FEBS Letters 346 (1994) 203–206

FEBS 14086

Sequential degradation of the neuropeptide gonadotropin-releasing hormone by the 20 S granulosa cell proteasomes

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Received 20 April 1994

Abstract

The decapeptide gonadotropin-releasing hormone (GnRH) is degraded by the 20 S multicatalytic proteinase complex (proteasome EC 3.4.99.46), purified from ovarian granulosa cells, at the Tyr^5–Gly^6 bond and to a lesser extent at the Gly^6–Leu^7 bond, when incubated for 2 h at 37°C. Further cleavage, at Trp^3–Ser^4 and Ser^4–Tyr^5 bonds of the neurohormone occurs only subsequently to the appearance of the initial N-terminal degradation products, (1–5)GnRH and (1–6)GnRH. Our results suggest that the sequential degradation of GnRH can serve as an important mechanism for the rapid termination of its biological activity in target cells.

Key words: Proteasome; GnRH; Protein degradation; Ovary; Pituitary

1. Introduction

The proteasome, a non-lysosomal multicatalytic proteinase complex, plays a key role in the selective cellular degradation of defective, poorly folded or short-lived regulatory proteins [1–3]. Moreover, proteasomes are implicated in the provision of antigenic peptides which are associated with the major histocompatibility complex class of molecules [4–6]. The barrel-shaped complex has a molecular mass of 700 kDa [7,8] and contains up to 14 different subunits, all encoded by members of one gene family [9].

Gonadotropin-releasing hormone, a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2; GnRH), is a primary regulator of the reproductive cycle. This neurohormone is synthesized in hypothalamic neurosecretory cells, released in a pulsatile pattern into the hypothalamo-pituitary portal system and triggers the synthesis and secretion of the gonadotropic hormones luteinizing hormone (LH) and fillicle stimulating hormone (FSH) from the anterior pituitary [10–14]. It has also been found that GnRH can act directly on the rat gonads to inhibit steroidogenesis in males and ovulation in females. Direct effect of GnRH on cultured rat and human granulosa cells via specific receptors located at the cell membrane have been demonstrated [15–20]. In cells obtained from rat preantral follicle, GnRH agonist block gonadotropin induced formation of LH receptors and progesterone production [18,20]. In contrast, GnRH agonist significantly stimulate progesterone production in granulosa cells obtained from preovulatory follicles [21]. Thus, GnRH can affect directly maturation and luteinization of granulosa cells. Since GnRH can initiate the cascade of the signal transduction pathway, involving the activation of phospholipase D, which leads to the hydrolysis of phospholipids [21,22], termination of such a signal also seems to be essential for cell viability. Moreover, since GnRH acts as a neurotransmitter or a neuromodulator [23,24] and since the pulsatile pattern of secretion of GnRH is crucial for the proper responsiveness of the pituitary gland [25], it is clear that mechanisms of efficient and rapid degradation of GnRH are needed.

GnRH, by virtue of the presence of an N-terminal pyroglutamate residue and a C-terminal amide bond, is resistant to degradation by most exopeptidases. The enzymatic degradation of GnRH must therefore be controlled by endopeptidases [26,27]. Proteasomes can degrade synthetic oligopeptides but their role in the degradation of natural existing bioactive peptides like neurotransmitters and hormones is still unclear. Internalization of the GnRH-receptor complex in target cells following its binding to the receptors was previously demonstrated [28]. Therefore, degradation of internalized cytosolic GnRH could be part of the GnRH inactivation mechanism. As we have previously demonstrated that GnRH degrading activity is present in the rat ovary [29] and since highly purified proteasomes were recently isolated from immortalized ovarian granulosa cells [30], the possible involvement of these enzymes in termination of the action of GnRH by proteolytic activity of proteasomes was examined. In the present communication we demonstrate, for the first time, that granulosa cell proteasome in microgram quantities can efficiently degrade physiological concentrations of GnRH (nM). Moreover, the progressive degradation of GnRH to

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SSDI 0014-5793(94)00472-8

small fragments (1–6), (1–5), 1–4) and (1–3)GnRH provides new insights into the mechanism of action of the proteasome.

2. Materials and methods

2.1. Isolation of proteasomes from POGRS1 granulosa cell line

Proteasomes were isolated from cultured POGRS1 cells as described recently [31]. Briefly, supernatant (13,500 × g) of cell lysates were chromatographed on DEAE Sephacel ion-exchange column followed by chromatography on Sephacryl HR-S-400 gel filtration column (Pharmacia, Sollentuna, Sweden). Fractions containing the main activity, as indicated by chymotripsin-like proteolytic activity (with Suc-Ala-Phe-7 aminomethyl coumarin) [30], were subjected to an additional cycle of ion-exchange chromatography using Mono Q fast protein liquid chromatography (FPLC) (Pharmacia). All proteolytically active fractions were examined by SDS-PAGE. The fractions containing the proteasomes as the major protein were pooled, concentrated and chromatographed on a Superose-6 gel filtration column (Pharmacia). The purity of the isolated proteasomes was verified both by gel electrophoresis and electron microscopy of negatively stained proteasomes.

2.2. Synthesis of GnRH marker fragments

The 1–3 and 1–4 fragments of GnRH were synthesized and used as markers for identification of GnRH degradation products. The fragments were synthesized using the solid phase method [31] on a manual solid phase peptide synthesizer. Optimized protection of side chain functional groups minimized their participation in undesirable side reaction bonds.

2.3. Assay for GnRH degrading activity in purified proteasomes

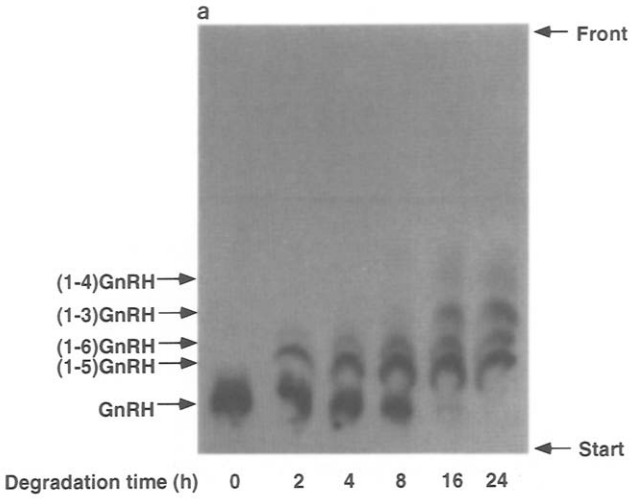
The assay for degradation was performed as follows: Aliquots of the proteasomes containing 15 μg protein (determined by the method of Bradford [32]) were incubated with 1.2 pmol of [^{3}H]GnRH labeled at the N-terminal residue (NEN-DuPont, Regensdorf, Switzerland). The final volume of the reaction was 50 μl of 20 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA. The reaction was carried out for 0, 2, 4, 8, 16, 24 and 48 h in a shaking water bath at 37°C, and was terminated by heating the samples in a boiling water bath for 5 min. Preheated proteasomes (5 min in a boiling water bath) incubated with [^{3}H]GnRH were used for control reactions.

2.4. Separation and identification of GnRH degradation products by TLC (RP-18)

The procedure was basically carried out according to the method developed by Leibovitz et al. [33]. Briefly, the GnRH degradation reaction mixtures were concentrated by Speed-Vac centrifugation (3,000 rpm; 0.5 mm) and the pellets were resuspended in 25 μl of methanol. Samples of the solutions (20 μl) were resolved on reversed-phase precoated glass plates (5 × 10 cm TLC RP-18) employing, as a solvent, 20% acetonitrile in water (v/v) containing 0.1% trifluoroacetic acid (TFA). This was followed by a second run using as a solvent a solution of 25% acetonitrile in water (v/v) containing 0.1% TFA. The TLC plates were prepared for autoradiography by spraying with EN^{3}HANCE and then exposed for 24 h at −70°C. Identification of intact labeled GnRH and labeled degradation products were accompanied by co-chromatography with synthetic GnRH, and its synthetic N-terminal fragments (1–2, 1–3, 1–4, 1–5, 1–6 and 1–7GnRH). The mobility of these synthetic markers was determined by spraying the plates with Pauly and Ehrlich's reagents [34]. The bands corresponding to the labeled intact GnRH and labeled degradation products were tentatively identified by their R_f values relative to the markers.

3. Results and discussion

When [^{3}H]GnRH was incubated with highly purified granulosa cell proteasomes at 37°C for different periods of time, progressive degradation of the decapeptide neurohormone was evident (Fig. 1). After two hours of reaction, the major detectable labeled product, by thin layer chromatography, was the N-terminal pentapeptide pGlu-His-Trp-Ser-Tyr, (1–5)GnRH. It was accompanied by smaller amounts of the N-terminal hexapeptide pGlu-His-Trp-Ser-Tyr-Gly, (1–6)GnRH. After 4 h of incubation, the amounts of (1–5) and (1–6)GnRH significantly increased and at 8 h of incubation a third degradation product, the N-terminal pGlu-His-Trp,



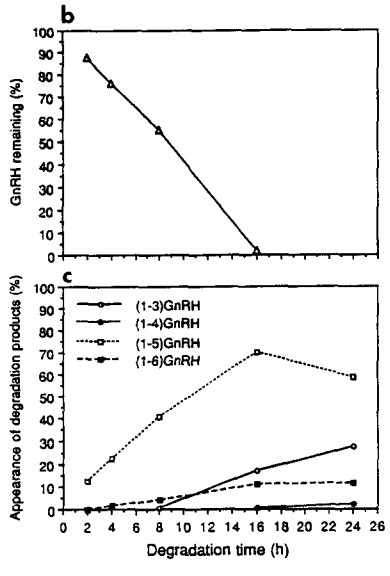


Fig. 1. The degradation pattern of GnRH as a function of time. Purified proteasomes (15 μg protein) were incubated for different time intervals (0, 2, 4, 8, 24 h) at 37°C with 1.2 pmol [^{3}H]GnRH. The incubation was terminated and the degradation products were separated as described in section 2. (a) A representative (out of three experiments) radiochromatograph TLC separation of the labeled GnRH and the N-terminal degradation products that were generated, is shown. The arrows indicate the position of the different synthetic GnRH fragment markers. (b) The percentage of GnRH remaining; and (c) the appearance of the degradation products, as quantitated by density measurements of the labeled GnRH and of the labeled degradation products on the TLC autoradiogram (Computing Densitometer, Model 30, Molecular Dynamics).

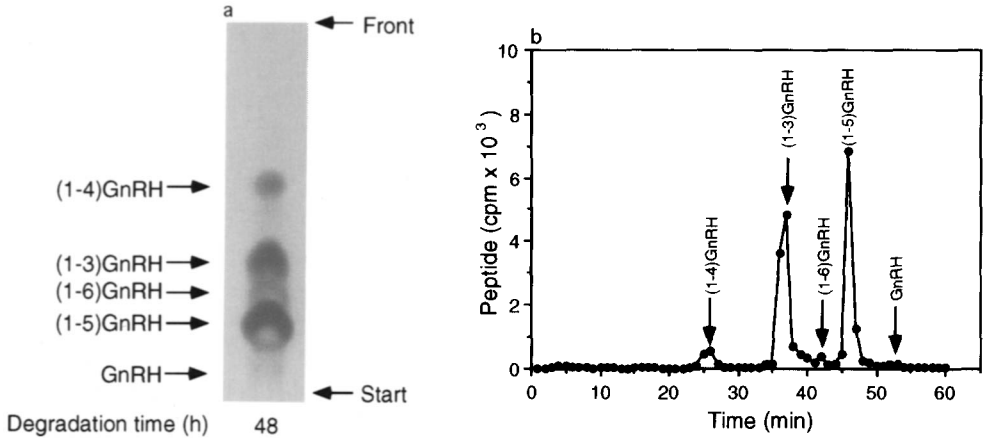


Fig. 2. Identification of GnRH fragments generated following degradation of the neurohormone by purified proteasomes. (a) TLC separation. Aliquots of purified proteasomes isolated from the POGS5 cell line (15 μg protein) were incubated with 1.2 pmol [^{3}H]GnRH for 48 h at 37°C. The incubation was terminated by heating the samples and the degradation products were separated by reversed phase RP-18 TLC, as described in section 2. The arrows indicate the positions of synthetic markers. This experiment was repeated three times. (b) HPLC separation. Following the degradation reaction, GnRH products were fractionated on a RP-C_{18} HPLC column and co-eluted with synthetic GnRH markers using a 60 min linear gradient of 10–60% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The absorbance at 280 nm was monitored. The radioactivity of each fraction (0.5 ml) was determined in a liquid scintillation spectrometer. The degradation products were identified by comparing their retention time to that of synthetic GnRH markers. The arrows indicate the retention time of the different markers.

(1–3)GnRH, was visible. Following 16 h of incubation, a pronounced rise in the quantity of (1–3)GnRH was evident and a fourth degradation product, the N-terminal pGlu-His-Trp-Ser, (1–4)GnRH, appeared. At 24 h of incubation there was a further increase in the formation of (1–3)GnRH product, concomitant with a decrease of the (1–5)GnRH degradation product (Fig. 1a). An inverse relationship between the appearance of GnRH degradation products and that of the intact GnRH was observed, namely the latter decreased sharply whereas the former increased as a function of time (Fig. 1b and c). To confirm the identity of the degradation products separated by the TLC method and visualized by autoradiography, we used high-pressure liquid chromatography (HPLC), which could demonstrate co-elution of the appropriate synthetic GnRH markers with the degradation products generated by the proteasomes (Fig. 2). The ratio between the different products fractionated by HPLC was identical to that quantified by the densiometric analysis of TLC autoradiography.

The results obtained indicate that the granulosa cell proteasomes can cleave peptide bonds between amino acids, Trp^3–Ser^4, Ser^4–Tyr^5, Tyr^5–Gly^6 and Gly^6–Leu^7 (Fig. 3). While the enzymatic degradation of GnRH by a multicatalytic proteinase complex from pituitary origin was briefly referred [35], this is the first detailed report on the proteolysis of the neurohormone GnRH by 20 S proteasomes. The kinetics of the generation of GnRH products suggests that the decapeptide is initially degraded to yield a pentapeptide and a hexapeptide which then become available to other catalytic sites within the granulosa proteasome complex (Fig. 3). Possibly the size of the polypeptide substrate is critical for its accommodation and cleavage at the different catalytic sites which are localized on distinct subunits of the 20 S proteasomes. Thus, the Trp^3–Ser^4 peptide bond may become available to the appropriate catalytic site(s) only after a smaller substrate (i.e. (1–5) or (1–6)GnRH) is generated. Indeed, the amount of these fragments was further increased after 24 h of degradation, although GnRH was already not available after 16 h of reaction (Fig. 1a and b). This is in line with the proposition that proteasomes possess a 'ruler' which determines the lengths of the degradation products [36]. This could explain the preferential appearance of the N-terminal (1–5) or (1–6)GnRH as an early event of GnRH proteolysis and the delayed appearance of the N-terminal (1–3) and (1–4)GnRH. The data are also in line with the suggestion that human erythrocyte proteasomes can degrade polypeptides progressively by channeling intermediates from one catalytic site to another as was concluded from experiments using oxidized insulin B chain as a substrate [37].

Proteasomes from pituitary [35] and from ovarian

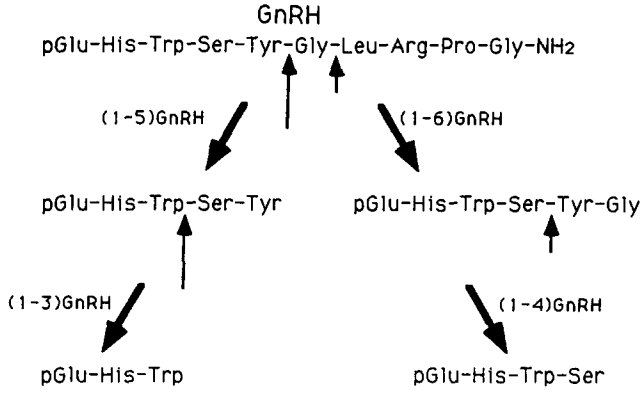


Fig. 3. The proposed pathways of GnRH degradation by purified proteasomes.

granulosa cells [30] were recently isolated and their proteolytic activity on synthetic polypeptides was examined. Both the pituitary gonadotropes and rat granulosa cells express receptors to GnRH, which, upon binding of the peptide regulate pituitary–gonadal function. It was already demonstrated that proteases of the anterior pituitary can efficiently degrade GnRH [27]. Since GnRH is internalized subsequent to the interaction with its specific receptor located on the cell membrane [28], it is tempting to suggest that following cell entry the 20 S proteasome can degrade GnRH and thus terminate its activity. It was clearly demonstrated that prolonged action of GnRH leads to desensitization of the pituitary gland, resulting in severe interference with gonadal function [15]. Continuous administration of high concentrations of GnRH to female rats has been recently shown to lead to programmed cell death in ovarian follicles [38]. Since proteasomes were found in all mammalian cells examined so far, it is possible that the 20 S proteasomes can serve as an efficient, general cellular tool for clearance of small natural and other bioactive peptides such as GnRH, neurotensin, etc., after their interaction with their specific receptor, and subsequent internalization. Undesirable, often pathological, consequences of overactivation by these molecules can thus be controlled.

Acknowledgements: We thank Mrs. Malka Kopelowitz for excellent secretarial assistance. The work was supported by a grant from the Minna and James Heinemann Foundation. Y.K. is the incumbent of the Adlai E. Stevenson III, Professorial Chair of Endocrinology and Reproductive Biology, M.F. is the incumbent of the Lester B. Pearson Professorial Chair of Protein Research and A.A. is the incumbent of the Joyce and Ben B. Eisenberg Professorial Chair of Molecular Endocrinology and Cancer Research.

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