SPECIFIC ANTISERUM TO LEU-ENKEPHALIN AND ITS USE IN A RADIOIMMUNOASSAY

B. A. WEISSMAN and H. GERSHON*

Departments of Pharmacology and Immunology, Technion – The Aba Khoushy School of Medicine, Haifa, Israel*

and

C. B. PERT

Adult Psychiatry Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland, USA

Received 15 September 1976

1. Introduction

Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) have recently been isolated from procine brain [1] and shown to exhibit morphine like activity in different paradigms [1–6]. For the lack of a direct, sensitive assay, indirect methods for the estimation of these morphine like peptides have been used [5–7]. These include various separation techniques in order to measure peptide levels after morphine administration [6]. This paper describes the preparation of an immunogenic protein-conjugate of Leu-enkephalin and the specificity of an antiserum generated to this antigen in rabbits. Such an antiserum, when used in a radioimmunoassay as described herein, should provide a useful tool for studying the role of both Leu- and Met-enkephalin in the 'Pain Pathway'. According to a recent hypothesis, enkephalin levels in the central nervous system should determine not only the pain threshold but phenomena such as tolerance, physical dependence and the withdrawl syndrome as well [8,9]. From this respect, the importance of a radioimmunoassay is obvious.

Send correspondence to: Dr B. A. Weissman, Dept. of Pharmacology, Technion – The Aba Khoushy School of Medicine, P.O. Box 9649, Haifa, Israel.

2. Methods and materials

2.1. *Preparation of Leu-enkephalin–BSA conjugate*

Twenty mg (3.3×10^{-2} mmol) Leu-enkephalin (Miles-Yeda Ltd., Rehovot, Israel) and 10 mg bovine serum albumin (BSA) (Fraction V, Sigma) were dissolved in 0.5 ml water and to them was added 0.25 ml of freshly prepared solution of 150 mg 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (Sigma) in water. This mixture was permitted to proceed with gentle agitation at room temperature for 15 min. The reaction mixture became turbid after few minutes [10] and remained so even after dilution. The reaction was terminated by dialysis against 1 liter of saline for 24 h. The antigen was stored in the cold until it was used for immunization.

2.2. *Immunization*

Three mature female rabbits were injected intradermaly in several sites along the flank, rump and nape with an emulsion of Leu-enkephalin–BSA (1 mg/rabbit) in complete Freund's adjuvant. Two weeks later they were given a similar immunization and after an additional two weeks they were bled and the sera were tested for presence of anti-Leu-enkephalin antibodies. Subsequently, the rabbits were boostered once every month and bled two weeks after boosting.

2.3. *Radioimmunoassay (RIA) of Leu-enkephalin*

To 0.1 ml of a known amount of unlabelled Leu-

enkephalin or of a heterologous peptide, or rat brain extract, in a total volume of 0.3 ml 50 mM Tris–HCl buffer (pH 7.0) containing 0.1% sodium azide was added 0.1 ml [^{3}H] Leu-enkephalin (50 Ci/mmol) (about 100 pg) in Tris buffer, followed by 0.1 ml of antiserum appropriately diluted in the same buffer. The dilution of antiserum was chosen so as to yield about 40% binding of the labelled hapten in the absence of an unlabelled peptide. This mixture was incubated for 4 h at 4°C. A saturated solution of ammonium sulfate (0.3 ml) was then added and the incubation continued for 30 min in the cold. The supernatants were discarded by aspiration and the precipitates resuspended in 1 ml water. Radioactivity was measured in the preciptates by sampling 0.9 ml aliquots and counting them in a xylene–triton X-100 'cocktail' [11] in a Packard Tri-Carb Spectrometer.

2.4. *Brain extracts*

Five rats of the Charles-River strain (220–260 g) were decapitated and their brains were immediately removed and homogenized in 5 ml ice cold 5% trichloroacetic acid. After centrifugation at 100 × *g* for 15 min the supernatants were extracted three times with 3 ml of benzene, lyophilized and reconstituted in 1 ml Tris–HCl buffer.

3. Results

3.1. *Formation of antibodies to Leu-enkephalin*

Antibodies binding [^{3}H] Leu-enkephalin appeared in the serum of the immunized rabbits one month after the first immunization. Three months later the serum of one immunized rabbit (No. 6) bound 50% of the labelled hapten (80 pg/assay tube) at a 1:750 dilution; the corresponding titers of the sera from the other immunized rabbits ranged from 1:200–1:400.

3.2. *Sensitivity and specificity of the antiserum to Leu-enkephalin*

The sensitivity of the RIA for Leu-enkephalin was found to be approximately 50 pg (fig.1). Whole brain extracts (from five rats) inhibited the binding of [^{3}H] Leu-enkephalin by the antiserum to Leu-

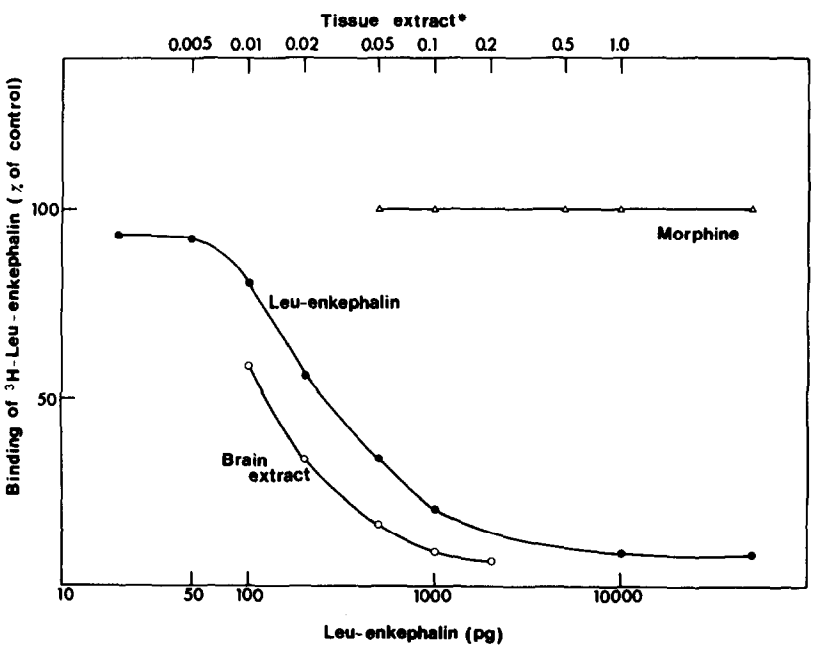

Fig.1. Inhibition of binding of [^{3}H] Leu-enkephalin to antiserum by unlabelled Leu-enkephalin, morphine or by rat brain extract. Anti-Leu-enkephalin serum used at 1:500 dilution.
* Units indicate the dilution of the extract. Original solution contained one brain per 1 ml Tris-buffer.

Table 1
Specificity of anti-Leu-enkephalin serum

No.	Compound	Relative activity (%)
1	Tyr-Gly-Gly-Phe-Leu	100.0
2	Tyr-Gly-Gly-Phe-Met	20.0
3	Dopa-Gly-Gly-Phe-Met	1.1
4	Tyr-Gly-Gly-Phe-Leu-NH_2	0.3
5	Arg-Tyr-Gly-Gly-Phe-Met	1.8
6	Lys-Arg-Tyr-Gly-Gly-Phe-Met	2.2
7	His-Gly-Gly-Phe-Met	2.7
8	Tyr-Gly-Gly-Phe-Met-NH_2	< 0.1
9	Trp-Gly-Gly-Phe-Met	< 0.1
10	Gly-Gly-Phe-Met	<< 0.01
11	Morphine	<< 0.01

Peptides and morphine were tested in doses ranging from 10 pg–10 mg. Anti Leu-enkephalin was used in a dilution of 1:500. Compound No. 1 and 2 were the products of Miles-Yeda Ltd., Israel. Compounds No. 3–10 were the products of Beckman Instruments Inc., Palo Alto.

enkephalin–BSA. The inhibition curve was parallel to that produced by synthetic Leu-enkephalin (fig.1). Morphine sulfate examined at various concentrations did not inhibit [^{3}H] Leu-enkephalin binding. The extent to which the antiserum to Leu-enkephalin crossreacted with a variety of other analogues to Leu-enkephalin is shown in table 1.

4. Discussion

The physiological function of Leu- and Met-enkephalin has not been determined yet. In order to facilitate the research in this area there is a need for a sensitive, specific and reproducible tool for estimating the levels of these peptides in biological extracts. The radioimmunoassay reported here offers such a procedure.

Based on chemical consideration, we decided to conjugate Leu-enkephalin to BSA by the soluble carbodiimide method [10] although, antigens generated in this fashion do not show high binding capacity ([10,12 and refs. cited therein]).

At a 1:500 dilution of the antiserum, one can detect the presence of 50 pg Leu-enkephalin per assay tube (fig.1). It should be noted that [^{3}H] Leu-enkephalin with a specific activity of 50 Ci/mmol is used in this RIA; it should be possible to lower the sensitivity limit of this assay by the use of an immunoreactive [^{125}I] -labelled peptide. It should be noted though, that from the results reported here and in a recent report [13], it appears that substitution on the aromatic ring of Tyr will render the peptide inactive.

Data shown in table 1 illustrate the fact that even minute chemical alterations in Leu-enkephalin bring about dramatic changes in the immunoactivity of the peptides, as demonstrated by reduced potency in the RIA. Met-enkephalin, the more potent of the two peptides in different biological tests [1,2,5], is 5-times less active than the homologues antigen Leu-enkephalin. Hydroxylation of the tyrosine residue, yielding the dopa-analogue of Met-enkephalin resulted in a much lower immunoreactivity compared to the parent compound (No. 3, 1.1%) while the amide derivative of Leu-enkephalin (No. 4) shows a marginal activity (0.3%). It is of interest that both the hexa- and hepta-peptides, No. 5 and No. 6, which are residues of the c-fragment of lipotropin [14] exhibit some activity in displacing antibody-bound [^{3}H] Leu-enkephalin. Subsitution of the tyrosine residue by histidine (No. 7) shows similar reactivity (2.7%). Peptides Nos. 8, 9 and 10, as well as morphine, did not demonstrate any measurable activity in the concentrations tested.

The inability of morphine to displace [^{3}H] Leu-enkephalin in this assay should simplify the protocols used in examining enkephalin levels after narcotics administration [6]. Hypotheses described in some recent reports [8,9] postulate that the phenomena of tolerance, physical dependence and withdrawal syndrome are consequences of narcotics – 'endogenous morphine' interactions. Thus, manipulations of the enkephalin(s) generating system(s) can now be quantitatively examined in order to better understand these phenomena as well as the 'pain pathway'.

The parallel [^{3}H] Leu-enkephalin-binding inhibition curves produced by unlabelled Leu-enkephalin and an extract of rat brain indicate immunochemical similarity if not identity between synthetic Leu-enkephalin and the endogenous peptides.

References

[1] Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergil, L. A., Morgan, B. A. and Morris, H. R. (1975) Nature 258, 577–579.
[2] Buscher, H. H., Hill, R. C., Romer, D., Cardinaux, F., Closse, A., Hauser, D. and Pless, J. (1976) Nature 261, 423–425.
[3] Bradley, P. B., Briggs, I., Gayton, R. J. and Lambert, L. A. (1976) Nature 261, 425–426.
[4] Gent, J. P. and Wolstencroft, J. H. (1976) Nature 261, 426–427.
[5] Chang, J. K., Fong, B. T. W., Pert, A. and Pert, C. B. (1976) Life Sci. 18, 1473–1482.
[6] Simantov, R. and Snyder, S. H. (1976) Nature 262, 505–507.
[7] Terenius, L. and Wahlstrom, A. (1975) Life Sci. 16, 1759–1764.
[8] Kosterlitz, H. W. and Hughes, J. (1975) Life Sci. 17, 91–95.
[9] Roy, A. C. and Collier, H. O. J. (1975) Life Sci. 16, 1857–1862.
[10] Goodfriend, T. C., Levine, L. and Fasman, G. D. (1964) Science 144, 1344–1346.
[11] Liao, S., Liang, T., Fang, S., Castaneda, E. and Shao, T. C. (1973) J. Biol. Chem. 248, 6154–6162.
[12] Koch, Y., Wilcheck, M., Fridkin, M., Chobsieng, P., Zor, U. and Lindner, H. R. (1973) Biochem. Biophys. Res. Commun. 55, 616–622.
[13] Terenius, L., Wahlstrom, A., Londeberg, G., Karlsson, S. and Ragnarsson, U. (1976) Biochem. Biophys. Res. Commun. 71, 175–179.
[14] Bradbury, A. F., Smyth, D. G. and Snell, C. R. (1976) Biochem. Biophys. Res. Commun. 69, 950–956.