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

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Received 10 February 1976

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 measurements 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°, c = 1, in 1 M acetic acid). Amino acid analysis of Met-enkephalin after digestion with aminopeptidase 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% CO_2 in oxygen at 37°C. The composition of the Krebs solution was as follows (in mM): NaCl 118; $NaHCO_3$ 25; glucose 11.5; KCl 4.7; KH_2PO_4 1.2; $CaCl_2$ 2.5 and $MgSO_4$ 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.

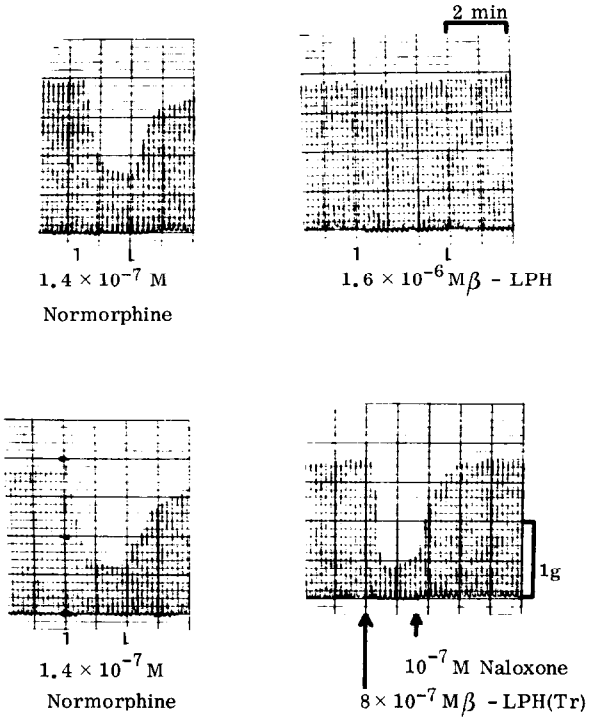

Fig.1. The depressant effects of normorphine, β-lipotropin (β-LPH) and a tryptic digest of β-lipotropin (β-LPH (Tr)) on the electrically induced contractions of longitudinal muscle strip from guinea-pig ileum.

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-

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 ± 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.

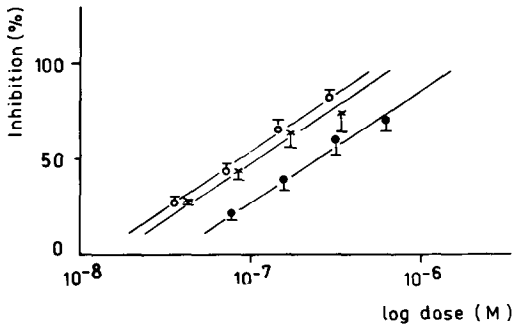

Fig.2. Dose–response curves for normorphine (○), synthetic Met-enkephalin (×) and LPH-(61–69)-peptide (●).

(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 treshold 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.

References

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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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