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A DIPEPTIDYL CARBOXYPEPTIDASE IN BRAIN SYNAPTIC MEMBRANES ACTIVE IN THE METABOLISM OF ENKEPHALIN CONTAINING PEPTIDES

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A dipeptidyl carboxypeptidase activity has been localized in synaptic plasma membranes which have been prepared from isolated rat brain cortical synaptosomes. The specificity of this proteolytic activity towards various synthetic and biological active peptides is compared to the peptidase activities of intact synaptosomes. In contrast to the synaptosomal peptidases which are capable of cleaving all peptide bonds of Met-enkephalin-Arg^6-Phe^7 the peptidase activity associated with the synaptic plasma membrane exclusively hydrolyses a dipeptide from the carboxyl terminus of all hepta- and hexapeptides tested. The fact that this dipeptidyl carboxypeptidase does not cleave the Gly^3-Phe^4 peptide bond of Met-enkephalin suggests that this enzyme is different from "enkephalinase". The synaptic membrane dipeptidyl carboxypeptidase is inhibited by metal chelating agents and thiols but is not affected by compounds known to inhibit serine proteases, thermolysin and "enkephalinase".

The discovery of enkephalins with C terminal extensions e.g. Met-enkephalin-Arg^6-Phe^7 and of enkephalin containing peptides in different organs such as adrenal medulla, intestinal mucosa and brain (1,2) has stimulated the interest in their conversion and inactivation. A variety of enzymes isolated from whole brain (3), pituitary (4), adrenal medulla (5) as well as from rabbit kidney and brain (6) are capable of generating Met-enkephalin from enkephalins with C terminal extensions. Furthermore, enzymes are described which are capable of removing C terminal dipeptides from enkephalin and other biological active peptides. Among these activities present in brain are angiotensin-converting enzyme, "enkephalinase" and neutral metalloendopeptidase (7-10).

The synapses, loci of the process of neurotransmission between nerve cells, are potential sites for processing and degradation of peptides acting

Abbreviations: PMSF, phenylmethansulfonylfluoride; PCMB, parahydroxymercuribenzoate; LeuHX, leucine-hydroxamate.

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as neurotransmitters or neuromodulators. Isolated nerve endings - synaptosomes - and synaptic plasma membranes may therefore be a suitable tool for studying such processing and degradation reactions.

In this study we show that in intact synaptosomes there are at least three different enzyme activities present hydrolyzing all peptide bonds of Met-enkephalin-Arg^{6}-Phe^{7} while synaptic plasma membranes isolated after hypoosmotic shock of synaptosomes retain only a dipeptidyl carboxypeptidase activity. The specificity of this enzyme activity towards various peptides and the sensitivity to a variety of inhibitors is also presented.

MATERIALS AND METHODS

Met-enkephalin-Arg^{6}, Met-enkephalin-Arg^{6}-Arg^{7}, Met-enkephalin-Arg^{6}-Lys^{7} were purchased from Peninsula Laboratories, Inc. Belmont, California, USA; Met-enkephalin-Arg^{6}-Phe^{7}, Leu-enkephalin-Arg^{6}-Arg^{7}, dithiothreitol, PCMB, puromycin, iodoacetic acid, 1,10-phenanthroline, EDTA were from Serva Heidelberg, FRG; Leupeptin, Bacitracin, Pepstatin, LeuHX, phosphoramidone were from Sigma, Munich, FRG; Captopril was kindly donated by Heyden, Munich, FRG. All other chemicals were of analytical grade.

The preparation of synaptosomes followed the method as described in (11) with minor modifications as described (12). If not used subsequently, the final synaptosomal pellet was resuspended in 4 ml of a buffer containing 124 mmol/l NaCl, 4 mmol/l KCl, 1.3 mmol/l $MgSO_4$, 16 mmol/l Na_2HPO_4 adjusted to pH 7.4 and saturated with O_2 (Krebs Ringer phosphate buffer) and stored at -20°C until use. The yield was 60 mg protein from 6 g of cerebral cortex. The extent of contamination of the synaptosomal preparation with free mitochondria has been followed by measuring NADH - cytochrome c reductase as mitochondrial marker enzyme according to (13). It was less than 5%, according to the value obtained by Both and Clark (14). Lactatedehydrogenase activity was assayed in the absence or presence of 0.1% Triton X-100 in order to assess the integrity of synaptosomes. A standard preparation of synaptosomes contained only 7 ± 3% lysed synaptosomes.

Synaptic plasma membranes were isolated essentially following the method of Matsuda and Cooper (15). Fractions 2 and 3 of the sucrose gradient were pooled, diluted with 0.1 mmol/l EDTA, 1 mmol/l sodium phosphate pH 7.4 and pelleted at 78000 x g for 30 min. The pellet was resuspended in 0.32 M sucrose, 4 mM Tris-HCl pH 7.6 and used as the synaptic membrane fraction. The enrichment of Na^{+}, K^{+}-ATPase was 7.6 fold compared to the homogenate (0.154 ± 0.032 µmol x mg^{-1} x min^{-1} from 6 experiments). This value is in agreement with the theoretical value (5-10 fold) estimated by Cotman and Matthews (16). The yield was about 6 mg protein from 6 g of cerebral cortex.

Reaction mixture contained synthetic peptides (56 nmol heptapeptides, 100 nmol of all other peptides tested), membranes or synaptosomes in 0.1 mol /l Tris-HCl buffer pH 7.8 (final volume 50 µl) in the presence or absence of inhibitor at 22°C. Incubation conditions were chosen such that degradation of the peptide substrates was linear with time and amount of protein. The reactions were terminated at the appropriate time by the addition of 6 N HCl to bring the pH values of the samples to pH 2.0. The samples were boiled and stored at -20°C. Sample blanks were included in each experiment. No spontaneous hydrolysis of the substrates occured during the incubation.

Products of enzymatic hydrolysis of synthetic peptides were separated by HPLC on a Kontron (Zürich, Switzerland) liquid chromatography 600/200 pump equipped with a Lichrosorb RP-18 (Merck AG Darmstadt, FRG) column 25 x 0.4 cm and an UVICORD detector with an analytical HPLC - cell (LKB, Bromma,

Sweden); emerging peaks were monitored at 206 nm. Samples were eluted with a 30 min linear gradient of 0-60% acetonitrile in 0.1% H_3PO_4 adjusted to pH 5.0 with NaOH. Column temperature was 30°C, flow rate of the solvent was 1 ml/min. Peak fractions were collected and their amino acid composition was determined after removal of the solvent and acid hydrolysis in a Biotronik LC 6000 E amino acid autoanalyzer. The kinetics of the individual reactions catalyzed by the peptidase activities present in rat brain cortical synaptosomes and synaptic plasma membranes were determined from the initial velocities of the disappearance of the substrate peaks. The rates were converted to nmoles x mg^{-1} x min^{-1} by calibrating the HPLC system with known amounts of standard peptides.

With respect to the diglycine peptide the liberated amounts of glycine were determined by amino acid analyses and calculated by the integration system connected to the amino acid autoanalyzer. They are expressed as nmol glycine per mg synaptosomal protein per minute.

Protein was measured according to the method of Lowry (17) as modified by Bensadoun and Weinstein (18).

RESULTS

Rat brain cortical synaptosomes, prepared by a standard procedure (11, 12) hydrolyze all peptide bonds of Met-enkephalin-Arg^6-Phe^7 in a sequential manner. Degradation begins with the hydrolysis of the molecule to the COOH-terminal dipeptide Arg^6-Phe^7 and Met-enkephalin, followed by the removal of the NH_2-terminal Tyr from the generated Met-enkephalin. The resulting tetrapeptide is then hydrolyzed at the Gly^3-Phe^4 bond and the dipeptides are hydrolyzed at a fast (Phe-Met) and a slow (Gly-Gly) rate to the free amino acids (Table 1). Purified synaptic plasma membranes, however, hydrolyze only

Table 1: Comparison of proteolytic activities in synaptosomes and synaptic membranes towards Met-enkephalin-Arg^6-Phe^7 and intermediate peptides

Substrate	Intact synaptosomes		Synaptic plasma membranes	
	Measured products	Specific activity ($nmol \times mg^{-1} \times min^{-1}$)	Measured products	Specific activity ($nmol \times mg^{-1} \times min^{-1}$)
Met-enkephalin-Arg^6-Phe^7	Met-enkephalin+Arg-Phe	3.52 ± 0.24	Met-enkephalin+Arg-Phe	3.6 ± 0.15
Tyr-Gly-Gly-Phe-Met	Tyr+Gly-Gly-Phe-Met	2.38 ± 0.07	negligible hydrolysis	
Gly-Gly-Phe-Met	Gly-Gly + Phe + Met	2.21 ± 0.13	negligible hydrolysis	
Gly-Gly	Gly	0.45	Gly	0.013

Reaction mixture contained substrate (0.25 mmol/l Met-enkephalin-Arg^6-Phe^7 or 0.5 mmol/l of the other peptides), Tris-HCl buffer (0.1 mol/l; pH 7.8) in a final volume of 50 µl. Products of reaction were separated by HPLC and identified as described under Materials and Methods.
Values are means ± SEM from 4-7 experiments.

the Met^5-Arg^6 peptide bond, releasing Met-enkephalin and the C terminal dipeptide, which are not further degraded even after prolonged incubation. From this result it appears that the aminopeptidase activity associated with intact synaptosomes is completely removed during the preparation of the membranes.

The dipeptidyl carboxypeptidase is also active in the same manner towards the heptapeptides Met-enkephalin-Arg^6-Arg^7, Met-enkephalin-Arg^6-Lys^7, Leu-enkephalin-Arg^6-Arg^7 and the hexapeptide Met-enkephalin-Arg^6 (Table 2). With all heptapeptides tested the corresponding enkephalins are generated as cleavage products whereas from the hexapeptide, the tetrapeptide Tyr-Gly-Gly-Phe is formed. The fact that the resulting enkephalins are not further degraded by synaptic plasma membranes indicates, that this preparation does not contain an "enkephalinase" activity as described by Llorens et al. (9) and the dipeptidyl carboxypeptidase found in intact synaptosomes (Table 1) cleaving the Gly^3-Phe^4 bond of enkephalins.

Table 2: Cleavage of Met-enkephalin containing peptides by synaptic membrane peptidase

Peptide	Site of cleavage	Product found	Specific activity nmol x mg^{-1} x min^{-1} Substrate metabolized
Met-enkephalin-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met, Arg-Phe	3,52
Met-enkephalin-Arg^6-Arg^7	Tyr-Gly-Gly-Phe-Met-Arg^6-Arg^7	Tyr-Gly-Gly-Phe-Met	2,54
Met-enkephalin-Arg^6-Lys^7	Tyr-Gly-Gly-Phe-Met-Arg^6-Lys^7	Tyr-Gly-Gly-Phe-Met	2,05
Leu-enkephalin-Arg^6-Arg^7	Tyr-Gly-Gly-Phe-Leu-Arg^6-Arg^7	Tyr-Gly-Gly-Phe-Leu	2,41
Met-enkephalin-Arg^6	Tyr-Gly-Gly-Phe-Met-Arg^6	Tyr-Gly-Gly-Phe, Met-Arg	1,5

Incubation conditions are described in the legend of Table 1. The dipeptides Arg-Arg and Arg-Lys were not retained on the RP - 18 column under the separation conditions used and could therefore not be detected.

The effect of inhibitors on the dipeptidyl carboxypeptidase activity in membrane preparations is summarized in Table 3. Inhibitors of serine proteases such as PMSF and Leupeptin as well as thiol blocking agents like PCMB and iodoacetic acid had no effect. The enzyme was strongly inhibited by metal chelating agents and thiols but not inhibited by phenobarbital a known inhibitor of "enkephalinase" (19).

The IC_{50} of Captopril, an inhibitor of angiotensin-converting enzyme for the synaptic plasma membrane activity was in the range of 250-500 nmol, which is more than ten-times higher than that of the rabbit lung enzyme (20).

Dipeptidyl carboxypeptidase activities have been shown to occur in a variety of tissues attached to membranes (7,9). In order to show the subcellular distribution in brain of the peptidase cleaving Met-enkephalin-Arg^6-Phe^7 to Met-enkephalin and the dipeptide Arg^6-Phe^7 we measured the activity

Table 3: Effect of inhibitors and chloride ions on the activity of dipeptidyl carboxypeptidase from synaptic membranes

Inhibitor	Concentration (mmol/l)	Percent inhibition
Phenobarbital	1.8	0
PMSF	0.8	0
Jodoacetic acid	5.0	8
PCMB	1.0	14
Bacitracin	100 µg/ml	2
Leupeptin	36 µg/ml	0
Pepstatin	45 µg/ml	0
LeuHX	2.0	0
Phosphoramidone	0.1	0
Puromycin	0.5	0
Phenanthroline	1.0	100
EDTA	1.0	100
Dithiothreitol	6.5	94
NaCl	300	54
KCl	300	40
$MgCl_2$	300	38

Peptidase activity was assayed with Med-enkephalin-Arg^6-Phe^7 as substrate as described in the legend of Table 1. Inhibition of the enzyme activity by Cl^- ions was performed in 0.1 mol/l HEPES pH 7.8.

Table 4: Distribution of dipeptidyl carboxypeptidase and Na^+, K^+-ATPase activities in subcellular fractions of rat brain cortices

Fraction	Dipeptidyl carboxypeptidase		Na^+, K^+-ATPase	
	Specific activity nmol x mg^{-1} x min^{-1}	%	Specific activity nmol x mg^{-1} x min^{-1}	%
Homogenate	2.46	70	0.154	13
Mitochondria I (Ficoll gradient)	1.4	40	0.11	10
Synaptosomes	3.52	100	0.275	25
Mitochondria II (sucrose gradient)	0.2	6	0.22	20
Synaptic plasma membranes	2.64	75	1.13	100

Dipeptidyl carboxypeptidase activity was measured with Met-enkephalin-Arg^6-Phe^7 as substrate as described in the Materials and Methods section. The Ouabain - sensitive Na^+, K^+-ATPase (ATP phosphohydrolase EC 3.6.1.3) was assayed as described in (15). Fractions were obtained during the preparation of synaptosomes and synaptic plasma membranes as described in the Materials and Methods section. Mitochondria I represents the pellet of the Ficoll gradient centrifugation and mitochondria II the pellet of the sucrose gradient centrifugation of the lysed synaptosomes. Both fractions were washed with 0.32 mol/l sucrose, 4 mmol/l Tris-HCl pH 7.4.

in different fractions of rat brain preparations (Table 4). The highest specific activity is associated with intact synaptosomes. A relative high specific activity of this peptidase could be detected also in the fraction containing free mitochondria (Mitochondria I), suggesting that this enzyme is either present in free brain mitochondria or the mitochondrial fraction is contaminated with synaptosomes.

Further purification of the synaptosomes from the Ficoll density gradient after osmotic shock by sucrose gradient centrifugation leads to a preparation (Mitochondria II) which contains only 6% of the specific activity found in synaptsomes. Synaptic plasma membranes retain dipeptidyl carboxypeptidase with a specific activity comparable to that of synaptosomes. The synaptic plasma membranes preparation contains only 10% of the protein of the synaptosomes from which they are derived. Therefore one can conclude that a

large part of the dipeptidyl carboxypeptidase activity is removed from the membranes during the preparation. Since extrasynaptosomal mitochondria are capable of cleaving the Arg^6-Phe^7 dipeptide from Met-enkephalin-Arg^6-Phe^7 too (Table 4, Mitochondria I fraction) care should be taken when using crude mitochondrial pellets without further purification to prepare synaptic membranes for studying peptide degradation activities.

DISCUSSION

The enkephalins are present in many tissues not only as the free pentapeptides, but also as internal sequences in larger polypeptides of varying sizes. In preproenkephalin the Met-enkephalin-Arg^6-Phe^7 sequence represents the carboxy terminus of the precursor molecule. It has been suggested that not the free enkephalins but the enkephalin containing peptides are the physiologic agents (21). Since Met-enkephalin-Arg^6-Phe^7 has a greater opiate activity than Met-enkephalin itself (22), Yang et al. (23) have raised the question whether the dipeptidyl carboxypeptidase acts as an Met-enkephalin-Arg^6-Phe^7 inactivating enzyme or Met-enkephalin forming enzyme. These authors and Benuck et al. (6) described the conversion of Met-enkephalin-Arg^6-Phe^7 to Met-enkephalin and subsequent release of Phe-Met and Tyr-Gly-Gly from the enkephalin molecule by dipeptidyl carboxypeptidase from striatum (23) and from brain and kidney (6) preparations resp. In contrast to their findings the enzyme discussed here does not attack the Gly^3-Phe^4 peptide bond in Met-enkephalin. Recently, Tonnaer et al. (8) described in rat brain synaptic membrane preparations the presence of a dipeptidyl carboxypeptidase being angiotensin-converting enzyme, functioning in the bioactivation of angiotensin I in the brain. We conclude that the dipeptidyl-carboxypeptidase localized in synaptic plasma membranes is distinct from angiotensin-converting enzyme for the following reasons: 1. the IC_{50} of Captopril for the angiotensin-converting enzyme from rabbit lung is 23 nM (20) but we found an IC_{50} for our enzyme in the range of 250-500 nM; 2. angiotensin-converting enzyme is capable of cleaving the Gly^3-Phe^4 peptide bond of enkephalins (24) whereas our membrane preparation does not further degrade enkephalins (Tables 1 + 2); 3.

angiotensin-converting enzyme activity has been shown to be strongly dependent on chloride ions (25) but we found a drastic reduction of the enzyme activity when chloride ions were present in the assay mixture (Tab. 3). This is in agreement with the results of Yang et al. (23).

A variety of different membrane bound dipeptidyl carboxypeptidase activities have been described to be present in brain (For review see 7). Most of the brain preparations studied contain more than one proteolytic activity and in a number of cases even more than one dipeptidyl carboxypeptidase activity. The advantage of our synaptic membrane preparation is the fact that it contains only one rather specific dipeptidyl carboxypeptidase and therefore is a suitable source for the isolation and further characterization of this enzyme.

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