DETERMINATION OF RELEASING HORMONES IN SUBCELLULAR FRACTIONS FROM PORCINE HYPOTHALAMIC TISSUE

by

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SUMMARY

In a study of possible biosynthesis of hypothalamic hormones in mitochondria, the activities of subcellular fractions from gradient Ficoll fractionation were assayed. Mitochondrial fractions identified by qualitative assays for oxygen uptake and phosphorylation, showed both respiratory activity and release of the luteinizing hormone. Purification of solvent extracts of mitochondrial fractions by Bio-Gel P-2 and Sephadex G-25 yielded fractions which released the luteinizing and follicle stimulating hormones and somatotropin. Labeled pGlu-His-Pro-NH_2 and the release of thyrotropin served as a control.

Johansson et al. (1) reported on the biosynthesis and evidence for the existence of the follicle stimulating hormone releasing hormone and gave citations to the biosynthesis of hypothalamic hormones by other investigators which are not herein repeated although pertinent.

We have investigated the subcellular localization of the activities of certain releasing hormones in preparations from porcine hypothalamic tissue. A mitochondrial pellet showing a pronounced activity for LHRH was purified by a Ficoll gradient. Subsequently, a solvent extract of mitochondrial fractions was chromatographically fractionated. Certain final fractions were assayed and found to release the luteinizing and follicle stimulating hormones (LH and FSH) and somatotropin (STH).

METHODS

Isolation and Extraction of Subcellular Fractions - Batches of 250 freshly removed hypothalamic fragments from swine were used. The tissue was packed in ice for shipping. All isolation steps were performed at 0-5°C.

*Hypothalamic Hormones LI.

Medium A, adjusted to pH 7.4 by KOH contained 0.3 M mannitol and 1 mM EDTA, and was used for the preparation of the first crude mitochondrial-synaptosomal fraction.

The fragments were homogenized in medium A at 15% w/v., by Potter-Elvehjem homogenizer, and the mixture was centrifuged at 900 x g for 15 min. The pellet was resuspended in medium A and centrifuged again at 900 x g for 10 min. The pellet was discarded, and the combined supernatant was centrifuged at 12,500 x g for 15 min. to give a mitochondrial-synaptosomal fraction P (pellet no. 1) and supernatant S. P was resuspended in medium A at 1 ml/gww original hypothalamic fragments.

Gradient 1 x 3 inch tubes were prepared by successive layering of 5 ml. each of 20%, 16%, 12%, 8%, and 2%, Ficoll in medium A. The prepared tubes were allowed to stand at room temperature for 60-90 min. and then in ice-water for 30-45 min. The suspension of P (5 ml) was layered on the top of (16) gradient tubes which were then centrifuged at 90,000 x g for 1 hr. in a Beckman L2-65B ultracentrifuge. Six sediments were obtained from each tube consisting of PA as the top layer; PB between 2 and 8% Ficoll; PC between 8 and 12% Ficoll; PD between 12 and 16% Ficoll; PE between 16 and 20% Ficoll; and PF as the pellet. The corresponding sediments from the tubes were pooled, diluted with about 100 ml of medium A and centrifuged at 90,000 x g for 15 min. The six pellets were suspended in medium A and after withdrawal for localization of mitochondria, the suspensions were resedimented as PA through PF.

PA through PF were assayed for oxygen uptake (Szarkowska (2)) and oxidative phosphorylation. A microplatinum oscillating electrode (Gilson Medical Electronics) in a chamber of 1.0 ml was used. Phosphorylation was determined polarographically; Voss et al. (3) and Stahl et al. (4). Protein was determined by the Lowry method (5).

Each fraction of PA through PF was lyophilized and extracted twice with 2N acetic acid in methanol at 5°C for 12 hrs. After filtration and evaporation of solvents, the residues were treated with methanol. Filtration and evaporation of the methanol yielded residues which dissolved in small volumes of water. Water-soluble extracts were similarly prepared from supernatant S and from a homogenate H from hypothalamic fragments.

In Vivo Assays - Sprague-Dawley female rats were used for in vivo assays of the activity of LHRH. 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; Ramirez and McCann (6). Under anesthesia, blood samples were taken from the jugular vein; the samples were injected into this vein. Assays on serum samples for LH were performed in duplicate by the double antibody radioimmuno assay; Niswender et al. (7).

Chromatographic Separations - Pooled preparations of PD, PE and PF of mitochondrial fractions were subjected to fractionation by purification on Bio-Gel P2 (exclusion limit 1800 Daltons, 2.5 x 90 cm column, 0.2 N acetic acid, 5.5 ml fractions). Tubes 50-90 were pooled and further fractionated by partition chromatography (Sephadex G-25 Fine, 2.0 x 90 cm column, 0.1% acetic acid-n-butanol-pyridine, 11-5-3, 10 ml fractions). Synthetic ^{3}H-pGlu-His-Pro-NH_2 was added as a "marker" in connection with this purification; the radioactivity was associated with release of TSH after the Bio-Gel P2 column. These purifications yielded fractions which were assayed in vitro for LHRH, FSHRH, TRH, and SRH.

In Vitro Assays - Pituitaries were obtained from female rats of the Sprague-Dawley strain which were 20-days old. Two pituitaries for each assay were incubated at 37°C in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10 ml Teflon beakers in a Dubnoff shaker. After preincubation for 1 hour, the medium was removed for a control assay and fresh medium was added. After the second hour of preincubation, the medium was removed for assay and replaced. During incubation, the medium was removed for assays of pituitary hormones and replaced four times at one-hour intervals. The total preincubation and incubation time was six hours.

The following radioimmunoassays were used: LH, Niswender et al. (7); FSH, Daane and Parlow (8); growth hormone and TSH, Parlow; TSH, Bowers et al. (9).

Reagents for the assays of FSH, growth hormone were from NIAMD of NIH. Dr. G. Niswender kindly supplied the anti-ovine LH serum No. 15. Dr. L.E. Reichert kindly supplied an ovine LH preparation for labeling and the reference standard of LH from the rat. Values were calculated in terms of ng of the standards: LH-LER-1240-2 (0.60 NIH-LH-S1 units/mg); GH (0.6 IU/mg); FSH (2.1 x NIH-FSH-S1 units/mg); TSH (0.22 U.S.P. bovine TSH units/mg).

RESULTS AND DISCUSSION

It was considered that the use of hypothalamic tissue for the study of subcellular localization of hormonal activity would be facilitated by using tissue within a day or two after removal from the brain and which had been stored only at ice temperature. A priority question in this study was whether there is mitochondrial biosynthesis of hypothalamic releasing and inhibiting hormones. To study this question, determinations of hypothalamic hormonal activities of mitochondrial and other subcellular fractions were made. Subcellular fractionation of a mitochondrial pellet from a hypothalamic homogenate was made by centrifugation on a gradient Ficoll layer system.

Qualitative determination of oxygen uptake and oxidative phosphorylation was made to localize mitochondria within the gradient layers, and to show the existence of active respiration in mitochondrial preparations made by such procedures.

TABLE I. Oxygen Uptake and Oxidative Phosphorylation of Subfractions From Ficoll-Layer Centrifugation of Mitochondrial-Synaptosomal Fractions

Fraction (a)	mg Protein	Relative Oxygen Uptake mg protein	ΔP/ΔO
P	580	1.00	
PA: Top fraction	13	0	-
PB: 2 and 8%	192	0.29	-
PC: 8 and 12%	152	0.54	-
PD: 12 and 16%	50	1.24	+
PE: 16 and 20%	50	1.35	+
PF: Pellet	120	1.52	+

The initial pellet consisting of a mitochondrial-"synaptosomal" fraction was prepared by a common procedure (10-15) for brain tissue. The qualitative data on oxygen uptake and phosphorylation in Table 1 show that the mitochondria were concentrated in gradient fractions PD, PE, and PF. Others conducted similar subcellular fractionations of rat brain (11-15) and of tissue from the hypothalamic-pituitary area of rats (16), cattle (17), and horses (18), and it was found that fractions analogous to PD, PE, and PF mainly contained mitochondria, myelin fragments and synaptosomes. Fractions analogous to PA, PB and PC consisted of myelin, synaptosomes and other particles.

The qualitative data in Table 1 on oxygen uptake and phosphorylation localized the mitochondria in gradient fractions PD, PE and PF. The gradient fractions PA-PF, the original homogenate (H) and the initial supernatant (S) were extracted with methanol and acetic acid. Data on release of LH, FSH and STH by these extracts are in Tables 2 and 3.

The data in Table 2 show the release of the luteinizing hormone, *in vivo*. The initial homogenate of the hypothalamic fragments showed maximal release of LH at a dose level of 0.5 h.f.e. This assay validates the activity of the initial tissue and is a control. Data on the release of LH by the gradient fractions of PA through PF and the initial supernatant (S) as well as synthetic LHRH for another control are in Table 2. The greatest activity for release of LH was in PE and PF where release was maximal although release of LH was not exclusive to PE and PF. However, the localization of mitochondria and pronounced release of LH appear to show some correlation. It is noted that the original homogenate (H) of the tissue was considerably more active for release of LH than the corresponding supernatant by a difference up to 25-fold.

It is understood that the release of LH from these preparations was probably due to the decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, which we consider as only LHRH and to the new FSHRH according to Johansson (1), Currie (19) and Bowers (20).

TABLE 2. RELEASE OF LUTEINIZING HORMONE (LH) IN VIVO BY EXTRACTS OF SUBCELLULAR FRACTIONS FROM PORCINE HYPOTHALAMIC FRAGMENTS

Fraction	ng LH/ml Serum							
	Dose Level h.f.e. (b)							
	0.1		0.5		1.0		2.5	
	Before	After (a)	Before	After	Before	After	Before	After
H(c)	5.2	181	< 4	> 286				
	8.6	> 286	< 4	> 286				
			6	> 286				
			6	> 286				
PA(d)	< 4	13.4			< 4	118		
	< 4	10			< 4	126		
PB			4	195	< 4	60.4	4.6	> 286
			< 4	206	< 4	88.4	4	> 286
					< 4	15.2		
					6.8	182		
PC					< 4	148		
					< 4	148		
PD			4	26	< 4	192		
			< 4	44	< 4	> 286		
PE			4	160	< 4	> 286		
			4	70	< 4	> 286		
			4	125				
			6.4	> 286				
			4.2	> 286				
			4.2	> 286				
PF			4	> 286			4.6	> 286
			4	236				
PD+PE	5.6	228	7.6	> 286	8	> 286		
+ PF	4	126	4.8	239	15.6	> 286		
	8.8	250						
	4.4	8.8						
S(e)	< 4	21.6	< 4	57.6			< 4	132
	< 4	8.8	8.0	46.6			< 4	110
			5.6	146			7	169
			5.0	100			4.6	> 286
Synthetic	1 ng		5 ng					
LHRH	7.2	216	4.2	266				
	< 4	162	4.0	238				
	16.8	168	4.0	> 286				
	4.0	109	< 4	205				

(a) Level of LH before and after addition of assay sample.

(b) Hypothalamic fragment equivalents

(c) H = Homogenate of whole hypothalamic fragments.

(d) PA-PF = Subfractions from Ficoll gradient centrifugation. See Table I.

(e) S = Supernatant after centrifuging a crude mitochondrial-synaptosomal fraction.

Combined extracts of gradient fractions PD, PE, and PF were chromatographed on Bio-Gel P2 and then on a Sephadex G-25 partition column. These purifications were carried out toward separation of hypothalamic hormones and for association of hormonal activities other than that of LH with subcellular components, particularly mitochondria.

TABLE 3. RELEASE OF LH, FSH, TSH and STH IN VITRO BY FRACTIONS FROM CHROMATOGRAPHIC PURIFICATION OF PD, PE AND PF

Fraction's(a)	Pre-Incubation 1 hr.	2 hr	3 hr	4 hr	5 hr	6 hr
			ng LH/ml medium			
I	-	-	0.2 h.f.e.	0.2 h.f.e.	1.0 h.f.e	1.0 h.f.e.
	20	28	> 714	> 714	> 714	> 714
	30	20	168	315	> 714	> 714
			ng FSH/ml medium (b)			
I	-	-	0.2 h.f.e.	0.2 h.f.e.	1.0 h.f.e.	1.0 h.f.e.
	1000	1250	13,500	17,750	46,000	36,650
	1850	1700	17,000	34,000	31,750	17,400
			ng TSH/ml medium			
II	-	-	0.1 h.f.e.	0.1h.f.e.	0.5h.f.e.	0.5h.f.e.
	-	1500	-	62,250	55,500	-
	-	2250	-	74,500	46,250	-
	-	6750	-	79,000	43,650	-
			ng GH/ml medium			
III	-	-	0.2h.f.e.	0.2h.f.e.	1.0h.f.e.	1.0h.f.e.
	605	398	1020	1265	1860	> 2560
	1430	1015	1670	2015	> 2560	2290

a) I: Tubes 50-90 from BioGel P-2 and 6-30 from Sephadex partition chromatography.

II: Tubes 50-90 from BioGel P-2 and 31-95 from Sephadex partition chromatography.

III: Tubes 126-160 from BioGel P-2 and 96-120 from Sephadex partition chromatography.

b) FSH may have been released by LHRH or FSHRH or both.

The assay data in Table 3 show that certain fractions from the Sephadex G-25 partition column release LH and FSH (from either or both LHRH and FSHRH) as well as thyrotropin (TSH) and somatotropin (STH). Some release of TSH must have been due to synthetic ^{3}H-pGlu-His- Pro-NH_2, which had been used as a marker. Maximal release of LH was observed at 0.2 h.f.e. The releases of FSH, TSH and LH were unambiguous at 0.1 to 0.2 and higher h.f.e. The release of STH was maximal at 1.0 h.f.e.

Mulder et al. (16) studied the subcellular localization of the corticotropin releasing hormone (CRH) and vasopressin in the median eminence of the rat, and concluded that the major part of both CRH and vasopressin is localized in nerve endings and stored in granules. It appeared that the production, transport and release of CRH may take place in separate neurones within the hypothalamus. Ishii (18) reported data which indicated that LHRH may be contained in vesicles of the median eminence.

Fink et al. (17) reported on release of LHRH and CRH in bovine stalk-

median eminence (SME). SME tissue was homogenized, etc.; ultimately, a sucrose-density gradient fractionation yielded a fraction which exhibited the activities of both LHRH and CRH, and contained myelin fragments and mitochondria, but the main components were structure-like granular vesicles and fiber profiles; the latter resembled nerve terminals or "synaptosomes". They believed that LHRH and CRH may be bound to the fibers or vesicles as consistent with data of Mulder and Ishii who found that fractions rich in "synaptosomes" exhibited the activities of LHRH and CRH.

Reichlin and Mitnick have currently described (21) the enzymatic biosynthesis by a non-ribosomal mechanism of the growth hormone releasing hormone by rat incubates and by extracts of rat and porcine hypothalamic tissue.

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