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A RADIOIMMUNOASSAY FOR γ_1-MELANOTROPIN AND EVIDENCE THAT THE SMALLEST PITUITARY γ-MELANOTROPIN IS AMIDATED AT THE COOH-TERMINUS.

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SUMMARY

Specific radioimmunoassays for γ_1-melanotropin (γ_1-MSH) and γ_2-melanotropin (γ_2-MSH) have been developed. The γ_1-MSH antibody recognizes the portion between His^5 and Phe^{11}-NH_2 of γ_1-MSH and shows no significant cross-reactivities with other related peptides. The γ_2-MSH antibody cross-reacts with γ_1-MSH and γ_3-MSH to the extent of 0.004% and 0.04%, respectively, on a weight basis. Using these two different antisera on bovine pituitary extracts, two γ_1-MSH-like peptides were detected only in the intermediate lobe, whereas γ_2-MSH-like peptides were not detectable. Furthermore, it is likely that the smallest γ-MSH produced in the bovine intermediate pituitary is a γ_1-MSH-like peptide with the COOH-terminus amidated.

A third melanotropin fragment, named γ-MSH, which shares a common amino acid sequence with α-MSH** and β-MSH was discovered by Nakanishi et al. (1) in the NH_2-terminal cryptic region of the ACTH/β-LPH precursor protein from bovine intermediate pituitary. Moreover, this γ-MSH fragment is located between pairs of basic amino acids at Arg^{-57}-Lys^{-56},-Arg^{-43}-Arg^{-42} and Lys^{-28}-Arg^{-27} (1).

To determine whether this γ-MSH fragment is also processed separately into one or more secretory products, we (2) and others (3) have previously developed a RIA for γ_3-MSH, a 27 amino acids synthetic peptide comprising the sequence between Tyr^{-55} and Gln^{-29} in the cryptic region of the ACTH/β-LPH preprohormone (1). Using the γ_3-MSH RIA we detected the presence of at least two γ_3-MSH-like peptides in the anterior as well as intermediate lobes of bovine pituitary and showed these γ_3-MSH-like peptides to be glycosylated (4).

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** Abbreviations: MSH, melanotropin; ACTH, Adrenocorticotropic hormone; β-LPH, β-lipotropin; RIA, Radioimmunoassay.

Symbols for amino acids and derivatives are according to IUPAC-IUB recommendations published in J. Biol. Chem. (1972) 247, 977-983.

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However, since the structures of γ-MSH-like peptides in the pituitary are still not known, we wished to determine whether there were other γ-MSH-like substances smaller than the ones with the γ_3-MSH antigenic determinant. For this purpose we have recently developed two other γ-MSH specific RIAs, one for γ_1-MSH, Tyr^{-55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe^{-45}-NH_2, and the other for γ_2-MSH, Tyr^{-55}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly^{-44}-OH, two other possible γ-MSH peptides in the cryptic region of the precursor (5). We report here the development of these two γ-MSH RIAs and the detection of two γ_1-MSH-like peptides in the bovine intermediate pituitary.

MATERIALS AND METHODS

Peptides. All peptides used were synthesized by solid phase methodology as previously described (5).

Induction of antibodies. Synthetic γ_1-MSH (18 mg) was coupled to bovine serum albumin (40 mg) through bis-diazotized benzidine (0.42 ml of a 0.025 M solution). Synthetic γ_2-MSH (30 mg) was coupled to ovalbumin (40 mg) through glutaraldehyde (0.18 ml of a 0.1 M solution). Each peptide-protein conjugate was used to immunize five New Zealand white rabbits as previously described (2).

Iodination of peptides. Synthetic γ_1-MSH and γ_2-MSH were iodinated by the method of Hunter and Greenwood (6). The iodinated peptides were purified on a 0.7 X 13 cm column (Vbed=5 ml) of CM-32 carboxymethyl cellulose (Whatman). Free iodine was eluted with 0.01 M ammonium acetate at pH 4.5 and the iodinated peptide was eluted with 1.0 M ammonium acetate at pH 6.5. Fractions containing the iodinated peptide were diluted to 5 times its volume with buffer D (see below), aliquoted and kept frozen at -20°C until use.

RIAs for γ_1-MSH and γ_2-MSH. Buffers A, B, C and D have been described (7). Standard solutions of peptide and unknown samples were added to glass tubes and diluted with buffer C to a volume 400 µl. Antiserum to γ_1-MSH or γ_2-MSH was diluted with buffer B (supplemented with 1% normal rabbit serum) to a concentration sufficient to give 30-40% maximum binding of the iodinated peptide and 50 µl of the diluted antiserum was added. A frozen aliquot of the iodinated peptide was thawed and diluted in buffer D to give an activity of ca. 5000 cpm/50 µl and 50 µl of the diluted tracer was added. The mixture was vortexed and incubated for 48 hours at 4°C. After the initial incubation 50 µl of goat anti-rabbit γ-globulin diluted in buffer B to a concentration sufficient for maximum precipitation was added. After another 24 hour incubation at 4°C, all tubes received 1.5 ml buffer A and they were centrifuged at 1500 g at 4°C for 45 minutes. The supernatant was aspirated and the pellets counted. In all experiments, reference standards and unknowns were run in duplicates.

Peptide extraction from bovine pituitary. Bovine pituitaries were obtained from a local abattoir and immediately frozen on dry ice and kept frozen at -70°C until use. After defrosting, a pituitary was dissected into its three lobes which were further dissected into small fragments. An eighty-one milligram fragment of the anterior lobe and a 40 mg fragment of the intermediate lobe were homogenized by Polytron in 8 ml and 4 ml, respectively, of cold 1 M acetic acid containing 20 mM HCl, 0.01% phenylmethyl sulfonyl fluoride and 130 KIU/ml Trasylol. The homogenates were centrifuged at 2500 g at 4°C for 30 minutes and the supernatant lyophilized.

Gel filtration chromatography. The lyophilized material was reconstituted in 1 M acetic acid (the anterior lobe extract in 0.5 ml, the intermediate lobe extract in 0.4 ml) and a 0.2 ml aliquot of the solution, corresponding to 32 mg of the anterior lobe or 20 mg of the intermediate lobe in wet weight, was applied to a 0.7 X 48 cm Sephadex G-75 column (Vbed=18.5 ml) pre-equilibrated with 1 M acetic acid and eluted with the same solvent at 1.4 ml/hr at 4°C. Fractions of 0.6 ml were collected and lyophilized. The residues were reconstituted in buffer C for RIA. The column was calibrated with ferritin, phenol red, and iodinated ovine-β-LPH, ovine ACTH, porcine β-endorphin, bovine γ_3-MSH and bovine γ_1-MSH.

RIA for γ_3-MSH. γ_3-MSH-like peptides were measured as previously described (2).

RESULTS AND DISCUSSIONS

Figure 1 shows a standard curve of the γ_1-MSH RIA and the cross-reactivities of the antiserum (RB 282) with other peptides. This antiserum is used at a final dilution of 1/500,000. The sensitivity of this RIA is 1 pg/tube with half maximal displacement at 15-20 pg. The usable range of the standard curve is from 1 pg to 150 pg. The intra-assay and inter-assay coefficients of variation are 7.0% and 12.1%, respectively. On a weight basis the antiserum shows cross-reactivities of 0.06% and 0.02% with γ_2-MSH and γ_3-MSH, respectively. However, it has no significant cross-reactivities with α-MSH, bovine β-MSH, bovine β-endorphin or human ACTH.

To determine the antigenic site of the γ_1-MSH molecule recognized by this antiserum, we have synthesized by solid phase methodology two series of γ_1-MSH fragments, one starting from the NH_2-terminus and progressively elongated towards the COOH-terminus, and the other from the COOH-terminus extending

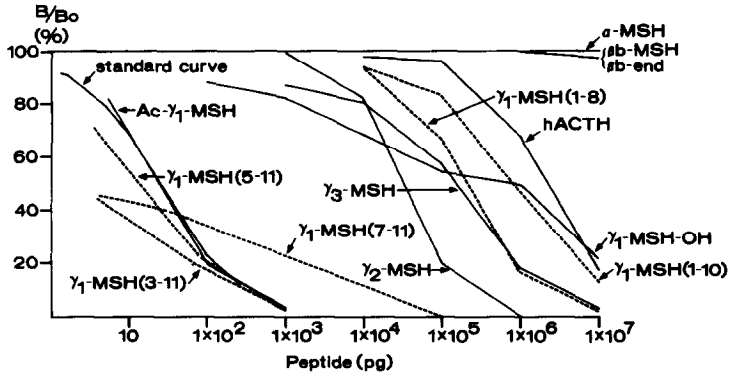

Figure 1. Cross-reactivities of the γ_1-MSH antiserum (RB 282) with related peptides.

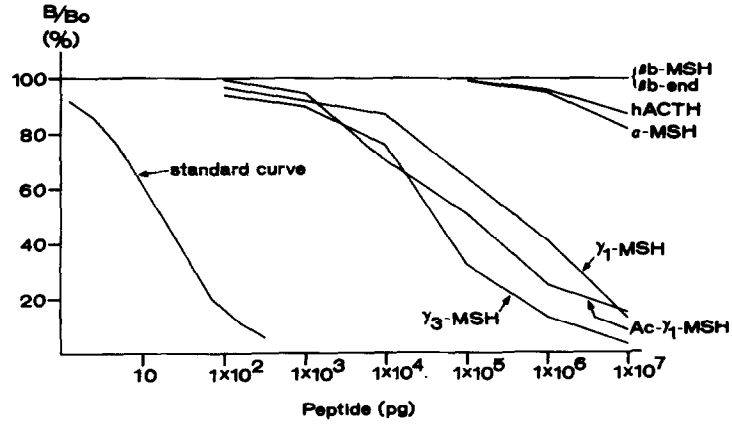

Figure 2. Cross-reactivities of the γ_2-MSH antiserum (RB 288) with related peptides.

towards the NH_2-terminus of γ_1-MSH. These synthetic peptides were characterized by amino acid analyses and their purity ascertained by high performance liquid chromatography. As shown in Figure 1, the NH_2-terminal fragments γ_1-MSH (1-8),*** γ_1-MSH (1-10) and γ_1-MSH-OH (the COOH-terminal carboxylic acid analog of γ_1-MSH) are hardly read by this antiserum, whereas in the COOH-terminal fragments except for γ_1-MSH (7-11) both γ_1-MSH (3-11) and (5-11) are completely read. As a result, it could be proposed that the specific recognition site of this antiserum towards γ_1-MSH is the region from His^5 to the Phe^{11}-NH_2 of γ_1-MSH. Ac-γ_1-MSH shows the same displacement curve as γ_1-MSH because it contains the same structure at the COOH-terminus as γ_1-MSH.

Figure 2 shows a standard curve of γ_2-MSH RIA and the cross-reactivities of the γ_2-MSH antiserum (RB 288) with other peptides. This antiserum is used at a final dilution of 1/500,000. The sensitivity of this RIA is 1 pg/tube and the usable range of the standard curve is between 1 pg to 150 pg. The antiserum does not cross-react with α-MSH, bovine ß-MSH, bovine ß-endorphin or human ACTH. On a weight basis the cross-reactivities of the RB 288 with γ_1-MSH, Ac-γ_1-MSH and γ_3-MSH are 0.004%, 0.016% and 0.044%, respectively.

*** The sequence of γ_1-MSH (1-8) is Tyr^1-Val-Met-Gly-His-Phe-Arg-Trp^8-OH which is equivalent to Nakanishi et al's. (1) numbering system from Tyr^{-55} to Trp^{-48}. Other fragments of γ_1-MSH are numbered accordingly.

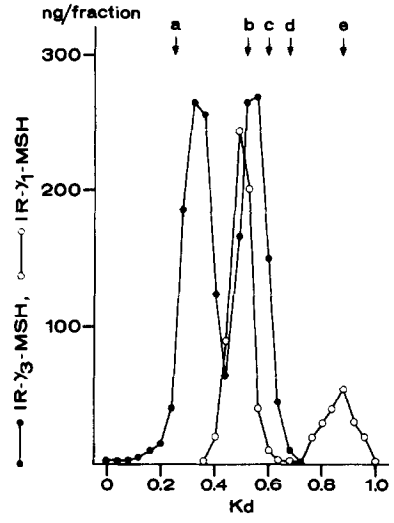

Figure 3. Sephadex G-75 gel permeation chromatography of the intermediate lobe extract of bovine pituitary. a. ^{125}I-β-LPH, b. ACTH, c. ^{125}I-β-endorphin, d. ^{125}I-γ_3-MSH, e. ^{125}I-γ_1-MSH.

The Sephadex G-75 gel filtration profiles of the γ-MSH-like peptides obtained from the intermediate lobe extract of bovine pituitary are shown in Figure 3. As reported earlier (2,3) two γ_3-MSH-like peaks are observed at Kd = 0.32 and 0.56. In addition to these γ_3-MSH-reactive peaks, two γ_1-MSH-like peaks, which have completely different mobilities from the γ_3-MSH-like substances are obtained. A large γ_1-MSH-reactive peak, the molecular size of which is about 5600, appears at Kd = 0.49 and a small γ_1-MSH-like peak at Kd = 0.88 which co-elutes with ^{125}I-γ_1-MSH. When the extract from the bovine anterior pituitary was subjected to the same gel filtration analysis, no significant peaks with γ_1-MSH-like immunoreactivity were detected. Furthermore, if the γ_1-MSH antiserum was replaced with the γ_2-MSH antiserum in the RIA, no significant γ_2-MSH-immunoreactive peaks were found in the same column fractions obtained from the gel filtration chromatography of either the anterior or intermediate lobe extracts of bovine pituitary.

Since the γ_1-MSH antiserum (RB 282) requires the phenylalanine carboxamide at the COOH-terminus for complete recognition, the larger γ_1-MSH-like peak at Kd = 0.49 is probably an NH_2-terminal extension peptide of γ_1-MSH. The apparent molecular size of this big γ_1-MSH is about 5600 which is smaller than that of

the whole NH_2-terminal extension fragment of γ_1-MSH from Phe^{-45} to Trp^{-105}. However, since gel filtration gives only approximate molecular weights and there is no obvious cleavage sites in the NH_2-terminal region beyond γ_1-MSH, it is not possible to speculate on the amino acid sequence of the big γ_1-MSH. On the other hand, it could be proposed that the small γ_1-MSH-like peptide is clearly related to the synthetic γ_1-MSH since it eluted at the same position as ^{125}I-γ_1-MSH. The COOH-terminus of this small native γ_1-MSH is amidated because the γ_1-MSH antiserum does not read the synthetic γ_1-MSH free acid. The biosynthesis of an amidated COOH-terminus in native γ_1-MSH is in analogy to the derivation of α-MSH from ACTH in the intermediate pituitary (8).

In a preliminary experiment using the RIAs for γ_3-MSH and γ_1MSH we have found that two forms of γ_3-MSH-like peptides as shown in Figure 3 were secreted from enzymatically dispersed intermediate lobe cells of bovine pituitary, whereas only one form of γ_1-MSH-like peptide which corresponds to the peak at Kd = 0.88 was secreted. The larger γ_1-MSH-like peptide which was found in the extract of the intermediate lobe was not secreted into the culture medium.

In this study, it is impossible to ascertain whether the NH_2-terminus of the small γ_1-MSH-like peptide is acetylated or not because the γ_1-MSH antiserum reads Ac-γ_1-MSH equimolarly as γ_1-MSH. However, we have recently isolated a γ_1-MSH-like peptide from the Pitressin intermediate (Parke-Davis and Co.) extract from porcine pituitary which is the Lys^{-56} extension of γ_1-MSH (paper in preparation). Whether this peptide represents the only small γ_1-MSH in the bovine intermediate pituitary extract will need further confirmation. Nevertheless, the finding of γ_1-MSH-like peptides only in the intermediate lobe reaffirms that the ACTH/β-LPH precursor is processed differently in the anterior lobe from the intermediate lobe (9,10).

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