High-Performance Liquid Chromatographic Determination of Peptides Released by Tryptic Degradation from Opioid Peptide Precursors in Rat Brain

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A high-performance liquid chromatographic method involving postcolumn fluorescence derivatization is described for the quantification of five fragment peptides (methionine-enkephalin-Thr-Ser-Glu-Lys, methionine-enkephalin-Lys, methionine-enkephalin-Arg, leucine-enkephalin-Lys and leucine-enkephalin-Arg) released by tryptic digestion from the opioid peptide precursors (proopiomeranocortin and proenkephalins A and B) in rat brain tissues. The tissue proteins containing the precursors are hydrolyzed with trypsin to the fragment peptides. The peptides are separated on an Asahipak ODP-50 column and on-line detected fluorometrically by using hydroxylamine, cobalt(II) and borate buffer reagents. The detection limits (S/N=3) for the peptides are 0.7–2.8 pmol per 100 μ l injected. The distribution of the precursors in the brain tissues was also discussed on the basis of the determined values of the fragment peptides.

Keywords opioid peptide precursor; brain tissue; tryptic degradation; fragment peptide; postcolumn fluorescence derivatization; high-performance liquid chromatography

It has been recognized that naturally occurring opioid peptides involving enkephalins, methionine-enkephalin (ME, Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (LE, Tyr-Gly-Gly-Phe-Leu), are derived from three different precursors, namely proopiomeranocortin, and proenkephalins A and B (Table I).^{1–4)} The opioid peptide moieties of these precursor proteins are typically flanked on both sides by basic amino acid residues which appear to be a processing signal for the trypsin-like enzyme.⁵⁾

Clearly, to arrive at an understanding of the details of the biosynthesis of opioid peptides in the brain, sensitive and convenient methods for estimating the distribution of the opioid peptide precursors in the brain are necessary. Radioimmunoassay (RIA) has usually been applied for the assay of the opioid peptide precursors. The production of antiserum directed against a purified bovine ME precursor (probably corresponding to proenkephalin A) was reported.⁶⁾ However, the antibody cross-reacted with other proteins having no enkephalin sequence. In the other RIA methods, a fragment peptide, ME-Arg released from proenkephalin A by tryptic hydrolysis was determined for the estimation of the precursor in rat brain.^{7,8)} However, no methods based on RIA are available to estimate the amounts of proopiomeranocortin and proenkephalin B. Recently, a method based on high-performance liquid chromatography (HPLC) with off-line mass spectrometric detection was utilized for the determination of ME and LE

yielded enzymatically from the precursor proteins in human cerebrospinal fluid, where two enzymes, trypsin and carboxypeptidase B, were used for the liberation of ME and LE.⁹⁾

We previously developed a simple method for determining opioid peptides in rat brain by reversed-phase HPLC with on-line post-column fluorescence detection.¹⁰⁾ The method allowed only N-terminal tyrosine-containing peptides to be derived to fluorescent compounds with reagents of hydroxylamine, cobalt(II) and borate buffer (pH 8.5).

In the present study, the HPLC method was applied to the quantification of fragment peptides released from the opioid peptide precursors in rat brain tissues by tryptic cleavage. Since trypsin catalyzes the hydrolysis of peptide bonds at the carboxyl side of Arg and Lys, the following fragment peptides should be produced from the precursors: ME-Thr-Ser-Glu-Lys from proopiomeranocortin, ME-Lys, ME-Arg and LE-Lys from proenkephalin A, and LE-Arg from proenkephalin B (Table I). These fragment peptides were separated on a reversed phase column and on-line detected fluorometrically. The five fragment peptides were also identified with further enzymatic degradation using carboxypeptidase B. [D-Ala²]ME was used as an internal standard. The distribution of the three opioid peptide precursors in the brain tissues was discussed.

Experimental

Chemicals and Solutions The following synthetic peptides were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification: Tyr-Gly, Tyr-Gly-Gly, Tyr-Phe, Tyr-Gly-Gly-Phe, ME, [D-Ala²]ME, ME-Arg, ME-Lys, ME-NH₂, ME-Arg-Phe, ME-Arg-Gly-Leu, ME-Thr-Ser-Glu-Lys, α -endorphin (α -EDP, ME-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr), LE, LE-Arg, LE-Lys and LE-NH₂. The peptide solutions were prepared in water and stored at –80°C. They were consumed within 3 weeks. Trypsin (EC 3.4.21.4, 12200 units/mg protein) from bovine pancreas, which has been added with L-1-tosylamido-2-phenylethylchloromethylketone, an inhibitor of chymotrypsin present as an impurity, and carboxypeptidase B (EC 3.4.17.2, 140 units/mg protein) from porcine pancreas were purchased from Sigma. Water was deionized and then distilled before use. The other chemicals were of the highest purity available.

Preparation of Rat Brain Protein and Tryptic Degradation Rat brain tissues were obtained from male Sprague-Dawley rats (220–280 g, 7 weeks). Brain areas were dissected according to the method previously described.¹⁰⁾ Each tissue (about 50 mg) was homogenized at 0–4°C with 0.5 ml of 0.1 M HCl. The homogenate was centrifuged at 350 g for 20 min.

TABLE I. Partial Amino Acid Sequences of the Opioid Peptide Precursors and the Fragment Peptides Released by Tryptic Digestion (Underlined)

Proopiomeranocortin
Lys-Arg ¹⁰³ -Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser
Proenkephalin A
-Lys-Arg ⁹⁹ -Tyr-Gly-Gly-Phe-Met-Lys-Arg-
Tyr-Gly-Gly-Phe-Met-Lys-Lys-
-Lys-Arg ¹³⁵ -Tyr-Gly-Gly-Phe-Met-Lys-Lys-
-Lys-Arg ¹⁸⁵ -Tyr-Gly-Gly-Phe-Met-Arg-Gly-
-Lys-Arg ²⁰⁹ -Tyr-Gly-Gly-Phe-Met-Arg-Arg-
-Lys-Arg ²²⁹ -Tyr-Gly-Gly-Phe-Leu-Lys-Arg-
-Lys-Arg ²⁶⁰ -Tyr-Gly-Gly-Phe-Met-Arg-Phe-
Proenkephalin B
-Lys-Arg ¹⁷⁴ -Tyr-Gly-Gly-Phe-Leu-Arg-Lys-
-Lys-Arg ²⁰⁸ -Tyr-Gly-Gly-Phe-Leu-Arg-Arg-
-Lys-Arg ²²⁷ -Tyr-Gly-Gly-Phe-Leu-Arg-Arg-

The supernatant was added with 1 ml of acetone, and centrifuged again at 1500 *g* for 10 min. The precipitate (about 8-mg protein, which contained the precursors) was suspended in 350 μ l of water. The suspension (50 μ l) was used to determine protein by the Lowry method.¹¹⁾ The suspension (300 μ l) was mixed with 50 μ l of 0.5 M Tris-HCl buffer (pH 7.8) containing 0.1 M CaCl₂ and 0.1 M NaCl, and 125 μ l of trypsin (24000 unit/ml). When an inactivated trypsin was required for use, the trypsin solution was heated at 100 °C for 3 min. Finally, the volume of the enzyme reaction mixture was adjusted with water to 500 μ l. The mixture was incubated at 37 °C for 60 min. Then 2.0 M HClO₄ (200 μ l) was added to the mixture to stop the enzyme reaction. A portion (15 μ l) of 5.0 nmol/ml [D-Ala²]ME (internal standard) was added to the enzyme reaction mixture. When calibration graphs were prepared, 10 μ l of a solution of synthetic peptides (5–10 nmol/ml each) was added to the mixture. The reaction mixture was centrifuged at 1500 *g* for 5 min. The supernatant was transferred to a mini-cartridge packed with 0.2 g of Asahipregel TC18 (particle size, 40 μ m; Asahi Chemical Ltd., Kawasaki, Japan). After loading the supernatant, the cartridge was washed twice with 2 ml of water. A fraction containing the fragment peptides was eluted with 1.5 ml of a mixture of acetonitrile and water (9:1, v/v). After evaporation *in vacuo* at approximately 30 °C, the residue was dissolved in water (total volume, 250 μ l). The solution (60 μ l) was diluted with water to 100 μ l and subjected to HPLC. In the case of degradation with carboxypeptidase B, the solution was used without dilution.

Degradation of Fragment Peptides with Carboxypeptidase B A portion (60 μ l) of the sample solution was mixed with 10 μ l of carboxypeptidase B (2 units/ml) in 50 mM phosphate buffer (pH 7.8). The mixture was incubated at 37 °C for 30 min. The reaction mixture was heated at 100 °C for 3 min to stop the enzyme reaction. The mixture was centrifuged at 1500 *g* for 3 min and the whole supernatant was diluted with water to 100 μ l and used for HPLC.

HPLC A Hitachi 655A-12 high-pressure pump fitted with a Rheodyne 7125 syringe-loading sample injector (100 μ l loop), programmed electronic valves for gradient elutions and a reversed phase column, Asahipak ODP-50 (150 $\times$ 6 mm i.d., particle size 5 μ m, Asahi Chemical Ltd.) was used. Eluents A and B were prepared for gradient elution. They were mixtures of acetonitrile, 50 mM sodium borate buffer (pH 7.0) and water (1:4:15 and 3:1:1, v/v, respectively). The gradient profile of acetonitrile in the mobile phase is indicated by a dotted line in Fig. 1. The flow rate was 1.0 ml/min. The column temperature was ambient (24 $\pm$ 4 °C). A 0.3 M sodium borate buffer (pH 9.0) and 8 mM hydroxylamine oxalate solution containing 0.2 mM cobalt(II) acetate were added to the eluate stream by Hitachi 655 reagent-delivery pumps at a flow rate of 0.4 ml/min each. The mixture was passed through a reaction coil (10 m $\times$ 0.5 mm i.d.; stainless-steel tube) in an electronic heater at 100 °C. The eluate was monitored at 430 nm emission and at 330 nm excitation with a Hitachi F 1000 fluorescence spectrophotometer fitted with a 12- μ l flow cell.

Results and Discussion

The conditions for the HPLC separation and fluorometric

detection were almost the same as previously reported.¹⁰⁾ Figure 1 shows a chromatogram of a standard mixture (10 pmol each) of seventeen N-terminal Tyr-containing synthetic peptides. The five synthetic fragment peptides were well separated between the retention times of 13.8–29.2 min.

The investigation of the effect of trypsin concentration and incubation time on the production of the fragment peptides from the opioid peptide precursors revealed that maximum and constant production was achieved at trypsin concentrations between 200–500 μ g (2400–6000 units)/tube, and that an incubation for 30 min or longer was necessary to obtain the maximal production (Fig. 2): incubation at 37 °C for 60 min in the presence of 250 μ g trypsin/tube was used in the procedure.

The synthetic five fragment peptides were not degraded with trypsin under the same reaction conditions: the peak

Fig. 1. Chromatogram Obtained with a Standard Mixture of Opioid Peptides and Related Peptides (10 pmol Each)

Peaks: 1, Tyr-Gly; 2, Tyr-Gly-Gly; 3, Tyr-Gly-Gly-Phe; 4, Tyr-Phe; 5, ME-Thr-Ser-Glu-Lys; 6, ME; 7, LE; 8, [D-Ala²]ME; 9, α -EDP; 10, ME-Lys; 11, ME-Arg; 12, LE-Lys; 13, LE-Arg; 14, ME-NH₂; 15, ME-Arg-Gly-Leu; 16, LE-NH₂; 17, ME-Arg-Phe.

Fig. 2. Effect of (A) Trypsin Concentration and (B) Incubation Time (at 37 °C) on the Formation of the Fragment Peptides from the Opioid Peptide Precursors in Striatum Tissue of Rat Brain

Curves: 1, ME-Lys; 2, ME-Arg; 3, ME-Thr-Ser-Glu-Lys; 4, LE-Lys; 5, LE-Arg.

Fig. 3. Chromatograms Obtained with Striatum Protein after Degradation with (A) Trypsin and (B) Inactivated Trypsin

Shaded peaks were due to the fragment peptides from the opioid peptide precursors. Peaks: 1, ME-Thr-Ser-Glu-Lys; 2, ME; 3, LE; 4, [D-Ala²]ME (internal standard); 5, ME-Lys; 6, ME-Arg; 7, LE-Lys; 8, LE-Arg.

Fig. 4. Chromatogram Obtained with the Fragment Peptides (from Striatum Protein) Treated with Carboxypeptidase B

Peaks: 1, unknown compound(s) and ME-Thr-Ser-Glu; 2, Tyr-Gly-Gly-Phe; 3, ME-Thr-Ser-Glu-Lys; 4, ME; 5, LE; 6, [D-Ala²]ME (internal standard); 7, ME-Lys; 8, ME-Arg; 9, LE-Lys; 10, LE-Arg.

height of the peptides was constant for the prolonged reaction period of 150 min.

Figure 3A depicts a chromatogram obtained with striatum protein after degradation with trypsin. Many peaks ascribable to N-terminal Tyr-containing peptides were detected: no peaks were observed when the brain protein was treated with inactivated trypsin (Fig. 3B). The shaded peaks corresponding to ME-Thr-Ser-Glu-Lys, ME-Lys, ME-Arg, LE-Lys and LE-Arg were identified on the basis of their retention times and also by co-chromatography with the synthetic peptides.

For additional identification, the same sample obtained after the tryptic digestion was further degraded by reaction with carboxypeptidase B. Carboxypeptidase B releases selectively a basic amino acid of Arg or Lys at the C-terminal end of the peptide. Therefore, the peaks corresponding to ME-Thr-Ser-Glu-Lys, ME-Lys, ME-Arg, LE-Lys and

LE-Arg in Fig. 3A disappeared, and the peaks for ME and LE derived from ME-Lys, ME-Arg, LE-Lys and LE-Arg were newly produced (Fig. 4). The peak for ME-Thr-Ser-Glu, which should be produced from ME-Thr-Ser-Glu-Lys, could not be completely identified because the peak was overlapped with an unknown large peak (the retention times, 5.4 min) which increased significantly in height during the carboxypeptidase B hydrolysis of the trypsin-hydrolyzate in comparison with the other unknown peaks. Similar chromatogram patterns to those of Figs. 3 and 4 were obtained with other brain tissues such as hypothalamus, hippocampus and cortex. Further identification of the produced fragment peptides based on the analyses of amino acid composition and amino acid sequence, and also on liquid chromatography-mass spectrometry is in progress.

The recoveries of synthetic ME-Thr-Ser-Glu-Lys, ME-Lys, [D-Ala²]ME, ME-Arg, LE-Lys and LE-Arg (50 pmol each) added to the reaction mixture of a striatum protein after digestion with trypsin were 50 ± 5 , 70 ± 5 , 85 ± 4 , 80 ± 4 , 65 ± 5 and $85 \pm 4\%$ (mean $\pm$ standard deviation, $n = 7$). The ratio of the recovery of each fragment peptide to that of the internal standard of [D-Ala²]ME was always constant ($n = 7$). The calibration graphs for the five synthetic peptides added to the reaction mixture of each tissue protein were linear up to at least 15 pmol/mg protein and passed through the origin ($\gamma = 0.997 - 0.999$, $n = 3$ each plot). The detection limits ($S/N = 3$) for the peptides were 0.7–2.8 pmol per 100 μ l injected. The precision was also examined using the striatum sample. The relative standard deviations ($n = 7$) were 3.4, 4.0, 3.0, 4.0 and 3.2% for 4.0 pmol ME-Thr-Ser-Glu-Lys, 4.4 pmol ME-Lys, 4.2 pmol ME-Arg, 2.1 pmol LE-Lys and 2.3 pmol LE-Arg per mg of protein, respectively.

The concentrations of the fragment peptides in the acetone-precipitated proteins from four regional tissues of rat brain were measured by the present method (Table II). The fragment peptides were found in all of the tissue proteins. ME-Lys, ME-Arg and LE-Lys appeared to be in the concentration ratio of approximately 3:3:1, which

TABLE II. Concentrations of the Fragment Peptides Produced from the Opioid Peptide Precursors in Rat Brain Tissues by Tryptic Degradation

Tissue	ME-Thr-Ser-Glu-Lys (from pro-opiomelanocortin)	ME-Lys (from proenkephalin A)	ME-Arg (from proenkephalin A)	LE-Lys (from proenkephalin A)	LE-Arg (from proenkephalin B)
	(pmol/mg protein)				
Striatum	4.7	5.0	3.8	2.6	2.1
	4.0	4.4	4.2	2.1	1.6
	3.5	3.9	4.1	1.6	2.3
Hypothalamus	5.5	4.6	4.5	1.8	1.8
	4.0	4.2	4.8	2.1	1.9
	5.2	4.4	4.3	2.5	3.1
Hippocampus	3.9	2.0	1.4	0.5	1.1
	3.0	1.6	1.6	0.6	0.8
	2.8	1.7	2.2	0.7	0.8
Cortex	2.2	1.8	0.8	0.4	0.8
	2.3	1.9	1.2	0.5	0.6
	2.7	2.3	2.0	0.8	1.1

was coincided with the ratio of the fragment peptide moieties in proenkephalin A (Table I). The concentrations of all fragment peptides were higher in the striatum and hypothalamus tissues than in the hippocampus and cortex tissues. This agreed with the fact that opioid peptides such as ME and LE in the striatum and hypothalamus tissues were at higher concentrations than other brain tissues.¹⁰⁾ These observations suggest that the amounts of the fragment peptides are proportional to their precursor concentrations in the tissues: the concentrations of proenkephalin A, proopiomeranocortin and proenkephalin B distributed in the four tissues are higher in that order.

The recommended method has sufficient sensitivity and selectivity for the quantification of the fragment peptides released from the opioid peptide precursors in rat brain tissues by tryptic digestion, and may permit all the precursors to be estimated. Therefore, this method should be useful for the investigation of opioid peptides in brain tissues.

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References

- 1) S. Nakanishi, A. Inoue, T. Kita, M. Nakamura, A. C. Y. Chang, S. N. Cohen and S. Numa, *Nature* (London), **278**, 422 (1979).
- 2) M. Comb, P. H. Seeburg, J. Adelman, L. Eiden and E. Herbert, *Nature* (London), **295**, 663 (1982).
- 3) H. Kakidani, Y. Furutani, H. Takahashi, M. Noda, Y. Morimoto, T. Hirose, M. Asai, S. Nakanishi and S. Numa, *Nature* (London), **298**, 245 (1982).
- 4) M. Noda, Y. Furutani, H. Takahashi, H. Toyosato, T. Hirose, S. Inayama, S. Nakanishi and S. Numa, *Nature* (London), **295**, 202 (1982).
- 5) K. Kangawa, H. Matsuo and M. Igarashi, *Biochem. Biophys. Res. Commun.*, **86**, 153 (1979).
- 6) D. R. Liston, J. J. Vanderhaeghen and J. Rossier, *Nature* (London), **302**, 62 (1983).
- 7) S. A. Girard, G. J. Dockray and R. G. Williams, *J. Neurochem.*, **43**, 1236 (1984).
- 8) A. Cupo, P. A. Pontarotti, Th. Jarry and M. Delaage, *Neuropeptides*, **4**, 375 (1984).
- 9) D. Liu, C. Dass, G. Wood and D. M. Desiderio, *J. Chromatogr.*, **500**, 395 (1990).
- 10) G.-Q. Zhang, M. Kai and Y. Ohkura, *Anal. Sci.*, **6**, 671 (1990).
- 11) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).