Localization of the thiorphan-sensitive endopeptidase, termed enkephalinase A, on glial cells

Hans Lentzen and Jochen Palenker

Pharmakologisches Institut der Freien Universität Berlin, Thielallee 69/73, 1000 Berlin 33, Germany

Received 21 December 1982

Degradation of tritiated Leu-enkephalin was studied in cultures of primary astrocytes from rat brain. The incubation experiments with a cell suspension revealed Tyr as the main tritiated metabolite; however, Tyr–Gly–Gly and Tyr–Gly were detectable as well. Using a crude membrane preparation of the astrocytes, we found about equal amounts of Tyr and Tyr–Gly–Gly but only trace quantities of Tyr–Gly. The production of Tyr was completely inhibited by bestatin, an inhibitor of aminopeptidases, that of Tyr–Gly–Gly by thiorphan, a specific inhibitor of enkephalinase A. The results prove the ability of glial cells to degrade enkephalin by aminopeptidase and a membrane-bound enkephalinase A.

Primary astrocyte *Leu-enkephalin* *Aminopeptidase* *Enkephalinase A*

1. INTRODUCTION

Soon after identification of the enkephalins, numerous authors showed that aminopeptidases present in the brain and in a variety of peripheral tissues are able to degrade these pentapeptides [1–5]. Enkephalinase A, a dipeptidylpeptidase activity present in crude membrane fractions of rat brains, which generates the tyrosine–glycine–glycine (Tyr–Gly–Gly) fragment from enkephalin, was reported in [6,7]. This enzyme appears to have the specificity of a physiologically relevant enkephalinase. The discovery of thiorphan as a reasonably specific inhibitor of enkephalinase A [8] permits a more thorough evaluation of the importance of this enzyme for the degradation of exogenous enkephalin.

Studies on neuroblastoma cells (N4TG1) [9] and cultured human endothelial cells [10] demonstrate that these cells are also able to degrade enkephalins effectively. However, only aminopeptidase activity, which cleaves the Tyr–Gly amide bond, was detected in these studies. Our investigations on glial cells in culture show that enkephalin degradation appears to result from the action of aminopeptidase, enkephalinase A and a Tyr–Gly producing mechanism.

2. MATERIALS AND METHODS

Tyrosyl-[^{3}H]Leu-enkephalin was obtained from New England Nuclear (Dreieich), all amino acids, peptides and bestatin from Sigma Chemical (München). Captopril was a gift of Heyden (München). Thiorphan was kindly donated by Professor B.P. Roques (Département de Chimie Organique, Université René Descartes, Paris).

2.1. *Cell culture*

Primary glial cultures of 2-day-old rats were cultured as in [11] with a slight modification [12]. The dissociated cells were grown in 150 cm^2 plastic flasks at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. The medium, which contained 20% and after 10 days 10% fetal calf serum, was changed twice a week and the experiments were performed with the suspended cells during the third week of cultivation. Cultures usually were observed by phase contrast or differential interference contrast (DIK) microscopy.

2.2. *Incubation experiments*

Cells, grown as monolayers, were cautiously scraped off and centrifuged at 150 × *g* for 10 min. They were washed thrice with Krebs–Ringer–Tris

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

(KRT) buffer (pH 7.4) containing NaCl (128 mM), $CaCl_2$ (1.4 mM), $MgCl_2$ (1.4 mM), KCl (5 mM), Tris–HCl (20 mM) and D-glucose (5 mM). In some experiments we prepared a crude membrane fraction according to [13]. The cells or the crude membranes were finally suspended in KRT buffer (~1 mg protein/ml suspension). Protein was determined by the Lowry method [14]. The suspension (40 μl) was incubated with 120 nM [^{3}H]Leu-enkephalin (20 μl) and 40 μl KRT buffer. The reaction was terminated by adding 10 μl 0.8 M HCl and freezing in liquid nitrogen. When the influence of drugs like bestatin, captopril or thiorphan was studied, the KRT buffer was replaced by the drug solution made in buffer.

2.3. *Analysis of the degradation products by HPLC*

HPLC was carried out with a Gynkotek constant flow pump (model 600/200). Generally, 20 μl samples were injected with a 50 μl Hamilton syringe through a stop flow injector (model Rheodyne). The initial flow rate was 1.0 ml/min and was increased to 2.0 ml/min after 4 min. The ^{3}H-labeled products were identified by their coelution with unlabeled standards, which were detected spectrophotometrically at 254 nm. For convenience, we used two isocratic systems instead of a gradient. The first isocratic system was used to separate Tyr, Tyr–Gly–Gly and Tyr–Gly, while Tyr–Gly–Gly–Phe and Leu-enkephalin were separated by the second one. The mobile phase had the following composition:

First system: citrate–phosphate buffer (0.01 M + 0.3 mM 1-octanesulfonic acid sodium salt monohydrate):methanol (90:10) pH 3;

Second system: phosphate buffer (0.1 M, pH 6.0):methanol (65:35).

The column (stainless steel, 12.5 cm length, 4.1 mm i.d.) was packed by the slurry technique with reversed phase material (Hypersil ODS Shandon, mean particle size 5 μm). First we eluted with system 1; after 6.8 min we used system 2. A chromatographic run was terminated after 13 min. Retention times of the metabolites were: Tyr 4 min, Tyr–Gly–Gly 6 min, Tyr–Gly 7 min, Tyr–Gly–Gly–Phe 9.5 min and Leu-enkephalin 12 min. After each chromatographic run methanol (2 ml/min, 10 min) and the mobile phase system 1 (4 ml/min, 15 min) had to percolate through the column.

3. RESULTS

3.1. *Hydrolysis of Leu-enkephalin by primary astrocytes in suspension*

The rates of enkephalin degradation by primary astrocytes in suspension at 0°C and 25°C are shown in fig.1. At 25°C, a 70% degradation is observed within 30 min. This enzymatic process is almost inactive at 0°C. Analysis of the degradation products performed after incubation of [^{3}H]Leu-enkephalin with the cell suspension, yields four radioactive peaks with retention times closely corresponding to those of Tyr, Tyr–Gly–Gly, Tyr–Gly and Leu-enkephalin. Fig.2 shows the curves representing the time-dependent formation of [^{3}H]Tyr, [^{3}H]Tyr–Gly–Gly and [^{3}H]Tyr–Gly. We found a very low level of the breakdown products Tyr–Gly–Gly and Tyr–Gly as compared to the main metabolite Tyr. For example, after 6 min ~30% of Leu-enkephalin was degraded. Of the degraded enkephalin 77% was accounted for by Tyr formation, 8% by Tyr–Gly and 15% by Tyr–Gly–Gly formation.

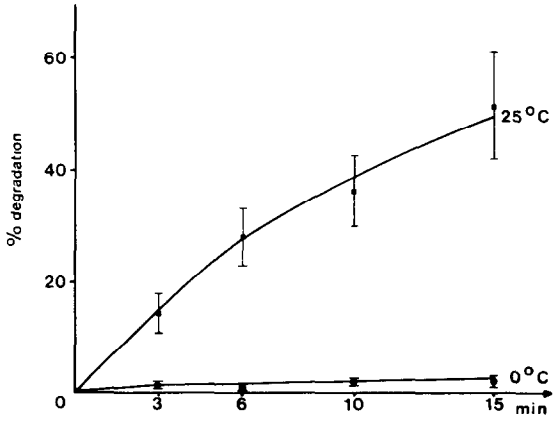

Fig.1. Time course of enkephalin degradation at 0°C and 25°C: 40 μl cell suspension (1 μg protein/1 μl) were incubated at 0°C and 25°C in 100 μl KRT buffer (pH 7.4) containing 120 nM [^{3}H]Leu-enkephalin. Enzyme activity was measured as in section 2. Mean values of 5 expts ± SD.

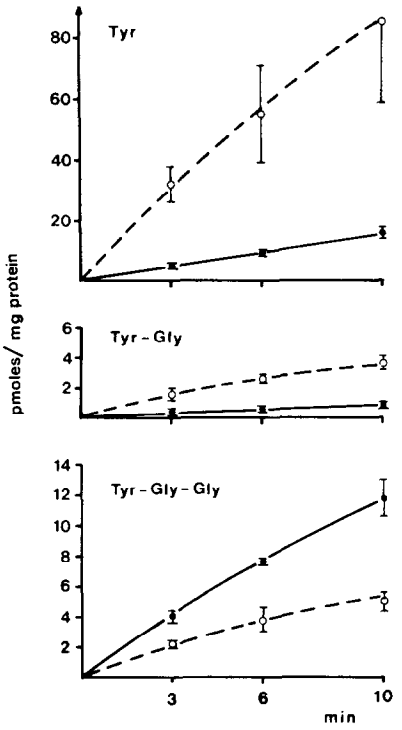

Fig.2. Time course of the formation of Tyr, Tyr–Gly and Tyr–Gly–Gly by enkephalin degradation at 25°C. Incubation was performed with the cell suspension (– – –) and with the crude membrane fraction (——) under the same conditions as mentioned in fig.1. Mean values of 5 expts ± SD.

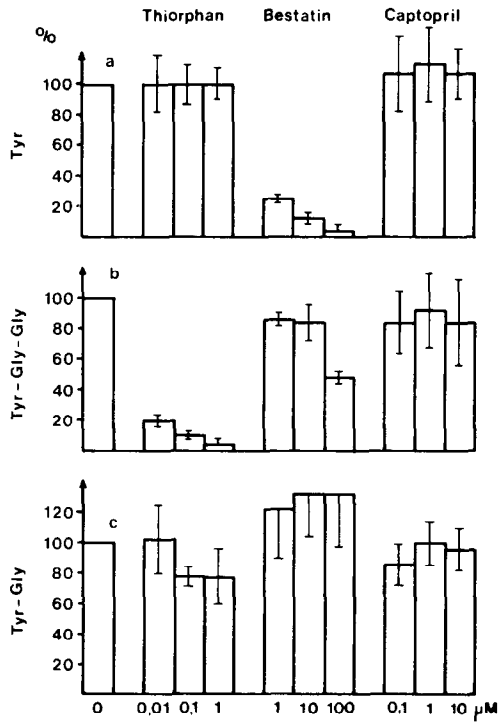

Fig.3. Influence of inhibitors on the formation of the Tyr-containing metabolites. The crude membranes were preincubated with the inhibitors for 15 min and then incubated in 100 μl KRT buffer (pH 7.4) containing 120 nM [^{3}H]Leu-enkephalin for 6 min at 25°C. Influence of thiorphan, bestatin and captopril on: (a) the Tyr-formation; (b) the Tyr–Gly–Gly formation; (c) the Tyr–Gly production. Mean values of 5 expts ± SD.

3.2. *Hydrolysis of Leu-enkephalin by crude membranes of primary astrocytes*

Proceeding on the postulation that a specific peptidase involved in the control of enkephalin concentration in the synaptic cleft ought to be membrane-bound, we used the crude membrane fraction to study the enkephalin breakdown. In this case, we found approximately equal amounts of Tyr and Tyr–Gly–Gly, but only trace quantities of Tyr–Gly (based on mg protein at 25°C) (fig.2).

3.3. *Effects of inhibitors on Leu-enkephalin degradation by crude membranes of primary astrocytes*

We used three inhibitors for further characterization of the enkephalin degradation pathways. Bestatin shows a concentration-dependent inhibition of Tyr formation. Only the highest concentration (10^{-4} M) of the inhibitor reduced the Tyr–Gly–Gly production. The Tyr–Gly formation seemed to be increased slightly in the presence of bestatin. Thiorphan (over 10^{-8}–10^{-6} M) has no effect on Tyr and Tyr–Gly formation, whereas it decreases the tripeptide production dramatically. Captopril (10^{-7}–10^{-5} M) was ineffective in all cases (fig.3).

4. DISCUSSION

In respect to the method used, this study shows a clear-cut separation of all possible Tyr containing breakdown products of the pentapeptide Leu-enkephalin with the help of HPLC. Another HPLC method has been described [15] which uses ligand exchange chromatography to separate all the possible breakdown products of enkephalin.

Our method using ion-exchange chromatography allows the separation of the metabolites Tyr, Tyr–Gly and Tyr–Gly–Gly with one isocratic system.

The brain peptides Leu-enkephalin and Met-enkephalin bind to opiate receptors and are thought to act as neurotransmitters in processes related to pain [16–18]. Several enkephalin degrading enzyme activities are known to be present in the brain: a soluble aminopeptidase cleaves the Tyr–Gly bond of enkephalin [1,15], a particulate endopeptidase (enkephalinase A) which hydrolyzes the Gly–Phe bond [6,15] and enkephalinase B, which cleaves the Gly–Gly bond [19]. However, their relative contribution to the regulation of enkephalin levels and in the termination of synaptic events has not yet been established [1,6,19–21].

The incubation experiments with primary astrocytes in suspension revealed Tyr as the main metabolite. If a crude membrane preparation of these glial cells is used, one finds a pronounced decrease in the specific activity of Tyr. This might be due to the removal of the Tyr producing enzyme, presumed to be associated with membranes, during the membrane preparation. Furthermore, it could not be excluded that some cells in the suspension are not intact. In this case, a part of the Tyr producing enzyme activity might be of intracellular origin. However, results with astrocytes grown on glass cover slides, which were neither scraped off nor suspended, demonstrate the same specific activity of the Tyr-producing enzyme as compared with a cell suspension (not shown).

Wagner and Dixon [22] examined 8 protease inhibitors of microbiological origin and found that bestatin was a potent inhibitor of a rat brain enkephalin aminopeptidase. The inhibitory effect of bestatin on Tyr formation of primary astrocytes observed in our experiments suggested that the cleavage of the Tyr–Gly bond is catalyzed by an aminopeptidase.

In respect to the Tyr–Gly formation we can only say that the enzyme probably involved is membrane-associated, not membrane-bound, similar to the Tyr producing aminopeptidase. Tyr–Gly formation in our experiments is probably not a product of the enkephalinase B reaction [19] as it is insensitive to *p*-chloromercuribenzoate. Furthermore, the existence of a Tyr–Gly producing dipeptidylaminopeptidase [23] is unlikely, because this enzyme is inhibited by *o*-phenanthrolin, a drug which was ineffective on primary astrocytes. Our special interest pertains to the formation of Tyr–Gly–Gly. Besides the particulate enkephalinase A [6], angiotensin-converting enzyme (ACE, EC 3.4.15.1, kininase II) [24,25] could also contribute to enkephalin degradation. Enkephalinase A and ACE are endopeptidases or dipeptidyl carboxypeptidases, which split the Gly–Phe bond. The use of selective inhibitors of enkephalinase A such as thiorphan [8], and of ACE such as captopril might, therefore, aid in evaluating the relative contribution of these enzymes to enkephalin degradation.

The Tyr–Gly–Gly producing enzyme is membrane-bound. Its specific activity in the membrane preparation is ~2–3-fold that of the cell suspension. Thiorphan over 10^{-8}–10^{-6} M, decreases the tripeptide production dramatically, whereas it has no effect on Tyr and Tyr–Gly production. However, captopril (10^{-7}–10^{-5}M) was ineffective in all cases.

From the observations that primary astrocytes show Tyr–Gly–Gly formation during enkephalin degradation, that this formation exhibits a very high sensitivity to thiorphan and that the other inhibitors used (except bestatin 10^{-4} M) have no influence on the production of this metabolite, we conclude that glial cells possess enkephalinase A. Due to the results obtained with the crude membrane fraction, we assume the localization of this enzyme to be mainly on cell membranes.

It is very interesting that the glial cells are able to degrade enkephalin so efficiently. There might be some doubts as to the validity of the conclusion that enkephalinase A is situated on neuronal membranes as these experiments have been conducted on synaptosome preparations [6,26,27] and a contamination with glial cells cannot be excluded. However, further experiments are being conducted with neurons and neuron-like cells to investigate the presence of enkephalinase on the neuronal membrane.

The role of the glial cells as a site of enkephalin degradation can at present only be a target of speculation. The putative biological relevance of this enzymatic process could depend on the lack of both enkephalins and their receptors on the glial cells. The presence of enkephalins in astrocytes is

presently being studied. To the best of our knowledge, no enkephalin receptors have as yet been located on astrocytes. Miller et al. [28] found no enkephalin receptors on C 6 glioma cells. The enkephalins can be more or less specifically degraded by a series of enzymes. If we assume that various types of cells possess such mechanisms in their synaptic regions, then the extracellular enkephalin concentration would be kept at a minimum. Thus, our results fit well in the general concept that one of the functions of glial cells is the prevention of the spread of neural (e.g., enkephalinergic) impulses into improper channels or target sites.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the expert technical assistance of Mrs I. Reinsch, Mrs U. Brandt and Mrs I. Monden.

REFERENCES

[1] Hambrook, J.M., Morgan, B.A., Rance, M.J. and Smith, C.F. (1976) Nature 262, 782–783.
[2] Marks, N., Grynbaum, A. and Neidle, A. (1977) Biochem. Biophys. Res. Commun. 74, 1552–1559.
[3] Meek, J.L., Yang, H.-Y.T. and Costa, E. (1977) Neuropharmacology 16, 151–154.
[4] Dupont, A., Cusan, L., Garon, M., Alvarado-Urbina, G. and Labrie, F. (1977) Life Sci. 21, 907–914.
[5] Vogel, Z. and Alstein, M. (1977) FEBS Lett. 80, 332–335.
[6] Malfroy, B., Swerts, J.P., Guyon, A., Roques, B.P. and Schwartz, J.C. (1978) Nature 276, 523–526.
[7] Sullivan, S., Akil, H. and Barchas, J.D. (1978) Commun. Psychopharmacol. 2, 525–531.
[8] Roques, B.P., Fournié-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C. and Schwartz, J.C. (1980) Nature 288, 286–288.
[9] Hazum, E., Chang, K.-J. and Cuatrecasas, P. (1979) Life Sci. 24, 137–144.
[10] Erdös, E.G., Johnson, A.R. and Boyden, N.T. (1978) Biochem. Pharmacol. 27, 843–848.
[11] Booher, J. and Sensenbrenner, M. (1972) Neurobiology 2, 97–105.
[12] Noske, W., Lentzen, H. and Herken, H. (1981) Cell. Mol. Biol. 28, 235–244.
[13] Gilbert, J.A., Knodel, E.L., Stenstrom, S.S. and Richelson, E. (1982) J. Biol. Chem. 257, 1274–1281.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Guyon, A., Roques, B.P., Guyon, F., Foucault, A., Perdisot, R., Swerts, J.-P. and Schwartz, J.C. (1979) Life Sci. 25, 1605–1612.
[16] Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. (1975) Nature 258, 577–579.
[17] Simantov, R. and Snyder, S.H. (1976) Proc. Natl. Acad. Sci. USA 73, 2515–2519.
[18] Fredrickson, R.C.A. (1977) Life Sci. 21, 23–42.
[19] Gorenstein, C. and Snyder, S.H. (1979) Life Sci. 25, 2065–2070.
[20] Swerts, J.P., Perdrisot, R., Malfroy, B. and Schwartz, J.C. (1979) Eur. J. Pharmacol. 53, 209–210.
[21] Benuck, M. and Marks, N. (1979) Biochem. Biophys. Res. Commun. 88, 215–221.
[22] Wagner, G.W. and Dixon, J.E. (1981) J. Neurochem. 37, 709–713.
[23] Hazato, T., Inagaki-Shimamura, M., Katayama, T. and Yamamoto, T. (1982) Biochem. Biophys. Res. Commun. 105, 470–475.
[24] Erdös, E.G., Johnson, A.R. and Boyden, N.T. (1978) Adv. Biochem. Psychopharmacol. 18, 45–49.
[25] Palenker, J. and Lentzen, H. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 943.
[26] Altstein, M., Mittman, S. and Vogel, Z. (1981) Life Sci. 28, 185–191.
[27] Fulcher, I.S., Matsas, R., Turner, A.J. and Kenny, A.J. (1982) Biochem. J. 203, 519–522.
[28] Miller, R.J., Chang, K.-J., Leighton, J. and Cuatrecasas, P. (1978) Life Sci. 22, 379–388.