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Proenkephalin A and proopiomelanocortin peptides in human cerebrospinal fluid

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ABSTRACT

Precursors to β-endorphin (BE) and methionine enkephalin (ME), and proteolytic enzymes that cleave those BE and ME precursors to BE and ME, were determined in several milliliters of human cerebrospinal fluid. Endogenous peptides were purified by reversed-phase high-performance liquid chromatography (HPLC), and were detected with radioreceptor assay (RRA), radioimmunoassay, and mass spectrometry (MS). Total opioid receptor activity measurements and the profile of HPLC–receptor activity of human CSF samples were both used to monitor neuropeptide metabolism. MS data linked the molecular ion of ME to a unique fragment ion. A later-eluting fraction (84 min) in a 90-min HPLC gradient appeared in all HPLC–RRA profiles, contained opioid receptor activity that discplaced [^{3}H]etorphine, and the quantitative and qualitative patterns of opioid receptor activity in those profiles both changed within the few minutes that elapsed between acquiring the first and second cerebrospinal fluid samples. That 84-min fraction contained precursors to opioid peptides and was fractionated further with a more shallow 120-min HPLC gradient into three sections that displayed δ-opioid receptor-preferring activity, using [^{3}H]ME as ligand. These three sections were hydrolyzed separately with human cerebrospinal fluid as the source for endogenous neuropeptides to yield products that correlated to immunoreactive BE in section I and immunoreactive ME in section III.

INTRODUCTION

This study was designed to test the hypothesis that opioid [1] and tachykinin [2] neuropeptides play a role in low back pain. Our working hypothesis is that the three opioid [proopiomelanocortin (POMC), proenkephalins A and B] and the three tachykinin (α, β, γ) neuropeptide pathways [3] play a homeostatic regulatory role in human cerebrospinal fluid (CSF), and that derangements in their

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metabolism are involved in several different pathophysiologies including low back pain (LBP) [1], orthodontic stress [4,5] and psychiatry [6]. A number of metabolic defects could occur at several different critical locations in the cascade that leads from the large gene product and its corresponding intermediate-sized precursors to a "working" peptide, to receptor binding of that peptide, and finally to metabolism of those peptides to inactive metabolites.

Although our preliminary data [1,2] substantiated this hypothesis, the data described in this manuscript indicated that neuropeptide metabolism in human lumbar CSF is even more complicated than expected, and that improvements in chromatographic separation and in detection are required. Therefore, in this study, we describe an inverse quantitative and qualitative relationship that was observed between earlier-eluting and later-eluting fractions in a reversed-phase high-performance liquid chromatographic (RP-HPLC) gradient in only a few milliliters of two lumbar CSF samples obtained within minutes of each other, the presence of precursors to methionine enkephalin (ME) and to β-endorphin (BE) (manifested through detection of ME and BE receptor-active products), the presence of neuropeptides of metabolize precursors to produce immunoreactive BE (ir-BE) and immunoreactive leucine enkephalin (ir-LE), and the use of mass spectrometry (MS) to link the $[M-H]^-$ ion of ME to a unique fragment ion (the loss of the Tyr side-chain). All of these data improve significantly the specificity of detecting peptides in CSF.

Several empirical observations made during our studies of opioid peptides in human CSF prompted this present study: the amount of total opioid receptor activity differed between the first and second lumbar CSF samples obtained within minutes of each other during the analysis of LBP; the sum of the HPLC-separated distribution of opioid receptor activity did not correlate with unfractionated opioid receptor activity (that situation is also similar to measurement of immunoreactive tachykinins [2]), and an inverse relationship was observed between later-eluting *versus* earlier-eluting HPLC peaks that contained opioid receptor activity. Human CSF was used as the source of endogenous opioid peptides, precursors, and precursor-processing enzymes; a more shallow HPLC gradient was developed [7]; radioreceptor assay (RRA) with two different ligands, [^{3}H]etorphine and [^{3}H]ME [8] and radioimmunoassay (RIA) were all used to detect peptides; MS was used to corroborate a structure link between the molecular anion of ME and a unique fragment ion.

EXPERIMENTAL

Cerebrospinal fluid

Samples of human CSF (generally 4 ml) were obtained by lumbar puncture from patients undergoing clinical evaluation of their LBP [1,2,9]. Patients who were relieved of pain with either a lumbar puncture (category 0) or with an injection of a volume of physiological saline (category 1) equivalent to that vol-

TABLE I

HPLC–RRA ANALYSIS OF OPIOID RECEPTOR ACTIVITY IN HUMAN CSF SAMPLES

Category[a]	Patient	Profile		Difference[b]
		First	Second	
0	L.H.	High	High	None
(needle)	R.V.	Low	High	Second increased
1	J.S.	Low	Low	None
(NaCl)	M.M.	High	High	None
2	S.C.	High	High	None
(0.5%)				
3	Y.M.	High	High	None
(1%)				
5-1	A.F.	Low	Low	None
(2%, 80 mg)				
5-2	C.G.	Low	High	Second increased
(2%, 100 mg)	G.H.	Low	High	Second increased
	L.K.	High	High	None[c]
6	S.V.	Low	High	None
(2%, 100 mg)	V.M.	Low	Low	None
(non-physiological responders)				

[a] See text for empirical definitions.

[b] Definitions: (1) If all receptor-active peaks in a HPLC–RRA profile were <10 pmol ME equivalents ml^{-1}, then that sample was defined as "low". (2) If any one peak in a HPLC–RRA profile was >25 pmol, then that sample was defined as "high". (3) If the first and second samples were either both "low" or both "high", then "no difference" was found between the first and second samples. (4) If the first sample was "low", but the second sample was "high", than that second sample was defined as "increased".

[c] Fig. 3.

ume of CSF removed by lumbar puncture were classified as placebo (see Table I). The volume of CSF removed was denoted as the first sample. If the patient was relieved of pain with any level of medication [level 2 = 0.5%; 3 = 1.0%; 4 = 1.5%; 5 = 2.0% (5A = 80 mg; 5B = 100 mg) lidocaine, respectively] injected into the lumbar region, then they were classified as physiological responders. A 5-min period elapsed between each injection set. The sample obtained just after pain relief was denoted as the second sample. Patients not relieved of pain by a full mid-thoracic spinal (lidocaine) anesthetic were classified as non-physiologic responders, all of whom were injected with the highest amount (100 mg) of lidocaine.

Preparation of receptor-rich P_2 fraction

A receptor-rich P_2 preparation [10] was prepared from a fresh canine limbic system, which contains one of the highest concentrations of opioid receptors in the brain.

Radioreceptor assay

For measurement of opioid receptor activity in individual HPLC fractions, each fraction was lyophilized, residue was re-dissolved in water (100–200 μl), and a portion (50 μl) was taken for RRA [10]. Following pre-incubation (45 min, 37°C) of the P_2 receptor preparation, sample and competing [^{3}H]etorphine ligand were added, and that mixture was incubated (0°C, 2 h). Unbound radiolabel was removed by filtering rapidly with a cell harvester (Skatron, Sterling, VA, U.S.A.) [11], the filter was transferred into a tube containing scintillation fluid, and radioactivity was measured with a liquid scintillation counter. Total binding (T) was measured in the absence of, and non-specific binding (NS) in the presence of, non-radioactive ME. Specific binding (S) was calculated as T − NS.

Specific binding measured in each HPLC fraction was compared to the specific binding measured for known amounts of synthetic ME, and therefore opioid receptor activity contained in each HPLC fraction was expressed as pmol ME equivalents per ml CSF. For the experiment described below and for the data contained in Fig. 3B, [^{3}H]ME was used as the RRA ligand to interact preferentially with the δ-opioid receptor [8]. Of course, RRA data cannot provide amino acid sequence information of a peptide [12].

To determine the amount of total opioid binding contained in an unfractionated CSF sample, a portion (150 μl) was purified with a Sep-Pak octadecylsilyl (ODS) disposable cartridge [13] to remove salts that interfered with RRA. Even though larger protein precursors may also have been lost unavoidably, important conclusions on metabolic relationships and the presence of precursors in lumbar CSF are not affected. The peptide-rich fraction that was eluted from the ODS cartridge was lyophillized, and the residue re-dissolved in water (150 μl). Duplicate RRA measurements were made on 50-μl samples.

Radioimmunoassay

Commercial RIA kits (IncStar, Stillwater, WI, U.S.A.) were used to measure ir-LE, ir-ME and ir-BE. RIA data also cannot convey amino acid sequence information [12].

Gradient reversed-phase high-performance liquid chromatography

A Varian microprocessor-driven pump system fitted with a C_{18} reversed-phase steel analytical HPLC column (15 cm x 0.46 cm I.D.; 85-Å pore size diameter; 10-μm particle diameter) was used [13]. UV absorption of the peptide bond at 200 nm was monitored. The flow-rate was 1.5 ml min^{-1}. The two gradients differed only after 72 min in the rate-of-change of organic modifier. The 90-min gradient

was constructed of several different segments: 0 min (10% acetonitrile), 18 min (15%), 48 min (18%), 72 min (30%), and 80–90 min (100%); the 120-min gradient after 72 min was 92 min (60%) and 112–120 min (100%) [7]. Therefore, the 90-min gradient of 8.75% min^{-1} was decreased in two steps to 1.5 and to 2.0% min^{-1} to produce a 120-min gradient.

Fractions of 1 min were collected, acetonitrile was used as organic modifier, and triethylamine–formic acid (TEAF, pH 3.15) as the volatile buffer [14]. To obtain a metabolic profile with RP-HPLC–RRA, a CSF sample (4 ml) was lyophilized; residue was re-dissolved in TEAF (500 μl), and injected onto the HPLC column.

In a separate experiment, a mixture of several synthetic opioid peptides was used to calibrate retention times of the HPLC system for ME-Lys-Lys (5 min), ME-Lys-Arg (7 min), ME-Lys (10 min), dynorphin 1-7 (12 min), α-neo-endorphin (14 min), LE-Arg (dynorphin 1-6) (16 min), dynorphin 1-9 (18 min), ME (21 min), dynorphin 1-13 (22 min), dynorphin 1-10 (27 min), dynorphin 1-8 (29 min), LE (30 min), dynorphin 1-12 (32 min), α-endorphin (36 min), dynorphin B (37 min), ME-Arg-Gly-Leu (40 min), Me-Arg-Phe (48 min), dynorphin 1-17 (50 min), substance P (SP) (56 min), "big" dynorphin (58 min), and β-endorphin (74 min).

Enzymolysis

CSF was used as the source of required endogenous enzymes (using only a first CSF sample to avoid any lidocaine contained in a second sample). Each one of the three HPLC-purified samples I, II and III (Fig. 4B) was incubated with CSF.

Table II contains the protocol for that set of enzymolysis experiments. Experiments 1 and 2 contained a control of either the added precursor (HPLC-purified samples I, II, and III from Fig. 4B) or of human CSF (enzymes), respectively. Experiment 3 included precursor plus CSF. If the immunoreactivity measured in experiment 3 exceeded the sum of immunoreactivity measured in experiments 1 and 2, then it could be concluded that CSF contained endogenous opioid precursors and precursor-processing enzymes.

TABLE II

ENZYMOLYSIS WITH HUMAN CSF ENZYMES OF HPLC-PURIFIED PRECURSORS (SAMPLES I–III) TO ir-ME AND ir-BE

Experiment No.	Precursor[a] (μl)	CSF (μl)	Water (μl)	Total volume (μl)
1	60	–	660	720
2	–	600	120	720
3	60	600	60	720

[a] 60 μl precursor (HPLC-purified fraction I, II, or III from Fig. 5B) is equivalent to 3 ml human CSF.

These experiments were performed three separate times, changing only slightly the conditions each time to optimize the experimental results. The three sets of data obtained were comparable. Because we were using such small amounts of human CSF, we selected one set of these data to represent these experiments.

For ME and LE, the three experiments listed in Table II were performed on each one of the three samples I, II, and III (see Fig. 4B for sample nomenclature). Experimental tubes were incubated (4 h, 37°C, with shaking), and enzymolysis was terminated by boiling (10 min). All samples were frozen, then lyophilized.

Mass spectrometry

A VG 7070E-HF mass spectrometer (Manchester, U.K.) was used to analyze two separate gradient RP-HPLC fractions (21 and 31) from the CSF of patient L.K. Corresponding experimental details of fast atom bombardment mass spectrometric (FAB-MS) analysis of peptides were published elsewhere [15,16].

Multiple reaction monitoring (MRM) data [17] link the metastable transition between a precursor ion, which in this case is the molecular anion $[M-H]^-$, and a unique fragment ion, and were obtained for patient L.K.'s HPLC fraction 21 (Fig. 3A), an HPLC "blank" (Fig. 3A, fraction 31) where no known opioid peptide is eluted, and a blank from glycerol, which was the matrix used to dissolve peptide samples for FAB-MS.

RESULTS

Experimental results presented here include: total opioid receptor activity measurements of unfractionated CSF samples; RP-HPLC–RRA profiles; qualitative and quantitative relationships observed among opioid precursors and peptides, and the products resulting from action of corresponding precursor-processing enzymes; and MS structural corroboration of ME.

Total opioid receptor activity measurements

To demonstrate the importance of using both types of analytical methods (unfractionated, HPLC-separated), total opioid receptor binding was measured in 108 unfractionated CSF samples obtained from 54 patients (first and second samples obtained from each patient). For the purpose of this present paper, it is not important to tabulate each individual measurement of total opioid receptor activity, but rather to summarize those measurements in a few broad categories. HPLC–RRA profiles of opioid receptor activity were determined for first and second samples from 12 selected patients (24 profiles) and three examples are given below in Figs. 1–3 from the six clinical categories described in Table I.

In 17 of the 54 patients, total opioid receptor activity was zero for both the first and second samples; in 32 patients, the first sample was relatively low (0–47 pmol ME equivalents ml^{-1} CSF), but high in the second sample (0.2–2000 pmol). This

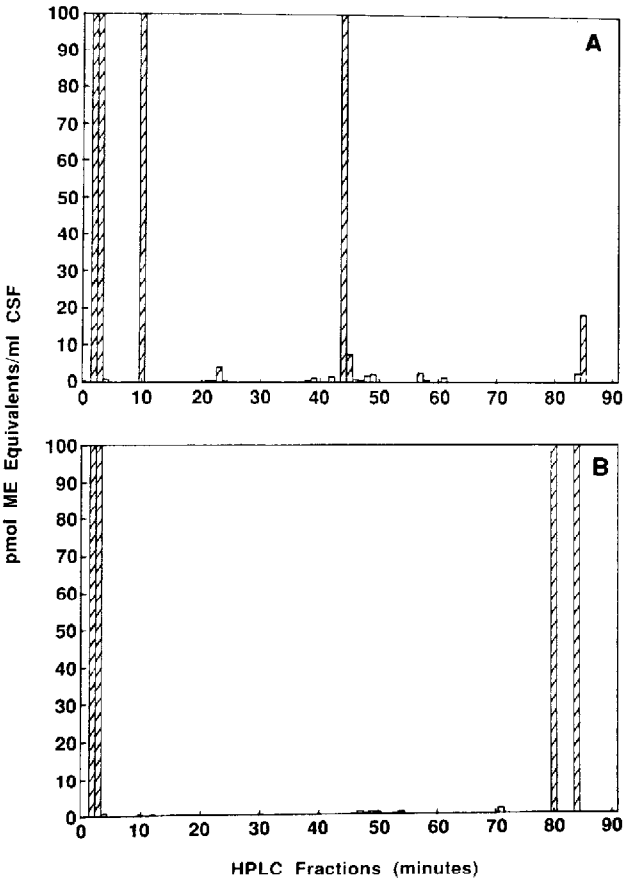

Fig. 1. HPLC–RRA gradient profiles of picomoles of opioid receptor activity, expressed as ME equivalents, for the first (A) and second (B) CSF samples for patient M.M. See text for experimental details.

distribution of opioid total receptor activity is similar to the substance P total immunoreactivity measured in human CSF [2]. Total opioid receptor activity of the second sample was higher than that of the first sample in some of these 32 patients, because lidocaine was injected into the patient's lumbar region during the period of time (5 min) that elapsed between acquiring the first and second samples.

In 4 of those 54 patients, total opioid receptor activity of the first sample was higher than that in the second sample. In 3 of those 4 patients, total opioid binding of the first sample was low (9–34 pmol), but one patient had a first sample measurement of >2000 pmol ME equivalents ml^{-1} CSF, which corresponded to 150 pmol, the upper limit of the RRA method used in this study. That particular patient's metabolic profile represented a remarkable situation that was reported elsewhere [9]. Finally, in 1 of the 54 patients studied here, measurement of total

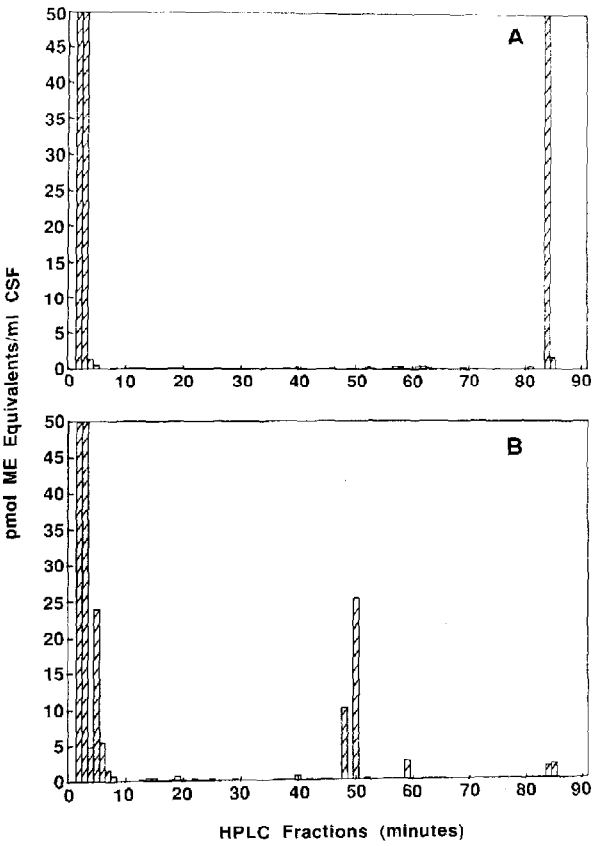

Fig. 2. HPLC-RRA metabolic profiles of opioid receptor activity for the first (A) and second (B) CSF samples for patient M.Y. See text for experimental details.

opioid receptor activity measured in the first and second samples indicated that both measurements were high (>2000 pmol ME equivalents ml^{-1} CSF).

As mentioned above, whereas it is important to measure total opioid receptor activity, it is also necessary to contrast that single measurement of unfractionated CSF to the qualitative and quantitative patterns of HPLC-separated receptor activity to demonstrate the analytical limitation of using only that measurement of total activity. For example, a measurement of zero total activity does not demonstrate necessarily only the absence of any peptide content, but may also be a possible mutual negation of activity by several different peptides competing for receptor types or for antibody binding. On the other hand, a positive measurement must be studied further by chromatographic separation methods to determine the number of specific active components. Those two phenomena have also been demonstrated clearly for RIA measurement of tachykinins in human CSF [2].

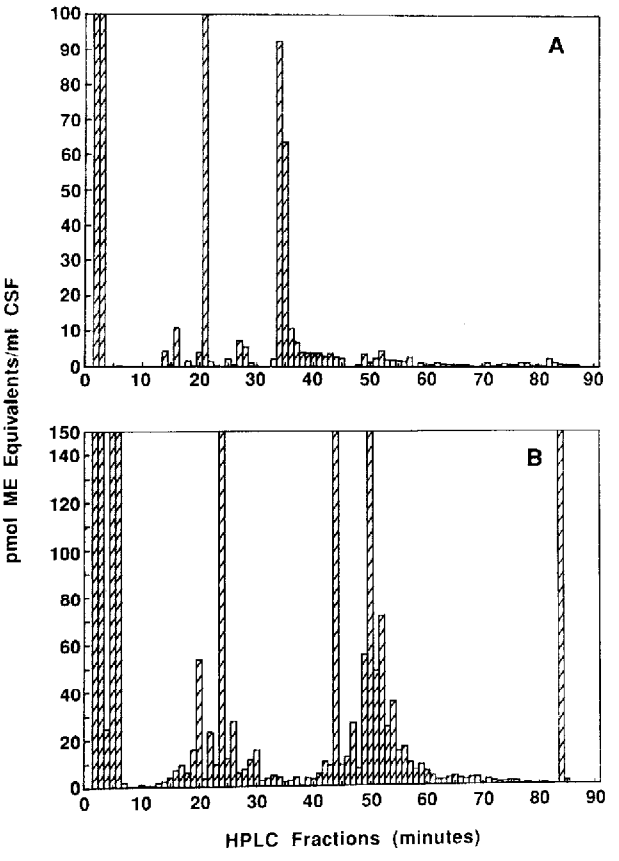

Fig. 3. HPLC–RRA metabolic profile of opioid receptor activity for the first (A) and second (B) CSF samples for patient L.K.

RP-HPLC–RRA profiles of CSF opioid peptides

No common opioid receptor-active peak was found among the 24 HPLC–RRA profiles (all profiles are not shown), except for those peaks observed normally in fractions 2–6 and 84 in Figs. 1–3. The peak at 84 min is significant for the purposes of this paper because it contains opioid precursor molecules [18] and because all 12 of the selected patient profiles (see below) had a peak at 84 min in their HPLC–RRA profiles. Salts and small polar peptides were eluted at 2–3 min. Lidocaine interacted with the opioid RRA preparation, because lidocaine (1.0, 0.5, and 0.2 μg or 4.27, 2.14, and 0.85 nmol, respectively) correlated with opioid receptor activity (3.5, 1.75 and 0.86 nmol, respectively). Similarly, sodium chloride is also opioid receptor-active, because 57 μmol of sodium chloride (3.3 mg) corresponds to 25 pmol ME equivalents of opioid receptor activity. Therefore, because 3.3 mg ml^{-1} sodium chloride occurs in human CSF, and because a large

amount of lidocaine was injected into the lumbar CSF of these patients, large opioid receptor-active peaks occurred at those two retention times of 2–3 and 4–6 min, respectively. Of course, lidocaine in these patients will be observed in the second but not in the first lumbar CSF sample of those patients in categories 2–5.

Relationships among precursors, opioid receptor-active peptides, and their corresponding precursor-processing enzymes

Because neuropeptides are excised from their precursor in a step-wise fashion, starting from a prepropeptide and proceeding through the propeptide and several different intermediate-sized peptide stages to the peptide and its metabolites [3], it was important to observe empirically where opioid receptor activity was eluted within the HPLC gradient (early, middle, or late, corresponding approximately to small, medium, or large peptide sizes, respectively). Thus, elution profiles of receptor activity indicated effectively the extent of metabolism and provided more comprehensive data compared to measuring only one immunoreactive peptide, because for example all three opioid systems can be monitored with RRA.

Experimental observation from HPLC RRA profiles

A correspondence between HPLC fractions and opioid receptor activity was observed when several patients' HPLC–RRA profiles of their first and second samples were compared. An inverse relationship was observed between the amount of opioid receptor activity found in the HPLC fraction 84 in a 90-min gradient. Later-eluting fractions contain generally larger peptides having a higher level of hydrophobicity [18] *versus* shorter peptides contained in earlier-eluting HPLC fractions. Specifically, we noticed that, when one area's level of receptor activity was high, the other was commensurately low, and *vice versa*. That inverse relationship is illustrated by the two representative RP-HPLC–RRA metabolic profiles shown in Figs. 1 and 2. In Fig. 1A (patient M.M., clinical category 1), opioid receptor activity measured in fraction 84 was lower than in fraction 44, whereas in the second sample (Fig. 1B), a converse relationship was observed. In Fig. 2A and 2B (patient M.Y., category 3), that quantitative relationship was reversed. It is important to note that these inverse relationships occurred in samples that were obtained within the 5 min that elapsed between acquiring the first and second CSF samples of those two patients.

These experimental observations were quite different qualitatively and quantitatively from most of the data obtained in our studies and indicated that fraction 84 contained precursors to opioid peptides, and appropriate enzyme systems may be present in CSF to cleave precursors into shorter, opioid receptor-active peptides that eluted earlier. Consequently, a set of enzymolysis experiments was developed to test directly that hypothesis.

Improved RP-HPLC resolution

However, before we could resolve those questions, it was necessary to develop first a more shallow HPLC gradient [7] to fractionate further the components contained in fraction 84. Even though isocratic elution at 100% acetonitrile had occurred for fraction 84 in Figs. 1 and 2, it was possible that fraction 84 contained probably more than one compound, because at that high concentration (and high rate of change) of organic modifier, chromatographic resolution was not optimum. Therefore, it was necessary to employ gradient elution to determine whether several different receptor-active components were contained in that fraction. Thus, the rate of change following fraction 72 of the percentage of organic modifier was decreased 5.8-fold, two gradients were used to resolve compounds contained in that section, and the time of HPLC separation was extended commensurately from 90 to 120 min. Figs. 4A and B contain the corresponding HPLC–RRA profile of opioid receptor-activity contained in the 80–120 min section, which is the only area in the chromatogram that was affected by the decreased rate of change of organic modifier in the 120-min chromatographic gradient.

Furthermore, a different RRA ligand was required for the experiments shown in Fig. 4B, because when [^{3}H]etorphine was used as the ligand (Fig. 4A), only one large area covering nearly 20 min of unresolved opioid receptor activity was found. Therefore, [^{3}H]ME was used as the ligand [8], because ME interacts preferentially with the δ-opioid receptor, whereas etorphine interacts with several different opioid receptors, including the μ-, κ-, ε-, σ-, and δ-receptors. Data in Fig.

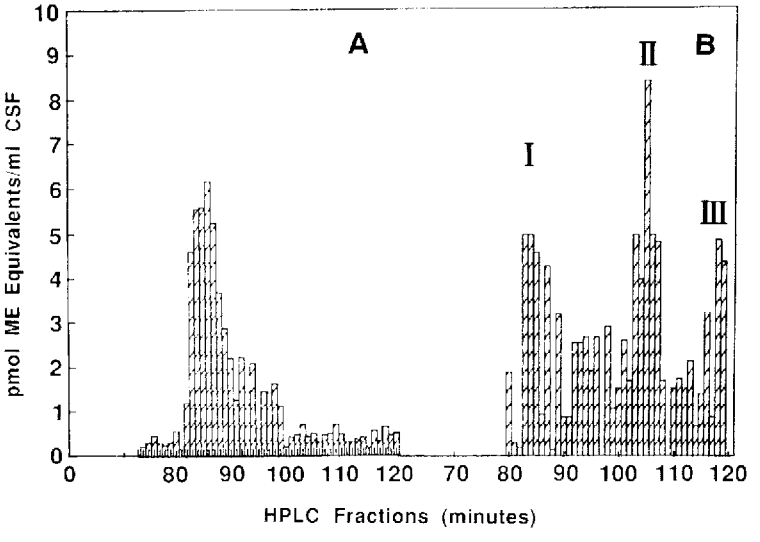

Fig. 4. Shallow HPLC gradient elution profile extended over 120 min to increase chromatographic resolution of later-eluting peaks into fractions 81–95, 96–110, and 111–120 (samples I, II, and III, respectively). (A) RRA data using [^{3}H]etorphine; (B) [^{3}H]ME as RRA ligand. See text for further experimental details.

4B indicated that the previously unresolved large peak (Fig. 4A) consisted of three distinct sections of opioid receptor activity, and that samples I (fractions 81–95), II (96–110), and III (111–120) contained components that interacted preferentially with δ-opioid receptors. HPLC fractions indicated within each section were combined, lyophilized, and dissolved in water to form three corresponding new samples (I, II, and III), that were treated with human lumbar CSF, which contains endogenous enzymes, to determine whether any opioid peptides were released.

Enzymolysis

The following experiment determined the types of opioid peptide precursors and precursor-processing enzymes that were present in CSF. If respective opioid immunoreactive peptides were measured in each one of those three incubates with an appropriate RIA antibody (RIA provides a higher level of detection sensitivity than RRA), then it could be concluded that opioid immunoreactive peptides were released from an appropriate precursor molecule contained in sample I, II, or III by the action of those endogenous CSF enzymes. Of course, other peptides, which might not be detected by the chosen RIA, could also be released from precursors by CSF enzymes, but our purpose here is to demonstrate that a precursor–product relationship exists specifically for opioids. Even though previous research used specific commercial enzymes for similar studies [19,20], our goal here was to simulate the endogenous human state as closely as possible, and, therefore, we used only 5 ml of human CSF, which contains all required endogenous neuropeptidases and buffers to cleave any precursor. The endogenous neuropeptidases were not purified or characterized here (that was not part of this study), but

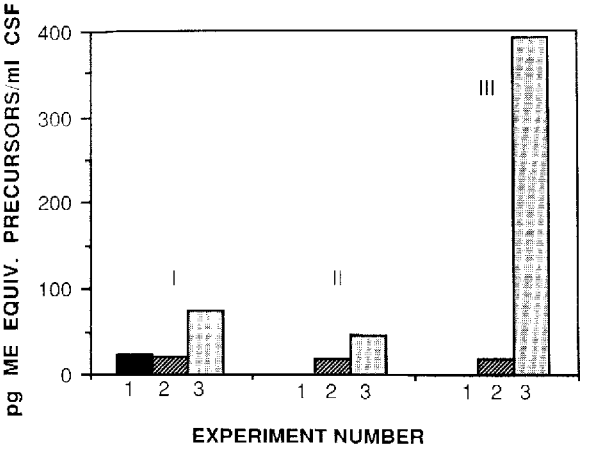

Fig. 5. Measurement of ME immunoreactivity in a series of experiments performed on samples I–III in Fig. 4B. The ordinate contains values (pg) for ir-ME of precursors (samples I, II, and III), which are equivalent to 3 ml CSF. For each indicated sample number (I, II, and III), the abscissa contains experiment numbers (1, 2, and 3). See text and Table II for further experimental details.

rather we demonstrated their effect on opioid precursors by demonstrating the formation of appropriate opioid peptide products.

Immunoreactivity measurements

ME RIA results. Fig. 5 contains ir-ME measurements of samples I, II, and III. The number at the base of each vertical bar in Fig. 5 corresponds to the number listed in Table II for that particular experiment. [Although equivalent experiments were done for LE, the amount of ir-LE measured was below the detection limit for that LE RIA kit, even when a larger amount (200 μl precursor, equivalent to 10 ml CSF) of CSF sample was used.] These data demonstrated that, for samples I and II, the amount of ir-ME measured in experiment 3 was nearly equivalent to the corresponding total amount measured in experiments 1 and 2, indicating that no measurable ir-ME was released from samples I and II by adding endogenous CSF enzymes. However, for sample III, experiment 1 (precursor) contains no detectable ir-ME (lower limit of ME RIA kit equals 5 pg) and experiment 2 (CSF) contains 17 pg. Therefore, if precursor-processing enzyme activity were present in CSF, then the ir-ME content measured in experiment 3 should be >17 pg. A significant increase of measured ir-ME (393 pg of ir-ME) following enzymolysis indicated that sample III contained a precursor to ir-ME, which could be the intact gene product proenkephalin A, or any one of several possible intermediate-sized precursors that could be derived by alternate processing of that precursor; and also that human CSF contains appropriate enzymes to cleave that precursor to corresponding immunoreactive opioid peptides, in this case, ir-ME. The other two sets of data corroborated these conclusions.

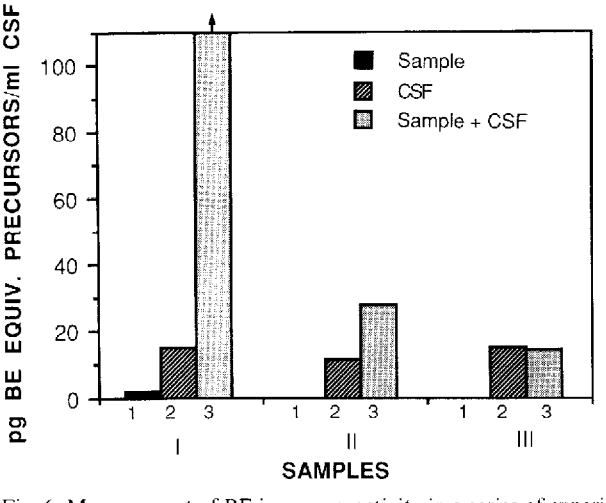

Fig. 6. Measurement of BE immunoreactivity in a series of experiments performed on samples I–III from Fig. 4B. See legend to Fig. 5 for the nomenclature used.

BE RIA data. For BE (Fig. 6) sample I (fractions 81–95), experiment 1 contained 2 pg ir-BE, experiment 2 contained 15 pg, and experiment 3 contained >110 pg. A significant increase in ir-BE was produced. The > sign indicates that the ir-BE activity of experiment 3 exceeded the upper limit (110 pg) of the BE RIA kit calibration curve. Even when a smaller amount (20 μl of sample I, equivalent to 1 ml of CSF) was used, the upper limit of the RIA curve was still exceeded. No significant increase in ir-BE was measured for samples I and III. These data demonstrated that endogenous human CSF enzymes cleaved one or more precursors in sample I to produce ir-BE, and also that sample I contains either the intact proopiomelanocortin precursor (POMC) of BE or an intermediate-sized precursor. The other two experiments confirmed these data.

Structural corroboration of ME with MS

To date, no amino acid sequence data are available for human CSF endogenous peptides. As a first step to provide those sequence data, MS data were obtained from one of the CSF samples for a peptide that co-eluted at the calibrated retention time of synthetic ME. HPLC–RRA metabolic profiles of patient L.K. (category 5-2) are shown in Fig. 3A and B. Data in Fig. 3A and B are in the "high" category (Table I).

CSF sample from patient L.K. had been separated by HPLC, collecting 90 1-min fractions, and one half of the volume of each fraction was analyzed by RRA (Fig. 3A). The other half of fractions 21 (ME) and 31 (blank, no ME receptor activity) were analyzed by tandem MS (MS–MS) and RIA. MS–MS monitored the metastable transition between the molecular anion $[M - H]^-$ and a unique fragment ion (namely, the loss of the Tyr side-chain from $[M - H]^-$, $[[M - H]^- - 107]^-$. The two FAB-MS–MS HPLC blank measurement bombardment times (1.0 and 1.5 min) corresponded to 141 and 208 MRM (digital-to-analog converter) units, respectively, and sample measurements were 161 and 245 units, respectively. Thus, sample measurements corresponded to 20 and 37 units, respectively. Because MS–MS data generally have low levels of ion currents, but are structurally very significant [21], they provide structural information to corroborate the presence of intact ME= YGGFM in that human CSF sample.

After MS–MS analysis of fractions 21 and 31 was completed, those two samples tubes were washed (TEAF) and lyophilized; remaining immunoreactivity was measured. ir-ME measured in fraction 21 was very high, exceeding the upper limit of 500 pg of the ME RIA kit's calibration curve; the corresponding blank was 107 pg.

DISCUSSION

This paper contains data that substantiate our hypothesis that opioid peptides may play a role in human LBP, and that describe the presence in human CSF of

opioid peptides and their precursors, intact opioid peptides (ME, BE), precursor molecules, and the enzymes required to process precursors to ME and BE. Furthermore, MS–MS correlated the molecular ion and a unique fragment ion for intact ME, a correlation that corroborated (beyond the RIA and RRA) the ME sequence.

These present data extend our previous study on the measurement of opioid receptor-active peptides in human CSF [1], where CSF samples from 99 patients were analyzed for total (unfractionated) endogenous opioid receptor activity. In that previous study, a correlation was observed between the amount of lidocaine injected into the lumbar region of those patients required to achieve pain relief and the content of the total receptor-active opioid peptide in that patient's CSF. However, this present subsequent research showed that it is necessary to measure more accurately the distribution over a gradient of HPLC-separated opioid receptor activity and not to conclude any significance from total (unfractionated) receptor activity. This present paper also expands upon data from previous CSF studies by other workers [19, 20, 22–27], who used large volumes of pooled CSF to study metabolic degradation of enkephalin and substance P using RIA or RRA.

Human CSF is an appropriate biologic fluid [28] in for the study of neuropeptide metabolism [1, 2, 19, 20, 24, 25, 27, 29] because, for example, cell bodies containing opioid peptide precursors are located in proximity [30]. Enzymes to degrade neuropeptides that are formed from their precursors [24–26, 31] are also present in CSF. However, no one to date has used only a few milliliters of human CSF to hydrolyze precursor molecules, nor used the combined analytical system described here.

CSF may play at least two roles in certain human pathophysiological conditions, and both possibilities were important to consider for our hypothesis. On one hand, if an anatomical lesion had occurred, then intracerebroventricular (ICV) circulation may collect peptide metabolic products resulting from that lesion and transport them to CSF. That situation might reflect a breach of the brain–CSF barrier [32]. On the other hand, a metabolic defect on the molecular level may have occurred, as discussed in the following section.

Endogenous neuropeptide systems, composed of several gene product precursor (intact and intermediate-sized) molecules and corresponding multiple neuropeptide products are required in the human to maintain homeostasis. Several workers [4,5,30] hypothesize that derangements in these neuropeptide-processing systems at several possible metabolic steps could account for several different human pathologies such as pituitary tumor formation [33], senile dementia of the Alzheimer's type [34], orthodontic stress [4,5], trigeminal nociception [35], and LBP [1].

The data in Fig. 4B demonstrate for the first time that immunoreactivity corresponding to BE (sample I) and to ME (sample III) was produced when these HPLC-purified samples (I–III) were incubated with endogenous human CSF en-

zymes. These data demonstrated the presence in CSF of proenkephalin A and POMC precursors (either intact of fragments thereof), and of precursor-processing enzymes to produce BE and ME.

To date, no amino acid sequence data are available for human CSF peptides, and most researchers have inferred the amino acid sequence from only HPLC, RIA, or RRA data, which (alone, or in combination), of course, cannot provide amino acid sequence information. Therefore, we used MS methods to analyze intact ME in CSF, which is a difficult biological fluid to use for this purpose because of the very low amounts of endogenous peptide. In general, abundant molecular ions ($[M + H]^+$ and $[M - H]^-$) of a peptide are produced with FAB-MS [36] and indicate that peptide's molecular weight, but not its amino acid sequence. Amino acid sequence information can be obtained readily [16] by a B/E (magnetic/electric field ratio) linked-field scan [17,37] to collect product ions from a precursor ion. Therefore, for a peptide $[M - H]^-$ as precursor ion, unique amino acid sequence-determining (and other fragment) ions are product ions. Accumulating ion current due to the metastable transition between $[M - H]^-$ of ME at 572 a.m.u. and $[[M - H]^- - 107]^-$ at 465 a.m.u., where 107 is the mass of the Tyr side-chain, with MRM computer programs provided a specific structure link between these two unique ions from ME [21]. These data, plus the HPLC and RRA data, corroborated the presence of intact ME in fraction 21 (Fig. 3A) from that patient. Because this MS experiment was performed at the instrumental detection limit, we must state here "corroboration", rather than "confirmed". This combination or RP-HPLC–RRA, RIA, and MS–MS is a powerful analytical method, and these results were the first data obtained for analysis of ME, which was extracted from only 2 ml of human CSF.

Although significant MS data were obtained here, it is nevertheless important to remember that limitations exist to these data. For example, the MS instrument used in this study was a two-sector, forward geometry MS instrument, which does not have the optimum precursor ion selectivity, detection sensitivity, nor the mass accuracy to detect (to within a fractional mass) the mass of the product ions that, for example, current four-sector instruments have [37]. Furthermore, human CSF contains only a very low amount (picomoles) of opioid peptides. Thus, we have "corroborated", not "determined" the amino acid sequence of an endogenous peptide. Nevertheless, the combination of HPLC–RRA, RIA, and MS analytical techniques provides here a set of significantly improved data beyond the type of data obtained to date [38].

Although proenkephalins A and B have been reported in CSF [19,25], no amino acid sequence data have been reported, and the specific enzyme system that cleaves that precursor has not yet been reported; and certainly not in the patient population studied here. It is clear that ME precursors found here are not known C-terminally extended ME peptides ME-Arg-Gly-Leu or ME-Arg-Phe because retention times of ME, ME-Arg-Gly-Leu and Me-Arg-Phe are 21, 40, and 48 min, respectively, compared to the precursor (111–120 min).

Enzymes from CSF were not purified specifically in this present work, but rather intact human CSF was used as the source of all required endogenous enzymes. Consequently, these present data demonstrated that human CSF contained enzymes that hydrolyzed opioid precursors to ir-ME and to ir-BE. Furthermore, this report is the first direct demonstration that human CSF contains the proenkephalin A and POMC precursors or their corresponding intermediate precursor(s).

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