Luteinizing hormone stimulates the formation of inositol trisphosphate and cyclic AMP in rat granulosa cells

Evidence for phospholipase C generated second messengers in the action of luteinizing hormone

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The following studies were conducted to determine whether luteinizing hormone (LH), a hormone which increases cellular levels of cyclic AMP, also provokes increases in 'second messengers' derived from inositol lipid metabolism (i.e. inositol phosphates and diacylglycerol). Rat granulosa cells isolated from mature Graafian follicles were prelabelled for 3 h with *myo*-[2-³H]inositol. LH provoked rapid (5 min) and sustained (up to 60 min) increases in the levels of inositol mono-, bis, and trisphosphates (IP, IP₂ and IP₃, respectively). Time course studies revealed that IP₃ was formed more rapidly than IP₂ and IP following LH treatment. The response to LH was concentration-dependent with maximal increases at LH concentrations of 1 µg/ml. LiCl (2–40 mM) enhanced the LH-provoked accumulation of all [³H]inositol phosphates, presumably by inhibiting the action of inositol phosphate phosphatases. The effectiveness of LH, however, was dependent on the concentration of lithium employed; maximal increases in IP were observed at 10 mM-LiCl, whereas maximal increases in IP₂ and IP₃ were observed at 20 mM- and 40 mM-LiCl, respectively. The stimulatory effects of LH on inositol phosphate and progesterone accumulation were also compared with changes in cyclic nucleotide levels. LH rapidly increased levels of inositol phosphates, progesterone and cyclic AMP, but transiently reduced levels of cyclic GMP. These results demonstrate that LH increases both cyclic AMP and inositol trisphosphate (and presumably diacylglycerol) in rat granulosa cells. Our findings suggest that two messenger systems exist to mediate the action of LH in granulosa cells.

INTRODUCTION

Ovarian cells possess a wide variety of receptors which, when activated, stimulate signal pathways that ultimately regulate ovarian function. Two major signal pathways exist in the ovary. The most extensively studied receptor-mediated signal pathway involves the conversion of adenosine triphosphate into cyclic AMP, which subsequently activates cyclic AMP-dependent protein kinase. More recently, it has been recognized that hormone-induced alterations in the metabolism of phosphoinositides functions as a signal-generating system in the ovary (Davis *et al.*, 1981, 1983*a,b*, 1984*a,b*, 1986; Naor & Yavin, 1982; Leung, 1985; Ma & Leung, 1985). Phospholipase C induced hydrolysis of phosphoinositides yields DAG and inositol phosphates, products which have recently received attention as second messengers. IP₃, one product of PIP₂ hydrolysis, has been proposed to be a second messenger for intracellular Ca²⁺ mobilization (Berridge, 1984; Berridge & Irvine, 1984). DAG, the other product of PIP₂ hydrolysis, can stimulate protein kinase C (Nishizuka, 1984*a,b*). In general, hormones which utilize cyclic AMP as their intracellular signal are not thought to provoke increases in phosphoinositide hydrolysis and IP₃ generation (Michell, 1975; Farese, 1983; Abdel-Latif, 1983; Fain, 1984; Berridge & Irvine, 1984).

It is well known that LH uses cyclic AMP as a major signalling mechanism for regulating the function of ovarian cells (Marsh, 1975; Williams *et al.*, 1978; Niswender *et al.*, 1980). We have previously reported that LH increases the phosphoinositide cycle by increasing synthesis of phosphoinositides in rat granulosa cells isolated from mature Graafian follicles (Davis *et al.*, 1983*a*, 1984*a*). However, we (Davis *et al.*, 1984*a,b*) were unable to demonstrate an effect of LH on the hydrolysis of prelabelled phosphoinositides. Thus, it is not known whether the effect of LH on phosphoinositide metabolism results in increases in IP₃ and DAG. In the present studies we have re-examined the effect of LH on the generation of second messengers and report that LH increases the formation of both IP₃ and cyclic AMP in rat granulosa cells.

MATERIALS AND METHODS

Pregnant mare's serum gonadotropin and ovine LH (oLH, S19) were kindly supplied by The National Hormone and Pituitary Program, National Institute of Arthritis, Diabetes and Digestive and Kidney Disease, National Institutes of Health. Synthetic GnRH was purchased from Beckman Instruments (Palo Alto, CA, U.S.A.) and the GnRH antagonist [Ac-Δ⁹Pro¹,

Abbreviations used: LH, luteinizing hormone; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; IP, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; DAG, 1,2-diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; GnRH, gonadotropin-releasing hormone; BSA, bovine serum albumin.

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p-F-D-Phe²,D-Trp^{3,6}]GnRH (ANTAG) was purchased from Bachem (Torrance, CA, U.S.A.). We purchased Medium 199 from Gibco (Grand Island, NY, U.S.A.), and AG 1-X8 ion exchange resin from Bio-Rad (Rockville Center, NY, U.S.A.). *myo*-[2-³H]inositol (15 Ci/mmol) was purchased from American Radio-labeled Chemicals (St. Louis, MO, U.S.A.). [1,2,6,7-³H] Progesterone (90 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Immature Sprague-Dawley rats (Charles River) were injected subcutaneously with 20 i.u. of pregnant mare's serum gonadotropin between 09:00 and 10:30 on day 27 and were killed 46 h later. Ovaries were removed, trimmed and placed in Medium 199 containing 25 mM-Hepes and 1% BSA. This modified medium was used in all subsequent incubations. Granulosa cells were harvested as described previously (Davis *et al.*, 1984a). Approx. 4×10^6 viable cells per ovary were obtained. Viability was determined by counting an aliquot of cells on a haemocytometer in the presence of Trypan Blue.

For studies of inositol metabolism, granulosa cell preparations (approx. 1×10^8 cells) were incubated for 3 h in modified medium containing *myo*-[2-³H]inositol (50 μ Ci/ml) under an atmosphere of O₂/CO₂ (19:1) at 37 °C. The cells were then washed twice with modified medium containing 10 mM-inositol and incubated without isotope or inositol for 15 min. After this incubation period, the cells were washed again and divided into siliconized glass tubes. Incubations were performed in triplicate with $(1-2) \times 10^6$ viable cells, in a final volume of 0.5 ml of Medium 199 containing 25 mM-Hepes and 1% BSA under an atmosphere of O₂/CO₂ (19:1) at 37 °C. After a 15 min preincubation with or without LiCl (0-40 mM), control media or hormones were added and the incubations were continued for up to 60 min. Incubations were terminated by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid. After 30 min at 4 °C, the samples were vigorously vortexed and acid-precipitable proteins and lipids were removed by centrifugation (1000 *g* for 15 min at 4 °C). The acid-soluble fraction was collected and extracted five times with 3 vol. of water-saturated diethyl ether to remove the trichloroacetic acid. After driving off the residual ether and increasing the sample volume to 5 ml with a solution of inositol (5 mM final concentration) the extracts were neutralized with a small volume of 0.1 M-NaOH. Extracts were placed onto columns (7 cm $\times$ 13 mm) of Bio-Rad AG 1-X8 ion exchange resin (formate form) pre-equilibrated with 5 mM-inositol. The method used thereafter was identical with that described by Berridge *et al.* (1982, 1983). Free [³H]inositol was washed through the columns with 5 mM-inositol. Glycerophosphoinositol was eluted with 5 mM-sodium borate in 60 mM-sodium formate. Inositol monophosphates (IP) were eluted with 100 mM-formic acid in 200 mM-ammonium formate. Inositol bisphosphates (IP₂) and trisphosphates (IP₃) were eluted with 100 mM-formic acid in 400 mM- and 1000 mM-ammonium formate, respectively. Aliquots were assayed for radioactivity by liquid-scintillation counting. The identification of IP₂ and IP₃ and verification of the chromatography procedures were accomplished using ³²P-labelled IP₂ and IP₃ prepared from human erythrocyte plasma membranes as described by Downes *et al.* (1982). It should be noted, however, that two naturally occurring isomers of IP₃ have been identified, inositol 1,4,5-trisphosphate and inositol 1,3,4-

trisphosphate (Irvine *et al.*, 1984, 1985; Burgess *et al.*, 1985). Because the 1,3,4 isomer has only recently been described and has no known function, the separation of the two isomers was beyond the scope of the present report. Hence, the measurements of IP₃ in these studies and others (see Berridge, 1984; Berridge & Irvine, 1984, for reviews) probably include both isomers.

In some experiments, the acid-precipitable pellet was extracted with 5 ml of chloroform/methanol/HCl (2:1:0.075, by vol.) to determine the [³H]inositol content of cellular phosphoinositides. Phosphoinositides were purified by t.l.c. as previously described (Davis *et al.*, 1984a).

The incubations of samples for cyclic nucleotide measurements were performed in duplicate with approx. 2×10^6 cells in 0.5 ml of modified medium. Incubations 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. The acidified and heat-treated samples were centrifuged at 1000 *g* for 10 min to sediment precipitated protein, and the supernatants were removed for inclusion in assays for cyclic AMP and cyclic GMP. Cyclic AMP was determined by competitive protein binding as described previously (Davis *et al.*, 1983b). Cyclic GMP was measured by radioimmunoassay (Steiner *et al.*, 1972) after acetylation (Harper & Brooker, 1975), employing reagents obtained from New England Nuclear. Progesterone was extracted from aliquots of incubation media and measured by radioimmunoassay (Davis *et al.*, 1981).

Data are presented as individual values and averages for duplicate values in each experiment, or as mean $\pm$ S.E.M. of triplicate values in a representative experiment or as the cumulative mean $\pm$ S.E.M. of experiment averages from three or more experiments. When appropriate, statistical analyses were carried out by analysis of variance followed by Duncan's Multiple Range testing or applying student's *t*-test. Comparisons with *P* < 0.05 were considered to indicate a statistical difference.

RESULTS

Temporal response to LH

Fig. 1 shows a time course of the stimulatory effect of LH on inositol phosphate accumulation in rat granulosa cells. Under control conditions, the levels of inositol phosphates were relatively unchanged in the presence or absence of 10 mM-LiCl (results not shown; also see Fig. 4). In the presence of 10 mM-LiCl, no early (< 2 min) changes in inositol phosphate levels were observed in response to LH. LH provoked significant increases in IP₃ and IP₂ after 5 min of incubation. The initial rate of formation of IP₃ appeared to be greater than the rate of formation of IP₂ ($15 \pm 4\%$ versus $9 \pm 3\%$ increase, respectively, over controls in 5 min incubations, mean $\pm$ S.E.M., *n* = 4). A slight, but insignificant, increase (10%) was also observed in IP levels after 5 min of LH treatment. Further increases in the levels of all inositol phosphates were observed in response to LH throughout the 30 min time course.

When the incorporation of [³H]inositol into phosphatidylinositol was examined under conditions identical with the prelabelling process used for the study of inositol phosphate metabolism (except that the isotope in

Fig. 1. Temporal profile of [³H]inositol phosphate accumulation in response to LH

The cells were preincubated with 10 mM-LiCl for 15 min prior to the addition of LH (1 μ g/ml). Incubations were continued for up to 30 min and were terminated by the addition of 10% (w/v) trichloroacetic acid. The Figure is a composite of four experiments each conducted in triplicate. Data are expressed as the means $\pm$ S.E.M. after expression as a percentage of the average control response in each experiment. *Earliest significant increase in [³H]inositol phosphate levels.

the media was not removed) treatment with LH provoked rapid (< 10 min) and sustained (> 60 min) increases in the levels of [³H]phosphatidylinositol (results not shown). LH also provoked rapid increases in progesterone accumulation in these incubations (results not shown).

Dose-response to LH

The concentration-dependence of the LH-induced stimulation of inositol phosphate accumulation was determined in incubations lasting 30 min (Fig. 2). Similar to previous results on ³²P incorporation into phosphatidic acid and phosphoinositides (Davis *et al.*, 1984a), LH at 1 μ g/ml provided maximal stimulatory effects, with half-maximal stimulation occurring at approx. 100 ng of LH/ml. A slight stimulatory response was also observed at 10 ng of LH/ml.

Effect of LH on [³H]inositol metabolites

The distribution of [³H]inositol in inositol phospholipids and their water-soluble metabolites, inositol phosphates, is shown in Table 1. Under control

conditions, most (67%) of the radioactivity was found as free [³H]inositol, with phosphatidylinositol containing the second largest quantity (30%) of radioactivity. The remaining components each contained less than 1% of the total radioactivity. Addition of LH (1 μ g/ml) provoked the following changes over a 30 min incubation period: [³H]-labelled IP, IP₂ and IP₃ were increased ($P < 0.001$) by 59, 96 and 101% over controls, respectively, levels of [³H]glycerophosphoinositol were slightly (16%) increased ($P < 0.05$) and levels of [³H]inositol and [³H]inositol phospholipids were unchanged.

Effects of lithium on inositol phosphate levels

The monovalent metal, lithium, prevents the recycling of inositol and allows for the accumulation of inositol phosphates. This action is presumably due to an inhibition of the phosphatase that cleaves inositol 1-phosphate to inositol (Hallcher & Sherman, 1980; Berridge *et al.*, 1982); however, lithium also increases the accumulation of inositol polyphosphates in some hormone-responsive tissues (Thomas *et al.*, 1984; Drummond *et al.*, 1984; Streb *et al.*, 1985; Burgess *et al.*, 1985; Davis *et al.*, 1986). Fig. 3 shows a time course of inositol phosphate accumulation in response to LH in the absence and presence of 10 mM-LiCl. Control levels of total [³H]inositol phosphates were unchanged in the presence and absence of lithium. No significant increases in the levels of total [³H]inositol phosphates (or IP, IP₂ or IP₃; results not shown) were observed in response to LH in the absence of LiCl. The addition of lithium simultaneously with LH or 30 min after LH treatment

Fig. 2. Dose-dependency of LH-induced increases in the levels of [³H]inositol phosphates in isolated rat granulosa cells

Cells were preincubated for 15 min with 10 mM-LiCl and were subsequently incubated for 30 min with 0.01–5 μ g of LH/ml. Results are mean and ranges of two experiments after expression as a percentage of the maximal response observed in each experiment.

Table 1. Effect of LH on [³H]inositol metabolites in isolated rat granulosa cells

Rat granulosa cells were prepared and prelabelled with *myo*-[2-³H] inositol as described in the Materials and methods section. Cells were preincubated for 15 min with 10 mM-LiCl and were further incubated for 30 min in the absence (control) or presence of LH (1 µg/ml). The incubations were terminated by the addition of ice-cold trichloroacetic acid. The acid-soluble [³H]inositol metabolites were purified by ion-exchange chromatography. [³H]Phosphoinositides were extracted from the acid-insoluble material and purified by t.l.c. The data shown are the mean ± S.E.M. from seven separate experiments, each conducted in duplicate or triplicate. *P* values are LH versus control, paired *t*-test; NS, not statistically significant.

[³ H]Inositol metabolite	Radioactivity in metabolite (c.p.m.)			<i>P</i>
	Control	LH	LH minus control	
Inositol	74077 ± 3522	70783 ± 3704	-3294 ± 2153	NS
Glycerophosphoinositol	456 ± 35	530 ± 57	74 ± 29	< 0.05
IP	970 ± 121	1540 ± 102	570 ± 48	< 0.001
IP ₂	122 ± 20	239 ± 23	117 ± 18	< 0.001
IP ₃	124 ± 21	249 ± 20	125 ± 12	< 0.001
Phosphatidylinositol	32901 ± 3342	33114 ± 3113	213 ± 997	NS
PIP	873 ± 158	906 ± 178	33 ± 59	NS
PIP ₂	1202 ± 116	1224 ± 88	22 ± 72	NS

Fig. 3. Effect of lithium on LH-induced increases in [³H]inositol phosphates in isolated rat granulosa cells

Cells were preincubated for 15 min and were subsequently incubated with (●, ■) or without (○, □) 10 mM-LiCl in the absence (○, ●) or presence (□, ■) of LH (1 µg/ml). In some samples LiCl was added to LH-treated samples at time zero or to LH-treated samples after 30 min of incubation. Data are expressed as means ± S.E.M. of triplicate incubations in a representative experiment.

resulted in similar increases in inositol phosphate accumulation. These data indicate that lithium rapidly inhibits inositol phosphate degradation and demonstrate that the effect of LH is continuous over a 60 min incubation period.

Fig. 4 shows the effects of lithium (0–40 mM) on LH-induced inositol phosphate accumulation. Lithium at concentrations of 2–10 mM had very little effect on

basal inositol phosphate levels. Increased concentrations of lithium raised levels of IP and IP₃, but had little effect on IP₂; IP and IP₃ were each increased 15% and 30% at 20 mM and 40 mM-lithium, respectively, whereas IP₂ was increased 15% at 40 mM-lithium. At all concentrations of lithium, dose-dependent increases in inositol phosphates were observed in response to LH. However, the maximal responsiveness to LH was determined by the concentration of lithium employed. Maximal increases in IP were observed at 10 mM-lithium and further increases reduced the effectiveness of LH. This was due to the increased basal levels of IP in cells incubated with higher lithium concentrations. Maximal increases in IP₂ and IP₃ were observed at 20 mM and 40 mM-lithium, respectively. These results indicate that lithium is a helpful tool for the study of phosphoinositide metabolism. The use of lithium to study LH-induced increases in inositol phosphate formation is somewhat analogous to the use of phosphodiesterase inhibitors to prevent cyclic AMP degradation in order to study LH effects on cyclic AMP accumulation.

Specificity of the response to LH

We (Davis *et al.*, 1986) and others (Ma & Leung, 1985) have recently demonstrated that gonadotropin-releasing hormone (GnRH) provokes very rapid (10 s) and substantial (20-fold) increases in inositol phosphate levels in rat granulosa cells. Therefore, experiments were conducted to determine whether the increases in inositol phosphates observed with LH could be due to a small amount of contamination with GnRH. We employed a specific GnRH antagonist to inhibit the action of GnRH. As shown in Table 2, the GnRH antagonist had no effect on inositol phosphate levels. Both LH and GnRH significantly increased inositol phosphate levels, albeit to greatly differing extents; LH provoked a 0.4-fold increase versus a 12-fold increase provoked by GnRH. The GnRH antagonist, however, completely inhibited the effect of GnRH without altering the effect of LH. In addition, the response to GnRH was not different when incubated in the presence of LH.

Fig. 4. Effects of increasing concentrations of LH and LiCl on $[^3\text{H}]$ inositol phosphate levels in rat granulosa cells

Cells were preincubated for 15 min with increasing concentrations (0–40 mM) of LiCl prior to the addition of control media ($\circ$) or LH ($\triangle$, 0.01 $\mu\text{g}/\text{ml}$; $\square$, 0.1 $\mu\text{g}/\text{ml}$; $\bullet$, 1 $\mu\text{g}/\text{ml}$). Incubations were terminated after 30 min by the addition of 10% (w/v) trichloroacetic acid. Results are expressed as the means of triplicate incubations (left panels) or as a percentage of the average control (absence of LH) response at each concentration of LiCl (right panels). Individual values varied less than 5% from the mean.

Effects of LH on cyclic nucleotide and progesterone levels

In many systems, increased inositol lipid metabolism is associated with increased cyclic GMP, but not cyclic AMP, levels (Michell, 1975; Berridge & Irvine, 1984; Fain, 1984). It is well documented that LH increases cyclic AMP, but little is known about cyclic GMP levels. Therefore, experiments were performed to assess the effects of LH on cyclic nucleotide accumulation in rat granulosa cells. Instead of increasing cyclic GMP, LH decreased it in incubations lasting 3 min: 2.0 ± 0.1 versus 0.8 ± 0.1 pmol of cyclic GMP/ 2×10^6 cells ($n = 5$, $P < 0.05$). In a time course study of the effect of LH, cyclic GMP levels were lowest at 3 min and increased toward control levels throughout 30 min of incubation (Fig. 5). The increases in cyclic AMP accumulation observed in these studies are consistent with previous results of LH-induced increases in cyclic AMP production in rat granulosa cells. LH also increased progesterone accumulation in these incubations. Thus, no increases in cyclic GMP were observed in conjunction with LH-induced increases in inositol phosphate levels.

DISCUSSION

The data presented here describe the changes in inositol phosphate levels which accompany hormone-stimulated inositol lipid metabolism in rat granulosa cells. Similar to the effects of LH on phosphoinositide synthesis (Davis *et al.*, 1983a, 1984a), LH provoked a relatively rapid accumulation of inositol phosphates in LH-treated rat granulosa cells. The initial effect of LH on cellular inositol phosphate levels appears to be the formation of IP_3 . Time-course experiments revealed that the levels of inositol polyphosphates were increased prior to increases in the levels of IP. In addition, the initial rate of formation of IP_3 was greater than the rate of formation of IP_2 . It is possible, however, that LH stimulates the formation of IP_2 directly from the breakdown of PIP, or that IP_2 is a product of IP_3 degradation. The increases in IP levels observed in these studies probably reflect the degradation of IP_3 and IP_2 , although formation of IP directly from phosphatidylinositol cannot be ruled out (Wilson *et al.*, 1985).

The increases observed in inositol phosphate levels in

Table 2. Effects of LH, GnRH and GnRH antagonist (ANTAG) on inositol phosphate levels in rat granulosa cells

Rat granulosa cells were preincubated for 15 min with 10 mM-LiCl and were further incubated for 30 min in the absence (control) or presence of LH (1 µg/ml), GnRH (100 ng/ml), GnRH antagonist (ANTAG, 100 ng/ml) or various combinations of each treatment. The results are means ± S.E.M. from one experiment conducted in triplicate. **P* < 0.05 versus control.

Treatments	[³ H]Inositol Phosphates (c.p.m.)
Control	1661 ± 39
ANTAG (100 ng/ml)	1837 ± 202
LH (1 µg/ml)	2270 ± 57*
ANTAG + LH	2344 ± 140*
GnRH (100 ng/ml)	21270 ± 1233*
ANTAG + GnRH	1660 ± 60
LH + GnRH	20332 ± 894*

Fig. 5. Effects of LH on cyclic nucleotide and progesterone accumulation by rat granulosa cells

Isolated cells (2×10^6) were preincubated for 15 min and were further incubated for up to 30 min in the absence (○) or presence (■) of LH (1 µg/ml). Results are expressed as the mean ± S.E.M., *n* = 5, **P* < 0.05 versus control.

LH-treated rat granulosa cells suggest that phospholipase C is involved in the action of LH. In fact, phosphodiesterase cleavage of PIP₂ is the only known mechanism for IP₃ formation in mammalian cells. It is not known, however, whether LH simply activates phospholipase C

or provides substrate (PIP₂) for the enzyme to act upon. In previous reports we were unable to demonstrate hydrolysis of inositol phospholipids in response to LH either by reductions in net levels (mass) of phosphoinositides (Davis *et al.*, 1983a, 1984a) or by decreases in levels of prelabelled phosphoinositides in steady-state (Davis *et al.*, 1984a) or pulse-chase labelling experiments (Davis *et al.*, 1984a,b). Thus, it seems likely that the increases observed in inositol phosphate production are at least partly due to LH-induced inositol lipid synthesis in granulosa cells (Davis *et al.*, 1983a, 1984a). This suggestion is also supported by the following observations. First, the time-course for LH-induced synthesis of inositol phospholipids (Davis *et al.*, 1983a, 1984a) and inositol phosphates are similar, both occurring within the first few minutes of LH action. Second, the maintenance of [³H]PIP₂ levels even after 30 min of LH treatment (Table 1) indicates that the formation of IP₃ from PIP₂ is complemented by an increased rate of synthesis from phosphatidylinositol and PIP. This is also supported by our observations of increases in ³²P-prelabelled PIP and PIP₂ following LH treatment (Davis *et al.*, 1984a). Third, the sustained increases in the accumulation of IP₃ and IP₂ in the presence of both LH and lithium, together with previous reports (Davis *et al.*, 1984a,b) of continued incorporation of radioactive precursors into phosphatidylinositol in the presence of LH suggest that LH-induced PIP₂ synthesis and breakdown is a continuous process.

Treatment of granulosa cells with lithium and LH greatly enhanced the accumulation of inositol phosphates. Although lithium reportedly acts to inhibit specifically the enzyme inositol-1-phosphate phosphatase (Hallcher & Sherman, 1980; Berridge *et al.*, 1982) and has little direct effect on the activity of purified preparations of inositol-1,4,5-trisphosphate 5-phosphatase (Connolly *et al.*, 1985), our results and those of others (Thomas *et al.*, 1984; Drummond *et al.*, 1984; Burgess *et al.*, 1985) demonstrate that lithium enhances hormone-induced accumulation of IP₃ and IP₂ in addition to IP. The reason for these differences is not clear, but may be due to: (1) differences in the sensitivities of inositol phosphatases to lithium in various cell types, (2) accumulation of inositol phosphates and their degradative byproducts which may also influence inositol phosphatase activity, and (3) the presence of multiple IP₃ phosphatases with differing sensitivities to lithium (Burgess *et al.*, 1985). The latter possibility is intriguing, since Burgess *et al.* (1985) have recently reported that lithium increases the accumulation of 1,3,4-IP₃, but not 1,4,5-IP₃, in hormone-stimulated cells. The identity of the IP₃ isomer formed in the presence of LH is unknown.

The presence of metabolic pathways for the synthesis and degradation of IP₃ and the ability of IP₃ to mobilize cellular Ca²⁺ (Berridge & Irvine, 1984; Oron *et al.*, 1985; Streb *et al.*, 1985; Williamson *et al.*, 1985) suggest that IP₃ may be a second messenger for Ca²⁺ mobilization in granulosa cells. Although Ca²⁺ appears to be important in the action of LH (Veldhuis *et al.*, 1984) it is not known whether LH, via IP₃, provokes increases in cellular Ca²⁺ mobilization. In keeping with a role for IP₃ in Ca²⁺ mobilization, we have observed that GnRH rapidly increases IP₃ levels and cytosolic free calcium concentrations in rat granulosa cells (Davis *et al.*, 1986). Alternatively, LH may only increase the 1,3,4-IP₃ isomer, which has no known cellular function, as a means to

increase DAG without altering Ca^{2+} mobilization (Burgess *et al.*, 1985).

Like cyclic AMP, DAG may play a central role in the action of LH. Increased levels of DAG probably occurred concomitantly with LH-induced increases in inositol phosphates. Diacylglycerols and the DAG-like tumour-promoting phorbol diester TPA stimulate protein kinase C by reducing the amounts of calcium and phospholipid required for activation (Castagna *et al.*, 1982; Nishizuka, 1984a,b). Similar to the effects of LH and GnRH, TPA stimulates prostaglandin production and progesterone synthesis in rat granulosa cells (Kawai & Clark, 1985) and rat Leydig cells (Lin, 1985). The effects of TPA occurred by a mechanism independent of increases in cyclic AMP or increases in cyclic AMP-dependent protein kinase activity. On the other hand, TPA (Welsh *et al.*, 1984; Kawai & Clark, 1985) and synthetic diacylglycerols (Kasson *et al.*, 1985a) inhibit gonadotropin-induced steroidogenesis in granulosa cells and Leydig cells (Papadopoulos *et al.*, 1985; Mukhopadhyay *et al.*, 1984). These studies indicate that TPA appears to exert biphasic effects on cellular function, depending on the presence or absence of hormones. It has been proposed that protein kinase C may phosphorylate and desensitize receptors that are coupled either to adenylate cyclase (Rebois & Patel, 1985; Nambi *et al.*, 1985) or phospholipase C (Drummond & MacIntyre, 1985; Cooper *et al.*, 1985), suggesting that increases in phosphoinositide metabolism, and subsequent increases in the levels of DAG and activation of protein kinase C, may be part of a negative feedback mechanism in the regulation of cellular activity. The documentation that protein kinase C is present in rat ovarian tissues (Clark *et al.*, 1985; Davis, 1985; Shinohara *et al.*, 1985; Noland & Dimino, 1985), and the increase in phosphoinositide metabolism (and presumably DAG) in LH-stimulated granulosa cells, point to the involvement of protein kinase C in the action of LH.

In general, the activation of receptors that stimulate phosphoinositide metabolism and increase Ca^{2+} mobilization is associated with increases in cellular cyclic GMP, but not cyclic AMP (Michell, 1975; Abdel-Latif, 1983; Farese, 1983; Berridge & Irvine, 1984; Fain, 1984). In our rat granulosa cell preparations, increases in cyclic GMP levels were not observed in conjunction with LH-induced increases in levels of inositol phosphates. Instead, cyclic GMP levels were transiently reduced. The results observed in this study with LH are very similar to those obtained previously with GnRH (Davis *et al.*, 1983b); increases in phosphatidylinositol turnover were associated with transient reductions in cellular cyclic GMP levels. Therefore, it appears that hormone-induced alterations in phosphoinositide metabolism observed in rat granulosa cells are not associated with increases in cyclic GMP. As expected, increases in cyclic AMP levels were observed in response to LH treatment.

Since the effect of LH on phosphoinositide metabolism does not appear to be mediated by increases in cyclic AMP (Davis & Clark, 1983; J. S. Davis, L. A. West & R. V. Farese, unpublished work), it is likely that receptor-mediated increases in cyclic AMP and phosphoinositide metabolism are initiated along separate pathways in rat granulosa cells. It may be that separate LH receptors exist for each type of response or that each response occurs in a specific subpopulation of granulosa cells. Indeed, subclasses of some hormone and neuro-

transmitter receptors (e.g., vasopressin and serotonin receptors) have been characterized which are linked to either phosphoinositide metabolism or cyclic nucleotide metabolism (Michell, 1975; Berridge & Irvine, 1984; Fain, 1984). More recently, subpopulations of granulosa cells have been isolated which have been characterized by their differing sedimentation properties and hormonal responsiveness (Kasson *et al.*, 1985b). Whether or not the action of LH on cyclic AMP and phosphoinositide metabolism is mediated by a single receptor in one cell type awaits further investigation.

In summary, we have demonstrated that LH stimulates specific, concentration-dependent increases in inositol phosphate levels in rat granulosa cells. The time course for these events suggests that phosphoinositide metabolism is an early event following LH receptor interaction. The metabolic by-products, IP_3 and DAG, may act as second messengers to mediate the action of LH in rat granulosa cells.

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