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CHARACTERIZATION OF A PLASMA FACTOR HAVING OPIATE AND IMMUNOACTIVITY LIKE BETA-ENDORPHIN

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SUMMARY: A factor from human plasma having opiate-like activity was characterized in the present study. In addition to its ability to inhibit the binding of [^{3}H]-methionine-enkephalin to opiate receptors, it also cross-reacted with two β-endorphin specific anti-sera. Compared with β-endorphin, the plasma factor had a shorter action on inhibiting the contraction of the guinea pig ileum. By gel-filtration chromatography, the size of this factor was intermediate between that of β-endorphin and methionine-enkephalin.

INTRODUCTION

The existence of compounds having pharmacological properties similar to morphine in the nervous system is well documented. Since the initial isolation of the enkephalins by Hughes *et al.*(1) in 1975, a number of peptides having opiate-like activities have been characterized (2, 3, 4). Structurally, these peptides are interrelated and all of them are believed to be derived from the parent molecular β-lipotropin (β-LPH, ref.5). Other than the β-LPH related peptides, two compounds having opiate-like properties were isolated by Pert *et al.*(6) in the blood and Gintzler *et al.*(7) and Shorr *et al.*(8) in the brain and CSF. In contrast to the enkephalins and endorphins, these compounds are relatively resistant to enzyme hydrolysis.

Recently, in the course of studying the effect of electroacupuncture on plasma endorphin level, we noticed the presence of a β-endorphin-like factor in the blood of human subjects. Because of its molecular size and other properties, this factor is unlikely to be either anodynin or the fast moving material (FMM) recently described by Schulz *et al.*(9). In this communication, we wish to report our findings on the characterization of this β-endorphin-like factor.

Abbreviations: β-LPH, β-lipotropin; β_h-EP, human β-endorphin; β-EP$_{x-y}$, the segment of the β-endorphin molecule between residues x and y; met-ENK, methionine enkephalin; leu-ENK, leucine enkephalin.

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MATERIALS AND METHODS

Chemicals. All chemicals used in the present study were reagent grade. [^{3}H]-met-ENK, [^{3}H]-leu-ENK and carrier free [^{125}I] were from the Radiochemical Center, Amersham, U.K. Synthetic peptides and their respective anti-sera were generously provided by Dr. C. H. Li of the Hormone Research Laboratory(β_h-EP and the anti-serum specific to β_h-EP$_{22-27}$) and Dr. N. Ling of the Salk Institute (β_h-EP, leu-ENK and the antisera specific to β_h-EP$_{14-27}$ and leu-ENK). Bio-gel P2 was purchased from Bio Rad Laboratory Richmond, California. Naloxone HCl was a gift from Endo Laboratory, Garden City, N.Y.

Fractionation of plasma samples. Blood was collected in plastic tubes containing bacitracin and heparin (100 ug and 0.1 mg per ml, respectively) from normal male and female subjects after overnight fasting. Immediately after collection, the samples were mixed and chilled in an ice-bath. Plasma was separated from the blood cells within an hour by centrifugation (1500 rpm, 20 min) at 4°C. The plasma obtained was aliquoted and boiled in a water-bath for 15 min. When samples were treated this way, they became solidified and formed a gel-like pellet. The gel pellet was first chilled in an ice-bath for 5 min and then an equal volume of cold 1N acetic acid added. The gel pellet was sliced into small pieces with a spatula and then homogenized. The homogenized material was centrifuged at 15,000 x g for 1 hour at 4°C and the supernatant obtained lypholized.

The crude extract obtained from 10 to 20 ml plasma was resuspended in 5 ml 0.1M ammonium acetate (pH7.4) and applied onto a Bio-gel P2 column (1.5 x 65 cm). Elution was carried out with the same buffer at a flow rate of 16 ml/hr. Fractions of 4 ml were collected and their absorption at 280 mu measured. After lypholization, the material in each fraction was dissolved in 0.5 ml distilled water. A 0.1 ml aliquot was taken for opiate-receptor binding assay while another was lypholized and resuspended in RIA buffer for radioimmunoassay.

Analytical assay. Radioreceptor binding was performed essentially as described by Simon *et al.* (10) except for the following modifications. [^{3}H]-met-ENK (specific activity, 25 Ci/mmol) was used as the tracer and incubation was carried out in an ice-bath for 90 min.

Radioimmunoassay using anti-sera against β-EP and leu-ENK were performed essentially as described by Li *et al.* (11) and Rossier *et al.* (12), respectively. All dilutions were made with a 0.01M phosphate buffered saline containing 33 mg/ml EDTA and 0.5% bovine serum albumin. [^{125}I] labeled β_h-EP was prepared according to the procedure of Ling *et al.* (13) and the labeled peptide was separated from free [^{125}I] by DEAE-Sephadex chromatography (0.5 cm x 20 cm).

The guinea pig ileum assay was performed essentially as described by Kosterlitz *et al.* (14).

RESULTS

Chromatography of materials extracted from boiled plasma. Fig.1 is a typical chromatogram obtained when a crude serum extract was passed through a Bio-gel P2 column. Based on the absorption profile at 280 mu, most of the materials applied were eluted either at the void or the hold-up volume. Although the intermediate fractions did not absorb strongly at 280 mu, some of them, parti-

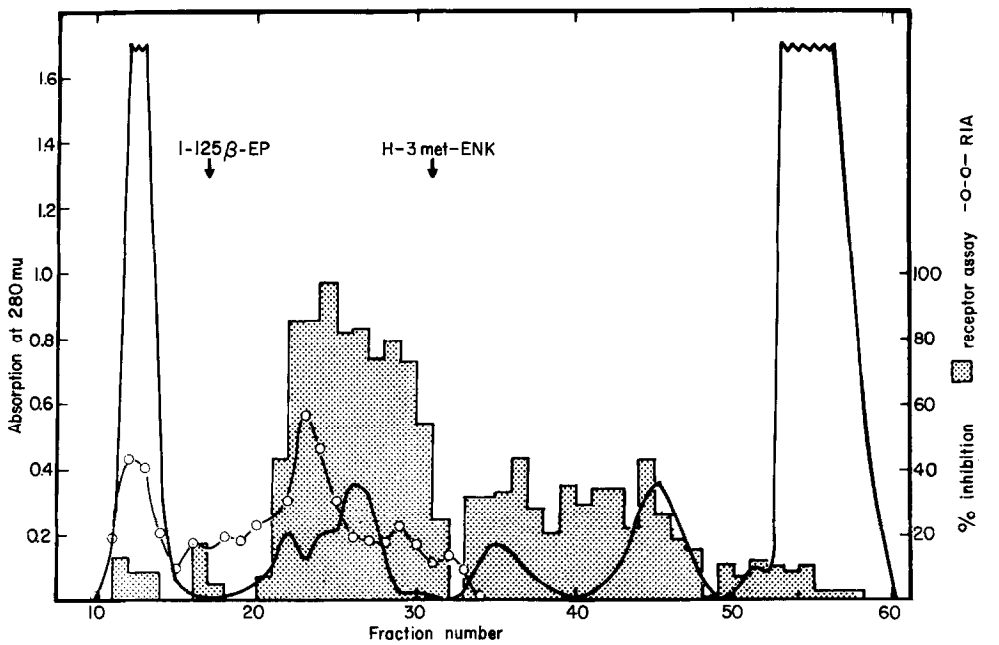

Fig. 1. Fractionation of crude plasma extract on a Bio-gel P2 column. Both receptor binding and radioimmunoassay (RIA) activities are expressed as the percent of inhibition of the maximum binding of the tracer ([^{3}H]-met-ENK or [^{125}I]-β-EP) to either opiate receptors or β-EP-specific antibodies. The conditions of elution are as described in Materials and Methods.

cularly fractions 20 through 30, contained a fair amount of whitish material after lypholization.

When the individual fractions were assayed for their ability to inhibit the binding of [^{3}H]-met-ENK to opiate-receptors, a significant peak of inhibitory activity was obtained around fraction 25. In addition to the major peak, some minor inhibitory activities were also noticed around fractions 36, 41 and 44. Inspite of this, the sum of these minor activities was never more than 50% of the major peak. Compared with the elution volume of [^{125}I]-β_h-EP and [^{3}H]-met-ENK, the major peak of inhibitory activity was eluted in a region between these two peptides.

To further explore the nature of the inhibitory activity, we scanned the fractions for their immunoactivity against a specific anti-serum for β_h-EP (kindly supplied by Dr. C.H. Li). As indicated in Fig.1, two major peaks of immunoactivity were observed. One at the region corresponding to the void volume and the other at the same region as the major receptor binding inhibitory peak. No appreciable amount of immunoactivity was observed at the position corresponding to β_h-EP. Since the anti-β_h-EP serum used presently slightly

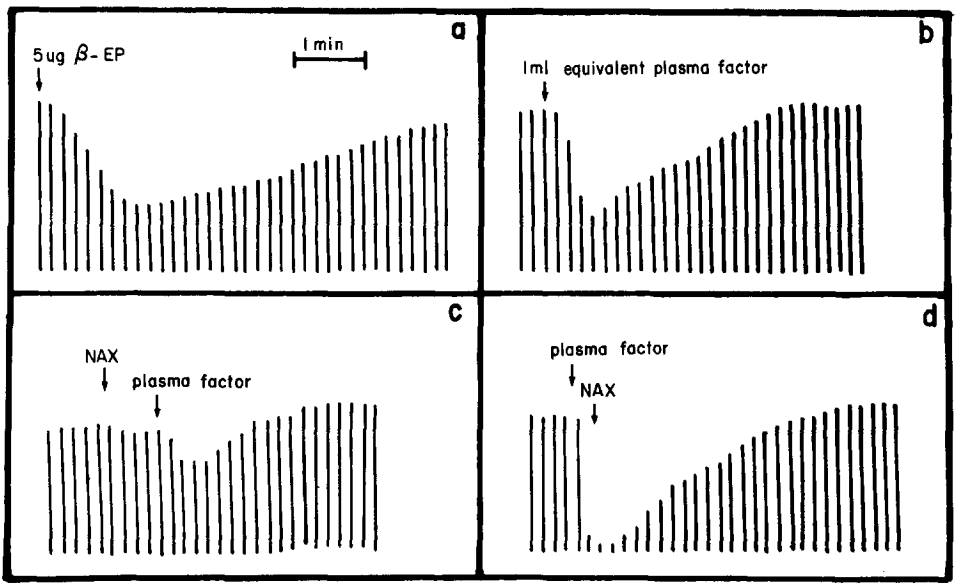

Fig. 2. Inhibition of guinea pig ileum contraction by plasma β-EP-like factor. a, 5ug β-EP; b, an amount of plasma factor obtained from roughly 1 ml plasma; c, 10^{-4}M naloxone applied before the plasma factor; d, 10^{-4}M naloxone applied after the plasma factor.

cross-reacts with β-LPH (C. H. Li, personal communication), the immunoactivity observed at the void volume was probably due to the presence of this peptide in the sample.

Characterization of the β-endorphin-like factor from Bio-gel P2 column. Fractions 22 to 26 were pooled and subjected to further analysis as described below. (See Fig.1)

Guinea pig ileum assay. The ability of the β-endorphin-like plasma factor in inhibiting the contraction of the guinea pig ileum is shown in Fig.2. Maximum inhibition was obtained with material roughly corresponding to 2 ml of plasma. In contrast to β_h-EP, the onset of the inhibition produced by the plasma factor was more rapid and its action lasted much shorter (3 min vs. 8 min). Naloxone was ineffective in antagonizing the plasma factor when applied concomitantly to the incubation medium. However, if it is applied 1 min prior to the plasma factor, partial antagonization was observed.

Immunoreactivity. In our initial experiment we obtained evidence indicating that the newly discovered plasma factor could interact with antibodies raised

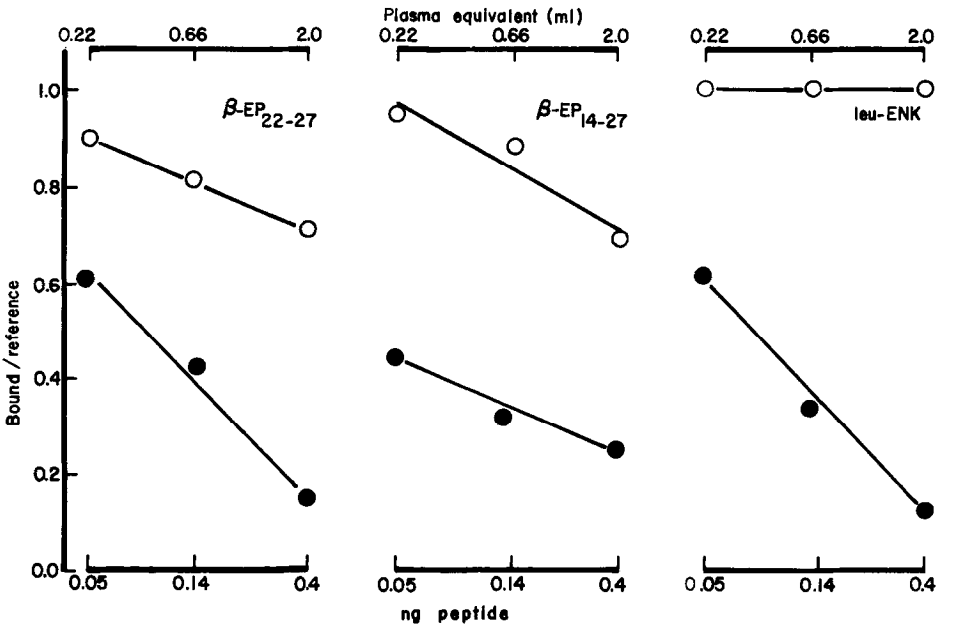

Fig. 3. Comparison of the immunoactivity of the plasma factor using different anti-sera. The plasma factor obtained from roughly 2 ml plasma was diluted three fold. 0.1 ml aliquots were then taken for the standard radioimmunoassay. -●-●-, inhibition curves obtained with synthetic peptides; -○-○-, inhibition curves obtained with the plasma factor. All determinations were done in duplicates and average values used.

specifically against β_h-EP. To further evaluate the structural similarity of this compound with β_h-EP, we tested its ability to interact with antisera that were specific to β_h-EP_{22-27}, β_h-EP_{14-27} and leu-ENK.

As indicated in Fig.3, the plasma factor cross-reacted with both anti-sera against β_h-EP but not with that against leu-ENK. Comparison of the displacement curves obtained with the two anti-β_h-EP sera indicates that the plasma factor behaved more closely to β_h-EP_{14-27} than β_h-EP_{22-27}.

DISCUSSION

In this study we have apparently discovered a factor in the human plasma which has immuno and opiate-receptor binding properties similar to β-EP. According to its elution profile on a Bio-gel P2 column, this compound probably has a molecular weight between one to two thousand. The two anti-β-EP sera used to characterize the plasma factor were specific to the 22 to 27 and 14 to 27 segment of the β-EP molecule. In addition, they do not cross-react with either α- or γ-endorphin. In view of these considerations, it is likely that

the plasma factor may have a configuration resembling at least a segment of β-EP. It has been claimed that the N-terminal tyrosine group of β-EP is essential for opiate-receptor binding activity (15, 16). Hence, by virtue of its activity in the opiate-receptor binding assay, the plasma factor may also contain this essential functional group.

The inability of the plasma factor to cross-react with the anti-leu-ENK serum indicates that either the enkephalin sequence is not present in this molecule or that it is hidden so that it cannot interact with the antibody (the anti-leu-ENK serum used presently cross-reacts with met-ENK to about 30%). Answers to these structural questions should be available when the plasma factor is further purified and characterized.

The inhibition of the contraction of the guinea pig ileum produced by our plasma factor lasted only 3 min while that produced by an equivalent dose of β_h-EP lasted for over 8 min. When naloxone was used to block the action of the plasma factor, it was only partially effective. Because of this, the plasma factor probably has a higher affinity for the opiate receptors than naloxone.

The origin of our plasma factor is still obscured. The possibility that the plasma factor was generated during sample preparation is remote because 1) all blood samples were collected in the presence of bacitracin and chilled immediately after collection, 2) the time period between collection and boiling of plasma is only one hour, and it is unlikely that much degradation can occur under these conditions, 3) in a control experiment, no appreciable amount of degradation was observed when synthetic β_h-EP was added to the blood sample and processed as described in Materials and Methods.

The presence of β-EP in the blood of normal human subject remains to be demonstrated. Both Jeffcoate *et al.*(17) and Suda *et al.*(18) were unable to detect any β-EP in human plasma using radioimmunoassay procedures sensitive down to 30 pg. In addition, Suda *et al.*(18) have shown that after the injection of 200 ug of β-LPH into the bloodstream of human subjects, no significant signs of increase of β-EP level was noticed. In view of these findings, it is unlikely that a significant amount of β-EP could be generated from the breakdown of β-LPH in the peripherial blood. However, since there is a fair amount of β-LPH in the bloodstream (18, 19), the possibility remains that our plasma factor may be derived from this molecule.

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