

OPIOID AGONIST ACTIVITY OF β -LIPOTROPIN FRAGMENTS: A POSSIBLE BIOLOGICAL SOURCE OF MORPHINE-LIKE SUBSTANCES IN THE PITUITARY

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1. Introduction

An endogenous peptide with opioid agonist activity, termed enkephalin, has been isolated from the porcine brain [1], and more recently the primary structure of this pentapeptide has been elucidated: H-Tyr-Gly-Gly-Phe-Met^{Leu}-OH [2]. Hughes et al. [2] pointed out that the complete sequence of the major form of enkephalin, Met-enkephalin, is contained in the primary structure of pituitary β -lipotropin between amino acid residues 61–65 [3–5]. This sequence overlap raises the possibility that β -lipotropin may be the biological precursor of enkephalin and/or other pituitary peptides with morphine-like activity. As an approach to this question the possible microheterogeneity of porcine β -lipotropin at sequence position 65, and the opiate agonist activities of β -lipotropin and some fragments of the hormone obtained by enzymic cleavages were investigated. The results of these studies are reported in this paper.

2. Experimental

2.1. Preparation and characterization of β -lipotropin and β -lipotropin fragments

Porcine β -lipotropin was isolated by a procedure described previously [6]. Prior to biological measure-

ments our preparation was subjected to chromatography on a Bio-Gel P-6 column in 10% acetic acid. Tryptic digestion was carried out in 0.05 M NH_4HCO_3 , pH 8.0 with an enzyme to peptide ratio of 1:50 (w/w) at 37°C for 2 h. Isolation of the tryptic fragment from residues 61–69 of the β -lipotropin sequence (LPH-(61–69)-peptide) was performed by preparative high voltage paper electrophoresis at pH 6.5 and subsequently at pH 2.0 [4]. The amino acid composition of a complete aminopeptidase M hydrolysate from LPH-(61–69)-peptide was as follows. Lys_{1.0}, MeSO_{0.2}, Thr_{1.0}, Ser_{0.9}, Glu_{1.0}, Gly_{1.9}, Met_{0.8}, Tyr_{0.9}, Phe_{1.0}. This composition is in agreement with that derived from the sequence proposed for this region of the lipotropin molecule: Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys [3–5]. It is worth mentioning that no trace of leucine could be detected in LPH-(61–69)-peptide.

Specific cleavage of the Lys-Ser bond at sequence positions 69–70 of β -lipotropin by a homogenate of fresh pituitary glands, and the isolation of the large NH_2 -terminal fragment (LPH-(1–69)-peptide) is published elsewhere.

2.2. Synthesis of Met-enkephalin

Boc-Tyr-Gly-Gly and Phe-Met-OMe were condensed by EEDQ to obtain the protected pentapeptide ester, I. Boc group of I was removed by EtOAc/HCl to yield Met-enkephalin methyl ester (m.p. 140–143°C with decomposition, R_f 0.52–0.57*). Saponification of I led to Boc-Tyr-Gly-Gly-Phe-Met, II (crystallized from ethyl acetate, m.p. 149–151°C with decomposition, R_f 0.59–0.64).

Deblocking of II by EtOAc/HCl gave Met-enkephalin hydrochloride (m.p. 139–142°C, R_f 0.29–0.34,

Abbreviations: LPH, lipotropic hormone (lipotropin); Boc, *t*-Butyloxycarbonyl; EEDQ, ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline; EtOAc, ethyl acetate.

* R_f values were obtained in thin-layer chromatography using an ethyl acetate-pyridine-acetic acid-water (60:20:6:11) system.

$[\alpha]_D^{20} + 18^\circ$, $c = 1$, in 1 M acetic acid). Amino acid analysis of Met-enkephalin after digestion with amino-peptidase M gave the following result: Gly_{2.0}, Met_{0.8}, Tyr_{1.0}, Phe_{1.0}.

2.3. Bioassay

The presence or absence of opioid agonist activity as well as the relative agonist potencies of different peptide fragments were determined on longitudinal muscle strip of guinea-pig ileum. It has been shown [7] that opiate receptors of the above muscle preparation, in their drug-binding properties, show close similarities to those of the opiate receptors in the central nervous system which may be involved in analgesic action of morphine-like drugs.

The longitudinal muscle strip of guinea-pig ileum was prepared as described in Paton and Vizi [8]. The experiments were carried out in Krebs' solution bubbled with 5% CO₂ in oxygen at 37°C. The composition of the Krebs solution was as follows (in mM): NaCl 118; NaHCO₃ 25; glucose 11.5; KCl 4.7; KH₂PO₄ 1.2; CaCl₂ 2.5 and MgSO₄ 1.2. Field electrical stimulation [8] was used by means of platinum wire or ring electrodes. The parameters of the stimulation applied were following: supramaximal (1.5 times the maximal voltage) rectangular stimuli of 1 msec duration delivered at a rate of 0.1 Hz. For the simultaneous measurement of agonist and antagonist activity, the method of Kosterlitz and Watt [9] was used.

3. Results

Highly purified β -lipotropin and LPH-(1-69)-peptide proved to be inactive as opioid agonist in longitudinal muscle strip of guinea-pig ileum at concentrations as high as 1.6×10^{-6} M and 8×10^{-7} M, respectively. However, tryptic digestion of both polypeptides led to the appearance of agonist activity as it is demonstrated for β -lipotropin in fig.1. The agonist activities of the tryptic digests were found to be 2-8 times less than that of normorphine or Met-enkephalin.

Considering that the Met-enkephalin sequence is contained between residues 61-65 of the β -lipotropin structure [2], theoretically the LPH-(61-69)-peptide could account for the morphine-like activity of the tryptic hydrolysates. Indeed, highly purified LPH-

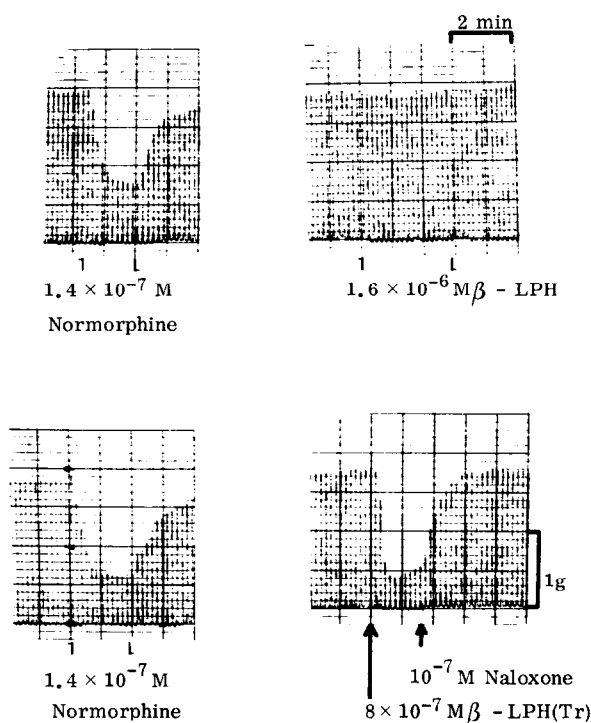


Fig.1. The depressant effects of normorphine, β -lipotropin (β -LPH) and a tryptic digest of β -lipotropin (β -LPH (Tr)) on the electrically induced contractions of longitudinal muscle strip from guinea-pig ileum.

Table 1
Relative agonist potencies of the peptides studied

Compound	Agonist potency ratios (relative to normorphine)	
Normorphine	1	(n = 8)
β -lipotropin	— ^a	
LPH-(1-69)-peptide	— ^b	
LPH-(61-69)-peptide ^c	0.4 ± 0.05	(n = 4) ^d
Met-enkephalin ^c	0.9 ± 0.2	(n = 4) ^d

^a Highest dose tested: 1.6×10^{-6} M.

^b Highest dose tested: 8.0×10^{-7} M.

^c Concentration of the peptides was determined spectrophotometrically.

^d Mean \pm SEM values are listed, the number of experiments are in parentheses. For the difference between LPH-(61-69)-peptide and normorphine $p < 0.02$. The difference between Met-enkephalin and normorphine is not significant.

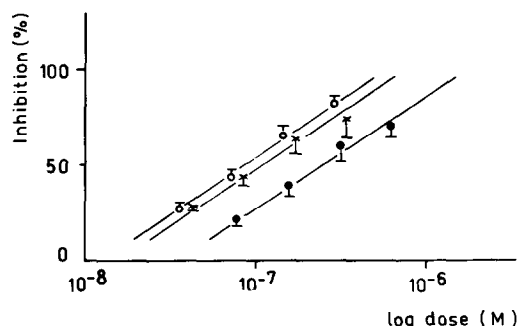


Fig. 2. Dose-response curves for normorphine (○), synthetic Met-enkephalin (×) and LPH-(61-69)-peptide (●).

(61-69)-peptide was found to possess an agonist potency 2.5 times weaker than that of normorphine. The relative agonist potencies of the peptides studied are listed in table 1. The dose-response curves for the active peptides, Met-enkephalin and LPH-(61-69)-peptide, were parallel to that of normorphine (fig. 2). The potency ratio obtained for normorphine: Met-enkephalin shows fair agreement with the results of Hughes et al. [2].

The inhibitory effect of peptides, tested at a depression of 70-90% could be completely antagonized by 10^{-7} M naloxone.

4. Discussion

The above data give some useful information on the structure-activity relationship in enkephalin. The fact that LPH-(61-69)-peptide shows comparable opiate agonist potency with that of enkephalin in guinea-pig ileum suggests that the free carboxyl group of methionine is not necessary for the morphine-like action. To support this assumption synthetic Met-enkephalin methyl ester gave identical biological response with that of Met-enkephalin as tested in a preliminary experiment. The COOH-terminal tetrapeptide portion of LPH-(61-69)-peptide however, seems to moderate the biological potency of the peptide as compared to that of Met-enkephalin (fig. 2, table 1). As to the lack of significant opioid properties of intact β -lipotropin and LPH-(1-69)-peptide, it may be speculated that the large NH_2 -terminal portion of these polypeptides sterically

hinders the opiate receptor-active site interaction.

The fact that only methionine was found at sequence position 65 of porcine β -lipotropin is inconsistent with the assumption that pituitary β -lipotropin may be the sole biological precursor of brain enkephalin. Porcine enkephalin has been shown to contain a mixture of methionine and leucine at the corresponding sequence position [2].

On the other hand, β -lipotropin may be one of the sources of pituitary substances with opiate agonist activity*. Tryptic digestion of β -lipotropin provides a reasonable model for the release of such a peptide. In fact, pituitary has been known to be rich in trypsin-like enzymes [11]. Whether or not such a cleavage mechanism really operates and has a physiological role in the pituitary, are open questions. It is tempting to speculate however, that LPH-(1-69)-peptide obtained by incubation of β -lipotropin with pituitary homogenate, might be a common intermediary precursor for both β -melanotropin and an enkephalin-like peptide.

In preliminary experiments the *in vivo* analgesic activity of some of the above peptides have been investigated. The peptides were administered intracerebroventricularly, and the pain threshold was measured by the 'tail-flick' method in rats [12]. Morphine in a dose of 20 μg per animal caused a strong analgesic effect, whereas Met-enkephalin and the tryptic digest of porcine β -lipotropin in equimolar dose were found to be practically inactive. Surprisingly, intact β -lipotropin in equimolar dose showed a considerable efficacy. To our present knowledge this is the first observation on the *in vivo* analgesic activity of a natural polypeptide.

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*Goldstein and co-workers [10] have also detected a peptide with opioid activity in bovine and porcine pituitary, but the trypsin sensitivity of this material differentiates it from a lipotropin fragment.

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