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N-TERMINAL DEGRADATION OF LOW MOLECULAR WEIGHT OPIOID PEPTIDES IN HUMAN CEREBROSPINAL FLUID

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Abstract—Opioid peptides are present in human cerebrospinal fluid (CSF), and their levels are reported to change in some pathologic conditions. However, less is known about their degradation in CSF. In the present study, human CSF was found to contain aminopeptidase activity which hydrolyzed alanyl-, leucyl- and arginyl-naphthylamides in a ratio of 100:28:27. Twelve CSF samples hydrolyzed alanyl-2-naphthylamide and degraded Met^5-enkephalin (N-terminal hydrolysis) at rates of 188 ± 38 and 420 ± 79 pmol/min/mL respectively. Further, the distribution of alanyl-naphthylamidase activity in individual samples (39–437 pmol/min/mL) was closely correlated with that of Met^5-enkephalin degradation (37–833 pmol/min/mL). Both alanyl-naphthylamidase and enkephalin degradation were optimal at pH 7.0 to 7.5 and were inhibited by the aminopeptidase inhibitors amastatin (IC_{50} = 20 nM), bestatin (4–7 μM) and puromycin (30–35 μM). Conversely, degradation was unaffected by inhibitors of neutral endopeptidase (phosphoramidon), carboxypeptidase N (MERGETPA) or angiotensin converting enzyme (captopril). The K_m of Met^5-enkephalin for the CSF aminopeptidase activity was 201 ± 19 μM (N = 4). Rates of hydrolysis of the Tyr^1-Gly^2 bond of larger opioid peptides decreased with increasing peptide length. Pooled, concentrated CSF hydrolyzed Leu^5-enkephalin, dynorphin A fragments [1–7], [1–10] and [1–13] and dynorphin A at rates of 2.05 ± 0.27, 1.76 ± 0.18, 0.94 ± 0.06, 0.55 ± 0.14 and 0.16 ± 0.03 nmol/min/mL respectively. When analyzed by rocket-immunoelectrophoresis against antisera to aminopeptidase M (EC 3.4.11.2), the concentrated CSF formed an immunoprecipitate which could be stained histochemically for alanyl-naphthylamidase activity. These data are consistent with a significant role for aminopeptidase M activity in the degradation of low molecular weight opioid peptides in human CSF.

Opioid peptides including dynorphins and enkephalins [1] are present in human CSF, and alterations in their levels have been reported in various pathologic conditions [2–5]. In addition, strong nociceptive stimuli increase the levels of opioid peptides in CSF [6, 7]. If such changes reflect physiologically significant alterations in endogenous opioid systems, it is likely that they relate not only to opioid peptide synthesis and release, but also to enzymatic degradation. CSF contains amino-, endo- and carboxypeptidase capable of processing and/or degrading opioid peptides [8–13], and altered rates of degradation have been reported in some pathologic conditions [14].

Evidence for a physiologically significant role for peptidases in modulating opioid-like effects comes from numerous studies. The analgesic effects of exogenous opioid peptides are potentiated after protection from catabolism by peptidase inhibitors [3, 8, 15–18]. Further, peptidase inhibitors alone can stimulate opioid-like effects, presumably via potentiation of endogenous opioids. For instance, intracerebral ventricular application of peptidase inhibitors reduces the severity of opiate narcotic withdrawal in rats [19] and is associated with naloxone-reversible antinociception in mice [17, 18]. In view of the potential importance of peptidase-modulated opioid activity, the present sudy was conducted to examine the degradation of low molecular weight opioid peptides in human CSF.

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MATERIALS AND METHODS

Materials. All opioid peptides, alanyl-, leucyl-, arginyl- and α-glutamyl-2-naphthylamides, alanyl-4-methoxy-2-naphthylamide (alanyl-MNA), *o*-phenanthroline, amastatin, bestatin, puromycin and phosphoramidon were obtained from the Sigma Chemical Co. (St. Louis, MO). The angiotensin converting enzyme inhibitor captopril was obtained from Squibb (Princeton, NJ) and the carboxypeptidase N inhibitor MERGETPA (D-L-mercaptomethyl-3-guanidino-ethylthiopropanoic acid) was obtained from Calbiochem-Behring (San Diego, CA). The MN 300 Uniplates used for TLC were from Analtech, Inc. (Newark, DE), and the amino acid standards, *o*-phthalaldehyde crystals and reagent solution (OPA) used for HPLC were from the Pierce Chemical Co. (Rockford, IL).

Human cerebrospinal fluid (CSF). Twenty-six samples of superfluous human CSF (0.5 to 5.0 mL) were obtained by lumbar puncture from neurologic subjects at Lincoln Hospital (Bronx, NY) and frozen until analysis. Samples were tested by Chemstrip® (Boehringer Mannheim, Indianapolis, IN) and grouped into those free of blood contamination

(twelve) and those containing trace amounts of blood (fourteen). In some instances, samples were pooled and dialyzed/concentrated (3- to 10-fold) using a Minicon B-15 concentrator (Amicon Corp., Danvers, MA) with a membrane cutoff of 15,000 mol wt.

Enzyme assays. Aminopeptidase activities in human CSF samples were determined as the rates of hydrolysis of alanyl-, leucyl- and arginyl-2-naphthylamides as previously described [20, 21] by recording the increase in fluorescence over 10-min intervals. Where appropriate, inhibitors were preincubated with enzyme and buffer (5 min) before addition of substrate. For peptide inhibitor experiments, peptides were added simultaneously with alanyl-2-naphthylamide substrate. Unless otherwise noted, specific activities are expressed as units per milliliter CSF where one unit equals the hydrolysis of one picomole substrate per minute.

Enkephalin metabolism. Qualitative analysis of enkephalin metabolism was carried out by TLC on MN 300 cellulose plates [22]. Quantitative analysis was performed by HPLC as described previously [20, 23]. Briefly, CSF aminopeptidase was determined as the rate of N-terminal Tyr released from enkephalins. The standard incubation consisted of mixing the peptide (250 μM) in 295 μL of 100 mM sodium phosphate buffer (pH 7.0) with 20 μL of CSF (37°). In some cases, captopril, phosphoramidon and/or MERGETPA (10 μM) were included in the incubation mixture. At sequential time intervals, 60-μL aliquots were immersed in a boiling water bath (5 min) to terminate the reaction, cooled on ice, and centrifuged (3 min), and the supernatant fractions were collected for analysis.

For determination of pH dependence, a 100 mM sodium acetate buffer was used over the pH range 4.0 to 6.0, and the above 100 mM phosphate buffer was used from pH 6.5 to 8.0. pH studies were also conducted with the Britton and Robinson type [21] universal buffer (pH 4.0 to 10.5). For inhibition studies, inhibitors were preincubated with enzyme and buffer for 10 min at 37°. For K_m determinations, measurements of the initial velocity of hydrolysis were determined over a range of substrate concentrations (25–500 μM). Data were plotted as $1/V$ vs $1/S$ and fit to the best straight line.

A high performance liquid chromatograph system (Waters Associates, Milford, MA) consisting of two model 6000A pumps, a model 730 Data Module, a model 721 System Controller, a model 712B WISP™ Autosampler and a model 420 Fluorescence Detector were employed for the HPLC analysis. Standards and unknowns (40 μL) were automatically derivatized with OPA solution (20 μL) 3 min prior to chromatography (Pre-column Derivatization Program, Waters 710B WISP™ Autosampler) and separated on a reverse phase column (Waters, 10 μm, C_{18}-Radial-PAK™, 8 mm × 10 cm) at a constant flow rate of 5.0 mL/min utilizing a linear gradient from 100% Buffer A to 40% Buffer A/60% Buffer B. Buffer A was 10 mM sodium phosphate (pH 7.0) and Buffer B was a 50/50 (v/v) mixture of Buffer A and acetonitrile. Integration of sample peak areas and quantitation of metabolite against the last-run standard were automatically calculated by the data module. Standards were run every sixth injection.

Table 1. Synthetic substrate hydrolysis by human CSF aminopeptidase activity

Substrate	Activity (pmol/min/mL)	Ratio
Ala-2-Naphthylamide	188 ± 38	100
Leu-2-Naphthylamide	53 ± 22	28
Arg-2-Naphthylamide	51 ± 12	27
Glu-2-Naphthylamide	34 ± 11	

Alanyl-, leucyl- and arginyl-naphthylamidase activities and aminopeptidase A-like α-glutamyl-naphthylamidase activity in twelve samples of human CSF were measured. Values are means ± SE.

The OPA solution was made fresh daily by mixing 3.8 mL *o*-phthalaldehyde reagent solution, 0.1 mL methanol, 0.1 mL β-mercaptoethanol and 16.8 mg *o*-phthalaldehyde crystals.

Immunoelectrophoresis. As previously described [21, 23], rocket immunoelectrophoresis of pooled, concentrated CSF against antisera to purified human aminopeptidase M (EC 3.4.11.2) was performed using 1.0 mm thick 1% (w/v) agarose gel containing 1% (v/v) Triton X-100. After electrophoreses (1.5 V/cm; 14 hr), the gel was repeatedly pressed and re-hydrated to remove soluble protein, and the aminopeptidase M immunoprecipitin line was stained using alanyl-MNA. The antisera to purified human aminopeptidase M [24] was supplied by Dr. Shigehiko Mizutani (Nagoya University School of Medicine, Nagoya, Japan).

RESULTS

In preliminary studies, pooled, concentrated human CSF was incubated with Met^5-enkephalin. Separation of metabolites by TLC revealed the production of N-terminal Tyr over time. Since hydrolysis was inhibited completely by amastatin (10^{-4} M) and no other product was detected, subsequent studies investigated the presence of aminopeptidase activity in human CSF.

Characterization of CSF naphthylamidase activity. Fourteen samples of human CSF containing trace levels of blood contamination hydrolyzed alanyl-2-naphthylamide at a rate of 273 ± 56 pmol/min/mL (22–690 units/mL). These levels were only slightly higher than the levels found in twelve human CSF samples containing no detectable blood contamination (188 ± 38 pmol/min/mL) (39–437 units/mL). As shown in Table 1, leucyl- and arginyl-naphthylamides were also hydrolyzed, whereas significantly less aminopeptidase A-like α-glutamyl-2-naphthylamidase activity could be detected.

CSF alanyl-naphthylamidase activity had a pH optimum of 7.5. Amastatin was the most potent inhibitor of the CSF activity with an IC_{50} of 20 nM, whereas both bestatin and puromycin were significantly less potent (IC_{50} = 7 and 35 μM respectively). The CSF activity was also inhibited by 1 mM *o*-phenanthroline (100%), but not by inhibitors of angiotensin converting enzyme (captopril), neutral endopeptidase (phosphoramidon) or carboxypeptidase N (MERGETPA) (less than 10% inhibition at a final concentration of 10 μM). As shown in Fig.

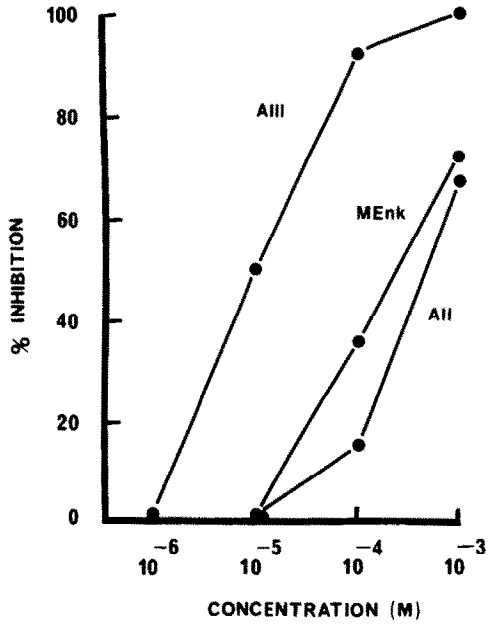

Fig. 1. Inhibition (%) of pooled human cerebrospinal fluid alanyl-2-naphthylamidase activity (201 pmol/min/mL) by a range of concentrations of angiotensin III, Met^5-enkephalin and angiotensin II. Peptides were added simultaneously with substrate. Values shown are the averages of two determinations.

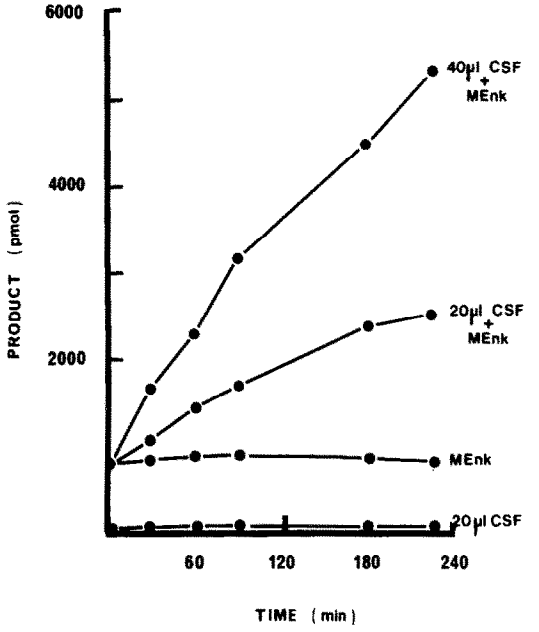

Fig. 2. N-Terminal hydrolysis of Met^5-enkephalin by 40 and 20 µL of pooled human cerebrospinal fluid. Tyrosine production was determined after separation and quantitation by high pressure liquid chromatography as described in Materials and Methods. No product was produced when Met^5-enkephalin and cerebrospinal fluid were incubated separately.

1, CSF alanyl-naphthylamidase activity was inhibited by angiotensin III (IC_{50} = 10 µM), whereas Met^5-enkephalin (IC_{50} = 250 µM) and angiotensin II (IC_{50} = 600 µM) were considerably less potent.

Enkephalin metabolism. N-Terminal hydrolysis of Met^5-enkephalin was directly proportional to both time of incubation and amount of CSF used (Fig. 2). Mean activity in blood-free CSF samples was 420 ± 79 pmol/min/mL (N = 12) and the activity in individual samples (37–833 units/mL) was closely correlated with the previously obtained rates of alanyl-2-naphthylamidase activity (Fig. 3). The fourteen samples containing trace amounts of blood hydrolyzed Met^5-enkephalin only slightly faster (540 ± 80 pmol/min/mL) (103–1158 units/mL).

As shown in Fig. 4, N-terminal hydrolysis of Met^5-enkephalin was optimal at pH 7.0 to 7.5. Amastatin was the most potent inhibitor (IC_{50} = 20 nM), whereas both bestatin and puromycin were significantly less potent (IC_{50} = 4 and 30 µM respectively) (Fig. 5). Enkephalin degradation was also inhibited by 1 mM *o*-phenanthroline (100%) but, as above, no inhibition was observed in the presence of captopril, phosphoramidon or MERGETPA (less than 5% inhibition at 10 µM final concentration). When the rates of hydrolysis of Met^5-enkephalin were determined over a range of substrate concentrations, the K_m obtained was 201 ± 19 µM (N = 4).

Since we had found previously that alanyl-2-naphthylamidases such as aminopeptidase M degrade opioid peptides at different rates [20, 23], the metabolism of larger opioid peptides were examined. As shown in Fig. 6, an initial experiment with pooled CSF indicated that the rates of hydrolysis of the N-terminal Tyr^1-Gly^2 bonds of C-terminally extended opioids decreased with increasing peptide length. When these studies were repeated with pooled, concentrated CSF, a clear relationship between rates of degradation and peptide length was apparent (Table 2). Leu^5-enkephalin was hydrolyzed most rapidly, whereas dynorphin A fragments [1–7] through [1–13] were hydrolyzed at progressively slower rates. The hydrolysis of dynorphin A (0.16 ± 0.03 nmol/min/mL) was less than one-tenth as rapid as Leu^5-enkephalin.

As shown in Fig. 7, rocket immunoelectrophoresis of increasing amounts of pooled, concentrated CSF against antisera to purified human aminopeptidase M [24] produced rocket-shaped immunoprecipitates when stained histochemically using alanyl-MNA. No immunoprecipitate was produced in the absence of aminopeptidase M antisera (Fig. 7/far right).

DISCUSSION

As recently reviewed by Dua *et al.* [17], aminopeptidase activity has been strongly implicated in the degradation of opioid peptides within the CNS. Consistent with this, the results of the present study demonstrate that, despite the reported presence of endopeptidases [10] and angiotensin I converting enzyme (EC 3.4.15.1) [13], human CSF preferentially degrades Met^5- and Leu^5-enkephalin by N-terminal hydrolysis. That this hydrolysis is due to aminopeptidase activity can be concluded from data demonstrating inhibition by aminopeptidase inhibitors, but not by inhibitors of neutral endopeptidase (EC 3.4.24.11), angiotensin converting enzyme or carboxypeptidase N (EC 3.4.17.3). Further, the rates

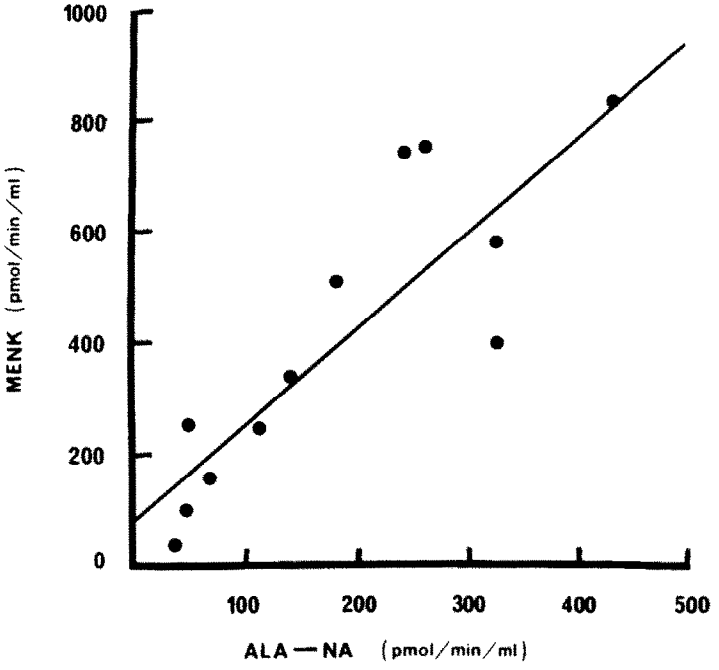

Fig. 3. Relationship of N-terminal Met^5-enkephalin hydrolysis and alanyl-2-naphthylamidase activity in twelve individual samples of human cerebrospinal fluid (Sigma Scan™ linear regression analysis; $r = 0.84$; $P < 0.005$).

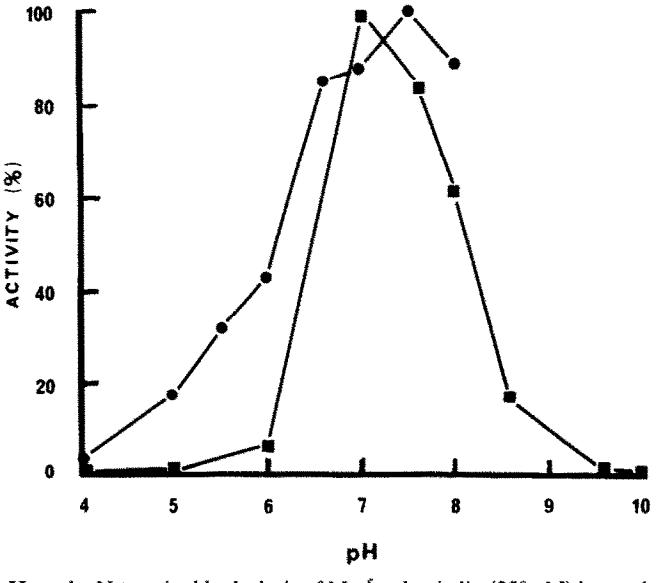

Fig. 4. Effect of pH on the N-terminal hydrolysis of Met^5-enkephalin (250 μM) by pooled, concentrated human cerebrospinal fluid. Activity is expressed as percent maximum in either sodium acetate/phosphate buffer (●) (5.2 nmol/min/mL) or Britton and Robinson universal buffer (■) (4.5 nmol/min/mL). Values shown are the averages of two or three determinations.

of N-terminal enkephalin hydrolysis in individual CSF samples were closely correlated with the levels of hydrolysis of the aminopeptidase substrate alanyl-2-naphthylamide.

Based on the substrate and inhibition profiles of the CSF aminopeptidase activity, several characterized aminopeptidases can be ruled out as the responsible enzyme. Aminopeptidase A (EC 3.4.11.7) and cytosolic aspartyl-aminopeptidase (EC 3.4.11.-) are unlikely candidates based on their substrate specificity for N-terminal acidic amino acids and resistance to inhibition by bestatin [25, 26]. Further, little glutamyl-2-naphthylamidase activity was detected in human CSF. Similarly, despite the presence of dipeptidyl(amino)peptidase IV (EC 3.4.14.5) in CSF (work in progress), it has a substrate specificity for N-terminal dipeptide (X-Pro) sequences and is not inhibited by either amastatin

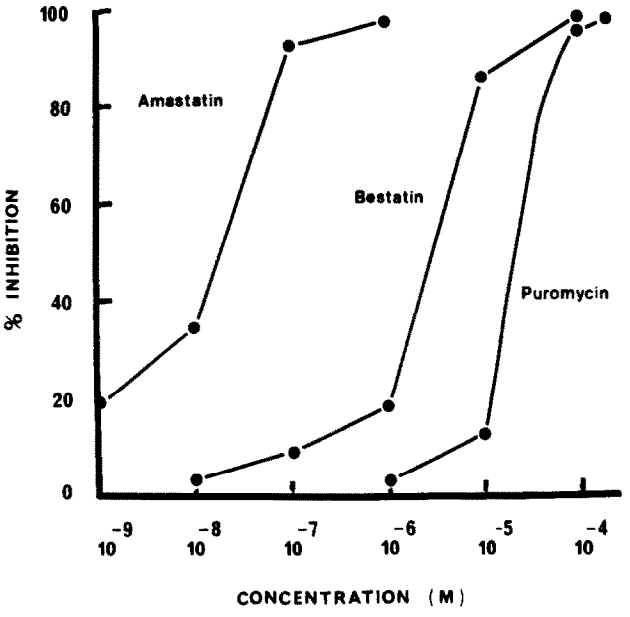

Fig. 5. Inhibition (%) of pooled, concentrated human cerebrospinal fluid N-terminal degradation of 250 μM Met^5-enkephalin (5.2 nmol/min/mL) by a range of concentrations of amastatin, bestatin and puromycin. Inhibitors were preincubated with buffer and enzyme for 10 min prior to the addition of substrate. Values shown are the averages of two or three determinations.

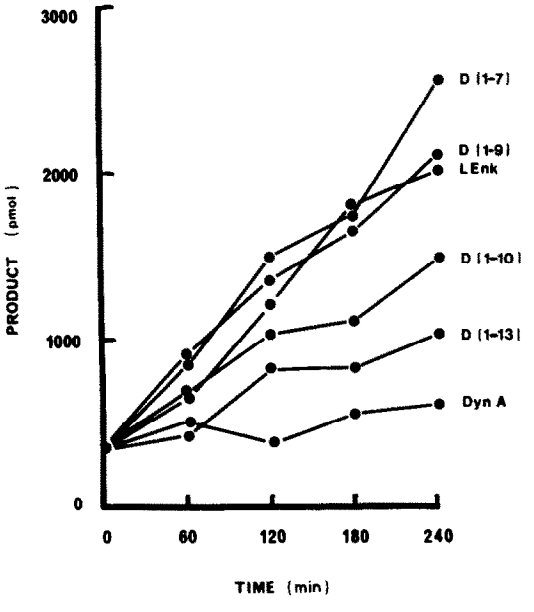

Fig. 6. Initial experiment examining the rates of N-terminal hydrolysis of low molecular weight opioid peptides (100 μM) by pooled human cerebrospinal fluid. Peptides include Leu^5-enkephalin, Dynorphin A fragments [1–7], [1–9], [1–10] and [1–13] and Dynorphin A.

Table 2. Rates of N-terminal hydrolysis of opioid peptides by cerebrospinal fluid aminopeptidase activity

Peptide		Activity (nmol/min/mL)
Leu^5-Enkephalin		2.05 ± 0.27
Dynorphin A	Fragment [1–7]	1.76 ± 0.18
	Fragment [1–9]	1.47
	Fragment [1–10]	0.94 ± 0.06
	Fragment [1–13]	0.55 ± 0.14
Dynorphin A		0.16 ± 0.03

Assays were performed using pooled, concentrated human CSF at a final substrate concentration of 100 μM. Values are means ± SE of three, or the average of two, experiments. Dynophin A = Leu^5-enkephalin-Arg^6-Arg^7-Ile^8-Arg^9-Pro^{10}-Lys^{11}-Leu^{12}-Lys^{13}-Trp^{14}-Asp^{15}-Asn^{16}-Gln^{17}.

or bestatin [27]. Unlike the CSF activity, cytosolic leucine aminopeptidase (EC 3.4.11.1) hydrolyzes leucyl-naphthylamide much more rapidly than alanyl- and arginyl-naphthylamides [26] and aminopeptidase B (EC 3.4.11.6) is reportedly unaffected by amastatin [26]. Two of the three membrane-bound aminopeptidases which have been identified in the CNS (MI and MII) [28, 29] can also be excluded as the responsible CSF enzyme. MI displays little alanyl- or leucyl-naphthylamidase activity and is relatively inactive regarding enkephalin metabolism [30]. MII activity degrades enkephalins but is sensitive to inhibition by puromycin [30].

Although characterization data on peptidases in unfractionated extracts must be interpreted with caution [26], the aminopeptidase activity in human CSF shares many characteristics with aminopeptidase M (AmM; EC 3.4.11.2). AmM has been identified in the CNS [23, 31–34], hydrolyzes alanyl-naphthylamide more rapidly than leucyl- and arginyl-naphthylamides [21, 35], has a similar inhibition profile for amastatin and bestatin [20, 21, 36], and is relatively resistant to inhibition by puromycin [21, 32, 36]. Further, immunoelectrophoretic studies using antisera to purified human AmM established the presence of immunoreactive AmM in pooled, concentrated CSF. Collectively, these data support

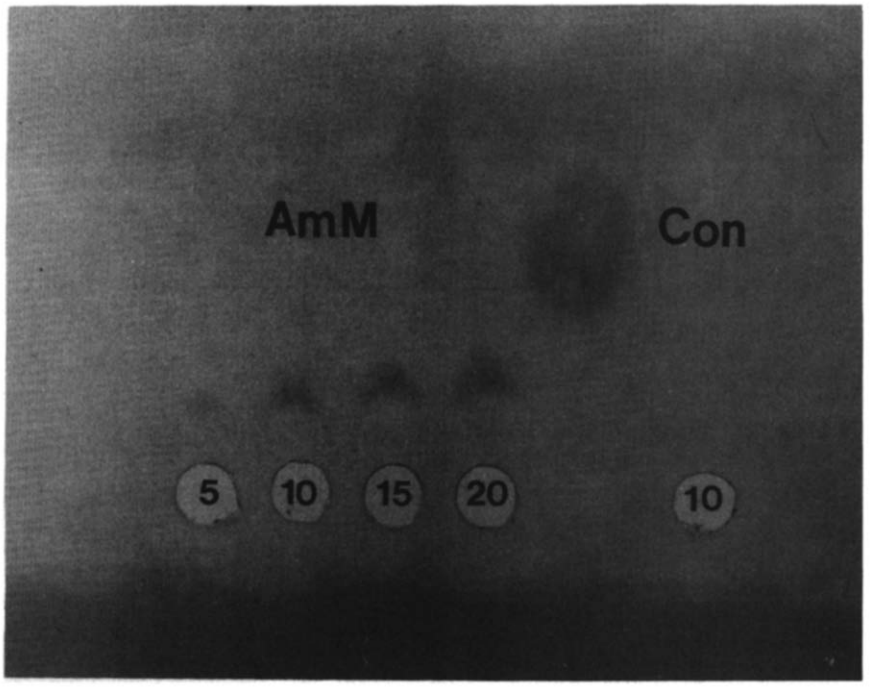

Fig. 7. Rocket immunoelectrophoresis of pooled, concentrated cerebrospinal fluid. CSF (5–20 μL) was electrophoresed into gel containing antisera to human aminopeptidase M (AmM) or gel alone (Con) at 1.5 V/cm for 14 hr. After repeated dehydration/hydration to remove soluble protein, the immunoprecipitate was visualized histochemically by staining for alanyl-MNA hydrolysis.

a significant role for AmM in CSF degradation of low molecular weight opioid peptides. Interestingly, immunoreactive AmM has also been identified in the choroid plexus [34] which is the principal source of CSF.

Both CSF and AmM [20, 23] degrade shorter opioid peptides more rapidly [20, 23], although this characteristic is not uncommon among aminopeptidases [37]. Consistent with these observations, Austen and co-workers [38] have reported that peptide length is a significant factor affecting metabolism by aminopeptidases. Thus, aminopeptidase-mediated metabolism may be more relevant to lower, rather than higher, molecular weight opioid peptides.

Hazato *et al.* [9, 39] have also reported aminopeptidase-mediated enkephalin degradation in human CSF. Although no data were given regarding CSF napthylamidase or enkephalin degradative activities in individual samples, an aminopeptidase (which they designated as C-AP_2) was found which was inhibited by both amastatin (IC_{50} = 8 nM) and bestatin (IC_{50} = 3.7 μM). These data are similar to that found in the present study (IC_{50} = 20 nM and 4 μM respectively). However, unlike Hazato *et al.* [9, 39], we found no evidence for the presence of a second aminopeptidase which was resistant to inhibition by both amastatin and bestatin.

As noted earlier, the present data do not exclude a role for endo- and carboxy-peptidases in CSF enkephalin hydrolysis, particularly for larger opioid peptides which are only slowly hydrolyzed at their N-termini. Although only N-terminal Tyr was detected in the present study, longer incubations may have revealed the production of other metabolites produced by CSF endopeptidases [10], angiotensin converting enzyme [13] and other peptidases [40]. However, since Tyr was clearly the initial metabolite produced in the present study, these data emphasize the importance of CSF N-terminal degradation and are consistent with the potency of analogs such as D-Ala^2-enkephalin [41] which are resistant to N-terminal hydrolysis [20, 23]. Although the K_m value of Met^5-enkephalin for the CSF aminopeptidase (201 ± 19 μM) is higher than that reported for neutral endopeptidase (20–90 μM), it is considerably lower than that for angiotensin converting enzyme (1000 μM) [18]. Although the relatively rapid aminopeptidase-mediated hydrolysis of Met^5-enkephalin in unconcentrated CSF (37–833 pmol/min/mL) would not occur at the opioid concentrations found in CSF (0.5–3 nmol/L) [1, 41], they may occur at or near the sites of release from spinal tissue. Thus, immediate inhibition or destruction of CSF aminopeptidase activity would be required if accurate levels of endogenous CSF opioid peptides are to be obtained. This may be particularly relevant to measurements of the pentapeptides which are subject to the most rapid N-terminal degradation. Such metabolism could account, at least in part, for the considerable variation in the levels of CSF Met^5- and Leu^5-enkephalin reported in the literature [4, 5]. Based on the inhibition profile obtained in the present study, amastatin would be a significantly better inhibitor than the more commonly used aminopeptidase inhibitors such as bestatin, bacitracin and puromycin.

Regarding inhibition profiles, it is interesting to note that the potency of amastatin (relative to puromycin) as an inhibitor of CSF enkephalin degradation is consistent with reports that amastatin produces antinociception stereoempimerically,

whereas puromycin is significantly less effective [17]. Another interesting feature is that angiotensin III, which was found in the present study to be the most potent peptide inhibitor of CSF aminopeptidase activity, has been reported to produce an opioid-dependent analgesia in mice through potentiation of endogenous opioids [42].

As noted earlier, altered levels of CSF opioid peptides in various pathologic conditions may relate to changes in the rates of opioid degradation. Dupont *et al.* [14] reported that schizophrenics display higher rates of degradation of receptor-assayed opioid activity in CSF, and such degradation is associated with lower levels of enkephalin-like material. Although the absence of control CSF samples from normal individuals precludes meaningful comparisons in the present study, studies examining possible changes in the activity of CSF aminopeptidases may be useful. Based on the similar characteristics of and close correlation between N-terminal enkephalin hydrolysis and alanyl-naphthylamidase activity, screening studies could be carried out using the relatively simple fluorometric assay of naphthylamidase activity.

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