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STIMULATORY EFFECTS OF ESTROGEN ON GONADOTROPIN-RELEASING HORMONE-INDUCED PHOSPHOINOSITIDE TURNOVER IN GRANULOSA CELLS*

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Summary—Gonadotropin-releasing hormone (Gn-RH) stimulates phosphoinositide metabolism in granulosa cells by binding to its specific receptor, and suppresses gonadotropin-induced steroidogenesis. Incubation of immature rat granulosa cells with Gn-RH stimulated time-sequential [^{32}P]phosphate incorporation into phosphatidic acid (PA) and phosphatidylinositol (PI) in a dose-dependent manner; EC_{50} was at 10 nM. Concurrent exposure to estradiol-17β (E_2) (100 nM) and Gn-RH (1 μM) augmented ^{32}P-labeling of PI by 5-fold, while Gn-RH alone induced 3.5-fold increase in PI-labeling. In cells preincubated with E_2 for 48 h, Gn-RH provoked a 7-fold [^{32}P]phosphate incorporation into PI, suggesting the induction by E_2 of Gn-RH-responsible phosphoinositide turnover. E_2 alone provoked a low but significant increase in basal labeling rate of PA and PI. Progesterone failed to mimic the action of E_2. Essentially similar results were also obtained in mature rat granulosa cells. These results indicate that E_2 augments Gn-RH-stimulated phospholipid turnover in granulosa cells, and suggest that estrogens within the microenvironment of the ovary may exert a local autoregulatory effect on their own production pathway through accelerating Gn-RH action to attenuate steroidogenesis.

INTRODUCTION

It is well recognized that gonadotropin-releasing hormone (Gn-RH) and its analogues can exert direct effects on the ovary by binding to specific gonadal receptors [1, 2], in addition to its actions on the anterior pituitary gland [3]. The extrapituitary actions of Gn-RH are either stimulatory or inhibitory on gonadotropin(Gn)-stimulated ovarian functions. In immature granulosa cells, Gn-RH and its analogues are known to suppress steroidogenesis and cellular differentiation induced by follicle-stimulating hormone (FSH) [4–9]. Clear evidence has been obtained that the hormone induces membrane phosphoinositide breakdown into inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DG), functioning as a second messenger for Gn-RH action in the ovary [10]. The former product mobilizes intracellular Ca^{2+}, while the latter activates protein kinase C. It has recently been demonstrated that the anti-Gn action of Gn-RH on the granulosa cells is mediated by protein kinase C [11–13].

Estrogen has been reported to enhance Gn-stimulated ovarian estrogen biosynthesis [14, 15]. Estrogen production in granulosa cells seems to be controlled via a positive feedback mechanism by direct enhancement of aromatase activity induced by FSH. Estrogen within the ovary may serve as an end-product amplifier of its own production [14, 15]. In view of the estrogen effect to augment FSH-dependent function, it is of interest to study the effect of estrogen on Gn-RH as an anti-Gn factor in granulosa cells. In the present communication, the interaction of estrogen with Gn-RH was investigated on phospholipid metabolism in rat granulosa cells.

EXPERIMENTAL

Preparation of granulosa cells

Mature and immature granulosa cells were obtained from the ovaries of immature rats treated with pregnant mare serum gonadotropin (PMSG) [12] and diethylstilbestrol (DES) [16, 17], respectively. Sprague–Dawley female rats (20–25 days old) were injected s.c. with PMSG or DES and were killed 3 or 4 days later. Ovaries were removed, trimmed, and

*To avoid further delay, this paper has been published without the authors' corrections.
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placed into Ham's F-12 medium. The granulosa cells were harvested by puncturing the ovarian follicles with fine needles under the dissecting microscope. The ovaries were then squeezed gently in the medium and the cells released into the medium were recovered by centrifugation for 10 min at 200*g*. The cells were washed and suspended in Ham's F-12 medium supplemented with antibiotics. Aliquots of the cell suspension were incubated for 2 days at 37°C under an atmosphere of 5% CO_2 in air.

Determination of phosphoinositide turnover

Phosphoinositide metabolism was observed by formation of [^{3}H]inositol phosphates from [^{3}H]inositol-labeled cells, and by [^{32}P]phosphate incorporation into phosphoinositides [18]. The cells were prelabeled with [^{3}H]inositol (1 μCi/ml medium) for 2 days, a time which was sufficient to reach isotopically steady-state. The cells were washed and resuspended in balanced salt solution (BSS) (135 mM NaCl, 4.5 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4) (2×10^6 cells/ml), and then exposed to the experimental agents. Steroids were dissolved in dimethyl sulfoxide (Me_2SO); Me_2SO concentration in the cell cultures were $<0.1\%$. The incubations were terminated by adding 4 vol of chloroform/methanol/HCl (1:2:0.001, v/v/v), followed by 1 vol of chloroform and 1 vol of H_2O. The upper and lower phases were separated. [^{3}H]Inositol sugars, which were contained in the upper phase, were analyzed by anion exchange column chromatography as previously described [18]. To examine [^{32}P]phosphate incorporation into phosphoinositide, the cell suspension (2×10^6 cells/ml) in BSS was preincubated with [^{32}P]phosphate (20 μCi/ml) at 37°C for 1 h, and then desired supplements were added. The reaction was terminated as described above, followed by extraction of phospholipid from the lower phase. The phospholipids were analyzed by thin-layer chromatography, and the individual phospholipids were scraped and counted [18].

Cell viability was estimated by determining the percentage of trypan blue-excluding cells. Treatment with hormones tested did not preferentially affect granulosa cell viability, compared to control cells.

Statistics

Statistical analysis was performed by *t*-test. Differences were considered significant if $P < 0.01$.

Materials

[^{32}P]Phosphate (carrier free) and *myo*-[2-^{3}H]inositol (20 Ci/mmol) were obtained from Amersham. Steroids and Gn-RH were purchased from Sigma. Silica gel plates and anion exchange resin (AG1 X-2) were from Merck and Bio-Rad, respectively. All other chemicals were of reagent grade.

RESULTS

When immature granulosa cells were exposed to Gn-RH, [^{32}P]phosphate incorporations into

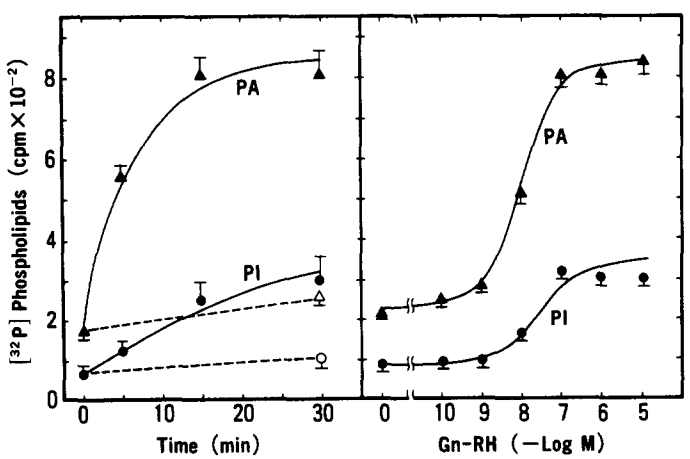

Fig. 1. Effects of Gn-RH on [^{32}P]phosphate incorporation into PI and PA in rat granulosa cells. *Left panel.* The rat immature granulosa cells (2×10^6 cells), preincubated with [^{32}P]phosphate (20 μCi/ml) for 1 h, were exposed to Gn-RH (1 μM) (●, ▲) or vehicle (○, △) for the indicated time intervals at 37°C. Each point represents the mean ± SD of two separate experiments performed in triplicate determinations. ▲, △ PA; ●, ○ PI. *Right panel.* The cells (2×10^6 cells), preincubated with [^{32}P]phosphate (20 μCi/ml) for 1 h, were exposed to various concentrations of Gn-RH for 30 min at 37°C. Each point represents the mean ± SD of two separate experiments performed in triplicate determinations. ▲ PA; ● PI.

phosphatidic acid (PA) and phosphatidylinositol (PI) were observed to be remarkably enhanced in a time-dependent and in a dose-dependent manner (Fig. 1). The PA-labeling seemed to reach maximal level by 30 min, while the rate of labeling in PI persisted for at least 30 min. EC_{50} for both lipid labelings was at 10 nM of Gn-RH. The stimulated PA and PI phosphorylations caused by Gn-RH occurred in parallel with stimulated IP_3 formation [17], suggesting stimulated phosphoinositide turnover in granulosa cells in response to Gn-RH.

As shown in Fig. 2 (left panel), where incubation of the cells with Gn-RH increased PI-labeling by 3.5-fold relative to the control, concurrent exposure with E_2 and Gn-RH augmented the ^{32}P-labeling of PI by 5-fold. E_2 alone caused a 1.5-fold increase in PI-labeling. When [^{3}H]inositol-labeled granulosa cells were incubated with Gn-RH alone or in the presence of E_2 for 10 min, IP_3 formation was increased by 5.0- or 7.3-fold relative to the control, respectively. E_2 alone had no significant effect on the IP_3 formation. At the concentrations used, the agents themselves did not affect cell viability. When the cells were preincubated with E_2 for 48 h, Gn-RH stimulated PI-labeling by 7-fold, as illustrated in the right panel of Fig. 2. In the cells pretreated with E_2, although the basal labeling rate of PI was observed to rise up to 2-fold, E_2 failed to show not only stimulatory action on PI-labeling but also synergistic action on Gn-RH-induced PI-labeling, perhaps resulting from down-regulation of E_2 receptor during preincubation. The E_2 alone-stimulated phosphoinositide metabolism may not be through the pathway associated with the classical action of E_2, as we suggested using human endometrial fibroblasts [19, 20]. Essentially identical results were obtained also in mature granulosa cells. Progesterone had no effects on basal or Gn-RH-stimulated phosphoinositide turnover.

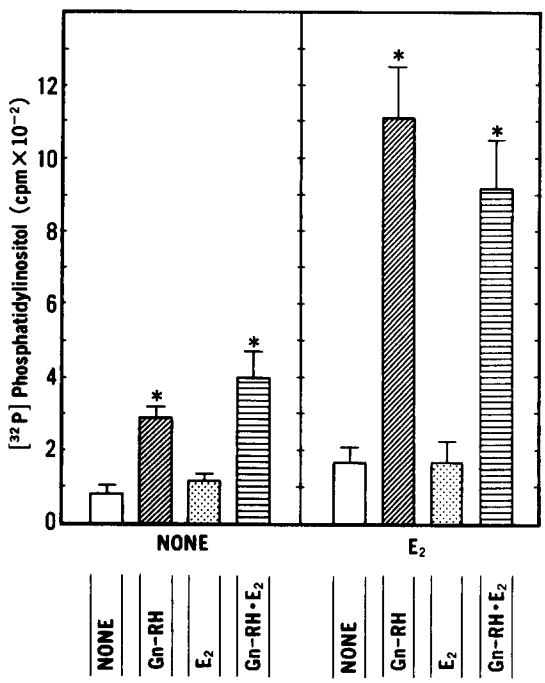

Fig. 2. Effects of E_2 on Gn-RH-stimulated [^{32}P]phosphate incorporation into PI. *Left panel.* The rat immature granulosa cells (2×10^6 cells), preincubated with [^{32}P]phosphate (20 μCi/ml) for 1 h, were exposed to Gn-RH (1 μM) and/or E_2 (100 nM) for 30 min at 37°C. The results represent the mean ± SD of two separate experiments performed in triplicate determinations; $^*P < 0.01$ vs none. *Right panel.* The rat immature granulosa cells (2×10^6 cells) were preincubated with E_2 (100 nM) for 48 h. The cells were then incubated with [^{32}P]phosphate (20 μCi/ml) for 1 h, and exposed to Gn-RH (1 μM) and/or E_2 for 30 min at 37°C. The results represent the mean ± SD of two separate experiments performed in triplicate determinations; $^*P < 0.01$ vs none.

DISCUSSION

In many cell types, activation of a variety of physiological responses by cell surface interacting stimuli is associated with a rapid disappearance and resynthesis of membrane phosphoinositide [21–23]. In these cells, a very rapid effect after occupancy of the receptor is enhanced by hydrolysis of PI 4,5-bis-phosphate, a minor polyphosphoinositide, by a phospholipase C to produce 1,2-DG and IP_3. It has been proposed that both 1,2-DG and IP_3 may serve as intracellular mediators (or second messengers) that transduce and amplify the initial signal, leading ultimately to stimulation of a physiological response, 1,2-DG appears to exert its effects by activating a Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) by a mechanism that does not depend on an elevation of cytoplasmic Ca^{2+} concentration. IP_3 appears to act by mobilizing Ca^{2+} from an intracellular pool(s) causing an elevation of cytoplasmic free Ca^{2+}.

In rat granulosa cells, Gn-RH and its analogues are known to bind to specific cell surface receptors and to suppress Gn-induced steroidogenesis [1–9]. The suppression by Gn-RH of FSH-stimulated steroidogenesis is tightly coupled to an increase in phosphoinositide metabolism during Gn-RH action [1, 2, 17]. The activation of protein kinase C as a result of phosphoinositide breakdown leads to the inhibition of Gn-RH-dependent steroidogenesis,

suggesting that protein kinase C could be involved in the inhibitory action of Gn-RH. In this communication, E_2, a product of FSH-stimulated steroidogenesis, was found to accelerate the phosphoinositide metabolism stimulated by Gn-RH. It is plausible that E_2 may serve as a product-attenuator of its own production through enhancement of the anti-Gn action of Gn-RH. The mechanism by which E_2 exerts its augmenting action on granulosa cell phosphoinositide metabolism is unknown so far. The site of action of E_2 may be distal to the Gn-RH receptor, judged from the synergistic effects of E_2 on the Gn-RH-dependent response including phosphoinositide metabolism. The findings that E_2 revealed its effect on the Gn-RH action even by acute incubation seems to be physiologically interesting, because the half-life of Gn-RH is extremely short *in vivo*.

In summary, E_2 activates intraovarian autoregulatory feedback mechanisms to control its own production by direct augmentation of aromatase activity induced by FSH in granulosa cells [14, 15]. Thus, estrogen production in granulosa cells is controlled via two independent antagonistic mechanisms: (1) direct enhancement of aromatase activity induced by FSH; and (2) enhancement of the anti-Gn action of Gn-RH through phosphoinositide turnover. E_2 within the microenvironment of the ovary may exert a local autoregulatory effect on its own production.

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REFERENCES

1. Clayton R. N., Harwood J. P. and Catt K. J.: Gonadotropin-releasing hormone analogue birds to luteal cells and inhibits progesterone production. *Nature* **282** (1979) 90–92.
2. Hsueh A. J. W. and Erickson G. F.: Extrapituitary action of gonadotropin-releasing hormone: direct inhibition of ovarian steroidogenesis. *Science* **204** (1981) 854–855.
3. Hsueh A. J. W. and Jones P. B. C.: Extrapituitary actions of gonadotropin-releasing hormone. *Endocr. Rev.* **2** (1981) 437–461.
4. Polan M. L., DeCherey A. H., Haseltine F. P., Mezer H. C. and Behman H. R.: Adenosine amplifies follicle-stimulating hormone action in granulosa cells and luteinizing hormone action in luteal cells of rat and human ovaries. *J. Clin. Endocr. Metab.* **56** (1983) 288–294.
5. Knecht M., Amsterdam A. and Catt K. J.: Inhibition of granulosa cell differentiation by gonadotropin-releasing hormone. *Endocrinology* **110** (1982) 865–872.
6. Darbon J. M., Knecht M., Ranta T., Dufan M. L. and Catt K. J.: Hormonal regulation of cyclic AMP-dependent protein kinase in cultured ovarian granulosa cells: effects of follicle-stimulating hormone and gonadotropin-releasing hormone. *J. Biol. Chem.* **259** (1984) 14,778–14,782.
7. Adashi E. Y., Resnick C. E., Svoboda M. E. and Van Wyk J. J.: A novel role for somatomedin C in the cytodifferentiation of ovarian granulosa cell. *Endocrinology* **115** (1984) 1227–1229.
8. Veldhuis J. D. and Kolp L. A.: Mechanisms subserving insulin's differentiating actions on progestin biosynthesis by ovarian cells: studies with cultured swine granulosa cells. *Endocrinology* **116** (1985) 651–659.
9. Tureck R. W., Mastroianni L., Jr., Blasco L. and Strauss J. F. III: Inhibition of human granulosa cell progesterone secretion by a gonadotropin-releasing hormone agonist. *J. Clin. Endocr. Metab.* **54** (1982) 1078–1080.
10. Leung P. C. K. and Wang J.: The role of inositol lipid metabolism in the ovary. *Biol. Reprod.* **40** (1989) 703–708.
11. Shinohara O., Knecht M. and Catt K. J.: Inhibition of gonadotropin-induced granulosa cell differentiation by activation of protein kinase C. *Proc. Natn. Acad. Sci. U.S.A.* **82** (1985) 8518–8522.
12. Wang J., Lee V. and Leung P. C. K.: Differential role of protein kinase C in the action of luteinizing hormone-releasing hormone on hormone production in rat ovarian cells. *Am. J. Obstet. Gynec.* **160** (1989) 984–989.
13. Veldhuis J. D. and Demers L. M.: An inhibitory role for protein kinase C pathway in ovarian steroidogenesis: studies with cultured swine granulosa cells. *Biochem. J.* **239** (1986) 505–511.
14. Adashi E. Y. and Hsueh A. J. W.: Estrogen augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. *J. Biol. Chem.* **257** (1982) 6077–6083.
15. Zhuang L. Z., Adashi E. Y. and Hsueh A. J. W.: Direct enhancement of gonadotropin-stimulated estrogen biosynthesis by estrogen and clomiphene citrate. *Endocrinology* **110** (1982) 2219–2221.
16. Erickson G. F. and Hsueh A. J. W.: Stimulation of aromatase activity by follicle-stimulating hormone in rat granulosa cells *in vivo* and *in vitro*. *Endocrinology* **102** (1978) 1275–1282.
17. Imai A., Iida K. and Tamaya T.: Direct action of gonadotropin-releasing hormone (LH-RH) analogue on ovary: an alternative acting mechanism of buserelin. *Archs Gynec. Obstet.* In press.
18. Imai A. and Gershengorn M. C.: Measurement of lipid turnover in response to TRH. *Meth. Enzym.* **141** (1987) 100–111.
19. Imai A., Iida K. and Tamaya T.: Phospholipase C in human endometrial fibroblasts and its regulation by estrogens. *Comp. Biochem. Physiol.* In press.
20. Iida K., Imai A. and Tamaya T.: Estradiol stimulation of inositolphospho-lipid metabolism in human endometrial fibroblasts. *Res. Commun. Chem. Path. Pharmac.* **66** (1989) 329–332.
21. Berridge M. J. and Irvine R. F.: Inositol phosphate and cell signalling. *Nature* **341** (1989) 197–209.
22. Sekar M. C. and Hokin L. E.: The role of phosphoinositides in signal transduction. *J. Membr. Biol.* **89** (1986) 193–210.
23. Majerus P. W., Connolly T. M., Deckmyn H., Ross T. S., Bross T. E., Ishii H., Bansal V. S. and Wison D. B.: The metabolism of phosphoinositide-derived messenger molecules. *Science* **234** (1986) 1519–1526.