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SPONTANEOUS INACTIVATION OF ENKEPHALIN

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SUMMARY

Dilute solutions of enkephalin are not stable upon prolonged storage. A new peak of UV absorption (at 325 nm) gradually appears. This change in spectral properties is accompanied by an alteration in the chromatographic pattern as well as the spontaneous modification of the tyrosyl residue of the enkephalin molecule. The A_{325} nm peak suggests that the tyrosine residue is converted into an indole derivative. The new material is biologically inactive as indicated by its failure to interact with opiate receptors or with antibodies against native enkephalin. In addition the modified peptide is not hydrolyzed by brain extracts or leucine aminopeptidase. This spontaneous inactivation of enkephalin should be taken into account, particularly in experiments utilizing dilute solutions of enkephalin.

INTRODUCTION

It is now well accepted that the two pentapeptides, Met-enkephalin and Leu-enkephalin (TyrGlyGlyPheMet or TyrGlyGlyPheLeu) bind to opiate receptors and, under appropriate conditions, mimic the effects of the opiates in vivo (1-4). The enkephalins are rapidly hydrolyzed by the enzyme, leucine aminopeptidase, as well as by enzymatic activity present in the brain (5,6). The peptide bond between the Tyr and the Gly is the preferential site of cleavage (5-7). Specific antibodies against the enkephalin molecules have been prepared and found useful for the radioimmunoassay of the enkephalins (8,9).

During the course of our studies we have detected a process of spontaneous inactivation of enkephalin upon storage. The observed modification in the enkephalin molecule was found to abolish the capacity of enkephalin to bind to the opiate receptor. In addition the antibody against native enkeph lin no longer recognized the modified molecule. Finally, neither leucine-

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aminopeptidase not the enzymatic activity present in brain homogenate was capable of hydrolyzing the modified enkephalin.

MATERIALS AND METHODS

Leu-enkephalin and rabbit anti Leu-enkephalin antiserum (prepared according to Ref. 8) were obtained from Miles-Yeda, Inc., Rehovot, Israel. [Tyrosyl-3,5-^{3}H]Leu-enkephalin was obtained from Amersham Radiochemical Centre (batches 2,4,5,7,9) and stored in the original vials at 0-4°C according to Amersham's recommendation. Leucine aminopeptidase (EC 3.4.1.1, 101 units/mg) was obtained from Worthington Biochemical Corp. and activated as described in the Worthington Manual.

Amino acid analyses were performed according to Moore and Stein (10). Protein was determined by the method of Lowry et al. (11).

Preparation of crude brain homogenate and membrane preparation: Crude brain homogenate was prepared as previously described (6). The brains (without the cerebellum) of adult male Sprague-Dawley rats were homogenized in 10 volumes of cold 0.3 M sucrose containing 10 mM Tris-HCl (pH 7.5). The supernatant was collected following centrifugation at 1000xg for 10 min. Brain membrane preparations were prepared as follows: Fifty ml of crude brain homogenate were centrifuged at 4°C for 30 min at 17,000xg. The pellet was resuspended in 100 ml of cold distilled water. The suspension was then homogenized in a glass-teflon homogenizer (800-1000 rpm) and centrifuged at 4°C for 10 min at 3,000xg. The upper 4/5 of the supernatant was centrifuged at 4°C for 30 min at 43,500xg, and the resulting pellet was resuspended in 15 ml of 50 mM Tris-HCl (pH 7.5) and stored in liquid nitrogen.

Enzymatic hydrolysis of enkephalin: The enzymatic hydrolysis of labeled enkephalin by leucine aminopeptidase or by crude brain homogenate was assayed as previously described, utilizing columns with polystyrene beads (Porapak Q) to which the enkephalin but not the tyrosine is adsorbed (6). The reaction mixture of the leucine aminopeptidase assay contained 1.6 µg leucine aminopeptidase, $1x10^{-7}$M labeled Leu-enkephalin, 2 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.5), in a final volume of 100 µl. The assay mixture for hydrolysis by crude brain homogenate contained 1.7 µg homogenate, $1x10^{-7}$M labeled Leu-enkephalin and 10 mM Tris-HCl (pH 7.5) in a final volume of 100 µl. The hydrolytic reactions were carried out at 30°C for 200 min (unless otherwise indicated) quenched by immersing the tubes in a boiling water bath, and assayed with the Porapak columns.

Binding of Leu-enkephalin to opiate receptor in brain membranes: Brain membrane preparations were preincubated for 30 min at 37°C (to destroy endogenous enkephalin). Aliquots containing 400 µg protein of this solution were then incubated for 10 min at 25°C with 1 pmole of labeled Leu-enkephalin in a solution containing 50 mM Tris-HCl (pH 7.5) and 1 mM $MnCl_2$ in a final volume of 0.25 ml. Following the above treatment, the reaction mixtures were diluted with 3 ml of Tris-HCl (pH 7.5) and filtered immediately through 25 mm GF/B (Whatman) filters. The filters were washed with Tris-HCl buffer and counted for radioactivity. Nonspecific binding was measured in the presence of 1 µM levorphanol and subtracted from the values obtained.

Binding of Leu-enkephalin to anti-Leu-enkephalin antibodies: The binding was assayed by a modification of the method of Weisman et al. (8). An aliquot of antiserum (4 µl) was added to a solution of labeled Leu-enkephalin (0.5 pmoles), containing 50 mM Tris-HCl (pH 7.0) and 0.1% NaN_3 in a final volume

of 0.5 ml. The solution was incubated for 4 h at 4°C. A solution of carrier rabbit immunoglobulins (50 µg in 10 µl) was added, followed by 0.5 ml of ice cold, saturated $(NH_4)_2SO_4$ solution. The mixture was centrifuged, and the pellet was dissolved in H_2O and counted for radioactivity. The amount of antiserum used was chosen in order to achieve 50% binding of the freshly obtained enkephalin added.

RESULTS

Stored solutions of radioactively labeled Leu-enkephalin gradually lost their capacity to be degraded at the Tyr-Gly peptide bond by crude brain homogenate (Fig.1). Newly obtained solutions of [Tyrosyl-3,5-^{3}H]Leu-enkephalin were quickly hydrolyzed, reaching 90% hydrolysis of the total labeled peptide within 1 h. The maximal level of hydrolysis obtained was reduced as a function of storage time, and labeled enkephalin solutions, stored at 4°C for 14 months, were completely resistant to hydrolysis. The same phenomenon was found with all commercially-obtained batches of tritiated Leu-enkephalin tested. As seen in Table 1, the hydrolysis of the peptide by the enzyme leucine aminopeptidase was similarly reduced upon storage.

The capacity of enkephalin to bind to the opiate receptor was also lost as a result of storage of the labeled enkephalin solution. Table 1 demonstrates that with enkephalin stored for 3 months 71 fmoles of enkephalin were specifically bound to receptor sites present in 1 mg of brain membranes. In comparison, after 12 months storage, only 15 fmoles enkephalin could be bound to the same membrane preparations. The interaction of the labeled peptide with antibodies prepared against the native enkephalin was concomitantly reduced.

An insignificant amount (less than 5%) of the tritium atoms were released from the peptide molecule during one year storage, indicating that the tyrosine moiety was retained as an integral part of the peptide. On the other hand, the capacity of the peptide to be cleaved by the enzyme leucine aminopeptidase was drastically reduced, suggesting that the amino group of the terminal residue was either blocked or eliminated. The mobility of the labeled peptide was assayed by thin-layer chromatography on silica plates in chloroform-

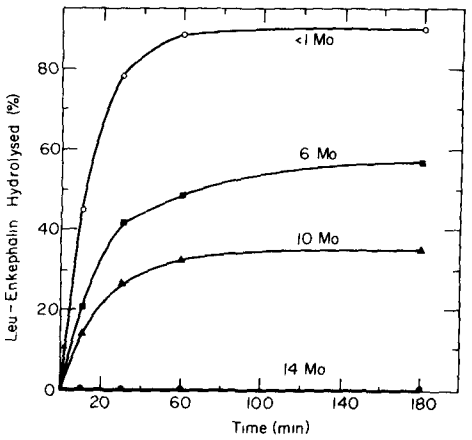
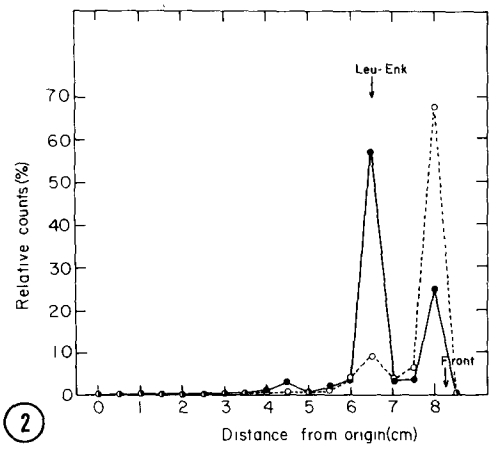


Figure 1. Effect of storage of labeled Leu-enkephalin on its time course of hydrolysis by crude brain homogenate. [Tyrosyl-3,5-^{3}H]Leu-enkephalin, stored at 4°C for the number of months indicated, was incubated with crude brain homogenate for 0-180 min and assayed for the relative amount of radioactivity released as tyrosine.

Table 1: Changes in the biological activities of stored solutions of labeled Leu-enkephalin

Storage (Months)	Max. hydrolysis (% of total) obtained by		Binding to brain membranes (fmoles/mg prot.)	Binding to anti Leu-enkephalin (% of total)
	Leucine amino-peptidase	Brain homogenate		
<1	-	91	-	50
3	71	71	71	39
12	18	22	15	19
14	-	-	-	3

methanol-acetic acid-water (45:30:6:9, v/v/v/v). Fig. 2 demonstrates that solutions of labeled Leu-enkephalin, stored for 3 months, contained about 75% native enkephalin (Rf=0.78) and about 23% of radioactive material with Rf=1. On the other hand, enkephalin stored for 14 months contained more than 80% of the unidentified spot (Rf=1), and only trace amounts of material which had the same Rf as Leu-enkephalin. The observed change in Rf indicated that the



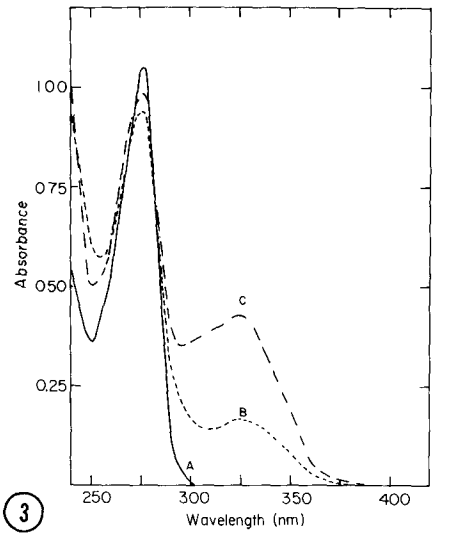


Figure 2. Thin layer chromatography of [Tyrosyl-3,5-^{3}H]Leu-enkephalin, stored for 3 and 14 months. Unlabeled Leu-enkephalin was added as carrier and the samples chromatographed on silica plates in chloroform-methanol-acetic acid-water (45:30:6:9). Half cm sections were counted for radioactivity. Labeled enkephalin stored for 3 months (●——●); labeled enkephalin stored for 14 months (o----o). Arrow shows the location of Leu-enkephalin marker determined by fluorescamine spray as previously described (6).

Figure 3. Absorption spectra of 0.5 mM unlabeled Leu-enkephalin solution stored at room temperature in capped vials. A. Fresh solution of Leu-enkephalin; B. Leu-enkephalin kept in 1 mM Tris-HCl (pH 7.5) for 50 days; C. Leu-enkephalin kept in 1 mM Tris-HCl (pH 7.5) and 0.5 mM dithiothreitol for 50 days.

product became more hydrophobic than native enkephalin, possibly due to a reduction of the charge of the molecule. Similar changes were also noticed with 0.5 mM solutions of synthetic, unlabeled Leu-enkephalin, kept at room temperature.

The UV spectrum of the Leu-enkephalin was also changed upon storage, and a new peak at 325 nm gradually developed (Fig.3). The presence of 0.5 mM dithiothreitol generated a larger UV peak at the same position. The appearance of the 325 nm peak indicated that an indole derivative was formed. This spectrum is similar to those found upon enzymatic or chemical oxidation of NH_2-terminal tyrosyl peptides (12).

Table 2: Amino acid analyses of Leu-enkephalin and the modified peptide eluted from thin layer chromatographs

	Amino acid (molar ratio found)			
	Tyr	Gly	Phe	Leu
Leu-enkephalin (Rf=0.78)	0.8	2.0	1.0	1.0
Modified Leu-enkephalin (Rf=1.00)	0	2.0	0.8	1.0

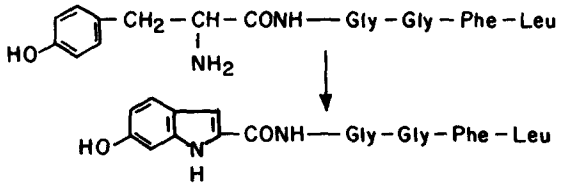
The compound with the 325 nm peak migrated with the solvent from (Rf=1) on thin-layer chromatography and could not be stained with ninhydrin reagent. Amino acid analysis of the peptides extracted from the silica plates showed that the compound with Rf=0.78 is pure enkephalin, exhibiting the expected amino acid content (Table 2). The compound, migrating with the solvent front, lacked tyrosine. The new N-terminal residue could not be identified since indole derivatives are destroyed during acid hydrolysis. Additionally, the latter compound lacks the free amino group.

DISCUSSION

Storage of diluted solutions of enkephalin resulted in a loss of biological activity. This loss was accompanied by changes in the mobility on thin layer plates, UV spectra and amino acid composition of the peptide. The amino acid analysis suggests that the NH_2-terminal tyrosine is converted into an indole derivative. The mechanism of this reaction is apparently similar to the chemical and enzymatic oxidation of tyrosine derivatives (12,13). Since the tyrosine is positioned at the NH_2-terminal of the enkephalin molecule, the dopachrome mechanism is implicated (see scheme 1).

It seems that the reaction is not generated via the introduction of a new hydroxyl group at positions 3 or 5, since the tritium atoms at these loca-

Scheme I.



tions are not lost during the reaction. Otherwise the cyclization mechanism is similar to the dopachrome oxidation mechanism (12).

This study also shows that the free amino group on the tyrosine is important for the binding to the opiate receptor as well as for hydrolysis by brain enzymes which cleave native enkephalin.

Tyrosines are seldom present at the NH_2-terminal position of proteins (14). This phenomenon may reflect the existence of chemical or enzymatic mediated cyclization of the NH_2-terminal tyrosine. Although this conversion is very slow upon storage, it would be interesting to determine whether this reaction might represent one of the mechanisms of enkephalin inactivation in vivo.

At any rate, it is cautioned that the spontaneous modification of the tyrosine residue in the enkephalin molecule be taken into account in the various studies utilizing dilute solutions of labeled and unlabeled enkephalin.

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