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γ-ENDORPHIN GENERATING ENDOPEPTIDASE IN RAT BRAIN: SUBCELLULAR AND REGIONAL DISTRIBUTION

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β-Endorphin is converted into the biologically active fragment γ-endorphin by an endopeptidase which we term "γ-endorphin generating endopeptidase". Subcellular and regional distributions of this endopeptidase activity in rat brain were studied by a newly developed assay. After subcellular fractionation of rat brain tissue γ-endorphin generating endopeptidase activity was predominantly recovered in the cytosolic fraction. A 10 to 15 fold lower activity was present in synaptosomes, mitochondria and synaptic membranes. Hardly any endopeptidase activity was detected in nuclei and myelin. The endopeptidase activity in cytosolic and particulate fraction was found throughout brain, pituitary and spinal cord in a rather homogeneous fashion. Cytosolic activity in all brain parts was 10 to 15 fold higher than the activity in the particulate fraction. It is suggested that rather the β-endorphin distribution than the endopeptidase is restricting for γ-endorphin production in certain brain parts.

γE is an endogenous peptide of the POMC system in pituitary, brain and testis (1-6). The peptide has distinct biological effects in behavioral and pharmacological test situations (7-10); its biological properties differ from αE and βE. γE is produced by a single proteolytic cleavage of the Leu^{17}-Phe^{18} bond of βE (12-14) by an endopeptidase that has originally been discovered in brain synaptic membranes. We have termed this endopeptidase activity "γE generating endopeptidase": γEGE (15). For further study of this endopeptidase an assay was developed based on cleavage of a synthetic substrate [Ac-Val^{15},(ε-$^{14}CH_3$)Lys^{19}-$NHCH_3$]βE-(15-19) (Fig. 1), that is derived from the βE midportion containing the cleavage site for γEGE activity (Leu^{17}-Phe^{18}) (15). Here we report the subcellular and regional distribution of γEGE activity in rat brain.

MATERIALS AND METHODS

Endopeptidase assay - Particulate or cytosolic fractions of the brain parts were incubated in a volume of 50 µl at a protein concentration (16) of 3.5

Abbreviations: αE, α-endorphin (β-endorphin-(1-16)), γE, γ-endorphin (β-endorphin-(1-17)); βE, β-endorphin-(1-31); γEGE, γ-endorphin generating endopeptidase; POMC, pro-opiomelanocortin.

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mg/ml in 50 mM Tris/HCl, pH 8.5 with 0.86 µM substrate (10,000 dpm) at 37°C for 30 min. The incubation was stopped by adding 50 µl 2 M acetic acid and subsequent boiling for 10 min. It was found not neccessary to remove particles from the digest previous to separation on the polystyrene columns. After the incubation with brain tissue [^{14}C]-labeled fragments derived by γEGE activity were separated from the intact [^{14}C]-labeled substrate by hydrophobic chromatography on Amberlite XAD-2 polystyrene beads . These polystyrene columns were packed in 1000 µl pipet tips and eluted stepwise with 10 % and 100 % ethanol. Radioactivity of the fragments and the intact substrate were determined separately by liquid scintillation counting.

Subcellular fractionation - The forebrains of 12 male Wistar rats (180-200 g.) were obtained and subcellular fractionation was performed as described previously (17,18). Briefly, the homogenate was centrifuged at 1000 g_{av} for 10 min. Nuclei were isolated by taking up the pellet (P_1) in 2 M sucrose and centrifugation at 12,500 g_{av} for 90 min. The nuclei were obtained as a pellet. The supernatant (S_1) was centrifuged at 10,000 g_{av} for 20 min. and the resulting pellet (P_2) was resuspended in 0.32 M sucrose. Mitochondria, synaptosomes and myelin were separated by centrifugation on a 1.2 M - 1.0 M - 0.85 M sucrose density gradient at 100,000 g_{max} for 60 min. Myelin accumulated on the top of the 0.85 M layer, the synaptosomes at the 1.0 M - 1.2 M interface and the mitochondria at the bottom of the tube. Synaptic membranes were obtained by lysis of the P_2 pellet and centrifugated at 10,000 g_{av} for 20 min. followed by fractionation of the supernatant on a discontinuous 1.0 M - 0.4 M sucrose density gradient. Synaptic membranes accumulated at the interface of the two sucrose densities after 100,000 g_{max} for 80 min. The S_2 supernatant was centrifuged at 17,000 g_{av} for 60 min. to obtain a supernatant from which microsomes and cytosol were obtained by centrifugation at 100,000 g_{max} for 60 min. All the particulate fractions were washed twice with 0.9% NaCl and stored at 4°C for 16 hours. The cytosolic fraction (2 ml) was dialysed against 1000 ml 0.9% NaCl for 16 hours. Incubations were performed immediately after storage or dialysis.

Regional distribution - Dissection of rat brain (Wistar, 180-200 g.) was performed as previously described (19). From the tissues cytosol and particulate fractions were prepared by homogenizing the different brain parts in 100 mM Tris/HCl buffer, pH 8.5, and centrifuging the 1000 g_{av} supernatant of this homogenate for 60 min. at 100,000 g_{max}. The pellet was washed twice in the same buffer. γEGE activity was determened immediately after preparation.

RESULTS

γEGE activity in cytosolic and particulate fractions was determined by the assay according to the principle depicted in fig 1. γEGE activity fulfilled all the criteria as previously drawn up for γEGE activity in synaptic membranes: the initial cleavage site of the substrate was at the Leu^{17}-Phe^{18} bond and substrate conversion was competitively inhibited by βE. There was a linear relationship between protein or time with the substrate conversion rate and the pH optimum of the endopeptidase activity for both the cytosolic and the particulate fraction was 8.5 (data not shown).

After fractionation of forebrain tissue the highest γEGE activity was recovered in the cytosolic fraction (Fig. 2). The activity was 10 to 15 fold higher than in the particulate fractions. Amongst the particulate preparations synaptosomes, mitochondria and synaptic membranes contained the

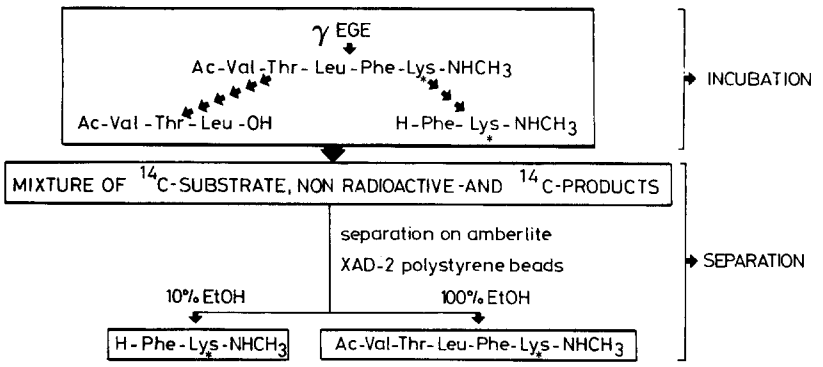

Fig. 1. The principle of the used assay. The substrate, which is radioactively labeled at the ϵ-NH_2 group of the Lys residue (indicated by *), is incubated with a tissue homogenate that contains γEGE activity. The reaction is stopped with acetic acid and subsequent boiling. The separation of labeled fragments from intact substrate is performed by hydrophobic chromatography. With 10 % ethanol the fragment Phe-Lys*-$NHCH_3$ elutes, and with the 100 % ethanol elution step the intact peptide elutes. Both fractions are collected in separate vials and radioactivity is determined by liquid scintillation counting.

highest endopeptidase activity. The endopeptidase activity in myelin and nuclei was very low.

For experiments on the regional distribution of γEGE activity, a cytosol and a a total particulate fraction, containing mitochondria, synaptosomes, synaptic membranes microsomes and myelin, were prepared from microdissected brain tissues. γEGE activity in both the cytosolic and the particulate fraction was detected throughout the brain and rather homogeneously distributed (Fig. 3). In all brain parts endopeptidase activity in the cytosolic fraction was 10 to 15 fold higher than in the particulate fraction. The highest cytosolic activity

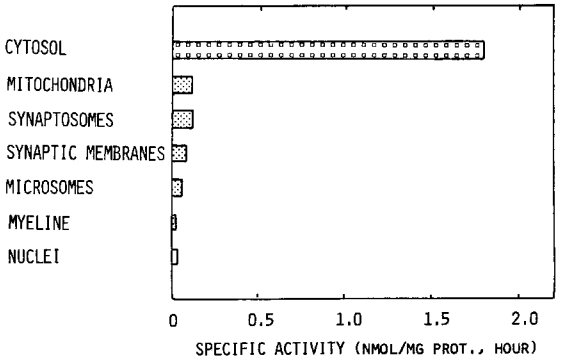

Fig. 2. Subcellular distribution of γ-endorphin generating endopeptidase activity. Data are expressed as mean of two experiments.

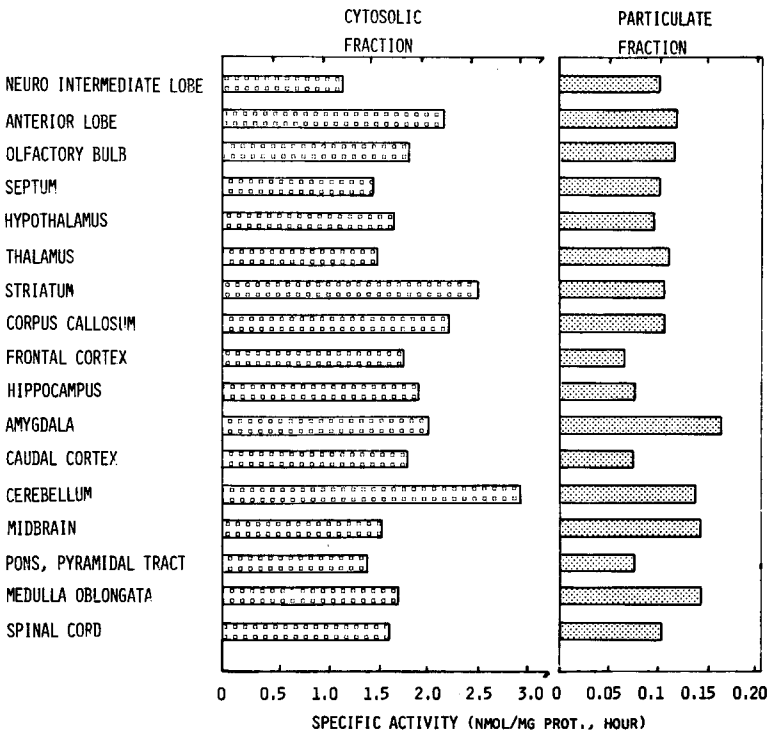

Fig. 3. Regional distribution of γ-endorphin generating endopeptidase activity in rat brain, pituitary and spinal cord. Data are expressed as mean of three experiments.

was present in the cerebellum and differed about 2 fold from the neuro intermediate lobe, the area that contained the lowest γEGE activity in the cytosolic fraction. The highest particulate activity, in amygdala was about 2.5 fold higher than the lowest activity in frontal cortex.

DISCUSSION

This paper focusses on an endopeptidase activity that converts βE directly into γE, a peptide with distinct behavioral effects. γEGE activity was originally found in studies on the proteolysis of βE by synaptic membranes (12-15). The present data reveal that the main endopeptidase activity appears in the cytosolic fraction after subcellular fractionation. The concentration of γEGE activity in the cytosolic fraction exceeds that in particulate preparations 10 to 15 fold, suggesting that γEGE activity is a true tissue cytosolic endopeptidase. It remains to be investigated whether γEGE activity in cytosol and membranes represents the same endopeptidase or not.

As components of the POMC system βE and its fragments αE and γE have a well defined distribution in the brain. They are most concentrated in the hypothalamus and to a lesser extent in septum, hippocampus and amygdala. Other brain areas contain only very little of these peptides. In the present study we found the EGE activity present throughout the brain, with only a 2-3 fold difference between areas with highest and lowest activity. This indicates that γEGE activity is not a component of the POMC system per se, but a cellular constituent of brain tissue in general. Therefore it may be suggested that all brain areas have the potential to convert βE into γE.

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