

## Influence of *N*-terminal acetylation and *C*-terminal proteolysis on the analgesic activity of $\beta$ -endorphin

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Removal of one, two and four amino-acid residues from the *C*-terminus of  $\beta$ -endorphin ('lipotropin C-Fragment', lipotropin residues 61–91) led to the formation of peptides with progressively decreased analgesic potency; there was no change in the persistence of the analgesic effects. The four *C*-terminal residues are thus important for the activity of  $\beta$ -endorphin, but not for the duration of action. Removal of eight amino-acid residues from the *N*-terminus provided a peptide that had no specific affinity for brain opiate receptors *in vitro* and was devoid of analgesic properties. The *N*-terminal sequence of  $\beta$ -endorphin is therefore necessary for the production of analgesia, whereas the *C*-terminal residues confer potency. The *N*<sup>α</sup>-acetyl form of  $\beta$ -endorphin had no specific affinity for brain opiate receptors *in vitro* and possessed no significant analgesic properties. Since lipotropin C'-Fragment (lipotropin residues 61–87) and the *N*<sup>α</sup>-acetyl derivative of  $\beta$ -endorphin occur naturally in brain and pituitary and are only weakly active or inactive as opiates, it is suggested that proteolysis at the *C*-terminus and acetylation at the *N*-terminus of  $\beta$ -endorphin may constitute physiological mechanisms for inactivation of this potent analgesic peptide.

The pentapeptide, [methionine<sup>5</sup>]enkephalin, was shown to exist in porcine brain and act as a ligand of the opiate receptor by Hughes *et al.* (1975). It was found to behave like morphine in peripheral opiate assays, but it had only a weak and transient analgesic action when administered intraventricularly in the rat (Beluzzi *et al.*, 1976), cat (Feldberg & Smyth, 1976) or mouse (Büscher *et al.*, 1976). With the elucidation of the primary structure, it became clear that [methionine<sup>5</sup>]enkephalin corresponds to five residues at the *N*-terminus of  $\beta$ -endorphin, a 31-residue peptide first isolated from porcine pituitary glands (Bradbury *et al.*, 1975).

$\beta$ -Endorphin, originally called the C-Fragment of lipotropin (lipotropin residues 61–91), shares with

enkephalin certain morphine-like actions in opiate assays *in vitro*, but it appears to have a higher affinity for brain opiate receptors (Bradbury *et al.*, 1976) and possesses strong analgesic properties (Feldberg & Smyth, 1976, 1977; Bradbury *et al.*, 1976; Van Ree *et al.*, 1976; Loh *et al.*, 1976). The relatively high analgesic potency and long duration of action of  $\beta$ -endorphin may be attributed to the peptide chain that extends from the *N*-terminal region, but it is notable that lipotropin C'-Fragment (lipotropin residues 61–87), which lacks four residues from the *C*-terminus of  $\beta$ -endorphin, has little analgesic potency (Feldberg & Smyth, 1977; Geisow *et al.*, 1977). In the present studies, carried out in the rat, an extensive investigation is reported on the

|   | 61  |     | 69  |     | 87  |           | 91                  |
|---|-----|-----|-----|-----|-----|-----------|---------------------|
| Lipotropin residues 61–91 ( $\beta$ -endorphin)                     | Tyr | Gly | Gly | Phe | Met | —————     | His Lys Lys Gly Gln |
| Lipotropin residues 61–89   | Tyr | Gly | Gly | Phe | Met | —————     | His Lys Lys         |
| Lipotropin residues 61–87 (C'-Fragment)                             | Tyr | Gly | Gly | Phe | Met | —————     | His                 |
| Lipotropin residues 69–91   |     |     |     |     |     | Lys ————— | His Lys Lys Gly Gln |
| Lipotropin residues 61–65<br>([methionine <sup>5</sup> ]enkephalin) | Tyr | Gly | Gly | Phe | Met |           |                     |

Fig. 1. Partial amino-acid sequence of  $\beta$ -endorphin and structurally related peptides

analgesic activity of  $\beta$ -endorphin (in terms of potency and duration) and detailed comparison is made between its properties and those of fragments that lack one (lipotropin residues 61–90), two (lipotropin residues 61–89) or four (lipotropin residues 61–87) of the C-terminal amino acids (Fig. 1). The results are discussed in relation to affinity for brain opiate receptors and resistance to enzymic degradation. For comparison, the properties of an enkephalin analogue protected against enzymic degradation, [D-Ala<sup>2</sup>]enkephalinamide, are also presented.

The N-terminal pentapeptide of  $\beta$ -endorphin ([methionine<sup>5</sup>]enkephalin) is able to produce a number of opioid activities. We have investigated whether the N-terminal region is essential for the activity of  $\beta$ -endorphin by examining the opiate properties *in vitro* and *in vivo* of a 23-residue peptide that lacks the eight N-terminal amino acids (lipotropin residues 69–91). In addition the N<sup>α</sup>-acetyl derivative of  $\beta$ -endorphin has been tested for opiate properties. The results of these studies demonstrate that modification of  $\beta$ -endorphin in the region of either the N- or C-terminus severe loss of analgesic activity.

## Materials and methods

### Surgery

Male Sprague–Dawley rats (250–300 g) were used. Lateral ventricular guide cannulae were chronically implanted with the tip immediately above the lateral ventricle. One week later, peptides or morphine were injected in a saline vehicle (5  $\mu$ l) through an internal cannula penetrating 1 mm deeper than the guide cannula into the lateral ventricle. The internal cannula was attached to a fine polythene tube containing the drug, which was connected to a Hamilton syringe. Injections were performed without anaesthetic while the rats were gently restrained.

### Analgesia

Rats were placed in specially designed cylindrical or square-tube Perspex restrainers sufficiently narrow to prevent the animal from turning around and with numerous ventilation holes and a hole through which the tail protruded. Animals were placed in restrainers 5 min before baseline tail-flick latency determination. Little distress ensued.

The tail-flick apparatus consisted of a 2500 W projector lamp mounted 33 mm above a groove into which the tail was placed. Switching the lamp on triggered a decimal counter indicating time in tenths of a second. When the animal flicked its tail the lamp and counter were switched off immediately. Three baseline tail-flick latencies were determined at 30 s intervals. After intraventricular injection, tail-flick

latencies were determined at 3, 10 and 30 min and in time-course studies at 30 min intervals thereafter. If the rats did not move their tails within 10 s the lamp was switched off to prevent tissue damage. No animal was used in more than two series of tests and the second test was always carried out 1 or more weeks after the first. In control experiments no tolerance to the analgesic effect of intraventricular  $\beta$ -endorphin or to high doses of systemic morphine was observed when the tests were separated by a week.

The tail-flick assay employed in these studies is widely used for the determination of analgesia. It is important to note that whereas the inhibitory motor effects commonly induced by opiate substances might interfere with tail-flick response, particularly at high doses, other drugs that cause muscular rigidity (for example, neuroleptics) do not increase tail-flick latency.

### Determination of affinity for brain opiate receptors

Binding studies were performed as described previously (Bradbury *et al.*, 1976). [<sup>3</sup>H]Naloxone (New England Nuclear; sp. radioactivity 20 Ci·mmol<sup>-1</sup>, 1 nM) was displaced by appropriate concentrations of unlabelled peptides from synaptosomal membranes in 50 mM-Tris/HCl (pH 7.4) and 100 mM-NaCl. Incubations were carried out at 30°C for 15 min.

### Materials

$\beta$ -Endorphin (lipotropin residues 61–91), lipotropin C'-Fragment (lipotropin residues 61–87) and their N<sup>α</sup>-acetyl derivatives were isolated in homogeneous form from porcine pituitary (Smyth *et al.*, 1978, 1979). The fragments comprising residues 61–89 and 61–90 of lipotropin were obtained by digestion of  $\beta$ -endorphin with carboxypeptidase A (Geisow *et al.*, 1977). The 23-residue peptide lipotropin residues 69–91 was prepared by digestion of  $\beta$ -endorphin (1  $\mu$ mol) with 5% of its weight of *Staphylococcus aureus* proteinase (Miles Laboratories) at 37°C for 16 h in 1 ml of 0.1 M-potassium phosphate (pH 7.4). The products were separated by gel filtration on a column (2 cm  $\times$  150 cm) of Sephadex G-50 suspended in 50% acetic acid. Two peptides detected by analysis of portions with ninhydrin were obtained and amino-acid analysis verified that the material in the less-retarded peak corresponded to lipotropin residues 69–91.

## Results

### Analgesic properties of $\beta$ -endorphin, lipotropin C'-Fragment (lipotropin residues 61–87) and related peptides

$\beta$ -Endorphin produced analgesia at all doses tested. As little as 0.1 nmol of the peptide produced a

2-fold increase in tail-flick latency measured 30 min after intracerebroventricular injection (Fig. 2) and 3 nmol caused all animals to reach the cut-off latency of 10 s. Removal of the C-terminal glutamine residue from  $\beta$ -endorphin, as shown by the properties of lipotropin residues 61–90, resulted in a considerable loss of potency at the 1 nmol dose, but significant analgesia still ensued. The 29-residue peptide lipotropin residues 61–89, which lacked the

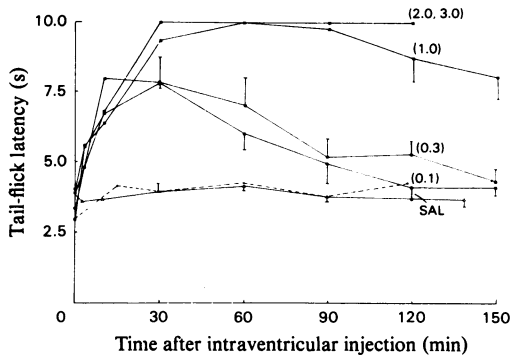


Fig. 2. Time course of analgesia produced by  $\beta$ -endorphin (lipotropin residues 61–91) in the rat. Doses in nmol are shown in parentheses, for four to seven animals per dose (bars represent S.E.M.). A group of five saline injected control tests is shown (SAL). The lack of effect of 10 nmol of  $N^{\alpha}$ -acetyl- $\beta$ -endorphin in four animals is shown by the broken line. The tail-flick latencies obtained with 20 nmol of  $N^{\alpha}$ -acetyl- $\beta$ -endorphin were not significantly different from the saline injected group.

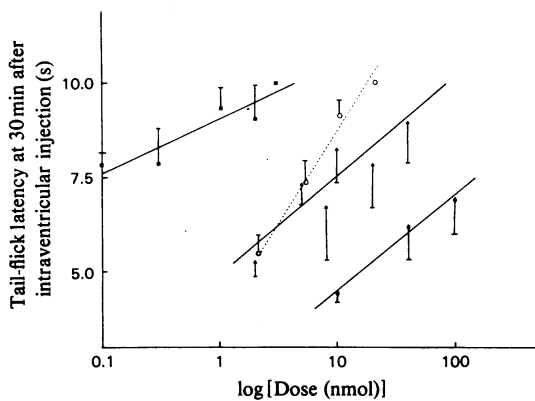


Fig. 3. Dose-response curves for  $\beta$ -endorphin (lipotropin residues 61–91) (■), lipotropin residues 61–89 (▲), lipotropin residues 61–87 (◆) and morphine sulphate (○)

The peptides and morphine were administered intraventricularly. Most points represent the mean values obtained for five rats, group sizes ranging from four to nine. Bars represent S.E.M., with some omitted for clarity.

two C-terminal amino acids of  $\beta$ -endorphin, was almost ineffective at a dose of 3 nmol; its dose-response curve showed an essentially parallel shift to the right, representing about a 100-fold decrease in potency. The dose-response curve of lipotropin C'-Fragment (lipotropin residues 61–87) was displaced further, representing a potency approx. 500 times less than that of  $\beta$ -endorphin (Fig. 3).

Although extensive behavioural observations were not carried out, morphine-like effects were noted at higher doses of all the peptides. 'Wet-dog' shakes were observed within 5 min of injection, the effects diminishing as analgesia developed. Later, rigidity and Straub tail (stiff, extended tail) developed and 90 min or more after peptide injection mild hyperactivity and stereotyped chewing movements were exhibited and continued after the animals were returned to their home cages.

The analgesic and cataleptic effects produced by  $\beta$ -endorphin (1 nmol) were reversed by 2.5  $\mu$ mol of naloxone/kg body wt. (intraperitoneal injection). In groups of four animals, 0.1, 0.5 and 3.0 nmol of  $\beta$ -endorphin produced at 30 min dose-response curves similar to that shown in Fig. 3. After intraventricular administration of naloxone (15 nmol/5  $\mu$ l) tail-flick latencies measured 5 min later decreased by 2 s at each dose, producing a parallel downward shift of the dose-response curve.

#### Study of affinity of lipotropin residues 69–91 for brain opiate receptors in vitro and examination for analgesic properties

Whereas  $\beta$ -endorphin at a concentration of 4.8 nM displaced 50% of bound naloxone, no displacement by lipotropin residues 69–91 was observed at a concentration of 30  $\mu$ M. Thus under these conditions there was no indication that lipotropin residues 69–91, a peptide derived from  $\beta$ -endorphin but lacking the enkephalin sequence at its N-terminus, has a specific affinity for the opiate receptor. The 23-residue peptide exhibited no analgesic effects at doses of 5 and 10 nmol. Further peptide was not available for the testing of high doses.

#### Analgesic action of [D-Ala<sup>2</sup>]enkephalinamide

[D-Ala<sup>2</sup>]Enkephalinamide produced significant analgesia. Unlike morphine and the above peptides, its peak effect occurred at 10 min rather than at 30 min (see Fig. 4); at 10 min its dose-response curve was similar to that shown for lipotropin residues 61–89. The dose-response curve of morphine sulphate (Fig. 3), shown for comparison, appeared slightly steeper than the dose-response curves of the peptides.

#### Time-course studies

The time-course of the analgesic effects of  $\beta$ -endorphin (lipotropin residues 61–91), lipotropin

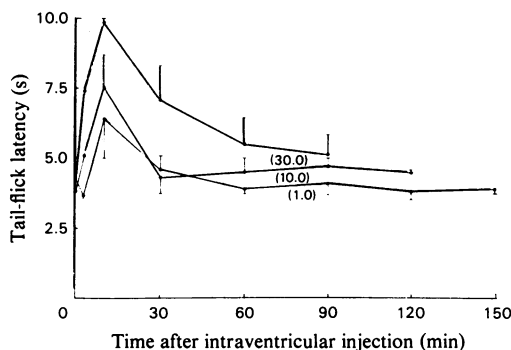


Fig. 4. Time course of [D-Ala<sup>2</sup>]enkephalinamide-induced analgesia

Doses (nmol) are shown in parentheses: results are means for five to seven animals per dose. Bars represent S.E.M.

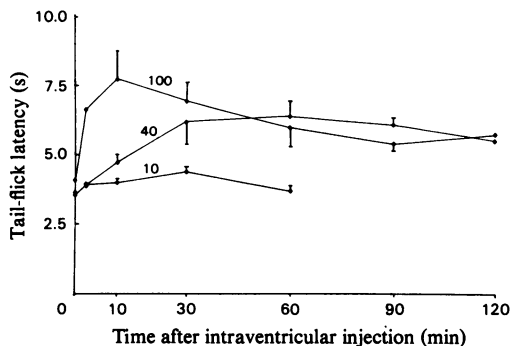


Fig. 5. Time course of analgesia induced by lipotropin C'-Fragment (lipotropin residues 61-87)

The doses (in nmol) are indicated against each series of tests. Bars represent S.E.M.

C'-Fragment (lipotropin residues 61-87) and [D-Ala<sup>2</sup>]enkephalinamide are shown in Figs. 2, 4, 5 and 6; also included is a control group of saline-injected animals. At most doses the analgesic effects of the larger peptides took at least 30 min to develop maximally, whereas [D-Ala<sup>2</sup>]enkephalinamide produced its greatest effect within 10 min. Fig. 6 shows the time course of analgesia of the different peptides at doses selected to produce the same maximum effect. The duration of action of the C'-Fragment (lipotropin residues 61-87), the 30-residue peptide lipotropin residues 61-90 and  $\beta$ -endorphin (lipotropin residues 61-91) were similar, tail-flick latencies remaining increased for at least 90 min. In contrast the analgesic action of [D-Ala<sup>2</sup>]enkephalinamide had ceased by 30 min. The analgesic effects of morphine followed a similar time course to those of the larger peptides, with peak effects taking 30 min to develop.

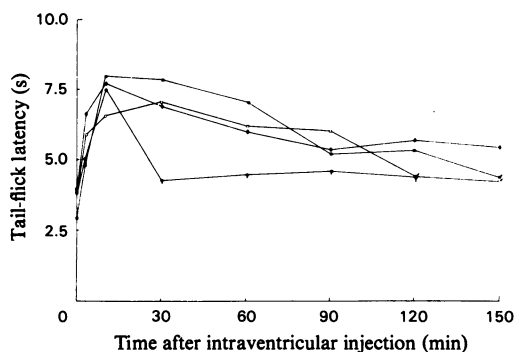


Fig. 6. Comparison of duration of action of peptides at doses selected to produce a similar maximum effect. Doses were as follows: lipoprotein residues 61-91 (■), 0.3 nmol; lipotropin residues 61-91 (□), 1.0 nmol; lipotropin residues 61-87 (◆), 100 nmol; [D-Ala<sup>2</sup>]enkephalinamide (●), 10 nmol. Tail-flick latencies indicated with a tagged point are not significantly different from baseline; 4-9 animals per group.

#### Properties of *N*<sup>α</sup>-acetyl- $\beta$ -endorphin

At concentrations of 50  $\mu$ M, *N*<sup>α</sup>-acetyl- $\beta$ -endorphin gave no significant displacement of [<sup>3</sup>H]naloxone from brain synaptosomal membranes *in vitro*, whereas the IC<sub>50</sub> value of  $\beta$ -endorphin was found to be 4.8 nM. Thus the acetylated derivative did not appear to bind specifically to the opiate receptor. The *N*<sup>α</sup>-acetyl derivative of  $\beta$ -endorphin was found to have no significant analgesic activity when tested in the rat at doses 100 and 200 times greater than a threshold dose of  $\beta$ -endorphin (Fig. 2).

#### Discussion

It is well known that the rank order of potency of certain opiates differs in different assays and it is generally accepted that this reflects the presence of different types of opiate receptor. In the guinea-pig ileum assay, for example, each of the naturally occurring peptides shown in Fig. 1 ( $\beta$ -endorphin, lipotropin C'-Fragment and [methionine<sup>5</sup>]enkephalin) exhibits a similar potency (Waterfield *et al.*, 1977); but with respect to the production of analgesia,  $\beta$ -endorphin is outstandingly active.

It has been suggested that the high potency of  $\beta$ -endorphin as an analgesic agent is conferred on the peptide by its 'address' sequence of amino acids, the address region being formed from the C-terminal residues, which potentiate the specific binding affinity between the *N*-terminal 'message' region and the complementary  $\beta$ -endorphin receptor (Smyth, 1980). The present study delineates some of the

structural requirements of the address sequence necessary for the high potency of  $\beta$ -endorphin as an analgesic agent. In addition it is demonstrated that the address sequence alone is unable to initiate an analgesic response.

$\beta$ -Endorphin was found to have potent analgesic properties when administered intraventricularly, in agreement with the results of several groups (see the Introduction for references) and the analgesic and behavioural effects were rapidly reversed after intraperitoneal or intraventricular naloxone administration, indicating an interaction with opiate receptors. It is notable that the dose-response curve for morphine was found to have a steeper slope than those of the peptides, which suggests that the alkaloid may act on a different population of opiate receptors. Such a possibility has been discussed by Lord *et al.* (1977), but more extensive investigation of dose and effect would be required to confirm that interpretation.

#### *The role of the C-terminus of $\beta$ -endorphin*

The present results show that removal of C-terminal amino acids from  $\beta$ -endorphin leads to a marked decrease in analgesic activity. Thus lipotropin C'-Fragment (lipotropin residues 61-87), which lacks the C-terminal tetrapeptide of  $\beta$ -endorphin, was approx. 500 times less potent (Figs. 3 and 6). When given in sufficient quantity to produce analgesia, however, the C'-Fragment exhibited analgesic effects that had a similar duration to those of  $\beta$ -endorphin (Fig. 6). This suggests that removal of the C-terminal amino acids does not cause loss of activity by rendering the molecule more readily destroyed by enzymes, a view supported by studies *in vitro* which have demonstrated that the C'-Fragment, like  $\beta$ -endorphin, is highly resistant to degradation by aminopeptidase enzymes (Austen & Smyth, 1977). The role of the C-terminal tetrapeptide would therefore seem to be to potentiate the interaction of  $\beta$ -endorphin with its complementary brain opiate receptors. This is in agreement with binding studies with rat brain membranes, which demonstrated that lipotropin residues 61-87 has a 40-fold lower affinity in displacing [ $^3$ H]naloxone from the receptors *in vitro* (Bradbury *et al.*, 1976).

#### *The role of the N-terminus of $\beta$ -endorphin*

The N-terminal region of  $\beta$ -endorphin contains the pentapeptide sequence of enkephalin, which itself

has morphine-like properties. It seems likely, therefore, that the N-terminus of  $\beta$ -endorphin provides a binding site for the analgesic receptors, whereas the remaining section of the peptide chain either enhances the affinity of the N-terminal region for the receptor or constitutes a second binding site. In addition the length of the peptide chain appears to protect the molecule from enzymic degradation (Austen *et al.*, 1979). The absence of activity in the truncated  $\beta$ -endorphin (lipotropin residues 69-91) and in the N $^{\alpha}$ -acetyl derivative of  $\beta$ -endorphin indicates that if there is a C-terminal receptor, then activation of that receptor does not lead to the production of analgesia. The evidence supports a view that the C-terminal tetrapeptide, though remote from the N-terminus in the linear sequence, potentiates the activity of  $\beta$ -endorphin by enhancing the affinity of its N-terminal region for the analgesic receptors.

The lack of potency but long duration of action of lipotropin residues 61-87 suggests that protection of the enkephalin sequence from enzymic degradation has increased the duration of action but not the potency. Previous results with a protected pentapeptide, [*N*-methyl-Tyr $^1$ ]enkephalinamide, are consistent with this observation (Bradbury *et al.*, 1977). However, while [*D*-Ala $^2$ ]enkephalinamide has an increased duration of action relative to the transient effects of enkephalin, it also appears to be a little more potent without having a significantly increased binding affinity for the opiate receptors (Pert *et al.*, 1976). This could indicate that factors in addition to affinity for a receptor may influence potency. It is possible, for example, that the agonists may vary in their transport properties (ability to penetrate the receptor) or having bound may possess different intrinsic efficacies. The *D*-alanine $^2$ - and *N*-amide modifications would appear to enhance the potency of enkephalin, albeit mildly, by affecting one of these parameters. It is also of interest that although the *D*-alanine $^2$ -modified peptide is relatively resistant to enzymic degradation and has a longer lasting analgesic action than enkephalin, its effects are less persistent than those of  $\beta$ -endorphin (Fig. 6). Other stabilized peptides have been reported (Walker *et al.*, 1977), but their properties have not been compared with those of the longer peptides.

The present results show that the potent analgesic activity of  $\beta$ -endorphin is severely impaired by loss of the four C-terminal residues or by acetylation at the N-terminus (Table 1). Since the peptides formed

Table 1. Analgesic activity of endogenous fragments of lipotropin

| Peptide                                   | Residue position in lipotropin | Relative potency in the rat |
|---|--------------------------------|-----------------------------|
| $\beta$ -Endorphin                        | 61-91                          | 500                         |
| N $^{\alpha}$ -Acetyl- $\beta$ -endorphin | (Ac) 61-91                     | <2.5                        |
| C'-Fragment                               | 61-87                          | 1                           |

by these molecular alterations occur naturally in pituitary and brain (Smyth *et al.*, 1979; Zakarian & Smyth, 1979), it seems likely that the reactions involved may contribute to physiological mechanisms for maintaining normal levels of opiate activity in the central nervous system.

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