BIOSYNTHESIS AND EVIDENCE FOR THE EXISTENCE OF THE FOLLICLE STIMULATING HORMONE RELEASING HORMONE

by

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Received November 13,1972

SUMMARY

Biosynthesis with ^{14}C-glutamic acid and ^{14}C-glutamine in a hypothalamic system followed by fractionation yielded fractions after defatting, Bio-Gel P2, CMC, and Sephadex G25 partition chromatography which released 40,000 to >128,000 ng/ml of FSH. The decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 released about 18,000 and rarely up to 35,000 ng/ml of FSH. Radioactivity was associated with these exceptionally active FSH-fractions. The apparent new hypothalamic hormone releases both FSH and LH and can be FSHRH which is different from the decapeptide which is apparently LHRH and not LHRH/FSHRH. The associated radioactivity and FSH-releasing activity indicate that FSHRH may have a pGlu-moiety.

Johansson et al. (1) reported the biosynthesis, in vitro, of the luteinizing hormone releasing hormone (LHRH) from ^{14}C-glutamic acid by porcine hypothalamic tissue. The use of synthetic LHRH as a carrier facilitated isolation of a Pauly-reactive area by tle which exhibited radioactivity. After further purification and acid hydrolysis, ^{14}C-glutamic acid was recovered.

Mitnick and Reichlin (2) had previously described the biosynthesis, in vitro, of the thyrotropin releasing hormone (TRH) according to chromatographic data, when rat hypothalamic fragments were used.

We have continued studies on the biosynthesis of hypothalamic hormones, and encountered in certain fractions an exceptionally high release of FSH which was not compatible with the activities of synthetic pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 which is now generally known to release both LH and FSH.

*Hypothalamic Hormones XLIII.

Many investigators for years have considered that one hypothalamic hormone releases LH and that another hypothalamic hormone releases FSH. After extensive studies on LHRH, Schally et al. and White jointly summarized (3) their evidence on both the isolated porcine and synthetic decapeptides which led them to introduce the name "gonadotropin-releasing hormone" for one polypeptide which regulates the secretion of LH and FSH. Additional citations to their proposal that one and not two releasing hormones control LH and FSH are in the accompanying paper by Currie et al. (4).

By this biosynthetic approach, we are continuing fractionation for isolation of this apparent hormone which stimulates such a high release of FSH, and has also been observed to release LH. Also, we are pursuing the isolation of the apparent FSHRH by direct fractionation of extracts of porcine hypothalamic tissue according to Currie et al. (4) and Bowers et al. (5).

After biosynthesis, fractionation yielded two different groups of fractions each of which showed activities for the release of both FSH and LH. However, the activities corresponding to one group indicated the presence of the known decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, particularly because of the ratio of the levels of FSH and LH which were released. The activities corresponding to the other group revealed the exceptionally high release of FSH which gives a different ratio of release of FSH/LH.

In a sense, this apparent recognition of one releasing hormone for FSH and another releasing hormone for LH, both of which release both FSH and LH, has been possible for the first time by the availability of the synthetic decapeptide or LHRH for comparison and differential assays.

Geiger et al. (6) reported in 1971 that during their attempts to isolate LHRH, they "obtained two well separated preparations with LHRH activity;" they reported only release of LH. At this time, any chemical relationship between the observations of Geiger et al. and our observations described herein and by Currie et al. (4) and by Bowers et al. (5) is unknown.

METHODS

Fragments of hypothalamic tissue in batches of 500 were homogenized and incubated in a buffered medium containing an amino acid mixture, $MgCl_2$, KCl, EDTA, succinate, ADP, mannitol ^{14}C-glutamic acid and ^{14}C-glutamine. After incubation for four hours at 32°C, the mixtures were lyophilized, repeatedly extracted with acetic acid in methanol, and defatted. The residues from four incubations, equivalent to 2,000 fragments, were subjected to gel filtration on Bio-Gel P2 (exclusion limit 1800 Daltons). The hormonally active fractions, equivalent to 4,000 fragments, were combined and passed again through a column of Bio-Gel P2. The samples for bioassay were taken after defatting (Stage SP) and after pooling appropriate fractions from each gel filtration (Stage P2).

TABLE I. ACTIVITIES OF PREPARATIONS FOR FSH- AND LH-RELEASE, IN VITRO

No.*	Stage	Pre-Incubation P_1	Pre-Incubation P_2	Incubation I_3	Incubation I_4	Incubation I_5	Incubation I_6
					ng FSH/ml		
		-	-	0.2 h.f.e.	0.2 h.f.e.	1.0 h.f.e.	1.0 h.f.e
1	SP	1,000	<1,000	12,500	52,000	60,000	52,000
2	P2	1,000	<1,000	26,000	37,500	51,000	42,000
3	P2	3,000	2,250	-	-	126,000	51,000
4	P2	2,000	1,900	121,000	112,500	38,000	30,750
5	P2	1,000	1,000	55,500	46,750	40,500	16,600
6	P2	3,000	3,500	20,000	-	76,750	68,500
7	P2	5,000	5,000	110,000	-	68,500	54,000
8	P2	5,000	4,500	64,000	-	59,500	39,250
9	P2	2,300	1,050	22,650	40,705	51,000	30,150
10	P2	-	3,250	-	37,500	53,400	-
				0.2	0.2	3.0	3.0
11	SP	1,250	<1,000	8,250	38,750	>128,000	46,250
12	P2	2,000	2,750	17,150	13,500	52,500	>128,000
13	P2	2,300	2,650	28,000	34,000	67,500	43,250
14	P2	5,300	4,300	10,150	8,900	>128,000	64,750
					ng LH/ml		
		-	-	0.2 h.f.e.	0.2 h.f.e.	1.0 h.f.e.	1.0 h.f.e.
1	SP	20	20	378	>714	>714	>714
2	P2	20	20	131	528	>714	>714
3	P2	148	63	-	-	>714	>714
4	P2	138	50	>714	>714	>714	>714
5	P2	30	40	>714	>714	>714	>714
6	P2	25	29	215	490	>714	>714
7	P2	126	208	405	683	>714	>714
8	P2	294	148	692	714	>714	>714
9	P2	125	55	195	423	>714	>714
		-	-	0.2	0.2	3.0	3.0
11	SP	20	32	165	>714	>714	>714
12	P2	50	60	155	258	>714	>714
13	P2	63	50	375	662	>714	>714
14	P2	205	88	80	115	>714	>714

h.f.e. = Hypothalamic fragment equivalent(s).
* = Samples having the same number are identical.

The aliquots for bioassay were lyophilized and dissolved in 0.05 M phosphate buffer, pH 7.1. Quantities of 10 and 50 µl of the solutions were added to the incubation medium at I_3, I_4 and I_5, I_6 respectively.

Hormonally active fractions at Stage P2 were further fractionated by chromatographic and partition methods as indicated in Table II, and it became possible to achieve a separation of the activity of FSHRH from that of LHRH. The bioassay data from these fractions are recorded in Tables II and III.

Assay Procedure In Vitro

The samples were assayed in vitro as described by Currie et al. (4) and by Bowers et al. (5). Two pituitaries from twenty-day old female rats of the Sprague-Dawley strain were used in each assay.

TABLE II. ACTIVITIES OF PREPARATIONS OF FSH-RH, RELATIVELY FREE OF LHRH, ON FSH- AND LH-RELEASE, IN VITRO

No.*	M*	Pre-Incubation		Incubation			
		P_1	P_2	I_3	I_4	I_5	I_6
		ng FSH/ml					
		-	-	0.5 h.f.e.	0.5 h.f.e.	2.5 h.f.e.	2.5 h.f.e.
1	A	3,500	5,000	52,000	-	40,000	28,250
2	A	3,300	4,500	33,000	-	67,000	47,500
3	A	4,000	4,000	5,000	-	25,000	55,500
4	B	-	2,000	-	33,250	43,500	-
5	A	-	1,400	-	33,400	69,500	-
6	A	-	3,100	-	46,500	46,850	-
		ng LH/ml					
		-	-	0.5 h.f.e.	0.5 h.f.e.	2.5 h.f.e.	2.5. h.f.e.
1	A	201	142	622	714	> 714	> 714
2	A	59	69	358	535	> 714	> 714
3	A	39	48	30	123	243	617

M* = Methods
A and B = Sephadex G25 partition chromatography systems
h.f.e. = Hypothalamic fragment equivalent(s)
* = Samples having the same number are identical

TABLE III. ACTIVITIES OF PREPARATIONS OF LH-RH ON FSH- AND LH-RELEASE, IN VITRO

No.*	M*	Pre-incubation		Incubation			
		P_1	P_2	I_3	I_4	I_5	I_6
		ng FSH/ml					
1	A	3,050	1,850	12,250	19,500	30,750	34,250
2	B	5,000	4,000	8,000	-	17,500	18,000
		ng LH/ml					
		-	-	0.5 h.f.e.	0.5 h.f.e.	2.5 h.f.e.	2.5 h.f.e.
1	A	121	50	283	> 714	> 714	> 714
2	B	94	79	179	168	460	555

M* = Methods
A = Carboxymethylcellulose chromatography
B = Sephadex G25 partition chromatography
h.f.e. = Hypothalamic fragment equivalent(s)
* = Samples having the same number are identical

RESULTS AND DISCUSSION

Preparations have been obtained by fractionation of the material resulting from the incubation of homogenates of porcine hypothalamic tissue in the presence of ^{14}C-glutamic acid and ^{14}C-glutamine which allow the biosynthesis of hypothalamic hormones. Samples of preparations were bioassayed after the defatting(Stage SP) and after purification on Bio-Gel P-2 (Stage P2). The activities of these preparations for release of FSH and LH are in Table I; two samples are from Stage SP and twelve samples are from Stage P2.

Bowers et al. (5) have shown that synthetic LHRH in this assay system at a dosage of 0.9 ng releases an average of 18,075 ng/ml of FSH. They also

observed that there was no significantly higher release of FSH even at dosages as high as 500 ng of synthetic LHRH. In only one of twenty-eight assays, was the level of FSH released by LHRH above 30,000 or specifically, 35,000 ng/ml.

The fourteen samples of Table I released 40,000 to >128,000 ng/ml of FSH in 30 of 39 assays. In comparison, the control levels of FSH during the two hours of pre-incubation ranged from <1,000 to 5,000 ng/ml. Similar data were obtained from preparations resulting from the direct fractionation of extracts of porcine hypothalamic tissue as described in the accompanying paper by Currie et al. (4).

In our experience, a release of FSH at levels of >128,000 ng/ml has not been observed by synthetic LHRH under the same assay conditions. Such exceptional activity for the release of FSH may be ascribed to the presence of an FSHRH which is different from the LHRH which has been identified as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. It is not expected that this exceptional release of FSH is due to a side effect of a hypothalamic releasing hormone other than FSHRH.

Appropriate fractions from Bio-Gel P2 columns which showed such exceptional release of FSH were further fractionated by partition chromatography utilizing Sephadex G-25, and also by CMC chromatography. Seven samples from such further chromatography were selected for bioassay, and the data are in Table II. Nine of fifteen assays revealed a release of FSH which was >40,000 ng/ml. Also, the ratio for FSH/LH release was greater than that observed with synthetic LHRH.

Other fractions exhibiting activities for release of FSH and LH had characteristics corresponding to those of synthetic LHRH. The assay data from two such fractions are in Table III. These data for the release of FSH and LH are similar to those obtained with synthetic LHRH. For example, when the level of LH-release is >714 ng/ml, and the FSH-release is in the range of 19,500 - 34,250 ng/ml, the activities of LHRH are indicated. Also, the ratio of release, FSH/LH, is similar to that for synthetic LHRH.

It is considered that the hormone responsible for the exceptionally high release of FSH as well as the release of LH is due to FSHRH. The possible presence of LHRH in the most advanced preparations of FSHRH is considered negligible.

Each one of the preparations which showed the exceptional release of FSH was associated with ^{14}C-radioactivity. However, during the studies described herein, greater emphasis has been given to the differential hormonal activities than to extensive characterization of radioactive fractions.

The dose levels of the biosynthetic material which release FSH may be a little lower than those for samples from tissue which has been directly used

for extraction. For example, preparations derived from biosynthesis have been observed to show a good release of FSH at a dosage corresponding to 0.2 hypothalamic fragment equivalent. A corresponding dosage was observed to be ca. 0.5 h.f.e. for a preparation at a comparable stage in direct fractionation according to Currie et al. (4).

It is considered likely, on the basis of the general association of ^{14}C-radioactivity from ^{14}C-glutamic acid and ^{14}C-glutamine that FSHRH could have a pGlu- moiety in its peptide structure.

ACKNOWLEDGMENT

Appreciation is expressed to The Robert A. Welch Foundation; to Dr. W.J. Aunan, American Meat Institute Foundation; to Dr. Roger Gerrits, United States Department of Agriculture, Plant Industry Station; to Dr. Robert Dudley, George A. Hormel Company; to Dr. David Isaksson and Dr. Bertil Åberg, Kabi Aktiebolaget, Stockholm; to Karen Frazer for technical assistance, and for the Population Council Grant No. M72.79.

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