DEGRADATION OF GONADOTROPIN-RELEASING HORMONE BY ANTERIOR PITUITARY ENZYMES

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Received 23 March 1981

1. Introduction

The secretion of gonadotropins from the anterior pituitary is regulated by the hypothalamic linear decapeptide gonadotropin-releasing hormone (GnRH). Peptidases that cleave GnRH may play a role in determining the physiological levels of the neurohormone at the sites of its release (hypothalamus) and of its action (anterior pituitary). Studies on the pathways of GnRH degradation may shed some light on the physiological mechanisms controlling the secretion and action of GnRH as well as assist in designing long-acting analogues of the neurohormone.

The initial site of degradation of GnRH by hypothalamic enzymes is the Gly^6–Leu^7 bond [1]. Here we describe the degradative pattern of GnRH by a soluble fraction of the anterior pituitary and compare some characteristics of the degradation processes caused by the cytosolic fractions derived from the pituitary and the hypothalamus.

2. Materials and methods

GnRH was a generous gift of Ayerst Research Laboratories (Montreal). [*pyroglutamyl*-3-^{3}H]GnRH was purchased from New England Nuclear (Boston). The following reagents were used: bacitracin (Sigma Chemical Co., St. Louis); diisopropylfluorophosphate, *p*-chloromercuribenzoate and *N*-ethylmaleimide (Merk, Darmstadt); 1,10-phenanthroline (Aldrich Chemical Corp., Milwaukee).

2.1. *Enzyme preparations*

Anterior pituitaries or the medial basal part of hypothalami from 5 rats (40–50 mg wet wt.) were detached immediately after decapitation and immersed in a Thomas homogenizer containing 1 ml of phosphate-buffered saline (PBS, pH 6.9). The tissue was homogenized by applying 10 strokes with a Teflon pestle and spun for 30 min at 100 000 × *g*. The resulting supernatant fraction was made up to a concentration of 30 mg wet wt. tissue/ml. Protein content of the preparation was determined as in [2].

2.2. *Assay of degradation*

Samples of different dilutions of the supernatant fractions were incubated as in [1] with 1 μg GnRH in 1 ml of 0.05 M PBS at 37°C; incubations were terminated by boiling for 3 min. The samples were then spun for 30 min at 17 000 × *g* and the supernatant was taken for determination of GnRH by radioimmunoassay or biological assay [3]. When the effects of pH on enzymic activities were studied, the pH was adjusted with 2 M HCl or 2 M NaOH.

For electrophoresis and amino acid analysis, the supernatant fractions (derived from 27 mg wet wt. anterior pituitaries) were incubated for 10 min with 0.8 μCi of [^{3}H]GnRH diluted with 1.0 mg of the unlabeled hormone. This short period of reaction was selected in order to enable the detection of the initial site of degradation of the neurohormone. After terminating the reaction by boiling, the samples (1 ml) were spun for 50 min at 100 000 × *g*, filtered through a Diaflo UM2 membrane (Amicon Corp. Lexington), lyophilized and redissolved in 0.2 ml distilled water. Electrophoresis was carried on Whatman No. 3 paper (prewashed with 10% acetic acid) in 2.5% formic acid and 7.5% acetic acid, pH 1.9, for 10 min at V/cm, followed by 40 min at 60 V/cm. Non-radioactive peaks were developed by using Pauli and ninhydrin reagents. The peaks were eluted with 10% acetic acid, lyophi-

lized, subjected to hydrolysis (6 M HCl, 18 h, 110°C) and analyzed for amino acid content using a Beckman Spinco Model 120C Amino Acid Analyzer.

2.3. *Determination of* M_r

Enzymic preparations derived from anterior pituitaries or hypothalami (150 mg wet wt. tissue) were gel filtrated on a Sephadex G-200 superfine column (2.5 × 70 cm) and eluted continuously with 10 mM phosphate buffer (pH 6.8) at a flow rate of 2 ml/h. The fractions eluted from the column were tested for enzyme activity. The column was calibrated by the following proteins: immunoglobulin, bovine serum albumin and ovalbumin.

3. Results

Incubation of GnRH with the 100 000 × *g* supernatant fraction of the homogenates from rat anterior pituitaries resulted in loss of the immunoreactivity as well as hormonal activity of the decapeptide. Using isolation and identification methods for the products of the enzymic degradation of GnRH, it was found that the preferred site of cleavage of GnRH by the pituitary enzyme was the Gly^6–Leu^7 bond. The supernatant fractions of the pituitary glands were incubated for 10 min with [^{3}H]GnRH diluted with unlabeled hormone. Electrophoresis of an aliquot of the incubation reactions (pH 1.9) revealed 7 different peaks (fig.1). 3 peaks (I–III) were radioactive, fluorescent and gave positive test with Pauli's reagent; peak II was also ninhydrin positive. The various peak regions were eluted from the paper with 10% acetic acid and the eluate was subjected to hydrolysis and amino acid analysis. Peak I was found to contain glutamic acid, histidine, serine, tyrosine and glycine in an apparent molar ratio of 1.2:1.0:1.0:0.97:0.8, respectively. Peak II had the same amino acid composition, with a molar ratio of 1.25:1.0:0.74:0.88:1.1, respectively, and its higher mobility might be due to an opening of the pyroglutamic ring. The third component (III) was identical to an authentic GnRH marker in amino acid composition and mobility. Of the 4 non-radioactive but ninhydrin-positive components, only region V had a composition which resembled a fragment of GnRH. It contained leucine, arginine, proline and glycine in a molar ratio of 0.92:1.06:1.01:1.00, respectively.

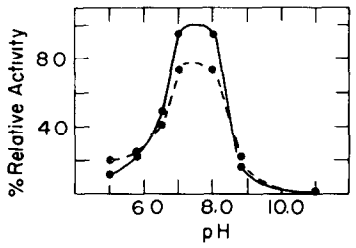

Fig.2. pH dependence of enzyme activity. •——•, pituitary enzyme; o– – –o, hypothalamic enzyme.

The inactivation of GnRH was time dependent and was proportional to the amount of enzyme preparation used. Equivalents of 0.6, 1.5 and 3.0 mg of wet wt. pituitary tissue caused 35.3 ± 12.8%, 84.6 ± 1.7% and 94.6 ± 0.5% degradation, respectively, of 1.0 μg GnRH after 1 h of incubation at 37°C. The following experiments were carried out under selected conditions (amount of enzyme and time of reaction) where about 50–60% of the substrate was degraded. This extent of degradation was arbitrarily defined as 100% and experimental effects (e.g. temperature, pH and inhibitors) were expressed relatively to this value.

The degradation reaction was pH and temperature dependent as illustrated in figs.2,3. Maximal proteolytic activity was evident in the pH range of 7.0–8.0 (fig.2) and at 37°C (fig.3) for both the hyporhalamic

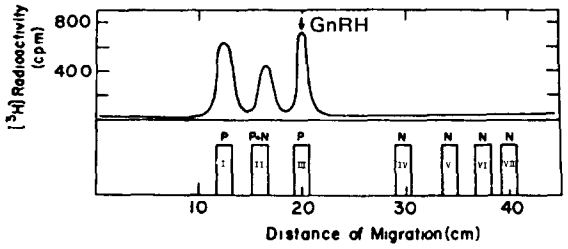

Fig.1. Electropherogram of the degradation products of GnRH. P., Pauli positive; N., ninhydrin positive.

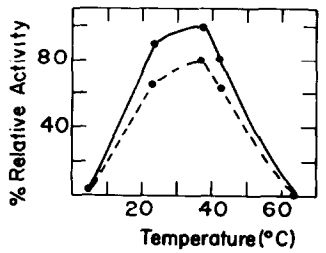

Fig.3. Temperature dependence of enzyme activity. •——•, pituitary enzyme; o– – –o, hypothalamic enzyme.

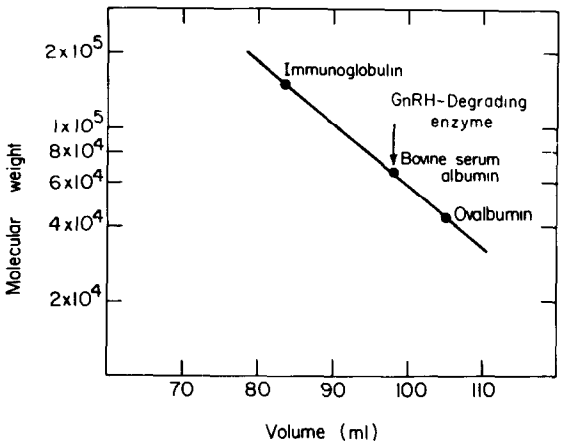

Fig.4. Chromatography of GnRH-degrading enzyme on Sephadex G-200. For experimental details see section 2.

and the pituitary enzymes. The elution patterns of the enzyme preparations, from both hypothalamus and anterior pituitary gland on a precalibrated Sephadex G-200 column, indicated a M_r of 67 000 ± 3000 for both enzymes (fig.4). Several enzyme inhibitors were examined for their ability to inhibit GnRH degradation. Non-active were the chelating reagents, 1,10-phenantroline and EDTA (10^{-3} M), trypsin and chymotrypsin blockers (TPCK, soybean trypsin inhibitor and benzamidine). Active reagents are enumerated in table 1.

4. Discussion

Relatively little is known about the mechanisms that regulate the secretion and the action of GnRH. Since both the hypothalamus and the pituitary gland contain enzymes that can degrade the neurohormone, it is possible that GnRH degradation participates in these processes. Changes in the activity of the GnRH-degrading enzymes were demonstrated after castration and replacement therapy with gonadal steroids [4–6]. High concentrations of dopamine have been demonstrated to enhance the degradation of GnRH upon its incubation with synaptosomes derived from the hypothalamus [7]. Substitution of the glycine at position 6 of GnRH by dextrarotatory amino acids results in analogues that are more potent and more resistant to enzymic degradation than GnRH itself [8–10].

Here, we examined the pattern of GnRH degradation by the 100 000 × *g* supernatant fraction of anterior pituitary homogenates. Electrophoresis of the incubation products, followed by hydrolysis and amino acid analysis suggested that the inactivation of GnRH by the pituitary was due to cleavage of the Gly^6–Leu^7 bond of the neurohormone similar to previous findings with the hypothalamic tissue [1,11]. Supporting evidence for this assumptions is the formaton of the hexapeptide pGlu-His-Trp-Ser-Tyr-Gly as the major radioactive product of [*pyroglutamyl*-3-^{3}H]GnRH and the formation of the tetrapeptide Leu-Arg-Pro-Gly-NH_2. The opening of the pyroglutamyl ring (fig.1, second component) must have occurred only after the initial degradation of GnRH and the formation of the N-terminal hexapeptide, since the only decapeptide that was detected was intact GnRH.

Additional evidence suggests that the GnRH-degrading enzymes of the hypothalamus and of the anterior pituitary are very similar, if not identical. Both have identical M_r-values (67 000), optimal activity at pH 7.0–8.0 and at 37°C. The neutral pH optimum suggests that these enzymes are not lysozomal cathepsins. The two enzymes were inhibited by bacitracin [12] and by crude preparations of kallikrein inactivator [1,13]. The enzymes were also inhibited by specific reagents for serine and thiol peptidases (table 1), suggesting the presence of serine or cysteine near or at the active site of the enzymes. The enzymes were not inhibited by metal-chelating reagents indicating that there are no metal requirements for the activity of the enzymes.

It seems likely that GnRH-degrading enzymes in the cytosolic fraction of the pituitary and the hypothalamus [1,8,11] as well as pituitary plasma membrane-associated enzymes [14,15] may act as a quenching mechanism that regulates the concentration and the activity of GnRH at the receptor site.

Table 1
Effect of various reagents on hypothalamic and anterior pituitary degrading enzymes

Reagent[a]	Concentration (M) required for 50% inhibition of GnRH degradation[b]	
	Hypothalamus	Pituitary
Diisopropylfluorophosphate	3.3×10^{-6}	4.0×10^{-6}
p-Chloromercuribenzoate	8.2×10^{-6}	9.9×10^{-6}
N-Ethylmaleimide	1.2×10^{-5}	1.6×10^{-5}

[a] All reagents were dissolved immediately before use
[b] Assays of degradation were performed as described in section 2

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