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CHARACTERIZATION OF ENDOPEPTIDASE 3.4.24.11 ("ENKEPHALINASE") ACTIVITY IN HUMAN PLASMA AND CEREBROSPINAL FLUID*

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Abstract—The presence of the neutral metallo-endopeptidase 3.4.24.11 ("enkephalinase") activity was investigated by fluorimetric assay in human body fluids. Although the enzyme was previously known to occur exclusively in membrane bound form in the human or animal central nervous system, its activity was detected in human cerebrospinal fluid (CSF), plasma and amniotic fluid. Although the endopeptidase 3.4.24.11 activity found in human body fluids has properties closely related to the membrane bound enzyme such as affinity constant for the inhibitors, optimal pH and K_m for the substrate, the V_{max} values were in CSF, plasma and amniotic fluid, respectively, 2×10^3, 10^2, 10 lower than mouse brain homogenate.

Endopeptidase 3.4.24.11 ("enkephalinase") is characterized as a membrane-bound metallo-enzyme which cleaves enkephalins at the level of the Gly-Phe amide bond and may be implicated in the biological inactivation of the endogenous opioid pentapeptides [1–3] and of the other peptides such as substance P, CCK, neurotensin, oxytocin and angiotensin [4]. Initially characterized in brain [5] where it is concentrated in synaptic membranes [3, 6], endopeptidase 3.4.24.11 is also found in high activity in a variety of peripheral tissues in both animals [7] and man [8–10]. Its purification from pituitary or kidney [11–13] led to the demonstration of its similarity with a neutral metallo-endopeptidase (EC 3.4.24.11) found earlier in the brush border of kidney and intestine [14] but whose presence in the brain as well as functional role had remained unknown. In the present study we evaluated the presence of endopeptidase 3.4.24.11 activity in human plasma, cerebrospinal fluid (CSF) and amniotic fluid. Such an activity is compared with those of mouse brain homogenate and the enzyme purified from rat kidney.

MATERIALS AND METHODS

Collection of human biological fluids. Blood was taken from the arm vein of 22 normal volunteers (14 males and 8 females, aged 20–70 years) and collected in heparinized glass tubes. Plasma was separated after a 2100 *g* centrifugation for 15 min at 4°, and frozen at −20° in plastic tubes until use. Plasma was thawed at room temperature and diluted 30-fold in 0.05 M HEPES pH 7.4 buffer just before the assay. CSF was sampled from 24 subjects (12 males and 12 females, aged 30–70 years) during diagnostic lumbar puncture, and stored at −80° in plastic tubes. After thawing at room temperature, the CSF were centrifuged at 8000 *g* for 10 min in a refrigerated centrifuge (Spinco, Beckman) and used for assay of peptidase activity without any dilution. Five samples of both plasma and CSF were ultracentrifuged at 100,000 *g* for 1 hr at 4° before assay. In eight subjects plasma and CSF were collected at the same time. Amniotic fluid was taken from six pregnant women (aged 30–45 years) at the 16th week of pregnancy, during diagnostic amniocenthesys. It was centrifuged at 2100 *g* for 15 min at 4°, filtered through paper and frozen at −20°. After thawing at room temperature, the amniotic fluids were diluted 100 times in HEPES buffer before assay of peptidase activity.

Purified rat kidney endopeptidase 3.4.24.11. The enzyme was purified from rat kidney as previously described [12]. The purified preparation of endopeptidase 3.4.24.11 had a specific activity of 6 nmol/mg protein/min as described by Malfroy *et al.* [12] corresponding approximately to a 2000-fold enrichment as compared to the starting kidney homogenate; its purity was of at least 80% as judged by SDS electrophoresis. The specificity constant for the substrate was 44 μM^{-1} min^{-1}.

Preparation of mouse brain homogenate. Mice were killed by cervical dislocation and their brain (minus cerebellum and brainstem) were individually homogenized into 10 mL ice-cold 0.05 M pH 7.4 HEPES/NaOH buffer, using a Braun homogenizer and a glass–Teflon Potter (5 up and down strokes at 1500 rpm). The homogenate was diluted 30-fold in HEPES buffer prior the assay. Protein content of the samples was evaluated by the Coomassie Blue method except for ultracentrifuged CSF and plasma where the method of Lowry *et al.* [15] was used.

Measurement of endopeptidase 3.4.24.11 *activity.* The enzyme activity was evaluated by means of a

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Table 1. Characterization of endopeptidase 3.4.24.11 activity in various preparations using Suc-Ala-Ala-Phe-AMC as substrate

Species source	Rat: Endopeptidase 3.4.24.11 purified from rat kidney	Mouse: Brain homogenate	Human: Plasma	Human: CSF	Human: Amniotic fluid
V_{max} (nmol/mg protein/min)	24,700 ± 0,600	26.5 ± 0.9	0.244 ± 0.086	0.014 ± 0.003	2.6 ± 0.2
K_m (μM)	52 ± 11	73 ± 11	58 ± 2	54 ± 8	43 ± 6
Optimal pH	7	7	7	7	7
K_i values of:					
Thiorphan (nM)	1.1 ± 0.2	2.5 ± 0.4	2.2 ± 0.5	2.3 ± 0.3	1.7 ± 0.1
Thiorphan–NH_2 (nM)	54 ± 2	57 ± 13	79 ± 4	35 ± 11	64 ± 2
Phosphoramidon (nM)	2.1 ± 0.4	3.7 ± 0.6	3.1 ± 0.8	3.7 ± 0.3	3.7 ± 0.2
Captopril (nM)	>100	>100	>100	>100	>100
(Met^5)-enk (μM)	6.9 ± 2.2	6.9 ± 0.1	17.1 ± 3.9	19.5 ± 3.9	11 ± 3.6
Leu^5)-enk (μM)	48 ± 6	134 ± 2	135 ± 62	87 ± 51	114 ± 3

Incubations were performed in the presence of 1 ng purified endopeptidase 3.4.24.11 from rat kidney [12] or a sample of mouse brain homogenate corresponding to 0.3 mg protein.

Human plasma and amniotic fluid were diluted in buffer (30 and 100 times, respectively) prior to the assay, whereas human CSF were assayed without any dilution. The enzyme activities were measured by the two step procedure described in Materials and Methods. Data represent means of 2–4 independent experiments (triplicate determinations). K_m and K_i values were established using at least four different concentrations of substrate or inhibitor, respectively, and calculated by least square analysis [24].

fluorimetric method in which 150 μL of samples of the various enzyme sources were incubated for 1 hr at 37° with 10^{-4} M succinyl-alanyl-alanyl-phenylalanyl (7-amido,4-methyl) coumarin (Suc-Ala-Ala-Phe-AMC; Bachem, Switzerland); blank values were obtained for each sample by addition of 1 μM thiorphan to the incubation medium as reported elsewhere [16].

The products of endopeptidase 3.4.24.11 activity were determined by the HPLC method, running in isocratic and using a C_{18} μBondapak column and 60% methanol as mobile phase. Such an analysis revealed endopeptidase 3.4.24.11 cleaving the substrate at the Ala-Phe bond while the fluorescent compound AMC was released from Phe-AMC after incubation with aminopeptidase M which does not attack the whole substrate.

RESULTS

Human body fluids hydrolyse the substrate in a time dependent manner (data not shown), and their kinetic parameters in this hydrolysis were identical to those found for the mouse brain homogenate and rat kidney purified enzyme except for the V_{max} values (Table 1). In fact while the K_m values were in the same range as shown by Eadie–Hofstee plots (Fig. 1) the V_{max} values ranged from 14 pmol/mg protein/min for CSF to 24.7 μmol/mg protein/min for purified enzyme. On the other hand the endopeptidase 3.4.24.11 activity in each enzyme source was similarly inhibited by various compounds including thiorphan, phosphoramidon and enkephalins (Table 1, Fig. 2).

Concerning the enzyme activity it shows considerable variations among the different preparations and a wide range in the enzyme activity in each enzyme source, as reported in Table 2. Similar results were obtained using 20 nM (^{3}H-Tyr^1, D-Ala^2, Leu^5) enkephalin as substrate (data not shown).

In eight subjects where plasma and CSF were simultaneously collected, no significant relationship is present between endopeptidase 3.4.24.11 activity in the two fluids ($r = 0.0008$). In three samples of both plasma and CSF endopeptidase 3.4.24.11 activity is, respectively, (mean ± SE) 90.7 ± 18 and 0.44 ± 0.05 pmol/mL/min before and 65.7 ± 15.7 and 0.31 ± 0.03 pmol/mL/min after ultracentrifugation, indicating a decrease of about 28% in the enzyme activity in both fluids. However in three CSF samples, where protein content was also evaluated no difference in endopeptidase 3.4.24.11 activity was found before and after ultracentrifugation (mean ± SE) (0.11 ± 0.012; 0.15 ± 0.015 pmol/mg protein/min) because a decrease of protein content was also detected.

DISCUSSION

The possibility of using a sensitive fluorimetric assay to evaluate endopeptidase 3.4.24.11 activity

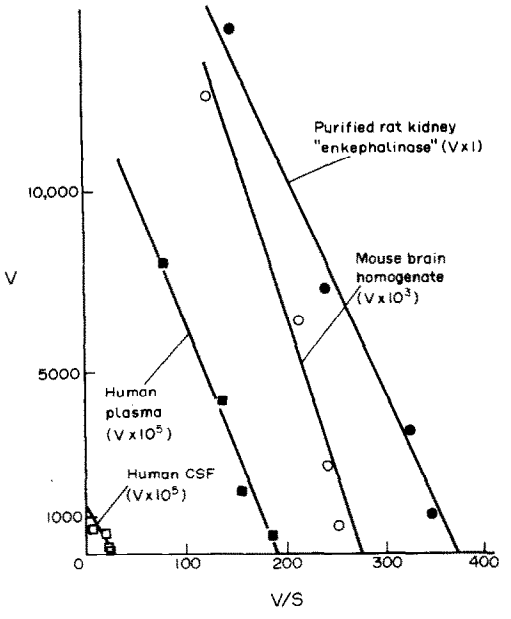

Fig. 1. Eadie–Hofstee plot of the hydrolysis of Suc-Ala-Ala-Phe-AMC by various enzyme sources. The hydrolysis of Suc-Ala-Ala-Phe-AMC in increasing concentrations by purified rat kidney endopeptidase 3.4.24.11 (●), mouse brain homogenate (○), human plasma (■) and human CSF (□) was measured as described in Materials and Methods. Data (mean from at least three independent experiments performed in triplicate) are represented as follows: (V) of the CSF, plasma and brain homogenate were multiplied by 10^5, 10^5 and 10^3, respectively, and the activity of the rat kidney endopeptidase 3.4.24.11 was kept unmodified (V in nmol/mg protein/min and S in μM). The data for amniotic fluid, which yielded a line parallel to the other ones, were omitted for clarity.

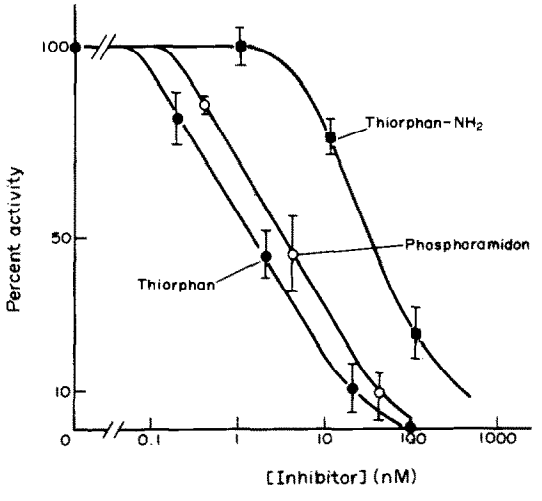

Fig. 2. Effects of various inhibitors on hydrolysis of Suc-Ala-Ala-Phe-AMC by human CSF. Human CSF were incubated with the substrate as indicated in Materials and Methods, in the presence of inhibitors in increasing concentrations. Data represent percentages of activity (means ± SE) from three independent triplicate assays. One hundred per cent activity corresponds to 1.15 pmol/mL/min.

allowed us to compare the activity of the enzyme present in human biological fluids with the purified rat kidney and mouse brain enzyme. When applied to a crude mouse brain homogenate the fluorimetric assay revealed an enzymatic activity that could be inhibited by 90% in the presence of 1 μM thiorphan, a selective endopeptidase 3.4.24.11 inhibitor [17]. In addition, the K_i values of the various endopeptidase 3.4.24.11 inhibitors tested (thiorphan, its amidified derivative and phosphoramidon) were in close agreement with corresponding values on the purified enzyme while captopril, a potent inhibitor of angiotension-converting enzyme (K_i in the nM range), was ineffective at 0.1 μM (Table 1).

Furthermore the K_i values of enkephalins were in close agreement with their respective K_m or K_i values on either the purified kidney enzyme (Table 1) or cerebral membranes [12].

Our results indicate that the endopeptidase 3.4.24.11 present in several biological fluids like blood plasma, CSF or amniotic fluid displays K_m values for the substrate and K_i values for the inhibitors and neutral optimal pH similar to the purified enzyme and mouse brain homogenate in spite of very large differences in specific activities. The enkephalins, particularly Leu-enkephalin, appeared slightly less potent on non-purified extracts than on the purified kidney enzyme, a small difference which might be due to their partial hydrolysis by enzymes distinct from endopeptidase 3.4.24.11 during incubation. Taken together these observations indicate that a soluble form of endopeptidase 3.4.24.11 or a closely related enzyme activity can be found in these various biological fluids. A support to the presence of a soluble form of the enzyme was obtained by the fact that the enzyme activity was little reduced after ultracentrifugation of the fluids and it does not change considering the activity in pmol/mg protein/min. This was rather unexpected since the enzyme in brain [5, 6] as in kidney [12, 13], pituitary [1] or other peripheral organs [7] seems entirely membrane-bound and requires the presence of proteolytic enzymes or detergent like Triton × 100 in a relatively high concentration to be solubilized. However, it cannot be excluded that a soluble isoenzyme representing a minor fraction of the total endopeptidase 3.4.24.11 activity in these tissues might have escaped detection. Alternatively the soluble endopeptidase 3.4.24.11 activity in plasma or CSF which is extremely low as compared to that found in cell membranes from tissues in contact with these extracellular fluids, might emanate from a slow release from the membranes, such as choroid plexus [18], neutrophylis and the particulate fraction present in blood flow. Further physicochemical or immunological studies, supported by the cloning of the enzyme [19] may help to clarify the origin of the enzyme, and the characteristic of its probable soluble form. The presence of endopeptidase 3.4.24.11 in CSF was apparently not related to a transfer from plasma since the levels of activities in the two body fluids were not correlated in individual subjects. The situation of endopeptidase 3.4.24.11 might be reminiscent of that of acetylcholinesterase which mainly occurs in membrane bound form in the CNS but of which a soluble isoenzyme also exists [20, 21].

Table 2. Endopeptidase 3.4.24.11 activity in various tissues and human biological fluids

Source of enzyme	Number of samples	Endopeptidase 3.4.24.11 activity	
		Means ± SE	Range
Mouse brain homogenate	7	2.5 ± 0.1*	2.2–2.7
Human CSF	24	1.8 ± 0.1†	0.7–3.2
Human amniotic fluid	6	1140 ± 345†	251–2482
Human plasma	22	94 ± 15†	13–318

Activity expressed in *nmol/mg protein/min or †pmol/mL/min.

The functional significance of the soluble and membrane-bound forms of endopeptidase 3.4.24.11 is still a matter of speculation and much more work is required to understand the possible role, if any, of endopeptidase 3.4.24.11 in human CSF and other extracellular fluids. Studies are in progress to assess the possible clinical significance of variations in enzyme activities in these fluids, in particular in view of the therapeutic effect of endopeptidase 3.4.24.11 inhibition in some pathological conditions [22, 23].

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