High enkephalyl peptide degradation, due to angiotensin-converting enzyme-like activity in human CSF

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The metabolism of enkephalin peptides was studied in human cerebrospinal fluid. The degradation rates of (Leu)-enkephalin and (Leu)-enkephalin-Arg^6 were compared and the latter was degraded at a 10-fold higher rate. The major enzyme activity was investigated by M_r determination and inhibition experiments, showing marked similarity with angiotensin-converting enzyme.

(Cerebrospinal fluid) Enkephalin peptide Degradation rate Angiotensin-converting enzyme Enzyme inhibitor HPLC

1. INTRODUCTION

Analysis of peptides in human cerebrospinal fluid (CSF) is becoming of clinical interest. Peptide levels are influenced by several factors including enzyme activities. We have previously found that human CSF contains greater amounts of the C-terminally extended, basic enkephalyl peptides than of the enkephalins themselves [1]. This may seem unexpected if the extended peptides are the precursors and the enkephalins are the active signal substances released at the synapses. One factor which might influence peptide levels as measured in CSF is the presence of enzymes in the CSF itself. Enkephalin degradation has previously been observed in CSF and ascribed to nonspecified aminopeptidase, dipeptidylaminopeptidase and dipeptidylcarboxypeptidase activities [2].

The aim of this study was to compare the metabolic stabilities of the enkephalins and the basic enkephalyl peptides in CSF. The peptides chosen for the comparison were (Leu)-enkephalin and (Leu)-enkephalin-Arg^6. These peptides and their metabolic products were more easily separated by HPLC, than the corresponding methionine-containing enkephalyl peptides (fig.1).

2. MATERIALS AND METHODS

The CSF was obtained by lumbar puncture from patients, undergoing investigation for suspected increased intracranial pressure. Total protein content was within the normal range. The tritiated peptides were obtained from Amersham, England, and all reference peptides from Bachem, Bubendorf, Switzerland. Chemicals and solvents were of reagent grade from usual sources.

The CSF material was desalted by a Sephadex G-25 M (Pharmacia) column, PD-10, bed volume 9 ml. The protein fraction was collected and lyophilized. It was redissolved in the original volume of 0.2 M Tris-HCl buffer, pH 7.4, and incubated with 5.9×10^{-8} M [^{3}H](Leu)-enkephalin or [^{3}H](Leu)-enkephalin-Arg^6 for different time periods at 37°C. The reactions were stopped by adding aliquots of the reaction mixture to 150 μl ice-cold EtOH, followed by centrifugation (Beckman microfuge B) for 5 min to remove the proteins. The supernatant was mixed in Eppendorf tubes with carrier peptides, including all potential metabolites from the substrate.

Separation of the degradation products was performed in a Hewlett Packard 1084 B liquid chromatograph with a Supelcosil LC-18-DB col-

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umn (250 × 4.6 mm) (fig.1). The smallest fragments Tyr, Tyr-Gly and Tyr-Gly-Gly were resolved by isocratic elution for 40 min with 0.04% trifluoroacetic acid/H_2O, followed by a 30 min linear gradient of methanol (15–45%) in 0.04% trifluoroacetic acid, which eluted the longer peptides. The flow rate was 1.0 ml/min and fractions of 1.0 ml were collected and the amount of radioactivity determined by liquid scintillation counting.

M_r determination was made on a Sephacryl S-200 column (2 × 60 cm), calibrated with proteins of known M_r: γ-globulin, M_r 160000; bovine serum albumin, M_r 68000; human β-casein, M_r 26000; cytochrome *c*, M_r 13000. The flow rate was 20 ml/h with 0.04 M NH_4HCO_3 as eluent. Fractions of 2 ml were collected. Lyophilized aliquots of the fractions were redissolved and incubated with 7 μM (Leu)-enkephalin-Arg^6 and tracer amounts of tritiated (Leu)-enkephalin-Arg^6. The metabolic products were analyzed as before.

Inhibition experiments were performed with 7 μM (Leu)-enkephalin-Arg^6 and tracer amounts of the tritiated peptide as substrate. The peptides used as inhibitors were added in concentrations of 700 μM; EDTA, 10 μM; phosphoramidone, 10 μM; captopril, 10 μM; MK-422 (the active diacid of enalapril), 0.1 or 100 μM. The incubation time was 60 min. Substrate and products as indicated were isolated by HPLC under the same conditions as in the previous series of experiments.

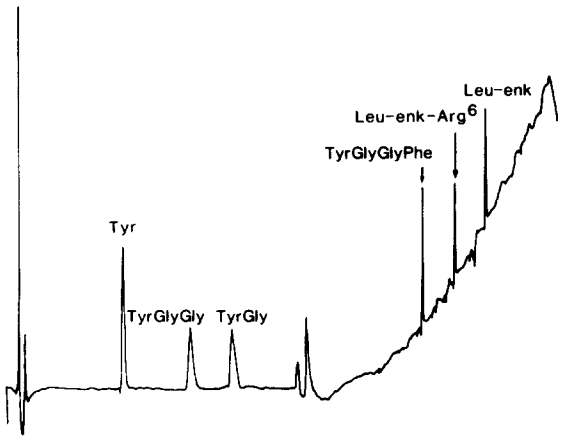

Fig.1. Separation of (Leu)-enkephalin, (Leu)-enkephalin-Arg^6 and their N-terminal fragments by HPLC.

3. RESULTS AND DISCUSSION

(Leu)-enkephalin was about 10-times more stable than (Leu)-enkephalin-Arg^6 in CSF (cf. fig.2a and b). The major degradation products from (Leu)-enkephalin were Tyr and Tyr-Gly-Gly, indicating aminopeptidase and dipeptidylcarboxypeptidase activities. The major route of metabolism of (Leu)-enkephalin-Arg^6 was the cleavage of the Phe^4–Leu^5 bond, giving rise to Tyr-Gly-Gly-Phe and Leu-Arg. Formation of these fragments was responsible for the higher degradation rate of the hexapeptide. Only small amounts of Tyr, Tyr-Gly and (Leu)-enkephalin were generated. Thus, the higher concentration of (Leu)-enkephalin-Arg^6 than (Leu)-enkephalin generally observed in CSF samples [1] cannot be explained in terms of differences in CSF degradation activities. Considering other possibilities for this difference, enzymes in the synaptic cleft may

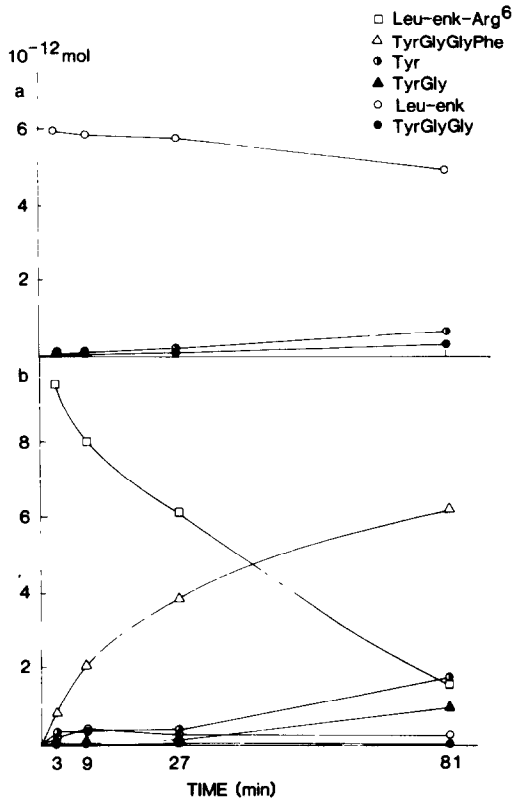

Fig.2. Time course of (a) (Leu)-enkephalin and (b) (Leu)-enkephalin-Arg^6 degradation in human CSF.

be more specific for the pentapeptides, allowing the C-terminally extended hexapeptides to diffuse out and reach the CSF in greater amounts.

A characterization of the enzyme activity was performed by adding different inhibitors to the incubation mixture (fig.3). EDTA, a metallo-enzyme inhibitor, completely blocked tetrapeptide formation. Phosphoramidone, which is an enkephalinase inhibitor, had a very small inhibitory effect, showing that enkephalinase is not a very important enzyme in CSF, for the degradation of the hexapeptide. The poor enkephalinase activity is also observed as a low rate of (Leu)-enkephalin degradation (fig.2a). The rate of formation of Tyr-Gly-Gly by enkephalinase or other enzymes is less than that of Tyr formation.

A possible candidate for the rapid degradation of the hexapeptide, angiotensin-converting enzyme (ACE), has previously been identified in human CSF [3]. The M_r of the CSF enzyme was assessed by chromatography and found to be 140000, which is the same as that reported for ACE in lung [4]. When captopril and MK-422, both relatively specific inhibitors of ACE [5], were present, no

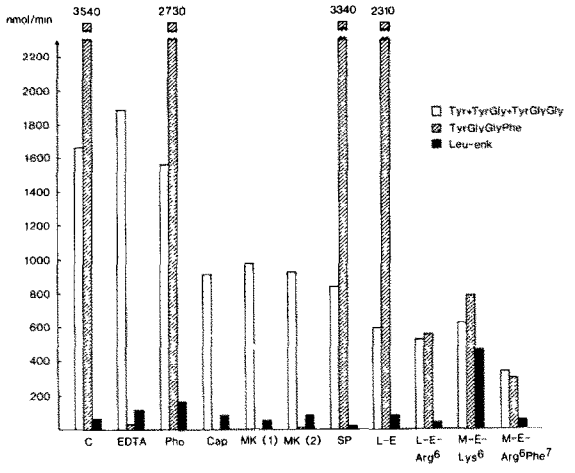

Fig.3. Inhibition experiments. Effects of various enzyme inhibitors and peptides on the enzymatic formation of (Leu)-enkephalin, Tyr-Gly-Gly-Phe and smaller N-terminal fragments of (Leu)-enkephalin-Arg^6. C, control; Pho, phosphoramidon (10 μM); Cap, captopril (10 μM); MK (1), MK 422 (100 μM); MK (2), MK 422 (0.1 μM); SP, substance P; L-E, (Leu)-enkephalin; L-E-Arg^6, (Leu)-enkephalin-Arg^6; M-E-Lys^6, (Met)-enkephalin-Lys^6; M-E-Arg^{6}Phe^7, (Met)-enkephalin-Arg^{6}Phe^7.

formation of Tyr-Gly-Gly-Phe was observed (fig.3). These results strongly support the contention that the enzyme responsible for the high degradation rate of enkephalyl peptides is ACE.

To investigate further the enzymatic conversion of (Leu)-enkephalin-Arg^6, related peptides such as (Leu)-enkephalin, (Met)-enkephalin-Lys^6, (Met)-enkephalin-Arg^{6}Phe^7 were added as inhibitors in a 100-fold molecular excess (fig.3). (Leu)-enkephalin had a very small inhibitory effect on the formation of the tetrapeptide from the hexapeptide, indicating that (Leu)-enkephalin-Arg^6 is a much better substrate than (Leu)-enkephalin, which has also been reported for ACE from rat brain [6]. (Met)-enkephalin-Lys^6 and (Met)-enkephalin-Arg^{6}Phe^7 were both good inhibitors and lowered tetrapeptide formation by 78 and 92%, respectively, as compared with the inhibition by (Leu)-enkephalin-Arg^6 (= 84%), when added in the same molecular excess.

The hydrolysis of substance P has been reported to be catalyzed by ACE [7,8]. Therefore it was tried as inhibitor, but it did not show any significant inhibitory effect on tetrapeptide formation. However, the rate of substance P conversion has been reported to be only about 1/60 that of angiotensin I.

Enzymes in CSF could potentially derive from tissues within the CNS, from choroid plexus and vasculature or even be of blood origin. However, in human choroid plexus the ACE activity is relatively low, while it is high in the caudate nucleus, even compared to the lung [9], favouring a neuronal origin of the ACE-like enzyme in CSF.

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