Mechanism of LH Release in Cultured Rat Pituitary Cells

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Abstract

To investigate the mechanisms of the synthesis and the release of gonadotropin, rat anterior pituitary cells were stimulated *in vitro* with luteinizing hormone releasing hormone (LH-RH), [D-Ser(tBu)]⁶ des-Gly-NH₂¹⁰ ethylamide (Buserelin) and 12-O-tetradecanoyl phorbol-13-acetate (TPA), and then the LH and LH- β subunit released into the medium were determined by radioimmunoassay. Buserelin showed its biological activity at a much lower concentration than LH-RH, but both of them caused the release of LH and LH- β subunit in a dose-dependent manner. Furthermore, intracellular LH synthesis from LH- β subunit by stimulation with LH-RH or Buserelin was also found. After inducing [various] degrees of desensitization by stimulation with LH-RH or Buserelin in a dose-dependent manner (the first stimulation), pituitary cells were stimulated with a fixed dose of TPA (the second stimulation) and the released LH was assayed. LH was released almost constantly by the second stimulation, regardless of the dose used for the first stimulation. These results suggest that the C-kinase pathway was unaffected by the desensitization induced with LH-RH or Buserelin.

Numerous studies have been performed concerning the mechanism of release of gonadotropins from pituitary cells in response to stimulation with luteinizing hormone releasing hormone (LH-RH). Nevertheless, many points still remain to be elucidated. The present study was designed to investigate the mechanism of the synthesis and the release of LH and the desensitization phenomenon in rat anterior pituitary cells by using LH-RH

and its agonist, [D-ser(tBu)]⁶ des-Gly-NH₂¹⁰ ethylamide (Buserelin). It is well-known that luteinizing hormone (LH) consists of two subunits, i.e., an α -subunit and a β -subunit. Especially the β -subunit possesses the biological activity of LH⁽⁸⁾. To examine whether the LH- β subunit can be converted into LH by exogenous stimulation, the pituitary cells were stimulated with LH-RH or Buserelin and the intracellular and the extracellular LH and LH- β subunit concentrations were determined. Many investigators have described the phenomenon of desensitization which occurs when pituitary cells are continuously stimulated with a secretagogue and fail to respond

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by releasing LH⁵⁾. However, the mechanism of LH desensitization has not been sufficiently elucidated, although down-regulation of LH-RH receptors has been suggested as one of the causes of desensitization⁶⁾. In this study, the influence of desensitization induced by LH-RH on the C-kinase pathway was investigated by using 12-O-tetradecanoyl phorbol-13-acetate (TPA) which activates C-kinase without mediation by LH-RH receptors⁴⁾.

Materials and Methods

Cell dispersion and Culture

Pituitary cell dispersion was performed by a modification of the method of Vale *et al.*¹⁰⁾. Sprague-Dawley rats weighing about 200–250 g were killed by decapitation at a low temperature using dry ice, and only the anterior lobes of the pituitary were removed for culture. The whole pituitary was collected into sterile 25 mM HEPES-buffered Medium 199 (Gibco) containing 0.3% BSA (fraction V, Sigma) and minced into 0.5-mm³ cubes. After washing the pituitary pieces twice with Medium 199/BSA, a prepared solution of 25 mg of Trypsin (Sigma)/5 ml of Medium 199 was added, and the suspension was incubated for 15 minutes at 37°C. The pieces were reincubated with 10 µg of deoxyribonuclease (Sigma, type 1)/5 ml of Medium 199 for 1 minute, and the reaction was terminated by incubation with 10 mg of trypsin inhibitor (Sigma)/5 ml of Medium 199. After chelation by treating with 2 mM EDTA for 5 minutes and 1 mM EDTA for 15 minutes and decantation, the pieces were suspended in Dulbecco's PBS which was adjusted to pH 7.3 and contained 0.3% BSA and 50 U/ml penicillin and 50 µg/ml streptomycin without Ca²⁺ and Mg²⁺. The pieces were then thoroughly dispersed into cells by inverting the test tube gently several times and pipetting. The dispersed pituitary cells were centrifuged at 470 × g for 5 minutes and resuspended in culture medium (Medium 199 adjusted to pH 7.3, containing 25 mM Hepes, 10% horse serum, 50 U/ml penicillin and 50 µg/ml streptomycin). Finally the cells were dispensed into the wells of a multiwell culture plate (Corning) at a density of 2 × 10⁵ cells per

dish and preincubated for 72 hours at 37°C under atmosphere of 5% CO₂ in air.

LH and LH-β subunit release in response to LH-RH and Buserelin

After the preincubation, the pituitary cells were washed twice with fresh culture medium and stimulated with 10⁻¹¹ M–10⁻⁷ M of either LH-RH or Buserelin for 3 hours. The amount of LH and LH-β subunit released into the medium and contained in the cell was determined by radioimmunoassay. After the preincubation and changing of the culture medium, other pituitary cells were incubated with either 10⁻⁸ M LH-RH or 10⁻⁸ M Buserelin for 0, 7, 5, 30, 60, 120, 180 and 240 minutes for a time-course study and the LH released into the medium was assayed.

Desensitization induced by LH-RH and Buserelin

Pituitary cells were incubated with 10⁻¹¹ M–10⁻⁷ M of LH-RH for 3 hours (the first stimulation) after the preincubation, and the released LH was assayed. After washing with the fresh medium, these stimulated cells were incubated again with 10⁻⁷ M of LH-RH or 10⁻⁷ M of TPA for 3 hours (the second stimulation), and an aliquot of each medium was kept for determination of LH. In the stimulation with Buserelin substituted for LH-RH, the same procedure was adopted. Preincubated pituitary cells were exposed to the first stimulation with 10⁻¹¹ M–10⁻⁷ M of Buserelin and then the second stimulation with 10⁻⁷ M of Buserelin or 10⁻⁷ M of TPA; each aliquot of medium was kept for LH quantitation.

Assay of LH and LH-β subunit

For the assay of the cell content of LH and LH-β subunit, the pituitary cells were washed twice with fresh medium, and complete cell lysis induced by adding 0.1 N HCl and subsequently neutralized with 0.1 N NaOH. Aliquots of the solution containing efflux of the cell content were kept for the determination of LH and LH-β subunit. For the determination of the LH and the LH-β subunit concentration, radioimmunoassay by the double antibody method was employed. The antiserum and standard hormone reagents were kindly provided by the Pituitary Hormone Distribution Program, NIADDKD (National Institute of Arthritis,

Diabetes and Digestive and Kidney Diseases), U.S.A. The standards used in this study were NIAMDD-rLH-RP2 for rat LH and AFP-A805 for LH- β subunit.

Statistical analysis

Each sample was tested in triplicate and the data are expressed as the means showing the standard error (n=3). Student's *t*-test was employed for statistical analysis and the significant difference was determined at the level of $P < 0.05$.

Results

LH release in response to LH-RH and Buserelin

As shown in Fig. 1, LH was released by LH-RH in a dose-dependent manner, and its increase was significant from 10^{-10} M of LH-RH compared with the control ($P < 0.01$). As LH released into the medium

increased, the cell content of LH showed a tendency to decline gradually, and it was significantly decreased at 10^{-7} M of LH-RH compared with the control ($P < 0.01$). The total amount of intracellular and released LH began to increase at an LH-RH concentration of 10^{-9} M, and showed a 46% increase at 10^{-8} M compared with the control. As shown in Fig. 2, similar results were obtained by stimulating with Buserelin. LH was released in a dose-dependent manner by Buserelin, but the cell content of LH at 10^{-11} M of Buserelin was significantly lower than in the control ($P < 0.01$). In contrast, the total LH was 19.2% higher than the control. Since 10^{-11} M of Buserelin released 80% of the maximum concentration of LH and 10^{-10} M of Buserelin released 91% of it, Buserelin showed the characteristic of being able to express biological activity at a lower concentration than LH-RH. Fig.

Fig. 1. LH release and cell content of LH in response to LH-RH after 3 hours of incubation.

Fig. 2. LH release and cell content of LH in response to Buserelin after 3 hours of incubation.

Fig. 3. Time-courses of LH release in response to LH-RH and Buserelin.

Fig. 4. Release and cell content of LH- β subunits in response to LH-RH after 3 hours of incubation.

3 shows that LH-RH and Buserelin had similar time-courses activity. The released LH increased along with the incubation time and reached a plateau after 180 minutes with both secretagogues. No significant differences were found in their time-course patterns except for a slightly larger increase in LH release by Buserelin than by LH-RH at 240 minutes ($P < 0.05$).

LH- β subunit release in response to LH-RH and Buserelin

As shown in Fig. 4 the pattern of release of the LH- β subunit in response to LH-RH was similar to the pattern of LH release. This dose-response pattern showed a significant increase at 10^{-9} M of LH-RH compared with the control ($P < 0.05$). Like the release of LH, as the release of the LH- β subunit increased, its cell content showed a tendency to decline and significantly decreased by 31.4% at 10^{-7} M of LH-RH compared with the control. However, it is interesting that the total amount of LH was significantly increased, whereas the total amount of the LH- β subunit was significantly decreased, showing a 15% decrease at 10^{-7} M compared with the control.

With the stimulation by Buserelin, the LH- β subunit was also released in a dose-

dependent manner, showing a significant increase at 10^{-11} M and reaching a plateau at 10^{-9} M (Fig. 5). When the LH- β subunit was released, its cell content declined, showing a 66.7% decrease at 10^{-7} M compared with the control. The total (i.e. released plus intracellular) amount of LH- β subunit showed a 37.8% decrease at 10^{-7} M compared with the control.

Desensitization induced by LH-RH and Buserelin

When pituitary cells were exposed to the first stimulation with LH-RH and to the subsequent second stimulation with the same secretagogue, the amount of LH released by the second stimulation was decreased compared to the control in inverse proportion to the dose used in the first LH-RH stimulation ($P < 0.01$), as demonstrated in Fig. 6. However, when the cells were stimulated with a fixed dose of TPA as the second stimulant, the amount of LH released almost unchanged. LH released by the

Fig. 5. Release and cell content of LH- β subunits in response to Buserelin after 3 hours of incubation.

Fig. 6. Effect of LH-RH or TPA on subsequent LH release in pituitary cells desensitized with LH-RH.

Fig. 7. Effect of Buserelin or TPA on subsequent LH release in pituitary cells desensitized with Buserelin.

second stimulation with Buserelin from pituitary cells previously stimulated with 10^{-11} M Buserelin was significantly decreased compared with the control ($P < 0.01$; Fig. 7). However, when pituitary cells were stimulated secondarily with the fixed dose of TPA, LH release remained unchanged, as in the LH-RH-stimulation experiment.

Discussion

It was previously reported that Buserelin had 30–60 times the biological activity of native LH-RH due to its resistance to metabolism in pituitary cytosol and its high affinity for the LH-RH receptors²³. In this study Buserelin showed its biological activity from a much lower concentration than LH-RH in a comparison of their dose-response curves, although the quantity of LH released during a certain period of incubation was almost the same as both secretagogues in

the same length of time.

As the LH release from pituitary cells by stimulating with LH-RH increased in a dose-dependent manner, the cell content of LH gradually decreased. If the released LH was derived only from intracellular LH which was already synthesized and new LH synthesis failed to occur in response to the exogenous LH-RH stimulation, the total amount of intra- and extracellular LH would not be changed. Our present study demonstrated that the total amount of LH increased by 46% over the control. A similar result was obtained by the stimulation with Buserelin. This suggests that following the stimulation with both LH-RH and Buserelin, LH synthesis in pituitary cells as well as its release occurred. A recent report described how amino-acids in pituitary cells could be converted into LH by exogenous LH-RH stimulation⁹. To prove our hypothesis that pituitary LH can probably be converted not only from free amino-acids

but also from LH-subunits, changes in the manner of the release and the cell content of LH- β subunits in response to LH-RH and Buserelin were investigated. Like LH, LH- β subunits were released in a dose-dependent manner by exogenous LH-RH, and the intracellular content of LH- β subunits decreased along with the LH release. However, the total amount of LH- β subunits was significantly decreased, although the total amount of LH was not decreased. This fact implies that LH- β subunits are released into the extracellular space without any modification, but some subunits can also be transformed into LH. Similar results were obtained by stimulating with Buserelin substituted for LH-RH. When pituitary cells were stimulated with various doses of LH-RH or Buserelin and then further stimulated with a fixed dose of the same secretagogues, since pituitary cells were desensitized by the first stimulation, LH release in response to the second stimulation was decreased. However LH released by the stimulation with a fixed dose of TPA as the second stimulant was almost constant. This implies that TPA activated C-kinase and released LH, regardless of the degree of desensitization induced by either LH-RH or Buserelin. LH release by LH-RH is mediated through the binding of LH-RH to LH-RH receptors on the cell surface and subsequent stimulation of the cyclic AMP pathway, Ca^{++} -calmodulin and C-kinase pathway, but LH release by TPA can be induced through direct stimulation of the C-kinase pathway without any binding to LH-RH receptors^{4), 7)}. Although details of the mechanism of desensitization have not yet been thoroughly elucidated, a decrease in the number of receptors i.e., down regulation, has been reported^{1), 3)}. Therefore, even when desensitization is induced by down regulation which is accompanied by stimulation with LH-RH or Buserelin, TPA can release LH without the participation of the LH-RH

receptor-mediated pathway. Although the mechanism of pituitary desensitization by LH-RH cannot be concluded to be only a down-regulation mechanism and some other changes can be induced in the post receptor mechanism, our present study demonstrated that the C-kinase pathway was preserved during this desensitization induced by LH-RH or Buserelin.

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