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CONVERSION OF DES-TYROSINE-γ-ENDORPHIN BY BRAIN SYNAPTIC MEMBRANE ASSOCIATED PEPTIDASES: IDENTIFICATION OF GENERATED PEPTIDE FRAGMENTS

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SUMMARY

Des-tyrosine-γ-endorphin, a β-endorphin fragment with neuroleptic-like properties, was digested with a cSPM fraction of rat brain. A profile of metabolites and a time course of conversion were obtained by HPLC analysis of the digests. Quantitative amino acid analysis and a second HPLC fractionation step which was designed to separate and to identify very similar des-tyrosine-γ-endorphin fragments, combined with dansyl end group determination allowed the characterization of β-LPH 65-77, β-LPH 66-77 and β-LPH 62-73 as main conversion products. In the digests the C-terminal leucyl peptides β-LPH 67-77 and β-LPH 68-77 as well as the N-terminal glycyl peptides β-LPH 62-74 and β-LPH 62-76 were minor components. The data indicate the involvement of several types of peptidase activities in the conversion process. It is suggested that these peptidases have a role in mediating in vivo des-tyrosine-γ-endorphin effects. In addition, this study points to the capacity of the brain to generate small peptides with neuroleptic-like properties.

INTRODUCTION

The endogenous opioid peptide β-endorphin (β-LPH 61-91) contains in its primary structure two classes of neuropeptides with distinctly different and opposite activities in several behavioural and psychopharmacological test systems (1,2). The two classes of β-endorphin fragments are represented by a) γ-endorphin (β-LPH 61-77) and related non-opioid peptides such as des-

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Abbreviations: β-LPH = β-lipotropin, DTγE = des-tyrosine-γ-endorphin, cSPM = crude synaptosomal plasma membrane, HPLC = high-pressure liquid chromatography, DNS- = dansyl-, 1-dimethylaminonaphthalene-5-sulfonyl-.
The amino acid residue numbers refer to the primary structure of human β-LPH.

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tyrosine-γ-endorphin (β-LPH 62-77, DTγE), and b) α-endorphin (β-LPH 61-76) and related peptides. The psychopharmacological profile of DTγE resembled that of neuroleptic drugs in various aspects (1,2) and this peptide reduced psychotic symptoms in schizophrenic patients (3). In recent structure activity studies in this laboratory it was found that the peptide β-LPH 66-77 was the shortest β-endorphin fragment, which possessed the full activity of DTγE (4). Thus, the β-LPH 66-77 sequence may be the active core for neuroleptic-like activity of γ-endorphin related peptides and the significance of β-LPH 66-77 as an endogenous neuroleptic-like neuropeptide was postulated.

In previous studies we have found evidence for the selective conversion of β-endorphin into peptides related to γ- and α-endorphin by peptidases associated with SPM preparations (5,6). DTγE was identified as one of the main β-endorphin metabolites and this peptide was shown to be present in the brain (7). The finding of β-LPH 66-77 as the shortest β-endorphin fragment with neuroleptic-like properties prompted us to extend our studies and to investigate the proteolytic conversion of DTγE.

MATERIALS AND METHODS

Synthetic β-LPH 62-77 (DTγE), β-LPH 65-77, β-LPH 66-77, β-LPH 67-77, β-LPH 68-77, β-LPH 69-77, β-LPH 70-77, β-LPH 62-76, β-LPH 66-76 and β-LPH 70-76 were kindly provided by Dr. H.M. Greven and Dr. J.W. van Nispen (Organon International B.V., Oss, The Netherlands).

A cSPM preparation from rat forebrain was obtained as described previously (8).

DTγE (4×10^{-5} M) was incubated with the cSPM preparation (3.5 mg protein) at 37°C in 1 ml 155 mM sodium chloride buffered with 25 mM sodium phosphate, pH 7.4. The reaction was terminated by heating the suspension in a boiling water bath for 10 min and the membranes were removed by centrifugation at 60,000 g_{av} for 30 min. The supernatant was fractionated by reversed-phase HPLC.

HPLC system I which was used for the fractionation of DTγE digests was carried out essentially as described previously (9). A μBondapak C_{18} reversed-phase column (0.39 x 30 cm) was eluted with a concave gradient of 30% to 75% acidified methanol (1.5 ml acetic acid per liter methanol) in 10 mM ammonium acetate, pH 4.15, over 45 min at a flow rate of 2 ml per min.

HPLC system II was designed for the separation of DTγE fragments containing the leucine-77 residue and was applied for the fractionation of peptide fractions obtained from system I. System II consisted of a concave gradient of 30% to 50% methanol in 10 mM sodium phosphate, pH 6.9, over 25 min at a flow rate of 2 ml per min using a μBondapak C_{18} reversed-phase column. The column used in system II was selected from several μBondapak C_{18} columns, as major column to column differences were encountered. These

differences did not occur in system I.

Freeze dried samples (circa 5 nmoles) were hydrolysed with 6 M hydrogen chloride, containing 1 µM thioglycolic acid to prevent losses of tyrosine and methionine, in evacuated glass tubes at 110°C in a constant boiling toluene bath for 22 hours as described by Zwiers et al. (10). The amino acid composition was determined by automated analysis (TSM, Technicon) using fluorescence detection with o-phtaldialdehyde (11). The amino acids of the unknown samples were quantified using a computing integrator (Pye Unicam DP101) in two ways: a) by comparison to norleucine as an internal standard, and b) by direct comparison to hydrolysates of reference peptides and to standard mixtures of amino acids, run in between. In this way, quantities of 20-200 pmoles could be determined in comparison to values of 0-5 pmoles in "blanc" HPLC-fractions.

Dansylation of N-terminal amino acid groups was essentially carried out as described by Gray and Smith (12). After hydrolysis and extraction dansylated amino acids were identified on micropolyamide thin layers (5 x 5 cm, Schleicher and Schüll) which were developed in a three solvent system (13).

RESULTS

HPLC fractionation of DTγE digests revealed the conversion of DTγE into several peptide fractions upon incubation with a cSPM preparation. In HPLC system I the two major fractions, I-C and I-D, co-migrated with a number of reference peptides such as β-LPH 66-77, β-LPH 67-77, β-LPH 68-77, β-LPH 69-77 (I-C) and β-LPH 65-77 and β-LPH 70-77 (I-D), respectively (fig. 1). Under the experimental conditions the conversion of DTγE was linear with time, while accumulation of the peptides in fractions I-C and I-D reached an optimum at 30 and 20 min, respectively (fig. 2). Fractions containing the DTγE fragments (I-A, I-B, I-C and I-D) were collected from the HPLC eluate and pooled for further analysis.

The amino acid composition of the fractions is presented in table I. The composition of the peptide in I-A was identical with that of β-LPH 62-73. The proline-73 residue of this peptide, which is not detected by column amino acid analysis, appeared to be present upon dansylation of the hydrolysate. The amino acid analysis indicated that fraction I-B contained the β-LPH 62-74 fragment. The analysis of fraction I-C suggested similarity to β-LPH 66-77, but the threonine content could account for 2 or 3 residues, when compared with the other analyses. Moreover, N-terminal end group determination revealed multiplicity of peptides in this fraction (table II).

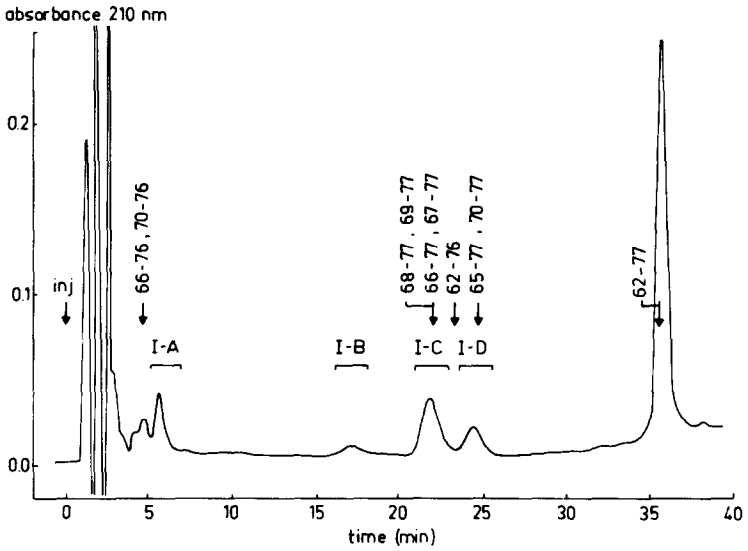

Figure 1. Reversed-phase HPLC of a digest of DTγE. Incubation of DTγE with a cSPM preparation was carried out for 45 min at pH 7.4. Chromatography was performed by gradient elution using an ammonium acetate buffer, pH 4.15, and acid methanol (system I). Experimental details are described in the text. The vertical arrows show the elution position of a series of synthetic peptides. Fractions were collected and pooled as indicated by the horizontal bars (I-A, I-B, I-C and I-D).

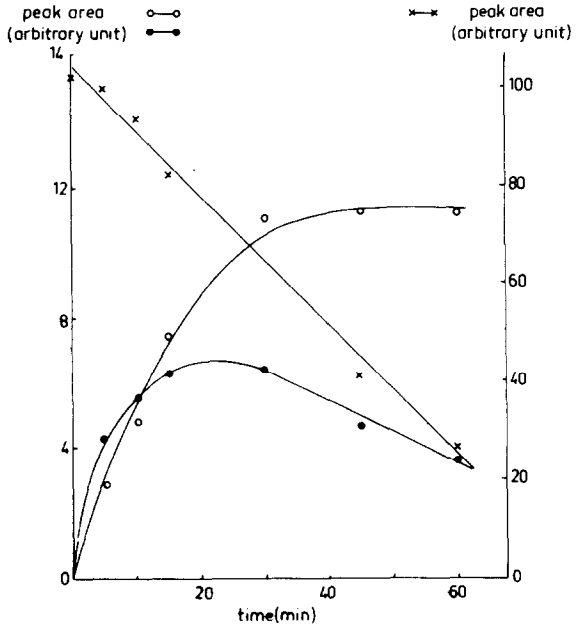

Figure 2. Time course of the conversion of DTγE by cSPM associated peptidases. The disappearance of DTγE (x) and the accumulation of fraction I-C (o) and I-D (•) were estimated from the peak areas in the HPLC profiles obtained at different time points after the start of the incubation.

Table I

AMINO ACID COMPOSITION OF HPLC FRACTIONS

OBTAINED AFTER DIGESTION OF DTγE

Amino Acid	Fraction I-A	I-B	I-C	I-D
Gly	2.4	2.2	0.1	0.2
Phe	1.1	0.7	0.0	0.0
Met	1.0	0.7	0.1	1.0
Thr+	1.7	1.5	2.2	2.5
Ser+	1.8	1.5	1.7	2.0
Glx	2.4	2.0	2.0	2.6
Lys	0.9	0.8	0.6	0.9
Pro++	-*	-*	-	-
Leu	0.2	1.0	1.7	2.0
Val	0.2	0.3	1.2	1.7
proposed sequence:	62-73	62-74	66/67/68-77	65-77

+ The values of threonine and serine are not corrected for destruction on hydrolysis.

++ Proline is not detected due to the o-phtaldialdehyde detection method; *present upon dansylation of the hydrolysate.

The indicated numbers of residues were calculated by dividing the pmole amounts obtained in the analyses by the highest common factor.

The amino acids are those contained in the primary structure of DTγE: Gly-Gly-Phe-Met^{65}-Thr-Ser-Glu-Lys-Ser^{70}-Gln-Thr-Pro-Leu-Val^{75}-Thr-Leu-OH.

Table II

N-TERMINAL AMINO ACID RESIDUES OF PEPTIDES ISOLATED

FROM DTγE DIGESTS

Fraction	N-Terminal End Group
I-C	Thr; Glu; Ser (trace)
I-D	Met; Gly (trace)
I-C1	Thr; Ser (trace)
I-C2	Glu
I-D1	Met

For the primary structure of DTγE see the legend to table I.

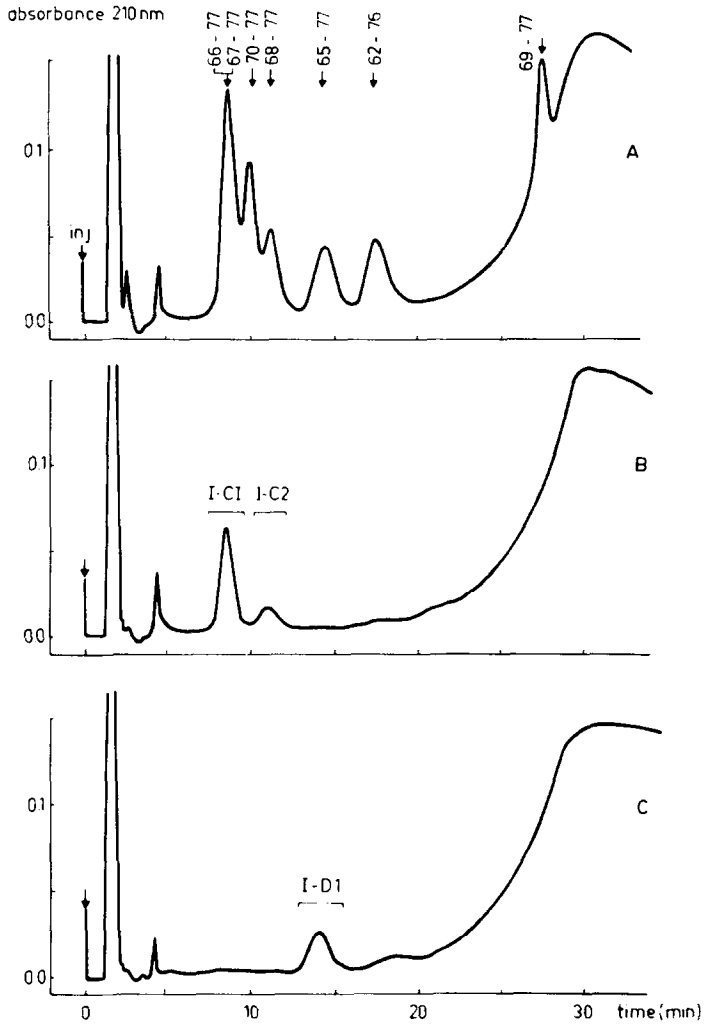

Figure 3. HPLC fractionation of a series of synthetic peptides related to DTγE (A) and the fractions I-C (B) and I-D (C) using a reversed-phase column eluted with a concave gradient of a sodium phosphate buffer, pH 6.9, and methanol (system II) as described in the text. The horizontal bars indicate the fractions which were collected from the eluate for N-terminal end group determination (I-C1, I-C2 and I-D1).

Based on composition and N-terminal end group, fraction I-D contained mainly β-LPH 65-77, but traces of an N-terminal glycyl peptide were present. Fractions I-C and I-D were subsequently subjected to refractionation. HPLC system II was designed for this purpose in order to achieve separation of the very similar leucyl-77 peptides (fig. 3A).

HPLC system II resolved I-C into two components, a major one comigrating

with β-LPH 66-77 and β-LPH 67-77 (I-C1), the other with β-LPH 68-77 (I-C2, fig. 3B). Fraction I-D eluted essentially as a single peak at the position of β-LPH 65-77 (I-D1). The presence of a minor component co-eluting with β-LPH 62-76 at a retention time of 18 min was indicated (fig. 3C). The identification of I-C1 as β-LPH 66-77 was established by the detection of DNS-threonine in N-terminal end group determination. A trace of DNS-serine indicated a minor amount of β-LPH 66-77 in this fraction. DNS-glutamic acid in I-C2 and DNS-methionine in I-D1 were in concordance with the presence of β-LPH 68-77 and β-LPH 65-77, respectively, in these fractions. The N-terminal amino acids detected in the various fractions are summarized in table II.

DISCUSSION

In the present study products of the proteolytic fragmentation of DTγE by cSPM associated peptidases have been characterized. The principal peptide fragments of DTγE are β-LPH 65-77, β-LPH 66-77 and β-LPH 62-73. In addition, β-LPH 67-77, β-LPH 68-77 and the N-terminal glycyl peptides, β-LPH 62-76 and β-LPH 62-74 are present in the DTγE digests in lower amounts.

The profile of the generated peptides is indicative of aminopeptidase as well as endopeptidase action. Firstly, the occurrence of β-LPH 65-77, β-LPH 66-77, β-LPH 67-77 and β-LPH 68-77 may suggest that these peptides originate by sequential removal of the N-terminal amino acid residues from DTγE by the sole action of aminopeptidases. Accordingly, we have previously detected aminopeptidase activity in this synaptic membrane preparation (5,8). However, the β-LPH 65-77 fragment appeared immediately upon incubation of DTγE and in the present experiments we did not detect the intermediate products β-LPH 63-77 and β-LPH 64-77. Therefore, β-LPH 65-77 may result from attack by an endopeptidase and the smaller fragments from successive aminopeptidase action on β-LPH 65-77. Finally, the direct formation of β-LPH 66-77 independently of the generation of β-LPH 65-77 seems likely in view of other

studies, which have demonstrated the formation of β-LPH 61-65 (methionine-enkephalin) by membrane bound brain peptidases (14,15,16). We have found accumulation of β-LPH 61-65 in digests of β-endorphin with brain cSPM fractions, when β-LPH 61-65 was protected against degradation by puromycin, bacitracin or acidic incubation conditions (Burbach, unpublished). Recently, one such membrane bound enzyme has been identified as a neutral endopeptidase cleaving the Met^{65}-Thr^{66} bond of [$Homoarg^{69}$]-β-LPH 61-69 (16). Either this or an endopeptidase with similar specificity may have been acting in our experiments, thus generating β-LPH 66-77. However, the ultimate demonstration of endopeptidase activity requires identification of the complementary peptide fragment.

In addition to the proteolytic events occurring in the N-terminal portion of DTγE, the presence of β-LPH 62-76, β-LPH 62-74 and β-LPH 62-73 is indicative of carboxypeptidase activity. These DTγE fragments were minor components in the digest, suggesting that carboxypeptidase action proceeds only with difficulty at pH 7.4. This is in agreement with previous findings that accumulation of α-endorphin in β-endorphin digests is relatively low at neutral conditions (5,14). Under these conditions β-endorphin is preferentially converted into γ-endorphin and related fragments (5,6,14).

The neuroleptic-like properties of DTγE have been established after in vivo administration of the peptide (1,2,3). DTγE reportedly inhibited ^{3}H-spiperone uptake in brain (17), but the peptide itself failed to interact with neuroleptic binding sites in vitro (18,19). Of several possibilities the in vivo effects have been proposed to be due to generation of a metabolite able to interact with binding sites (17). This was emphasized when in a preliminary experiment displacement of ^{3}H-spiperone occurred in vitro by incubates of DTγE with brain homogenates. The main metabolite was tentatively identified as β-LPH 66-77, but no evidence was provided that this peptide actually displaced ^{3}H-spiperone in vitro (17). These provisional data and the assessment of the active core of neuroleptic-like activity in β-LPH 66-77 (4)

suggests that this peptide is a potential candidate in the mediation of DTγE effects.

Using HPLC and amino acid analysis techniques we have actually demonstrated in this study the proteolytic conversion of DTγE into β-LPH 65-77 and β-LPH 66-77 by cSPM associated peptidases. Thus, the brain possesses the enzymatic capacity to generate short peptides with neuroleptic-like properties. This may favour the significance of β-LPH 65-77 and β-LPH 66-77 as endogenous neuropeptides. However, additional arguments such as the identification of these peptides in brain tissue and the demonstration of neuroleptic-like activities at a cellular level are prerequisite for the designation of β-LPH 65-77 and/or β-LPH 66-77 as endogenous neuroleptic-like neuropeptides.

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