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EVIDENCE FOR AN OPIATE-INACTIVE N-ACETYLATED DERIVATIVE OF LEUCINE-ENKEPHALIN IN THE RAT NEUROINTERMEDIATE PITUITARY

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SUMMARY: By use of a radioimmunoassay in combination with a variety of chromatographic separation procedures, evidence is provided for the existence of N-acetyl-leucine-enkephalin in the neurointermediate pituitary of the rat. N-acetyl-leucine-enkephalin accounts for ca. 20-25% of total leucine-enkephalin immunoreactivity. In the guinea-pig ileum bioassay, N-acetyl-leucine-enkephalin was found to be virtually opiate-inactive. This finding indicates that the process of N-acetylation in the rat pituitary is not restricted to ß-endorphin and may be of general importance as a mechanism for the modification and/or inactivation of opioid peptides.

In contrast to the neurointermediate pituitary, no N-acetylated derivative of leucine-enkephalin could be detected in the hypothalamus, striatum, midbrain and pons-medulla of rat brain.

N-acetylated forms of ß-endorphin (C-fragment, ß-lipotropin$_{61-91}$) and C'-fragment (ß-lipotropin$_{61-87}$) have recently been isolated from porcine pituitaries (1). These N-acetylated derivative, in which the N-acetyl-group is localized at the N-terminal tyrosine residue of the peptides, are virtually devoid of opiate activity in a variety of test systems (1).

Within the rat intermediate pituitary, a substantial amount of immunoreactive (ir-) ß-endorphin (comprised of C- and C'-fragment) was found to be N-acetylated (2). We have further recently provided direct evidence for the occurrence of N-acetylation of ir-ß-endorphin in the rat intermediate pituitary by the use of an in vitro biosynthesis technique: More than 70% of the newly synthesized ir-ß-endorphin was shown to subsequently undergo N-acetylation (3).

The present paper investigates the possibility as to whether the opioid pentapeptide leucine-enkephalin, which belongs to a peptide

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system differing from that of ß-endorphin (4,5), might also possess an N-acetylated opiate-inactive counterpart in the pituitary or brain of the rat.

METHODS AND MATERIALS

Extraction procedure

Male Sprague-Dawley rats (200-220 g) were decapitated, their pituitaries divided in situ, into anterior and neurointermediate lobes and the brains dissected according to Glowinski and Iversen (6). 50 neurointermediate pituitaries (1 n. p. $\cong$ 1.2 $\pm$ 0.2 mg, mean $\pm$ S.D., n=10, wet weight) and the tissue from 4 brain areas (1.3 g hypothalamus, 1.0 g striatum, 2.4 g midbrain, 4.5 g pons/medulla, wet weight) were incubated in 2 v/w (but not less than 300 µl) 0.1 M HCl for 10 min at 96°C, homogenized and centrifuged (140.000 x g, 45 min, 4°C). The enkephalins in the supernatants were adsorbed onto 100 mg (dry weight) Bio-Beads SM-2 20-50 mesh (Bio-Rad, Munich, FRG) per 1.5 ml acidic supernatant (respectively 100 mg Bio-Beads for 300 µl acidic supernatant from the extract of 50 neurointermediate pituitaries), by use of a modification of a method described previously (7). The beads were washed with 0.1 M HCl and distilled water. The enkephalins, desorbed with ethanol, were evaporated and redissolved in 200 µl 0.1 M acetic acid for chromatography on high-performance liquid chromatography (HPLC).

N-acetylation of synthetic leucine-enkephalin

2 mg leucine-enkephalin (a gift from Dr. Wünsch, Munich, FRG) were dissolved in 1.5 ml distilled water. 30 µl acetic acid anhydride was added immediately and again after a period of 5 hrs. The reaction (at 20°C) was stopped after 24 hrs by freezing and lyophilizing the sample. The sample was redissolved in 200 µl 0.1 M acetic acid and injected into a HPLC reverse phase column by that method as described in the legend to figure 1. As evaluated by UV-absorbance, $\sim$90% of the injected material eluted $\sim$4 min later than synthetic leucine-enkephalin. This more lipophilic compound was hypothesized to be N-acetyl-leucine-enkephalin. In order to obtain internal standards, 5 x 10^6 cpm ^{3}H-leucine-enkephalin (Amersham, Braunschweig, FRG, 43 Ci/mmole) without addition of unlabelled leucine-enkephalin was subjected to the same N-acetylation procedure, but by use of only 2 x 5 µl acetic acid anhydride. HPLC-separation showed that the main peak of ^{3}H-radioactivity ($\sim$90%) again eluted $\sim$4 min later than synthetic leucine-enkephalin.

Further investigations of the main radioactive peak provided confirmatory evidence for the generation of N-acetyl-leucine-enkephalin: putative ^{3}H-N-acetyl-leucine-enkephalin (10^4 cpm) was dissolved in 30 µl distilled water and adjusted to pH 8.2 by acetic acid and ammonium. 100 µg bovine α-chymotrypsin (Sigma, Taufkirchen, FRG) was added at time 0, after 6 and at 20 hrs. The cleavage products were subjected to thin-layer cellulose chromatography with fluorescence at 254 nm (DC Plastikfolien, Cellulose F 254, Merck, Darmstadt, FRG), the plates were developed by use of two different running buffers: butanol:acetic acid:water (4:1:1) and chloroform:methanol:ammonium (20:20:6). 5 mm strips of the thin-layer plates were dissolved in 15 ml scintillation fluid (Aqualuma plus, Baker, Groß-Gerau, FRG) and counted for ^{3}H-radioactivity. As expected for chymotryptic cleavage of ^{3}H-N-acetyl-leucine-enkephalin, which is tritiated at the tyrosine residue, tritium radio-

activity was found in position of synthetic N-acetyl-tyrosine (Sigma, Taufkirchen, FRG) but not in position of tyrosine in both running buffers.

Methionine-enkephalin (Beckman Instruments, Fullerton, CA, USA) and ^{3}H-methionine-enkephalin (Amersham, Braunschweig, FRG) were subjected to the same N-acetylation procedure as described above.

Radioimmunoassay (RIA) procedure

The leucine-enkephalin-RIA was performed by the use of the same RIA protocol and highly specific leucine-enkephalin antiserum as described elsewhere (8). (^{125}I)-monoiodinated leucine-enkephalin (NEN, Dreieich, FRG, 800 Ci/mmole) was used as the radioactive tracer. N-acetyl-leucine-enkephalin and leucine-enkephalin were equally well recognized by the antiserum. Cross-reactivities to other opioid peptides and opioid peptide fragments were negligible, see (8).

The methods, employed for HPLC and paper electrophoresis, are described in the legends to figure 1 and 2.

RESULTS

Occurrence of ir-N-acetyl-leucine-enkephalin in the neurointermediate pituitary

The extract of 50 neurointermediate pituitaries was adsorbed onto Bio-Beads SM-2, the desorbed enkephalins further separated by a HPLC reverse phase column, which yielded two leucine-enkephalin-related ir-peaks. Figure 1 shows the major peak eluting in the position of synthetic ^{3}H-leucine-enkephalin, the minor peak coeluting with synthetic ^{3}H-N-acetyl-leucine-enkephalin. During the extraction and chromatography of the rat neurointermediate pituitaries, ^{3}H-leucine-enkephalin, used as an internal standard, eluted in a single position and no radioactivity emerged in the position of N-acetyl-leucine-enkephalin. Moreover, no arteficial degradation or de-acetylation of ^{3}H-N-acetyl-leucine-enkephalin as internal standard was observed. Arteficial degradation of N-acetyl-ß-endorphin to N-acetyl-leucine-enkephalin was impossible, because the sequence of the pentapeptide leucine-enkephalin is not contained in the structure of ß-endorphin, though they share a common N-terminal amino acid sequence. It is, therefore, likely that ir-N-acetyl-leucine-enkephalin occurs naturally and is not formed as

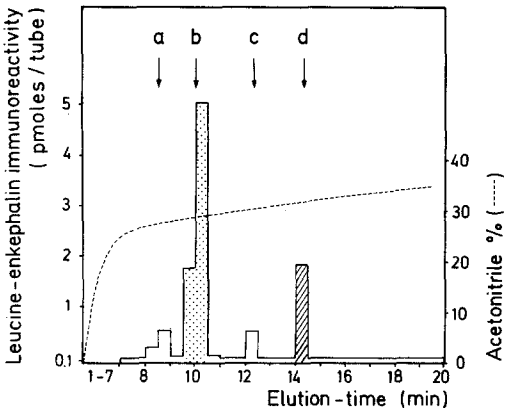


Figure 1

Chromatography of leucine-enkephalin-related peptides from the rat neurointermediate pituitary on a HPLC reverse phase column

Enkephalins from the extract of 50 rat neurointermediate pituitaries were separated on a μ-Bondapak C_{18} reverse phase column (3.9 x 300 mm, Waters 6000A HPLC). The column was eluted with a 30 min non linear gradient (Nr. 4) of 100% 0.1 M acetic acid to 0.1 M acetic acid:acetonitrile (60:40) at a flow rate of 2 ml/min. 1 ml fractions were collected, and 10% aliquots were assayed for ir-leucine-enkephalin. ^{3}H-leucine-enkephalin and ^{3}H-N-acetyl-leucine-enkephalin (both purified on HPLC) were used as internal standards (40-50% recovery for both peptides for extraction plus chromatography). Immunoreactivities are not corrected for recoveries. a = methionine-enkephalin; b = leucine-enkephalin; c = N-acetyl-methionine-enkephalin; d = N-acetyl-leucine-enkephalin.

an artefact of the extraction and separation procedures. Endogenous ir-N-acetyl-leucine-enkephalin represented ∿25% (22 ± 7%, mean ± S.D., n=5) of the total ir-leucine-enkephalin in the rat neurointermediate pituitary (total ir-leucine-enkephalin: 1.5 ± 0.4 pmoles/mg, mean ± S.D., n=5, corrected for recovery). In order to provide further evidence for the existence of N-acetyl-leucine-enkephalin in the rat neurointermediate pituitary, ir-N-acetyl-leucine-enkephalin, obtained from the HPLC C_{18} reverse phase column, was subjected to two further complementary separation systems: to a HPLC μ-Porasil column (Fig. 2A) and to paper electrophoresis (Fig. 2B). In both cases, endogenous and synthetic N-acetyl-leucine-enkephalin comigrated.

Lack of ir-N-acetyl-leucine-enkephalin in the rat brain

Four rat brain areas (hypothalamus, striatum, midbrain, and pons/medulla), known to contain the highest concentrations of enkephalins

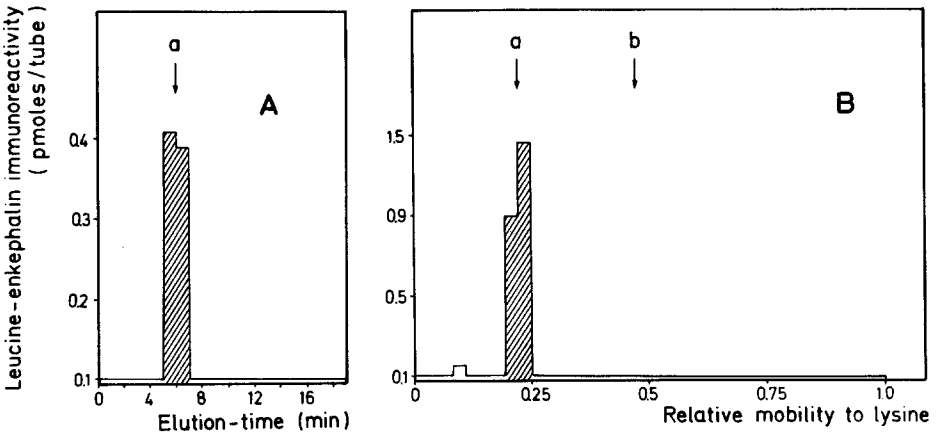


Figure 2

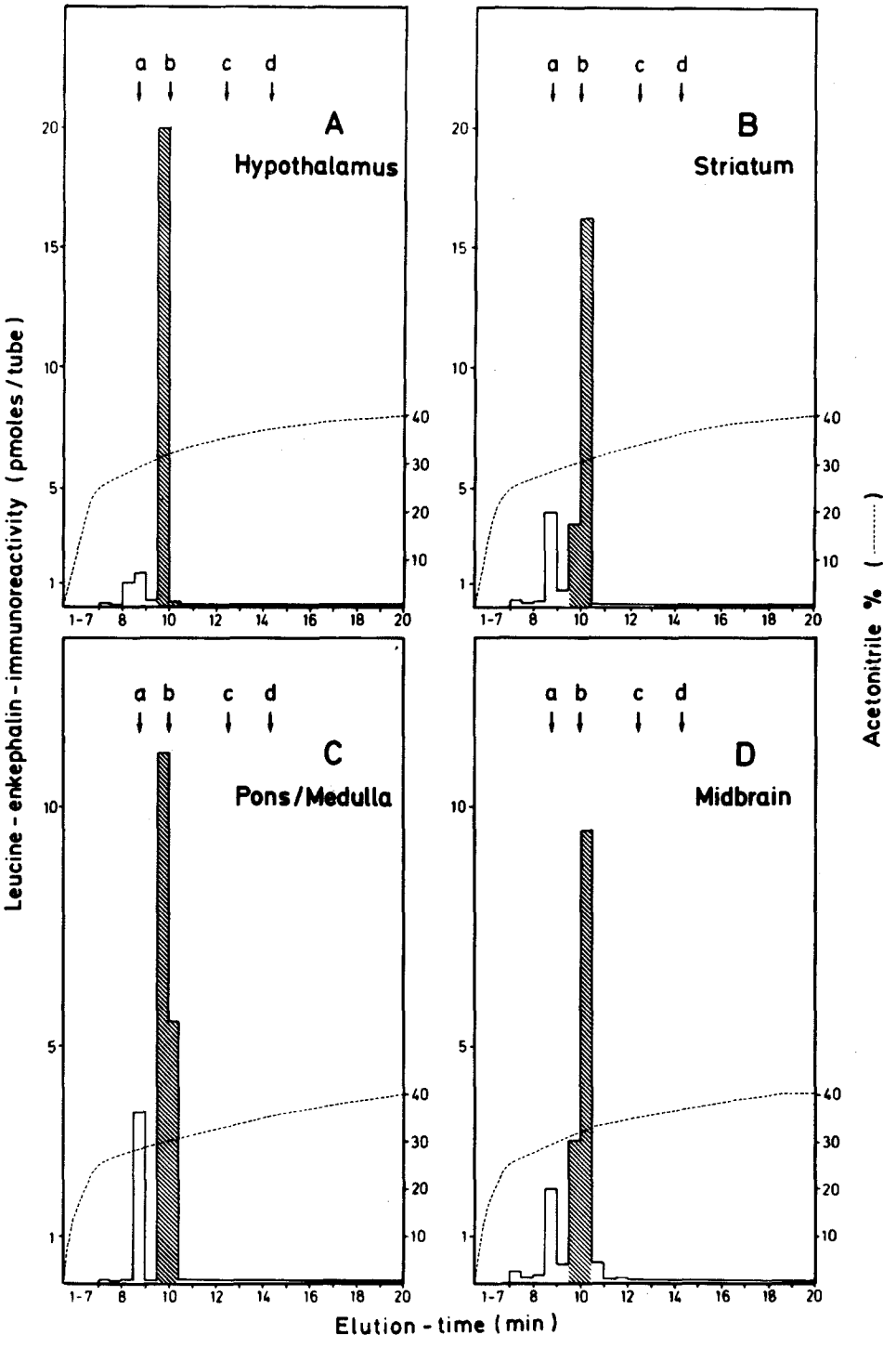
Chromatography of immunoreactive N-acetyl-leucine-enkephalin from the rat neurointermediate pituitary on a HPLC μ-Porasil column and on paper electrophoresis

(A) The enkephalins from 50 rat neurointermediate pituitaries were chromatographed on HPLC as described in the legend to figure 1. 80% of the ir-N-acetyl-leucine-enkephalin-containing fraction was subjected to a Waters HPLC μ-Porasil column (GPC 60 Å). The column was eluted with chloroform:methanol (80:20) at a flow rate of 1 ml/min. 1 ml fractions were collected, 80% was assayed for ir-leucine-enkephalin. ^{3}H-N-acetyl-leucine-enkephalin, the internal standard, displayed a recovery of 10% on the μ-Porasil column.

(B) The enkephalins from 50 rat neurointermediate pituitaries were chromatographed on HPLC as described in the legend to figure 1. 40% of the ir-N-acetyl-leucine-enkephalin-containing fraction was subjected to paper electrophoresis with Beckman paper (Type S+S2043A mgl) at 500 V/30 cm (3 hrs, 6°C). Formic acid:acetic acid:water (2:8:90) was used as running buffer. Paper strips were cut into 5 mm sections and peptides eluted with ethanol. 80% of the eluate fractions were assayed for ir-leucine-enkephalin. ^{3}H-N-acetyl-leucine-enkephalin, the internal standard, displayed a recovery of 50% for paper electrophoresis and elution from the strip sections. The peptide mobilities are expressed in terms of mobilities relative to that of lysine.

Immunoreactivities of the two figures are not corrected for recoveries. Abbreviations: see figure 1.

within the brain (9), were investigated for the presence of N-acetyl-leucine-enkephalin by the use of the same extraction and separation procedures as described for the neurointermediate pituitaries. Figure 3 depicts leucine-enkephalin-related peptides obtained from these brain areas after separation on a HPLC reverse phase column. In contrast to the rat neurointermediate pituitary, only ir-leucine-enkephalin, but no ir-N-acetyl-leucine-enkephalin could be detected (detection limit for ir-N-acetyl-leucine-enkephalin: ~1% of the ir-leucine-enkephalin peak


a b c d
A
Hypothalamus
B
Striatum
C
Pons/Medulla
D
Midbrain
Leucine - enkephalin - immunoreactivity (pmoles / tube)
Acetonitrile % (----)
Elution - time (min)
1-7 8 10 12 14 16 18 20

obtained). The concentrations of total ir-leucine-enkephalin found in the 4 brain areas examined were in agreement with earlier findings (9).

Lack of opiate-like activity of N-acetyl-leucine-enkephalin

The guinea-pig ileum isolated longitudinal muscle preparation showed a 50% inhibition (IC_{50}) of electrically induced contractions upon application of 0.32 µM methionine-enkephalin or 1.4 µM leucine-enkephalin. 100 µM synthetic N-acetyl-leucine-enkephalin, the highest concentration applied, caused, however, only a 4% inhibition. In comparison, a 4% inhibition was obtained with 40 nM leucine-enkephalin. Thus, N-acetyl-leucine-enkephalin is virtually inactive in this bioassay (at least 2000 times less active than leucine-enkephalin).

DISCUSSION

In addition to the N-acetylated forms of ß-endorphin and C'-fragment, the present investigation provides evidence for the existence of a N-acetylated derivative of the opioid pentapeptide leucine-enkephalin in the neurointermediate pituitary of the rat. Since the N-acetylated forms of these peptides are devoid of opiate activity, N-acetylation may be of general importance for the inactivation of opioid peptides.

N-acetylated forms of opioid peptides have only been found to occur in substantial quantities in the pituitary, since very small amounts of

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Figure 3

Chromatography of leucine-enkephalin-related peptides from various areas of the rat brain on a HPLC reverse phase column

Enkephalins from the extract of 1.3 g rat hypothalamus (A), 1.0 g striatum (B), 4.5 g pons/medulla (E) and 2.4 g midbrain (D) were separated on a HPLC reverse phase column and 30% of the column eluate assayed for ir-leucine-enkephalin as described in the legend to figure 1. ^{3}H-leucine-enkephalin and ^{3}H-N-acetyl-leucine-enkephalin, used as internal standards, displayed a recovery of 20-30% for extraction and chromatography. Immunoreactivities are not corrected for recoveries. Abbreviations: see figure 1.

N-acetylated derivates of ß-endorphin and C'-fragment and no N-acetylated leucine-enkephalin has been detected in various areas of the brain (2; the present paper).

It remains to be elucidated as to whether the N-acetylated form of leucine-enkephalin exists in the intermediate lobe and/or neural lobe of the neurointermediate pituitary. Although immunohistochemical studies have localized ir-leucine-enkephalin in fibres of the neurohypophysis but not in the intermediate tissue (10), there is biochemical evidence for the existence of ir-leucine-enkephalin in the intermediate lobe (8,10). It might be possible that N-acetylation of leucine-enkephalin, as i the case with that of ß-endorphin and C'-fragment (2), occurs exclusively in the intermediate lobe. However, this hypothesis can only be addressed by means of biosynthesis studies.

It should be pointed out that the N-acetylation of hormones does not necessarily result in their biological inactivation, as in the case of the α-melanocyte-stimulating hormone it even results in the production of the melanotropic activity of this peptide (11). Thus, it cannot be discounted that N-acetylated opioid peptides are not merely opiate-inactive derivative of their unmodified counterparts, but may be of quite distinct, as yet unknown, biological significance.

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