BIOSYNTHESIS IN VITRO OF THE LUTEINIZING RELEASING HORMONE BY HYPOTHALAMIC TISSUE

by

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SUMMARY

The biosynthesis, in vitro, of the luteinizing releasing hormone (LRH) from ^{14}C-glutamic acid by hypothalamic tissue has been demonstrated. After fractionations of the incubation mixture, the use of synthetic LRH as a carrier made it possible to isolate by electrophoresis a Pauly-reactive area which exhibited radioactivity. The areas in front of and behind this spot were not radioactive. The radioactive and Pauly-reactive area was further purified by tlc. Acid hydrolysis of the apparently homogeneous Pauly-reactive area, which contained all the expected radioactivity, gave about 75% of the expected ^{14}C-glutamic acid. Assays, in vivo, showed release of LH and FSH at three appropriate stages of fractionation.

The primary structures of the porcine and ovine luteinizing releasing hormone (LRH) have been elucidated by degradation by Matsuo et al. (1), and Burgus et al. (2,3), respectively. The porcine and ovine LRH have the same amino acid sequence, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. This decapeptide was synthesized by Folkers (4), Sievertsson et al. (5), Monahan et al. (6), Geiger et al. (7) and Matsuo et al. (8). The hormonal activity of this decapeptide is shown by the release of LH and FSH in several mammalian species including man (4-8 and 9, 10, 11).

Mitnick and Reichlin (12) described the biosynthesis, in vitro, of the thyrotropin releasing hormone (TRH) by rat hypothalamic fragments. We have also been studying the biosynthesis of the hypothalamic releasing hormones,

*Hypothalamic Hormones XLI.

and have observed the biosynthesis of the luteinizing releasing hormone from ^{14}C-labeled glutamic acid by appropriate incubation of porcine hypothalamic tissue; the procedure and data are described herein.

METHODS - Radioactivity was determined by using low background glass vials to which was added 10 ml of a toluene counting solution of Beckman Bio-Solv solubilizer (BBS-3) and Fluor-Alloy. A Beckman LS-150 Scintillation counter was used for counting.

The in vivo determinations of LRH-activity were performed by using Sprague-Dawley female rats. After ovariectomy and 72 hours before the administration of the samples, the rats were injected with 50 μg of estradiol benzoate and 25 mg of progesterone according to Ramirez and McCann (13). Under anesthesia, control blood samples were taken from the jugular vein, and then the samples for assay were injected into this vein. Blood samples were taken again after 15 minutes. The determination of the level of LH in the serum was performed in duplicate, by the double antibody radioimmuno assay of Niswender et al. (14). The determination of the level of FSH was performed as described by Schalch et al. (15).

Fragments (125) of hypothalamic tissue were sliced and added to a phosphate buffer solution containing 20 μC of ^{14}C-glutamic acid, a 0.5 mM amino acid mixture, 4 mM $MgCl_2$ 50 mM KCl, 0.16 mM EDTA, 0.15 M mannitol, 5 mM ADP and 10 mM succinate. After incubation with shaking for four hours at 37°C, the mixture was frozen and lyophilized. The mixture was defatted with light petroleum ether, and extracted with 2 N acetic acid in methanol at 5°C for 12 hours. After filtration, the solvent was evaporated. The residue was treated with methanol, and the mixture was filtered and the methanol was evaporated. After repetition of this methanol treatment, the residue was dissolved in water.

RESULTS AND DISCUSSION

The water-soluble extractives obtained after the two methanol steps showed release of LH, in vivo, at a dosage of 0.5 fragment-equivalent. (Table 1).

A fraction containing the radioactive LRH was separated from the bulk of other peptidic material by chromatography on polyacrylamide gel (Bio-Rad Bio-Gel P-2, exclusion limit 1800 Daltons, column size 2.5 x 90 cm; 0.2 M acetic acid in water; 5 ml fractions were collected). Bio-assay of the material in tubes 90-130 showed the release of LH at a dosage of 1.5 fragment- equivalents (Table 1).

The material in combined tubes 90-130 was chromatographed on two preparative cellulose thin-layer plates (Munktell celluose, 0.8 mm layer, 20 x 20

TABLE I. BIOASSAYS FOR LRH AND FSH-RH ACTIVITIES

Preparation from	Dosage Fragment-Equivalents	ng/LH/ml Serum Before	After
Water solution after extraction with methanol. (no carrier LRH)	0.5	4	47.8
		< 4	96.0
		< 4	30.4
		4	37.8
Chromatography on polyacrylamide gel; Tubes 90-130. (no carrier LRH)	1.5	4	242
		8	130
		ng FSH/ml Serum Before	After
Preparative cellulose TLC. Band with Rf 0.42-0.58 (Synthetic LRH, Rf=0.50). (no carrier LRH)	2.0	1870	2730
		1790	2230
		Other areas of the plate were devoid of FSH-RH activity. (no carrier LRH)	

plates, nBuOH-acetic-water, 4-1-5, upper phase). Synthetic LRH and TRH were separately chromatographed on one side of the plates. The marker side of the plate was sprayed with Pauly reagent (LRH, Rf 0.50; TRH, Rf 0.37). The area containing the biosynthesized LRH, which was adjacent to the synthetic LRH spot, was extracted with an excess of 0.2 N acetic acid in water, and the solution was frozen and lyophilized. This material released FSH, in vivo, at a dosage of 2.0 fragment-equivalent (Table 1).

The hormonally active fraction from the tlc purification was then purified by electrophoresis on cellulose thin-layer plates (Munktell cellulose, 0.8 mm layer, 20 x 20 cm plates, pyridine-acetic acid-water, 100-4-900, pH 6.4, 900 V, 1.2 hours). By using synthetic LRH as a reference compound, it was observed that no Pauly or ninhydrin positive spot was present in the area which showed hormonal activity after electrophoresis. About 100 μg of synthetic LRH was then added to the hormonally active fraction, and aliquots of this material were subjected to electrophoresis. The plates were sprayed with Pauly reagent and eluates of sections of the plates were placed in vials, treated with 0.25 ml of water and scintillation solution, and radioactivity was checked. The LRH which had reacted with Pauly reagent was radioactive, but the areas immediately in front of and behind this spot were not radioactive (Table 2).

The Pauly-reactive area corresponding to LRH from preparative electrophoresis was obtained by extraction with water. After evaporation of the water, the residue was dissolved in methanol, and an aliquot with a determined amount of radioactivity was chromatographed on silica (phenol-formic acid-water, 500-13-167). There was a single Pauly-spot (R_f 0.33) in this system, and it contained all the expected radioactivity (Table 3).

TABLE II. CELLULOSE TLE OF SAMPLES WITH LRH AND FSH-RH ACTIVITY. SYNTHETIC LRH ADDED AS A MARKER

Distance from anode in mm.	^{14}C cpm
78 - 105	10 - 11 (background)
105 - 120 (Synthetic LRH-Pauly reactive spot)	22
120 - 148	10 - 11 (background)

TABLE III. RADIOACTIVITY OF LRH AND GLUTAMIC ACID

	Sample	Part of total LRH from cellulose TLC	^{14}C cpm
A.	Pauly spot from prep. electrophoresis	1/10	37.8
B.	Silica TLC of A Rf = 0.33	3/10	124.3
C.	^{14}C Glutamic acid from hydrolysis A	1/10	28.0

Note. - ^{14}C-Radioactivity of derivatives of LRH biosynthesized in vitro. An extract from porcine hypothalamic glands incubated in vitro was purified by chromatography on polyacrylamide gel and cellulose thin-layer plates. Synthetic, non-radioactive LRH was added to the LRH active fraction from these separations and this mixture was analyzed by electrophoresis on cellulose thin-layer plates. The plates were sprayed with the Pauly reagent and the Pauly derivative of LRH was recovered. See Text.

Another sample with a determined amount of radioactivity was hydrolyzed with 6 N HCl in a sealed ampoule at 110°C for 24 hours. Unlabelled glutamic acid was added, and the hydrolyzed mixture was analyzed by 2-dimensional chromatography on a cellulose thin-layer plate (Munktell cellulose, 1st dimension: n-BuOH-acetic-water, 4-1-5, upper phase; 2nd dimension, n-PrOH-water 7-3). The plate was sprayed with ninhydrin, and the area corresponding to glutamic acid was eluted. About 75% of the radioactivity was recovered in this glutamic acid (Table 3).

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