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N^{α}-ACETYL-γ-ENDORPHIN IS AN ENDOGENOUS NON-OPIOID NEUROPEPTIDE WITH BIOLOGICAL ACTIVITY

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Summary

N^{α}-acetyl-γ-endorphin (AcγE) was identified in the rat neurointermediate pituitary, based on its immunological properties, comigration with synthetic AcγE on HPLC and resistance to aminopeptidase-M degradation. The peptide appeared to be the main form of γ-endorphin (γE) in this tissue and in brain areas remote from the hypothalamus (hippocampus, septum, amygdala). The anterior pituitary, the hypothalamus and the thalamus contained almost exclusively the non-acetylated form of γE. In contrast to γE, AcγE was completely devoid of specific affinity for brain opiate binding sites. Yet, the peptide mimicked γE in that it potently attenuated passive avoidance behaviour in rats, when injected topically into the nucleus accumbens. It is concluded that AcγE is an endogenous neuropeptide with non-opioid biological activity. N^{α}-acetylation may not merely represent a mechanism for the inactivation of opioid activities of endorphins, but rather allow the organism to select specific sets of biological activities that reside in the endorphin structure.

The β-endorphin (βE) fragments α-endorphin (βE-(1-16); αE) and γ-endorphin (βE-(1-17); γE) have originally been identified as opioid peptides in porcine hypothalamo-neurohypophyseal extracts (1). Since, they have been established as neuropeptides with distinct effects on avoidance behaviour (2). Their des-Tyr^{1}- and des-enkephalin congeners, that are opiate-inactive, share these activities, indicating that the behavioural properties of αE and γE are independent of their opioid character (3,4). In particular, γ-type endorphins attenuate passive avoidance behaviour in rats, when injected subcutaneously or topically into the nucleus accumbens (2,5). αE and γE have also been detected in brain tissue (6,7). Their distribution in this organ closely parallels that of βE (8, 9). *In vitro* studies have demonstrated that brain peptidases can convert βE into αE and γE (10-12). This suggests that βE is a natural precursor for these neuropeptides. A considerable portion of the βE immunoreactivity present in rat brain is due to N^{α}-acetylated forms of the peptide (13). It has been shown that such acetylated β-endorphins are devoid of analgesic activity (14). Therefore, it was thought of interest to investigate the presence of N^{α}-acetylated fragments of βE in rat pituitary and brain tissue. We have now identified AcγE as an endogenous peptide in rat brain and pituitary, and evaluated its biological properties.

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Materials and Methods

Biochemical studies

Tissue dissection and extraction of endorphins. Male Wistar rats (180 - 200 g; Cpb: WU, TNO, Zeist, The Netherlands) were used. Immediately after decapitation brain and pituitary were removed. The pituitary was dissected into anterior and neurointermediate lobes and the brain in anatomically defined regions (15). Tissues were immediately frozen on dry ice. Pooled tissues from 18 animals were heated for 10 min in 1 M acetic acid (1 : 10, w/v) in a boiling water bath and homogenized by ultrasound. The resulting precipitate was removed by centrifugation. The supernatant was lyophilized and the residue dissolved in 0.01 M ammonium acetate buffer (pH 4.15).

HPLC fractionation of tissue extracts. Insoluble material was removed from the extracts by centrifugation. Then, the extracts were fractionated by reverse phase HPLC on a μ-Bondapak C-18 column as described previously (16). Chromatography was performed with a 45 min concave gradient running from 30% to 75% methanol in 0.01 M ammonium acetate (pH 4.15) at a flow rate of 2 ml/min. Fractions (0.5 min; 1 ml) were collected, dried and redissolved in 12.5 mM phosphate buffered saline (pH 7.5), containing 0.25% BSA and 0.2% sodium azide RIA buffer).

Endorphin radioimmunoassay systems. Radioimmunoassays (RIAs) were performed in essence as described previously (16). γE-Immunoreactivity (-IR) was measured using an antiserum (L_2) specifically recognizing the sequence βE-(8-17). γE was used as standard and ^{125}I-γE as tracer. The sensitivity of the assay was 1 pg per tube at 10% displacement. The following cross-reactivity data (on mass basis) were obtained: camel βE(β_cE), 4%; human βE (β_hE), 4%; AcγE, 150%; des-Tyr^1-γE (DTγE), 120%; des-enkephalin-γE (DEγE), 120%; βE-(2-19), 7%; αE, 8% and des-Tyr^1-αE (DTαE), 0.6% (see also 16). N^α-acetylated endorphins were assayed with antiserum raised against AcγE (Nancy Beth 51-6; generous gift of Dr. H. Akil, Ann Arbor, MI., USA). This antiserum is specifically directed against the acetylated N-terminal Tyr-residue of endorphins. AcγE was used as standard and ^{125}I-AcγE as tracer. The sensitivity of this system was 3 pg per tube. No cross-reactivity was observed with non-acetylated αE, γE, β_cE, β_hE, enkephalins, dynorphin, ACTH, α-MSH and β-LPH. αE-IR was determined with antiserum A_2, αE as standard and ^{125}I-αE as tracer. The sensitivity of the assay was 5 pg per tube. The cross-reaction with γE and AcγE was 2%, with β_hE 1% and β_cE 2%. βE-IR was assayed in a system employing antiserum B_3, β_hE as standard and ^{125}I-β_hE as tracer with a sensitivity of 2 pg per tube. AcγE cross-reacted 6%, αE and γE both 4% and β_cE 61%.

Incubation with aminopeptidase-M. Aliquots of HPLC fractions containing 10 ng γE-IR material were diluted with 0.1 M Tris-HCl buffer (pH 8.0) to 112.5 μl. The incubation was started by addition of 12.5 μl aminopeptidase-M solution (1 μg/10 μl in 0.9% NaCl containing 10% glycerol), continued for 90 min at 37 °C and terminated by dilution with 1 ml 0.01 M ammonium acetate buffer (pH 4.15). The incubates were stored frozen until further processing. To monitor the recovery of γE-IR after the incubation, samples were fractionated by HPLC and radioimmunoassayed for γE.

Preparation of a total particulate brain fraction. A total particulate fraction of brain tissue was prepared according to Pasternak et al. (17). Immmediately after decapitation of the animals (male Wistar rats, 180 - 200 g) their brains (minus cerebellum) were removed and placed in 100 volumes (w/v) ice-cold 50 mM Tris-HCl buffer (pH 7.4). The tissue was homogenized (Polytron; 30 sec, setting 5) and the homogenate centrifuged at 32,000 x g for 15 min (4 °C). The pellet

was rehomogenized in buffer, and incubated at 37 °C for 30 min. Subsequently the incubate was centrifuged (32,000 xg; 15 min at 4 °C) and the pellet resuspended in 100 volumes (w/v) Tris-buffer. The protein content of this fraction was determined according to Lowry et al. (18).

Opiate binding assays. Incubations (30 min at 25 °C) were started by the addition of 1.8 ml total particulate brain fraction (approx. 0.65 mg protein/ml) to 0.2 ml 50 mM Tris-HCl buffer (pH 7.4), containing the following chemicals (final concentrations): ^{3}H-naloxone (2.2 nM), BSA (0.1%), bacitracin (0.4%) and peptides. Non-saturable binding was determined in the presence of 2 μM unlabelled naloxone. The incubations were terminated by filtration over Whatman GF/B glass filters. The filters were washed twice with 5 ml ice-cold buffer, and counted for ^{3}H-radioactivity. Specific binding was calculated by subtraction of non-saturable binding from total binding. Under these conditions the K_D for naloxone was 3 nM.

Behavioral studies

Animals and surgery. Male Wistar rats (130 - 140 g; Cpb: WU) were used. The animals were housed under controlled conditions with 14 h light/10 h dark cycle (light on at 7.00 a.m.) and had free access to food and water. Before surgery the rats were anesthetized with Hypnorm®. Using a stereotactic instrument, a stainless steel cannula (0.6 mm ID; 0.3 mm OD) was implanted unilaterally at the right side of the brain, and aimed at the nucleus accumbens. The coordinates according to Pellegrino and Cushman (19) were: 2.6 mm anterior to bregma, 2.7 mm lateral to the midline, and 6.1 mm below the dura at the point of penetration. Cannulae were inserted at an angle of 12 °C with the sagittal plane. After the operation the animals were housed in separate cages, and allowed to recover for at least 7 days.

Passive avoidance behaviour. Passive avoidance behaviour was studied in a one trial learning, step-through situation (20). The test apparatus consisted of a small illuminated platform, attached to a dark compartment equipped with a grid floor. The rats were habituated to the dark compartment (2 min) and subsequently placed on the illuminated platform to enter the dark. On the following day three more trials were given. At the end of the third trial, upon entering the dark compartment, the animals received an unescapable footshock (0.25 mA for 2 sec) delivered through the grid floor, or no shock. Then at 24 and 48 hrs after the learning trial, retention was measured by the latency to re-enter the dark compartment to a maximum of 300 sec after placing the animals on the illuminated platform. One hour before the first retention test, the animals were treated with peptide or saline (1 μl), injected unilaterally into the nucleus accumbens through the cannula. All injections were performed blind.

Histological control. After experimentation the rats were sacrificed and the brains were fixed in 4% formalin. Serial sections (100 μm) were prepared on a cryostate, and the exact positions of the tips of the cannulae were microscopically determined using the atlas of Pellegrino and Cushman (19). Data from animals with injection sites outside the nucleus accumbens were discarded from further analysis.

Results and Discussion

HPLC-chromatograms of rat neurointermediate lobe extracts showed two major peaks with γE-IR (antiserum L_2; Fig. 1A). The first peak (fraction 73) eluted in the same position as synthetic γE. The second γE-IR peak (fraction 78) was not appreciably recognized by antisera specific for αE or βE (A_2 and B_3), but did react in a RIA specifically detecting N^{α}-acetylated-endorphins (antiserum

Nancy Beth; data not shown). Synthetic AcγE showed 150% cross-reactivity in the γE-RIA (L_2). The peptide in fraction 78 also appeared to be more efficiently detected (160%) in this RIA- system than with the Nancy Beth antiserum. This suggests the presence of AcγE in fraction 78.The presence of a blocked N-terminus in the peptide was confirmed by its resistance to aminopeptidase-M degradation. After incubation of a 10 ng aliquot from fraction 78 with aminopeptidase-M and subsequent processing on HPLC, the γE-IR in the sample was completely recovered with unaltered retention time (data not shown). The identity of the peptide in fraction 78 as AcγE was confirmed by its comigration on HPLC with synthetic AcγE (Fig. 1A). Similarly, based on immunological properties in the four RIA systems used, sensitivity for aminopeptidase-M (complete degradation) and comigration on HPLC with synthetic γE, fraction 73 was identified as non-acetylated γE.

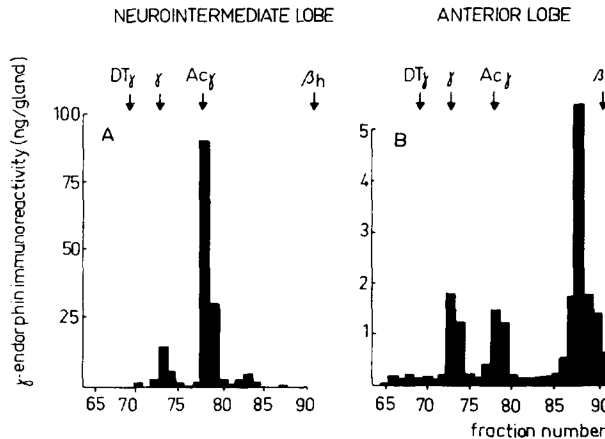

Fig. 1

HPLC of γE-IR extracted from neurointermediate (A) and anterior lobes (B) of rat pituitary glands. Data are presented as γE-IR recovered and are not corrected for differential cross-reactivity of the peptides in the γE-RIA. The elution positions of synthetic DTγE, γE, AcγE and β_hE were determined in consecutive HPLC runs and are indicated by arrows.

The regional distribution of the two forms of γE in pituitary and brain was investigated by HPLC fractionation and quantitation in RIA using the L_2-antiserum. In the neurointermediate lobe of the pituitary, most of the γE-IR occurred as AcγE and γE, the former being the predominant peptide (ratio acetylated/non-acetylated = 5.5; Fig. 1A). In the anterior lobe, however, AcγE appeared to be minor (ratio acetylated/non-acetylated = 0.6; Fig. 1B). In addition to acetylated and non-acetylated forms of γE, a third peak was resolved around fraction 88, containing considerable immunoreactivity. Preliminary data suggest the presence in this peak of a much larger peptide with γE-IR, possibly β-LPH-(1-77). Grāf and Kenessey (21) have shown that β-LPH-(1-77) can be specifically generated from β-LPH by enzyme activity present in pituitary homogenates, and β-LPH-(1-77) has recently been detected in human pituitary glands (22). The presence of this large molecular weight form of γE in the anterior pituitary may well be indicative for an alternative pathway, specific for the intracellular formation of γE, as has been suggested previously (22,23). No γE-IR was observed to elute with retention time of synthetic [Met-O]-γE, indicating that sulphoxides do not represent significant components under the experimental con-

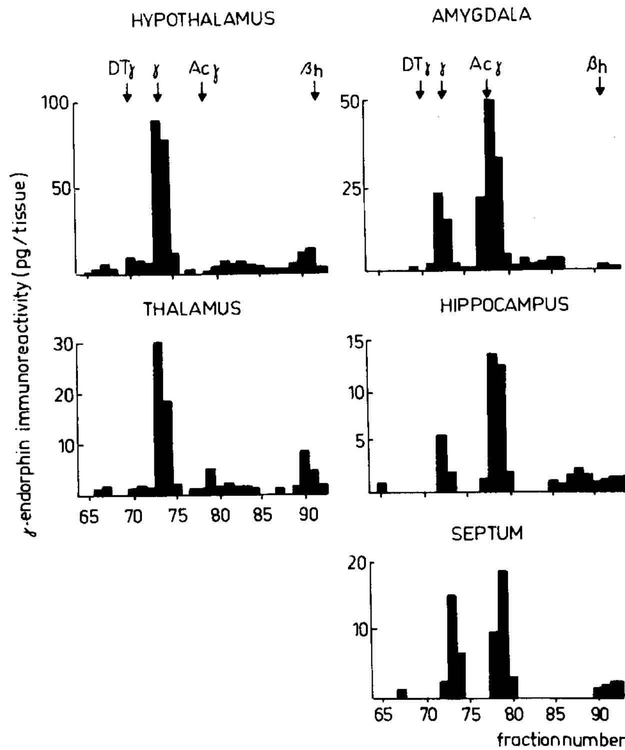

Fig. 2
HPLC of γE-IR extracted from dissected regions of rat brain. The data shown represent γE-IR recovered and are not corrected for differential cross-reactivity of the peptides in the γE-RIA. The elution positions of synthetic marker peptides are indicated by arrows (see legend to Fig. 1).

ditions employed. HPLC profiles of various dissected brain regions showed marked differences in the concentration of γE and AcγE, and in the ratio acetylated/non-acetylated γE (Fig. 2). The highest concentration of γE was found in the hypothalamus, a region containing negligible amounts of AcγE (ratio 0.04). Also in the thalamus the γE-IR existed almost exclusively in the non-acetylated form (ratio 0.1). However, in regions further removed from the hypothalamus (septum, amygdala, hippocampus) the acetylated form appeared to be predominant (ratios 1.4, 2.7 and 4.1, respectively). Of all regions analyzed, the amygdala contained the highest total amount of AcγE. The peptides presently identified in pituitary and brain tissue accounted for 80 - 90% of the total γE-IR recovered. Earlier observations have demonstrated the presence in the brain of considerable amounts of DTγE (9,24) and DEγE (24). In the present experiment DTγE occurred as a minor component and no immunoreactivity was found to elute with the retention time of DEγE. These differences may reflect different states of the animals used. For, it has been shown recently that physiological variables such as age and stress are important determinants of the processing of βE (25, 26).

The present experiments identify AcγE as the major endogenous form of γE in brain regions remote from the hypothalamus. Others (27) maintain that acetylated endorphins are confined to the intermediate lobe of the pituitary and do not exist in rat brain. However, they used whole- brain extracts and did not analyse dissected (extrahypothalamic) brain regions where the acetylated endorphins have been specifically localized. In view of the high levels of endorphins in the hypothalamus and the absence of N^{α}-acelated forms of these peptides in this region, it is likely that the latter escape detection in whole-brain extracts.

The pattern of distribution of acetylated and non-acetylated γE observed in brain and pituitary parallels that of acetylated and non-acetylated forms of αE and βE (28,29). This suggests that similar to the formation of αE and γE from βE by brain peptidases (11), the acetylated forms of αE and γE may be enzymatically generated from AcβE.

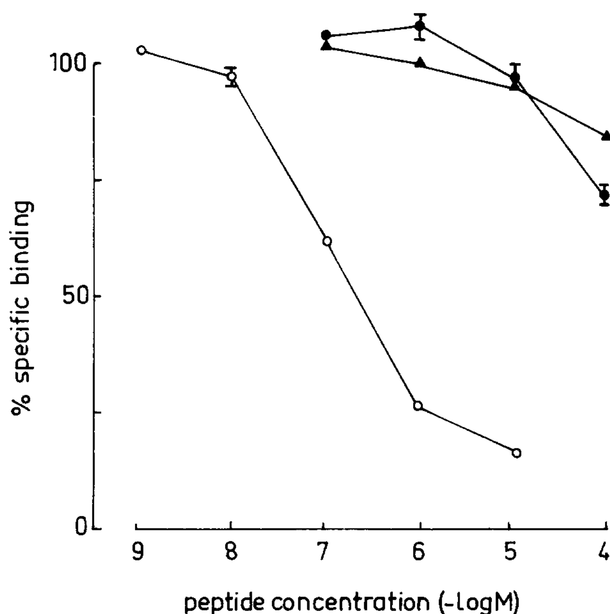

Fig. 3

Displacement of ^{3}H-naloxone (2.2 nM; K_D ∿ 3 nM) from its specific binding sites in a total particulate fraction of rat brain by various concentrations of γE (o), $AcγE_3$(●) and DTγE (▲). Values are expressed as percentage of specific H^3-naloxone binding under control conditions, and represent mean ± SEM of triplicate incubations.

The abundance of AcγE in the brain prompted us to investigate its biological activities. In the *in vitro* opiate binding assay, γE displaced ^{3}H-naloxone from its specific bindings sites in a concentration-dependent manner (IC_{50} = 2 x 10^{-7} M; Fig. 3). AcγE did not interfere with ^{3}H-naloxone binding, even when high concentrations were used (IC_{50} > 5 x 10^{-5} M). Similar observations were made with DTγE, a non-opioid congener of γE (IC_{50} > 5 x 10^{-5} M; Fig. 3). These

TABLE I

Effect of N^{α}-Acetyl-γ-Endorphin Injected in the Nucleus Accumbens on the Retention of a One Trial Learning Passive Avoidance Response in Rats.

Dose (pg)	N^{α}-acetyl-γ-endorphin			γ-endorphin			saline (1 μl)		
	n	24 h	48 h	n	24 h	48 h	n	24 h	48 h
0.3	6	63 (17-89)	39 (8-70)	6	79 (17-112)	57 (18-79)	30	75(31-121)	49(21-100)
1	6	15 (9-45)	15 (10-37)	6	38*(11- 79)	39 (10-59)			
3	6	8**(7-20)	11* (6-22)	6	17*(5- 56)	16*(6-61)			
10	6	7**(3-37)	15 (9-39)	6	24*(7- 61)	21 (8-51)			
30	6	13* (5-21)	12* (3-25)	6	45 (26- 81)	32 (10-57)			
60	6	45 (19-61)	37 (12-57)	6	47 (18- 79)	26 (20-48)			
10, no shock	6	6.5(3-10)	5.5(3- 8)	6	7 (4- 10)	6 (3- 7)	6	6(3- 10)	3(2- 6)

Entrance latencies are given as median in seconds, with the 25th and 75th percentile shown between brackets. N represents the number of animals in each treatment group. Combined data from five separate experiments are shown. The data of each of five experiments were first analyzed with a Kruskall-Wallis ANOVA and subsequently with Mann-Whitney U-tests.

Different from the concomitantly tested saline group ($p < 0.02$; ** $p < 0.005$).

results are in line with those of Deakin et al. (14), who reported that N^{α}-acetylation destroys the analgesic activity of endorphins.

Previous work from our laboratory showed that the effect of γE on the retention of a passive avoidance response is shared by its non-opioid des-Tyr^{1}- and des-enkephalin-congeners (3-5). We studied the activity of γE and AcγE in the passive avoidance paradigm. Both peptides were active, and significantly reduced the entrance latency of shocked rats when injected topically into the nucleus accumbens one hour prior to the retention test (Table I). This effect is likely not due to non-specific locomotor effects, since no effect of peptide treatment was observed on the entrance latency of rats that did not receive footshock in the training session (Table I). A U-shaped dose-response relationship was observed for both peptides, AcγE being active over a somewhat broader dose range (1 - 30 pg) than γE (1 - 10 pg). U-shaped dose response curves have been reported for effects of a large variety of peptides in different test systems. Although not completely understood as yet, such U-shaped curves may be the result of interaction of the peptide with more than one substrate (30).

These data indicate that by N^{α}-acetylation the opioid properties of γE are lost, resulting in the selective retention of its non-opioid, behavioural activities. These activities are not limited to attenuation of passive avoidance behaviour. They also include other non-opioid central activities as exerted by DTγE and DEγE. This is evidenced by findings of Van Ree et al. (31,32), who reported that AcγE antagonized apomorphine-induced hypolocomotion by interaction with dopaminergic systems in the nucleus accumbens. The sensitivity of the nucleus accumbens to low doses of γ-type endorphins suggests that this region is a site of action for these peptides. This contention is supported by recent observations showing the presence of γE-IR and AcγE in the vicinity of this area (8, 24). The observation that the profile of action of AcγE sofar parallels that of other non-opioid endogenous γ-type endorphins (DTγE, DEγE), evokes the question as to the natural ligand of the putative γ-type endorphins receptor. It should, however, be noted that the relative concentrations of the γ-type endorphins in the brain show marked regional differences (8,9,24 and present results) and are apparently dependent on the state of the animal (25). Thus the present data do not allow definite conclusions in this respect.

It is concluded that AcγE is an endogenous, biologically active neuropeptide. More in general, our data provide arguments for a functional significance of N^{α}-acetylation of endorphins beyond its role as mere inactivation process. For N^{α}-acetylation selectively eliminates the opioid character of endorphins, and leaves non-opioid activities intact. Recently, we have identified AcαE as an endogenous peptide in rat brain and pituitary (28). Preliminary data indicate that this peptide retains non-opioid behavioural activities of α-type endorphins (Van Ree et al., unpublished). In this respect AcβE may function as a precursor for shorter N^{α}-acetylated endorphins with differential biological activities.

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