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ENZYMIC DEGRADATION OF LUTEINIZING HORMONE-RELEASING
HORMONE (LH-RH) BY HYPOTHALAMIC TISSUE

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SUMMARY

Synthetic luteinizing hormone-releasing hormone (LH-RH) lost both its immunoreactivity and hormonal activity on incubation with hypothalamic or cerebrocortical slices or homogenates. This inactivation was shown to be due to degradation of the decapeptide by soluble enzyme(s) present in the 100,000 x g supernatant fraction of the homogenates. The supernatant derived from one rat hypothalamus was capable of destroying 1 μ g of exogenous LH-RH within 5 min. The hexapeptide pGlu-His-Trp-Ser-Tyr-Gly was identified as the major radioactive breakdown product of [pGlu-3-³H]LH-RH, and tentative evidence for the formation of the tetrapeptide Leu-Arg-Pro-Gly-NH₂ was obtained by sequential electrophoresis and paper chromatography. These findings suggest that the Gly-Leu bond may be the preferred site of cleavage.

A decapeptide has been isolated from the hypothalamus of several mammalian species, which stimulates the secretion of gonadotropic hormones from the pituitary gland *in vivo* and *in vitro*, and its synthesis has been accomplished (1, 2). Although designated "luteinizing hormone-releasing hormone" (LH-RH), the use of specific antibodies generated against the synthetic peptide (3) established that it has a physiological role in regulating the secretion of follicle-stimulating hormone (FSH) as well as luteinizing hormone (LH) (4). The mechanisms regulating hypothalamic LH-RH synthesis and release are not fully understood. Studies aimed at their elucidation employ both *in vivo* and *in vitro* systems; the latter commonly involve the incubation of hypothalamic fragments and the determination of LH-RH by either biological or immunological assays. Hypothalamic and other cerebral tissues, however, have been shown to contain peptidases (5), that may seriously interfere with such studies.

We wish to report the existence of enzymic activity, in the soluble fraction of hypothalamic and cerebrocortical extracts, which causes structural alteration of the LH-RH molecule, resulting in loss of both its immunological properties and biological activity.

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MATERIALS AND METHODS

Materials. LH-RH was a generous gift of Hoechst A. G. (Frankfurt). [Pyroglutamyl-3-³H] LH-RH was purchased from New England Nuclear (Boston). The following proteinase inhibitors were used: Kallikrein inactivator (Trasylol; SK/T22/3, 5100 KIU/mg and GOS 746/31, 5700 KIU/mg, Bayer A. G., Frankfurt); soybean trypsin inhibitor (Worthington Biochemical Corp., N. J.); benzamidine (Eastman Kodak Co., Rochester) and L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK, Sigma Chemical Co., St. Louis).

Animals and tissue preparation. Two- to four-month-old male and female Wistar-derived rats of the Departmental colony were used. The animals were killed by decapitation, and hypothalami and slices of cerebral cortex were excised immediately. Hypothalamic tissue from five animals (about 150 mg) or an equivalent weight of cortical tissue was homogenized in 1 ml 0.01 M phosphate-buffered saline (PBS), pH 6.9, by applying seven strokes in a Thomas teflon homogenizer immersed in crushed ice. The supernatant fraction, collected after 30 min centrifugation at 17,000 x g or 100,000 x g at 4°C, was used.

Incubation and extraction. Portions of the supernatant fractions were incubated in the presence of 1 µg LH-RH in 1 ml PBS at 37°C in a Dubnoff shaking bath. Incubations were terminated by boiling for 3 min; control experiments indicated that LH-RH is not damaged by this treatment. The samples were then spun for 30 min at 17,000 x g and the supernatant fraction was taken for determination of LH-RH by radioimmunoassay (3) or biological assay. For electrophoresis and amino-acid analysis the supernatant fraction, derived from 105 mg wet brain tissue (equivalent to 3.5 hypothalami), was incubated for 10 min with 0.4 µCi [³H] LH-RH diluted with 1 mg of the unlabeled hormone. After terminating the reaction by boiling, the samples (1 ml) were spun for 50 min at 100,000 x g, filtered through a Diaflo UM2 membrane (Amicon Corp., Lexington), lyophilized and redissolved in 0.2 ml distilled water.

Assay of LH-releasing activity was performed according to Ramirez and McCann (6), using ovariectomized estrogen- and progesterone-primed rats. LH was determined by radioimmunoassay (7).

Electrophoresis was carried out on Whatman No. 3 paper (prewashed with 5% acetic acid) in pyridine acetate buffer, pH 3.5, for 10 min at 10 V/cm, followed by 90 min at 60 V/cm. The region of this electropherogram carrying a slow-moving peak (Fig. 1a; Peak II) was excised, the segment sewn onto another strip of Whatman

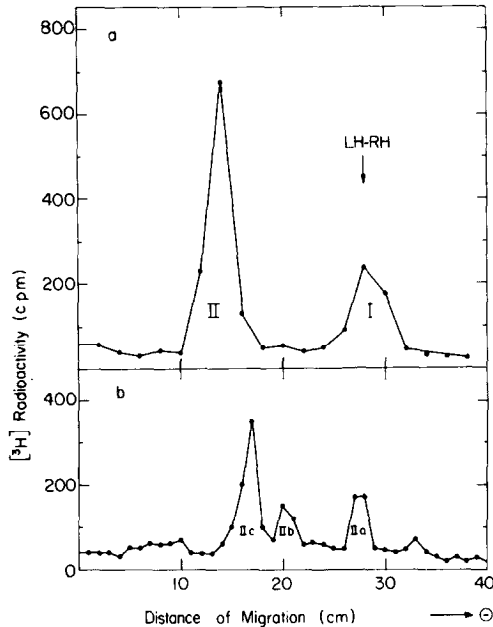


Fig. 1. Electropherogram of the degradation products of LH-

No. 3 paper and subjected to further high voltage electrophoresis (60 V/cm for 45 min in 2.5% formic acid and 7.5% acetic acid, pH 1.9).

Amino-acid analysis. The radioactive and fluorescent electrophoretic components were eluted with 10% acetic acid, lyophilized, subjected to exhaustive hydrolysis (6N HCl, 18h, 110°C) and to amino-acid analysis according to Spackman et al. (8).

RESULTS

Enzymatic inactivation of LH-RH. Incubation of LH-RH with the 17,000 x g or the 100,000 x g supernatant fraction of homogenates from rat hypothalamus or cerebral cortex resulted in progressive loss of the immunoreactivity and hormonal activity of the decapeptide.

The velocity of LH-RH inactivation was assessed by exposing 1.2 μ g of the peptide to the supernatant fraction, derived from one hypothalamus, for various periods. As shown in Table 1, less than 20% of the LH-RH added was measurable by radioimmunoassay after 5 min incubation; after 15 min less than 1% of the intact decapeptide was recoverable. Inactivation of LH-RH was proportional to the amount of enzyme preparation used (Table 2). Inactivation was temperature-dependent in that the supernatant

Table 1. Time course of LH-RH inactivation

Incubation Time (minutes)	LH-RH Recovered (ng \pm SEM; N=7)
0	1200 \pm 28.8
5	248 \pm 37.0
10	15.3 \pm 5.2
15	7.0 \pm 1.0
30	4.3 \pm 0.8
60	1.8 \pm 0.5
120	undetectable

LH-RH (1.2 μ g) was incubated with the supernatant fraction of one hypothalamus. The reaction was terminated by boiling and LH-RH determined by radioimmunoassay.

Table 2. Recovery of LH-RH after exposure to varying amounts of enzyme preparation

Amount of tissue homogenized		LH-RH recovered after incubation with:	
wet weight (mg)	Equivalents of Hypothalamus	Hypothalamus (% \pm S. E. M.)	Cortex (% \pm S. E. M.)
0	0	100	100
0.3	0.01	not determined	85.7 \pm 1.58
0.6	0.02	64.7 \pm 12.86	70.0 \pm 16.45
1.5	0.05	16.4 \pm 1.72	not determined
3.0	0.1	5.4 \pm 0.46	11.7 \pm 0.95
6.0	0.2	0.4 \pm 0.05	0.7 \pm 0.05
30	1	<0.01	<0.01

LH-RH (1 μ g) was incubated for 1 h with sequential dilutions of the supernatant fraction of homogenates from one rat hypothalamus (30 mg wet weight) or an equivalent amount of cerebral cortex. The reaction was stopped by boiling and LH-RH determined by radioimmunoassay. N = 6.

fraction from 0.05 hypothalami destroyed 83% of the LH-RH ($1 \mu\text{g}$) during 1 h incubation at 37°C , whereas at 2°C only 31% of the LH-RH was lost.

The effect of the enzyme-treated LH-RH on serum LH levels in ovariectomized, estrogen- and progesterone-treated rats was compared to that of the intact hormone. As shown in Figure 2, the LH-releasing activity of LH-RH ($1 \mu\text{g}$) disappeared after 1 h incubation with the supernatant fraction from 6 mg of hypothalamic tissue.

Inhibitors of LH-RH degradation. Several proteinase inhibitors were assayed for their ability to prevent the degradation of LH-RH by slices or homogenates of cerebral tissue. As shown in Table 3, Kallikrein inactivator (K. I.) at high concentrations (0.2-1 mg/ml) prevented the enzymic inactivation of LH-RH as measured by radioimmunoassay, whereas a more purified preparation of this inhibitor (GOS 746/31) as well as soybean trypsin inhibitor, TPCK and benzamidine were ineffective. K. I. (SK/T22/3) also prevented loss of the LH-releasing activity of LH-RH during 1 h incubation with the supernatant from 6 mg of hypothalamic tissue (Fig. 2).

Identification of degradation products by electrophoresis, chromatography and amino-acid analysis. Tritiated LH-RH was incubated for 10 min with the hypothalamic supernatant preparation. Electrophoresis of an aliquot of the incubation products at

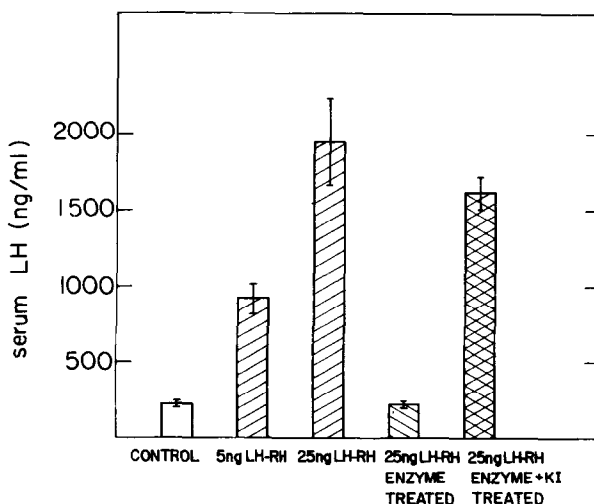


Fig. 2. Effect of enzyme treatment on the biological activity of LH-RH. Groups of five ovariectomized estrogen- and progesterone-primed rats, were injected intravenously with: saline (control); LH-RH (5 or 25 ng); LH-RH (25 ng portions of $1 \mu\text{g}$) with the enzyme preparation in the presence or absence of Kallikrein inactivator (K. I.). Blood samples were collected, by heart puncture, 15 min later and LH was measured by radioimmunoassay. Results are expressed in terms of NIAMD-Rat LH-RP-1. Vertical bars represent standard errors of the mean.

Table 3. Effect of proteinase inhibitors on LH-RH inactivation

Inhibitor	Recovery of immunoreactive LH-RH from the incubation medium of:	
	Excised Hypothalamus	Homogenized Hypothalamus
-	42.5 ± 4.98*	0
Kallikrein-inactivator 2 µg/ml	53.7 ± 2.92	-
Kallikrein-inactivator 200 µg/ml	92.0 ± 1.74	33.1 ± 2.10
Kallikrein-inactivator 1 mg/ml	-	99.3 ± 0.68
Soybean trypsin inhibitor 1 mg/ml	-	0.8 ± 0.11
Benzamidine 15 mg/ml	-	0.4 ± 0.14
TPCK 3 µg/ml	52.8 ± 0.72	-

*
% ± S. E. M. ; N = 14

LH-RH (1 µg) was incubated for 1 h with one hypothalamus or with the supernatant fraction of one homogenized hypothalamus. Where indicated, inhibitors were added to the incubation medium. The reaction was stopped by boiling and LH-RH determined by radioimmunoassay.

pH 3.5 revealed two radioactive peaks (Fig. 1a). One of these (I) corresponded to the authentic LH-RH marker. The slower-moving peak (II) was eluted, hydrolyzed and subjected to amino-acid analysis. It was found to contain all the amino acids of LH-RH, except for arginine, in the molar ratios present in the native hormone. The absence of arginine in this electrophoretic fraction was confirmed by a negative Sakaguchi's test. Under these conditions of electrophoresis, however, free proline, leucine and glycine comigrate with the slow radioactive peak (II), and free arginine leaves the paper. Peak II was therefore subjected to further electrophoresis at pH 1.9. In this system, the material was resolved into three radioactive peaks, all of which were fluorescent, indicating the presence of tryptophan (Fig. 1b). These peaks were eluted and subjected to hydrolysis and amino-acid analysis. The slowest component (IIc) contained glutamic acid, histidine, serine, tyrosine and glycine in the apparent molar ratio of 1.0 : 0.7 : 0.7 : 0.4 : 1.1. In addition, the presence of tryptophan in this peptide was established by using p-toluenesulfonic acid, instead of hydrochloric acid, for hydrolysis. The second component (IIb) had the same amino-acid composition, and its higher mobi-

Jity might be due to opening of the pyro-glutamic ring. The third component (IIa) contained glutamic acid, histidine and serine (1.0 : 0.7 : 1.0), and its fluorescence indicated the presence of tryptophan.

Another portion of the incubation products was subjected to two-dimensional separation by sequential electrophoresis and chromatography, alongside the following marker substances : Gly-NH₂; Gly; Pro; Arg; Leu; Pro-Gly; Arg-Pro-Gly-NH₂; Arg-Pro-Gly; Leu-Arg-Pro-Gly-NH₂ and Leu-Arg-Pro-Gly. Electrophoresis was done at pH 1.9 (15 min at 10 V/cm and 30 min at 60 V/cm). Chromatography was done on Whatman No. 3 paper in the system: n-butanol/acetic acid/water/pyridine (15:3:12:10; v/v) at room temperature for 15 h. Components containing free amine groups were detected by use of the ninhydrin-cadmium reagent. The ninhydrin-reactive products that corresponded in mobility to one of the marker substances were in the positions occupied by Leu-Arg-Pro-Gly-NH₂ and the free amino acids leucine and possibly arginine (trace). Several other ninhydrin-reactive components were present, but could not be identified.

DISCUSSION

Inactivation by brain tissue of several oligopeptide hormones, such as oxytocin (5) and thyrotropin-releasing hormone (9), has been attributed to enzymic degradation. In the case of oxytocin, one of the resulting fragments, claimed to possess hormonal activity of its own, was in turn destroyed by an aminopeptidase (10, 11). LH-RH was shown to be inactivated by homogenates from liver and kidney (12), pituitary gland (Koch & Baram, unpublished) and hypothalamic extracts (13), but the nature of this inactivation has not been elucidated.

In this study, the presence of potent LH-RH-inactivating enzyme(s) in rat hypothalamus and cerebral cortex was demonstrated. The enzymic activity responsible was associated with the 100,000 x g supernatant fraction of the homogenates, and passed into the medium during incubation of hypothalamic or cortical slices. Analysis of the incubation products suggested that the inactivation of LH-RH was due to cleavage of the Gly-Leu bond. This interpretation is supported by (i) identification of a radioactive and fluorescent hexapeptide, pGlu-His-Trp-Ser-Tyr-Gly by electrophoresis in two different systems and amino acid analysis, as the major breakdown product of [pyroglutamyl-3-³H] LH-RH; (ii) tentative identification of the tetrapeptide Leu-Arg-Pro-Gly-NH₂ by sequential electrophoresis and paper chromatography alongside a synthetic marker; (iii) failure to detect any of the di- or tripeptides, or free amino acids other than leucine and possibly arginine, that would be formed if degradation

were due to the action of an exopeptidase attacking the C-terminal of the peptide. It would be desirable to confirm the identification of the tetrapeptide as a degradation product by use of LH-RH labeled near the C-terminal.

The use of several proteinase inhibitors indicated that the degrading activity is not trypsin- or chymotrypsin-like. Sufficient activity was present in the supernatant fraction from a single rat hypothalamus to destroy 1 μ g exogenous LH-RH within 5 min. The LH-RH content of the hypothalamus of a normal mature female rat is about 5 ng. Prima facie, these findings could suggest a very high turnover rate of LH-RH in the hypothalamus. However, it appears that endogenous LH-RH, stored in the neural tissue, is in some way protected from degradation (Koch & Baram, unpublished). While the physiological significance of this degradative pathway of LH-RH in the hypothalamus thus remains uncertain, its existence cannot be ignored when interpreting the results of in vitro experiments. A varying extent of LH-RH degradation under different in vitro conditions may help explain the inconsistent results obtained in studies of the effects of neurotransmitters on LH and FSH release by pituitaries co-incubated with hypothalamic tissue (14, 15, 16, 17).

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