal chelating agents. The enzyme was not inhibited by captopril (SQ 14225)or by SQ 20881. Kinetic studies indicated a Km for this enzyme of 0.14 mM with Leu-enkephalin and 0.12 mM with bradykinin as substrates. Present data indicate that more than one enzyme is present in brain membrane fractions acting as dipeptidyl carboxypeptidases inactivating enkephalin; these data suggest multiple roles for such enzymes in the regulation of peptide metabolism.

INTRODUCTION

Inactivation of enkephalin by membrane bound dipeptidyl carboxypeptidases has been reported by several groups (1-6), and claims have been made that brain contains a distinct C-terminal cleaving enzyme recognizing only enkephalins (2,4). To establish clearly if brain contains a unique dipeptidyl carboxypeptidase we chose to exploit the availability of an immunoaffinity procedure to separate the angiotensin converting enzyme (EC 3.4.15.1) (ACE) from other membrane bound enzymes inactivating Met- or Leu-enkephalin by releasing the C-terminal dipeptide. In the present paper we report on the presence of a second dipeptidyl carboxypeptidase which is inactive towards angiotensin-I but which cleaves enkephalin and bradykinin with release of the C-terminal dipeptide and exhibits properties that are distinct from ACE.

Abbreviations used: ACE, Angiotensin converting enzyme ; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; HEPES, N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid.

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METHODS

Enzyme purification. The purification procedure is a modified version of that previously described (1). Briefly, a crude mitochondrial fraction was isolated from 20 g of rabbit brain and extracted with 10 vol of 40 mM Tris-HCl buffer, pH 7.6, containing 0.2% Triton X-100. This extract was applied to a DEAE cellulose column (15 x 2 cm) previously equilibrated with the same buffer containing detergent. After elution of unadsorbed material, a gradient of 0 to 0.15 N NaCl was applied to the column according to the procedure of Marks et al. (7), followed by elution with 0.2 N NaCl. Fractions collected were assayed using Met-enkephalin as a substrate. Active fractions were pooled and concentrated by ultrafiltration using an Amicon diaflo unit with a XM 100 filter (Amicon Instr., Lexington MA) and then passed through an IgG-Sepharose column prepared by coupling antibody directed towards purified rabbit lung ACE as described previously (1,8). Enzyme immobilized by the column together with the effluent material was then assayed with several peptide substrates, as described below.

Enzyme assays. Angiotensin converting enzyme was assayed in the various fractions using hippuryl-His-Leu as a substrate; His-Leu release was determined by a fluorometric procedure as previously described (9). Enkephalin degradation was determined using an automated ninhydrin procedure (10) capable of detecting enzymatic activity acting either on the N-terminus or internally. In the latter case, 50 nmol of Met-enkephalin was incubated with an appropriate aliquot of enzyme in 50 mM HEPES buffer, pH 8.0, containing 60 μmol of NaCl in a total volume of 0.2 ml; the reaction was stopped after 1-2 h by addition of 0.4 M sodium acetate buffer, pH 5.5. Aminopeptidase activity was assayed using either Leu-Gly-Gly or leucyl-β-napthylamide as substrate, followed by ninhydrin analysis or by fluorometric detection of released napthylamine (7,11). For comparative studies on the breakdown of oligopeptides, 50-100 nmol of substrate (Leu- or Met-enkephalin, angiotensin-I, or bradykinin) was incubated with enzyme in 0.2 ml of 40 mM Tris-HCl buffer, pH 7.6, containing 60 μmol of NaCl. Incubations were terminated after 2 h by boiling; products were analyzed by thin layer chromatography or high performance liquid chromatography. In the former case, samples were developed on silica gel plates in isopropanol : ethyl acetate : 5% acetic acid (2:2:1), visualized by spraying with fluorescamine, and identified by the use of known standards.

Dipeptides and other products were also separated and quantitated by reverse phase high pressure liquid chromatography using a C-18 Bondapack column (Waters, Assoc., Milford MA). Products such as Tyr, Tyr-Gly-Gly, His-Leu, or Phe -Arg were eluted isochratically using either 0.1 M potassium phosphate buffer, pH 3.0, or 0.05 M potassium phosphate buffer, pH 2.1, containing 15% methanol. Other peptides (enkephalin, bradykinin, angiotensin-I) were eluted using a 0-60% acetonitrile gradient, generated using the programming device of Lewis (12). Peaks were monitored at 210 nm and identified by their retention time as compared to known standards, or by amino acid analysis following hydrolysis with 6 N HCl for 18 h at 105°C. Protein was measured by the Folin-Lowry procedure with bovine serum albumin as a standard (13).

RESULTS AND DISCUSSION

Enkephalinases (defined as enzymes degrading Met- or Leu-enkephalin) were purified from rabbit brain particulates by extracting with a hypotonic buffer containing

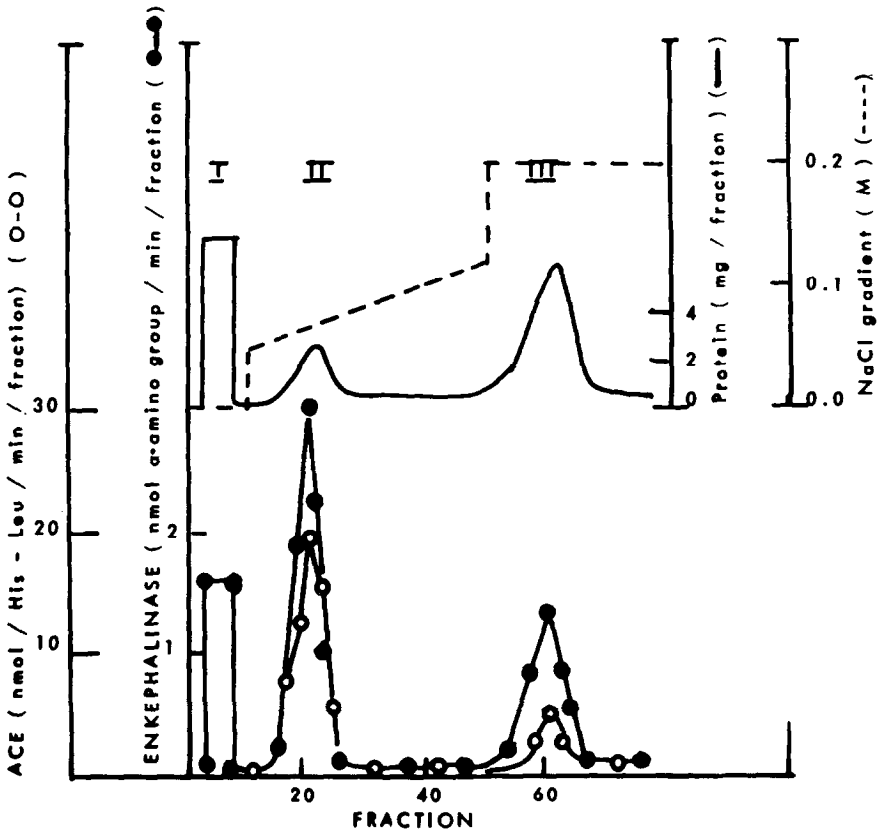

Figure 1: Elution profile of membrane bound enzymes degrading enkephalin (●———●) as compared to the hydrolysis of the ACE substrate hippuryl-His-Leu (o———o). Enzyme was extracted from the P2 fraction of rabbit brain with 40 mM Tris-HCl buffer, pH 7.6, containing 0.2% Triton X-100 and placed on a DEAE cellulose column equilibrated with the same buffer. Elutions were made with a salt gradient (- -). Protein present in the peaks is indicated in the upper half of the figure. Activity present in peak II was selected for immunoaffinity chromatography utilizing a Sepharose-IgG column prepared with antibody to rabbit lung ACE as described in the text.

detergent followed by ion-exchange chromatography on DEAE cellulose. Incubation of fractions with Met-enkephalin revealed three peaks of activity, eluted with the starting buffer, 0.05-0.1 N and 0.2 N NaCl respectively (peaks I to III, Fig. 1). Of these, peak II contained the larger amount of enzyme (s) hydrolyzing the ACE-substrate hippuryl-His-Leu. To purify the ACE further and to decide whether peak II contained other dipeptidyl carboxypeptidases distinct from ACE, the enzyme preparation was subjected to immunoaffinity chromatography by the procedures described above.

In agreement with previous findings, immunoaffinity removed all ACE-like enzymes from peak II, based on absence of activity in the unadsorbed effluent material

towards angiotensin-I, and hippuryl-His-Leu. The effluent of peak II, however, still retained activity towards Met- and Leu-enkephalin, as shown by the release of Tyr-Gly-Gly and the C-terminal dipeptide (Phe-Leu or Phe-Met) as products (detected by both TLC and HPLC). In addition to the pentapeptides, the effluent also cleaved bradykinin, with release of the C-terminal dipeptide Phe-Arg. With enkephalin as substrate, the rate of degradation found under the conditions of the assay used was 0.7nmol per min per mg protein, with a Km of 0.14mM; for bradykinin the Km was 0.12mM. From the rates of Tyr-Gly-Gly release as measured by HPLC for bound and unbound enzyme, the ratio of the ACE-like to the non-ACE dipeptidyl carboxypeptidase was 3:1. Comparison of the properties of the two enzymes revealed a number of differences, the chief ones being the absence of activity with hippuryl-His-Leu and angiotensin-I for the unbound enzyme and the absence of effects by ACE-inhibitors SQ 14225 and SQ 20881 at 0.001-0.005mM concentrations (Tables 1 and 2). With respect to other added materials, both enzymes were inhibited by the metal sequestering agents EDTA and o-phenanthroline, and the unadsorbed enzyme was inhibited more strongly by bacitracin. No effects were observed on addition of puromycin, and no effects were noted on the hydrolysis of enkephalin in the presence of added chloride ion (Table 2).

Examination of the three DEAE-cellulose peaks for other potential enkephalinases indicated the presence in peaks I and III of two different aminopeptidases both capable of cleaving the Tyr-Gly bond with release of Tyr and Gly-Gly-Phe-Met as products (as detected by TLC and HPLC). Enzyme in peak I hydrolyzed Leu-Gly-Gly and that in peak III hydrolyzed aminoacylated napthylamides such as Leu-β-napthylamide. Recently, it has been shown that aminopeptidases purified by means of an arylamide substrate can act as enkephalinases by cleavage of the Tyr-Gly bond (14,15) and thus this enzyme could account for the enkephalinases found in the IgG-unadsorbed enzyme present in peak III. The aminopeptidase in peak I has not previously been described and

Table 1. Cleavage of Polypeptides by Dipeptidyl Carboxypeptidases A and B.

Substrate	Product Assayed	Dipeptidyl Carboxypeptidase Activity (nmol/h) A	B
Hippuryl-His-Leu	His-Leu	26	< 1
Angiotensin-I	His-Leu	1.6	< 0.1
Bradykinin	Phe-Arg	1.8	0.8
Leu-enkephalin	Tyr-Gly-Gly	1.1	0.8

Dipeptidyl carboxypeptidase A and B refers to immobilized ACE and a second enzyme cleaving C-terminal dipeptide of enkephalin respectively. Enzyme was purified from rabbit brain and incubated with the polypeptides noted above. The reaction mixture of 0.2 ml contained approximately 0.2 μg of immunoaffinity purified ACE or 20 μg of protein from the unadsorbed effluent of peak II containing dipeptidyl carboxypeptidase B, incubated for 2 h at 37°C, and the products then assayed as described in the text. Products were assayed either fluorometrically (hippuryl-His-Leu) or by HPLC. Values are the means of 3-5 determinations agreeing within 15%.

Table 2: Effect of Inhibitors on Activity of Dipeptidyl Carboxypeptidases A and B

Additions	Conc. (mM)	Relative Activity A	B
Chloride	300	100	100
EDTA	1.0	40	60
o-Phenanthroline	0.1	40	50
SQ 20881	0.001 - 0.005	10	100
SQ 14225	0.001 - 0.005	0	90
Puromycin	1.0	95	90
Bacitracin	1.0	50	30
	0.1	100	50

Values are expressed relative to assays performed without any additions (see Table 1). A and B refer to ACE and a separate dipeptidyl carboxypeptidase cleaving Leu-enkephalin with release of Tyr-Gly-Gly (for details see Table 1 and text). The values for effects of SQ 20881 and SQ 14225 represent those found for the two concentrations listed. Values were determined by the quantitative measurement of Tyr-Gly-Gly by HPLC and are the means of 2-4 determinations agreeing within 15%.

represents an alternative mechanism for enkephalin degradation. The effluent of peak II also contained an aminopeptidase that could be assayed with Leu-Gly-Gly, but, in this case, the enzyme did not degrade enkephalin. As such it might be comparable to an aminotripeptidase purified in this laboratory from the cytosol using Leu-Gly-Gly as substrate, and which is inactive when incubated with various polypeptides including enkephalins, kinins and angiotensins (16).

The present study has demonstrated that rabbit brain contains two distinct dipeptidyl carboxypeptidases, one of which resembles the angiotensin converting enzyme (dipeptidyl carboxypeptidase A is proposed for an alternative nomenclature) and one which is distinct in its properties but which degrades enkephalin by a similar mechanism (dipeptidyl carboxypeptidase B). A role for dipeptidyl carboxypeptidase B in the central nervous system has not been established but account must be taken of the fact that it is one of many potential membrane bound and soluble enzymes that can degrade enkephalin. Others include membrane bound aminopeptidases and ACE itself. Information is required on the localization of all these enzymes in order to evaluate their role in cerebral peptide regulation. It might be noted that the dipeptidyl carboxypeptidase B preparation is not specific for enkephalin but also degrades bradykinin. It will be of interest, therefore, to decide whether dipeptidyl carboxypeptidase B contains the same site recognizing the terminal dipeptides Phe-Leu or Phe-Met of enkephalins or Phe-Arg of bradykinin as that recently proposed by Cheung et al. (17) for dipeptidyl carboxypeptidase A purified from lung.

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