Acta Neurobiol. Exp. 2002, 62: 1-5

Proteolysis of endomorphin-1 by brain synaptic membrane-associated peptidases

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Abstract. The biotransformation of endomorphin-1 (EM-1) by brain synaptic membranes has been studied. Peptide fragments of EM-1 that were formed during incubation with membrane preparations *in vitro* were isolated by High-performance liquid chromatography (HPLC) and characterized using determination of amino acid composition. At pH 7.4, two degradation products, EM-1(1-3) and EM-1(1-2), were identified. EM-1 was degraded 77.5% at 30 min incubation with synaptic membranes. The time course of the experiments and the effect of carboxypeptidase inhibitor (CPI) demonstrated that the proteolysis reaction involves the participation of carboxypeptidase activity.

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Key words: endomorphin-1, proteolysis, degradation, brain synaptic membranes

INTRODUCTION

Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM-1) has been identified as an opiate peptide in mammalian brain (Hackler et al. 1997, Zadina et al. 1997), which produces potent and prolonged analgesia in mice. EM-1 is an endogenous agonist for the μ receptor, for which it has a high affinity (K_i = 360 pm) and selectivity (4,000- and 15,000- fold preference over the δ and κ receptors). Its analgesia activity is much stronger than the μ-selective analogue DAMGO (enkephalin analogue) *in vitro* (Zadina et al. 1997). A study by Hackler et al. (1997) suggests that the content of EM-1 in human brain is higher than in bovine brain, as determined by a specific and sensitive radioimmunoassay with an antibody generated against EM-1. Studies also have demonstrated that EM-1 has other biological activities, including spinal analgesic action in acute inflammatory and neuropathic pain in the rat, involvement in 2 Hz but not 100 Hz electroacupuncture analgesia in the rat and inhibitory modulation of the electrical activity of rostral ventrolateral medulla neurons which is mediated through the stimulation of μ-opioid receptors as well (Chu et al. 1999, Han et al. 1999, Przewlocka et al. 1999, Soignier et al. 2000). At the same time, EM-1 exerts antinociceptive effects and causes hypotension by blocking sympathetic vascular activity. Furthermore, it potentiates HIV expression in human brain cell culture (Rialas et al. 1998, Peterson et al. 1999, Sugimoto--Watanabe et al. 1999). It is known that stimulation of opioid receptors activates various intracellular signaling pathways responsible for alterations in gene expression (Bilecki and Przewlocki 2000). The structure-activity relationship of naturally occurring and synthetic opioid tetrapeptides acting on locus coeruleus neurons has also been studied (Yang et al. 1999). We synthesized EM-1 by liquid-phase method and got pure product. Bioactivity determination in mice (i.c.v.) shows analgesic activity at a maximum of 87%, 20 min after administrating a dose of 5 μg. Additionally, our study also shows that the analgesia activity of EM-1 declines quickly in 1 h after administration, which was in accordance with the literature (Zadina et al. 1997). This finding suggests that EM-1 could be quickly degraded by peptidase activity of brain synaptic membranes.

The degradation of EM-1 *in vitro* has been demonstrated (Peter et al. 1999), but up to now no reports about the proteolysis of EM-1 by brain synaptic membranes are available. In the present study, we have aimed to define sites of cleavage of EM-1 by brain synaptic membrane-associated peptidase *via* characterization of EM-1 fragments and to ascertain the proteolysis time course of EM-1 *in vivo*.

METHODS

Materials

EM-1 was synthesized by our lab and purified by using HPLC C-18 reverse-phase column. All chemicals were of analytical grade. Trifluoracetic acid (TFA) was obtained from Merck Company, Sweden. Acetonitrile was purchased from Fisher Company, U.S.A. Carboxypeptidase inhibitor (CPI, Potato, M.W. 4200) was purchased from Calbiochem Corporation, USA. Experimental mice (Kungming species) weighing 18-20 g were used.

Preparation of brain synaptic membranes

Whole brains from randomly selected male or female mice were homogenized in 4 volumes (v/w) of buffer A containing 1 mM $NaHOCO_3$ and 5 mM β-sulfenyl-ethanol. Homogenates were diluted in 25 volumes (v/w) of buffer A and centrifuged at 2,000 g for 30 min. The pellets were suspended in 4 volumes (v/w) of buffer A in a glass homogenizer and centrifuged at 1,200 g for 20 min. The supernatants were combined and centrifuged at 30,000 g for 30 min. The final pellets were suspended in 1 ml of double-distilled H_2O (Poirier 1976). The whole procedure was performed at 4°C.

EM-1 proteolysis

EM-1, in a final concentration of 100 μg/ml, was incubated at 37°C with the brain synaptic membranes (1.13 mg/ml) in 3.0 ml of 0.08 M phosphate buffer (pH 7.4). During the incubation, at 0, 0.5, 1, 2, 4, 6 h of the experiment, 0.4 ml aliquots of the reaction solution were collected and the reaction was stopped in 100°C water bath for 3 min, then the membranes were removed by centrifugation at 10,000 g for 10 min and the supernatant was lyophilized for high performance liquid chromatography analysis (HPLC) (Yuan et al. 1988). In order to get enough degradation products for determination of amino acid composition, 0.5 ml aliquots of the reaction were collected at 0.5 h and 1 h, and processed as described above. When the effect of carboxypeptidase in-

hibitor (CPI) on EM-1 proteolysis was studied, CPI was introduced into the reaction solution before EM-1. The final concentrations of EM-1, synaptic membrane protein and CPI in these experiments were 100 μg/ml, 0.213 mg/ml, 0.1 mg/ml, respectively.

HPLC analysis

High-performance liquid chromatography equipment (Waters™ 600) with a uv detection system was used. Chromatography was performed on a Bondapak C18 reversed-phase column (0.5 x 20 cm, USA) at ambient temperature. The samples were dissolved in 50 μl of double-distilled H_2O and centrifuged before injection. Upon injection of EM-1 digests, elution was performed with a linear gradient of 1% TFA in double-distilled water (v/v)(solvent A) and acetonitrile containing 1% TFA (solvent B). The gradient ran from initial conditions of A/B = 90:10 (v/v) to final conditions of A/B = 55:45 (v/v) in 25 min, at a flow rate of 1.0 ml/min. The eluate was monitored by uv absorbance at 280 nm during HPLC fractionation of the digests. Fractions were collected from the HPLC eluate and the samples were lyophilized after removal of acetonitrile *in vacuo* at 60°C.

Determination of amino acid composition

Lyophilized samples were hydrolyzed with 5.7 M HCI containing 0.1% thioglycolic acid in evacuated glass tubes at 110°C for 16 h. After evaporation of HCI in vacuum, the amino acid composition was determined using general microdansylation method. Amino acid residues were identified on micropolyamide thin layers (7 x 7 cm), which were developed in a double solvent system (Zhang et al. 1997).

RESULTS

Purification of degradation fragments and determination of amino acid composition

After incubation of EM-1 synthesized by liquid-phase method with brain synaptic membranes for 1 h at 37°C (pH 7.4), four fragment peaks (I -IV) were detected (Fig. 1). Peak I was identified as $PheNH_2$, the C-terminal residue of EM-1. The amino acid composition of peak II is the same as EM-1(1-3) (Tyr-Pro-Trp), whereas determination of the amino acid composition of peak III indicates that this fragment is EM-1(1-2) (Tyr-Pro) (Fig. 2). According to either retention time in HPLC or amino acid composition assay, peak IV is the substrate, EM-1.

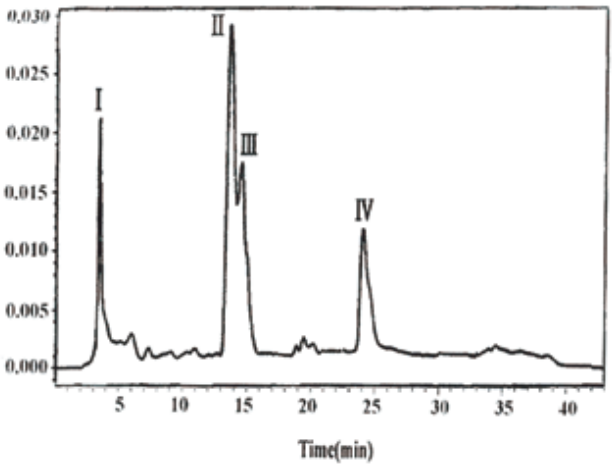

Fig. 1. HPLC profile of endomorphin-1 digests by brain synaptic membrane, using C-18 reverse-phase column, a linear gradient elution. Solvent A: double-distilled H_2O containing 1% TFA; solvent B: acetonitrile containing 1% TFA. The gradient runs from A/B=90:10 (v/v) to A/B = 55:45 in 25 min. There are four fragments: I, II, III, IV.

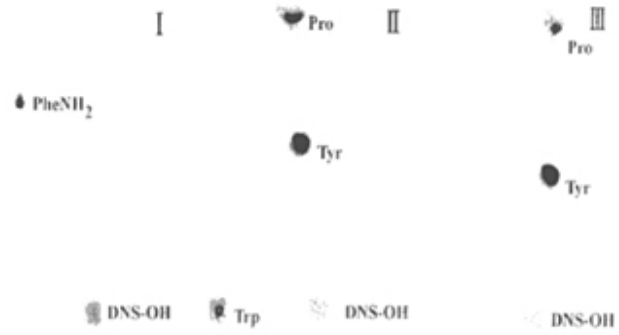

Fig. 2. Chromatography of dansylated (DNS-) amino acids of endomorphin-1 digests by mouse brain synaptic membrane, peak I, II, III, on micropoly-amide thin layers. The polyamide sheets were developed in the first dimension in formic acid (1.5:100, v/v), dried carefully and subsequently developed in the second dimension in benzene-acetic acid (9:1, v/v).

Time course of EM-1 degradation by brain synaptic membrane

The dynamics of EM-1 degradation under physiological conditions provides information on rates and mechanism of EM-1 hydrolysis *in vitro* by the target tissue. At physiological pH 7.4, the fragment EM-1

(1-3)(Tyr-Pro-Trp) was maximally accumulated after 0.5 h incubation, and subsequently decreased to a relatively low level. The fragment EM-1 (1-2) (Tyr-Pro) increased gradually within 1 h and reached maximum at 1 h, then was slowly reduced. The C-terminal residue, $PheNH_2$, accumulated slowly for the whole time of the experiment. The substrate EM-1 was degraded by 77.5% during 0.5 h and almost completely disappeared from the system within 1 h (Fig. 3).

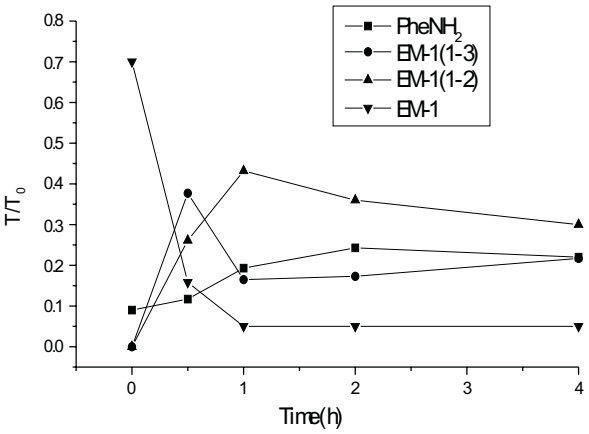

Fig. 3. Degradation time course of endomorphin-1(EM-1) without carboxypeptidase inhibitor (CPI^-) by brain synaptic membrane at pH 7.4. There are four fragments: -■-, $PheNH_2$; -●-, Tyr-Pro-Trp; -▲-, Tyr-Pro; -▼-, Tyr-Pro-Trp-Phe-NH_2. T/T_0 indicates the relative content of each fragment in total digests during the experimental process.

The effect of carboxypeptidase inhibitor on EM-1 proteolysis

As presented in Fig. 4, the proteolysis of EM-1 by brain synaptic membrane-associated peptidases in the presence of CPI was inhibited, especially within 0.5 h incubation time, but in the presence and absence of CPI both the degradation fragments were detected.

DISCUSSION

The result of HPLC elution and amino acid assay shows that, among the four peaks, peak I was identified as $PheNH_2$, peak II was identified as EM-1(1-3) (Tyr-Pro-Trp), and peak III was identified as EM-1(1-2) (Tyr-Pro). Furthermore, peak II appears earlier than peak III; subsequently, the relative quantity of peak III is increasing slowly and of peak II is decreasing gradually. The relationship of proteolysis fragments and their relative quantity at various times shows that the first step of degradation of EM-1 by brain synaptic membrane takes place in the peptide bond between Trp^3 and $PheNH_2{}^4$. The Tyr-Pro-Trp formed by this step can be further degraded into Tyr-Pro.

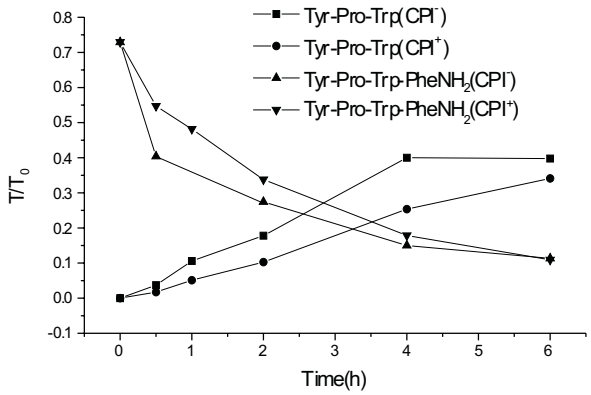

Fig. 4. The effect of carboxypeptidase inhibitor on time course of endomorphin-1 (EM-1) and EM-1(1-3)(Tyr-Pro-Trp) by brain synaptic membrane at pH 7.4. -■-, Tyr-Pro-Trp(CPI^-); -●-, Tyr-Pro-Trp(CPI^+); -▲-, Tyr-Pro-Trp-Phe-NH2(CPI^-); -▼-, Tyr-Pro-Trp-Phe-NH_2(CPI^+). T/T_0 indicates the relative content of each fragment in total digests during the experimental process.

The degradation pathway of EM-1 by brain synaptic membrane-associated peptidase is very similar with the degradation site of EM-1 by proteinase A and carboxypeptidase Y (Peter et al. 1999), though we haven't observed the presence of fragment Tyr-Pro-Trp--Phe-COO^-, which suggested that the proteolysis reaction of EM-1 may include the participation of carboxypeptidase and proteinase A. The experimental result of a carboxypeptidase inhibitor effect supports this opinion because CPI certainly inhibits the proteolysis of EM-1.

The bioactivity determination (i.c.v.) in mice shows the analgesic effect reaches apex at 20 min, then decreases quickly in the following 1 h (data not shown). At the same time, according to the proteolysis time course of EM-1 by brain synaptic membrane, it was degraded 77.5% at 30 min and almost completely metabolized by 1 h. These results give an explanation about the relationship between the content of EM-1 in brain and the analgesic effect of EM-1.

CONCLUSIONS

The results of this study, particularly the time-course of EM-1 degradation and the effect of CPI, demonstrate

that carboxypeptidase participates in EM-1 proteolysis by brain synaptic membranes.

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Received 20 September 2000, accepted 3 January 2002