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α-NEO-ENDORPHIN : A "BIG" LEU-ENKEPHALIN WITH POTENT OPIATE ACTIVITY FROM PORCINE HYPOTHALAMI

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SUMMARY : α-Neo-endorphin, a new morphinomimetic peptide was isolated in a yield of 50 μg from the extracts of 30,000 pig hypothalami. Its partial structure was determined by dansyl-Edman method in a nano mole scale as : Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-(Pro,Gly,Tyr_2,Lys_2,Arg). On the basis of our structural data, it is concluded that α-neo-endorphin is a "big" Leu-enkephalin, which has never been discovered and its structure shows no relationship with β-lipotropin. The occurence of α-neo-endorphin in brain might suggest the possible existence of a separate precursor to Leu-enkephalin distinct from that to Met-enkephalin and the other known endorphins.

INTRODUCTION

The isolation from mammalian brain of two pentapeptides, named enkephalins(Tyr-Gly-Gly-Phe-X: X=Met or Leu), with morphinomimetic activity has stimulated a number of studies in neurophysiological and neuropharmacological fields (1). Endorphins (α-, β-, γ- and δ-), structurally related to Met-enkephalin, have also been characterized as endogenous ligands of opiate receptors (2-6).

What is remarkable is that, with the exception of Leu-enkephalin, all the known endogenous opioid peptides have C-terminal subsequences of β-lipotropin. Thus, the known endorphins belong to

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a class of "big" Met-enkephalin and they are considered to be derived from β-lipotropin or a bigger ancestor, i.e. 31K protein or pro-opiocortin, which is proved to be a common precursor to Met-enkephalin and corticotropins (7-9).

To the contrast with Met-enkephalin, neither a precursor to Leu-enkephalin, nor a "big" Leu-enkephalin has been isolated as yet. Biosynthetic pathway of Leu-enkephalin has also remained to be clarified.

During the course of our study on the hypothalamic peptides, a novel opioid peptide, which we have named α-neo-endorphin, has recently been isolated in a pure state from porcine hypothalami. On the basis of our structural data, α-neo-endorphin is proved to be a "big" Leu-enkephalin, which has never been discovered. The present paper describes a method for obtaining α-neo-endorphin in pure form, its amino acid composition and its NH_2-terminal sequence. These data serve to distinguish α-neo-endorphin from other known opioid peptides. Morphinomimetic activity of this peptide is also discussed.

METHODS AND MATERIALS

Porcine hypothalami (30,000 pigs) were excised within 1 hr. of killing and frozen immediately. Fluorescamine was kindly donated by Dr.S. Udenfriend of Roche Institute of Molecular Biology. Met- and Leu-enkephalins, neurotensin and substance P were obtained from Protein Research Foundation, Mino, Japan. [Arg^6]-Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu-Arg) was synthesized by Dr.S. Sakakibara, Protein Research Foundation. Its synthesis will be reported elsewhere in the nearest future. Trypsin, treated with TPCK, was purchased from Worthington. 4N Methanesulfonic acid was a product of Pierce. Pre-coated cellulose thin layer plate (20x20 cm; 0.1 mm thick) was obtained from Merck.

On chromatography and gel-filtration, column eluates were monitored by measuring their absorbance at 280 nm in earlier stages of purification and by fluorescamine analyses in manual manipulations (10).

Thin layer chromatography on cellulose plate was performed in the solvent system; n-BuOH:pyridine:AcOH:water = 15:10:3:12 (v/v).

Electrophoresis on cellulose plate was performed at 200 volt in a buffer of pH 3.5; pyridine:AcOH:water = 2.6:30:867 (v/v). Ninhydrin solution (0.25%) in n-BuOH saturated with water and fluorescamine solution (0.02%) in acetone were used as chromogenic reagents.

Amino acid analysis was performed after hydrolysis of 5 μg of α-neo-endorphin in 4N methanesulfonic acid (24 hrs., 110°C).

Sequence analyses were carried out with 5-μg aliquot of α-neo-endorphin by dansyl-Edman method in a nano mole scale, mainly according to the reported method (11).

Tryptic digestion of α-neo-endorphin (1μg) was done with 50 ng of trypsin in a buffer of 0.2M N-ethylmorpholin-acetate (pH 8.0) at 37°C for 4 hrs.

In all analyses, control experiments were made under exactly the same conditions, except that the peptide was omitted.

Opiate activity was assayed by the use of electrically stimulated myenteric plexus-longitudinal muscle from guinea-pig ileum (12). Naloxon used was provided by Sankyo Co., Tokyo, Japan.

RESULTS AND DISCUSSION

Isolation of α-Neo-endorphin : Frozen fragments of 30,000 pig hypothalami were pulverized, defatted and then extracted with 2N AcOH. Lyophilized extracts (400 g) were submitted to gel filtration on Sephadex G-25, which had been equilibrated with N AcOH, to collect a small peptide fraction that eluted in a range of 1.4-2.3 x hold-up volume. Lyophilized material (150 g) was dissolved in 30% AcOH and treated batch-wise with SP-Sephadex C-25 (free form). Adsorbed material, which was eluted with N NH_4OH, was lyophilized. This material (13 g) was chromatographed on a column of SP-Sephadex C-25 (Fig.1). α-Neo-endorphin fraction, which was emerged in tubes No.149-160, was collected. The lyophilized material (555 mg) was then submitted to gel filtration on Sephadex G-25 (Fig.2), followed by ion-exchange chromatography on SP-Sephadex C-25 (Fig.3). Major opiate activity by guinea-pig ileum assay was emerged in the hatched area, as shown in Figs.1-3. Thus, purified α-neo-endorphin was obtained in a yield of 50 μg from 30,000 hypo-

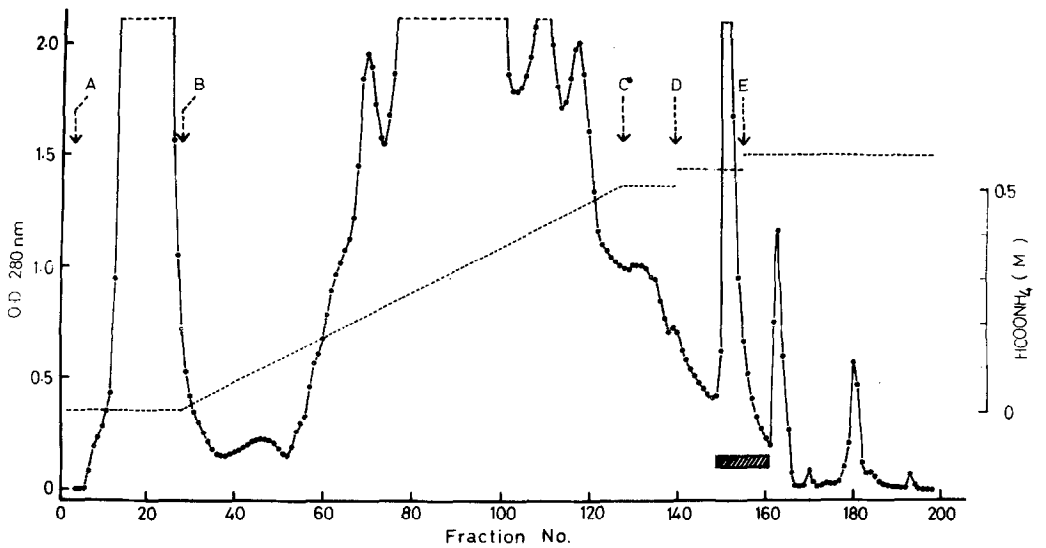

Fig.1 SP-Sephadex C-25 chromatography of a small peptide portion obtained after Sephadex G-25 gel filtration of hypothalamic extracts, followed by batch-wise treatment with SP-Sephadex C-25 (free form)

Column size: 4.2 x 26 cm. Pre-equilibrated with 0.001N HCO_2H.
Sample loaded: Lyophilized material (13 g) in 0.1N HCO_2H (130 ml)
Fraction size: 20 ml/tube
Elution system: (A): 0.001N HCO_2H (B)-(C): linear gradient, starting from water to solution (C) (C): 0.5M ammonium formate (pH 6.5) (D): 0.5M ammonium formate (pH 9.5) (E): N NH_4OH
Opiate activity by guinea-pig ileum assay was observed in the hatched area (tube No. 146-160)

thalami. This preparation showed only a single spot (Rf 0.68), positive to Ninhydrin and fluorescamine reagents on the thin layer chromatogram as shown in Fig.4-(a), and also gave one spot on the electrophoretogram on thin layer cellulose plate. These data verified that the α-neo-endorphin preparation was pure and homogeneous enough for further structural analyses.

Structure of α-Neo-endorphin : Due to the very limited amount, all analyses had to be performed in a nano mole scale. Amino acid composition of α-neo-endorphin after methanesulfonic acid hydrolysis was found to be : Pro_1, Gly_3, Leu_1, Phe_1, Tyr_3, Lys_3, Arg_3.

The NH_2-terminal sequence analyses with 5-10 μg of α-neo-endorphin were successfully performed to the 8th step by dansyl-Edman

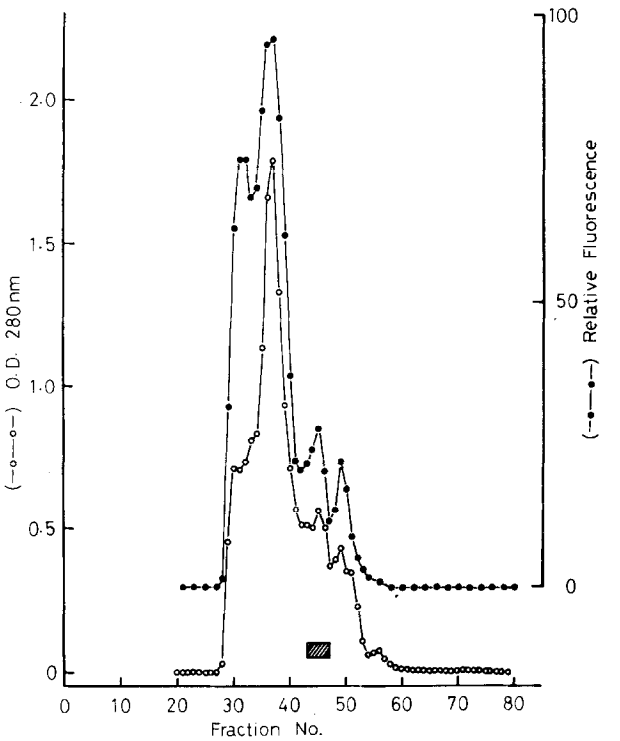

Fig.2 Gel filtration on Sephadex G-25

Column size: 1.8 x 130 cm. Pre-equilibrated with N AcOH.
Sample loaded: Opiate-active region in Fig.1 (555 mg).
Fraction size: 5 ml/tube Eluent: N AcOH
Opiate activity was observed in the hatched region.

procedure in a nano mole scale. The partial sequence thus determined is shown and compared with that of the known endorphin in Fig. 5. As clearly seen from the sequence, α-neo-endorphin has a primary structure identical to that of Leu-enkephalin at its NH_2-terminus. In order to ascertain this, tryptic digestion of α-neo-endorphin was carried out with the expectation of the formation of [Arg^6]-Leu-enkephalin fragment by the tryptic cleavage of the bond of -Arg^6-Lys^7-. Thin layer chromatogram of the tryptic digests revealed that an NH_2-terminal hexapeptide thus formed was identical with synthetic [Arg^6]-Leu-enkephalin.(Fig.4-(b))

From these structural data, it is unambigously verified that

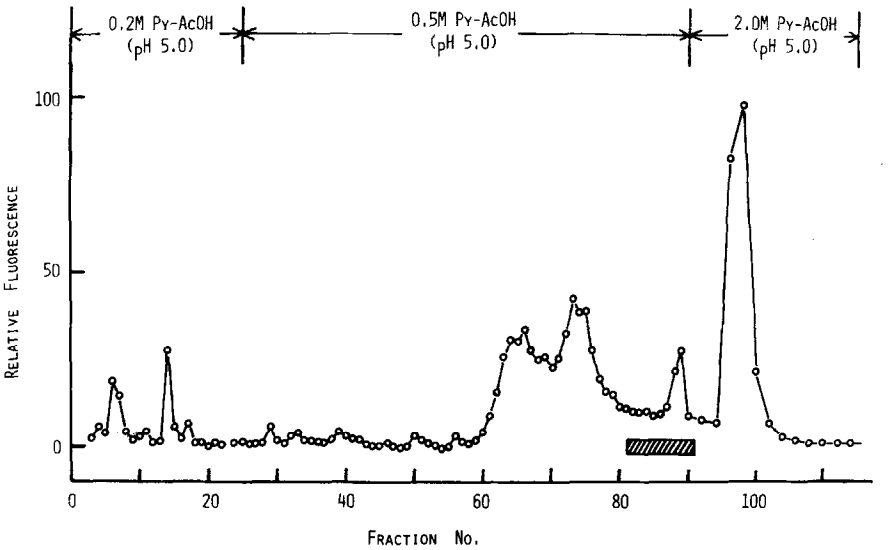

Fig.3 Rechromatography on SP-Sephadex C-25 of opiate-active fraction
Column size: 1.0 x 20 cm. Pre-equilibrated with 0.2M pyridine-acetate at pH 5.0.
Sample loaded: Opiate-active fraction (4.5 mg) in Fig.2.
Fraction size: 3 ml/tube
Elution system: Step-wise elution as indicated in Fig.3.
Opiate activity was observed in the hatched area.

Table 1. Opiate Activities of α-Neo-Endorphin and other Opioids (Guinea-Pig Ileum Assay)

Opioid Peptide	Relative Potency*1)
Met-enkephalin	100
Leu-enkephalin	25
β-Endorphin	130*2)
[Arg^6]-Leu-enkephalin	8
α-Neo-endorphin	670

*1) Expressed as percentage of Met-enkephalin
*2) Taken from ref. (13).

α-neo-endorphin is a "big" Leu-enkephalin, which has never been discovered. Further, it should be also noted that Leu-enkephalin structure in α-neo-endorphin is followed by a characteristic tri-

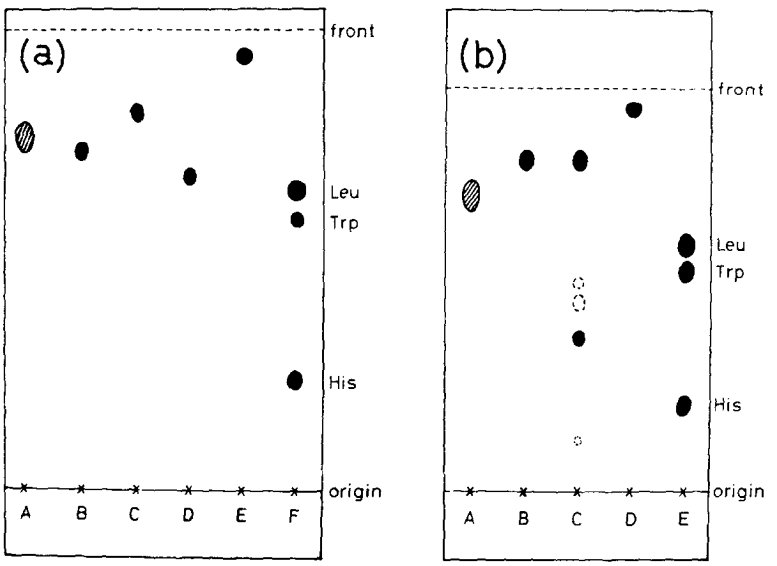

Fig.4 Thin layer chromatography on cellulose plate
(Solvent system: n-BuOH:pyridine:AcOH:water=15:10:3:12)

(a) A: neutral red B: substance P C: neurotensin D: α-neo-endorphin (the hatched area in Fig.3) E: Leu-enkephalin F: amino acid mixture as standard.

(b) A: neutral red B: synthetic [Arg^6]-Leu-enkephalin C: tryptic digests of natural α-neo-endorphin D: Leu-enkephalin E: amino acid mixture as standard

α-Neo-endorphin :

Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-(Pro,Gly,Tyr_2,Lys_2,Arg)

Endorphins (C-terminal subsequence of β-lipotropin)

61 65 70 75

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val---

Fig.5 Proposed partial structure of α-neo-endorphin, compared with endorphins

ad of -Arg-Lys-Arg-, which often occurs in pro-peptide sequences, but not in the known endorphins related to Met-enkephalin. These facts mentioned above obviously distinguish α-neo-endorphin from the known endorphins. Accordingly, occurence of α-neo-endorphin in brain might suggest the possible existence of a separate precursor to Leu-enkephalin distinct from that to Met-enkephalin.

Opiate Activity of α-Neo-endorphin : As summarized in Table 1, α-neo-endorphin showed very potent opiate activity in guinea-pig ileum assay, 6.7 times as high as Met-enkephalin and 5 times as high as β-endorphin (13). Therefore, α-neo-endorphin seems to be the most active of the endogenous opioids, which have been isolated. And, it should be mentioned that [Arg^6]-Leu-enkephalin, which corresponds to tryptic NH_2-terminal fragment, showed very low activity, not more than 10% of Met-enkephalin.

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