Vol. 94, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1980 Pages 211-217

EXISTENCE OF γ-MELANOTROPIN (γ-MSH)-LIKE IMMUNOREACTIVITY IN BOVINE AND HUMAN PITUITARY GLANDS

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Received March 20,1980

SUMMARY A radioimmunoassay for γ-melanotropin (γ-MSH) was designed with an antiserum obtained in a rabbit immunized with synthetic γ_3-MSH. The antiserum cross-reacts with synthetic γ_1-MSH and γ_2-MSH and slightly with β-lipotropin, but not with α-MSH, β-MSH, ACTH, and β-endorphin. Using this radioimmunoassay, γ-MSH-like immunoreactivity (γ-MLI) was detected in bovine and human pituitary glands. Gel chromatographic studies on Bio-Gel P-60 revealed a single component of γ-MLI in the bovine and human anterior pituitary, whereas an additional peak of small γ-MLI was observed in the bovine intermediate lobe.

ACTH and β-lipotropin (β-LPH) are known to be derived from an ACTH-β-LPH common precursor protein (1-3). Nakanishi et al (4) have recently determined the primary structure of the precursor protein based on the nucleotide sequence of complementary DNA for the bovine precursor, utilizing a newly developed recombinant DNA technique. This sequence demonstrates the existence of a melanotropin-like peptide containing the amino acid sequence of His-Phe-Arg-Trp in the cryptic N-terminal portion of the precursor protein, which has been termed γ-melanotropin (γ-MSH). The γ-MSH is located between pairs of consecutive basic amino acids, Arg^{-57}-Lys^{-56} and Arg^{-43}-Arg^{-42} or Lys^{-28}-Arg^{-27}, all possible sites of enzymatic cleavage. Because the termini of this proposed hormone are not definitive, Ling et al synthesized four possible γ-MSH peptides by solid phase methodology (5). These peptides, especially γ_3-MSH, had weak skin darkening and steroidogenic activities (5, 6). The present study was an attempt to demonstrate the

Abbreviations used in this paper: β-EP, β-endorphin; β-LPH, β-lipotropin; γ-MLI, γ-melanotropin-like immunoreactivity; MSH, melanotropin.

0006-291X/80/090211-07$01.00/0

existence of γ-MSH-like immunoreactivity (γ-MLI) in the pituitary glands, utilizing a radioimmunoassay for γ-MSH.

MATERIALS AND METHODS

Peptides. γ_1-MSH (Tyr^{-55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe^{-45}-NH_2), γ_2-MSH (Tyr^{-55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly^{-44}-OH), and γ_3-MSH (Tyr^{-55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln^{-29}-OH) were gifts from Ling and Guillemin.

Antiserum. The antiserum (JNFT 911) was produced in a rabbit immunized with synthetic γ_3-MSH conjugated with bovine serum albumin (Sigma Chemical Company) (7).

Iodination of γ_3-MSH. γ_3-MSH was labelled with Na^{125}I (The Radiochemical Center) using the chloramine T (Kanto Chemical Co. Inc., Tokyo, Japan) method of Hunter and Greenwood (8). The labelled γ_3-MSH was purified by adsorbing to silicic acid (100 mesh, Malinckrodt Inc.) (9). The specific activity was calculated to be 120 μCi/μg.

Radioimmunoassay for γ_3-MSH. Radioimmunoassay was performed by the method of Berson and Yalow (9). Unlabelled γ_3-MSH as standard or unknown samples were incubated with the antiserum diluted to 1/100,000 for 1 day at 4°C. Labelled γ_3-MSH was then added and the mixture was incubated further for 2 days at 4°C (10). The separation of antibody-bound from free labelled peptide was effected by adsorbing the free fractions to 50 mg of talc. γ-MLI was expressed as weight of γ_3-MSH.

Preparation of samples. Three bovine pituitaries obtained at the time of slaughter were promptly separated into the anterior and intermediate-posterior lobes. Each lobe was homogenized in a siliconized glass tube in 25 volumes of 0.2 N HCl. The homogenate was centrifuged at 10,000 x g, for 30 minutes at 4°C and the supernate was stored at -20°C. Protein concentrations were determined on an aliquot of unspun homogenate (11). Human pituitary was obtained from a patient with hepatoma 2 hr after death and frozen at -70°C. This preparation was subjected to the same extraction procedure as mentioned above. The samples were neutralized with 0.1 N NaOH immediately before assay. For chromatography, the pituitary extracts were lyophilized and reconstituted in 1 N acetic acid preheated to 95°C. After 10 min in the hot bath, the samples were chilled in ice and diluted with 0.05 M phosphate buffer, pH 7.4, containing 0.5 % human serum albumin (Fraction V, ICN Pharmaceuticals, Inc.), 500 kallikrein inactivator units/ml of Trasylol (Delbay Pharmaceuticals, Inc., Div. Schering Corp.), and 0.4 % 2-mercaptoethanol (Nakarai Chemicals, Ltd., Kyoto,Japan) (standard diluent). The recovery of synthetic γ_3-MSH was 80 % during the extraction procedure.

Gel filtration. Gel filtration was performed on a Bio-Gel P-60 (Bio-Rad Laboratories) column (0.7 x 52 cm) equilibrated and eluted with the standard diluent at a flow rate of 2.8 ml/hr. The fraction volume was 0.72 ml. Recovery of ^{125}I-γ_3-MSH in gel chromatography was 85 %. Markers used were blue dextran for void volume, human β-LPH, ^{125}I-human β-endorphin (β-EP) for β-EP, γ_3-MSH, and ^{125}I for the salt peak.

Radioimmunoassays for β-EP and ACTH. β-EP was radioimmunoassayed as described previously (12), with an antiserum which has the same affinity for human β-EP and human β-LPH, on a molar basis. ACTH was determined by the method of Berson and Yalow (9), utilizing an antiserum (West) directed towards the midportion of the ACTH molecule.

RESULTS

A typical standard curve with γ_3-MSH is shown in Fig. 1. Significant inhibition of the binding of ^{125}I-γ_3-MSH to antibody was evident with as little as 5 pg of γ_3-MSH. This antiserum showed a cross-reactivity with synthetic γ_1-MSH (21 %), γ_2-MSH (10 %), and human β-LPH (0.1 %), whereas it was not reactive with α-MSH, porcine β-MSH, ACTH, and human β-EP, even when quantities as large as 10 ng were added. The intra- and inter-assay coefficients of variation were 7.0 %, and 10.5 %, respectively. The parallelism of displacement curves with extracts of bovine anterior pituitary, intermediate-posterior pituitary, and human pituitary is depicted in Fig. 2A.

Gel chromatographic patterns of extracts of bovine anterior pituitary and human pituitary contained only one peak of γ-MLI, emerging near the elution position of β-LPH (Fig. 3A and Fig. 3C). On the other hand, gel chromatography of an extract of bovine intermediate-posterior pituitary revealed two peaks of γ-MLI (Fig. 3B) with the first peak eluting near the elution position of β-LPH, and the second peak emerging near the elution position of β-EP. There was no peak of γ-MLI at the position of synthetic γ_3-MSH. Serial dilutions of the fraction number 15 (the first

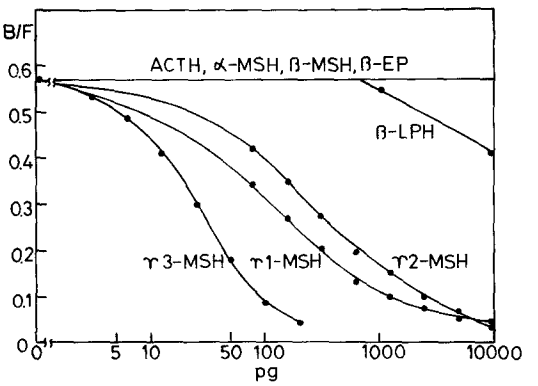

Fig. 1. Specificity of anti-γ_3-MSH antiserum. The standard curve with synthetic γ_3-MSH shows that 5 to 100 pg/tube of γ_3-MSH are measurable with this antiserum. γ_1-MSH, γ_2-MSH, and β-LPH cross-react 21 %, 10 %, and 0.1 %, respectively, whereas ACTH, α-MSH, β-MSH, and β-EP are not reactive.

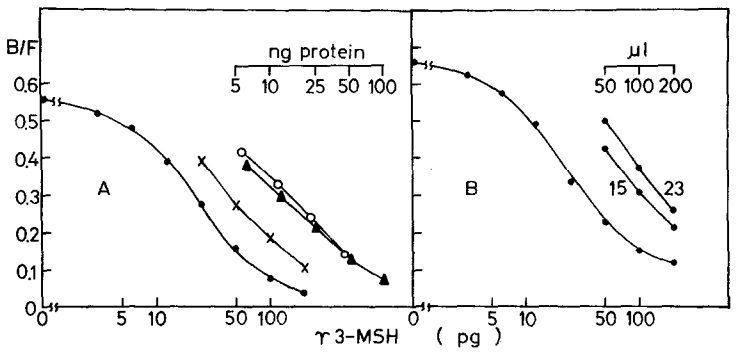

Fig. 2. A: Dilution curves of extracts of bovine anterior pituitary (▲——▲), intermediate-posterior pituitary (X——X), and human pituitary (O——O), exhibiting parallel inhibition with that of the standard γ_3-MSH (●——●).
B: Dilution curves of fraction No. 15 and 23 obtained by gel filtration of the extract of bovine intermediate-posterior pituitary, exhibiting parallel inhibition with that of the standard γ_3-MSH.

peak) and number 23 (the second peak) obtained from gel filtration of an extract of bovine intermediate-posterior pituitary gave parallel curves to the standard curve of γ_3-MSH as shown in Fig. 2B. The contents of γ-MSH-like, β-endorphin-like, and ACTH-like immunoreactivities in bovine anterior pituitaries, intermediate-posterior pituitaries, and human pituitary are shown in Table 1.

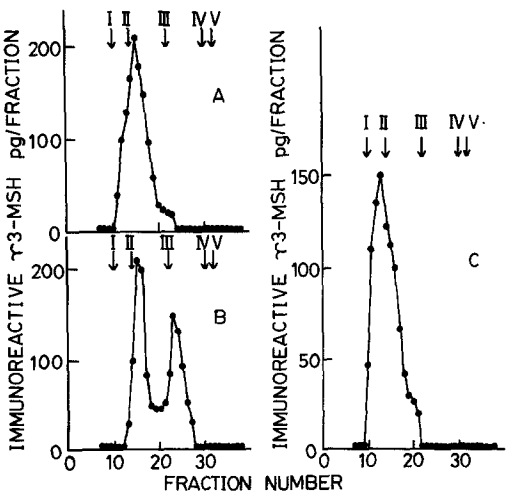

Fig. 3. Gel filtration profiles on a Bio-Gel P-60 column, (0.7 x 52 cm) of the extracts of bovine pituitary and human pituitary.
A: Bovine anterior pituitary.
B: Bovine intermediate-posterior pituitary.
C: Human pituitary. Arrows: I, void volume (blue dextran), II, β-LPH; III, β-EP; IV, γ_3-MSH; V, Iodine.

TABLE 1.

γ-MSH-like, β-endorphin-like, and ACTH-like immunoreactivities in bovine and human pituitaries

	No.	γ-MSH-like immunoreactivity* ng/100 μg protein	β-endorphin-like immunoreactivity ng/100 μg protein	ACTH-like immunoreactivity ng/100 μg protein
Bovine Pituitary				
Ant.	3	93 $\pm$ 24.4	463 $\pm$ 86.0	712 $\pm$ 139.1
Int.-Post.	3	560 $\pm$ 86.4	1041 $\pm$ 287.9	204 $\pm$ 12.1
Human Pituitary	1	135	2250	1920

mean $\pm$ S. E.

*γ-MSH-like immunoreactivity is expressed as ng of γ_3-MSH/100 μg protein.

DISCUSSION

Using synthetic γ_3-MSH, we were able to obtain a sensitive antiserum and to set up a radioimmunoassay for γ-MSH. The absence of cross-reactivity with α-MSH, porcine β-MSH, and ACTH is of significance, since these peptides share the tetrapeptide sequence. A slight cross-reaction (0.1 %) of β-LPH may represent either an actual cross-reaction or a contamination of γ-MLI in the native β-LPH preparation used.

The parallelism of dilution curves obtained with γ_3-MSH and with extracts of bovine anterior pituitary, intermediate-posterior pituitary, and human pituitary suggests the existence of a substance(s) immunologically indistinguishable from γ_3-MSH. This γ-MLI in these extracts cannot be explained by cross-reaction of β-LPH, even when native β-LPH is assumed to actually cross-react with γ-MSH antiserum.

The γ-MSH concentration is 7.7 times and 14 times lower than ACTH in bovine anterior pituitary and human pituitary, respectively, whereas it is 2.7 times higher than ACTH and 1.9 times lower than β-EP in bovine intermediate-posterior pituitary, when compared on a weight basis. The unexpectedly low γ-MLI content in the

bovine anterior and human pituitaries can be explained by low cross-reactivity of γ-MLI in the anterior pituitary. In fact, gel chromatographic study on Bio-Gel P-60 of the anterior pituitary extract failed to demonstrate any peak corresponding to γ_3-MSH, but revealed a peak near the elution position of β-LPH. This big γ-MLI is assumed to correspond to the complete or almost complete amino acid sequence of the N-terminal portion of the precursor based on the molecular weight assessed from gel filtration study. However, our unpublished observation suggests that γ-MLI contains carbohydrate moieties and, therefore, the exact size of the big γ-MLI remains uncertain.

On the other hand, gel chromatography of a bovine intermediate-posterior pituitary which contained relatively higher concentrations of γ-MLI than bovine anterior pituitary showed one additional peak which emerged near the elution position of β-EP, although it did not co-elute with synthetic γ_3-MSH. Further studies to characterize the γ-MLI in the pituitary gland are ongoing in our laboratory. In conclusion, the presence of peptides immunologically indistinguishable from synthetic γ_3-MSH, a peptide whose presence is predicted from the nucleotide sequence of cDNA determined by Nakanishi et al (4), has been demonstrated by radioimmunoassay in bovine and human pituitaries. Size heterogeneity of γ-MLI has been observed only in the intermediate lobe.

ACKNOWLEDGEMENT

We are greatly indebted to Dr. R. Guillemin and Dr. N. Ling, the Salk Institute, for the gift of synthetic γ_1-MSH, γ_2-MSH, and γ_3-MSH, to Professor C. H. Li, University of California at San Francisco, for the gift of human β-LPH and human β-EP, to Professor H. Yajima, Kyoto University, for the gift of α-MSH and porcine β-MSH and to M. Ohara for comments on the manuscript. ACTH and its specific antibody were gift of NIAMDD National Pituitary Agency. We thank Misses Y. Mitsuda and K. Horii for the secretarial assistance.

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