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STUDIES ON THE ENZYMIC DEGRADATION OF LUTEINIZING HORMONE RELEASING HORMONE BY RAT PITUITARY PLASMA MEMBRANES

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SUMMARY

Degradation of luteinizing hormone releasing hormone (LH-RH) by purified plasma membranes from rat pituitaries was investigated. Synthetic LH-RH (0.5 mg/ml) was incubated (20 min, 37°C)with pituitary plasma membranes (750 µg protein/ml). The reaction was stopped by centrifugation at 4°C. The degradation products were isolated by high pressure liquid chromatography using a reversed-phase column. Amino acid analysis of the degradation products indicated that the N-terminal tripeptide (pGlu-His-Trp) and the N-terminal hexapeptide (pGlu-His-Trp-Ser-Tyr-Gly) sequence of LH-RH are the main degradation products. These results suggest that the main cleavage sites of LH-RH by the pituitary plasma membrane-bound enzymes are the Gly^6-Leu^7 and the Trp^3-Ser^4 bonds of the neurohormone.

INTRODUCTION

Luteinizing hormone releasing hormone (LH-RH), a decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, regulates the secretion of both luteinizing hormone and follicle stimulating hormone from the anterior pituitary (1). The neurohormone is synthesized in the hypothalamus and carried via the hypophysial portal system to the anterior pituitary where it exerts its stimulatory effects on hormone release. Relatively little is known about the mechanisms controling the secretion and action of LH-RH but it is possible that its enzymic degradation participates in these processes.

In the hypothalamus, the 1-6 hexapeptide sequence of LH-RH (pGlu-His-Trp-Ser-Tyr-Gly) has been identified as the major degradation product of LH-RH suggesting that its enzymic cleavage occurs initially at the Gly^6-Leu^7 bond (2,3,4). An additional site of cleavage may be the Pro^9-Gly^{10} bond (3,5). The enzymic degradation of LH-RH by the cytosol fraction of the

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pituitary gland has also been reported. The 1-6 hexapeptide sequence of LH-RH was again found to be the major cleavage product by the soluble pituitary peptidases (6,7). These findings have been indirectly confirmed by the demonstration that analogs of the neurohormone modified at the site of degradation, namely the 6th position, are less susceptible to enzymic degradation and possess increased biological activity (6,8,9,10).

The first step in the induction of gonadotropin secretion from the pituitary gland by LH-RH involves its binding to specific membrane receptors (11,12). However, it has been demonstrated that LH-RH can be inactivated by pituitary plasma membranes of bovine (11) and of rat (13,14,15) origin. It thus seems possible that pituitary membrane-bound LH-RH degrading enzymes may serve as a quenching mechanism which determines the duration and the amount of the neurohormone available at the receptor site. We have therefore investigated the mode of LH-RH cleavage by purified rat pituitary plasma membranes. Recognition of the degradative pathways of LH-RH may lead to the chemical synthesis of stable, long-acting and potent analogs having important clinical applications.

MATERIALS AND METHODS

LH-RH was a generous gift of Hoffmann-La Roche (Basel, Switzerland). [2-^{3}H]Adenosine 5'-monophosphate, ammonium salt was purchased from Amersham (Buckinghamshire, England).

Subcellular fractionation of anterior pituitary glands: Wistar derived adult female rats were sacrificed by decapitation. Anterior pituitaries were immediately excised and immersed in cold sucrose buffer (0.25 M) containing 10 mM HEPES, 1 mM DTT; pH 7.0. The glands were then minced, homogenized (100 mg wet tissue/ml) by a motor driven teflon-glass homogenizer (clearance 0.026 inches) at 1000 rpm. The homogenate was then filtered through four layers of cheese-cloth and the filtrate centrifuged for 10 minutes at 1500 x g. The pellet obtained was washed with the above buffer and under identical conditions. Purification of the pituitary plasma membranes proceeded according to the procedure described by Fleischer and Kervina (16) for the subcellular fractionation of rat liver. 5'-Nucleotidase activity was used as a plasma membrane enzyme marker, and was determined by a modification of the procedure given by Aronson and Touster (17). A subcellular fraction containing mainly mitochondria was prepared as described by Fleischer and Kervina (16). Succinate-cytochrome C reductase, an enzyme marker for the mitochondrial fraction, was determined according to Tisdale (18).

Electron microscopy of membranes: Fresh samples of membrane fractions were mixed with Karnofsky reagent (19), kept for 15 minutes at room temperature and

then centrifuged in polyethylene tubes for 15 minutes at 10,000 x g. Thin disks were cut from the tubes and placed in Karnofsky reagent for an additional 30 minute period. The disks were post fixed in 1% OsO_4 in Na-cacodylate buffer (0.1 M pH 7.2) for one hour. The preparations were dehydrated in ethanol and propylene oxide and embedded in Epon (20). Thin sections were stained with Uranyl acetate and lead citrate. The sections were examined under a JEM-100B (Jeol, Tokyo, Japan) electron microscope at 30,000 magnification.

Assay for LH-RH-degrading activity in plasma membranes: The reaction was performed in 10 mM Trisma buffer (Tris [hydroxymethyl] aminomethane and hydrochloride) pH 7. Plasma membranes (750 µg protein/ml) were incubated in a shaking bath with synthetic LH-RH (0.5 mg/ml) in a final volume of 0.3 ml, for 20 minutes at 37°C. The degradation reaction was stopped by cooling the tubes to 4°C and immediate centrifugation for 15 minutes at 100,000 x g. The supernatant obtained was applied on the high pressure liquid chromatography column after millipore filtration. Control tubes containing the same amount of plasma membranes with or without LH-RH were kept at 4°C during the enzymic reaction.

Isolation of degradation products by HPLC: The degradation products were isolated by high pressure liquid chromatography using a reversed-phase Lichrosorb RP-18 column (Merck, Darmstadt, Germany, 0.4 x 2.5 cm, particle size 10 µ). All solvents and samples were filtered through 0.22 µ Millipore filters before use.

Portions (200 µl) of the above LH-RH degradation mixture were injected. They were eluted with a linear gradient of isopropanol in 0.05 M Ammonium acetate, pH 5.5 starting with 5% isopropanol and ending with 20% isopropanol. The flow rate was 1 ml/min, and the gradient lasted for 1 hour. Elution was followed continuously by monitoring UV absorbance at 230 nm. The different peaks were collected, lyophilized and repurified on HPLC under isocratic conditions. Peaks I, II, III were repurified by elution with 5% isopropanol in 0.05 M ammonium acetate; pH 4.5 and Peaks IV, V, VI and VII with 10% isopropanol in 0.05 M ammonium acetate at pH 4.5. Controls of plasma membrane preparations without LH-RH were incubated, treated and analyzed under identical conditions.

Amino acid analysis of the degradation products: The HPLC purified peaks were collected, lyophilized and repeatedly (5 times) redissolved in water and lyophilized, and finally subjected to exhaustive hydrolysis (6N HCl, containing 4% phenol or in 4N methane sulfonic acid for determination of Tryptophane; 20 hours at 110°C) in vacuum sealed tubes. Amino acid analysis was performed according to Spackman et al. (21). Tryptophane was determined by eluting the samples with Li-E buffer pH 5.3 (Dionex, U.S.A.).

RESULTS

Purification of plasma membranes

A modification of the procedure originally designed by Fleischer and Kervina (16) for the subcellular fractionation of rat liver was applied for purification of pituitary plasma membranes. The preparations thus obtained were free of other identifiable subcellular components, as demonstrated by electron microscopy (Fig. 1). Determination of marker enzymes (Table 1) also confirms an enrichment of the plasma membrane fraction. A 7-fold increase was reached

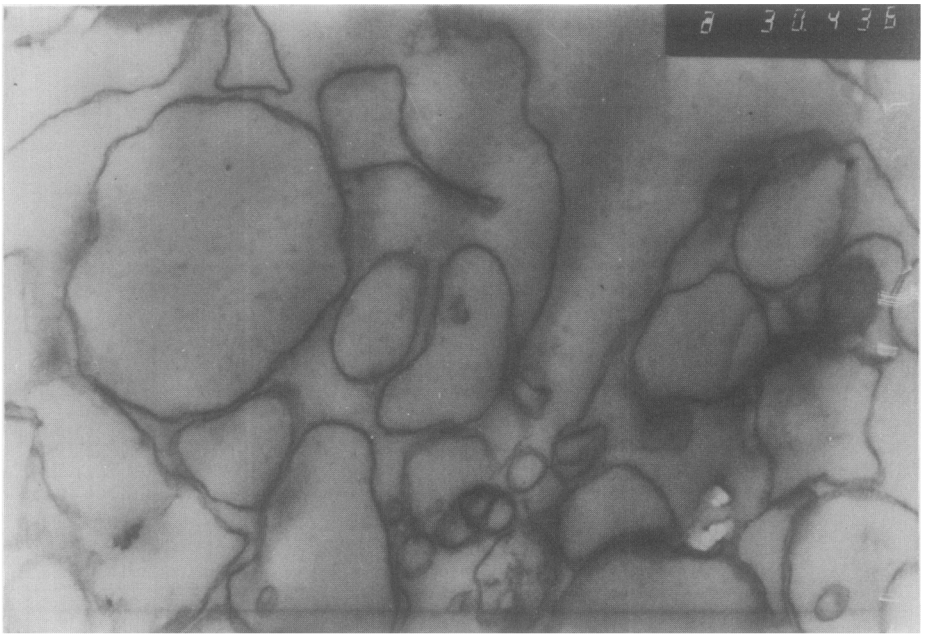


Fig. 1. Electron micrograph of purified plasma membranes from rat anterior pituitaries.

in the specific activity of 5'-nucleotidase in the plasma membrane fraction as compared to the total homogenate, without any appreciable contamination by a mitochondrial enzyme marker.

Identification of degradation products by HPLC and amino acid analysis

Incubation of pituitary plasma membrane preparations with LH-RH resulted in the degradation of 20-30% of the neurohormone. Several fragments of LH-RH

Table 1. Specific activity of marker enzymes in starting homogenate and subcellular fractions of pituitaries. The 5'-nucleotidase data is expressed as μM phosphate hydrolysed/mg protein/hour. Specific activity of succinate-cytochrome C reductase is reported in μM Cytochrome C reduced/mg protein/min.
n.d* - not determined.

	5'-nucleotidase	Succinate-cytochrome C reductase
Homogenate	0.51 ± 0.06	0.12 ± 0.02
Plasma membranes	3.6 ± 0.7	0.007 ± 0.002
Mitochondrial fraction	n.d*	0.44 ± 0.04

Table 2. Elution time and amino acid composition (molar ratios) of the different peaks isolated by HPLC. Peak II was further resolved into two peaks (here designed as IIa and IIb) upon repurification under isocratic conditions.

Peak Number	Elution Time (min)	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro
I	6.8	-	-	-	-	-	2.4	1.0	1.1	1.0
IIa	9.8	0.9	0.8	1.0	0.8	-	-	-	-	-
IIb	9.8	1.1	1.0	1.0	-	-	-	-	-	-
III	10.4	4.4	4.2	5.6	-	-	-	-	-	-
IV	26.6	2.0	1.7	2.5	2.0	2.0	2.5	-	-	-
V	50.7	0.9	0.5	0.6	0.8	0.5	1.6	0.7	0.6	0.7
VI	52.7	2.4	1.6	2.1	1.9	1.6	4.0	1.8	1.8	2.0
VII	54.5	20.0	19.0	25.0	18.5	19.0	40.0	19.0	21.5	20.8

could be resolved by high pressure liquid chromatography. Figure 2 shows a typical chromatogram obtained by the reversed-phase HPLC system, and Table 2 presents the time of elution and the amino acid composition of the different peaks. Amino acid analysis was performed only after repurification of the resulting peaks under isocratic conditions as indicated in the Materials and Methods Section. This procedure resulted in a better purification and separation of the different substances and even in the resolution of peak II (Fig. 2) into two separate components. Amino acid analysis showed that the (1-3) N-terminal tripeptide pGlu-His-Trp (Peaks IIb and III) and the (1-6) N-terminal hexapeptide pGlu-His-Trp-Ser-Tyr-Gly are the main degradation products of LH-RH. The (1-4) N-terminal tetrapeptide pGlu-His-Trp-Ser and the (6-10) C-terminal pentapeptide Gly-Leu-Arg-Pro-Gly were also identified but in smaller quantities. The last three peaks (V, VI and VII) contained the complete amino acid composition of LH-RH. Peak VII corresponds in its elution time to intact LH-RH and is the only peak observed in control experiments when the degradation reaction was carried out at 4°C or when standard LH-RH was used. Peaks V and VI may represent modifications of the decapeptide at the C and N

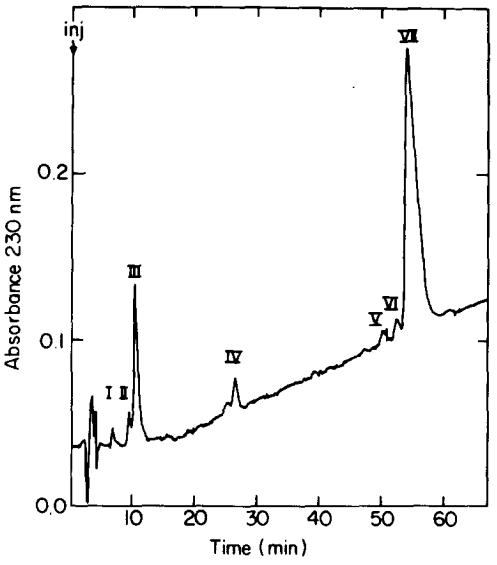
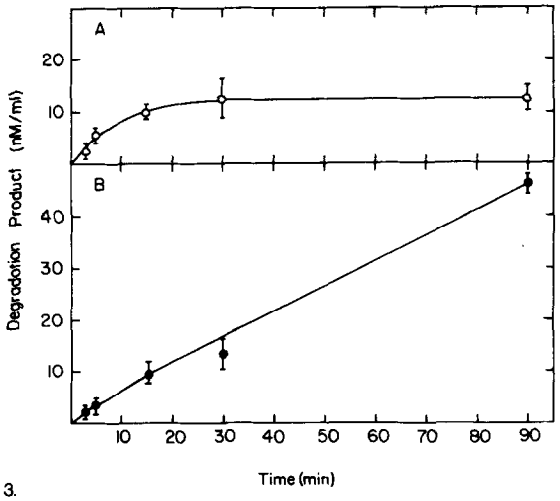


Fig. 2. High pressure liquid chromatography of the degradation products of LH-RH. Synthetic (1-6) N-terminal hexapeptide and LH-RH were chromatographed, as authentic markers, under identical conditions. Their time of elution corresponded to peak IV and Peak VII respectively. No additional peaks were observed when a mixture of the sample and the markers were cochromatographed.

terminals (deamidation or opening of the pyroglutamic ring).

Kinetic studies on the production of the different degradation products are demonstrated in Figures 3 and 4. When pituitary plasma membranes (750 µg protein/ml) were incubated with 0.5 mg/ml of LH-RH at 37°C for periods of 3, 5, 15, 30 and 90 minutes, the N-terminal hexapeptide product showed a five fold increase from 3 to 30 minutes and reached a steady state, while the other degradation product, the N-terminal tripeptide, continued to accumulate and was increased by 20 fold after 90 minutes of reaction. When plasma membrane preparation (750 µg protein/ml) was incubated for 20 minutes with increasing concentrations of LH-RH (Fig. 4), it was observed that the N-terminal tripeptide was the only degradation product when the substrate concentration was limited (Range: 10-50 nM/ml). Only when the concentration of LH-RH was further increased (50-500 nM/ml), was the formation of the N-terminal hexapeptide initiated.



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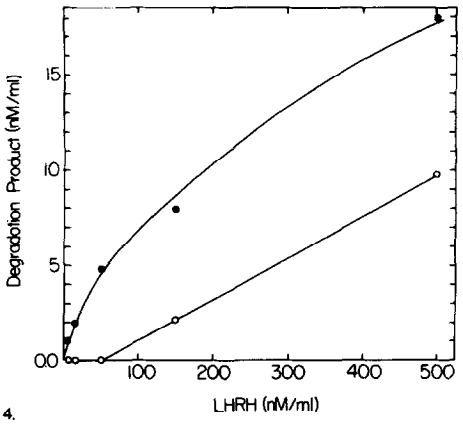

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Fig. 3. Time course of LH-RH degradation by pituitary plasma membrane-bound enzyme.
A. Production of (1-6) N-terminal hexapeptide
B. Production of (1-3) N-terminal tripeptide.

Fig. 4. Dose response of LH-RH degradation by pituitary plasma membrane-bound enzyme. (o—o) 1-6 N-terminal hexapeptide; (●—●) 1-3 N-terminal tripeptide.

DISCUSSION

We have previously reported that LH-RH can be degraded by rat crude pituitary plasma membrane-associated enzymes (13,15). This enzymic activity was shown to be intrinsic to the membrane preparation and thorough washings did not lead to reduction in the intensity of the enzymic activity. In this study we have purified membranes and characterized the involvement of the enzymes in the initial cleavage of LH-RH. This has been achieved by incubation of LH-RH with purified rat pituitary plasma membrane preparations, followed by isolation of the degradation products by HPLC and determination of their amino-acid composition.

The N-terminal tripeptide (pGlu-His-Trp) and the N-terminal hexapeptide (pGlu-His-Trp-Ser-Tyr-Gly) fragments of LH-RH were identified as the main degradation products of the neurohormone (Fig. 2, Table 2). These findings may suggest that there is more than one proteolitic enzyme that is associated with the pituitary plasma membrane. These enzymes cleave the decapeptide at

different sites. Alternatively, it can be argued that the initial site of cleavage is the Gly^6-Leu^7 bond of LH-RH and that the resulting hexapeptide is further processed by a secondary enzyme to yield the N-terminal tripeptide. However, the identification of the C-terminal pentapeptide (Table 2) and under different experimental conditions (results not shown) also of the C-terminal heptapeptide, suggest that the formation of the N-terminal tripeptide fragment of LH-RH is not necessarily dependent on the formation of the N-terminal hexapeptide. This assumption is further supported by the kinetic studies (Figs. 3 and 4) which suggest the existence of an enzyme cleaving LH-RH at the Trp^3-Ser^4 bond. Its affinity to LH-RH may be even higher than that of the enzyme cleaving at the Gly^6-Leu^7 bond.

Acknowledgements

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