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Gonadotropin-releasing hormone (GnRH) stimulates phosphatidylinositol metabolism in rat granulosa cells: Mechanism of action of GnRH*

(phospholipids / steroidogenesis / cyclic nucleotides)

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ABSTRACT **This report describes the effects of gonadotropin-releasing hormone (GnRH; gonadoliberin) and an agonist, [D-Ala^6,des-Gly^{10}]GnRH ethyl amide (GnRHa), on phospholipid metabolism in rat granulosa cells isolated from mature Graafian follicles. As indicated by the incorporation of $^{32}PO_4$, GnRHa rapidly (less than 2 min) stimulated the labeling of phosphatidic acid and phosphatidylinositol but had no effect on the labeling of other phospholipids. Increased phosphatidylinositol labeling was also observed when *myo*-[2-^{3}H]inositol was incubated with granulosa cells in the presence of GnRHa. Increases in labeling were dependent on the dose of GnRH and time of incubation. Thyrotropin-releasing hormone and a specific GnRH antagonist had no effect on labeling, but a GnRH antagonist prevented the stimulatory action of GnRH. In addition, treatment with GnRHa slightly increased the levels of phosphatidylinositol (15%) in 60-min incubations but had no effect on the levels of other phospholipids. Significant increases in progesterone accumulation were observed after 30 min of incubation with GnRHa, and further increases were correlated with the time of incubation. The stimulatory action of GnRH on phospholipid metabolism and progesterone accumulation was not related to increases in cyclic nucleotide accumulation. In incubations lasting up to 30 min, GnRHa had no effect on cAMP accumulation. However, a transient decrease in cGMP levels was observed in response to GnRHa. These studies suggest that the rapid and specific effects of GnRH on phospholipid metabolism in rat granulosa cells represent early events in the action of GnRH.**

Gonadotropin-releasing hormone (GnRH; gonadoliberin) and its agonistic analogs have been found to exert direct effects on ovarian follicular function (1–8). Many of these actions of GnRH are inhibitory [e.g., reduction of follicle-stimulating hormone-induced cyclic nucleotide accumulation (2, 3) and steroidogenesis (2–4) in cultured rat granulosa cells], whereas other actions are stimulatory [e.g., induction of oocyte maturation (9, 10) and ovulation (10, 11), stimulation of progestin (3, 5, 6), cyclic nucleotide (3), and prostaglandin (6, 7) accumulation, and stimulation of phosphodiesterase activity in granulosa cells (8)]. It has been suggested that the stage of follicular development determines whether GnRH has stimulatory or inhibitory effects (1). However, the mechanism by which GnRH or its agonists exerts these effects is unknown.

In preovulatory follicles, where GnRH increases progesterone and prostaglandin accumulation (6, 7) and stimulates follicular maturation and ovulation (9–11), acute inhibitory effects of GnRH have not been observed. The stimulatory effects are intriguing because they mimic the action of luteinizing hormone (LH; lutropin) on the preovulatory follicle, yet they occur by a mechanism distinct from LH (6, 7). While the actions of LH are predominantly mediated by increases in cellular cAMP, GnRH action in granulosa cells from mature Graafian follicles does not involve similar changes in cAMP levels (7). In the pituitary, increased phospholipid turnover has been proposed in the mechanism of GnRH-induced LH release (12, 13). The recent demonstration of GnRH receptors in the ovary (14), together with the association of phospholipid turnover with many forms of receptor-mediated cell activation (15, 16), prompted us to examine the involvement of phospholipid metabolism in the action of GnRH in ovarian granulosa cells. In this report we describe a rapid and marked effect of GnRH on phosphatidylinositol (PtdIns) metabolism in rat granulosa cells that may well represent an early event in the action of GnRH.

MATERIALS AND METHODS

Pregnant mare's serum gonadotropin (PMSG, Equinex) was obtained from Ayerst (New York). [D-Ala^6,des-Gly^{10}]GnRH ethyl amide (GnRHa), synthetic GnRH, and thyrotropin-releasing hormone (TRH; thyroliberin) were obtained from Beckman. The GnRH antagonist [Ac-Δ^3Pro^1,D-*p*-F-Phe^2,D-Trp^{3,6}]GnRH was donated by J. Rivier (The Salk Institute). [1,2,6,7-^{3}H]Progesterone and *myo*-[2-^{3}H]inositol (10 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) were purchased from New England Nuclear. $^{32}PO_4$ (carrier-free, 285 Ci/mg) was purchased from ICN. Phospholipids were obtained from Sigma.

Granulosa cells were isolated from immature rats 48 hr after a subcutaneous injection of 20 international units of PMSG as described previously (6), with a minor modification to facilitate cell dispersal (16). Incubations were performed in duplicate with approximately 4×10^6 viable cells, each incubation mixture in a final volume of 0.5 ml of medium 199 (GIBCO) containing 25 mM Hepes, 1% bovine serum albumin, and approximately 50 μCi of $^{32}PO_4$ or 10 μCi of *myo*-[2-^{3}H]inositol under an atmosphere of 95% O_2/5% CO_2 at 37°C. The incubations of samples for progesterone and phospholipid measurement were stopped

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Abbreviations: PtdIns, phosphatidylinositol; Ptd-OH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; GnRH, gonadotropin-releasing hormone (gonadoliberin); GnRHa, [D-Ala^6,des-Gly^{10}]GnRH ethyl amide (GnRH agonist); LH, luteinizing hormone (lutropin); PMSG, pregnant mare's serum gonadotropin; TRH, thyrotropin-releasing hormone (thyroliberin).

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by freezing cells and medium at −100°C. Those for cyclic nucleotide measurements were terminated by the addition of 0.5 ml of 100 mM sodium acetate, pH 4.0, followed by immersion in a water bath at 100°C for 10 min. Further experimental details are given in the text and figure legends.

Progesterone was extracted from 0.05-ml aliquots of incubated cells and media and total progesterone was measured by radioimmunoassay (18). The remaining cells and incubation media were extracted with 10 vol of $CHCl_3/CH_3OH$, 2:1 (vol/vol), as described (18). Individual classes of phospholipids were separated on Supelco thin-layer plates containing 5% magnesium acetate in $CHCl_3/CH_3OH/4.3$ M NH_4OH, 90:65:20 (vol/vol) (18–21), or on Whatman LK5D plates in $CHCl_3/CH_3CO_2C_2H_5/CH_3CH_2CH_2OH/CH_3OH/0.25\%$ KCl, 25:25:25:13:9 (vol/vol) (22). Phospholipids were localized by iodine staining, charring, or radioscanning (18–21). Cellular phospholipid concentrations were quantified in terms of phospholipid phosphorus (19) and phospholipid radioactivity was determined by scintillation counting (18).

The acidified and heat-treated samples for cyclic nucleotide measurement were centrifuged at 1,000 × *g* for 10 min to sediment precipitated protein, and the supernatants were removed for inclusion in assays for cAMP and cGMP. cAMP was determined by competitive protein binding as described (7). cGMP was measured by radioimmunoassay (23) after acetylation (24), employing reagents obtained from New England Nuclear.

Data are presented as individual values and averages for duplicate values in each experiment or as the cumulative mean ± SEM of the experimental averages. When appropriate, statistical analyses were carried out by analysis of variance or applying Student's *t* test to paired comparisons. Comparisons with $P < 0.05$ were considered to indicate a significant difference.

RESULTS

Treatment of granulosa cells with GnRHa for 15 min increased the incorporation of $^{32}PO_4$ into phosphatidic acid (Ptd-OH) 65% and into PtdIns 201% ($P < 0.05$; Fig. 1). However, treatment with GnRHa did not have a significant effect at 15 min on ^{32}P labeling of total phospholipids or on the labeling of the phosphatidylcholine and phosphatidylethanolamine fraction (PtdCho/PtdEtn), which contained greater than 80% of the total radioactivity. Incubation with GnRHa for an additional 60 min (Fig. 1) resulted in an increase in the labeling of total ^{32}P-labeled phospholipids (20% increase over control, $P < 0.05$). These increases were due to enhanced labeling only in specific phospholipids, Ptd-OH (122%, $P < 0.05$) and PtdIns (308%, $P < 0.05$). GnRHa also stimulated progesterone accumulation at 75 min of incubation from a control of 1.5 ± 0.3 to 3.4 ± 0.8 ng of progesterone per 4 × 10^6 cells (mean ± SEM, $n = 3$).

The concentration dependence of the GnRHa-induced stimulation of phospholipid labeling was determined (Fig. 2). In 1-hr incubations GnRHa at concentrations as low as 0.1 ng/ml stimulated PtdIns labeling. Similar to previous results on prostaglandin (7) and progesterone (6) accumulation, GnRHa at 10–100 ng/ml provided maximal stimulatory effects.

Treatment of granulosa cells with synthetic GnRH (100 ng/ml) for 60 min resulted in phospholipid labeling patterns similar to those observed with GnRHa (Fig. 3). Major increases due to GnRH were also observed only in the Ptd-OH and PtdIns fractions (note logarithmic axis). The stimulation of PtdIns labeling appeared to be specific for GnRH and GnRHa, because a GnRH antagonist had no effect on this parameter (Table 1). On the other hand, the GnRH antagonist eliminated the stimulation of PtdIns labeling by GnRH. Similarily, TRH did not exert any stimulatory effects on PtdIns labeling or progesterone accumulation (Fig. 4).

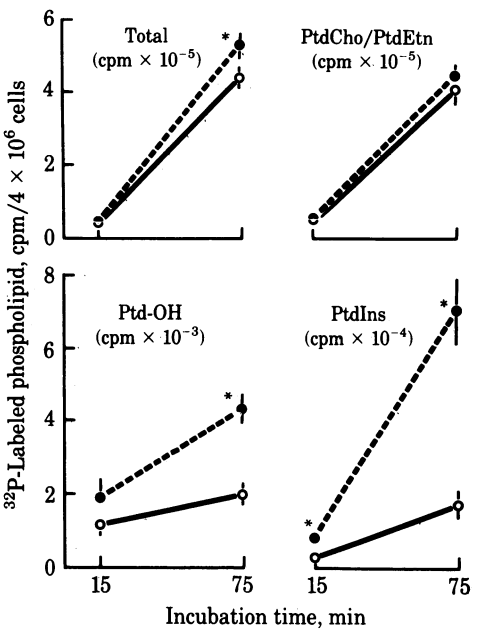

FIG. 1. Effect of GnRHa on phospholipid labeling by rat granulosa cells. Isolated granulosa cells were incubated in medium containing $^{32}PO_4$ for 15 and 75 min in the absence (○) or presence (●) of GnRHa at 100 ng/ml. Extracted phospholipids were separated by thin-layer chromatography. Data are expressed as the mean ± SEM of three experiments each performed in duplicate. Ptd-OH, phosphatidic acid; PtdCho/PtdEtn, combined phosphatidylcholine and phosphatidylethanolamine zone; total, total ^{32}P-labeled phospholipids.
*$P < 0.05$ vs. control.

To gain further insight into the changes in PtdIns metabolism, the effect of GnRHa on the incorporation of *myo*-[2-^{3}H]-inositol into PtdIns was examined. Fig. 5 depicts the time course for inositol labeling of PtdIns and progesterone accumulation in response to GnRHa at 100 ng/ml. Similar to the effect of GnRHa on ^{32}P labeling of PtdIns, marked increases in incorporation of label from inositol into PtdIns were observed (75% over control after 15 min of incubation). Further increases in labeling were observed in incubations lasting up to 120 min. GnRHa also increased progesterone accumulation in a time-dependent manner (Fig. 5 *Inset*). In addition, the effect of GnRHa on [^{3}H]inositol incorporation was maximal at 10 ng/ml (data not shown).

Our initial experiments demonstrated that the stimulation of phospholipid labeling by GnRHa was already evident within 15 min (Figs. 1 and 5). In other tissues, similar alterations in phospholipid metabolism have been observed within the first few

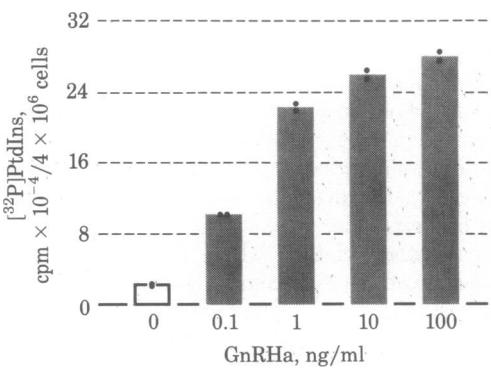

FIG. 2. Stimulation of ^{32}P incorporation into PtdIns in rat granulosa cells by increasing concentrations of GnRHa. Isolated granulosa cells were incubated for 60 min in medium containing $^{32}PO_4$ with GnRHa (0–100 ng/ml). The individual values (•) and averages (bars) of duplicate incubations from one experiment are shown.

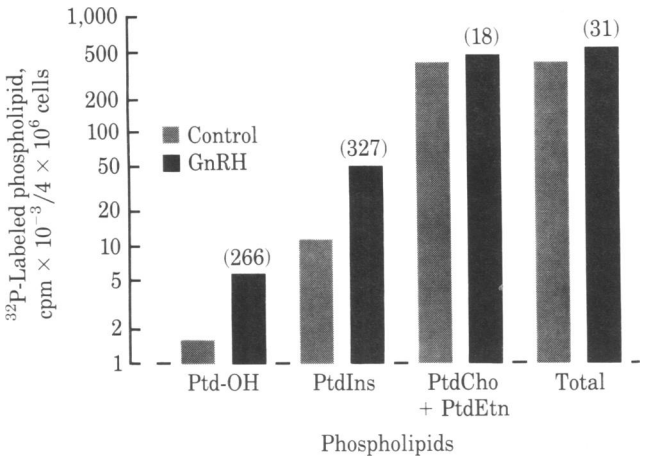

FIG. 3. Effect of GnRH on phospholipid labeling by rat granulosa cells. Isolated granulosa cells were incubated for 60 min in the absence (control) or presence of GnRH (100 ng/ml). Data are means from duplicate incubations. Individual values varied ≤ 5% from the mean. The numbers in parentheses represent the percent increase in ^{32}P incorporation due to GnRH (note logarithmic axis).

minutes after ligand receptor binding (15, 16). Therefore, we conducted an experiment to more clearly demonstrate the early onset of GnRH action. After a 30-min prelabeling period, incubations of granulosa cells were stopped for determination of zero-time phospholipid labeling or were continued in the absence or presence of GnRH (100 ng/ml). Using this protocol (Fig. 6), we observed that the incorporation of $^{32}PO_4$ into Ptd-OH was increased over 2-fold after 2 min of incubation with GnRH [4,540 (4,310–4,770) cpm/4 × 10^6 cells (mean and range) for GnRH compared to 1,600 (1,570–1,630) for control incubations]. Labeling of PtdIns was also increased (15%) after 2 min. In samples treated with GnRH for 10 min, both Ptd-OH and PtdIns labeling were increased 3-fold. Except for a slight decrease after 2 min of incubation, PtdEtn and PtdCho labeling were not altered by treatment with GnRH.

In many tissues, the stimulation of PtdIns labeling occurs as a result of PtdIns hydrolysis to form diacylglycerol followed by rephosphorylation of diacylglycerol by diglyceride kinase to form Ptd-OH and subsequent resynthesis of PtdIns (15, 16). Alternatively, the formation of diacylglycerol from the breakdown of other lipids or the enhancement of PtdIns synthesis *de novo* may result in enhanced PtdIns labeling. Experiments were performed, therefore, to examine the effects of GnRH on phospholipid levels in granulosa cells. Measurements of phospholipid phosphorus in three separate experiments revealed that GnRH slightly increased net PtdIns levels (15%, $P < 0.05$) but had no significant effects on other phospholipids (Table 2).

Table 1. Effect of GnRH and GnRH antagonist on ^{32}P incorporation into phospholipids by rat granulosa cells

Treatment*	^{32}P incorporation, cpm × 10^{-3}/4 × 10^6 cells† PtdIns		PtdCho	
Control	11.6 9.2	(100)	308 247	(100)
GnRH antagonist	9.6 9.2	(90)	269 263	(96)
GnRH	53.2 57.6	(533)	338 331	(120)
GnRH + GnRH antagonist	10.9 10.1	(101)	285 297	(105)

*Granulosa cells were incubated with GnRH (100 ng/ml) and [Ac-Δ^3Pro^1,D-*p*-F-Phe^2,D-Trp^{3,6}]GnRH (GnRH antagonist, 100 ng/ml) alone or in combination for 60 min.

†Phospholipids were separated by thin-layer chromatography. Individual values from duplicate samples are shown. Average values for ^{32}P incorporation (numbers in parentheses) are expressed as a percentage of the average control values.

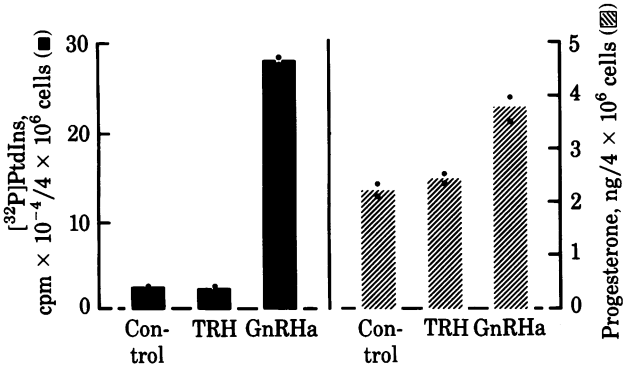

FIG. 4. Specificity of ^{32}P incorporation into PtdIns and progesterone synthesis by rat granulosa cells. Isolated granulosa cells were incubated with TRH (100 ng/ml) or GnRHa (100 ng/ml) for 60 min. The individual values (•) and averages (bars) of duplicate incubations from one experiment are shown.

Activation of receptors that elicit a PtdIns response in other tissues has also been associated with an increase in cGMP but not cAMP (16, 25). In the pituitary, GnRH induces a PtdIns response (13) and increases cGMP (26). Therefore, experiments were performed to assess the effect of GnRHa on cyclic nucleotide accumulation in granulosa cells. Instead of increasing the concentration of cGMP, GnRHa decreased it in incubations lasting 3 min: 2.0 ± 0.1 vs. 1.3 ± 0.1 pmol of cGMP per 2 × 10^6 cells ($n = 5$, $P < 0.05$). In a time course study of the effect of GnRHa, cGMP levels were lowest at 3 min and increased toward control levels throughout 30 min of incubation (Fig. 7). GnRHa had no effect on cAMP accumulation at any time studied (Fig. 7). These results on cAMP accumulation are consistent with previous results demonstrating a lack of effect of GnRHa (10–2,000 ng/ml) on cAMP levels in incubations of granulosa cells lasting between 1.5 min and 5 hr (7). GnRHa significantly increased progesterone accumulation after 30 min of incuba-

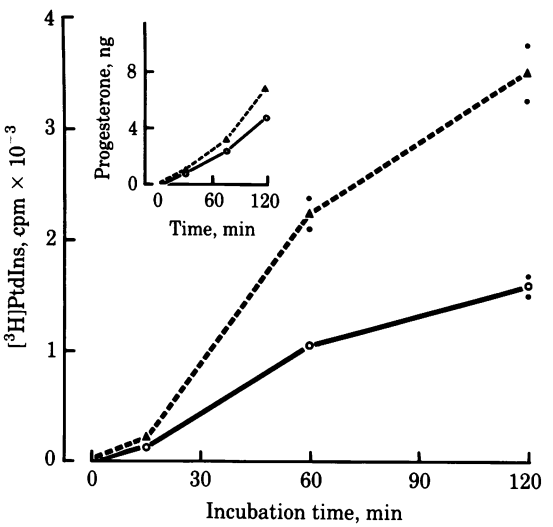

FIG. 5. Temporal profile of *myo*-[2-^{3}H]inositol incorporation into PtdIns and (*Inset*) progesterone accumulation in response to GnRHa (100 ng/ml). Isolated granulosa cells (4 × 10^6 cells) were incubated in medium containing *myo*-[2-^{3}H]inositol in the absence (○) or presence (▲) of GnRHa at 100 ng/ml. The individual values (•) and averages (○ and ▲) of duplicate samples from one experiment are shown.

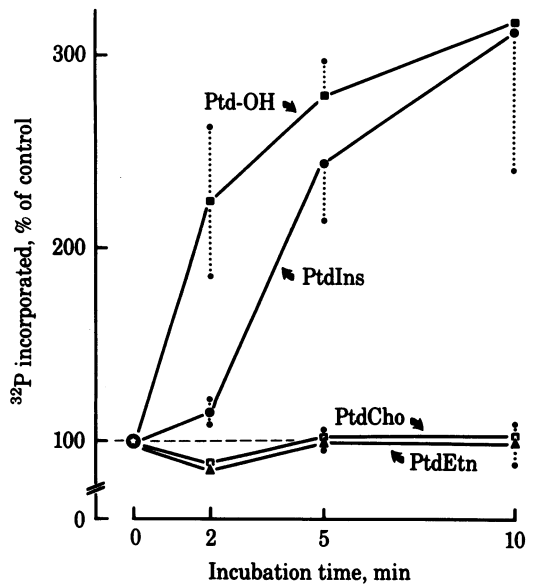

FIG. 6. Temporal profile of ^{32}P incorporation into phospholipids by rat granulosa cells. Isolated granulosa cells were incubated in medium containing $^{32}PO_4$ for 30 min and were further incubated for various periods of time in the absence or presence of GnRHa (100 ng/ml). Ptd-OH (■), PtdIns (●), PtdCho (□), and PtdEtn (△) were separated by thin-layer chromatography. Phospholipid labeling due to GnRH is represented as percent of control labeling. The range of duplicate determinations is indicated by dotted vertical lines or was obscured by the symbol.

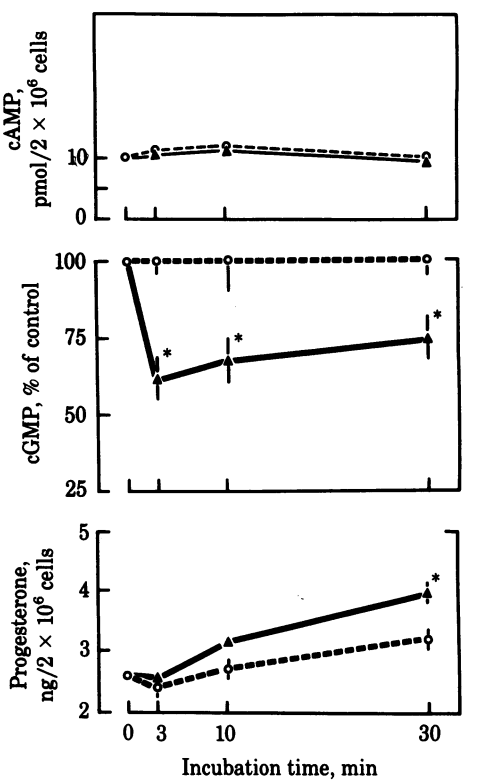

FIG. 7. Effects of GnRHa on cyclic nucleotide and progesterone accumulation by rat granulosa cells. Isolated granulosa cells (2 × 10^6) were incubated for 15 min and were further incubated for up to 30 min in the absence (○) or presence (▲) of GnRHa (100 ng/ml). Results are expressed as the mean ± SEM, $n = 5$.
*$P < 0.05$ vs. control.

tion, although slight increases were observed at earlier times (Fig. 7). Thus, no increases in cyclic nucleotide levels were observed in conjunction with the GnRH-induced increases in phospholipid metabolism and progesterone accumulation.

DISCUSSION

Granulosa cells respond to GnRH and GnRHa by rapidly increasing incorporation of $^{32}PO_4$ into Ptd-OH and PtdIns while the labeling of other phospholipids is unaffected. This effect on phospholipid labeling may reflect an early and possibly initiating action of GnRH in granulosa cells. This event appeared to be mediated by the characterized GnRH receptor on rat granulosa cells, because it exhibited similar specificity and a good correlation between the concentrations required for biological activity and receptor binding (1, 14). The increased phospholipid labeling occurred more rapidly than any other identified action of GnRH in the ovary except receptor binding. This event was quite prominent within 2 min, whereas the other known effects of GnRH on the ovary require hours to days to express themselves (1–8). Similar alterations in phospholipid metabolism have been shown to occur as rapidly in other tissues (15, 16, 25). GnRHa also decreased cGMP (3 min), although it is not clear if this effect is subsequent to or unrelated to changes in phospholipid metabolism. These observations indicate that changes in phospholipid metabolism precede most other actions of the decapeptide and suggest that this event is very proximal to receptor occupancy and could represent a second step in GnRH action.

Table 2. Effect of GnRHa on phospholipid synthesis by rat granulosa cells

	Phospholipid phosphorus,† ng/10^7 cells		
Treatment*	Ptd-OH	PtdIns	PtdCho + PtdEtn + PtdGro
Control	26 ± 5.1	305 ± 70	2,370 ± 310
GnRHa	33 ± 3.9	355‡ ± 75	2,610 ± 360

* Rat granulosa cells were incubated for 60 min in the absence (control) or presence of GnRHa (100 ng/ml). Phospholipids were separated by thin-layer chromatography and phospholipid phosphorus was quantified. PtdGro, phosphatidylglycerol.
† Mean ± SEM, $n = 3$.
‡ $P < 0.05$ vs. control, paired t test.

The stimulatory effects of GnRH and GnRHa on phospholipid labeling may simply reflect the net increases observed in PtdIns synthesis. However, the possibility exists that both *de novo* synthesis of PtdIns and turnover (PtdIns hydrolysis and subsequent resynthesis) of a hormone-sensitive pool of PtdIns are occurring simultaneously. Farese *et al.* (27) have recently demonstrated that both of these events occur simultaneously in the rat adrenal zona glomerulosa in response to angiotensin II. Although increased PtdIns labeling occurs in response to GnRH in the pituitary (13, ¶) and granulosa cells, a direct effect of GnRH on PtdIns hydrolysis in either tissue has not been demonstrated.

Whether or not the action of GnRH in granulosa cells is similar to the action of GnRH on its principal target, the pituitary, is open to question. Early reports implicated cyclic nucleotides in the stimulation of LH release, but more recent studies have suggested the involvement of increased phospholipid turnover (12, 13, ¶) and calcium translocation (28) in GnRH action. In the ovary, Ca^{2+} ionophore A23187 and GnRH exert similar effects on rat luteal cell responsiveness (29). In a recent abstract, Leung and Raymond (‖) reported that GnRH

¶ Raymond, V., Veilleux, R. & Leung, P. C. K., 64th Annual Meeting of the Endocrine Society, June 16–18, 1982, San Francisco, p. 285 (abstr.).
‖ Leung, P. C. K. & Raymond, V., 64th Annual Meeting of the Endocrine Society, June 16–18, 1982, San Francisco, p. 180 (abstr.).

and A23187 also generated a PtdIns response in isolated rat luteal cells. However, a specific role for Ca^{2+} in GnRH action or phospholipid metabolism in granulosa cells is unknown. Of considerable interest are recent reports suggesting that newly synthesized Ptd-OH may mediate Ca^{2+} translocation by acting as an ionophore (16, 30, 31).

The mechanism of action of membrane-active hormones that fail to stimulate cAMP accumulation is unknown. Several models have been described that link hormonal activation to changes in specific membrane phospholipids. In general, the activation of receptors that stimulate PtdIns metabolism and increase Ca^{2+} translocation is associated with increases in cellular cGMP but not cAMP (15, 16, 25). Indeed, in our granulosa cell preparations, GnRH has no effect on cAMP levels. However, increases in cGMP levels were not observed in conjunction with GnRH-induced increases in PtdIns labeling. Instead, cGMP levels were transiently reduced. Therefore, it appears that the rapid alterations in PtdIns metabolism observed in response to GnRH are not associated with increases in cyclic nucleotide accumulation. Other investigators have demonstrated that a PtdIns response can be generated when cGMP levels were either unchanged or reduced (25) and that alterations in cGMP levels in intact cells may be related to Ca^{2+} translocation (32). Clearly, more work is needed to establish whether or not there is a link between PtdIns metabolism and cGMP formation.

Other roles for PtdIns metabolism in ovarian function have been suggested recently. Farese *et al.* have proposed that increased synthesis of phospholipids in the phosphatidate-phosphoinositide pathway may, in part, mediate the action of steroidogenic hormones (19–21, 27). Increased levels of phospholipids in this pathway have been closely correlated with corticotropin (ACTH)-induced steroidogenesis in the rat adrenal. In addition, inositide-containing phospholipids stimulate cholesterol side-chain cleavage and corticosterone production when added to adrenal mitochondria and cells, respectively. Recent studies in our laboratory have shown a similar relationship between LH action and PtdIns metabolism in ovarian cells (18, 33, **). A good correlation also exists between angiotensin II-induced increases in Ptd-OH and PtdIns metabolism and aldosterone synthesis in rat (27) and bovine (34) adrenal zona glomerulosa. In keeping with this proposal, GnRH may stimulate ovarian progesterone synthesis by a mechanism involving PtdIns metabolism.

Kishimoto *et al.* (35) have recently suggested that the PtdIns response is coupled to the activation of a cyclic nucleotide-independent, calcium- and phospholipid-dependent protein kinase. This enzyme is selectively activated by the simultaneous presence of Ca^{2+} and phospholipid. Diglyceride sharply increases the affinity of this protein kinase for Ca^{2+}, as well as for phospholipid, and thereby renders the enzyme active at lower Ca^{2+} concentrations. We have recently demonstrated the presence of such an enzyme in ovarian cytosol from PMSG-treated immature rats (††). Protein kinase activity was stimulated only by the simultaneous presence of Ca^{2+} and phospholipid. PtdIns and phosphatidylserine were most active in supporting enzyme activity. The activation of a protein kinase by specific changes in membrane phospholipids may partially explain the actions in the ovary of hormones that do not produce changes in cAMP.

We suggest that GnRH-induced changes in phospholipid metabolism represent an early event in the mechanism of action of GnRH. These changes may be important links between GnRH binding and altered Ca^{2+} translocation, enzyme activities, and ultimately granulosa cell function.

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†† Davis, J. S. & Clark, M. R., 64th Annual Meeting of the Endocrine Society, June 16–18, 1982, San Francisco, p. 179 (abstr.).