STUDIES OF ENZYMATIC DEGRADATION OF LUTEINIZING HORMONE-RELEASING HORMONE BY DIFFERENT TISSUES

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1. Introduction

The isolation and structure identification of luteinizing hormone-releasing hormone (LH-RH) [1,2] together with its synthesis had led to the use of this hormone for many biochemical and physiological studies [3]. In the course of our research on the in vitro biosynthesis of LH–RH in the rat hypothalamus, we observed that this hormone undergoes a rapid inactivation as determined by radioimmunoassay. Subsequently, the examination of the incubates by thin layer chromatography allowed us to observe the presence of several degradation products.

There are some reports on inactivation of LH–RH by plasma [4], brain tissue and hypothalamus [5–7]. Lipmann has also recently reported the degradation of another hypothalamic hormone TRH by hypothalamic tissue [8].

The aim of this work was to measure the inactivation of LH–RH by homogenates of median eminence, of hypothalamic tissue without median eminence, of pituitary gland and of brain cortex and to find inhibitor(s) preventing its inactivation.

2. Materials and methods

2.1. *Reagents*

Synthetic LH–RH was provided by Drs R. O. Studer and D. Gillessen (Hoffmann-La Roche, Basel, Switzerland). Kallikrein inhibitors (KI): two batches of Trasylol (Bayer A. G., Frankfurt, Germany) were used, SK/T22/3, 5100 KIU/mg and GOS 746/31, 5700 KIU/mg. These two products were provided by Dr Y. Koch, Rehovot, Israel. One batch of Zymofren, another Kallikrein inhibitor (JMi 1297, 1032 KIU/ml) was obtained from Société des Usines Chimiques 'Rhône-Poulenc' (Paris, France).

2.2. *Tissue preparations and analytical methods*

Normal male rats (Wistar, laboratory strain) weighing 300 to 350 g were used. The animals were killed under light ether anaesthesia, tissues were rapidly collected in ice-cold 0.01 M Tris–HCl buffer, pH 7.6, containing 50 mM KCl + 12 mM $MgCl_2$ (Tris buffer) 8 mg/ml and homogenized in an all glass homogenizer, immersed in crushed ice. Starting from this point, two types of procedures were employed.

(a) For analysis by thin-layer chromatography, homogenates of the region of median eminence (4 mg) and of anterior pituitary glands were filtered through Sartorius membranes in a cold room in order to remove endogenous amino acids and peptides. 25 μl of filtered homogenate were mixed with 25 μl of LH–RH (50 μg) solution in Tris buffer.

Incubation was performed at 30°C for one or two hours. Reaction was stopped by adding 75 μl of absolute ethanol. Unincubated tubes served as controls (zero time). After centrifugation, 5 μl aliquots of supernatant were applied to thin-layer cellulose plates. Chromatograms were developed in the following solvent: *n*-butanol–acetic acid–water (75:10:25). Spots were detected by spraying with 0.25% ninhydrin in acetone or with Pauly reagent.

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(b) For radioimmunoassay of LH–RH, homogenates of median eminence, entire hypothalamus without median eminence (40 mg) anterior pituitary (5–6 mg) and brain cortex, were centrifuged at 1000 *g* for 10 min to remove cell debris. Only supernatants were taken for incubation (30°C, 30 and 60 min). Incubation tubes contained: 0.5 ml of supernatant (equivalent to 4 mg of tissue), 0.5 ml of Tris buffer (with or without KI), 0.1 ml of LH–RH solution (5 ng) in Tris buffer. KI was added to some tubes in amounts of 250 μg per tube.

Radioimmunological assay (RIA) of LH–RH was performed according to the method developed in our laboratory [9,10]. Results were expressed as percentage of zero time incubation.

3. Results

Fig.1 shows the results of measurements of LH–RH activity in the incubation media using RIA. The most important inactivation of LH–RH occurred in the presence of homogenates of brain cortex (100% inactivation in 30 min), the inactivation was less for hypothalamus without median eminence and pituitary gland and still less for median eminence. In the presence of Trasylol SK/T22/3, LH–RH inactivation was in all cases partially but noticeably diminished. This batch of Trasylol inhibited LH–RH inactivation at the highest level in brain cortex incubates (about 90% of recovery); inhibition was less for hypothalamus and median eminence incubates (respectively 75 and 65% of recovery) and still less for pituitary incubates (about 41% of recovery).

Figs.2 and 3 show thin-layer chromatograms, revealed with ninhydrin, of incubates of median eminence and pituitary with LH–RH in absence and in presence of two batches of Trasylol, SK/T22/3 and GOS 746/31, respectively. Intact LH–RH is ninhydrin positive spot, while incubated with LH–RH for 60 and 120 min the same homogenate showed

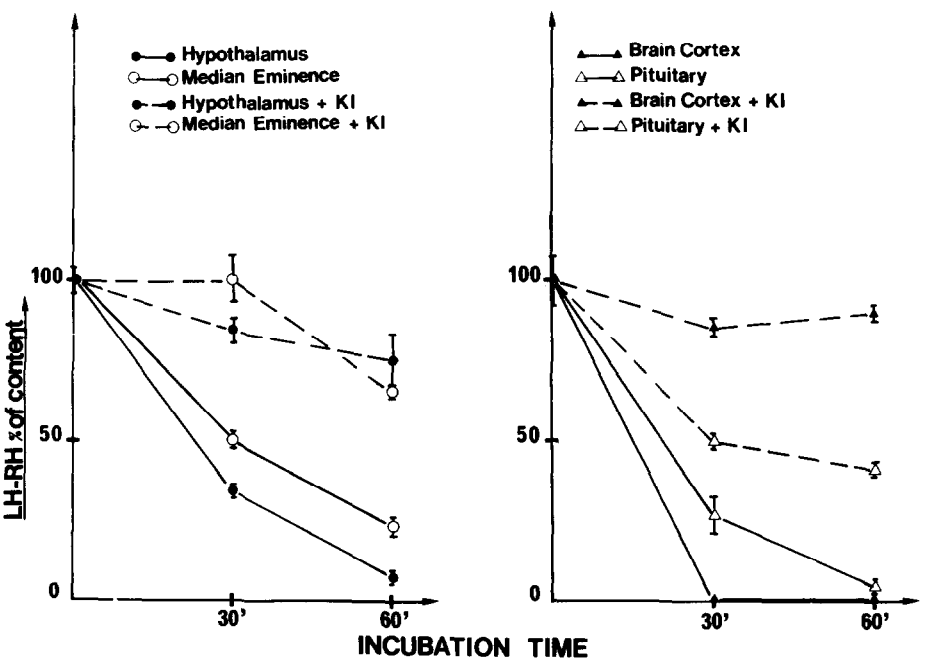

Fig. 1. Effect of incubation of LH–RH with homogenates of rat median eminence, of hypothalamus without median eminence, of pituitary and brain cortex. Equivalents of 4 mg of tissue in 1.1 ml of Tris buffer were incubated at 30°C with 5 ng of LH–RH in the presence or absence of a Kallikrein inhibitor (KI, Trasylol batch No. SK/T22/3). 40 and 160 μl aliquots of the incubation media were essayed using RIA and results were expressed as percentage of LH–RH content. Vertical bars represent standard of error of the mean.

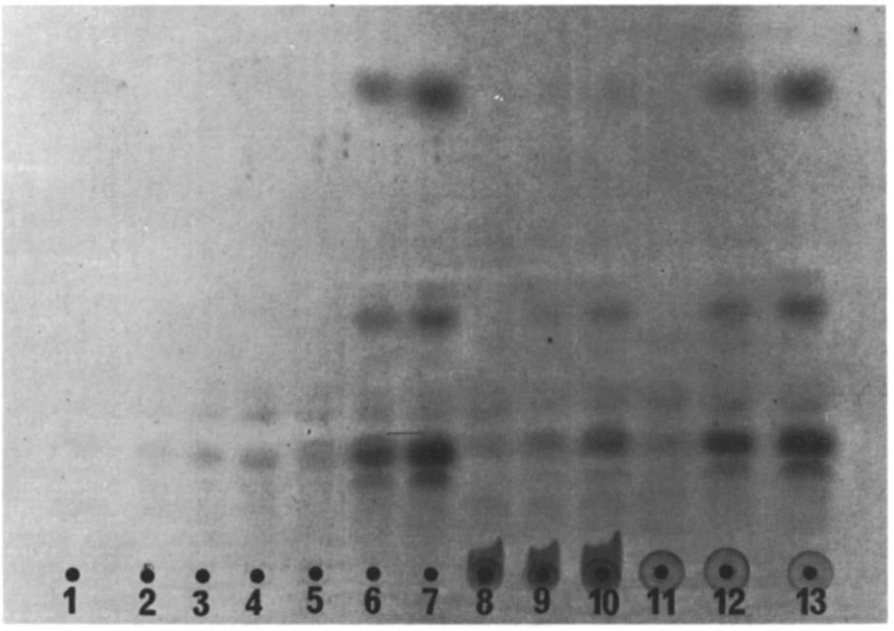

Fig.2. Thin-layer chromatography of LH–RH incubated for 1 or 2 hr with homogenates of rat median eminence (ME). Trasylol SK/T22/3 (KI_1) or Trasylol GOS 746/31 (KI_2) was added to some tubes. Chromatogram was revealed with ninhydrin (see text for details). 1. LH–RH; 2. ME zero time; 3. ME incubated for 1 hr; 4. ME incubated for 2 hr; 5. LH–RH + ME zero time; 6. LH–RH + ME incubated for 1 hr; 7. LH–RH + ME incubated for 2 hr; 8. LH–RH + ME + KI_1 time zero; 9. LH–RH + ME + KI_1 incubated for 1 hr; 10. LH–RH + ME + KI_1 incubated for 2 hr; 11. LH–RH + ME + KI_2 zero time; 12. LH–RH + ME + KI_2 incubated for 1 hr; 13. LH–RH + ME + KI_2 incubated for 2 hr.

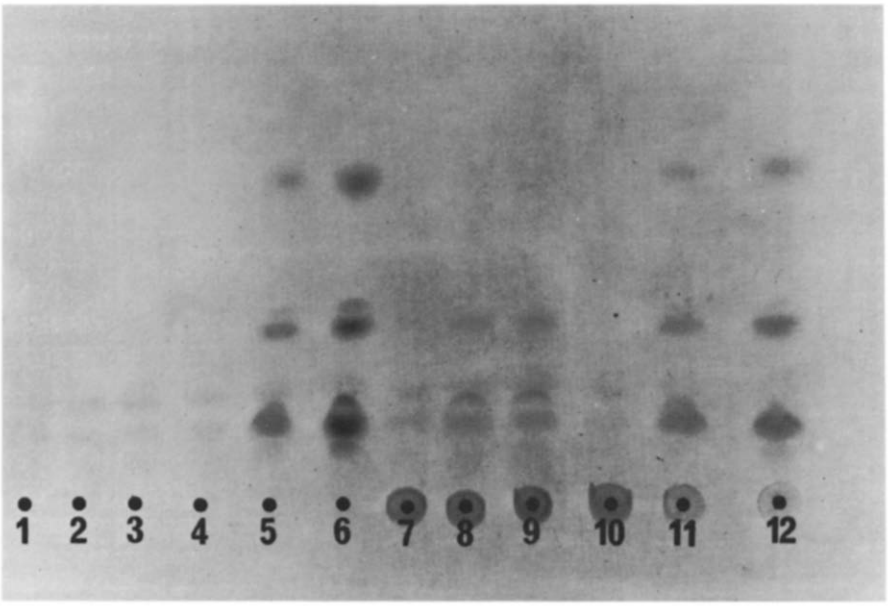

Fig.3

appearance of three new ninhydrin positive spots deriving from LH–RH. Trasylol SK/T22/3 partially prevented degradation of LH–RH while Trasylol GOS 746/31 and Zymofren were without effect.

LH–RH incubated with pituitary homogenates also showed several ninhydrin positive spots. Intensity of these spots was greatly diminished in the presence of Trasylol SK/T22/3. Again Trasylol GOS 746/31 and Zymofren were without action.

Very similar results were obtained when chromatograms were sprayed with Pauly reagent. After incubation of LH–RH with homogenates of median eminence and pituitary gland two spots appeared in addition to that of LH–RH which was also positive with Pauly reagent.

4. Discussion

The rat hypothalamus has been already shown to contain peptidases capable of inactivating oxytocin [11,12], TRH [8] and LH–RH [5,6]. Griffith and Hooper [6] suggested that the same enzymes in rat hypothalamus which inactivate oxytocin may also inactivate LH–RH. Oxytocin is inactivated by cleavage of C-terminal glycinamide and leucylglycinamide. It is possible that in LH–RH the same bonds are split as in oxytocin. Pyroglutamic acid also could be split from LH–RH, since pyrrolidonecarboxylyl peptidase which specifically cleave pyroglutamic acid from NH_2-terminal position of certain peptides, was isolated from bovine pituitary gland and other tissues [12].

Radioimmunoassay of LH–RH activity in the incubation media containing homogenates of different tissues appears to be an adequate method for measurements of enzymatic inactivation of the decapeptide. This method is highly specific of LH–RH as was shown in a study of many structural analogues of this decapeptide [14,15]. Only LH–RH possessing an intact structure gave a 100% response. Peptides shorter than LH–RH had no activity in our RIA system.

In the present work the RIA method combined with thin layer chromatography method was chosen. By the RIA method we were able to show a rapid inactivation of LH–RH during incubation with homogenates and supernatants of all examined tissues. Our results suggest that LH–RH is split at least into three peptidic fragments because three ninhydrin positive spots appeared on the plates. Inhibition of LH–RH degradation by only one batch of Trasylol suggests that only this preparation contained substances capable of inhibiting enzymes present in tissue homogenates and responsible for the partial hydrolysis of LH–RH. It is possible that inhibition was due not to the Kallikrein inhibitor itself, but to some impurities present in one commercial preparation of this substance (SK/T22/3).

Results obtained in the present work are in agreement with other publications [5,6] in which the inactivation of LH–RH was reported. Our observation that Trasylol may inhibit proteases involved in the degradation of LH–RH is also in agreement with a report by Koch et al. [7].

In short our results and those reported by others show that it is possible, at least partially, to prevent degradation of LH–RH by enzymes present in homogenates from median eminence and thus to use this method for in vitro study of the biosynthesis of LH–RH.

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Fig.3. Thin-layer chromatography of LH–RH incubated for 1 or 2 hr with homogenates of rat anterior pituitary (AP). Trasylol SK/T22/3 (KI_1) or Trasylol GOS 746/31 (KI_2) was added to some tubes. Chromatogram was revealed with ninhydrin (see text for details). 1. AP zero time; 2. AP incubated for 1 hr; 3. AP incubated for 2 hr; 4. LH–RH + AP zero time; 5. LH–RH + AP incubated for 1 hr; 6. LH–RH + AP incubated for 2 hr; 7. LH–RH + AP + KI_1 zero time; 8. LH–RH + AP + KI_1 incubated for 1 hr; 9. LH–RH + AP + KI_1 incubated for 2 hr; 10. LH–RH + AP + KI_2 zero time; 11. LH–RH + AP + KI_2 incubated for 1 hr; 12. LH–RH + AP + KI_2 incubated for 2 hr.

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