CHEMICAL CHARACTERISTICS OF ENDORPHINS IN HUMAN CEREBROSPINAL FLUID

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1. Introduction

For several years we have used a radio-receptor assay to investigate the levels of endorphins in the cerebrospinal fluid (CSF) of patients. Correlations were established to, e.g., disease characteristics of chronic pain syndromes and various psychiatric disorders [1]. It became apparent that the chemical identity to the active material was different from previously characterized endorphins: Here we present more extensive observations and chemical characteristics, particularly of fraction I (FI) as in [2,3]. The strategy for the isolation has been to use the following procedures in sequence:

(i) Analytical runs of FI, isolated from the CSF of cases with well-defined clinical characteristics;
(ii) Preparative scale separation of unspecified CSF obtained at neurosurgical procedures.

It was found that the receptor-active material of FI could be resolved into several ($\geqslant 3$) components by ion-exchange, molecular sieving and high-pressure liquid chromatography (HPLC). One component co-eluted with the dynorphin (1–13) fragment and reacted with antibodies raised against that peptide; on a quantitative bases the receptor assay and the radioimmunoassay gave similar results. Also confirming the opioid character of material isolated from FI, was the observed activity on the guinea-pig ileum myenteric-plexus preparation.

2. Material and methods

Lumbar CSF from patients with various psychiatric disorders was the starting material in analytical runs while ventricular CSF obtained at neuro-surgical procedures was used in preparative runs. Met^5-enkephalin and Leu^5-enkephalin were purchased from Bachem, β-endorphin and dynorphin (1–13) from Peninsula. Leu^5–Arg^6-enkephalin was synthesized and kindly donated by Dr Ulf Ragnarsson, Department of Biochemistry, University of Uppsala. [^{3}H]Dihydromorphine at 75 Ci/mmol was purchased from the Radiochemical Centre, Amersham. All other chemicals were of reagent grade.

The fractions were tested for opioid activity by a radio-receptor assay (RRA) using partially purified synaptic plasma membrane preparations from rat brain [4]. Labelled dihydromorphine was the competing radioligand. The activity was read as relative units; one relative unit being defined as the concentration of Met^5-enkephalin giving a 50% inhibition of dihydromorphine binding.

Radioimmunoassay was performed with the charcoal absorption technique. The antisera used were prepared at this laboratory for the enkephalins and β-endorphin. The antiserum directed against dynorphin (1–13) was kindly donated by Professor Avram Goldstein Department of Pharmacology, Stanford University, Palo Alto, CA [5].

Analytical runs isolating FI endorphins were performed as in [6]. The purification steps included ultrafiltration and chromatography on a Sephadex G-10 column (50 × 2 cm) in 0.2 M acetic acid (fractionation step A). For preparation scale purification of endorphins, a pool of CSF (30–300 ml) from several patients was lyophilized and reconstituted in 0.2 M pyridine acetate buffer of pH 5. Material from analytical or preparative runs was fractionated on a SP-Sephadex C-25 ion-exchange column (fractionation step B) with step-wise elution with 100 ml 0.2 M (fraction Ba), 150 ml 0.5 M (fraction Bb) and finally 150 ml 2 M (fraction Bc) pyridine-acetate buffer solutions of pH 5.0.

The active material from each fraction was further purified on a Biogel P2 column (160 × 1 cm), which

was eluted with 0.1% trifluoroacetic acid (TFA) (fractionation step C) yielding two major fractions of activity (fractions Ca and Cb, respectively, cf. fig.1). The final fractionation step (D) was HPLC chromatography on a reversed-phase-μ-Bondapak C_{18} column (3.9 X 300 mm, Waters) in a methanol/water/TFA gradient (starting buffer 30% methanol in water titrated to pH 3; final buffer 80% methanol in water titrated to pH 3; gradient time 30 min). Three different peaks of activity Da, Db and Dc with retention times 12.5, 16.5 and 21.5 min, respectively, were isolated (cf. fig.2).

Bioassay of opioid material in the guinea-pig ileum longitudinal muscle myenteric-plexus preparation followed standard procedures [7]. The preparation was placed in a 5 ml bath stimulated supramaximally at 0.1 Hz. Contractions were recorded isotonically. In each run a standard curve with graded concentrations of normorphine was run.

3. Results

During this work, availability of CSF samples varied. The preparative runs omitted the isolation of FI from the Sephadex G-10 column (step A).

Table 1
Identification of fractions Bc and Ca as functionally relevant, and related to activity measured in FI

Fractionation step	Fraction	CSF sample[a]					
		1	2	3	4	5	6
A.							
Sephadex G-10	FI	42	–[b]	–	4.4	7.2	–
B.							
SP-Sephadex	Bb	9	36	20	–	–	40
	Bc	31	176	63	–	–	60
C.							
Biogel P2	Ca	–	–	–	4.3	7.5	48
	Cb	–	–	–	0.4	0.6	7.5

[a] Origin of CSF samples: (1) schizophrenics (patient number, n=10; total vol. 30 ml); (2,3) schizophrenics (n=3), before (2) and after (3) treatment with neuroleptics (22 ml each); (4,5) patients with chronic pain (n=3), before (4) and after (5) transcutaneous nerve stimulation (14 ml each); (6) preparative run of ventricular CSF (n=2; 200 ml)
[b] Not tested

A preparative run (6) not starting from FI was included for comparison. The table gives endorphin activity in total relative units measured in receptor assay

3.1. *Analytical runs of FI*

It was necessary to introduce separation steps of high capacity before high resolution procedures like HPLC. A number of CSF samples from patients with specified clinical diagnoses were included. In two series, with schizophrenics and patients with chronic pain, respectively, each patient delivered samples before and after a treatment procedure known to change FI content. Fractionation step B (table 1) isolated most receptor active material in the 2 M pyridine acetate fraction while most of remaining salts and UV-absorbing material was removed. Receptor-active material in the Ba and Bb fractions showed no distinct pattern on further chromatography; they were therefore not studied further. The material in the Bc fraction was run on a Biogel P2 column for further fractionation. The fractionation pattern revealed the presence of several components with most activity eluting in the 1500–2000 M_r range (fig.1). The activity recovered at this step seems to represent most of the activity present in the original FI fraction (cf. CSF samples 4, 5). Also in the preparative run (sample 6) most of the activity from step B, fraction Bc, was recovered in fractions 40–45 (Ca) on Biogel P2.

3.2. *Preparative scale separation*

The active material in fraction Ca was subjected to further purification on a reversed phase HPLC column. A linear gradient was run in methanol/water/

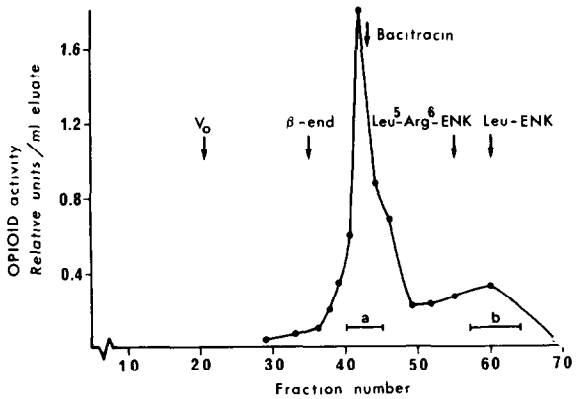

Fig.1. Chromatography of material from ventricular CSF on a Biogel P2 column (160 X 1 cm) in 0.1% TFA. The fractions were assayed for opioid activity in a receptor assay The arrows indicate elution volumes of standard substances. bovine serum albumin, M_r 60 000; β-endorphin M_r 3500; bacitracin, M_r 1411; Leu⁵–Arg⁶-enkephalin, M_r 712; Leu⁵-enkephalin, M_r 574.

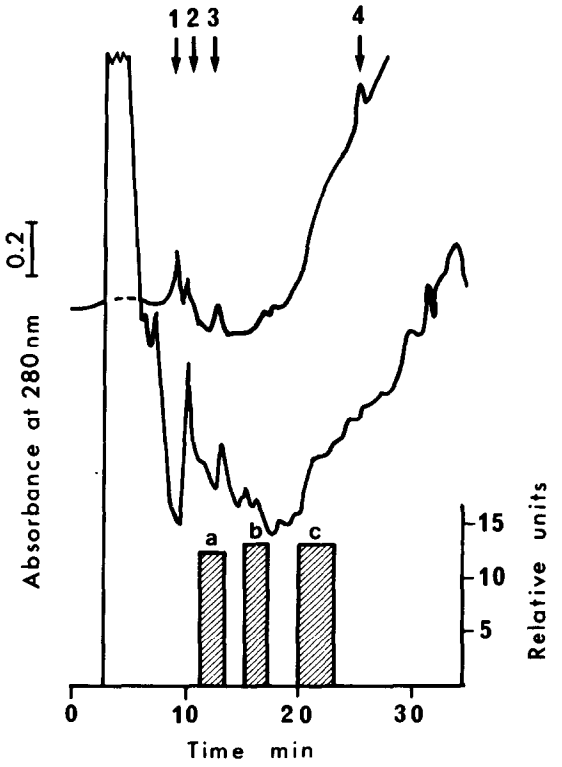

Fig.2. Chromatography of material from ventricular CSF on a μ-Bondapack C_{18} column. The samples were eluted in a linear gradient methanol/water/TFA at pH 3 for 30 min. Fractions of 1 ml (flowrate 1 ml/min) were individually assayed for opioid activity in a receptor assay and in a radioimmunoassay against dynorphin (1–13) directed antibodies. The upper curve shows the elution pattern of standard substances: (1) Leu^5–Arg^6-enkephalin; (2) Met^5-enkephalin; (3) dynorphin (1–13); (4) β-endorphin.

Table 2
Fractionation of opioid activity from ventricular CSF on a preparative scale

Fractionation step	Fraction	Run	
		1	2
B. SP-Sephadex	Bc	60	57
C. Biogel P2	Ca	48	30
D. HPLC	Da	4.6	12
	Db	1.6	13
	Dc	2.8	13

The table gives endorphin activity in total relative units in receptor assay

Table 3
Relative amounts of receptoractive material (RRA) or material active in dynorphin radioimmunoassay (RIA) in fractions isolated by HPLC (step D)

HPLC fraction	Activity in RRA / Activity in RIA		
	Preparative runs		Fraction I
	1	2	
D. a	0.61/0.63	0.72/0.60	0.23/0.20
b	0.67/<0.2	–[a]	<0.1/ –
c	0.68/<0.2	0.39/0.39	0.17/ –

[a] Not tested

All values are expressed as pmol dynorphin (1–13)/sample which was used as the standard

TFA and three peaks with active material were eluted (fig.2). Table 2 shows the distribution of the active material in two runs of ventricular CSF. The reason for the poor total recovery in the first run is not known.

3.3. *Identification of receptor-active material in radioimmunoassay*

The different HPLC fractions were tested against antibodies for Met^5- and Leu^5-enkephalin, β-endorphin and dynorphin (1–13). The only positive results were obtained with antibodies directed against dynorphin (table 3). Two preparative runs and one run starting from FI material gave consonant findings for fraction Da, which in receptor assay and radioimmunoassay gave comparable results on a molar basis, using dynorphin (1–13) as the standard. The situation for the Db and Dc fractions is less clear – in the second preparative run, the active material may be related to dynorphin.

3.4. *Bioassay of the Biogel P2 fractions from ventricular CSF*

Material from fraction Ca was tested on the electrically stimulated guinea-pig ileum, where it showed inhibitory activity as the standard, normorphine. This activity was partially reversed by naloxone (fig.3). Material from 75 ml ventricular CSF gave an inhibition equal to that produced by 2×10^{-7} M normorphine, which on this preparation gave 50% inhibition of the twitch.

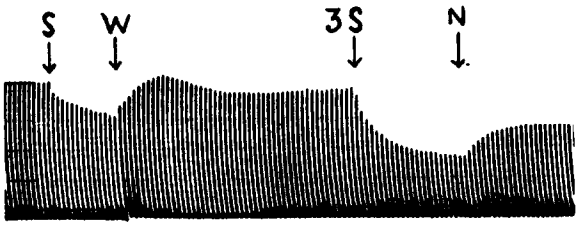

Fig.3. Inihibition of the electrically induced twitch of the guinea-pig ileum longitudinal muscle myenteric-plexus preparation by fraction Ca eluting from the Biogel P2 column: S (sample) marks the addition of fraction Ca: 3 S the addition of the same fraction at 3-times higher concentration; W marks washing; and N the addition of naloxone to final conc. 10^{-6} M.

4. Discussion

The purpose of this study was to find out, which opioid receptor-binding components are present in FI, defined in a radio-receptor assay with dihydromorphine as the radioligand. As a guideline, we compared samples where we had observed correlations between FI levels and different clinical classifications or disease characteristics with or without treatment modifications. We have found that transcutaneous nerve stimulation in chronic pain patients increases [8], and neuroleptic medication in schizophrenics decreases [9] total FI activity. Pre- and post-treatment CSF from patients where such therapy had been carried out was therefore compared. The analytical runs revealed that fraction Ca from the Biogel P2 column retained most FI activity. This fraction gave no reaction with antibodies directed against the enkephalins or β-endorphin, which was expected since these peptides are known to be eliminated in previous steps of the fractionation procedure. The estimated M_r is ~1500–2000 confirming [2,3]. The material could also be characterized as opioid on the guinea -pig ileum – although the inhibition of the twitch was not completely reversed by naloxone.

The HPLC step revealed ⩾3 major fractions of activity. The fraction Da might well be dynorphin or a fragment thereof; it gave very similar calculated molar quantities in the radio-receptor assay or radioimmunoassay when using dynorphin (1–13) as the standard and it also co-migrated with this compound. The antibody shows no cross-reactivity with enkephalins, α-, β- or γ-endorphin [5]. The possible existence of dynorphin in CSF is of considerable interest. This peptide has been isolated from porcine brain and pituitary. It shows extraordinary potency in most bioassays [10] and it may therefore be functionally relevant even at very low concentrations. The identity of the other HPLC fractions is unclear as is their relation to known opioid peptide systems. Work is in progress to isolate enough material of these subfractions to facilitate chemical characterization. CSF samples need to be collected from different patient categories to establish which pattern of activity is characteristic for each category.

Acknowledgements

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