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CO-IDENTITY OF BRAIN ANGIOTENSIN CONVERTING ENZYME WITH A MEMBRANE BOUND DIPEPTIDYL CARBOXYPEPTIDASE INACTIVATING MET - ENKEPHALIN

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SUMMARY: The distribution in rat brain of angiotensin converting enzyme (EC 3.4.15.1) using hippuryl-His-Leu as substrate was identical to a dipeptidyl carboxypeptidase present in membranes assayed with Met-enkephalin as substrate. Highest activity occurred in pituitary, followed by cerebellum, corpus striatum, midbrain, pons-medulla, hypothalamus, cerebral cortex and spinal cord. The ratio of products His-Leu/Tyr-Gly-Gly was identical for all regions but differed from His-Leu/Tyr. Angiotensin converting enzyme purified by immunoaffinity chromatography gave a K_m for hippuryl-His-Leu of 0.5mM and for Met-enkephalin of 0.1mM. In the presence of the specific inhibitor of angiotensin converting enzyme, SQ 14,225, the Ki value was 10^{-7}M. Present data point to the co-identity of brain angiotensin converting enzyme with the dipeptidyl carboxypeptidase inactivating enkephalin.

INTRODUCTION

The potent behavioral and analgesic actions of opiate peptides have led to considerable interest in the role of proteolytic enzymes in regulating their diverse activities. In the case of the pentapeptides Leu and Met-enkephalin that occur in brain and pituitary, two mechanisms have been suggested: 1) inactivation by cytoplasmic and membrane bound aminopeptidases leading to liberation of Tyr, and a tetrapeptide, followed by release of all constituents (1), and 2) inactivation by a membrane bound dipeptidyl carboxypeptidase with release of Phe-Met and Tyr-Gly-Gly (2-3). Purified forms of cytoplasmic aminopeptidase that inactivate enkephalins

Abbreviations used: ACE, Angiotensin converting enzyme; HEPES, N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid; BANA, benzoyl-D, L-arginyl-β-napthylamide; βNA, β-naphthylamide.

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have been shown to be identical to arylamidase of brain purified previously (4-6), but the nature of the membrane bound dipeptidyl carboxypeptidase is unclear.

We present information herein that the dipeptidyl carboxypeptidase inactivating enkephalin and present in the membranes of rodent brain is identical to angiotensin converting enzyme (ACE) (EC 3.4.15.1) recently purified from rat brain by Benuck and Marks (7). Our conclusion on the co-identity of ACE with the membrane bound enzyme inactivating enkephalin is based on the anatomical distribution in rat brain regions, and the properties of a dipeptidyl carboxypeptidase purified by immunoaffinity chromatography.

METHODS

Membrane preparation. Tissues were dissected from different anatomical areas of adult male rats by the method of Glowinski and Iversen (8), homogenized in 0.32 M sucrose and membranes prepared by the method of Knight and Klee (9).

Enzyme purification. Brain tissue (20 g) from rabbit was homogenized in 10 vol. of 40 mM Tris-HCL buffer, pH 7.6. The particulate fractions were collected by centrifugation at 15,000 x G for 20 min and the enzyme was solubilized by homogenization in 80 ml buffer containing 0.2% Triton X-100 (v/v). After centrifugation at 100,000 x G for 30 min, the residue was rehomogenized in the same detergent medium and recentrifuged to remove insoluble material. The Triton X-100 soluble extract was applied to a DEAE cellulose column (15 x 2 cm) previously equilibrated with 40 mM Tris-HCl buffer pH 7.6, containing 0.2% (v/v) Triton X-100. Following removal of unadsorbed material, enzyme was eluted with 0.1 N NaCl, as described previously (7).

Goat serum containing antibodies directed against angiotensin converting enzyme of rabbit lung supplied by Richard L. Soffer (Dept. of Biochemistry, Cornell Medical College, New York) was subjected to heat treatment and ammonium sulfate fractionation and the IgG antibodies were isolated by DEAE cellulose chromatography, according to the procedure of Das and Soffer (10). Immunoaffinity columns were prepared by coupling 3 g of activated Sepharose 4B with 75 mg of purified IgG and equilibrated in 10 mM Tris HCl buffer, pH 7.6, containing 0.15 M NaCl (11). The DEAE cellulose fractions were applied to the Sepharose-IgG column and unadsorbed material removed by washing with the same buffer. The enzyme was not eluted by 1-2 M NaCl, or 4-5 M urea but was stable when stored in the bound form in 40 mM Tris buffer, pH 7.6, at -20° for two months or longer.

Enzyme assays. Angiotensin converting enzyme was assayed in membrane preparations using hippuryl-His-Leu as substrate; His-Leu release was determined by a fluorometric procedure as previously described (7,12). In the case of enkephalin, aliquots were incubated under the same conditions with the addition of 50 nmoles of

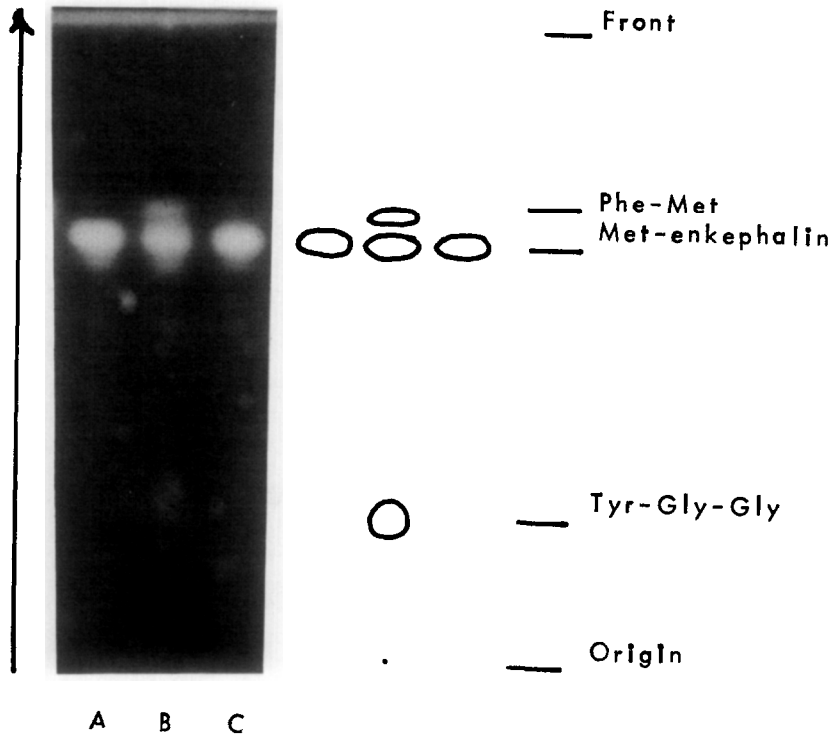

FIGURE 1. Separation of products of hydrolysis of Met-enkephalin by immobilized angiotensin converting enzyme of rabbit brain. Peptide (A) was incubated with the enzyme for 2 hr. (B) and also in the presence of the inhibitor SQ 14,225 (10^{-7} M) (C). Products were separated on silica gel plates using the solvent system butanol: acetic acid:ethyl acetate:water (1:1:2:1). The plates were sprayed with fluorescamine.

Met-enkephalin and 1 μCi of [^{3}H-3,5 Tyr] -Met-enkephalin (Amersham-Searle, Arlington Heights, Ill.); the reaction was terminated after 30 min by addition of 0.1 N HCl and heating. Aliquots were spotted on silica gel plates which were then developed in butanol:acetic acid:ethyl acetate:water (1:1:2:1) (13). The R_f values for the products of incubation were Met-enkephalin 0.67, Phe-Met 0.75, Tyr-Gly-Gly 0.39 and Tyr 0.55. Products were localized by spraying with fluorescamine, scraped off the plates and counted (see Fig. 1). For the immobilized enzyme, the incubation mixture of 0.2 ml contained 10 μmoles HEPES buffer pH 8.6, 60 μmol NaCl, 1-5 mg of the enzyme-Sepharose complex, and either 70 nmoles angiotensin I, one μmole of hippuryl-His-Leu, or 50 nmoles of Met-enkepahlin. His-Leu was determined fluorometrically; release of Tyr-Gly-Gly from Met-enkephalin was determined either by chromatography on silica gel or by the use of Porapak Q column (Waters Assoc.) as described elsewhere (2).

The absence of contaminating enzymes in the immobilized preparation of dipeptidyl carboxypeptidase was established using the following substrates: Z-Phe-Leu (carboxypeptidase A), BANA (tryptic-like), Z-Gly-Gly-Arg-βNA (tryptic-like), Leu-βNA (aminopeptidase) incubated and analyzed according to the procedures of Serra et al. (14). The inhibitor SQ 14,225 was prepared fresh for each experiment and was supplied by David Cushman (Squibb Institute, Rahway, N.J.). Values for Ki using this inhibitor were calculated according to procedures previously described (12).

Table 1: Cleavage of Hippuryl-His-Leu and Met-Enkephalin by Membrane Preparations of Rat Brain.

	Activity (nmoles per min per mg protein) ± S.D.			Ratio
Tissue	His-Leu	Tyr	Tyr-Gly-Gly	$\frac{\text{His-Leu}}{\text{Tyr-Gly-Gly}}$
Pituitary	11.8 ± 2.2	5.7 ± 0.8	6.1 ± 1.5	1.9
Cerebellum	8.7 ± 0.6	2.9 ± 0.2	4.1 ± 1.1	2.1
Corpus Striatum	3.6 ± 0.9	2.5 ± 0.7	1.9 ± 0.3	1.9
Midbrain	3.7 ± 0.6	1.0 ± 0.3	1.6 ± 0.2	2.3
Pons-Medulla	2.8 ± 0.6	2.3 ± 0.4	1.2 ± 0.3	2.3
Hypothalamus	1.5 ± 0.2	1.6 ± 0.2	0.73 ± 0.15	2.0
Cerebral Cortex	0.94 ± 0.05	1.7 ± 0.5	0.42 ± 0.18	2.2
Spinal Cord	0.95 ± 0.05	0.9 ± 0.2	0.42 ± 0.14	2.3

Membrane fractions were prepared from the various anatomical areas by the method of Knight and Klee (9). Incubations were performed with either hippuryl-His-Leu or Met-enkephalin as described in the text. Values represent means ± S.D. for 3-5 experiments.

RESULTS AND DISCUSSION

The distribution of membrane bound dipeptidyl carboxypeptidase was compared for different anatomical regions of rat brain using hippuryl-His-Leu, a known substrate of ACE and [^{3}H-3,5,Tyr] -Gly-Gly-Phe-Met (Met-enkephalin). The release of His-Leu and Tyr-Gly-Gly was highest in the pituitary, followed by cerebellum, corpus striatum, midbrain, pons-medulla, hypothalamus, cerebral cortex and spinal cord (Table 1). Acutal rates with hippuryl-His-Leu were 1-12 nmole per min per mg protein and for Met-enkephalin 0.5-5 nmole per min per mg protein. For all regions the ratio of His-Leu/Tyr-Gly-Gly was approximately 2.0. These data point to a similarity for the enzyme releasing His-Leu and Tyr-Gly-Gly from their respective substrates.

To examine further the possible co-identity of ACE with membrane bound dipeptidyl carboxypeptidase degrading enkephalin, the K_m values were determined for the two substrates, and the effects of the specific ACE inhibitor SQ 14,225 compared. Enzyme prepared by an earlier procedure contained relatively high levels of aminopeptidase and was unsuitable for kinetic measurements using labelled enkephalin (7). This contamination was removed by immunoaffinity chromatography using an antibody directed towards purified rabbit lung ACE coupled to a Sepharose 4B column as described. Brain enzyme was strongly bound and was not eluted by salt, or urea, but retained good activity in the bound form. The immobilized preparation represented an enrichment of approximately 1000 fold, based on the retention of activity on the wet gel, and the loss of protein after elution. Incubation of the bound form with substrate under the conditions described led to a linear release of end-product (His-Leu or Tyr-Gly-Gly) with time and provided a basis for kinetic measurements. Bound enzyme displayed all the properties associated with ACE such as augmentation of activity with addition of Cl^-, cleavage of angiotensin-I with release of His-Leu, and inhibition by SQ 14,225. It is known that in cleavage of other peptides such as bradykinin there is less dependence on addition of Cl^-. In the present study the effect of Cl^- addition on enkephalin breakdown was not pronounced.

The values for K_m calculated from double reciprocal plots were 0.5 mM for hippuryl-His-Leu and 0.1 mM for Met-enkephalin (Fig. 2). The value found for Met-enkephalin corresponds with that of ACE using angiotensin-I. This value is 10^3 fold higher than the value (90 nM) found for enzyme of mouse striatal membranes by Malfroy et al. (2) using Leu-enkephalin and termed "high affinity enkephalinase". Reasons for these differences in K_m are unclear and are unlikely to have arisen from the use of Met rather than Leu-enkephalin since both substrates are rapidly degraded *in vitro* and have short half-lives *in vivo* (1). Moreover, the K_m found in this study

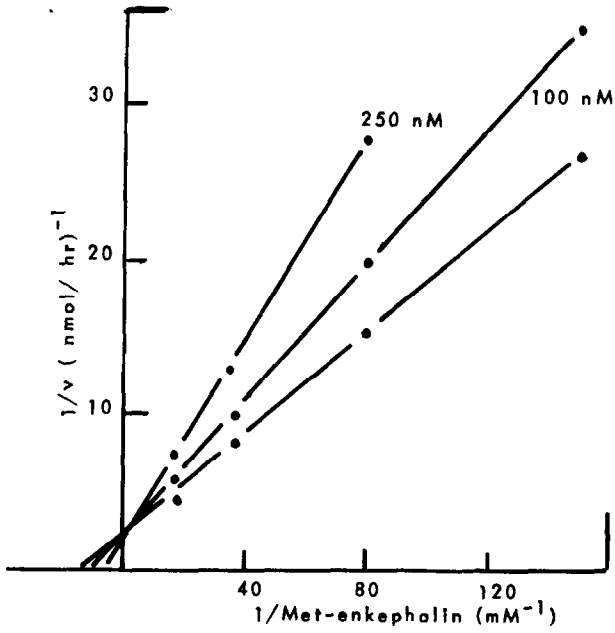

FIGURE 2. Double reciprocal plot of the activity of immobilized angiotensin converting enzyme of rabbit brain in the presence of the inhibitor SQ 14,225, with Met-enkephalin as substrate. Concentrations of inhibitor are shown in the figure. The velocity of reaction was measured by the release of [^{3}H-3,5-Tyr] -Gly-Gly as described in the Methods section.

is in the same range as that found for ACE of brain and other tissues using hippuryl-His-Leu as the substrate (12,15). Another fact pointing to the similarity of the enzyme degrading enkephalin with that of ACE was the inhibition of breakdown in presence of SQ 14,225 yielding a Ki value of 10^{-7}M (Fig. 2). This inhibitor is known to potently block the action of ACE (12,16). The value found for Ki using enkephalin as the substrate is comparable to that of enzyme purified from rat brain (12).

Present data on the anatomical distribution and properties of ACE indicate that it is identical to the membrane bound dipeptidyl carboxypeptidase degrading enkephalin. The high concentration in pituitary may be related to a role in the turnover of opiate peptides at this site. The distribution in rat brain of ACE is different from that mentioned by Malfroy et al. (2) for mouse brain. As such, it

does not appear to parallel that of opiate receptors which are generally low in cerebellum (17) and provides another example where sites of degradation are distinct from those of opiate binding (18). The cleavage of enkephalin by ACE provides an example of the non-specific nature of this enzyme and suggests that it is unlikely to act as a specific "enkephalinase".

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