BIOSYNTHESIS OF THE LUTEINIZING HORMONE RELEASING HORMONE IN MITOCHONDRIAL PREPARATIONS AND BY A POSSIBLE PANTETHEINE-TEMPLATE MECHANISM

by

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SUMMARY

Data show that the luteinizing hormone releasing hormone (LHRH) is biosynthesized by mitochondrial preparations from hypothalami which were obtained by centrifugation of a Ficoll gradient system. Incubation, extraction, use of carrier synthetic LHRH, and chromatography, yielded fractions showing associated hormonal activity and radioactivity. Other investigators reported biosynthesis of the thyrotropin and the growth hormone releasing hormones by soluble enzymes, but these enzymes might have been derived from mitochondria and/or synaptosomes. These particles appear implicated, but the true site of biosynthesis of these hormones is unknown. The mechanism at the site could possibly involve a pantetheine-protein template.

Johansson, et al. (1) reported evidence for the biosynthesis, in vitro, of the luteinizing hormone releasing hormone (LHRH), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, by homogenates of porcine hypothalamic fragments.

Johansson, et al. (2) also reported on the purification of solvent extracts of mitochondrial preparations, utilizing Bio-Gel P2 and Sephadex G-25, which yielded fractions which released the luteinizing hormone and the follicle stimulating hormone, as well as somatotropin. Labeled pGlu-His-Pro-NH_2 and the release of thyrotropin had served as controls in these studies on activities of releasing hormones in subcellular fractions from porcine hypothalamic tissue.

The association of the hormonal activity of the luteinizing hormone releasing hormone as well as that of other releasing hormones provided a basis

*Hypothalamic Hormones LIII.

for determining if mitochondria and/or synaptosomes are possible cellular sites for the actual biosynthesis of certain hypothalamic releasing and inhibiting hormones.

We have now investigated incubation in the presence of ^{14}C-glutamic acid of mitochondrial-synaptosomal preparations from porcine hypothalamic fragments, and summarize the data showing apparent biosynthesis of the luteinizing hormone releasing hormone (LHRH) by such mitochondrial preparations.

METHODS

A Beckman LS-150 scintillation counter was used for determining the radioactivities of samples in low background vials. The samples were dissolved in 10-ml of a toluene-counting solution containing Beckman Bio-Solv solubilizer (BBS-3) and Fluor Alloy.

Samples were assayed for the activity of the luteinizing hormone releasing hormone, in vivo, as described (1).

Isolation and Incubation of a Mitochondrial Fraction. - Freshly collected hypothalamic fragments from swine were received packed in ice. Batches of 250 fragments were homogenized and a crude mitochondrial fraction was isolated as described by Johansson et al. (2). This fraction was further purified by centrifugation in a gradiant Ficoll system consisting of concentrations of 20%, 16%, 12%, 8% and 2% (2). The pellet and the subcellular fractions between the layers of 16-20% and 12-16% were combined and resedimented. This sediment was incubated in the medium for 3 hours at 37° under an atmosphere of 95% air and 5% CO_2. The incubation medium consisted of a phosphate buffer, pH 7.4, and was 4 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.15 M mannitol, 5 mM ADP, 10 mM succinate, 0.5 mM amino acid mixture (1,6) and the medium contained 20 μC of ^{14}C-glutamic acid. After incubation, the mixture was lyophilized, and a water-soluble extract was prepared (2) and assayed in vivo for the activity of LHRH (Stage SP).

Separation Procedures. - The extract at Stage SP from the incubated fragments was chromatographed on Bio-Gel P2 (exclusion limit 1,800 Daltons, 2.5 x 100 cm column) using 0.2 N acetic acid (5.5 ml fractions). The fractions (60-100) which showed the activity of LHRH in the assay were combined and further purified by partition chromatography on Sephadex G-25 (Fine, 2 x 90 cm column, using 0.1% Acetic acid-n-Butanol-Pyridine, 11-5-3, 3.8 ml fractions). Fractions were bioassayed for the presence of LHRH and ^{14}C radioactivity (Stage PC). Fractions 15-45 showing the presence of LHRH were combined and purified by electrophoresis on cellulose thin-layer plates (Munktell cellulose, about 1 mm layer on a 20 x 20 cm plate, pH 6.4, 900 V, 1.2 hrs. The buffer was Pyridine-Acetic acid-Water (100-4-900).

TABLE I. RELEASE OF LH IN VIVO BY SAMPLES FROM AN INCUBATED MITOCHONDRIAL-SYNAPTOSOMAL FRACTION

Preparation	Dosage h.f.e.	ng LH/ml serum Before	After
Incubated mitochondrial-synaptosomal preparation Stage SP	0.5	< 4	> 286
		< 4	> 286
		6	66
		7	38
		5	> 286
Chromatography on Bio-Gel P2 of SP. Stage P2	0.5	4.2	> 286
		4.0	125
		4.0	138
		6.4	> 286
Chromatography of Stage P2 on a partition column Stage PC	0.5	4	48
		< 4	16
		< 4	26
		< 4	26
	1.0	9	136
		8	164

h.f.e. = hypothalamic fragment equivalents.

A small portion of the combined fractions containing LHRH had been first electrophoretically compared to synthetic LHRH. Spraying with Pauly reagent did not reveal LHRH in comparison with the control, presumably because the level was below the sensitivity of the reagent as expected.

About 20 μg of synthetic LHRH was added to the combined fractions and the material was subjected to electrophoresis in comparison with synthetic LHRH. Spraying with the Pauly reagent showed the reaction with LHRH in the zone corresponding to the control LHRH. The appropriate areas were removed from the plate and ^{14}C was determined.

RESULTS AND DISCUSSION

An initial mitochondrial cell fraction was prepared from porcine hypothalamic fragments. When this preparation was centrifuged in a discontinuous Ficoll gradient system as described (2), the mitochondria became localized primarily in the pellet and in conjunction with the two fractions which were closest to the pellet, i.e., the layers between 16-20% and 12-16% Ficoll. These three fractions were combined, resedimented, and used in incubations.

Other investigators using similar subcellular fractions of rat brain (3-7) and tissue from the hypothalamic-pituitary area of rats (8), cattle (9),

and horses (10) found that cellular fractions analogous to these three from porcine hypothalami contained mitochondria, synaptosomes, and myelin.

The results of assays, in vivo, of samples derived by purification from the mitochondrial preparations are in Table I. The purification steps which yielded the fractions included extraction of the incubated material and appropriate chromatography. Hormonal activity based on hypothalamic-fragment-equivalents (h.f.e.) was reduced at stage PC, but the reduction was compensated by the substantial reduction in weight of the fractions required for activity.

During electrophoresis, synthetic LHRH moved from the anode toward the cathode. Data are in Table II on electrophoresis of the fraction at stage PC to which synthetic LHRH had been added. The Pauly spot corresponding to synthetic LHRH was associated with radioactivity, but the areas in front and behind of this spot were not radioactive.

The data show that LHRH is biosynthesized in the in vitro system consisting of the mitochondrial preparations from porcine hypothalamic tissue.

These data on the indicated mitochondrial biosynthesis of LHRH may be appraised in relationship to studies by Mitnick and Reichlin (11) on the biosynthesis of the thyrotropin releasing hormone, or pGlu-His-Pro-NH_2. They (11) described evidence for the enzymatic biosynthesis of TRH which was based on using freeze-dried porcine fragments which had been homogenized in a Virtis Omnimixer in 0.01 M phosphate buffer, and then centrifuged at 100,000 x g for 13 hours to remove particulate entities. They utilized the supernatant in the incubation which provided the evidence for the biosynthesis of TRH. Reichlin and Mitnick (12) have also described the enzymatic biosynthesis of the growth hormone releasing hormone (GHRH) by incubation of rat tissue, and by incubation of extracts from both rat and porcine hypothalamic tissue.

Mitnick and Reichlin (11) reported that their data on the biosynthesis of TRH was by soluble enzymes. Their data on biosynthesis by soluble enzymes and ours on mitochondrial preparations are not necessarily in conflict. Of alternative explanations, their soluble enzyme might not necessarily be of soluble nature, and might be derived from mitochondria due to homogenization in a Virtis Omnimixer of freeze-dried tissue in a hypotonic buffer. Klingenberg (13) cites precautions for maintaining the intactness of mitochondria; even isolation of mitochondria can cause leakage of enzymes from these organelles. de Duve (14) discussed "the true localization of an enzyme". We use the expression "mitochondrial preparation", and recognize de Duve's comment (14) that "there are many more things than mitochondria in mitochondrial fractions".

Biosynthesis of these hormones by synaptosomes is not excluded; it is noted that even to achieve "a considerable degree of enrichment of synaptosomes" vigorous homogenization can be detrimental (15,16).

TABLE II. CELLULOSE THIN LAYER ELECTROPHORESIS OF AN LHRH-ACTIVE FRACTION FROM PARTITION COLUMN CHROMATOGRAPHY. RADIOACTIVITY IN CPM.

Distance in mm from origin	^{14}C cpm
34 - 61	Background (10.5 - 10.8)
62 - 76 (containing synthetic LHRH; Pauly spot)	85
77 - 170	Background (10.2 - 10.4)

The true localization of the biosynthetic site of these hormones is not established, and may be resolved by the aides of marker enzymes and a "balance sheet" (14).

Mitnick and Reichlin (11) also stated that their data on the biosynthesis of TRH indicated that the mechanism is not by DNA-dependent and RNA-directed reactions. Consequently, it is of interest to determine the nature of the biosynthetic mechanism which is involved. Of the alternatives, it is possible that the enzyme which forms these hypothalamic peptide hormones is one which utilizes pantetheine which forms the peptide bonds through a thioester mechanism. The linkage of precursor peptides as thioesters to an enzyme has been found by Gevers, Kleinkauf and Lipmann (17) and Frøyshov, Zimmer and Laland (18). A mechanism involving pantetheine was described by Laland et al. (19,20) for the biosynthesis of certain peptides on a protein template.

This pantetheine mechanism for biosynthesis of these hypothalamic hormones is seemingly supported by the inhibition of TRH-biosynthesis by iodoacetamide and by mercuric chloride (11), which are thiol reagents. We are now studying whether this pantetheine mechanism is or is not involved.

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