In vitro interaction of enkephalin with serum and chromaffin granule components[1]

Ruth Hogue-Angeletti and L. G. Roda

Laboratory of Neuropathology, University of Pennsylvania School of Medicine, Philadelphia (Pennsylvania 19104, USA), 19 May 1980

Summary. Incubation of radioactively labeled enkephalin with serum components or chromaffin granule components in vitro provided evidence that there are specific constituents which bind enkephalins and may affect their degradation. Evidence for carboxy-terminal degradation was found.

In recent months, the adrenal medulla chromaffin granule has been shown to contain not only high concentrations of catecholamines, but also enkephalin[2,3], enkephalin precursors[4], and other peptides[5]. Enkephalins are released into the bloodstream after stimulation of the adrenal[3]. However, their serum half-lives have been estimated at 2 sec[6] to several min[3,7]. Since some effects of the enkephalins are thought to be persistent[8], the source of these longer term effects remains a question. Some prolonged actions could result from continued release by proteolytic digestion of more stable precursor molecules. On the contrary, serum components could bind enkephalin as has been observed for biologically active molecules such as nerve growth factor[9] and norepinephrine[10]. In this paper, we confirm that degradation of leu-enkephalin by serum is rapid in vitro, but further provide evidence that serum proteins and chromaffin granule proteins may interact with enkephalins to prolong their active life. In order to demonstrate this, 2 types of experiments were performed: a) the proportions of intact enkephalin and free tyrosine remaining after incubation of enkephalin with serum or serum plus chromaffin granule lysate were measured; b) these incubation mixtures were analyzed by gel filtration in order to detect interactions of the enkephalin with other molecules.

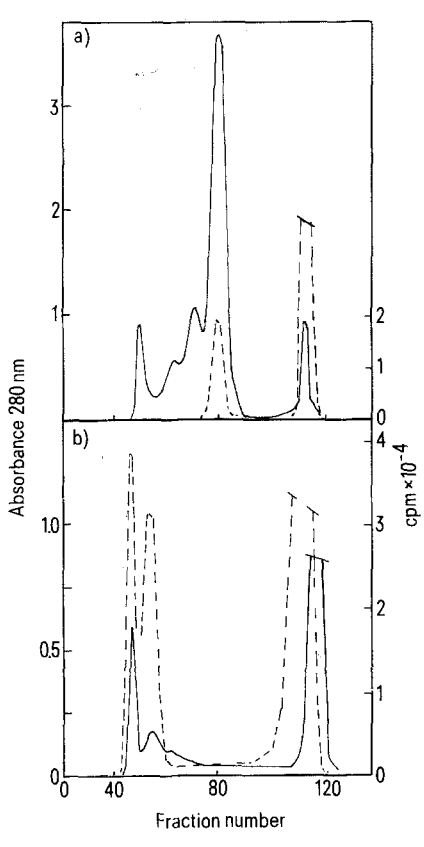

Fig. 1. Gel filtration of serum and chromaffin granule lysate after incubation with [^{3}H]-enkephalin. A 0.9× 100 cm column of Biogel A 1.5-m was equilibrated with 10 mM Tris HCl pH 7.2 containing 0.1 M NaCl and eluted at a flow rate of 5 ml/h. Fractions of 0.8 ml were collected. Solid lines show the absorbance at 280 nm. Broken lines represent the total cpm per fraction. *a* Elution profile of 1 ml rabbit serum incubated for 15 min at 37 °C. *b* Elution profile of a ml of bovine chromaffin granule lysate[4] (5 mg protein/ml) incubated for 1 h at 37 °C.

First, radioactive leu-enkephalin (20 Ci/mmole) was incubated with control rabbit serum at 37 °C for varying times. The reaction mixtures were then acidified to pH 2.2 and applied to an amino acid analysis column whose effluent was assayed for radioactivity. Since the tritium label is located in the tyrosine moiety and the elution volumes for tyrosine and enkephalin are quite distinct, the liberation of free tyrosine is easily monitored. As seen in the table, the enkephalin is rapidly degraded. However, the rapid appearance of a peak of radioactivity at an elution volume intermediate to enkephalin and tyrosine indicates that the removal of the tyrosyl residue is not the first step in hydrolysis as previously reported[7]. When the reaction is slowed by lowering the incubation temperature to 4 °C, this product becomes even more evident. When similar samples were analyzed in the protein sequencer, it was found that the products formed are the result of carboxyl-terminal degradation.

The data in the table (lower portion) were obtained from similar experiments in which the radioactive enkephalin was preincubated with bovine chromaffin granule lysate for 1 h at 4 °C before the addition of serum. The lytic process appears to be slowed. In both sets of experiments, there exists a finite amount of enkephalin remaining at the end of 30 min of incubation (1.7 and 3.9%). When chromaffin granule lysate is present, there is residual enkephalin remaining even after 1 h (data not shown).

In order to determine whether leu-enkephalin might interact with serum protein, labeled enkephalin was incubated as above in normal serum for 15 min at 37 °C. When the reaction mixture was applied to a gel filtration column, the elution profile shown in figure 1, a, is seen. Although most of the radioactivity is found among the low mol.wt material, a peak of radioactivity is also consistently found at an elution volume corresponding to an apparent mol.wt of 70,000. When aliquots of this peak were monitored by the amino acid analysis column as described above, we found that over 90% of the total radioactivity in the peak emerges at the position of intact enkephalin. The amount of enkephalin retained by this protein peak corresponds approximately to the percent shown to be remaining in the table. Purified serum albumin, when incubated and analyzed in the same manner, was also found to bind labeled enkephalin.

Similarly, labeled enkephalin was incubated in the presence of bovine chromaffin granule lysate[5] for 1 h at 37 °C. The elution profile for the gel filtration of this sample on the same Biogel A 1.5-m column is shown in figure 1, b. Again, most of the radioactive material is eluted at volumes corresponding to a low mol.wt. However, there are 2 radioactive peaks found at higher mol.wts corresponding to the positions of chromolipin (soluble lipoprotein) and

Degradatation of [^{3}H]-leu-enkephalin by serum in vitro in the absence and presence of chromaffin granule lysate.

Incubation	Fraction	5 min	15 min	30 min
Serum, 37 °C	1	18.2	55.1	68.1
	2	–	–	–
	3	11.5	23.6	19.7
	4	22.6	14.1	10.5
	5	47.7	7.2	1.7
Serum, 4 °C	1		–	
	2		–	
	3		5.2	
	4		56.5	
	5		38.3	
Preincubation with lysate	1	12.9	37.8	57.5
	2	2.3	2.3	1.0
	3	15.4	13.4	12.9
	4	21.3	33.6	24.7
	5	48.1	12.9	3.9

Labeled enkephalin was incubated with rabbit serum for the quantity of time indicated. At the end of this period, the incubation mixture was diluted 1:10 with 0.2 M sodium citrate, pH 2.2, and applied to the column of a Dionex amino acid analyzer. The effluent was collected and analyzed for radioactivity. Fractions 1–5 represent successive fractions, the only ones containing radioactivity. Free tyrosine emerges from the column at position 1. Intact enkephalin emerges last, at fraction 5. Fractions 2–4 represent the 3 intervening fractions, and thus, are peptide degradation products of enkephalin which contain the labeled tyrosine. Serum incubations were performed at 37 °C, except for the 1 time point at 4 °C. The same incubations were then repeated, except that the labeled enkephalin was first preincubated with 10 µg of chromaffin granule lysate.

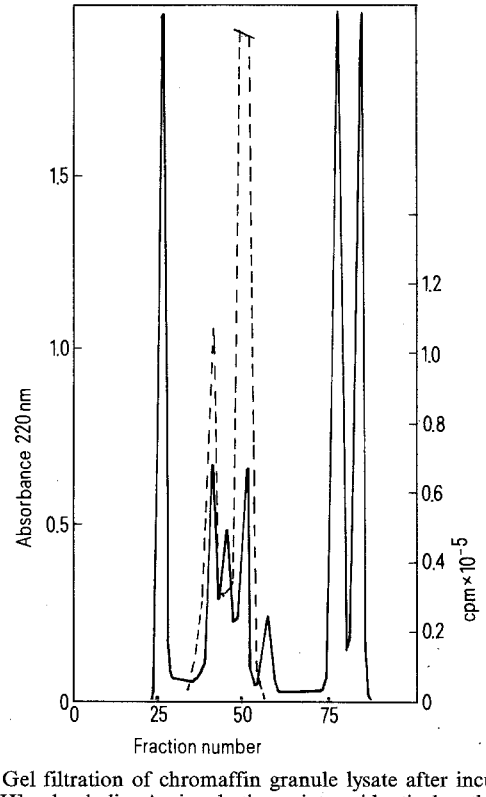

Fig. 2. Gel filtration of chromaffin granule lysate after incubation with [^{3}H]-enkephalin. An incubation mixture identical to that used in figure 2, b, was incubated for 1 h at 37 °C. The sample was then applied to a 0.9× 100 cm column of Biogel P2 equilibrated with 10 mM sodium citrate pH 5.2 eluted at a flow rate of 3 ml/h. Fractions of 0.7 ml were collected. Solid line: absorbance at 220 nm. Broken line: cpm in each fraction.

chromogranin A. At lower temperatures of incubation, less radioactivity is found associated with the chromolipin peak. Analysis revealed that the bound enkephalin was still intact.

When the incubation mixture containing the chromaffin granule lysate was instead applied to a Biogel P2 column (figure 2) in order to analyze the lower mol.wt material, a large proportion of radioactivity was found associated with a peptide of approximately mol.wt 1800, distinct from the large peak of free enkephalin which follows. Amino acid analysis of this peak indicates that two of the peptides recently found in the chromaffin granule[5] are eluted at this position. When these peptides were purified and then incubated with the labeled enkephalin, the label was again found to be associated with the peptide material.

The physiological role of the secretion of enkephalins from the adrenal in massive doses can be seriously questioned because of the short half-life. The results above provide evidence that the fate of enkephalins after release from the adrenal medulla may be quite complex. As shown, the degradation of enkephalin is very rapid in vitro. However, liberation of free tyrosine does not appear to be the first step in hydrolysis as has been described. Instead, a carboxyl-terminal degradation was observed. It is clear that some intact enkephalin can survive in vitro in serum. The experiments described above indicate that components of serum or the chromaffin granules may bind enkephalins, thereby retarding their degradation. A serum component with an apparent mol.wt of 70,000 apparently binds labeled enkephalin. Furthermore, 2 high mol.wt components and 1 low mol.wt component within the chromaffin granule appear to bind the labeled enkephalin. The binding to one of these chromolipin, is temperature dependent, and probably indicates only the increased solubility of enkephalin in lipid at physiological temperatures where the lipid is more fluid. The apparent binding of enkephalin to the peak containing chromogranin A and the peptide are consistently reproducible. Since the mixture used for binding contains the endogenous enkephalin, it may be expected that binding is underestimated in these experiments.

Quantitative measurements of specific binding with purified proteins and peptides are being performed to clarify to what extent they contribute to the physiological action of the enkephalins.

1 This work was supported by the NIH Grants NS-13201 and NS-00201.

2 M. Schaltzberg, J.M. Lundberg, T. Hökfelt, L. Terenius, J. Brandt, R.P. Elder and M. Goldstein, Neuroscience *3*, 1169 (1978).
3 H.O. Viveros, E.J. Di Liberto, E. Hazum and K.J. Chang, Molec. Pharmac., in press.
4 R.V. Lewis, A.S. Stern, J. Rossier, S. Stein and S. Udenfriend, Biochem. biophys. Res. Commun. *89*, 822 (1979).
5 L.G. Roda and R.A. Hogue-Angeletti, FEBS Lett. *107*, 393 (1979).
6 A. DuPont, L. Cusan, M. Garon, G. Alvarado-Urbina and F. LaBrie, Life Sci. *21*, 907 (1977).
7 J.M. Hambrook, B.A. Morgan, M.J. Rance and C.E.C. Smith, Nature *262*, 782 (1976).
8 A.J. Kastin, R.D. Olson, A.V. Schally and D.H. Coy, Life Sci. *25*, 401 (1979).
9 R.H. Angeletti, Brain Research *12*, 234 (1969).
10 B.L. Mirkin, D.M. Brown and R.A. Ulstran, Nature *212*, 1270 (1966).