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# β-ENDORPHIN BIOTRANSFORMATION IN BRAIN: FORMATION OF γ-ENDORPHIN BY A SYNAPTOSOMAL PLASMA MEMBRANE ASSOCIATED ENDOPEPTIDASE DISTINCT FROM CATHEPSIN D

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SUMMARY

cSPM preparations of rat brain contain a peptidase activity which generates γ-endorphin from β-endorphin. Some properties of this enzyme were studied and compared with those of cathepsin D. Maximal accumulation of γ-endorphin upon digestion of β-endorphin with a cSPM preparation was found at neutral pH values. The activity of cathepsin D, forming γ-endorphin and β-LPH 78-91 was limited to acidic pH values. The SPM associated peptidase was not inhibited by the specific cathepsin D inhibitor pepstatin. The peptidase activity remained associated with SPM preparations, which were purified extensively by sucrose density centrifugation. It is concluded that the SPM associated peptidase which generates γ-endorphin from β-endorphin is distinct from cathepsin D. Such an enzyme may have a physiological function in the formation of β-endorphin fragments in the brain.

Recent studies have shown that β-endorphin (β-LPH 61-91) may serve as a precursor for neuropeptides, which are involved in the regulation of behavioural adaptation (1,2). In a number of behavioural paradigms, β-endorphin fragments displayed distinct activities which were not mediated by opiate receptor sites (3). γ-Endorphin (β-LPH 61-77) and, in particular, the non-opioid peptide des-tyrosine-γ-endorphin (β-LPH 62-77) displayed activities resembling those of neuroleptic drugs (4,5). α-Endorphin (β-LPH 61-76) exerted effects opposite to those of γ-type endorphins: its activity was comparable to that of psychostimulant drugs (1,6). Therefore, it was suggested that a balance between γ- and α-type endorphins plays a significant role in the control of adaptive behaviour (1,7). Studies demonstrating the preferential formation of

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Abbreviations: β-LPH = β-lipotropin, (c)SPM = (crude) synaptosomal plasma membrane, HPLC = high-pressure liquid chromatography, $t_r$ = retention time. The amino acid residue numbers correspond to the primary structure of human β-LPH.

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either γ-endorphin and des-tyrosine-γ-endorphin or α-endorphin and des-tyrosine α-endorphin (β-LPH 62-76) from β-endorphin by a cSPM preparation supported this concept (8). Recently it was reported, that cathepsin D, an acid proteinase of lysosomal origin, cleaving specifically the $Leu^{77}$-$Phe^{78}$ bond of β-endorphin, generates γ-endorphin (9,10). This prompted us to compare the properties of the γ-endorphin generating peptidase in the SPM preparation with those of cathepsin D with respect to pH optimum and susceptibility to pepstatin, a specific cathepsin D inhibitor (11).

## MATERIALS AND METHODS

Synthetic human β-endorphin and related peptides were kindly supplied by Dr. H.M. Greven and Dr. J. van Nispen (Organon International B.V., Oss, The Netherlands). Pepstatin A was obtained from the Peptide Institute Inc. (Osaka, Japan) and cathepsin D (EC 2.4.23.5) from bovine spleen was a commercial preparation from Sigma Chemical Co. (St. Louis, USA).

A cSPM preparation from rat forebrain was obtained as described previously (12). When indicated membranes were further purified by sucrose density centrifugation according to Zwiers et al. (13). Briefly, the cSPM suspension was layered over a sucrose density gradient consisting of 1.0 M and 0.4 M sucrose. Centrifugation was carried out at 100,000 $g_{max}$ for 80 minutes and synaptosomal plasma membranes were collected from the 1.0-0.4 M interface. The membranes were washed and stored at -20°C.

β-Endorphin ($2 \times 10^{-5}$ M) was incubated with the SPM preparations (3.5 mg protein/ml) at 37°C for 30 min in 1 ml saline buffered with either 25 mM sodium phosphate (pH 7.4, 6.7 and 5.9) or 25 mM sodium acetate (pH 5.0 and 4.0). In experiments concerning enzyme inhibition, pepstatin dissolved in ethanol was included in the incubation mixture at a concentration of $10^{-4}$ M. In the absence of pepstatin equivalent amounts of ethanol were added to the incubation mixtures. The reaction was stopped by heating the suspension at 95°C for 10 min and the membranes were removed by centrifugation. The supernatant was fractionated by HPLC.

Cathepsin D (5 µg/ml; 12.5 U/mg) was incubated with β-endorphin ($2 \times 10^{-5}$ M) at pH 6.7 or pH 4.0 in the absence or presence of pepstatin ($10^{-4}$ M) at 37°C for 60 min. The reaction was terminated by heating and the digest was subsequently fractionated by HPLC.

HPLC fractionation of the digests was carried out essentially as described previously (14). A µBondapak C18 reversed-phase column was eluted with a concave gradient of 30% to 75% acidified methanol (1.5 ml acetic acid/l methanol) in 10 mM ammonium acetate pH 4.15 at a flow rate of 2 ml/min. The column was calibrated with a series of synthetic peptides related to β-endorphin as indicated in fig. 1 and fig. 3.

γ-Endorphin present in the digests was identified and quantitated by combined application of HPLC and radioimmunoassay. Fractions of the HPLC eluates containing γ-endorphin were collected. Methanol was evaporated in vacuo at 60°C and aliquots of the residual solution were subjected to a radioimmunoassay system for γ-endorphin as described elsewhere (14).

RESULTS

Digestion of β-endorphin by a cSPM preparation of rat brain resulted in the accumulation of a small number of peptide fragments (fig. 1A). Among other peptides γ-endorphin ($t_r$ = 36.8 min), des-tyrosine-γ-endorphin ($t_r$ = 35.4 min) and α-endorphin ($t_r$ = 26.8 min) were detectable by UV absorbance at

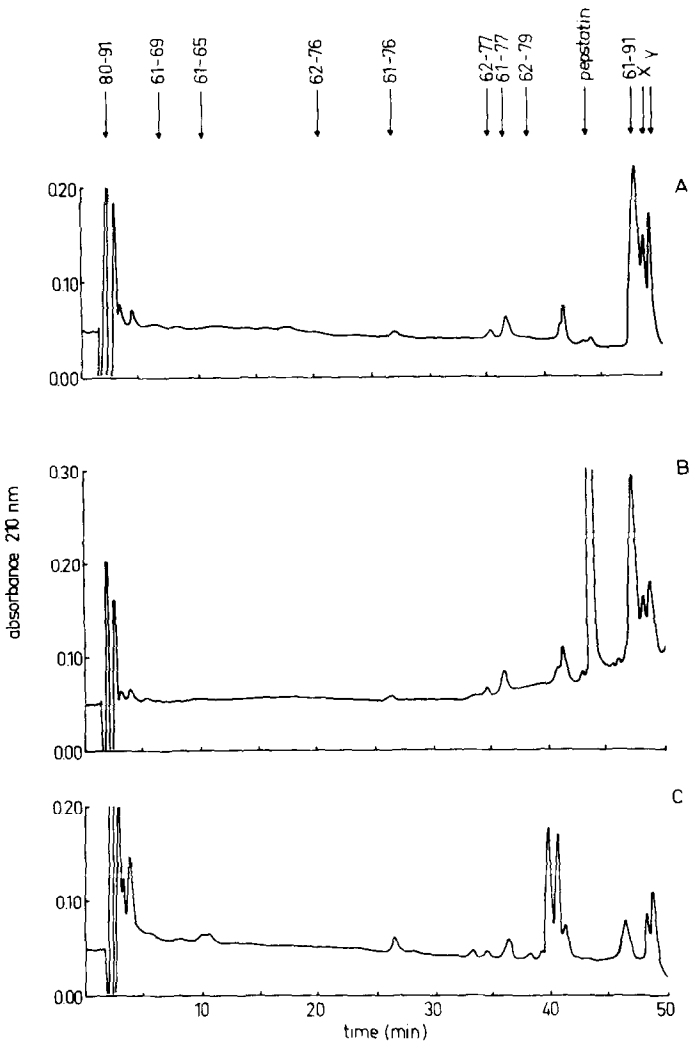


Figure 1:

HPLC separation of peptides accumulated during digestion of β-endorphin with SPM preparations. β-Endorphin was incubated with a cSPM fraction in the absence (A) or presence (B) of pepstatin at pH 6.7. The HPLC fractionation of a β-endorphin digest obtained with a purified SPM preparation is shown (C). Experimental details are described in the text. The elution positions of a series of synthetic β-endorphin fragments and pepstatin are indicated at the top. X and Y represent organic contaminants of the mobile phase.

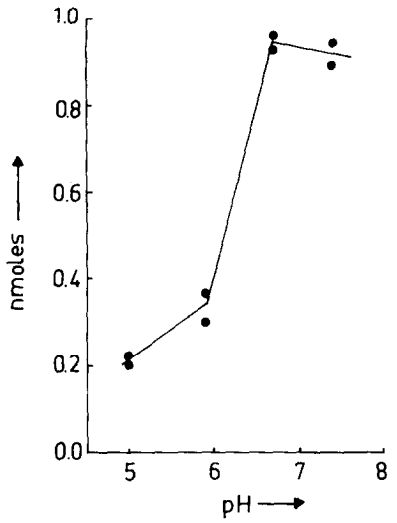


Figure 2:

pH dependency of the accumulation of γ-endorphin upon incubation of β-endorphin with a cSPM preparation. γ-Endorphin was quantitated after HPLC fractionation by radioimmunoassay as described in the text.

210 nm during HPLC fractionation of the digest. The identification of these β-endorphin fragments has been reported previously (8). The accumulation of γ-endorphin upon incubation of β-endorphin with the cSPM preparation was pH dependent. The largest amounts of γ-endorphin were detected at pH values around pH 7, while at acidic pH values only small amounts were present (fig. 2). At pH 4.0 no accumulation of γ-endorphin was detectable in the HPLC profile.

The fractionation profile of a digest obtained by incubation of β-endorphin in the presence of pepstatin is depicted in fig. 1B. The digest contained identical amounts of γ-endorphin and of residual β-endorphin when compared to the digest in fig. 1A. This clearly demonstrates that pepstatin did not inhibit the peptidase activity which converts β-endorphin to γ-endorphin.

Incubation of β-endorphin with cathepsin D at pH 4.0 resulted in the formation of γ-endorphin and the complementary C-terminal β-endorphin fragment β-LPH 78-91 (fig. 3A). The presence of $10^{-4}$ M pepstatin inhibited the activity of cathepsin D completely (fig. 3B). At pH 6.7 no activity of cathepsin D was observed (fig. 3C).

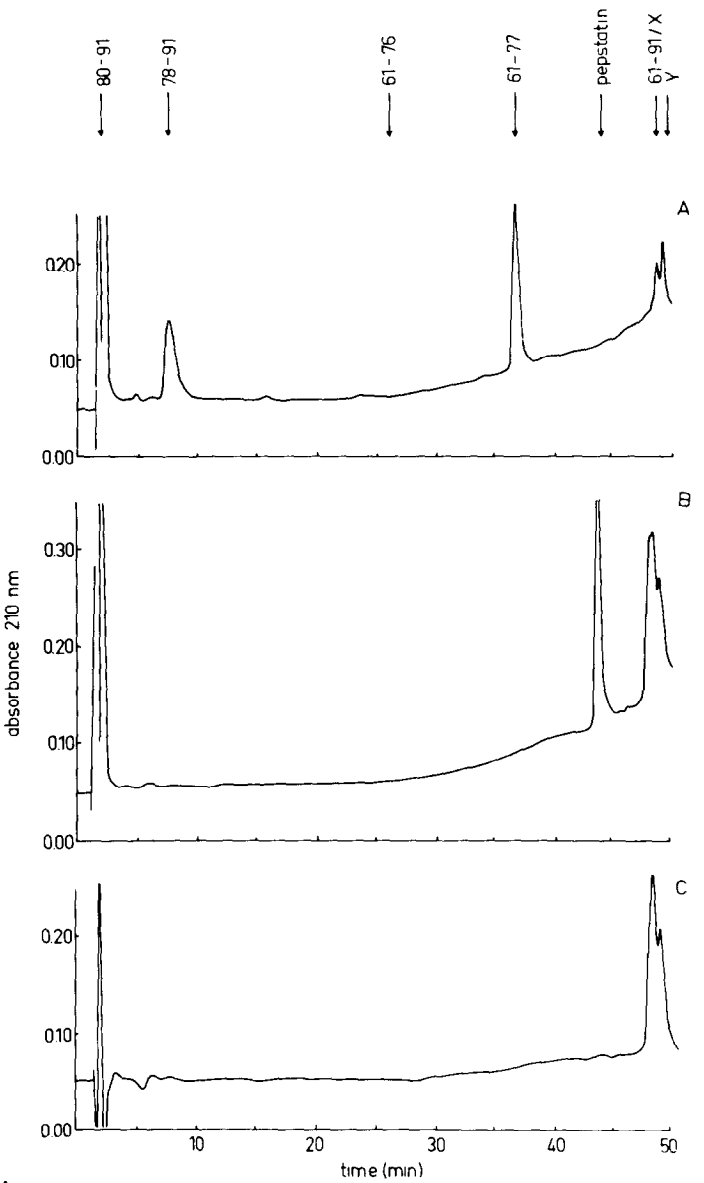


Figure 3:

HPLC separation of peptides formed by digestion of β-endorphin with cathepsin D in the absence (A) or presence of pepstatin at pH 4.0 (B) and at pH 6.7 (C). The elution positions of a number of synthetic β-endorphin fragments, pepstatin and organic contaminants of the mobile phase (X and Y) are indicated at the top. The incubation conditions are described in the text.

During digestion of β-endorphin by a SPM which was purified over sucrose density gradients γ-endorphin accumulated among other β-endorphin fragments such as des-tyrosine-γ-endorphin and α-endorphin (fig. 1C). Apparently the γ-endorphin generating activity was contained in the SPM.

DISCUSSION

The present study demonstrates that β-endorphin biotransformation in brain is mediated by a peptidase which converts β-endorphin to γ-endorphin. Some properties of this enzyme, such as pH dependency, resistance to inhibition by pepstatin and intimate association with SPM preparations are distinct from those of cathepsin D.

Cathepsin D, a lysosomal acid proteinase, has been reported to generate γ-endorphin from β-endorphin (9,10). Its pH range, however, is limited to acidic pH values, although different values have been reported depending on the substrate used (15). Human pituitary cathepsin D acts optimal at pH 3.2 using hemoglobin as substrate and at this pH value it cleaves the $Leu^{77}$-$Phe^{78}$ bond of β-LPH and β-endorphin readily (9). Cathepsin D in a crude extract of porcine anterior pituitary was found to cleave the same peptide bond of β-LPH at a pH optimum of pH 3.5-4.5, while the range of this reaction was extended to pH 7.0 (10). In our experiments the activity of cathepsin D was limited to acidic conditions. Using cathepsin D from bovine spleen, β-endorphin was cleaved at pH 4.0, but no activity of cathepsin D was detectable at pH 6.7 even during prolonged incubation at high enzyme concentrations. Moreover, cathepsin D action was completely inhibited by pepstatin. In experiments with SPM maximal amounts of γ-endorphin accumulated around pH 7, whereas pepstatin did not have any inhibitory effect in this proteolytic process. Although subsequent proteolytic mechanisms following the formation of γ-endorphin may have affected the final accumulated amount of γ-endorphin in the digests, the present study clearly demonstrates that the SPM associated peptidase activity cleaves β-endorphin at neutral pH values.

Cathepsin D is a soluble enzyme, which is liberated upon hypo-osmotic shock of the lysosomal fraction. In our membrane preparations the γ-endorphin generating enzyme activity remained associated with the membranes after extensive washing in 0.9% NaCl. This may be compatible with data of Austen et al. In experiments with washed brain membranes and $^{125}I$-labeled β-endorphin

these authors have found formation of [$^{125}$I]-γ-endorphin at neutral pH (16). In addition, we observed that the peptidase activity was preserved even after purification of the cSPM fraction indicating that such proteolytic activity may be an intrinsic component of synaptosomal plasma membranes. The here described experiments lead us to conclude that the SPM associated formation of γ-endorphin from β-endorphin is not due to contamination by cathepsin D but is mediated by a peptidase with distinct properties. Previously we have detected the complementary C-terminal fragment of β-endorphin, β-LPH 78-91, together with γ-endorphin in β-endorphin digests (unpublished). This observation suggests that the γ-endorphin generating enzyme is an endopeptidase and that it is involved in the initial, rate limiting step in the biotransformation of β-endorphin in the brain.

Recently γ-endorphin, α-endorphin and their des-tyrosine fragments have been found in rat brain and pituitary gland (17). It seemed unlikely that these β-endorphin fragments were produced artificially due to post-mortem degradation and the extraction procedure. Their presence persisted upon sacrifice of the rats either by microwave irradiation or decapitation followed by rapid heat inactivation of peptidases in the intact tissues and subsequently by different extraction methods. Moreover, β-endorphin fragments are present in human lumbar CSF (18) while in CSF no β-endorphin converting peptidase activity could be demonstrated (19). These data provided suggestive evidence that γ-endorphin, α-endorphin and their des-tyrosine fragments are endogenous peptides formed by selective cleavage of β-endorphin. The endopeptidase described in this study may play a key role in the generation of these neuropeptides. We have observed that depending on the pH of the in vitro system γ-endorphin is rapidly converted to des-tyrosine-γ-endorphin by aminopeptidase action or to α-endorphin by a presumed carboxypeptidase activity (unpublished). In view of the localization of neuropeptide receptor sites in synaptosomal plasma membranes (20-24) enzymatic conversion of β-endorphin at these sites may be of physiological

importance. Further studies are required to establish the significance of the γ-endorphin generating endopeptidase as a regulatory factor in endorphin homeostasis in brain.

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