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Characterization of an aminopeptidase in cerebrospinal fluid

Structure elucidation of enzyme hydrolysis products of synthetic methionine-enkephalin by reversed-phase high-performance liquid chromatography and mass spectrometry

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ABSTRACT

An aminopeptidase was found in canine cerebrospinal fluid via the presence of two products: Y, which has an $[M+H]^+$ ion at m/z 182; and GGFM, which has an $[M+H]^+$ ion at m/z 411. The linked scan at a constant ratio of the magnetic field to the electric field of the GGFM $[M+H]^+$ ion at m/z 411 generates product ions at m/z 120, 150, 266, 297, 354, 357, and 411. That aminopeptidase was bestatin-sensitive (BSAP = bestatin-sensitive aminopeptidase), and had a half-time for disappearance of 60 min, maximum velocity of 1.08 nmol ml^{-1} min^{-1}, and Michaelis constant of 0.26 n*M*.

INTRODUCTION

Cerebrospinal fluid (CSF) is in close contact with and surrounds the brain's extracellular compartment. It represents a unique and important space for the study of neuropeptide metabolism [1,2]. The amount of a neuropeptide in CSF reflects the balance between that peptide's synthesis and degradation, indicates the metabolic activity of cells lining the choroid plexus [3] and ventricles of the human brain, and may reflect the actual homeostatic status and express pathophysiological changes [4,5]. Neuropeptides derive from a multi-step metabolic cascade including DNA, RNA, prehormone, the neuropeptide itself, and inactive metabolites. Enzymes act at each step in that metabolic scheme, and the neuropeptides interact with a receptor to effect a cellular event.

The opioid and tachykinin neuropeptide content in human CSF was analyzed in studies of low back pain [6–11], senile dementia of the Alzheimer type (SDAT) [12], epilepsy [13], and severe head trauma [14]. Whereas CSF neuropeptides, enzymes, and precursors [11] have been

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analyzed, the purpose of this paper is to describe a fast and facile analytical method to characterize those enzymes that act on exogenously added opioid peptides such as methionine enkephalin (ME = YGGFM). By monitoring the yield of peptides produced by enzyme degradation of the exogenous peptide and by elucidating the amino acid sequence of the peptide product, the peptidases can be more accurately characterized [half-time for disappearance ($t_{1/2}$), maximum velocity (V_{max}), inhibitors, product formation]. The CSF enzyme system is a much more robust system compared to the rapidly metabolized neuropeptides. At present, no one has elucidated the amino acid sequence of the peptide products from enzymatic degradation using canine and human CSF.

Enzyme activities in the CSF were analyzed and characterized by monitoring the rate of disappearance of exogenous ME and by elucidation of the time-course for the production of, as well as the structural analysis of several enzymatic degradation products. The amino acid sequence of the enzymatic degradation products was obtained by producing the protonated molecule ion, $[M+H]^+$, by fast atom bombardment mass spectrometry (FAB-MS) by elucidating the amino acid sequence-determining fragment ions in a tandem MS (MS–MS) experiment.

EXPERIMENTAL

Collection of CSF

Canine CSF was obtained following exsanguination, collected rapidly into liquid nitrogen to avoid metabolism, and stored at −70°C until analyzed. Human CSF was collected by shock-freezing in liquid nitrogen during studies on low back pain [6–11] and was stored at −70°C until analyzed.

Chemicals

Synthetic ME (YGGFM) and des-[Tyr^1]-ME (GGFM) were purchased from Sigma (St. Louis, MO, USA) and were used without further purification. For ME, 50 μg (87 nmol) were used for each experiment; for GGFM, 50 μg (122 nmol) were used.

Triethylamine was purchased from Pierce (Rockford, IL, USA), formic acid from Mallinckrodt (Paris, KY, USA), acetonitrile from J. T. Baker (Phillipsburg, NJ, USA), glycerol from Sigma, and Sep-Pak cartridges from Waters Division of Millipore (Milford, MA, USA).

Thiorphan (DL-3-mercapto-2-benzylpropanoyl glycine, $M_r = 253$, an enkephalinase inhibitor) [15] and bestatin [(2*S*,3*R*)3-amino-2-hydroxy-4-phenylbutane-L-leucine, $M_r = 308$, an aminopeptidase inhibitor] were purchased from Sigma, and 20 μl of a solution of 4.9 m*M* (98 nmol) and 4.1 m*M* (82 nmol) were used, respectively. Four types of experiments were performed. CSF (200 μl) plus synthetic ME (50 μg) were mixed with either: no inhibitors (experiment 1), thiorphan (experiment 2), bestatin (experiment 3), or thiorphan plus bestatin (experiment 4).

Incubation conditions

The mixture was incubated at 37°C for 5 h. When inhibitors were used (experiments 2–4), samples were pre-incubated (10 min) before adding the synthetic peptide ME or GGFM.

Reversed-phase high-performance liquid chromatographic separation

Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to separate the exogenously added synthetic peptide from its enzymatic degradation products. RP-HPLC analyses used either a Waters (for the kinetic study) or a microprocessor-driven Varian-5000 (for product identification) HPLC system. A μBondapak C_{18} column (300 mm × 3.9 mm I.D., 10 μm particle size, 30 Å pore size) purchased from Waters, triethylamine formate buffer (40 m*M*, pH 3.14) [16], and acetonitrile as the organic modifier were used. The gradient profile was 0% acetonitrile at 0–2 min, 0.39% min^{-1} from 2 to 40 min, and 15% at 40–50 min. The flow-rate of the mobile phase was 1.5 ml min^{-1}. Samples were collected manually at the retention time ($\Delta t = \pm 0.5$ min) of the peptides of interest. Peptide bond UV absorbance at 200 nm was monitored. Memory effects by contamination of the column by peptides could be prevented by a column rinse with 100% acetonitrile. MS analysis revealed that a clean column effluent was obtained.

Mass spectrometry

FAB-MS generated the protonated molecule ion $[M+H]^+$ of each peptide, and MS–MS collected the amino acid sequence-determining product ions from each individual $[M+H]^+$ ion [17–20]. A Finnigan MAT 731 mass spectrometer was used for $[M+H]^+$ determination. Glycerol was used as the FAB matrix, and an accelerating voltage sweep over a fixed mass range ($[M+H]^+$ ± 5%) was applied [20]. A linked scan at constant B/E (B = magnetic field; E = electric field) [21] on a VG 7070E-HF instrument was used to collect the product ion spectrum [17,18,22,23].

Kinetic data

Time course of the enzymolysis of ME and des-[Tyr¹]-ME in canine CSF. To 600 μl of canine CSF, 37.5 μl of an ME solution (0.2 μg/μl; total amount 13 nmol) were added. The final concentration of ME was 21.8 nmol/ml (21.8 μ*M*). The samples were incubated at 37°C until the ME concentration was reduced by approximately 98% to <0.43 nmol/ml. Every 0.5 h, a sample (40 μl) was removed, acidified with trifluoroacetic acid (TFA) to stop enzymatic degradation, and injected onto the HPLC column for ME, des-[Tyr¹]-ME, and peptide product analysis.

Lineweaver–Burke plot for ME in dog CSF. The rate of ME enzymolysis was measured for seven different concentrations (*i.e.*, 21.3, 31.5, 41.5, 60.9, 79.3, 113.8, and 145.4 nmol/ml). To 200 μl of dog CSF, the required amount of synthetic ME was added to obtain the needed concentration. The samples were incubated at 37°C for 30 min and acidified with TFA; an aliquot was analyzed for ME content by RP-HPLC.

RESULTS

The experimental data obtained in this study derive from HPLC, MS, MS–MS, and measurements of $t_{1/2}$, Michaelis constant (K_M), and V_{max} of the BSAP (bestatin-sensitive aminopeptidase).

RP-HPLC data

Fig. 1 shows the RP-HPLC profile of the separation of ME from the products that were formed by incubating synthetic ME with canine CSF. That chromatogram corresponds to the aliquot taken from the incubation mixture at 5 h. The UV absorbance (200 nm, 0.32 a.u.f.s.) is plotted *versus* elution time (min). For comparison of sensitivities, 1 μg of ME (1.7 nmol) corresponds to approximately 0.1 a.u.f.s. The chromatogram in Fig. 1 shows peaks at the retention times of ME (44.5 min), GGFM (32 min), FM (21.5 min), F (8 min), and Y (5 min).

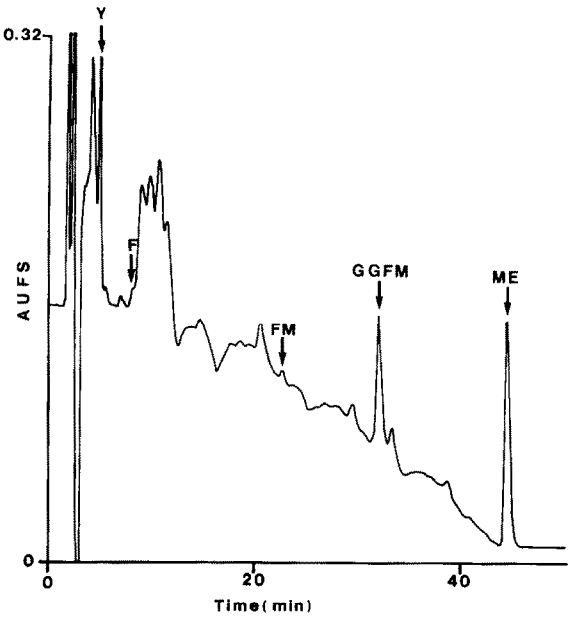

Fig. 1. Reversed-phase HPLC separation of exogenously added ME from its metabolites formed by incubating synthetic ME with canine CSF. This sample corresponds to an incubation time of 5 h. UV absorbance at 200 nm is plotted *versus* time. Peaks are shown at the retention times for ME (44.5 min), GGFM (32 min), FM (21.5 min), F (8 min), and Y (5 min).

Mass spectrometric data

FAB-MS corroborated the $[M+H]^+$ ions of YGGFM at m/z 574, GGFM at 411, FM at 297, Y at 182, and F at 166. The FAB-MS spectrum of the synthetic ME (YGGFM) that was added to and extracted from CSF demonstrates an abundant $[M+H]^+$ ion at m/z 574. The corresponding MS–MS spectrum (Fig. 2) of the m/z 574 precursor ion contains amino acid sequence-determining fragment ions at m/z 425 (B_4), 397 (A_4), 354 (Y''_2), 297 (Y''_3), 221 (B_2), and 136 (A_1), and establishes the amino acid sequence of ME [18]. Published nomenclature for product ion spectra was used [24].

Fig. 3 contains the product ion spectrum of the

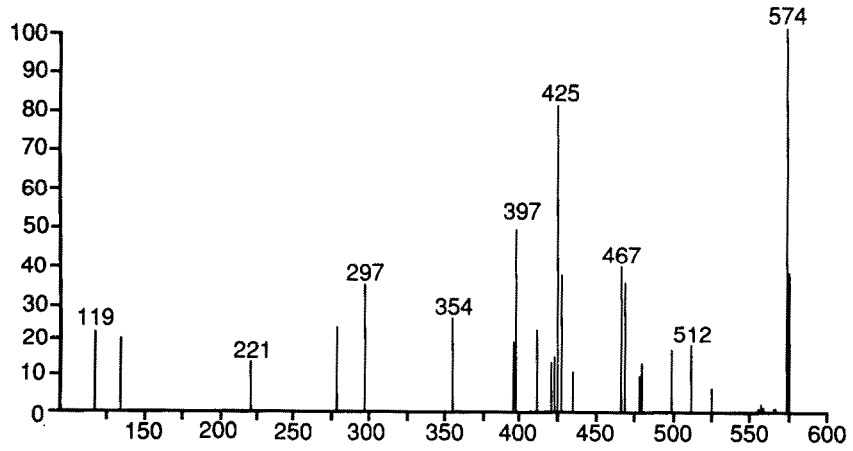

Fig. 2. Product ion spectrum derived from the precursor ion m/z 574. The scale on the x-axis is in mass-to-charge (m/z) units, and on the y-axis, relative abundance (%). Amino acid sequence-determining fragment ions are found at m/z 136 (A_1), 221 (B_2), 297 (Y''_3), 354 (Y''_2), 397 (A_4), and 425 (B_4). The ion at m/z 467 corresponds to the loss of the tyrosine side-chain (107 a.m.u.) from m/z 574.

tetrapeptide GGFM formed by the enzymolysis of synthetic ME. The product ion spectrum from the precursor ion at m/z 411 contains amino acid sequence-determining fragment ions at m/z 150 (Y''_3), 262 (B_3), 297 (Y''_2), and 354 (Y''_1). The ion at m/z 393 corresponds to the loss of H_2O from the $[M+H]^+$ ion at 411. Therefore, in addition to the production of the $[M+H]^+$ ion at m/z 411, the product ion spectrum in Fig. 3 unequivocally established the amino acid sequence of that tetrapeptide degradation product as GGFM.

In a similar fashion, the product ion spectrum (Fig. 4) of the precursor ion $[M+H]^+$ at m/z 297 contains amino acid sequence-determining fragment ions at m/z 120 (A_1), 150 (B''_1), and 177 (X'_1); those data confirm the sequence FM.

During the incubation of the sample (ME-spiked CSF), bestatin almost completely inhibited the production of GGFM (Fig. 5). Therefore, it seems likely that a BSAP was responsible for the conversion of synthetic YGGFM to Y and GGFM. On the other hand, when thiorphan was added, the amount of released FM was not affected. That result may be surprising, because FM could have been produced by the action of enkephalinase (whose thiorphan sensitivity is well known) [15] on YGGFM to produce YGG and FM. Therefore, the thiorphan-refractive en-

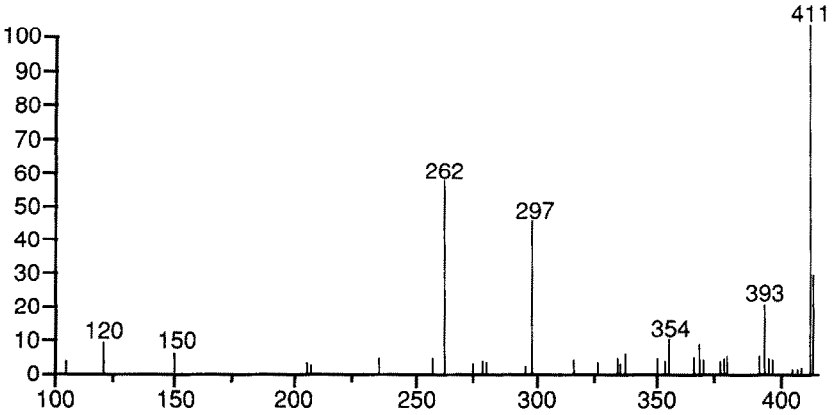

Fig. 3. Product ion mass spectrum of the precursor ion m/z 411. The scale on the x-axis is in mass-to-charge (m/z) units, and on the y-axis, relative abundance (%). Amino acid sequence-determining fragment ions are found at m/z 150 (Y''_3), 262 (B_3), 297 (Y''_2), and 354 (Y''_1).

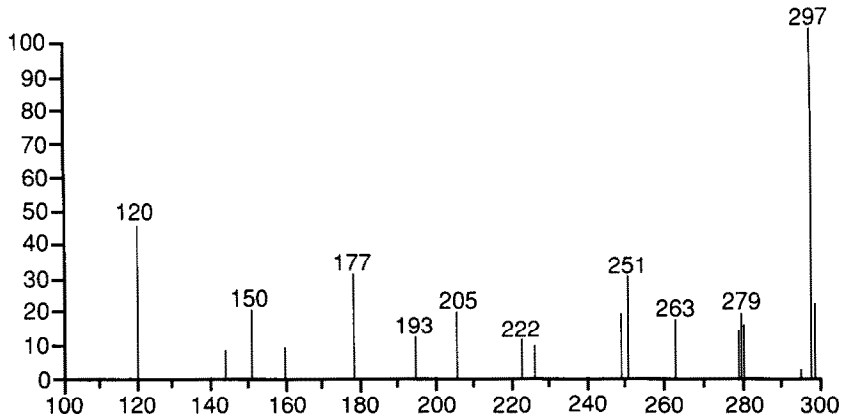

Fig. 4. Production ion spectrum of the precursor ion m/z 297. The scale on the x-axis is in mass-to-charge (m/z) units, and on the y-axis, relative abundance (%). Amino acid sequence-determining fragment ions are found at m/z 120 (A_1), 150 (B_1''), and 177 (X_1').

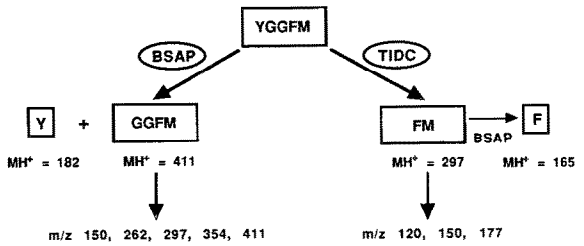

Fig. 5. Conversion of YGGFM to Y and GGFM by a bestatin-sensitive aminopeptidase (BSAP) and to FM and F by a thiorphan-insensitive dipeptidyl carboxypeptidase (TIDC).

zymatic activity discovered in this study may presumably represent a thiorphan-insensitive dipeptidyl carboxypeptidase (TIDC) that acts on synthetic YGGFM to produce FM.

To further characterize this enzyme activity, synthetic GGFM was added to CSF. The two isolated products found corresponded to the dipeptide FM and to the amino acid F (Fig. 6). No tripeptide GFM was found, perhaps due to the rapid subsequent cleavage of the two G residues in the starting substrate by the aminopeptidase activity. Thiorphan had little effect on GGFM degradation. In contrast, bestatin did not affect the production of FM, but did inhibit the production of F. Thus, the FM→F products are linked via a BSAP.

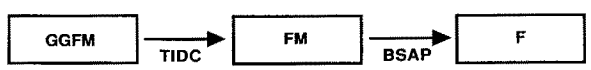

Fig. 6. GGFM degradation to the dipeptide FM and to the amino acid F. TIDC = thiorphan-insensitive dipeptidyl carboxypeptidase.

Kinetic plots

Fig. 7 contains a plot of the time-course for the disappearance of synthetic ME, and for the appearance and disappearance of the GGFM product. The $t_{1/2}$ was 60 min.

Lineweaver–Burke plot

The lineweaver-Burke plot for the enzymatic degradation of synthetic ME is shown in Fig. 8,

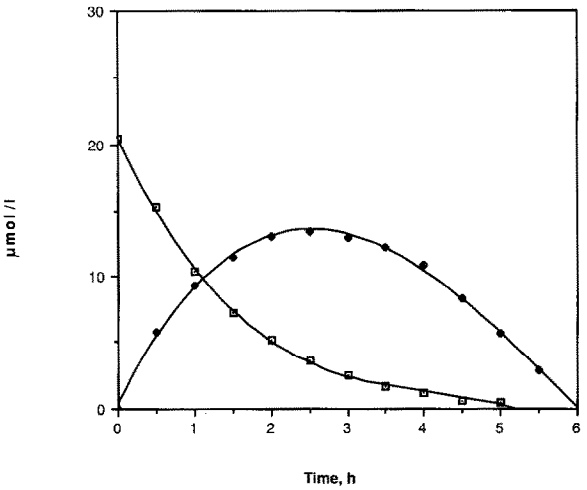

Fig. 7. Plot of the time-course for the disappearance of synthetic ME (open box), and for the appearance and disappearance of the tetrapeptide product GGFM (closed box). The amount of peptide is given as μM, and the time-axis in h. The $t_{1/2}$ value for the disappearance of synthetic ME was 60 min.

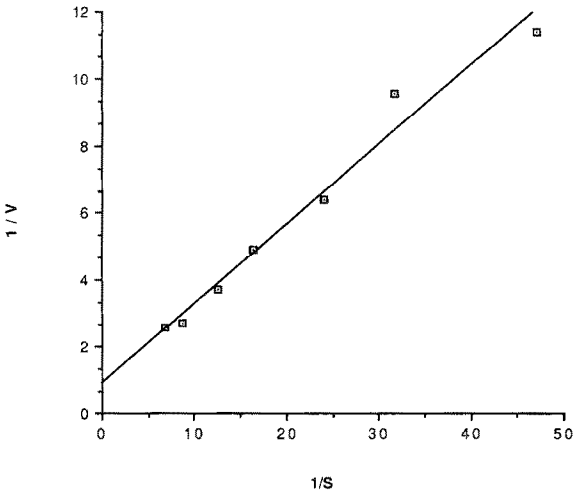

Fig. 8. Lineweaver–Burke plot for the enzymolysis of synthetic ME. V_{max} = 1.08 nmol ml^{-1} min^{-1}; K_M = 0.26 nM.

and the following kinetic data are obtained: V_{max} = 1.08 nmol ml^{-1} min^{-1} and K_M = 0.26 nM.

Human CSF

To a sample of human CSF (400 μl), 15 μl of a solution of synthetic ME (0.5 μg/μl = 13 nmol total) were added to produce a final concentration of 31.3 nmol/ml. The peptide products were analyzed by HPLC, and $t_{1/2}$ = 9.1 h was observed for ME degradation. The peptide products from the human CSF studies were also analyzed by MS, and GGFM was found as the main enzymatic degradation product.

DISCUSSION

A BSAP in canine CSF has been found, and its properties have been elucidated. That BSAP was characterized by adding a synthetic peptide (ME) to CSF, following the time course of the disappearance of the synthetic peptide, monitoring the production and subsequent disappearance of a tetrapeptide product, analyzing the amino acid sequence-determining fragment ions of the products via MS–MS, and describing the $t_{1/2}$, K_M, and V_{max} values for the enzyme. In addition to the characterization of that BSAP, another enzyme activity was described that exhibited the properties of a TIDC.

For the first time, these experiments used MS–MS to elucidate the amino acid sequence of the products formed by the action of endogenous CSF enzymes on exogenously added ME. A BSAP and a TIDC were found, but no degradation products generated by the action of thiorphan-sensitive enzymes such as enkephalinase were found.

Our experiment represents a fast and facile assay for the study of endogenous enzymatic activity in CSF and has been successfully applied to the cleavage of several different synthetic peptides. We intend to use this test to assay human CSF peptide-degrading enzyme activity in controls and pathophysiological changes such as low back pain [6–11,25], epilepsy, SDAT [12], and head trauma. For low back pain, we have been studying lumbar CSF, whereas samples of ventricular fluid were withdrawn for analysis of epilepsy and head trauma. We hypothesize that different pathophysiologies will express different enzymes and enzyme activity (V_{max}).

Other authors have analyzed human CSF for the presence of enkephalin-degrading enzymes [26]. Thin-layer chromatography and HPLC indicated the presence of Y, YG, and YGG; those data imply that an aminopeptidase, a dipeptidyl aminopeptidase, and a dipeptidyl carboxypeptidase, respectively, were present in lumbar CSF. The limitations to that study are that co-elution of a peptide standard is of limited molecular specificity and cannot indicate an amino acid sequence. The second limitation is the fact that apparently [^{3}H-]Tyr in the exogenously added ME was used; that N-terminal radiolabel limited the study of carboxypeptidases. For example, the presence of YGG does not indicate exclusively the possibility of a dipeptidyl carboxypeptidase without also firmly establishing the presence of the corresponding FM product.

The CSF activity of dynorphin-converting enzyme transforming prodynorphin-derived peptides to leucine-enkephalin-Arg^6 was measured in twelve women at term pregnancy before cesarean section and in eight non-pregnant, non-puerperal controls [27]. In the pregnant women, the amount of dynorphin-converting enzyme activity was significantly lower than in non-pregnant controls. Those data indicate that a reduced activity of the opioid peptide-degrading enzymes

might contribute to an increased resistance to pain at term pregnancy. One of the limits in that study is that the authors stated that HPLC data clearly indicated that the major product released by the analyzed activity was leucine-enkephalin-Arg^6. Of course, HPLC retention time cannot indicate amino acid sequence information [28].

An endopeptidase in human CSF that hydrolyzed substance P to substance P(1–7) and substance P(1–8) was studied [29]. Apparently, a peptidyl α-amidating monooxygenase has been demonstrated to be present in human CSF [30]. To measure its activity, synthetic substance P-Gly^{12} was added to CSF, and SP-like immunoreactivity (SP-li) was measured following HPLC separation.

Angiotensin-converting enzyme-like activity was demonstrated in human CFS [31]. Leucine-enkephalin (LE = YGGFL) was approximately ten times more stable in CSF than LE-Arg^6, and the major degradation products from LE were Y and YGG, suggesting the presence of aminopeptidase and depeptidyl carboxypeptidase activities, respectively.

A study of eleven peptidases was performed with membrane fractions prepared from porcine choroid plexus [3]. Those peptidases, which are present on the choroid plexus, may play a role in metabolizing neuropeptides at that locality. The enzymes included endopeptidases, peptidyl dipeptidases, aminopeptidases, and carboxypeptidases. These enzymes may play a key role in the regulation of neuropeptide concentrations in the CSF, and also may exert a protective role by guaranteeing that peptides that enter the CSF from within the central nervous system would be prevented from reaching, by the CSF circulation, distant but possibly responsive sites.

Aminopeptidase M activity in human CSF was studied because it may play a significant role in the degradation of low-molecular-mass opioid peptides in human CSF. The rates of hydrolysis of the ^{1}Y–^{2}G bond of larger opioid peptides decreased with increasing peptide lengths [32]. The mean activity in blood-free CSF samples was 0.42 nmol min^{-1} ml^{-1}, which is a value similar to the 1.08 nmol min^{-1} ml^{-1} rate found in this present study.

Some of the limitations of the preceding reports include several important experimental factors. In several cases, the authors state that the identity of the peptide was established by co-elution on an HPLC column; however, co-elution does not constitute amino acid sequence determination, and therefore those claims for the identity of a peptide cannot be made. The second limitation is that radioimmunoassay (RIA) of a peptide was used; RIA cannot establish the amino acid sequence of a peptide [28,33,34]. A third limitation of the previous data is the fact that an iodinated peptide was used in the RIA. Of course, the introduction of a large hydrophobic atom such as iodine in a peptide alters significantly the structural and hydrophobic characteristics of that peptide. The only way to establish the presence of the enzymes in CSF is to unambiguously establish the amino acid sequence of the peptide produced by the action of the CSF peptidase.

The study of CSF neuropeptidases must include the amino acid sequence of each product peptide so that confidence is available on specific enzymes responsible for product formation. Clearly, the only datum that defines a peptide is the amino acid sequence, which can only be provided by gas- or liquid-phase sequencers, X-ray crystallography, or MS–MS methods [20]. RIA, receptor assay, RP-HPLC, *in situ* hybridization, mRNA measurements, cDNA studies, etc. cannot provide the amino acid sequence of the final, post-translationally modified neuropeptide [28,35]. Therefore, sequence determination is required for each and every peptide in each and every experiment.

CONCLUSIONS

In summary, an aminopeptidase in CSF was characterized that cleaves exogenously added ME into Y and GGFM. We used FAB-MS to produce the $[M+H]^+$ ion of peptides and MS–MS to produce amino acid sequence-determining product ions in order to elucidate the amino acid sequence of peptides formed by the action of various CSF peptidases on endogenously added synthetic peptides (ME, GGFM). The existence of a BSAP and a TIDC is shown for canine CSF. The kinetic parameters of the canine BSAP were $t_{1/2}$ = 60 min, V_{max} = 1.08 nmol ml^{-1} min^{-1}, and

K_M = 0.26 nM. The $t_{1/2}$ value for canine CSF is nine times greater than that of human CSF.

These data are important for studies requiring the analysis of neuropeptides in CSF for the elucidation of the molecular mechanisms involved in low back pain, SDAT, severe head trauma, and epilepsy.

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