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RADIOIMMUNOASSAY FOR γ-ENDORPHIN

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SUMMARY.

A double antibody radioimmunoassay technique for γ-endorphin has been developed. The antisera have been raised in rabbits against synthetic γ-endorphin coupled to bovine serum albumin by carbodiimide. The best antibody has a working titer of 1/35,000 and can detect less than 9 pg of peptide. The usable range of the standard curve is between 9 to 2400 pg. This antiserum probably binds the Glu^{8} - Leu^{17} region of γ-endorphin and shows only weak cross-reactivity with α-endorphin, β-endorphin and β-lipotropin. Parallelism is observed between the standard curve and the inhibition curves obtained with rat neurohypophysis-pars intermedia extracts or rat plasma.

The pituitary gland and the central nervous system contain endogenous peptides with naloxone-reversible morphinomimetic activity (1-6). Two of these peptides, termed enkephalins, were isolated and characterized as methionine-enkephalin (Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH). Four other peptides which act as agonists at opiate receptor sites were later isolated from the hypothalamus-pituitary extracts. These peptides, termed endorphins, were characterized as the fragments 61-76 (9), 61-91 (10,12), 61-77 (11) and 61-87 (12) of β-lipotropin (β-LPH) and were named α-, β-, γ- and δ-endorphins, respectively. The ability of all the enkephalins and endorphins to bind to opiate receptors and to mimic morphine bioactivity precludes the identification and measurement of each of these endogenous opioid

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peptides by those bioassays. Furthermore, the assessment of regional endorphin contents in brain and in endocrine tissues or the measurement of plasma endorphin levels requires specific assays which would differentiate each peptide. The present report describes the production and evaluation of γ-endorphin antibodies and the development of a sensitive and specific radioimmunoassay technique for the assessment of γ-endorphin in rat plasma or tissue homogenates.

MATERIALS AND METHODS

Preparation of immunogen. Synthetic γ-endorphin prepared by one of us (13) was covalently linked to bovine serum albumin (BSA, Serva Feinbiochemica, Heidelberg) with 1-ethyl-3(3-dimethylaminopropyl carbodiimide-HCl) (ECDI) (14): 5.48 mg of synthetic γ-endorphin and approximately 2.5 ng of ^{125}I labelled γ-endorphin (10^6 cpm) were dissolved in 0.3 ml of 0.1 mM HCl and conjugated to 10 mg BSA by 100 mg of ECDI (Sigma Chemical Co.) in a final volume of 0.9 ml. Once the reaction was completed (5 days, 4°C, in the dark), the precipitate was suspended in 4 ml chilled distilled water.

A small volume (50 µl) was set aside to determine the initial radioactivity, and the mixture was dialyzed against 2 l distilled water at 4°C. The water was changed 2 times in 2 days and no radioactivity was found in the water at the end of dialysis. The final volume of the dialyzed conjugate was measured and a 50 µl aliquot was removed to assess the retained radioactivity. The coupling efficiency of this method, as measured by isotopic dilution, was 31%. Approximately 6 molecules of γ-endorphin were conjugated to one BSA molecule.

The dialyzed conjugate was mixed with 20 ml 0.17 M pH 7.5 phosphate sodium buffer (PS buffer) containing 0.002% tween 20, aliquoted in 1 ml doses and stored frozen.

Raising antibodies. Rabbits were injected intradermally in 50 sites of the interscapulo-vertebral region (15) with 1 dose of immunogen per rabbit (85 µg of γ-endorphin conjugate in 1 ml) emulsified in an equal volume of Freund's adjuvant. They were boosted with the same amount of conjugate at monthly intervals. Rabbits were bled 10 days after each injection, sera were separated in aliquots and stored at -25°C.

Iodination of peptide. Two microgram of γ-endorphin diluted in 10 µl PS buffer were labeled with 1 mCi of ^{125}I using 20 µg of chloramine-T in 20 µl 0.5 M pH 7.6 phosphate buffer for 15 sec and the excess chloramine-T decomposed with 60 µg of $Na_2S_2O_5$ in 20 µl of the same buffer The labeled peptide was purified by Sephadex G-25 gel filtration. The column was equilibrated and eluted with the PS buffer.

Radioimmunoassay procedure. The first incubation (2 days, 4°C) was performed in 5 ml polystyrene test tubes. The labeled peptide (3000 cpm/tube), antibody and standard peptide or unknown samples were diluted up to 500 µl in PS buffer containing 0.002% tween 20. To separate the antibody-bound γ-endorphin, 200 µl of normal rabbit serum (1/100 dilution) and 200 µl of anti-rabbit sheep γ-globulins (1/50 dilution) in 0.02 M pH 8.6 veronal buffer were added and the mixture was incubated at 4°C for another 2 days. After the second incubation, the tubes were centrifuged (20 min, 4°C, 3100 g), the supernatant was aspirated and the precipitate counted.

Animals. Ten female Sherman rats weighing 200-230 g were used

for this study. They were caged in groups of 6 animals in a temperature controlled (24 ± 1°C) room with a light (07.00 - 19.00 h) dark (19.00 - 07.00 h) cycle for 10 days and were fed ad libitum commercial chow and water.

The animals were killed by decapitation. Systemic blood was collected in 5 ml chilled polystyrene tubes containing 100 µl of 5% EDTA. The tubes were centrifuged at 4°C, plasma was separated and immediately assayed by the γ-endorphin RIA.

The roof of the skull and the brain were removed from the second group of rats. The neuro-intermediate lobe of the pituitary gland (PIN) was carefully separated in situ, weighed, and immersed in 1 ml of chilled PS buffer. The tissues were homogenized (30 sec, 4°C, high speed homogenizer), centrifuged (3100 g, 4°C, 20 min) and the supernatant stored at -25°C.

Serial dilution (r = 1/2) of plasma or pituitary homogenates were performed in PS buffer and radioimmunoassayed for γ-endorphin in order to check the parallelism between the inhibition curves and the standard curve.

RESULTS

Titre. Two of the four rabbits immunized against γ-endorphin gave, after the third injection, antibodies usable at titers of 1/3,000 and 1/10,000 respectively. After the fourth injection, antiserum coded 86-1301, used at a final dilution of 1/35,000, binds 40% of the labeled hormone. The sensitivity of this antiserum is ≤9 pg/tube. Use of the logit/log graphic representation makes it possible to linearize the standard curve. Under these conditions, usable range of the inhibition curve is between 9 to 2400 pg γ-endorphin (Fig. 1).

Specificity. The specificity of the 86-1301 antiserum was evaluated by determining its cross-reactivity with various β-LPH short-chain analogs synthesized by one of us (N.L.). Figure 2 shows the results obtained for doses ranging from 1 to 10^6 pg of each peptide. The antiserum probably reads the region comprising Glu^{68} -Leu^{77} of the β-LPH molecule. It does not cross-react with the Tyr^{61}-Glu^{68} fragment which includes the pentapeptide methionine-enkephalin. Very weak cross-reactions are observed with α-endorphin. The cross-reactivities of β-endorphin and β-LPH with the antiserum, calculated from the ratios at IC_{50}, are 4.9% and 1.3% respectively on a weight basis.

Immunological identity between synthetic γ-endorphin and plasma or pituitary γ-endorphin. The displacement of antibody-bound ^{125}I-

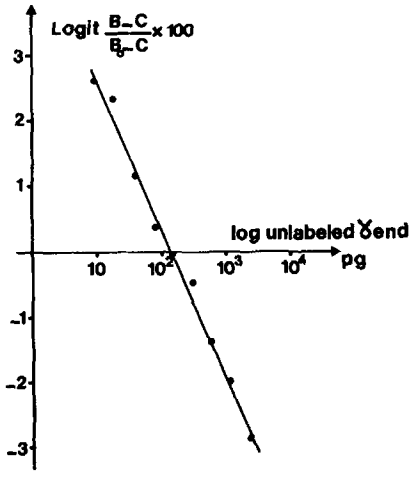

FIGURE 1.
Standard curve for γ-endorphin, linearized by means of logit/log transformation.

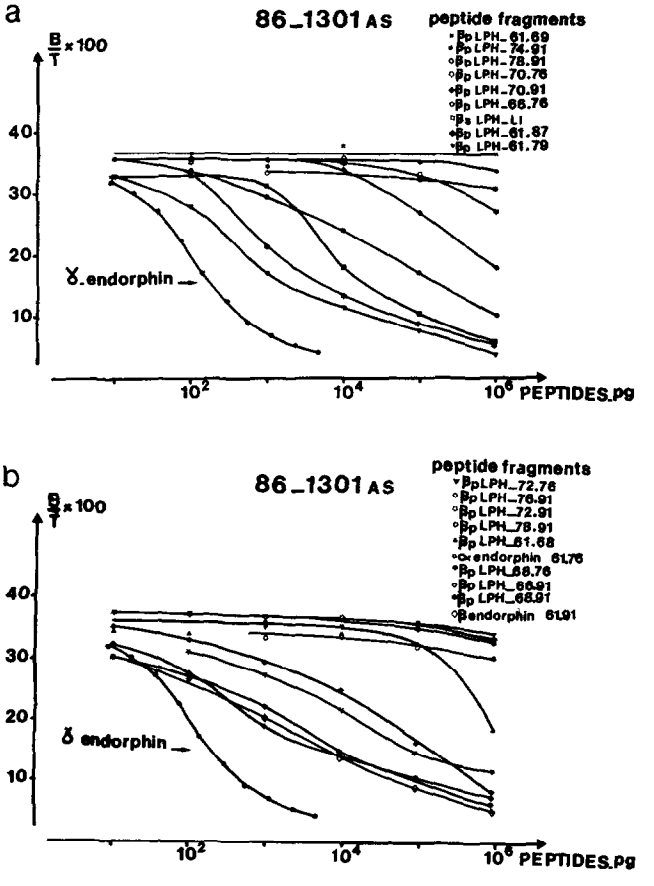

FIGURE 2. Competitive inhibition of ^{125}I-labeled γ-endorphin binding to antibodies to γ-endorphin by synthetic γ-endorphin and various β-LPH short chain analogs.

labeled γ-endorphin by synthetic γ-endorphin and by plasma dilutions or by various dilutions of PIN extracts from normal female rats yielded parallel binding curves (Fig. 3).

DISCUSSION

Even though it is theoretically possible to measure very small amounts of enkephalins and endorphins with bioassay methods, based on the competition with either [^{3}H]opiates or [^{3}H]Leu-enkephalin for brain opiate receptors (16), radioimmunoassays alone make it currently possible specifically to assess samples' contents in one endorphin or another. Since it has been recently shown that many brain areas, including hypothalamus, striatum and hippocampus may contain both enkephalins and endorphins (17-20), the development of specific radioimmunoassays will be useful for the determination of the regional distribution of each peptide within brain tissues.

Radioimmunoassay methods for met-enkephalin and leu-enkephalin have already been developed (18-19). Concurrently, double antibody radioimmunoassays for α- and β-endorphins, which make possible the assessment of these peptides in peripheral blood or tissue homogenates, have been described (21,22). The method reported in this study for γ-endorphin antibody production made it possible to obtain antisera with working titers ranging from 1/15,000 to 1/35,000. Among the antibodies produced, 86-1301 is particularly sensitive since it can detect less than 9 pg of synthetic γ-endorphin. On a molar basis, the cross-reactivity with α-endorphin, β-endorphin and β-LPH are 0.37%, 9.4% and 7.1%, respectively. The absence of significant cross-reactions between α-endorphin and the antiserum to γ-endorphin is of great interest since α- and γ-endorphin differ only by the Leu^{77}-residue. The cross-reactions observed with β-endorphin and with β-LPH indicate that these molecules will interfere in the assay. However, the amounts (expressed as the weight of peptide) of β-endorphin and β-LPH required to obtain a signifi-

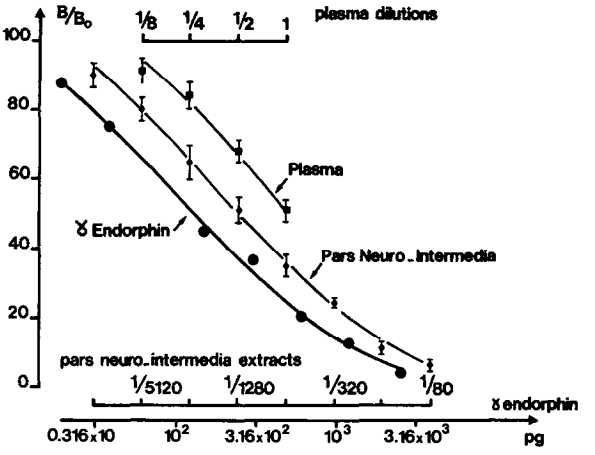

FIGURE 3 Competitive inhibition of ^{125}I-labeled γ-endorphin binding to antibodies to γ-endorphin by synthetic γ-endorphin, rat plasma dilutions and rat pars neuro-intermediate extracts.

cant decrease in antibody-bound labeled γ-endorphin are 20 and 77 times higher than those of γ-endorphin. The study of the different cross-reactions obtained with various synthetic short-chain analogs leads us tentatively to conclude that the 86-1301 antiserum reads the region between Glu^{8}-Leu^{17} of the γ-endorphin molecule.

The dilution curves obtained with PIN extracts and with plasma samples from normal rats are parallel to those obtained with synthetic γ-endorphin. These data provide the first evidence that rat PIN and rat plasma contain a peptide immunologically similar to synthetic γ-endorphin.

The concentration of γ-endorphin in rat PIN is 110.5 ± 14.4 ng/mg fresh tissue (59.4 ± 7.7 pmole). This concentration is 14 times lower than α-MSH concentration in rat PIN (23) but is similar to β-endorphin concentration in rat pituitary (20). The plasma concentration of γ-endorphin in normal rats is 1430 ± 350 pg/ml. This concentration is about ten times higher than plasma α-MSH levels as measured by radioimmunoassays (23-24) but almost the same as β-endorphin on a molar basis (25). Availability of this antiserum specifically to measure γ-endorphin concentra-

tions in tissue fluids or extracts is particularly of interest in view of the recent demonstration by Graf (26) of the existence of specific enzymes in pituitary and brain tissues that cleave β-endorphin at the Leu^{17}-Phe^{18} bond to generate γ-endorphin.

Further experiments are in progress to determine the endocrine and (or) nervous mechanisms which may control the release of γ-endorphin from rat PIN in vivo and in vitro.

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