# β-ENDORPHIN: STRUCTURE-ACTIVITY RELATIONSHIPS IN THE GUINEA PIG ILEUM AND OPIATE RECEPTOR BINDING ASSAYS

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SUMMARY: The opiate activities of some derivatives and enzymatic digests of camel and human β-endorphin were determined in the guinea pig ileum and rat brain opiate receptor binding assays. Derivatives of β-endorphins altered within the amino-terminal five residues showed pronounced losses in activity. Anisylation of the C-terminal glutamic acid residue of $\beta_h$-endorphin produced only small reductions in activity. Chymotryptic digestion greatly weakened the opiate activities of $\beta_h$-endorphin, whereas carboxypeptidase A, tryptic and leucine aminopeptidase digests showed only small losses in potency. The C-terminus of β-endorphin appears to contribute little directly to opiate activity. Amino acid analysis and assay of the leucine aminopeptidase digests suggest that the larger potency of β-endorphin relative to Met-enkephalin may be a consequence of its greater resistance to exopeptidase attack.

Several peptides which are endogenous ligands for the opiate receptors of brain have been isolated. Two pentapeptides, named Met- and Leu-enkephalin, have been purified from porcine and bovine brain (1, 2). Li and Chung (3) reported the isolation and structure of a thirty-one amino acid peptide (β-endorphin) from the camel pituitary with potent opiate activities. The homologous untriakontapeptides have also been identified in the pituitary glands of the pig (4), sheep (5), and human (5, 6). α- and γ-Endorphins have been characterized from the pig neurohypophysis-hypothalamus (7). The amino acid sequences of Met-enkephalin, α- and γ-endorphins, and β-endorphins are contained within the sequence of pituitary β-LPH (8, 9)and correspond to β-LPH-(61-65), -(61-76), -(61-77), and -(61-91), respectively. Each of these peptides possess substantial morphine-like

Abbreviations: Met-enkephalin, methionine enkephalin; $\beta_c$-endorphin, camel β-endorphin; $\beta_h$-endorphin, human β-endorphin; $\beta_c$-MSH, camel β-melanotropin; $\beta_s$-LPH, sheep β-lipotropin; β-LPH-(61-65), -(61-76), etc., that peptide corresponding to residues (61-65), -(61-76), etc., of the sequence of β-lipotropin.

activities when measured in vitro as in the guinea pig ileum assay, or as specific binding to brain opiate receptors (1, 2, 6, 7). However, they display widely different opiate activities when measured in vivo. Met- and Leu-enkephalins and α-endorphin display weak transient analgesic action after intracranial injection in mice or rats (10-13). In contrast, β-endorphin has potent analgesic action when injected centrally (12) or intravenously (13).

Structure-activity relationships have shown the importance of the $Tyr^1$, $Phe^4$ and $Met^5$ or $Leu^5$ residues for the in vitro opiate activities of enkephalins (14-17). We have analyzed for the first time the structure-activity relationships of β-endorphin by guinea pig ileum and rat brain opiate receptor assays. Results of these studies are reported herein.

## Materials and Methods

$\beta_c$-Endorphin (18), $\beta_h$-endorphin (19) and $\beta_c$-MSH (20) were synthesized by the solid-phase method. $\beta_s$- and $\beta_h$-LPH were purified as previously described (8,9). $\beta_h$-LPH-(61-69) was one of the tryptic fragments (T-17) utilized in the amino acid sequence determination of $\beta_h$-LPH (9). Met-enkephalin was purchased from Bachem Laboratories (Marina del Reyes, Ca.).

Acetone-treated $\beta_h$-endorphin was prepared by reaction of 12.8 mg of the peptide in 50% aqueous acetone (20 ml) for 21 hr at 24°. Partition chromatography on Sephadex G-50 in 1-butanol-pyridine-0.6 M $NH_4OAc$ (5:3:10) gave essentially one peak with $R_f$ 0.48 ($R_f$ of $\beta_h$-endorphin, 0.37) and 9.1 mg of product. On paper electrophoresis (pH 3.7, 400 V, 4 hr) the substance behaved as a more acidic material ($R_f$ 0.48 relative to lysine as compared to $R_f$ 0.54 for β-endorphin). A sample heated in 0.1% acetic acid for 10 min at 100° liberated acetone. All these properties parallel the behavior of acetone-oxytocin whose structure (21) consists of an isopropylidene bridge between the N-terminal amino group and the nitrogen of the peptide bond between sequence positions 1 and 2.

(Anisylated-$Glu^{31}$)-$\beta_h$-endorphin was isolated as a by-product in the synthesis of $\beta_h$-endorphin (19) and was further purified by partition chromatography on Sephadex G-50 in 1-butanol-pyridine-0.1% acetic acid (5:3:11) in which it gave a $R_f$ of 0.53. Amino acid analysis, ultraviolet spectral analysis and peptide mapping indicated an anisylated glutamic acid residue (22) at the C-terminus.

(Homoserine$^5$)-$\beta_h$-endorphin was produced by dissolving 10 mg $\beta_h$-endorphin in 0.5 ml 70% formic acid followed by addition of 20 mg CNBr (Eastman Organic). After stirring at 24° for 24 hr in the dark, the solution

was diluted with a large volume of water and lyophilized. Peptide mapping and end-group analysis showed that no cleavage occurred. The Met-Thr bond has previously been shown to be resistant to CNBr cleavage in 70% formic acid (23). The amino acid analysis indicated that $Met^5$ was converted to homoserine by CNBr. Formic acid treated-$\beta_h$-endorphin was produced as above except no CNBr was added.

Carboxypeptidase A digestion of $\beta_h$-endorphin was done by dissolving 1.6 mg of $\beta_h$-endorphin in 0.1 ml, pH 8.5, Tris-HCl buffer and adding 0.05 mg carboxypeptidase A (Worthington). The suspension was incubated at 37° for 8 hr and the peptide recovered by lyophilization.

Tryptic digestion of $\beta_h$-endorphin was performed by dissolving 1.1 mg $\beta_h$-endorphin in 0.2 ml 0.2 M $NH_4$ OAc buffer (pH 8.0). Trypsin (Serva), 0.02 mg, was added and the solution incubated at 37° for 8 hr. The reaction was stopped by addition of 1.8 ml 0.05 N HCl. Chymotryptic digestion of $\beta_h$-endorphin was done as above except that chymotrypsin (Calbiochem) was used.

Leucine aminopeptidase digested peptides were prepared as follows: $\beta_c$-endorphin (0.34 mg) and Met-enkephalin (0.06 mg) were each dissolved in 0.1 ml pH 8.5 Tris buffer. Leucine aminopeptidase (0.007 mg, Worthington) was added to both samples, and the solutions incubated for 2 hr at 37°. The digests were acidified to pH 3, lyophilized, and portions removed for amino acid analysis.

Narcotic agonist activities were measured from the depression of electrically-stimulated contractions of the guinea pig ileum prepared as previously described (24). Briefly, a 2-3 cm segment of ileum was mounted between internal and external platinum electrodes in a 25 ml bath containing Kreb's solution (with 125 nM diphenyamine as antihistamine) at 37° and aerated with 95% $O_2$/5% $CO_2$. Tension changes evoked by rectilinear electrical pulses (0.4 msec duration, 0.1 Hz, 80 V) were recorded by a Grass Isometric Pressure Transducer (tension, 2 g) coupled to a model 7 Grass Polygraph. Peptides and normorphine were assayed on two ileum preparations at 3-5 concentrations.

Opioid activity was also assayed as the inhibition of stereospecific [$^3$H]-morphine (New England Nuclear, 10 Ci/mmole) binding to lysed rat synaptic plasma membranes prepared essentially as described (25). Synaptic plasma membranes (200 μg protein) were preincubated in 2 ml sodium-free HEPES buffer (26 mM, pH 7.5) at 25° for 10 min with peptides, morphine sulfate or dextrorphan. Next [$^3$H]-morphine was added (4 nM) and the incubation continued for 15 min. Assays were terminated by filtration followed by three 4 ml rinses with ice-cold buffer on glass fiber filters (Whatman GF/c). Filters were dried and counted by liquid scintillation spectrophotometry. Stereospecific opiate binding, the difference between [$^3$H]-morphine binding in the presence of dextrorphan ($1 \times 10^{-6}$M) and morphine sulfate ($1 \times 10^{-6}$M) was 45% of the total binding. Peptides were assayed in quadruplicate; standard errors of the mean c.p.m. were always less than 6%.

Potencies of peptides in both assays were calculated as the ratio of the $IC_{50}$ (inhibiting molar concentration - 50%) to that of $\beta_c$-endorphin.

Table I

Opiate Activities of $\beta_c$-Endorphin and Related Peptides

| Preparation | Relative Potency* in | |
|---|---|---|
| | Guinea pig ileum | Brain opiate receptor binding |
| $\beta_c$-endorphin | 1.00 | 1.00 |
| $\beta_h$-endorphin | 1.00 | 1.00 |
| Met-enkephalin | 0.25 | 0.34 |
| Normorphine | 0.65 | --- |
| $\beta_s$-LPH | < 0.01 | < 0.01 |
| $\beta_h$-LPH | < 0.01 | < 0.01 |
| $\beta_c$-MSH | < 0.01 | < 0.01 |
| $\beta_h$-LPH-(61-69) | 0.20 | 0.26 |
| Acetone treated -$\beta_h$-endorphin | 0.01 | 0.02 |
| (anisylated-$Glu^{31}$)-$\beta_h$-endorphin | 0.76 | 0.75 |
| Formic acid-treated-$\beta_h$-endorphin | 1.00 | 1.00 |
| (5-homoserine)-$\beta_h$-endorphin | < 0.01 | < 0.01 |
| $\beta_h$-endorphin: Carboxypeptidase A digest | 1.00 | 1.00 |
| $\beta_h$-endorphin: Chymotryptic digest | 0.13 | 0.03 |
| $\beta_h$-endorphin: Tryptic digest | 0.71 | 0.61 |
| $\beta_c$-endorphin: Leucine aminopeptidase digest | 0.97 | 0.93 |
| Met-enkephalin: Leucine aminopeptidase digest | < 0.01 | < 0.01 |

* Potency of $\beta_c$-endorphin is defined as 1.00. Other preparations were compared to $\beta_c$-endorphin standard on a molar basis. $IC_{50}$ of $\beta_c$-endorphin: 2.5-8.0 x $10^{-8}$ M in the guinea pig ileum assay; 1.2-3.7 x $10^{-7}$M in the rat brain opiate receptor binding assay.

## Results and Discussion

Opiate activities of the various peptides and enzymatic digests relative to $\beta_c$-endorphin as measured in the guinea pig ileum and rat brain receptor assays are summarized in Table I. In the ileum assay $\beta_c$- and $\beta_h$-endorphins were each

55% more potent than normorphine, and 4-times more active than Met-enkephalin. Similarly in the opiate receptor assay, Met-enkephalin had reduced potency (34%) compared to β-endorphins. $\beta_h$-LPH-(61-69), corresponding to $\beta_h$-endorphin-(1-9), was somewhat less potent than Met-enkephalin, as previously observed (11, 26). In contrast, $\beta_s$- and $\beta_h$-LPH and $\beta_c$-MSH showed no activity in either assay, even when tested at the high concentration of $5.0 \times 10^{-6}$M (see also 26). $\beta_s$-LPH and $\beta_c$-MSH also failed to show naloxone-like antagonism to normorphine or $\beta_c$-endorphin in the ileum (data not shown).

Acetone treated $\beta_h$-endorphin, cyclized between $Tyr^1$-$Gly^2$ by a bridge which incorporates the amino group of $Tyr^1$, displayed only 1% (ileum) and 2% (opiate receptor assay) of $\beta_h$-endorphin activity. $\beta_h$-Endorphin, anisylated at its C-terminal glutamic acid residue, showed a modest decline in opiate activities. Whereas its amino-terminal Tyr may be essential, the carboxyl-terminal glutamic acid residue appears to contribute little directly to the interaction of $\beta_h$-endorphin with opiate receptors. In addition, conversion of $Met^5$ to 5- homoserine abolished the opiate activities of $\beta_h$-endorphin.

Hydrolysis of $\beta_h$-endorphin with chymotrypsin diminished considerably its potency in both assays. Chymotrypsin can be expected to cleave $\beta_h$-endorphin at $Phe^4$ as well as at other aromatic residues. On the other hand, digestion of $\beta_h$-endorphin with enzymes having no specificity within the amino-terminal five residues had no (carboxypeptidase A) or limited (trypsin) effects on in vitro opiate activities. Carboxypeptidase A has been shown to cleave the $Gly^{90}$-$Gln^{91}$ dipeptide from porcine LPH-(61-91) (4), indicating again the non-essential nature of these residues for opiate action. Trypsin digestion, however, reduced somewhat $\beta_h$-endorphin potency in ileum (71%) and opiate receptor binding (61%). Interestingly, these reduced activities were still greater than the activities of the nonapeptide $\beta_h$-LPH-(61-69), which can be expected to be produced by the action of trypsin on

$\beta_h$-endorphin. Therefore, the trypsin digest contains undigested $\beta_h$-endorphin or some other tryptic fragment, or fragments in combination, having opiate activity.

$\beta_c$-Endorphin was susceptible to very limited hydrolysis with leucine aminopeptidase as indicated by liberation of only trace amounts of tyrosine as detected by amino acid analysis. As shown in Table 1, this enzyme digest of $\beta_c$-endorphin showed only slight dimunition of opiate activities; in contrast, Met-enkephalin, which showed stoichiometric hydrolysis of the $Tyr^1$-$Gly^2$ bond, lost all activity after leucine aminopeptidase digestion. Therefore, the amino terminal residue of $\beta_c$-endorphin appears to differ from that of Met-enkephalin by being protected from exopeptidase attack. Enzymes in brain and plasma are known to rapidly inactivate enkephalins (27), but digest less readily the more potent [ D-$Ala^2$] -Met-enkepahlin (28). The greater analgesic potency of β-endorphin compared to Met-enkephalin in vivo may result in part from its greater resistance, especially at its amino-terminus, to enzymic digestion.

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## References

1. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. and Morris, H. R. (1975) Nature 258, 577-579,
2. Simantov, R. and Snyder, J. H. (1976) Proc. Natl. Acad. Sci. USA 73, 2515-2519.
3. Li, C. H. and Chung, D. (1976) Proc. Natl. Acad. Sci. USA 73, 1145-1148.
4. Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M. and Hulme, C. E. (1976) Nature 260, 793-795.
5. Chrétien, M., Benjannet, S., Dragon, N., Seidah, N. G. and Lis, M. (1976) Biochem. Biophys. Res. Commun. 72, 472-478.
6. Li, C. H., Chung, D. and Doneen, B. A. (1976) Biochem. Biophys. Res. Commun. 72, 1542-1547.
7. Guillemin, R., Ling, N. and Burgus, R. (1976) Séances Acad. Sci. Ser. D. 282, 783-785.

8. Li, C. H., Barnafi, L., Chrétien, M. and Chung, D. (1966) Excerpta Med. Int. Congr. Ser., 111, 349-364.
9. Li, C. H. and Chung, D. (1976) Nature 260, 622-624.
10. Belluzzi, J. D., Grant, N., Garsky, V., Sarantakis, D., Wise, C. O. and Stein, L. (1976) Nature 260, 626-627.
11. Gráf, L., Romai, A. Z., Bajusz, S., Cseh, G. and Székely, J. I. (1976) FEBS Letters 64, 181-184.
12. Loh, H. H., Tseng, L. F., Wei, E. and Li, C. H. (1976) Proc. Natl. Acad. Sci. USA 73, 2895-2898.
13. Tseng, L. F., Loh, H. H. and Li, C. H. (1976) Nature 263, 239-240.
14. Chang, J-K., Fong, B. T. W., Pert, A. and Pert, C. B. (1976) Life Sci. 18, 1473-1482.
15. Day, A. R., Luján, M., Dewey, W. L., Harris, L. S., Redding, J. A. and Freer, R. J. (1976) Res. Commun. Chem. Path. Pharmacol. 14, 597-603.
16. Terenius, L., Wohlström, A., Lindeberg, G., Karlsson, S. and Ragnarsson, U. (1976) Biochem. Biophys. Res. Commun. 71, 175-179.
17. Ling, N. and Guillemin, R. (1976) Proc. Natl. Acad. Sci. USA 73, 3308-3310.
18. Li, C. H., Lemaire, S., Yamashiro, D. and Doneen, B. A. (1976) Biochem. Biophys. Res. Commun. 71, 19-25.
19. Li, C. H., Yamashiro, D., Tseng, L-F., and Loh, H. H. (1976) J. Med. Chem. (in press).
20. Li, C. H., Yamashiro, D. and Lemaire, S. (1975) Biochem. 14, 953-956.
21. Hruby, V. J., Yamashiro, D. and du Vigneaud, V. (1968) J. Amer. Chem. Soc. 90, 7106-7110.
22. Feinberg, R. S. and Merrifield, R. B. (1975) J. Amer. Chem. Soc. 97, 3485-3496.
23. Schroeder, W. A., Shelton, J. B. and Shelton, J. R. (1969) Arch. Biochem. Biophys. 130, 551-556.
24. Kosterlitz, H. W., Lydon, R. T. and Watt, A. F. (1970) Brit. J. Pharmacol 39, 398-413.
25. Whittaker, V. P. and Sheridan, M. N. (1965) J. Neurochem. 12, 363-372.
26. Cox, B. M., Goldstein, A. and Li, C. H. (1976) Proc. Natl. Acad. Sci. USA 73, 1821-1823.
27. Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, C. F. C. (1976) Nature 262, 782.
28. Pert, C. B., Pert, A., Chang, J-K., and Fong, B. T. W. (1976) Science 194, 330-332.