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SPECIFIC ACTIVITY OF LHRH AND TRH DEGRADING ENZYMES IN VARIOUS TISSUES OF NORMAL AND CASTRATED MALE RATS

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Summary : Specific activity and apparent kinetic constants of peptidasic enzymes degrading LHRH (luteinizing hormone releasing hormone) and TRH (thyrotropin releasing hormone) were evaluated in cerebral cortex, hypothalamus and pituitary homogenates and in the serum by determining the disappearance rate of saturating concentrations of the immunoreactive neuropeptides. Apparent affinity of the enzymes ranged from 2 to 3×10^{-5} M for LHRH in all tissues tested. It was somewhat lower in the pituitary for TRH (3.8×10^{-5} M vs 1.4 to 2x 10^{-5} M for hypothalamus and cortex respectively). Specific activity of LHRH degrading enzymes was comparable in cortex, hypothalamus and pituitary (15µg/min/mg protein), and lower in the pituitary for TRH inactivation (269ng/min/protein vs 650 ng/min/protein for the cortex). Castration did not affect any of these parameters under our experimental conditions.

The existence in the Central Nervous System (CNS) of a potent enzymatic activity that readily degrades neuropeptides is now well documented (1-4). Several tissues have been shown to contain an apparently heterogeneous population of peptidasic enzymes. However, under certain conditions, their action results in cleavage of peptides at discrete and reproducible sites (1,3,4), an observation which suggests a certain degree of peptide specificity.

Some authors have postulated that peptidasic degradation may be involved in the metabolic regulation of brain peptides, and, thus, play a physiological role in neuroendocrine functions (5,6). If this were the case, one would anticipate at least a certain degree of correlation between the regional distributions of a physiological relevant enzymatic activity and of the peptides themselves or their targets. This has proven difficult to demonstrate as yet, mainly because the specific enzymatic activity of different tissues or brain areas could not always be satisfactorily quantified.

In the present work, we attempted to determine the experimental conditions under which the specific activity of LHRH and TRH degrading enzymes could be evaluated. We then investigated the distribution of these enzymatic activities in the cortex, the hypothalamus and the pituitary, as well as in the serum. Finally, we compared their apparent kinetic constants in tissues sampled from

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castrated male rats, since studies using different evaluation methods reported steroid (5,6) or thyroid hormones (7) induced changes in LHRH and TRH degradation, respectively. Comparison of the degradation rates of both peptides after castration, a condition which affects preferentially the turnover rate of LH-RH, was undertaken in order to further assess a possible specific role of these enzymatic activities in peptide regulation.

MATERIAL AND METHODS

Male rats (Sprague-Dawley, 150g) kept under controlled light conditions and fed ad libitum were divided in a control and a castrated group. Six to 9 weeks after castration the animals were killed by decapitation. Trunk blood was collected and kept at 4°C for 3 hrs, centrifuged at 4000xg for 10 min and the serum obtained used for enzymatic determination. The tissues (mediobasal hypothalamus, frontal cortex, pituitary) were dissected, kept on ice until all animals were sacrificed, and homogenized in Tris 50mM (pH 7.2) on a 10% w/v basis. The homogenates were centrifuged at 4000xg for 3 min and the supernatant further centrifuged for 1 hr at 100 000xg. The resulting supernatant was kept in ice and diluted with the same buffer immediately before incubation.

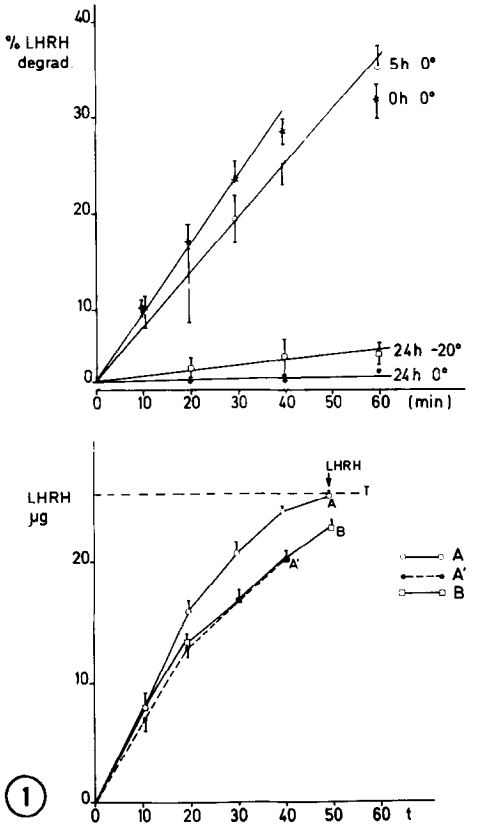
Incubations were performed in Eppendorf tubes placed in a shaking water bath at 37°C for varying periods of time indicated in Figure legends. Incubation volumes varied from 50 to 200µl of supernatant solution. The neurohormones were diluted in the same buffer and introduced in volumes of 5 to 10µl, after 5 min preincubation. In each experiment the same enzyme preparation was used for both LHRH (Hoechst) and TRH (Hoechst) and their degradation was followed in parallel. The releasing factors were not added together to avoid possible interference in their degradation (8). Immediately after the introduction of the releasing factor, 10µl of incubation mixture were removed and added to 500µl of HCl 0.1N. This represented the 0'time control for each sample. At the end of the incubation period, 10µl were likewise acidified. Each experimental point consisted of 5 tubes. All samples were kept frozen until assayed. The concentrationsof LHRH and TRH were measured in duplicate by specific radioimmunoassays.

The radioimmunoassay of LHRH was performed as previously described (9). The samples were diluted in 0.01N so as to give about 30 pg/tube and 10µl were introduced into the assay. The standard curve carried 10µl of the same acid solution. TRH was assayed using our own antibody (10) which shows no cross-reactivity with the proposed metabolites of TRH-degradation : the free acid or p-glu-his. Dilution of the samples was performed in the same immunoassay buffer (0.05M phosphate-0.25% BSA) and 50µl introduced into the assay. Each radioimmunoassay carried an internal standard prepared from acid extracts of rat median eminence.

Protein content was measured in all homogenates used by the method of Lowry.

RESULTS AND DISCUSSION

Experimental conditions : Storage of the 100 000 g-Tris-homogenate at 0 or -20°C for 12 hrs led to an important loss in enzymatic activity (Fig. 1). In contrast, when the homogenate was kept up to 5 hrs at 4°C, the degrading activity of the enzyme preparations was maintained. Stability of the enzyme during the time of incubation was also studied (Fig. 1) ; a slight decrease in activity was observed after 70 min of incubation, which is not due to inhibition by endproducts, since equivalent amounts of hormone are degraded du-



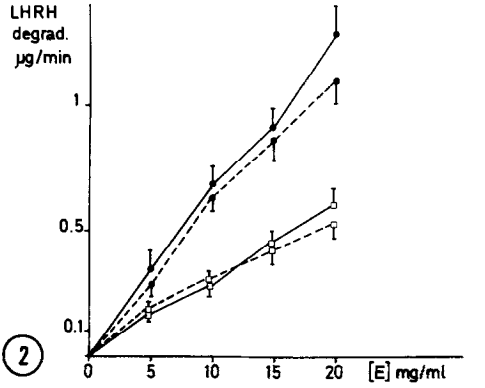


FIGURE I : a) Stability of the enzyme. 100 000 x g cortex supernatant was incubated with 25µg of LHRH at concentrations of homogenate of 4mg/ml wet weight. Before incubation the homogenate was kept : in ice for 5 h (○) ; incubated immediatly after centrifugation (✱) ; at 0°C (●) or at -20°C (□) for 24 h.
b) Effect of preincubation or reaction products on enzymatic activity. After 5 min preincubation, LHRH was added and samples taken every 10 min ; A : control incubation ; A' : to the tubes of A new LHRH solution was added and samples taken each 10 min for 40 min;B:after 50 min of preincubation LHRH was added and the reaction followed for further 40 min.

FIGURE 2 : Rate of LHRH and TRH degradation. 70µg of LHRH or 7.5µg of TRH were incubated in 200µl of rat cortex.
100 000 x g supernatant (Tris 50mM pH 7.2) at concentrations of 40, 12 and 4 mg wet weight/ml.

ring two successive periods of incubation (Fig. 1).

It is usually accepted (11) that optimal determination of kinetic constants requires the best possible evaluation of initial velocity. Fig. 2 shows degradation rates for TRH and LHRH as a function of enzyme concentrations. At 4mg wet weight tissue, little variation in the velocity is observed in the

TABLE I

		DEGRADING ACTIVITIES FOR LHRH		DEGRADING ACTIVITIES FOR TRH	
		NORMAL	CASTRATED	NORMAL	CASTRATED
SPECIFIC ACTIVITY (mole/min/mg/Prot)	MEDIAN EMINENCE	1.3×10^{-8}	1.4×10^{-8}	1.7×10^{-9}	1.8×10^{-9}
	CORTEX	1.2×10^{-8}	1.2×10^{-8}	1.8×10^{-9}	1.7×10^{-9}
	PITUITARY	1.4×10^{-8}	1.3×10^{-8}	0.7×10^{-9}	0.7×10^{-9}
	SERUM	(NOT DETECTABLE)		1.9×10^{-10}	1.6×10^{-10}
APPARENT K_M VALUES	MEDIAN EMINENCE	2.1×10^{-5}	2.0×10^{-5}	1.4×10^{-5}	NOT MEASURED
	CORTEX	2.4×10^{-5}	2.8×10^{-5}	2.2×10^{-5}	NOT MEASURED
	PITUITARY	3.3×10^{-5}	3.1×10^{-5}	3.8×10^{-5}	NOT MEASURED
	SERUM	(NOT DETECTABLE)		2.8×10^{-5}	NOT MEASURED

Enzymatic activity was measured in a 4 point assay (5,10,15,20mg wet weight/ml) of a 100000 g supernatant (Tris 50mM, pH 7.2). TRH was incubated for 20 min at concentrations of 1.70µg/50µl in all regions studied. LHRH (13µg/50µl) was incubated for 10 min in pituitary and 20 min in other regions.
The apparent K_M was measured in 100000g supernatant of brain regions at concentrations of 4mg wet weight/ml of tissue. ^{125}LHRH was added at a concentration range of 23-105µg/50µl ; TRH, 5-50µg/50µl. Regression line of the individual points was calculated by least squares method and a confidence limit of 0.95 of the variation obtained for the slope.

first 20 min for TRH and 10 min for LHRH. In contrast, at high enzyme concentration (40mg) the rate of degradation is considerably diminished.

Apparent kinetic constants : The apparent Km values obtained for LHRH have been found similar in all brain structures or tissues studied (Table I) ; They are higher for TRH in the pituitary than in cortex or median eminence (Table I).

Specific activities, as determined by measuring the rate of degradation at four different enzyme concentrations in presence of high amounts of substrate (3-10xKm), are shown on Table I. Fig. 3 shows that under these conditions, velocity is proportional to the concentration of enzymes.

Expressed as mg wet weight (Fig. 3), enzyme activity degrading LHRH is considerably higher in the pituitary than in the median eminence or cortex extracts. However, the regional distribution of the specific activity of LHRH peptidases is homogeneous. No detectable activity is present in the serum.

On a molar basis, TRH is less degraded than LHRH ; pituitary presents the lowest activity. In contrast to the data obtained with LHRH, TRH is readily degraded by serum samples. This however may be species specific, since in another experiment, low concentrations of TRH were almost not degraded by guinea pig or rabbit serum (unpublished data).

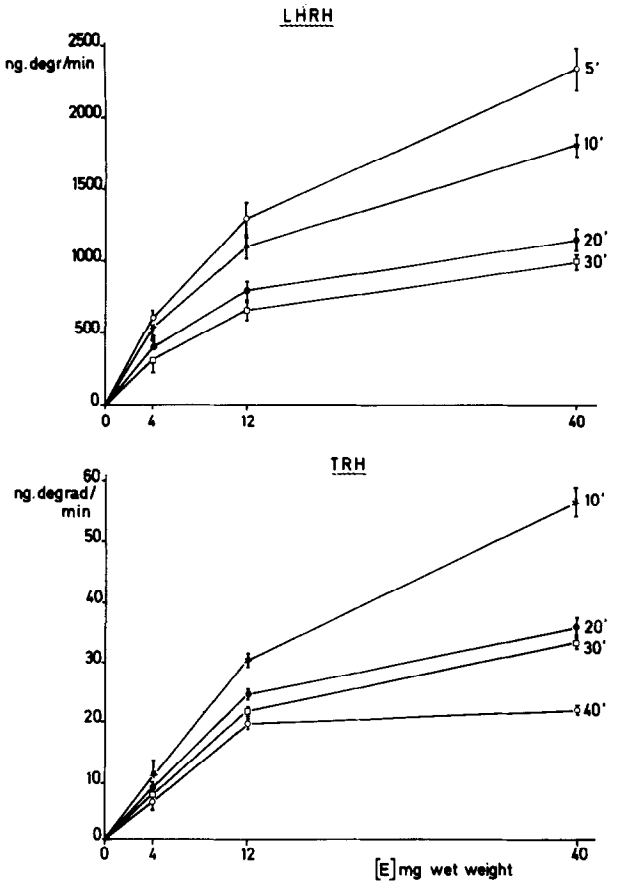


FIGURE 3 : Enzymatic activity for LHRH degradation. LHRH (13µg/50µl) was incubated for 10 min with 100 000 x g supernatant of o———o pituitary from normal, o---o castrated male rat, and 20 min with □———□ median eminence from normal and □-------□ castrated male rat. Enzyme concentration (E) expressed as mg wet weight tissue/ml.

Effect of castration : No effect of castration was found on any of the parameters studied. The inability of castration to alter the affinity of the soluble enzymes for LHRH or their specific activity in hypothalamic homogenates is not in agreement with previous observations (5,6) reporting changes in LHRH degradation by brain homogenates after castration or treatment with sex steroids. The reasons of this discrepancy are still unclear, but may depend upon the experimental preparation used to calculate the enzyme activity. In one of these observations, enzyme activity was measured with non saturating concentrations of the substrate (5), a procedure which does not permit as accurate an evaluation that calculation of kinetic constants. In the other, the method used was indirect and relied on conversion of a synthetic substrate

(6). At any rate, under conditions which permit one to calculate kinetic constants, the characteristics of the enzymes do not seem to depend upon peripheral sex steroid levels.

Our experimental procedure does not permit to differenciate, among the highly heterogeneous population of peptidases present in tissue homogenates (4) between enzymes cleaving specifically LHRH or TRH from other less peptide specific enzymatic activities. At the present stage, however, ineffectiveness of castration to affect either LHRH or TRH degradation under our experimental conditions does not substantiate the hypothesis that the overall pool of peptidasic activity which can be evaluated by radioimmunological methods plays an important role in regulating endogenous neurohormone levels.

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