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# Morphinomimetic activity of synthetic fragments of β-lipotropin and analogs

(peptides/endorphins/enkephalins/myenteric plexus/hypothalamus)

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ABSTRACT **In the myenteric plexus-longitudinal muscle bioassay, β-endorphin, i.e., β-lipotropin (β-LPH)-[61–91], has a potency of 450 with confidence limits of 281-966 when Met$^5$-enkephalin is used as a reference standard with a potency of 100. The primary amide and the ethylamide of Met$^5$-enkephalin have potencies statistically overlapping with that of β-endorphin. The primary amide of α-endorphin has twice the potency of the free acid form of α-endorphin. An intact $NH_2$-terminal tyrosine is not necessary for full intrinsic activity. The shortest fragment of β-LPH with morphinomimetic activity is β-LPH-[61–64].**

Two laboratories have recently reported the isolation and primary structure of novel peptides with morphine-like activity isolated from whole brain or hypothalamus-neurohypophysis extracts (1, 2). Hughes *et al.* (1) recognized that *Met$^5$-enkephalin* isolated from whole pig brain has a primary structure identical to that of the fragment Tyr$^{61}$-Met$^{65}$ of the β-lipotropins (β-LPH) (3), while Leu$^5$-enkephalin would share at least the sequence Tyr$^{61}$-Phe$^{64}$ of the known β-lipotropins. Guillemin *et al.* (2) similarly called attention to the fact that the *α-endorphin* isolated from extracts of porcine hypothalamus-neurohypophysis was identical to the sequence Tyr$^{61}$-Thr$^{76}$ of the various β-lipotropins, Met$^5$-enkephalin thus being the $NH_2$-terminal pentapeptide of α-endorphin. These results and early evidence (2, 4) that synthetic β-LPH-[61–69] and β-LPH-[61–91] showed opiate-like activity led to the proposal (1, 2, 4) that β-LPH could be a prohormone for the various endorphins and enkephalins. Indeed, Lazarus *et al.* recently reported (5) that while β-LPH-[1–91] has no morphinomimetic activity in the bioassay or opiate-receptor binding assays classically used in these studies, its incubation with the high-speed supernatant of an aqueous extract of rat brain rapidly generates (peptide fragments with) morphinomimetic activity. Moreover, we now have characterized *γ-endorphin* as being identical to β-LPH-[61–77]. The purpose of this short note is to describe the primary structures of a series of fragments of β-LPH and several analogs, all prepared by synthesis, and to report their morphinomimetic activity in the myenteric plexus bioassay.

## MATERIALS AND METHODS

**Bioassay.** The assay for opiates is the myenteric plexus-longitudinal muscle of the guinea pig's ileum as described by Paton and Zar (6); a response reversed or prevented by the opiate-antagonist naloxone is classically recognized to indicate specific involvement of "opiate receptors" (6, 7).

**Synthetic Peptides.** All peptides were synthesized as described previously (8, 9) by the solid phase technique; β-endorphin, i.e., β-LPH-[61–91], was prepared with a Beckman synthesizer model no. 990 (with the collaboration of Dr. Jean Rivier).

Abbreviations: β-LPH, β-lipotropin; β-LPH-[61–91], etc., the peptide consisting of residues 61–91 of β-LPH.

**Statistical Analysis of Bioassays.** Potencies shown in Table 1 were calculated, in multiple four-point assays, by factorial analysis, following an analysis of variance of all the responses obtained on several tissue strips.

## RESULTS AND DISCUSSION

Results of the various bioassays are presented in Table 1. The smallest peptide sequence related to β-LPH, with morphine-like activity in the assay described here, is the tetrapeptide III, H-Tyr-Gly-Gly-Phe-OH, in agreement with the data of Bradbury *et al.* (10). While its specific activity (potency) is *ca.* $1 \cdot 10^{-3}$ that of the next longest peptide, V, i.e., Met$^5$-enkephalin, the tetrapeptide III has full intrinsic activity.

Three peptides, VI, VII, and XVII, have strikingly higher potency than any other of the series reported here; the most potent substance in this series is β-endorphin, XVII, i.e., β-LPH-[61–91], a substance isolated earlier by several groups from extracts of the pituitary gland (3, 13) on the basis of its chemical behavior. Our finding of a statistically greater potency for β-endorphin (β-LPH-[61–91]) than for Met$^5$-enkephalin is at variance with the results recently reported by Cox *et al.* (4) using the same bioassay as used here. Greater potency of β-endorphin than that of α- and γ-endorphins as seen by us here is in keeping with earlier results (in Fig. 2 of ref. 5) using displacement of [$^3$H]etorphine from a synaptosomal preparation of rat brain and is also in agreement with the recent results and conclusions of Bradbury *et al.* (10) using a similar opiate-receptor binding assay.

The other two most potent substances in the series reported here are VI and VII, the two synthetic analogs of Met$^5$-enkephalin amidated at the COOH-terminal. Considering the overlapping of fiducial limits of the bioassays, the two amidated pentapeptides are statistically of similar potency as β-LPH-[61–91]. Similarly, the COOH-terminal amide of α-endorphin (XV) has increased potency when compared to that of the free acid form (XIV).

It is somewhat puzzling to observe (Table 1) that extending the peptide chain at the COOH-terminal end of the tetrapeptide III would produce a considerable (1000×) increase in potency as in V (Met$^5$-enkephalin), followed by a lowering of potency (½, ⅓) upon further extension (XIII, + 4 residues; XIV, + 11 residues; XVI, + 12 residues), but producing a new increase in potency (5×) upon more extension (XVII, + 26 residues). Before attempting to explain these variations of potency, it may be wise to ascertain their existence on a statistical basis in a large number of quantitative assays, in several laboratories.

Discussions have recently appeared in the literature (4, 11, 12, 17) emphasizing the requirement of a tyrosine as the $NH_2$-terminal for any peptide with opiate-like activity, in view of its similarity to the A-ring of morphine. The lower specific

Table 1. Comparative potencies, *M*, on an equimolar basis, with 95% fiducial limits, of synthetic peptides with morphinomimetic activity as tested in the myenteric plexus bioassay

| Code | Relation to β-LPH | Primary structure | *M* (lower–upper 95% fiducial limits). Met-enkephalin = 100 |
|---|---|---|---|
| I | β-LPH-[61-62] | H-Tyr-Gly-OH | 0 |
| II | β-LPH-[61-63] | H-————Gly-OH | 0 |
| III | β-LPH-[61-64] | H-————————Phe-OH | 0.1 |
| IV* | | H-————————————Leu-OH | 37 (24–53) |
| V* | β-LPH-[61-65] | H-————————————Met-OH | 100 |
| VI | | H-———————————— -$NH_2$ | 302 (179–649) |
| VII | | H-———————————— -NHEt | 246 (178–335) |
| VIII | | H-————————————Met(O)-OH | 20 (13–29) |
| IX | | (OMe)Tyr-————————Met-OH | 17 (9–27) |
| X | | Ac-———————————-OH | Divergence |
| XI | | H-Phe-Gly-Gly-Phe-Met-OH | 0.2 (0.1–0.3) |
| XII | | H-Tyr-——D-Ala-——- -OH | 4 (2–8) |
| XIII | β-LPH-[61-69] | H-————Gly———— -Thr-Ser-Glu-Lys-OH | 60 (38–85) |
| XIV* | β-LPH-[61-76] | H-———————— ————————Ser- -Gln-Thr-Pro-Leu-Val- -Thr-OH | 36 (19–53) |
| XV | | H-———————— ———————— — -$NH_2$ | 72 (47–102) |
| XVI* | β-LPH-[61-77] | H-———————— ———————— ———————— — -Leu-OH | 23 (11–37) |
| XVII | β-LPH-[61-91] | H-———————— ———————— ———————— ——— -Phe-Lys-Asn- -Ala-Ile-Val-Lys-Asn- -Ala-His-Lys-Lys-Gly- -Gln-OH | 450 (281–966) |

* Indicates peptides isolated from crude extracts based on a bioassay for morphinomimetic substances. IV*: Leu-enkephalin (whole brain), pig (1), calf (14). V*: Met-enkephalin (whole brain), pig (1), calf (14). XIV*: α-endorphin (hypothalamus-neurohypophysis) pig (2). XVI*: γ-endorphin (hypothalamus-neurohypophysis), pig (2).

activity of [(OMe)$Tyr^1$]-$Met^5$-enkephalin (IX) and [$Phe^1$]-$Met^5$-enkephalin (XI) when compared to that of the native peptide $Tyr^1$-$Met^5$-enkephalin is in keeping with these theoretical considerations. On the other hand, it must be emphasized that both analogs IX and XI with (OMe)$Tyr^1$ or $Phe^1NH_2$-terminals have full intrinsic activity. [Ac-$Tyr^1$]-$Met^5$-enkephalin (X) has low biological activity; the function relating its activity to log-dose is divergent from that obtained for $Met^5$-enkephalin in the same assay, thus precluding the calculation of a true potency for X. No evidence was obtained that either IX, X, XI, or XII would act as competitive antagonist of $Met^5$-enkephalin (V) or α-endorphin (XIV).

Though somewhat difficult to ascertain with this type of bioassay without a very large series of assays for statistical analysis, it appears that all peptides longer than $Tyr^{61}$-$Met^{65}$-OH (V) have longer duration of activity than the free acid form of the pentapeptide (V). This is also true for $Tyr^{61}$-$Met^{65}$-$NH_2$, (VI) and H-Tyr-Gly-D-Ala-Phe-Met-OH (XII). In our hands, β-endorphin (XVII), i.e., β-LPH-[61–91], has the longest duration of biological activity at equimolar concentrations. This is probably in keeping with its greater binding affinity to the synaptosomal opiate-receptors when compared to other morphinomimetic peptides (4, 5, 10). It is actually quite remarkable how β-endorphin (β-LPH-[61–91]) differs from all other morphinomimetic peptides when they are tested in the myenteric plexus bioassay at equimolar concentrations: all peptides longer than $Met^5$-enkephalin take slightly longer (than $Met^5$-enkephalin) to reach their maximal inhibitory effects; β-endorphin takes the longest; while they "wash out" rapidly (seconds), again in inverse relation to their size, β-endorphin requires several "washing outs" to remove its effects on the tissue incubated *in vitro*. Moreover, while the biological effects of $Met^5$-enkephalin and α-endorphin are readily reversed by naloxone, similar amounts of naloxone produce only partial reversal of the activity of β-endorphin: 5 to 10 times larger concentrations of naloxone are required to produce complete reversal of the biological activity of β-endorphin, with variations from one tissue preparation to another.

All the peptides with opiate-like activity (i.e., naloxone reversible) which have been isolated from whole brain, hypothalamus and neurohypophysis, or whole pituitary gland (see Table 1) have been shown to possess a primary structure identical to that of one of several fragments of the [61–91] COOH-terminus of β-LPH, in agreement with the hypothesis proposed in the first two notes reporting the isolation of the enkephalins (1) and of the endorphins (2). $Leu^5$-enkephalin, reported in extracts of pig brains (1) and calf brains (14), does not fit entirely that pattern, since no sequence of β-LPH has

been reported to possess Leu in the 65th position from the $NH_2$-terminal residue. Neither $Met^5$-enkephalin nor $Leu^5$-enkephalin has been observed, so far, in the various steps of the purification sequence that led to our isolation of several endorphins in large quantities from an extract of hypothalamus-neurohypophysis of pig brain origin. Perhaps this raises the question of the authentic presence of the pentapeptides in the living tissues; the ultimate enzymatic degradation product of the endorphins, with biological activity, $Met^5$-enkephalin could be the artefactual result of protracted chemical manipulations. In such a hypothesis, already proposed for the genesis of $\beta$-melanotropin (13), the same question could be asked about $\alpha$- and $\gamma$-endorphins; $\beta$-endorphin with its greatest potency would thus be the most favored candidate for a physiological role should it be unquestionably demonstrated to be available *in vivo*, in reasonable concentrations.

Terenius *et al.* have reported (15, 16) that fragments of adrenocorticotropin (ACTH) bind to synaptosomal opiate-receptors. In contradistinction to the results reported here with the fragments of $\beta$-LPH, we find that the fragments of ACTH have no opiate-like activity in the bioassay used here or, for those which reduce slightly the amplitude of the muscle twitch in the myenteric plexus preparation, that the effect is not sensitive to naloxone. Moreover, several of the ACTH fragments tested by us have actually naloxone-like activity, inhibiting or reversing the effects of $\alpha$-endorphin or normorphine.

It is becoming more and more apparent that availability of various "opiate-like" peptides is already leading to a remarkable pharmacological dissection of the mechanisms involved in the multiple biological activities which, until now, were considered as being mediated *en bloc* by "opiate receptors." Obviously, much information still has to be obtained about these opiate-like peptides before they can be accepted as genuine physiological carriers of information.

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