FACTOR C-LHIH WHICH INHIBITS THE LUTEINIZING HORMONE FROM BASAL RELEASE AND FROM SYNTHETIC LHRH AND STUDIES ON PURIFICATION OF FSHRH

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SUMMARY

A new entity, factor C-LHIH, inhibits the basal release of the luteinizing hormone, and inhibits the release of LH and FSH from synthetic LHRH. Factor C-LHIH has been assayed in fractions which were and were not derived from biosynthesis. Factor C-LHIH and FSHRH (not LHRH or GnRH) initially fractionated together. The latter obscured the former, and FSH assays guided fractionation. After partition chromatography on Sephadex G-25 in two systems, factor C-LHIH and FSHRH were separated by Sephadex LH-20, purified by DEAE Sephadex and high pressure liquid chromatography to give fractions of factor C-LHIH which inhibited LH-release at 100-500 nanograms, and did not inhibit the TRH-TSH response.

INTRODUCTION

Hypophysiotropic peptide hormones (also called factors) which either release or inhibit the release of hormones of the anterior lobe of the pituitary gland are now widely recognized. For years, three such inhibitory hormones designated as a growth hormone inhibitory hormone, the prolactin inhibitory hormone, and the melanocyte inhibitory hormone have been studied. Recently, a peptide was isolated from ovine hypothalamic tissue, which inhibits the release of the growth hormone; it was structurally elucidated, synthesized, and named somatostatin (1).

Apparently, there are no data described in the literature on the possible existence of a hypothalamic luteinizing hormone inhibitory hormone, although the luteinizing hormone releasing hormone (LHRH) is now established by structure, synthesis, and hormonal activities.

Our studies on the biosynthesis of the hypothalamic peptide hormones by Johansson et al. (2,3), particularly led to evidence (4) for the existence of the follicle stimulating hormone releasing hormone (FSHRH). These studies on

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biosynthesis, utilizing ^{14}C-glutamic acid, also led to data indicative of the existence of a luteinizing hormone inhibitory hormone. We now report data on a new entity which inhibits the release of the luteinizing hormone (LH) and which we currently designate as factor C-LHIH. These data appear to constitute the first literature report supporting the possible existence in hypothalamic tissue of a natural inhibitor of the release of LH. However, there has been a report that a pineal fraction increased the hypothalamic FSH and LHRF content *in vitro* and *in vivo*, which might have been a consequence of a decrease in the secretion of the releasing factors in the presence of the pineal fraction (5).

Many current research programs on structural modifications of LHRH as a new approach toward the control of conception and population are now widely recognized. A naturally occuring inhibitor of the release of LH in the human hypothalamus could offer clinically important advantages over synthetic analogs of LHRH as medicinals, and perhaps even in conjunction with the current steroids which are used in birth control.

METHODS

The *in vitro* assays were carried out with pituitary glands from female rats of the Sprague-Dawley strain. Two pituitaries from 20-day old rats were used for each assay. The incubation was done at 37°C in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10-ml Teflon beakers in a Dubnoff shaker. The medium was removed at 1-hour intervals during incubation for assay of LH, and then replaced. The total pre-incubation and incubation period was 6 hours. The radioimmunoassays of Niswender *et al.* (LH) (6) and NIAMDD (FSH and TSH) were used. The latter were kindly supplied by Dr. Parlow.

Porcine hypothalamic fragments which had been freshly collected were received packed in ice. Generally, batches of about 500 fragments, but occasionally 700-800 fragments were homogenized with a Sorval Omnimixer in the Krebs-Ringer bicarbonate buffer (ca. 900 ml). More buffer (500-800 ml) was added and the mixture was allowed to stand for 5-min pre-incubation. An amino acid mixture (2) was added to make the solution 1 mM; with respect to each amino acid, there were also added: ^{14}C-glutamic acid (50 µl), ATP (0.1 mM), DPN (0.1 mM), phosphoenolpyruvate (0.2 mM), pyruvate kinase (200-300 units) and cyclic AMP (1 mg). The incubation was terminated by the addition of acetic acid. Then, the mixture was lyophilized, extracted with acetic acid and methanol, and defatted by extraction with methylene chloride. The residues of lyophylization were dissolved in 1 M acetic acid and subjected to gel filtration on Bio Gel P-2 (exclusion limit 1800 Daltons). The material from this step was purified (100-fold weight reduction) on Sephadex LH-20, which had been swelled in n-butanol/water (6:100) and was eluted with the same solvent. Partition chromato-

graphy was then performed on Sephadex G-25 with system A; 0.1% acetic acid, n-butanol, pyridine (11:5:3) and system B; n-butanol, acetic acid, and water (4:1:5). The gel was swelled and packed in the aqueous phase and equilibrated on the column with the organic phase. The applications were made in a minimum volume of the aqueous phase and then eluted with the organic phase. Anion exchange was performed with DEAE-Sephadex which had been washed with 2 mM ammonium acetate. Application was made in the same solvent, and the elution continued with an ammonium acetate/pH gradient. High pressure liquid chromatography was performed on a Bondapak Phenyl-Corasil column using the instrument ALC 202 of Waters Assoc. Inc., which was equipped with a 6000 psi pump and a U.V. detector monitoring at 254 nm.

RESULTS AND DISCUSSION

Factor A-GHRH and factor B-GHRH have been described from our related fractionations (7,8). This tentative nomenclature was based on the general recognition that even hypothalamic peptide hormones do have more than one biological activity. pGlu-His-Pro-NH_2 (TRH) releases both thyrotropin and prolactin, and pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LHRH) releases both the luteinizing and follicle stimulating hormones. In addition, more than one peptide in the hypothalamus may alter the release of a given pituitary hormone. Consequently, these two unknown entities were designated by the letters A and B, which were hyphenated to the name of the hormonal activity which is presently guiding the fractionation.

The data in Table 1 are on relatively highly purified fractions which were all derived from tissues subjected to incubation to permit biosynthesis of these releasing and inhibitory hormones. It is relatively well established

TABLE 1. IN VITRO ASSAYS OF FACTOR C-LHIH

Column	Fraction	Dose(μg)	ng LH/ml	
			P_2	I_3
P-2	201-297	300	166	144
P-2	200-300	300	131	67
LH-20	62-100	25	103	18
DEAE-Sephadex	28-32	1-5	277	105
DEAE-Sephadex	26-32	1-5	118	48
Phenyl-Corasil	3	0.1-0.5	161	57
Saline Control			33	42
			158	227
			56	50
			99	140

that this general approach to biosynthesis, as studied by ourselves and others, does indeed result in the biosynthesis of TRH (9),LHRH (3), and FSHRH (4).

The separate and new entity, described herein, is designated factor C-LHIH because an in vitro assay, which measures a diminution in the release of LH, is being used to guide the fractionation of the new entity, factor C.

Extraction of porcine hypothalamic tissue with methanol-acetic acid, followed by defatting, generally constituted the first steps. Gel filtration on Bio Gel P-2 often gave a partial separation of factor C-LHIH from the LH-releasing activity of FSHRH (10). More frequently, the major part of factor C-LHIH appeared to be eluted in some steps in the same fractions as FSHRH and was obscured by the concomitant presence of FSHRH.

In some cases, factor C-LHIH, which was derived from biosynthetic experiments, was totally obscured at the Bio Gel P-2 stage, and apparently by FSHRH. This same obscuration has also been observed during steps of fractionation of extracts from hypothalamic tissue which had not been subjected to incubation for biosynthesis.

Fractions 170-300 (14-ml fractions) from the Bio Gel P-2 column (5 x 140 cm column) were combined and rechromatographed on Bio Gel P-2. Fractions 140-400 were combined and subjected to partition chromatography utilizing system A on Sephadex G-25 and followed by rechromatography on the same column. The apparent activity of FSHRH was observed in fraction 46-112 (10-ml fractions) from a column of 2 x 90 cm. Subsequent partition chromatography utilizing system B on a column (2 x 90 cm) of Sephadex G-25 again showed the activity ascribed to FSHRH in fractions 25-40. Then, extraction with butanol-water, followed by chromatography of the butanol phase on Sephadex LH-20 (2.5 x 95 cm column and 10-ml fractions) gave a partial separation of FSHRH and factor C-LHIH. FSHRH was less strongly retained.

TABLE 2. IN VITRO ASSAYS OF FSHRH AND FACTOR C-LHIH

			ng LH/ml		ng FSH/ml	
Background	Fraction	Dose (μg)	P_2	I_3	P_3	I_3
BuoH extraction	H_2O-phase	1500	155	33[a]	1100	22000[a]
BuoH extraction	BuoH phase	2000	100	24[a]	1750	78000[a]
LH-20	#37-47	10	670	755[b]	2550	3350[b]
LH-20	#48-61	2000	53	>714[c]	2200	63000[a]
LH-20	#62-100	25	103	18[a]	2350	2150[b]

[a] active entity is present.
[b] active entity is absent.
[c] active entity is present, but obscured.

TABLE 3. FACTOR C-LHIH ON THE RELEASE OF LH AND FSH BY SYNTHETIC LHRH

	LH Δ ng/ml Medium			FSH Δ ng/ml Medium		
	I_3	I_4	I_5	I_3	I_4	I_5
1.	39±19	63±24	19±11	1,862±303	729±348	-254±273
2.	401±91	627±29	628±29	16,191±1,538	17,149±1,633	12,288±985
3.	162±23	454±95	604±20	10,438±814	10,438±576	10,805±686
4.	167±21	397±98	183±23	8,786±656	6,869±756	1,854±227

Corresponding p-Values

	I_3	I_4	I_5	I_3	I_4	I_5
1.						
2.	vs. 1; <0.001	vs. 1; <0.001	vs. 1; <0.001	vs. 1; <0.001	vs. 1; <0.001	vs. 1; <0.001
3.	vs. 2; <0.05	vs. 2; ns	vs. 2; ns	vs. 2; <0.02	vs. 2; <0.01	vs. 2; -
4.	vs. 2; <0.05	vs. 1; -	vs. 1; -	vs. 2; <0.01	vs. 1; -	vs. 1; -

Additions to Medium

	I_3	I_4	I_5
1.	I_2	-	-
2.	L	L	L
3.	I_1+L	I_1+L	L
4.	I_2+L	-	-

I_1= 50 µl/ml factor C-LHRH
I_2= 50 µl/ml factor C-LHRH
L = 3 ng/ml LHRH
Δ values = P_2-I_3.
Each value = mean 4±SEM

Fractions 62-100 were then chromatographed on DEAE-Sephadex (1.5 x 30 cm column) with an ammonium acetate - pH gradient. Factor C-LHIH was eluted with 80 mM NH_4OAc (pH $\simeq$ 4.5).

It is evident that such incubations for biosynthesis may or may not result in the achievement of a labeled new hormone depending upon whether the amino acid (in this case, Glu) which is labeled is actually present in the hormone. Also, glutamic acid could be incorporated not only into releasing and inhibitory hormones, but into other peptides and proteins. These and other variables limit the usefulness of biosynthesis as an aid to isolation.

The active fractions (factor C-LHIH) from the DEAE-Sephadex separations coincided with the finding of a small radioactive peak. The material from these fractions showed bioactivity in the dosage range of 1-5 µg. (Table 1).

This preparation of factor C-LHIH from this peak was further purified by high pressure liquid chromatography on Bondapak Phenyl Corasil. Elution with

TABLE 4. EFFECT OF FACTOR C-LHIH ON TSH RELEASE BY TRH

Additions to Medium		TSH △ng/ml Medium	p-Values	
	I_3	I_3		
1.	-	862±	vs. 1	vs. 2
2.	T	5,699±1,447	p <0.001	-
3.	T+I_1	5,456±86	p <0.001	ns
4.	T+I_2	6,708±1,286	p <0.001	ns

T = 0.3 ng/ml TRH.
I_1= 50 µl/ml factor C-LHIH.
I_2= 50 µl/ml factor C-LHIH.
△ values = P_2-I_3.
Each value = mean 4±SEM.

water gave a slightly radioactive fraction which inhibited the release of LH in a dosage range of 100-500 ng.

The fraction in Table 1 showing activity in the range of 100-500 ng stemmed from a peak of fractions from DEAE Sephadex. Both "peaks" showed radioactivity only twice that of background. In contrast, a large and immediately following peak from DEAE-Sephadex showed radioactivity which was ca. 100-fold that of background. This area showed no hormonal activities.

Data on the activity of fractions which apparently contain FSHRH are in Table 2. Again, it is evident that levels of 63,000-78,000 ng/ml FSH are above that expected for the synthetic LHRH (15). Furthermore, LHRH had been previously separated from these fractions containing FSHRH by chromatography.

The data in Table 3 show that a fraction of factor C-LHIH purified from hypothalamic fragments without prior biosynthesis will inhibit the LH and the FSH in vitro response of synthetic LHRH. The data in Table 4 show that this fraction of factor C-LHIH did not inhibit the release of TSH by synthetic TRH which indicates some specificity of inhibition by factor C-LHIH.

The reductions in the basal levels of LH at stage I_3 in comparison with stage P_2 were generally not large, and perhaps can not be expected to be otherwise. This degree of diminution of basal LH-release is perhaps over-shadowed by the chemical experience of the fractionations yielding factor C-LHIH. Many procedures of fractionations, which have been repeated and modified many times, and stemming from many batches of nonincubated tissues and as well as incubated tissues for biosynthesis, led to confidence in predicting and finding the activity of factor C-LHIH on a reasonably reproducible basis.

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REFERENCES

1. Burgus, R., Ling, N., Butcher, M., Guillemin, R., Proc. Nat. Acad. Sci. USA., 70, 684 (1973).
2. Johansson, K.N.G., Hooper, F., Sievertsson, H., Currie, B.L., Folkers, K., Bowers, C.Y., Biochem. Biophys. Res. Commun., 49, 656 (1972).
3. Johansson, K.N.G., Currie, B.L., Folkers, K., Bowers, C.Y., Biochem. Biophys. Res. Commun., 53, 502 (1973).
4. Johansson, K.N.G., Currie, B.L., Folkers, K., Bowers, C.Y., Biochem. Biophys. Res. Commun., 50, 8 (1973).
5. Moszkowska, A., Scemama, A., Lombard, M.N., Héry, M., J. Neural. Transm., 34, 11 (1973).
6. Niswender, G.D., Midgley, Jr., A.R., Monroe, S.E., Reichert, Jr., L.E., Proc. Soc. Exp. Biol. Med., 128, 807 (1968).
7. Currie, B.L., Johansson, K.N.G., Greibrokk, T., Folkers, K., Bowers, C.Y., Biochem. Biophys. Res. Commun., 60, 605 (1974).
8. Johansson, K.N.G., Currie, B.L., Folkers, K., Bowers, C.Y., Biochem. Biophys. Res. Commun., 60, 610 (1974).
9. Mitnick, M.A. and Reichlin, S., Endocrinology, 91, 1145 (1972).
10. Bowers, C.Y., Currie, B.L., Johansson, K.N.G., Folkers, K., Biochem. Biophys. Res. Commun., 50, 20 (1973).