RADIOIMMUNOASSAYS FOR α-ENDORPHIN AND β-ENDORPHIN

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SUMMARY

This note describes the technical details of double antibody radioimmunoassays for the two peptides α- and β-endorphin and the specificity characteristics of the antisera. Antisera raised in rabbits to synthetic α-endorphin measure quantitatively α-endorphin; usable range is 50 pg to 5 ng. Their specific recognition site is toward the C-terminal region (Ser^{10}-Thr^{16}) of α-endorphin. Other antisera, raised in rabbits to synthetic β-endorphin measure quantitatively β-endorphin; usable range is 50 pg to 5 ng β-endorphin. Their specific recognition site is toward the C-terminal region (Asn^{20}-His^{27}) of β-endorphin; these antibodies recognize β-endorphin i.e. β-LPH-(61-91), β-lipotropin i.e. β-LPH-(1-91), and the precursor molecule to LPH-ACTH with molecular weight ca. 31 x 10^3 (31K-precursor). Neither antiserum binds enkephalins.

Characterization in hypothalamic and hypophysial extracts of several endorphins (α, β, γ, δ) (1-4) and two enkephalins in extracts of whole brain (5,6) has led to the realization that the tests used so far to assess their biological activity (guinea pig ileum and mouse vas deferens bioassays, competition for the binding of some labelled opiate agonist or antagonist on synaptosomal preparations, modification of cAMP content of a neuroblastoma-glioma hybrid), could not and would not differentiate qualitatively each of these peptides. All are active in the in vitro assays, all are active in the synaptosomal binding assays, though admittedly with variable specific activities. Furthermore, several of these tests will give positive results with totally unrelated substances such as N-terminal fragments of ACTH*(7). Such nonspecific responses limit considerably the significance of the many reports based on these types of assays claiming modifications

*Abbreviations: ACTH = Adrenocorticotropic hormone, β-LPH = β-lipotropin, RIA = radioimmunoassay, MW = molecular weight, NRS = Normal rabbit serum

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of "opiate-like substances" in one or another experimental protocol. Hence the interest in developing and obtaining antisera of well defined specificity.

MATERIALS AND METHODS

a. Peptides. All peptides used here, either replicates of native (isolated) molecules or fragments or analogs thereof designed for studies of the specificity of the antisera were prepared by one of us (NL) using solid phase methodology (8) as previously described; all these peptides were obtained in a state of high purity by the methodology used routinely here (9).

b. Preparation of immunogens. Synthetic α-endorphin (48 mg) was coupled to bovine thyroglobulin (80 mg) with bis-diazotized benzidine (1.1 ml of a 7 mg/ml solution) in 0.16 M sodium borate - 0.13 M sodium chloride buffer at pH 9.0 (10 ml) for 2 hours at 0°. The resulting reddish solution was dialyzed against 1 liter H_2O for 10 hours five times to remove the unconjugated peptide. Coupling of β-endorphin to bovine serum albumin was similarly performed with bis-diazotized benzidine at ratios of 41.8 mg peptide, 80 mg protein and 0.45 ml benzidine solution as above.

c. Raising antibodies. Rabbits were injected in the dermis of the back at multiple sites with the conjugate (2 mg total dose) mixed with Freund's adjuvant; they were boosted with 1.0 mg of the conjugate at monthly intervals. Sera containing antibodies were vialed in 1 ml aliquots and kept frozen at -20°C.

d. Iodination of peptides. α- and β-endorphins were iodinated using chloramine-T (10), the reaction being stopped with sodium metabisulphite. The labelled peptides were purified by chromatography on Sephadex G-25.

e. Radioimmunoassays for α- and β-endorphin. Complete description of the procedures follows.

Buffer solutions used in RIA for α-endorphin or β-endorphin:

Buffer A: Phosphate buffered saline: 0.02 M sodium phosphate, 0.15 M NaCl, 0.01% thimerosal, pH 7.5.
Buffer B: 0.1% gelatin (purified calfskin, Eastman 1099) in buffer A.
Buffer C: 0.01% crystalline bovine serum albumin (Miles Labs. 81-001-3) in buffer B.
Buffer D: Buffer C containing 0.1% Triton X-100, pH adjusted to 7.5 with 5 N NaOH.

RIA for α-endorphin.

Samples for testing are kept on ice but RIA tubes are at room temperature during pipetting of samples and reagents. All incubations of antisera, labelled and cold peptides are conducted at 5-8°C.

Standard solutions of α-endorphin and unknown samples are added (normally in 100 µl volumes) to test tubes and diluted with buffer B to a volume of 500 µl; 500 µl of buffer B are added to "maximum binding" and "non-specific binding" tubes.

Preincubation with first antibody. Antiserum to α-endorphin is diluted with buffer B to a concentration sufficient to give 30-40% uncompeted binding of trace. (For the antiserum coded RB66-5/10 a starting dilution of 1/4000 is used). Normal rabbit serum (NRS) is also added to the first antibody solution to give a total rabbit serum concentration of 0.5%; 100 µl of this solution are added to all except "non-specific binding" tubes which

receive 100 µl of 0.5% NRS in buffer B. Reagents are mixed and the tubes incubated for 24 hours.

Addition of trace. A frozen aliquot of ^{125}I-labelled α-endorphin prepared as above (d) is thawed and diluted in buffer B to give ca. 12,000 cpm/100 µl; 100 µl are added to all tubes. Reagents are mixed and the tubes incubated for 72 hours.

Addition of second antibody. Fifty microliters of goat antirabbit γ-globulin diluted with buffer B to concentration sufficient for maximal precipitation are added to all tubes. Reagents are mixed and the tubes incubated for 24 hours. After incubation with the second antibody all tubes receive 2 ml of buffer A and are centrifuged for 30 minutes at 3000 rpm at 4°C. The supernatant is aspirated and pellets are counted.

RIA for β-endorphin.

Standard solutions, samples for testing and RIA tubes are kept on ice during assay pipetting. All incubations are done at 5-8°C.

Note: The presence of bovine serum albumin in the RIA system is recommended as it greatly increases the binding of labelled β-endorphin to the specific antibody and increases the sensitivity of the assay. In this RIA system 500 µl of 0.01% crystalline bovine serum albumin is sufficient; increasing amounts have no further effects. Some batches of Cohn's Fr. V have been observed to cause the rapid disappearance of β-endorphin probably due to some contamination with proteolytic enzymes. Even in the presence of albumin, iodinated β-endorphin sticks avidly to plastic and concentrates at air/liquid interfaces and is therefore difficult to pipet reproducibly. The use of disposable borosilicate glass tubes and of Triton X-100 in the trace solution alleviates these problems.

Standard solutions of β-endorphin in 100 µl volumes and unknown samples in 10-100 µl volumes are added to test tubes and diluted with buffer C to a volume of 500 µl. "Maximum binding" and "non-specific binding" tubes receive 500 µl of buffer C.

Addition of trace. A frozen aliquot of concentrated ^{125}I-labelled β-endorphin prepared as above (d) in buffer C is thawed and diluted with buffer D to give ca. 5000 cpm/100 µl; 100 µl are added to all tubes. Reagents are mixed.

Addition of first antibody. Antiserum to β-endorphin is diluted with buffer B to a concentration sufficient to give 30-40% uncompeted binding of trace (for the antiserum coded RB100-10/76 a starting dilution of 1/5000 is used). Normal rabbit serum is also added to the first antibody solution to give a total rabbit serum concentration of 0.5%; 100 µl are added to all tubes. Reagents are mixed and tubes are incubated for 4-24 hours.

Addition of second antibody. Fifty microliters of goat antirabbit γ-globulin diluted with buffer B to a concentration sufficient for maximal precipitation is added to all tubes. Reagents are mixed and incubated for 16-24 hrs. At the end of the incubation all tubes receive 2 ml of buffer A and are centrifuged for 30 minutes at 3000 rpm at 4°C. The supernatant is aspirated and pellets are counted.

In all experiments, reference standards are always run in triplicates; unknowns are run in duplicate at three doses.

RESULTS AND COMMENTS

With antiserum RB66-5/10 raised against α-endorphin, at a final dilution of 1/21,000, sensitivity is ≤ 50 pg; half maximal displacement is usually

TABLE 1

Specificity of α-Endorphin Antiserum RB66-5/10

α-Endorphin	†Y G G F M T S E K S Q T P L V T	100*
α-Endorphin-NH_2	Y G G F M T S E K S Q T P L V T·	5
[Gln^8]-α-Endorphin	Y G G F M T S Q K S Q T P L V T	100
γ-Endorphin	Y G G F M T S E K S Q T P L V T L	0.1
γ-Endorphin-NH_2	Y G G F M T S E K S Q T P L V T L·	0.1
Met^5-Enkephalin	Y G G F M	0
β-LPH-(61-69)	Y G G F M T S E K	0
β-LPH-(70-76)	S Q T P L V T	50
β-LPH-(71-76)	Q T P L V T	15
β-LPH-(72-76)	T P L V T	< 0.05
β-LPH-(73-76)	P L V T	< 0.05
β_o-Endorphin	β-LPH-(61-91)	0.05
β_o-LPH-(1-91)		0.08

*Numbers show molar ratios x 100 of compound to the left when compared to α-endorphin, for 50% competition of trace binding to the antibody (EB_{50}). Any number different from zero implies that at no less than 3 doses each compound tested gives a binding curve parallel to that of α-endorphin reference standard.
†Peptide sequences shown in the one-letter code of Dayhoff (see Atlas of Protein Sequence and Structure, M.O. Dayhoff, Nat. Biomed. Res. Fdt., Silver Springs, Md., Publ. 1975).
Subscripts o: ovine; p: porcine (Table 2 only).

obtained at 200-300 pg; usable range of the displacement curve is 50 pg to 5 ng α-endorphin. Table 1 shows the specificity of this antiserum; it recognizes a portion of the C-terminal of α-endorphin comprised between Ser^{10} and Thr^{16} or β-LPH-(70-76). It does not bind either Leu^5- or Met^5-enkephalin, nor, in practice, β-endorphin or β-LPH.

With antiserum RB100-10/27 raised against β-endorphin, at a final dilution of 1/28,000, sensitivity is ≤50 pg; half maximal displacement is usually obtained at 200-300 pg; usable range of the displacement curve is ≤50 pg to 5 ng β-endorphin. Table 2 shows the specificity of this antiserum as investigated so far. It can be proposed that it recognizes a specific conformation of the C-terminal of β-endorphin comprised between Asn^{20} and His^{27} which is stabilized by an NH_2-terminal fragment between Thr^6 and Lys^{19} or of longer chain length. It binds neither enkephalins nor α- or γ-endorphin.

Ovine β-lipotropin (β-LPH) of two different sources (gifts of C.H. Li and M. Chrétien) competes in parallel with the binding of β-endorphin. This

TABLE 2

Specificity of β-Endorphin Antiserum RB100-10/27

β_o-Endorphin	β-LPH-(61-91)	61 Y G G F M T S E K S 71 Q T P L V T L F K N 81 A I I K N A H K K G Q	100*
β_p-Endorphin		... A I V K N A H K K G Q	100
[Leu^5]-β_p-Endorphin	[Leu^5]-β-LPH-(61-91)	61 Y G G F L T S E K ...	100
Met^5-Enkephalin	β-LPH-(61-65)		0
α-Endorphin	β-LPH-(61-76)		0
γ-Endorphin	β-LPH-(61-77)		0
δ_o-Endorphin	β-LPH-(61-87)		100
	β-LPH-(62-91)		100
	β-LPH-(66-91)		100
	β-LPH-(78-91)		0
β_o-Lipotropin	β-LPH-(1-91)		100
31K-Precursor			100
1N HOAc Extract (rat)	Whole Pituitary		parallelism
1N HOAc Extract (rat)	Whole Brain		parallelism
Morphine Sulfate			1×10^{-7}

*Legend for Table 2 as in Table 1

is easily explained: β-endorphin is the whole C-terminal (61-91) fragment of β-LPH and antiserum RB100-10/27 is directed to the C-terminal region of β-endorphin. On a weight basis, β-LPH is ca. 3 X less potent than β-endorphin in the displacement assay. On a molar basis, the two molecules are equipotent, again in keeping with knowledge that one molecule of β-LPH-(1-91) (MW ca. 9200) contains one molecule of β-endorphin, i.e. β-LPH-(61-91) (MW ca. 3400). Thus we can conclude that in aqueous solution, the configuration of β-LPH is such that its C-terminal region is readily exposed and available for binding to the antibody.

Crude extracts (1 N acetic acid) of (rat) whole pituitary gland and whole brain contain substances which compete in parallel with the binding of β-endorphin. Mains, Eipper and Ling have recently shown that pituitary β-LPH and ACTH have a common biosynthetic origin, in the form of a large glycoprotein molecule of MW ca. 31×10^3 (11), referred to here as 31K-precursor. In the course of these studies we have observed that the β-endorphin antiserum RB100-10/27 does bind 31K-precursor (sent to us by Mains and Eipper) in parallel to β-endorphin. The subsequent studies of Mains, Eipper and Ling have

shown that there is one molecule of β-endorphin/molecule of 31K-precursor (11). We have preliminary evidence that sizing by gel filtration can separate 31K-precursor, β-LPH and β-endorphin from crude (rat) pituitary extracts.

The antisera for α- and β-endorphins described here have already been used in radioimmunoassays on extracts of bovine brain and pituitary by Krieger et al. (12), for immunoprecipitation in studies on the biosynthesis of LPH-ACTH by the AtT-20 rat cell line by Mains et al. (11), for immunocytochemistry of rat pituitary and brain by Bloom et al. (13). Physiological studies by our laboratory using these antibodies will be reported separately; they show that these antisera can be used to measure, in the rat, levels of α-, β-endorphin in peripheral blood and tissue extracts.

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