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Physiology

β-Lipotropin as a prohormone for the morphinomimetic peptides endorphins and enkephalins

(lipotropin/morphine/brain/endogenous ligand/pituitary)

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ABSTRACT The hypophysial homomeric peptide β-lipotropin (β-LPH-[1-91]) has no morphinomimetic activity in a bioassay (myenteric plexus-longitudinal muscle of the guinea pig's ileum) or binding assays with stereospecific opiate-receptors of rat brain synaptosome preparations. Incubating β-LPH-[1-91] at neutral pH with the supernatant aqueous extracts of rat brain generates (fragments of β-LPH with) morphinomimetic activity in the same assay systems. These results are related to the recently recognized structural relationships between β-LPH, the newly isolated peptides met-enkephalin (β-LPH-[61-65]) and α-endorphin (β-LPH-[61-76]) and also to the biologically active fragments or analogs: β-LPH-[61-64], β-LPH-[61-65]-NH$_2$, (Met(O)^{65})-β-LPH-[61-65], β-LPH-[61-69], and β-LPH-[61-91]. Enzymatic biogenesis of these morphinomimetic peptides would preclude localizing them as such in cellular or subcellular elements with currently available methodology.

Two laboratories have recently reported the isolation and primary structure of novel oligopeptides of whole brain or hypothalamus-neurohypophysis origin, endowed with biological characteristics similar to those of the alkaloids of opium in bioassays *in vitro* and stereospecific binding assays on synaptosomal opiate-receptors: Hughes *et al.* (1) characterized *enkephalin*, extracted from whole (pig) brain, to be in fact a mixture in the ratio 4/1 of the two pentapeptides H-Tyr-Gly-Gly-Phe-Met-OH (met-enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH (leu-enkephalin). Guillemin *et al.* (2) isolated several *endorphins* from an extract of (pig) hypothalamus-neurohypophysis and characterized one of them, *α-endorphin*, as the hexadecapeptide H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH. Hughes *et al.* recognized that met-enkephalin is identical to the sequence which extends from Tyr^{61} to Met^{65} of the various β-lipotropins, and Guillemin *et al.* observed that the primary structure of α-endorphin is identical to that of the sequence from Tyr^{61} to Thr^{76} of the β-lipotropins, met-enkephalin thus being the NH$_2$-terminal pentapeptide of α-endorphin. Moreover, in their original report (2) Guillemin, Ling, and Burgus stated that the (synthetic) nonapeptide H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-OH, i.e., β-LPH-[61-69] also had considerable morphinomimetic activity. It was then obvious that the relationship between β-lipotropins (β-LPHs) and the opioid peptides was more than accidental. In fact, after the statement of Hughes *et al.* appeared (1), and before the structure of α-endorphin was completed, both our group and Goldstein's (see 3) observed that while β-LPH had no morphinomimetic activity even at high doses, the COOH-terminal fragment β-LPH-[61-91], available from previous isolation studies (4, 5), had considerable activity in the morphine-bioassay and opiate receptor binding assays (in ref. 4, the proposal is made to name β-LPH-[61-91] as β-endorphin).

Isolated in 1964 from sheep pituitary glands (6), β-LPH was subsequently isolated and characterized from the pituitary of a variety of species; in all cases it is a homomeric 91-residue polypeptide with minor species variances in its amino acid sequence (see ref. 7 and references therein). The physiological significance of β-LPH has remained obscure to this day. It has lipolytic activity in several systems and minimal melanophoretic and adrenocorticotropic activity (7). It has been reported in blood (8) and has been located in discrete cells of the anterior and intermediate lobes of the pituitary by immunofluorescence (9). Long recognized (6) to be a possible source of β-melanocyte stimulating hormone β-MSH (β-LPH-[41-58]) and perhaps its only artefactual source (10), β-LPH was investigated here as a possible prohormone for the morphinomimetic peptides in view of the relationships of primary structures recalled above. There is ample evidence that *brain* extracts contain endopeptidases with the ability to produce the expected or suspected fragments (11, 12).

The morphinomimetic activity of several synthesized fragments of β-LPH and analogs are also reported here.

Abbreviation: β-LPH, β-lipotropin; β-MSH, β-melanocyte stimulating hormone.

MATERIALS AND METHODS

(*a*) For bioassays (myenteric plexus-longitudinal muscle of the guinea pig's ileum) (13), ovine β-LPH (25 μg/25 μl of H$_2$O) was incubated, in several experiments, up to 2 hr at 37° with 25 μl of the supernatant of a sucrose (0.32 M) extract of whole rat brain centrifuged at 100,000 × *g* for 1 hr. At chosen times, aliquots of the total incubation mixture (see Fig. 1) were added to the incubation fluid (5 ml chamber) of the bioassay.

(*b*) For studies of binding to synaptosomal opiate-receptors, ovine β-LPH (2 μg/2 μl of H$_2$O) was incubated for up to 2 hr at 37° with 10 μl of the same rat brain extract as above. At chosen times, aliquots of the mixture were removed and boiled for 5 min. Rat brain synaptosomal fractions were prepared by discontinuous gradients (14). Aliquots of these fractions (513 μg or 189 μg of protein for P$_2$ or F fraction respectively) were incubated with 0.025 μCi of [^{3}H]etorphine (41 Ci/mol, Amersham-Searle), 10 mM Tris·HCl at pH 7.4, 1 mg of bovine serum albumin (BSA) and β-LPH (0.5 μg), or the same weight of β-LPH after incubation with the aqueous brain extract, in a total volume of 0.1 ml, for 20 min at 37° or 60 min at 0° in a dimly lit room (15). For measurement of specific binding, 10 μg of normorphine or 10 μg of levorphanol was added to the reaction mixtures. Two ml of ice-cold isotonic buffer containing 0.2% BSA was added to the above mixture at the end of incubation, the solution was filtered on Whatman glass fiber discs (GF/C), and rinsed once. The entire operation took less than 10 sec. The filters were dried and the radioactivity was determined with a standard toluene based scintillation fluid. Specific binding was determined by the difference between [^{3}H]etorphine displaced by either the normorphine or levorphanol and the total [^{3}H]etorphine bound. Nonspecifically bound [^{3}H]-

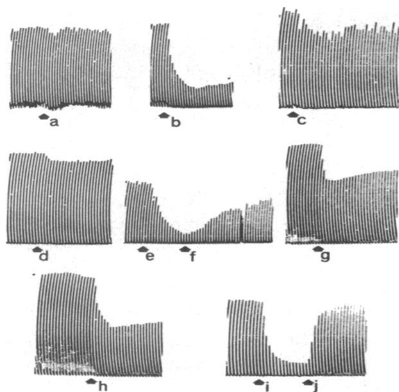

FIG. 1. Electrically evoked contractions of the myenteric plexus-longitudinal muscle of guinea pig ileum. a, β-LPH-[1-91], 1 μM; b, normorphine, 30 nM; c, 0.5 μM β-LPH-[1-91] incubated with brain extract for 30 min; d, as in c, but incubated for 2 hr—the small decrease in amplitude is identical to that produced by brain extract alone; e, β-LPH-[61-91], 0.6 μM; f, naloxone, 1 μM; g, β-LPH-[61-69], 1 μM; h, met-enkephalin, (β-LPH-[61-65]), 0.1 μM; i, α-endorphin, (β-LPH-[61-76]), 50 nM; j, naloxone, 1 μM.

etorphine amounted to 55–70% of the total bound. In other experiments, solutions (10 μg/ml) of synthetic peptides were diluted in aqueous BSA (1 mg/ml) and incubated as above with [^{3}H]etorphine.

(*c*) Synthetic peptides were prepared by solid phase methodology, purified, and their homogeneity ascertained by the methods routinely used in this laboratory (16). The peptides so prepared were: H-Tyr-Gly-Gly-OH, i.e., β-LPH-[61-63]; H-Tyr-Gly-Gly-Phe-OH, i.e., β-LPH-[61-64]; H-Tyr-Gly-Gly-Phe-Met-OH, i.e., β-LPH-[61-65] also known as met-enkephalin (1); H-Tyr-Gly-Gly-Phe-Met-NH_2, i.e., (met)-enkephalinamide; (Met(O)^{65})-β-LPH-[61-65]; H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-OH, i.e., β-LPH-[61-69]; and α-endorphin, i.e., β-LPH-[61-76].

RESULTS

Even at the high doses tested (up to 10 μg/ml), β-LPH has no morphinomimetic activity (Fig. 1a), in a bioassay sensitive to 10 ng of normorphine per ml (Fig. 1b). Incubation of β-LPH with the aqueous extract of rat brain generates opioid activity (Fig. 1c) which disappears after a 2 hr incubation (Fig. 1d). The morphine-like activity of the mixture in the bioassay is increased rather than reversed by naloxone, indicating an unusual involvement of the opiate-receptors of the myenteric plexus tissue; such unmasking of agonist activity of naloxone has been seen occasionally by us when dealing with mixtures of peptides or with some peptides related to sequences of ACTH. In the opiate receptor binding assay, competing activity displacing [^{3}H]-etorphine is observed after incubation of β-LPH with aqueous brain extract, while intact β-LPH has no statistically significant activity (Table 1); as in the bioassay, prolonged incubation with the brain extract (2 hr) leads to disappearance of the opiate-receptor binding activity. As shown in Fig. 2, the peptides compete with [^{3}H]etorphine in the synaptosomal binding assay in a manner that parallels the effects of levorphanol or normorphine. The minor activity of the highest dose of β-LPH, occasionally observed, is best explained by the ample possibility for peptide cleavage during the incubation with the synaptosomal fraction known to carry proteases.

Fig. 1e and g–i show the morphine-like activity of some fragments of β-LPH of known structure, and its reversal by naloxone (Fig. 1f and j). The tripeptide H-Tyr-Gly-Gly-OH, i.e., β-LPH-[61-63] has no opioid activity in the bioassay at doses as high as 10 μg/ml (0.1 mM). The tetrapeptide H-Tyr-Gly-Gly-Phe-OH has definite activity at the same doses, its specific activity being thus *ca.* 1/100 that of met-enkephalin. H-Tyr-Gly-Gly-Phe-Met-NH_2 has considerable activity in the bioassay, its specific activity being *ca.* 5 times that of the free acid form of the pentapeptide, while the methionine sulfoxide analog has *ca.* ½ the specific activity of the free acid of the pentapeptide. The duration of activity of met-enkephalinamide is considerably increased over that of the free acid form.

DISCUSSION

Though far from exhaustive, these experiments show that incubation of β-LPH with aqueous brain extracts at neutral pH generates (peptides with) morphinomimetic activity. It will be of interest to determine the primary structure of the fragments produced to relate them to those of characterized peptides with opioid activity (no attempt was made here even to assess the cleavage points of β-LPH). The opioid activity of many syn-

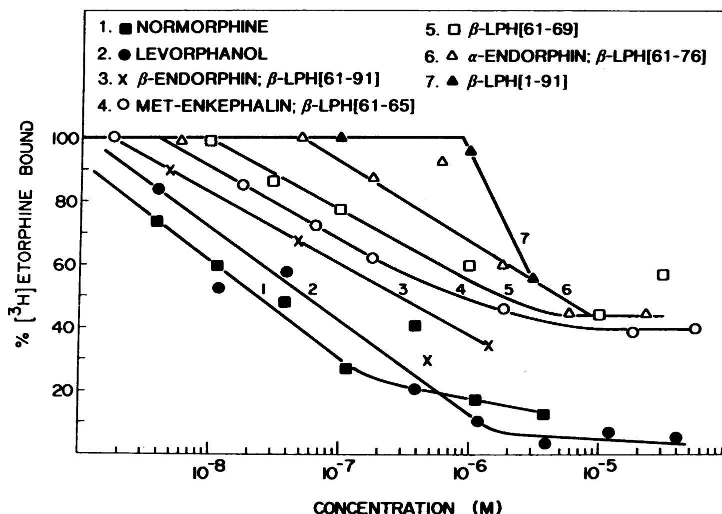

FIG. 2. Competition of the binding of [^{3}H]etorphine by opiates and morphinomimetic peptides. Stock solutions of all compounds were diluted in water containing 1 mg of bovine serum albumin/ml. Each point represents the average value of assays carried out in triplicate as given in *Materials and Methods* (0° for 60 min with a synaptosomal membrane preparation P_2).

Table 1. Stereospecific binding of [^{3}H]etorphine in the presence of various competitors

Condition	Addition	Radioisotope bound (cpm) No. 1	No. 2	No. 3	P*
(I)					
	Control	2655	2676	2913	
	3×10^{-5} M normorphine	1623	1687	1791	0.01
	1×10^{-6} M β-LPH	2497	2339	2729	—
(II)					
	Control	3801	3773	4061	
	3×10^{-5} M normorphine	2606	2601	2431	0.01
	3×10^{-5} M levorphanol	2445	2442	2514	0.01
	5×10^{-7} M β-LPH preincubated with brain extract for 30 min	3166	3366	2613	0.01
	5×10^{-7} M β-LPH	3986	3986	3374	—

Condition	Addition	No. of replicates	Radioisotope bound (cpm)†	
(III)				
	Control	5	9897 ± 295	
	3×10^{-5} M normorphine	5	3488 ± 89	0.01
	5×10^{-7} M β-LPH preincubated with brain extract for 30 min	3	8222 ± 295	0.05
	5×10^{-7} M β-LPH preincubated with boiled brain extract for 5 min	3	10,433 ± 895	—
	5×10^{-7} M β-LPH	5	10,828 ± 315	—

Conditions I and III were a 20 min incubation at 37° with synaptosomes. Condition II was a 60 min incubation at 0° with synaptosomes.
* Values of P were calculated by multiple comparison tests of Dunnett or Duncan after analysis of variance on original data.
† Values are the mean ± SEM.

thesized fragments of the COOH-terminal [61-91]-portion of β-LPH, as reported here, is in keeping with these observations.

Thus, β-LPH, containing the sequences of met-enkephalin (β-LPH-[61-65]), of α-endorphin (β-LPH-[61-76]), of β-endorphin (β-LPH-[61-91]), of β-MSH (β-LPH-[41-58]), and also of the fragment Met[4] to Gly[10] of adrenocorticotropin (β-LPH-[47-53]) may well be involved in those circumstances, physiological and experimental in which enkephalin, α-endorphin, β-MSH and ACTH-[4-10] have been reported to have profound and diverse neurotropic or behavioral activities (review in ref. 17). The various opioid peptides so far characterized from whole brain, hypothalamus or pituitary all have primary structures related to fragments of β-LPH; so far the only recognized source of β-LPH is the pituitary gland. These results are, thus, in agreement with and probably explain the earlier work of Goldstein *et al.* (18) who originally reported the presence of substances possibly of peptide nature with opioid activity in extracts of the pituitary. The results reported here, along with knowledge of the primary structure of enkephalins and endorphins raise a remarkable possibility: while the biogenesis of the hypothalamic hypophysiotropic peptides thyrotropin- releasing factor (TRF), luteinizing hormone-releasing factor (LRF), and somatostatin is probably by protein synthesis of the oligopeptides or more likely of proto-forms from ribosomal templates, the likely biogenesis of the neurotropic peptides discussed here is reminiscent of that of the angiotensins (19). With β-lipotropin as clearly the circulating and available substrate, it will be of major physiological importance to characterize, locate, and study the regulation of the several enzymes involved in the multiple cleavages of β-lipotropin that yield the neurotropic peptides. The resemblance with the biogenesis of angiotensins is even more striking considering the demonstration of angiotensin-converting enzyme in discrete locations of the brain (caudate nucleus) (ref. 20 and references therein). There is also renin-like activity in aqueous extracts of the brain of several species (21, 22), though a purely lysosomal origin of such brain-renin, as recently proposed (23), would raise doubts about its physiological availability. Should the various neurotropic peptides be generated by enzymatic cleavages of β-LPH as a substrate available in extracellular spaces, attempts to localize them by the currently available methods of immuno-histochemistry would be futile. The search will have to be for the converting enzymes after they have been characterized. By that time, the physiological significance, *if any*, of these peptide-ligands of the opiate receptors should have been critically evaluated.

Note Added in Proof. The primary structure of γ-*endorphin* isolated from hypothalamus-neurohypophysis extracts (2) has now been established as being identical to that of β-LPH-[61-77]; also β-LPH-[61-91] has been isolated from the same extracts on the basis of the bioassay described here for opiate-like activity.

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