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ACTION OF PEPTIDASES IN BRAIN SYNAPTIC MEMBRANES ON THE NH_2-TERMINUS OF ADRENOCORTICOTROPIN USING ACTH-(1-16)-NH_2 AS A MODEL SUBSTRATE

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The action of brain peptidases on NH_2-terminal sequences of adrenocorticotropin was studied by incubation of ACTH-(1-16)-NH_2 under different pH conditions. Profiles of metabolites and time course of product formation were obtained by HPLC analysis of the digests. Fragments of ACTH-(1-16)-NH_2 were isolated and characterized by their amino acid composition and NH_2-terminal groups. Both at pH 7.4 and pH 8.5 the following fragments were found: ACTH-(3-16)-NH_2, ACTH-(4-16)-NH_2, ACTH-(5-16)-NH_2, and ACTH-(7-16)-NH_2. At pH 7.4 the major products were ACTH-(4-16)-NH_2 and ACTH-(7-16)-NH_2, while the peptide ACTH-(3-16)-NH_2 was the main metabolite at pH 8.5. The nature of identified peptides and the time course of their formation demonstrates that aminopeptidase activities predominate in the conversion of the NH_2-terminus of adrenocorticotropin and related peptides by brain synaptic membranes.

Various forms of adrenocorticotropin (ACTH-(1-39)) occur as endogenous neuropeptides in the CNS. In addition to the complete ACTH-(1-39) peptide, ACTH-(18-39) (CLIP), α^1-N-acetyl-ACTH-(1-13)-NH_2 (α-MSH), and ACTH-(1-13)-NH_2 (des-acetyl-α-MSH) have been identified (1-10). Recent reports suggest that ACTH-(1-13)-NH_2 is the most abundant ACTH related peptide in the brain (10). During studies on the proteolytic conversion of ACTH-(1-39) by brain peptidases we found that the NH_2-terminal peptide ACTH-(1-16) was formed by an endopeptidase activity. Structure-activity studies on the CNS effects of ACTH-(1-39) have indicated that most, if not all, of the different CNS activities of ACTH are located in the ACTH-(1-16) sequence (11,12), suggesting that proteolytic conversion of such an NH_2-terminal ACTH sequence could result in production of

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Abbreviations: ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate lobe peptide; MSH, melanocyte stimulating hormone; HPLC, high pressure liquid chromatography; CNS, central nervous system; DNS-, dansyl-, 1-dimethylaminonaphthalene-5-sulfonyl-.

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peptides with more selective CNS activities. In the present study we have investigated in detail the action of brain peptidases on the NH_2-terminus of ACTH using the COOH-terminally protected peptide ACTH-(1-16)-NH_2. The nature of principal peptide fragments of ACTH-(1-16)-NH_2 and the time course of their formation indicates the predominant role of aminopeptidase activities in the conversion of peptides related to the NH_2-terminus of ACTH.

MATERIALS AND METHODS

Synthetic ACTH-(1-16)-NH_2 was prepared and kindly supplied by Dr. H.M. Greven and Dr. J. van Nispen (Organon, Oss, The Netherlands).

Synaptic membranes were obtained from male Wistar rat forebrain tissue as described previously (13).

ACTH-(1-16)-NH_2 (2×10^{-5} M) was incubated with synaptic membranes (2.5 mg/ml medium) at 37°C in 1.0 ml of 40 mM Tris-HCl buffer (pH 7.4) or 40 mM ammonium bicarbonate (pH 8.5). The reaction was terminated by addition of 80 µl 6 N HCl. The membranes were removed by centrifugation at 10,000 g_{av} for 30 min and the supernatant was stored at -20°C for analysis by HPLC.

HPLC was performed on a µBondapak C18 column (Waters Associates, Milford, USA), eluted with mixtures of 10 mM ammonium acetate (pH 4.15) and acidified methanol (0.15% acetic acid, v/v) with a flow rate of 2.0 ml/min (14). Digests of ACTH-(1-16)-NH_2 were fractionated by a linear gradient which ran from 10% to 50% acidified methanol in 30 min. The fractions were collected and lyophilized after removal of methanol in vacuo at 60°C. The amount of ACTH-(1-16)-NH_2 was quantitated by its peak height in the UV absorbance (210 nm) profile of the column effluent.

Freeze dried samples were hydrolysed with 6 N HCl containing 0.1% thioglycolic acid in evacuated glass tubes at 110°C for 16 h. The amino acid composition of samples was determined using precolumn derivatization with o-phthaldialdehyde, HPLC separation and fluorescence detection as described by Burbach et al. (15). HPLC of o-phthaldialdehyde derivatives was performed on a Cp_tm_Spher C18 column (250 x 4.6 mm i.d., particle size 8 µm, Chrompack, The Netherlands) using the previously described gradient of 100 mM sodium citrate, pH 6.5 and methanol (15).

Dansylation of NH_2-terminal amino acids was carried out according to Gray and Smith (16). After hydrolysis and extraction dansylated amino acids were identified on micropolyamide F-1700 sheets (5 x 5 cm; Schleicher & Schüll, Dassel, Germany) which were developed in a three solvent system (17).

RESULTS

HPLC fractionation and characterization of ACTH-(1-16)-NH_2 fragments

Fig. 1A shows the HPLC profile of ACTH-(1-16)-NH_2 fragments obtained after incubation with brain synaptic membranes at pH 7.4 for 3 h. Five major UV absorbing fractions were collected (fraction I - V, fig. 1) and analyzed. Fraction I co-migrated with ACTH-(1-16)-NH_2 on HPLC. Based on amino acid analysis and NH_2-terminal residue (DNS-Ser), fraction I was identified as intact ACTH-(1-16)-NH_2. Fraction II was repurified using the same HPLC system. It had the amino acid composition and NH_2-terminal group (DNS-Ser) of ACTH-

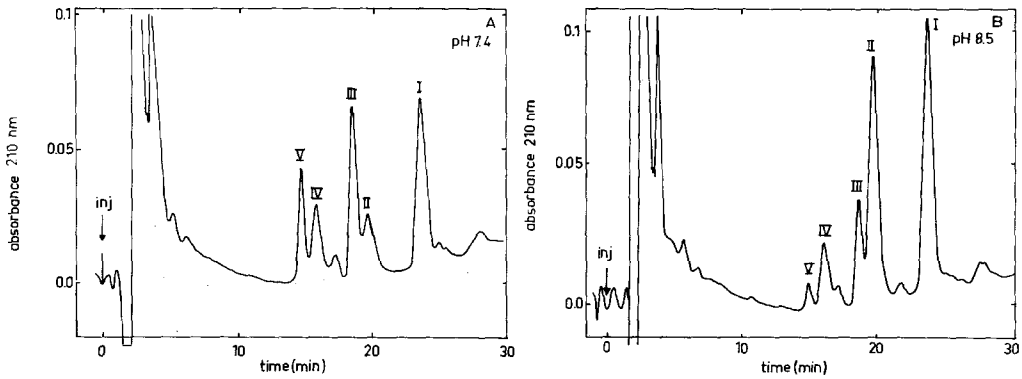

Figure 1. HPLC profiles of ACTH-(1-16)-NH_2 digests. ACTH-(1-16)-NH_2 (2×10^{-5} M) was incubated with rat brain synaptic membranes (2.5 mg/ml medium) at 37°C for 3 h. HPLC was performed as described in Materials and Methods. The eluate was monitored by UV detection at 210 nm. A: incubation was carried out at pH 7.4 in 40 mM Tris-HCl buffer; B: incubation was carried out at pH 8.5 in 40 mM ammonium bicarbonate buffer.

(3-16)-NH_2. The amino acid composition of fraction III showed that it contained ACTH-(4-16)-NH_2, which was in agreement with the NH_2-terminal methionine

TABLE I

ANALYSES OF HPLC FRACTIONS OF ACTH-(1-16)-NH_2 DIGESTS

Amino acid residue	Fraction[a] I	II	III	IV	V	ACTH-(1-16)-NH_2[d]
Glu	1.14 (1)	1.10 (1)	1.17 (1)	1.08 (1)	0.29 (0)	1
Ser	1.95 (2)	1.10 (1)	0.12 (0)	0.23 (0)	0.14 (0)	2
His	0.99 (1)	1.00 (1)	0.92 (1)	1.07 (1)	0.09 (0)	1
Arg	0.99 (1)	1.00 (1)	0.98 (1)	1.18 (1)	1.00 (1)	1
Gly	2.28 (2)	2.00 (2)	2.14 (2)	2.20 (2)	2.14 (2)	2
Tyr	0.90 (1)	0.11 (0)	0.07 (0)	0.01 (0)	0.04 (0)	1
Met	0.89 (1)	0.94 (1)	0.93 (1)	0.00 (0)	0.03 (0)	1
Val	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	0.81 (1)	1
Phe	0.92 (1)	1.00 (1)	1.00 (1)	1.00 (1)	0.83 (1)	1
Lys	2.91 (3)	3.00 (3)	2.70 (3)	2.66 (3)	2.80 (1)	3
Pro[b]	(1)	(1)	(1)	(1)	(1)	1
Trp[c]	(1)	(1)	(1)	(1)	(1)	1
DNS-derivative	DNS-Ser o-DNS-Tyr	DNS-Ser	DNS-Met		DNS-Phe	
proposed sequence	1-16	3-16	4-16	5-16	7-16	

The values in parentheses are the theoretical number of residues based on the composition of the proposed sequence.
a: fraction coding corresponds to the coding in fig. 1.
b: not determined because of destruction during acid hydrolysis.
c: not detected by the amino acid analysis technique.
d: the number of amino acid residues as based on the primary structure of synthetic ACTH-(1-16)-NH_2, presented in Fig. 3.

residue. The components in fractions IV and V were characterized as ACTH-(5-16)-NH_2 and ACTH-(7-16)-NH_2, respectively. The analyses data are summarized in table I.

HPLC fractionation of ACTH-(1-16)-NH_2 digests by brain synaptic membranes at pH 8.5 showed the accumulation of five metabolites eluting with similar retention times as those formed at pH 7.4 (Fig. 1B). Chemical analyses revealed that all these ACTH fragments were similar to those formed at pH 7.4 (not shown).

Time course of proteolysis of ACTH-(1-16)-NH_2

Although identical metabolites were generated at pH 8.5 and pH 7.4, considerable differences in their quantities were observed. In order to investigate the differences in peptidase action underlying this phenomenon, the conversion process of ACTH-(1-16)-NH_2 was followed with time under the two pH conditions.

Under the applied incubation conditions, ACTH-(1-16)-NH_2 was degraded with a half-life of about 30 min at pH 7.4 (Fig. 2A). As ACTH-(1-16)-NH_2 decreased, the fragments ACTH-(3-16)-NH_2, ACTH-(4-16)-NH_2, ACTH-(5-16)-NH_2, and ACTH-(7-16)-NH_2 accumulated. The amounts of ACTH-(3-16)-NH_2 remained low during incubation. ACTH-(4-16)-NH_2 accumulated as predominant initial product and its accumulation was maximal at 1 h. As ACTH-(4-16)-NH_2 decreased, ACTH-(5-16)-NH_2

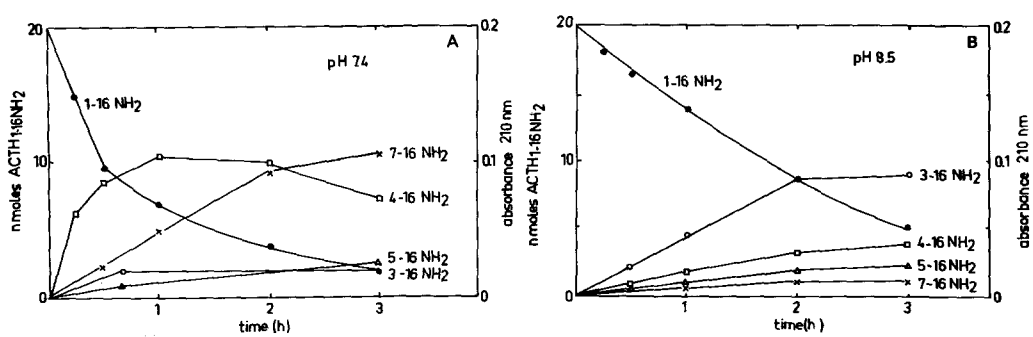

Figure 2. Time courses of proteolysis of ACTH-(1-16)-NH_2 and the accumulation of peptide fragments. The incubation conditions were the same as in Fig. 1; A: at pH 7.4, B: at pH 8.5. Aliquots were taken at various time intervals. The samples were subjected to HPLC analysis. The decrease of ACTH-(1-16)-NH_2 and formation of peptide fragments was quantitated by UV absorbance at 210 nm in the HPLC eluate. The amounts of intact ACTH-(1-16)-NH_2 are expressed in nmoles.

and ACTH-(7-16)-NH_2 accumulated steadily. The amount of ACTH-(5-16)-NH_2 was very small, while ACTH-(7-16)-NH_2 remained relatively high with time.

Following incubation of ACTH-(1-16)-NH_2 at pH 8.5, the degradation rate of ACTH-(1-16)-NH_2 was about 3 times as low as at pH 7.4 (Fig. 2B). The accumulation of metabolites decreased proportionally. ACTH-(3-16)-NH_2 appeared as initial fragment and remained the predominant product during the tested time range. Sequential accumulation of ACTH-(4-16)-NH_2, ACTH-(5-16)-NH_2, and ACTH-(7-16)-NH_2 was observed.

DISCUSSION

The present study demonstrates that the predominant mechanism of proteolysis of ACTH-(1-16)-NH_2 by a brain synaptic membrane fraction involves sequential cleavage of peptide bonds from the NH_2-terminus onwards, indicating the action of aminopeptidases (Fig. 3). This mechanism is based on the nature of characterized peptide fragments as well as on the course of proteolytic events. The peptides which were generated during proteolysis at pH 7.4 and 8.5 were ACTH-(3-16)-NH_2, ACTH-(4-16)-NH_2, ACTH-(5-16)-NH_2, and ACTH-(7-16)-NH_2. The fragments ACTH-(2-16)-NH_2 and ACTH-(6-16)-NH_2 were not found; apparently they did not accumulate in sufficient amounts. This may be due to rapid removal of the Tyr^2 and His^6 residues by the aminopeptidase activity immediately after formation of ACTH-(2-16)-NH_2 and ACTH-(6-16)-NH_2, respectively. However, the involvement of dipeptidyl aminopeptidases cannot be excluded (18,19). Our results may relate to data obtained by Reith and Neidle (20), who showed that neutral arylamidases and a dipeptidylaminopeptidase in soluble brain extracts were responsible for degradation of ACTH-(1-4).

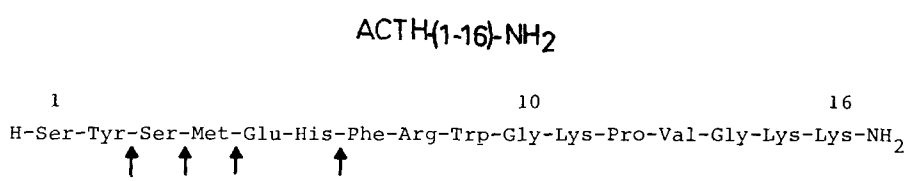

Figure 3. Primary structure of ACTH-(1-16)-NH_2 and main cleavage sites by peptidase activity in brain synaptic membranes. Indicated cleavage sites are based on the fragments identified and the time course of their formation.

Although the qualitative patterns of peptides generated at pH 7.4 and pH 8.5 were identical, the quantities of accumulated peptides differed markedly. At pH 7.4 ACTH-(4-16)-NH_2 was the main product during the initial period of incubation, while ACTH-(7-16)-NH_2 predominated at longer incubation times. At pH 8.5, however, ACTH-(3-16)-NH_2 remained the predominant product. These observations are in agreement with previous data on the conversion of ACTH-(1-39) showing that ACTH-(7-20) and ACTH-(7-21) were formed at pH 7.4 and ACTH-(3-15) at pH 8.5 (in preparation), and indicate that the NH_2-terminus of these ACTH fragments were similarly cleaved by aminopeptidase activities. The differences in product accumulation under the different pH conditions may indicate that multiple aminopeptidases with different pH dependencies are involved in the conversion process, although it cannot be ruled out that a single aminopeptidase with pH dependent substrate specificity is responsible for the observed cleavage pattern.

ACTH-(1-16)-NH_2 has been used as a model substrate representative for the NH_2-terminus of adrenocorticotropin and naturally occurring ACTH-related peptides. Moreover, the ACTH-(1-16)-NH_2 sequence is of interest since it contains the various CNS activities of ACTH (11,12). The aminopeptidase conversion could generate shorter peptides with more selective CNS activities. Indeed, the peptides ACTH-(4-16)-NH_2 and ACTH-(7-16)-NH_2 which were main products at neutral pH,seem to have a more specific CNS effect in various tests (12,21). Moreover, the ACTH-(4-16)-NH_2 has been the basis of an ACTH analogue which is about 10^6 times more potent in inhibiting the extinction of an active avoidance response than ACTH-(4-10) (22). Our results also suggest that ACTH-(1-13)-NH_2 (des-acetyl-α-MSH) which reportedly is the most abundant ACTH related peptide in the brain (10), is converted similarly by the aminopeptidase activity.

The presently observed aminopeptidase action on the NH_2-terminus of ACTH-related peptides may be involved in inactivation as well as production of neuropeptides derived from adrenocorticotropin in brain.

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