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Meiotic resumption in response to luteinizing hormone is independent of a G_i family G protein or calcium in the mouse oocyte

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Abstract

The signaling pathway by which luteinizing hormone (LH) acts on the somatic cells of vertebrate ovarian follicles to stimulate meiotic resumption in the oocyte requires a decrease in cAMP in the oocyte, but how cAMP is decreased is unknown. Activation of G_i family G proteins can lower cAMP by inhibiting adenylate cyclase or stimulating a cyclic nucleotide phosphodiesterase, but we show here that inhibition of this class of G proteins by injection of pertussis toxin into follicle-enclosed mouse oocytes does not prevent meiotic resumption in response to LH. Likewise, elevation of Ca^{2+} can lower cAMP through its action on Ca^{2+}-sensitive adenylate cyclases or phosphodiesterases, but inhibition of a Ca^{2+} rise by injection of EGTA into follicle-enclosed mouse oocytes does not inhibit the LH response. Thus, neither of these well-known mechanisms of cAMP regulation can account for LH signaling to the oocyte in the mouse ovary.

Keywords: Meiotic resumption; Oocyte maturation; Luteinizing hormone; Heterotrimeric G proteins; Intracellular calcium; Follicle-enclosed oocytes; Mouse

Introduction

Luteinizing hormone (LH) acts on the somatic cells of the mammalian ovarian follicle to stimulate ovulation (Richards et al., 2002), as well as the progression of meiosis in the oocyte from prophase to metaphase, in preparation for fertilization (Eppig et al., 2004; Jones, 2004; Mehlmann, 2005b). Through a series of incompletely understood steps, LH causes a decrease in cAMP in the oocyte (Schultz et al., 1983; Sela-Abramovich et al., 2006), which is necessary and sufficient for the prophase-to-metaphase transition (see Eppig, 1993). Particularly strong support for this conclusion is provided by the recent finding that oocytes from mice that lack PDE3A, an isoform of cAMP phosphodiesterase present in mouse oocytes, have elevated cAMP, and although LH causes ovulation in these mice, the oocytes remain arrested in prophase (Masciarelli et al., 2004). Conversely, application of the cAMP antagonist Rp-cAMPS, causes meiosis to resume within oocyte-somatic cell complexes grown in vitro (Eppig, 1991).

In prophase-arrested mouse oocytes, cAMP is maintained at a high level by a constitutively active orphan receptor, GPR3, that is coupled to a G_s G protein, thus stimulating the adenylate cyclase AC3 to generate cAMP (Mehlmann et al., 2002, 2004; Horner et al., 2003; Kalinowski et al., 2004; Freudzon et al., 2005; Hinckley et al., 2005; Ledent et al., 2005; Mehlmann, 2005a). The closely related orphan receptor, GPR12, serves an analogous function in rat oocytes (Hinckley et al., 2005). The follicle cells have also been considered as a source of cAMP, which could enter the oocyte through gap junctions (Anderson and Albertini, 1976; Dekel et al., 1981; Eppig et al., 2004; Mehlmann, 2005b; Sela-Abramovich et al., 2006), but conflicting results have been obtained as to whether cAMP in the cumulus cells equilibrates with cAMP in the oocyte (Eppig and Downs, 1984; Bornslaeger and Schultz, 1985). In a recent

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study, elevating cAMP in the cumulus cells elevated cAMP in the oocyte, but with a delay of several minutes, which is not easily explained by diffusion through gap junctions (Webb et al., 2002). Whether or not the follicle cells contribute cAMP to the oocyte, this source of cAMP is not sufficient to maintain meiotic arrest if the function of GPR3, G_s, or AC3 is eliminated (see references above).

Much is known about LH signaling at the somatic cell level (Richards et al., 2002; Tsafriri et al., 2005; Conti et al., 2006). LH causes the outer somatic cells to produce ligands that activate epidermal growth factor (EGF) receptors on the cumulus cells that directly surround the oocyte, and this EGF receptor activation is necessary and sufficient to stimulate meiosis (Park et al., 2004). Cumulus cell MAP kinase activity is also essential (Su et al., 2003). However, the events at the oocyte level that decrease cAMP are poorly understood. Inhibition of the oocyte's receptor/G_s/adenylate cyclase system, or stimulation of its cAMP phosphodiesterase, are possibilities. Another alternative is a change in permeability of the gap junctions between the oocyte and somatic cells (Dekel et al., 1981; Eppig, 1993; Eppig et al., 2004; Conti et al., 2006; Mehlmann, 2005b; see Discussion).

In this study, we tested two hypotheses for how LH acts to lower cAMP in mouse oocytes. First, we examined whether LH action requires a G_i family G protein in the oocyte because G_i inhibits adenylate cyclase, and because hormonal stimulation of the prophase-to-metaphase transition in starfish and fish oocytes requires G_i (Shilling et al., 1989; Chiba et al., 1992; Jaffe et al., 1993; Pace and Thomas, 2005). G_i is not required for the spontaneous meiotic resumption that occurs in isolated mouse oocytes (Jones and Schultz, 1990; Faerge et al., 2001), but pertussis toxin (PTX), which specifically inhibits G_i family G proteins (see Gierschik, 1992), inhibits the meiotic resumption seen in some oocytes when the peptide INSL3 is applied to rat follicles (Kawamura et al., 2004). The role of G_i in LH signaling has not been previously investigated.

G_i family G proteins inhibit multiple adenylate cyclase isoforms (Simonds, 1999), including AC3, which is essential for maintaining meiotic arrest (Horner et al., 2003). In addition, some G_i family G proteins stimulate cyclic nucleotide phosphodiesterases (see Fields and Casey, 1997). Either of these actions would lower oocyte cAMP. Although G_i family G proteins have not been shown to directly regulate the PDE3A isoform present in mouse oocytes, these G proteins are known to stimulate cGMP phosphodiesterases in the visual and olfactory systems (Fields and Casey, 1997); in particular, the G_i family member G_t (transducin) stimulates PDE6 (Granovsky and Artemyev, 2001). Because cGMP competitively inhibits PDE3A (Hambleton et al., 2005), stimulation of a G protein-sensitive cGMP phosphodiesterase could indirectly lower oocyte cAMP. To test a requirement for a G_i family G protein, we injected oocytes within antral follicles with PTX and determined if meiotic resumption in response to LH was inhibited.

The second possibility that we tested is that a rise in free Ca^{2+} in the oocyte mediates the reinitiation of meiosis in response to LH. In some studies (Carroll and Swann, 1992; Carroll et al., 1994), but not others (Tombes et al., 1992; Mehlmann and Kline, 1994; Hyslop et al., 2004), Ca^{2+} oscillations, lasting 10–60 s and occurring every 1–3 min, have been seen during the spontaneous resumption of meiosis that occurs when mouse oocytes are removed from their follicles. Possible reasons for the variable occurrence of the oscillations include the type of Ca^{2+} indicator, the strain of mouse, and other experimental variables.

A rise in oocyte Ca^{2+} has been reported to occur when LH is applied to cumulus-enclosed sheep oocytes (Mattioli et al., 1998), but not when EGF is applied to cumulus-enclosed mouse oocytes (O'Donnell et al., 2004). The membrane permeant AM form of the Ca^{2+} chelator Quin 2 inhibits spontaneous meiotic resumption of mouse oocytes (DeFelici et al., 1991), but toxic effects of Quin 2-AM have been reported (Carpenter-Deyo et al., 1991). Another chelator, BAPTA-AM, does not inhibit spontaneous meiotic resumption (Carroll and Swann, 1992; Tombes et al., 1992; Mehlmann and Kline, 1994; Coticchio and Fleming, 1998). BAPTA-AM (Coticchio and Fleming, 1998), or inhibitors of calcium/calmodulin-dependent protein kinase II (Su and Eppig, 2002), do inhibit the meiotic resumption that can be stimulated by applying follicle stimulating hormone to cumulus-oocyte complexes, but in these experiments, Ca^{2+} was chelated in both the oocyte and the cumulus cells. Thus, some of these studies suggest that Ca^{2+} may function in controlling meiotic arrest, but it has not been directly examined whether a rise in Ca^{2+} is necessary for meiotic stimulation by LH. The pathway by which LH acts could differ from that occurring during spontaneous meiotic resumption.

A role for Ca^{2+} in regulation of oocyte cAMP is also suggested by the inhibitory effect of Ca^{2+} on the adenylate cyclase isoform AC3 (Wang and Storm, 2003), which is present in mouse oocytes (Horner et al., 2003). Thus, a rise in oocyte Ca^{2+} could result in a fall in oocyte cAMP. In addition, although PDE3A is Ca^{2+} insensitive (see Hambleton et al., 2005), oocytes also contain a cAMP phosphodiesterase that is activated by Ca^{2+} (Bornslaeger et al., 1984; see Rybalkin et al., 2003). To test a requirement for Ca^{2+}, we injected follicle-enclosed oocytes with EGTA and determined if meiotic resumption in response to LH was inhibited.

For these experiments, we used a recently developed technique for injecting oocytes within antral follicles (Mehlmann et al., 2002; Kalinowski et al., 2004). In contrast to the inhibition of both somatic cell and oocyte signaling events that results when membrane permeant inhibitors are applied to follicles, this technique allowed us to examine the consequences of specific inhibition of G_i activation or Ca^{2+} elevation within the oocyte on LH signaling.

Materials and methods

Follicle and oocyte isolation, microinjection, and culture

B6SJLF1 mice (Jackson Laboratories, Bar Harbor, ME) were used for these studies. Except for the caged IP_3 experiments, the mice were injected intraperitoneally with 5 I.U. equine chorionic gonadotropin (eCG) (National Hormone and Peptide Program, Torrance, CA), in order to stimulate LH receptor synthesis (Camp et al., 1991; Peng et al., 1991), and sacrificed 40–46 h later by cervical dislocation or CO_2 asphyxiation. The mice were 21–25 days old on the

day of use. Antral follicles (~320–500 μm in diameter) were isolated using Dumont #5 forceps or mini forceps (#11200-14; Fine Science Tools, Foster City, CA) and 30-gauge needles. In the course of these experiments, we found that oocytes in follicles from eCG-primed mice that were ~320 μm in diameter had a higher probability of resuming meiosis spontaneously within the follicle, compared with those in follicles that were ≥360 μm in diameter. A possible explanation for the spontaneous meiotic resumption seen in the ~320 μm follicles is that these follicles had failed to respond to eCG stimulation and were in the process of undergoing atresia (Hirshfield, 1991). Therefore, in subsequent experiments using eCG-primed mice, we used 360–500 μm diameter follicles. The medium used for dissecting and culturing the follicles was MEMα (#12000-022; Invitrogen, Carlsbad, CA) supplemented with 5% or 10% fetal bovine serum (#16000-044; Invitrogen) and equilibrated with 5% CO_2 and 95% air (Su et al., 2003). Following isolation, the follicles were placed in 2 ml of this medium and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air until the series of microinjections was completed.

Follicle-enclosed oocytes were microinjected as previously described, using an injection chamber in which the follicles were compressed between 2 coverslips separated by a spacer (Mehlmann et al., 2002; Kalinowski et al., 2004; Jaffe and Terasaki, 2004). We used an ~200 or ~300 μm spacer composed of 2 or 3 layers of double-sided tape (Scotch #137, 3M, St. Paul, MN), or an ~250 μm spacer composed of 2 layers of double-sided tape and 1 layer of double-coated tape (Scotch #667). This assembly was mounted on a U-shaped plastic slide over a reservoir of MEMα with fetal bovine serum, and observed using an upright microscope (Axioskop, Carl Zeiss Inc., Thornwood, NY) with a 20× lens (0.5 or 0.75 N.A.). A micropipet was used to roll the follicle in order to position the oocyte near the upper surface for optimal viewing. Only oocytes in which a nucleolus could be seen were injected and used for experiments. 2–4 follicles were placed in the microinjection chamber and were kept there at ~22°C for ~10–20 min. An equal number of follicles, equivalent in size and appearance to those chosen for microinjection, were placed in a dish to serve as uninjected controls. Injections were performed within 1–2 h after follicle isolation. The injection volumes were quantitated as previously described (Jaffe and Terasaki, 2004), and concentrations of injected substances in the oocyte were calculated based on an oocyte volume of 200 pl.

Following microinjection, follicles were removed from the chambers and placed in 2 ml of medium. When all microinjections were completed, injected and uninjected follicles were transferred, in a small amount of medium, onto Millicell culture plate inserts (PICMORG50, Millipore Corp., Bedford, MA) in 35 mm Petri dishes containing 1.6 ml of medium in the reservoir underneath the filter. 4–10 follicles were placed on each 4.2 cm^2 membrane. Injected and uninjected follicles were treated with LH (oLH-23 or oLH-26; National Hormone and Peptide Program). LH was purified from ovine pituitaries, and analytic gel filtration, SDS gel electrophoresis, and NH2-terminal analysis revealed the purity to approximate 98%. The contamination with other anterior pituitary hormones was negligible, as determined by radioimmunoassay (GH, <0.1% by weight; TSH, <0.5% by weight; FSH, <0.06% by weight; PRL, <0.1% by weight; vasopressin, <0.5% on a molar basis).

The LH was applied by removing 50 or 75% of the solution beneath the culture plate membrane and replacing it with an equal amount of LH-containing medium, resulting in a final concentration of 1 μg/ml LH. Follicles were cultured in a humidified atmosphere of 5% CO_2 and 95% air, at 37°C, for 3–4 h; oocytes were then isolated from the follicles using 30 gauge needles. Cumulus cells were removed using a small-bore mouth pipet, and the oocytes were scored for the presence or absence of a prophase nucleus (germinal vesicle).

For experiments with isolated mouse oocytes, the culture medium was either HEPES-buffered MEM (Mehlmann and Kline, 1994), or MEMα with 5% fetal bovine serum, as indicated; oocytes were collected and injected as previously described (Mehlmann and Kline, 1994). For experiments with isolated starfish oocytes (*Asterina miniata*), the culture medium was natural seawater; oocytes were collected and injected as previously described (Jaffe et al., 1993; Jaffe and Terasaki, 2004).

Pertussis toxin experiments

Pertussis toxin (PTX) was prepared from the supernatant of culture medium of *Bordetella pertussis*, strain 165-143, as previously described (Cronin et al., 1983) and stored in 1 mM HEPES, 154 mM NaCl, pH 8, at 4°C, at a stock concentration of 132 μg/ml. For some initial experiments, we used lyophilized PTX obtained from EMD Biosciences (La Jolla, CA); however, the lyophilized toxin was not completely soluble. For injection into antral follicle-enclosed mouse oocytes, the PTX was mixed with a fluorescent marker (calcium green–10 kDa dextran; #C-3713, Molecular Probes, Eugene, OR), in order to check that the injection was successful (Mehlmann et al., 2002). The stock mixture contained 129 μg/ml PTX and 250 μM calcium green. Mouse oocytes were injected with 14 pl of this stock, corresponding to 1.8 pg of PTX per oocyte. Concentrations in the mouse oocyte cytoplasm were 9 μg/ml PTX and 17 μM calcium green. For control experiments, the concentration of PTX in the starfish oocyte cytoplasm was 4–10 μg/ml.

For control experiments using mouse oocytes that co-expressed the muscarinic m2 acetylcholine receptor and the chimeric G protein G_{qi}, we used constructs and reagents as previously described (Kalinowski et al., 2003). Isolated oocytes were injected with 1 pg of RNA encoding the m2 Ach receptor RNA, and 5 pg of RNA encoding G_{qi}, in HEPES-buffered MEM containing 250 μM dibutyryl cAMP (dbcAMP), to prevent spontaneous meiotic resumption. 5–75 min later, the oocytes were washed into MEMα with fetal bovine serum, but without dbcAMP, and were incubated overnight to allow protein expression. The removal of dbcAMP also caused the oocytes to undergo the process of maturation, in which they progress to second metaphase and become more sensitive to inositol trisphosphate stimulation of Ca^{2+} release (Mehlmann and Kline, 1994). The mature oocytes were then injected again with either 1.8 pg of PTX + 17 μM calcium green–10 kDa dextran, or 17 μM calcium green–10 kDa dextran alone. Calcium green fluorescence was measured at 37°C, in the presence of 5% CO_2, using a photodiode coupled to an inverted microscope, as previously described (Mehlmann et al., 1998). The oocyte was held on a suction pipet in a 10-μl droplet of medium under mineral oil, and 10 μl of 20 μM acetylcholine was added to the droplet. Recordings were started at least 90 min after PTX injection, and ~19–20 h after washing the oocytes out of dbcAMP.

Immunoblotting

Immunoblotting was performed as previously described (Mehlmann et al., 1998). The antibody against the alpha subunit of G_z, which was made against amino acids 93–112 of the human sequence, was obtained from Santa Cruz Biotechnology (sc-388) and was used at a concentration of 0.2 μg/ml. Mouse brain lysate was used as a positive control because α_z is highly expressed in this tissue (Fields and Casey, 1997); the lysate was prepared by homogenizing and sonicating the brain tissue, then centrifuging at 1000×*g* for 1 min to remove nuclei and debris. Precision Plus protein standards (BioRad, Hercules, CA) were used for molecular weight standards. The blot was developed using the ECL Western Blotting Analysis System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

EGTA and BAPTA experiments

EGTA was obtained from Sigma Chemical Company (St. Louis, MO) and was prepared as a 200-mM stock in 200 mM HEPES, pH 7.3 (the measured pH in a fully grown mouse oocyte; Erdogan et al., 2005). 60 mM $CaCl_2$ and 20 mM $MgCl_2$ were included in the solution in order to buffer free Ca^{2+} at ~30 nM (calculated using the program "EGTA calculator"; see entropy.brneurosci.org/egta.html). 400 μM calcium green–10 kDa dextran was also included in order to monitor free Ca^{2+} in control experiments and to confirm that the injections were successful. Injection of 10 pl of this solution into a mouse oocyte resulted in cytoplasmic concentrations of 10 mM of the EGTA buffer (~7 mM free EGTA), 1 mM $MgCl_2$, 10 mM HEPES, and 20 μM calcium green dextran.

RNA encoding the m1 acetylcholine receptor was made as previously described (Williams et al., 1998); isolated oocytes were injected with 2 pg of this RNA and matured as described above for the m2 acetylcholine receptor. The mature oocytes were then injected again with either the 10-mM EGTA buffer described above, or with a control buffer (10 mM HEPES, pH 7.3, and 20 μM calcium green dextran in the cytoplasm). Calcium green fluorescence before and after acetylcholine addition was measured as described for the m2 acetylcholine receptor.

Caged IP_3 (Ins 1,4,5P_3, nitrophenylethyl ester, #I-23580) was obtained from Molecular Probes. It was prepared as a 20-μM stock, and mixed with 400 μM

calcium green–10 kDa dextran, with or without 200 mM of the EGTA buffer described above. A 10-pl injection of this stock resulted in cytoplasmic concentrations in the antral follicle-enclosed oocytes of 1 μM caged IP_3, 20 μM calcium green dextran, and 10 mM of the EGTA buffer (or the corresponding control buffer). For these experiments, we used antral follicles from mice that had not been primed with eCG (290–360 μm diameter) because these follicles are more optically clear than follicles from primed mice. Follicles from unprimed mice do not yet express LH receptors in their mural granulosa cells (Camp et al., 1991; Peng et al., 1991) but are like those from primed mice in terms of signaling from the somatic cells to maintain meiotic arrest. After injection, the follicles were incubated on Millicell membranes for 3–4 h at 37°C, then transferred to a chamber comprised of 2 coverslips separated by a 200-μm double-sided tape spacer. This compressed the follicle such that the oocyte could be clearly imaged. Calcium green fluorescence was measured using a photodiode coupled to an upright microscope, with the preparation at 25°C in air, and caged IP_3 was released by exposing the follicle to light from a mercury arc lamp through a 330±40 nm filter, for a period of 2 s.

BAPTA (tetrapotassium salt, #B1204) was obtained from Molecular Probes. For injection of a cytoplasmic concentration of 10 mM, BAPTA was prepared as a 200-mM stock in 20 mM HEPES, pH 7.3, and 400 μM calcium green–10 kDa dextran; 26 mM $CaCl_2$ was included in the solution, in order to buffer free Ca^{2+} at ~30 nM (based on a K_d of 0.2 μM for BAPTA, taking into consideration temperature and ionic strength, see Harrison and Bers, 1987). For injection of a cytoplasmic concentration of 1 mM, the BAPTA stock contained 25 mM BAPTA and 500 μM calcium green–10 kDa dextran. Calcium green–3 kDa dextran (#6765) was obtained from Molecular Probes.

Photographs of BAPTA- and EGTA-injected oocytes were obtained using a Pascal laser scanning microscope, with a 20×, 0.75 N.A. objective (Carl Zeiss, Inc.). The oocytes were placed in 10-μl drops of medium under mineral oil, within dishes with a coverslip bottom (MatTek Corp., Ashland, MA).

For measurement of intracellular Ca^{2+} during LH exposure, follicle-enclosed oocytes were injected with 12 μM calcium green–10 kDa dextran, and placed on Millicell culture plates, on the stage of an inverted fluorescence microscope. The stage was held at 37°C, and 5% CO_2 in air, saturated with water vapor, was flowed over the dish surface. Calcium green fluorescence was excited using a Xenon arc lamp and measured at 1-s intervals using a CCD camera system (IonOptix, Milton, MA).

Results

Concentration dependence of LH-induced meiotic resumption in antral follicle-enclosed mouse oocytes

Previous studies have shown that mouse oocytes within isolated antral follicles undergo nuclear envelope breakdown (the first sign of meiotic resumption that is detectable by transmitted light microscopy) in response to 1–10 μg/ml LH (Hashimoto et al., 1985; Su et al., 2003; Park et al., 2004; Jamnongjit et al., 2005). To determine the minimal concentration of LH required to cause meiotic resumption, we exposed antral follicles to various concentrations of highly purified LH. 4–5 h after applying LH, the oocytes were dissected from their follicles and scored for the presence or absence of an intact nuclear envelope. 0.1 μg/ml of LH was found to be sufficient to cause almost all oocytes to resume meiosis, whereas 0.01 μg/ml had a partial stimulatory effect (Fig. 1). In comparison with previous determinations of the amount of LH needed to cause meiotic resumption in isolated rat follicles (Tsafriri et al., 1972; Törnell et al., 1995), the amount of LH that we found to be needed for isolated mouse follicles was somewhat less. Using 1 μg/ml of LH, we determined that a 3-h exposure was sufficient to cause almost all oocytes to resume meiosis (24/25).

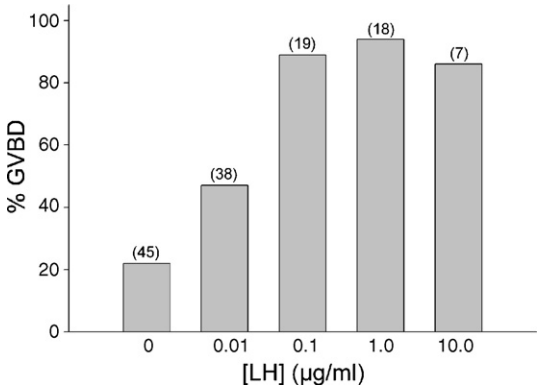


Fig. 1. Concentration dependence of LH stimulation of meiotic resumption in follicle-enclosed oocytes. Antral follicles were exposed to various concentrations of LH for 4–5 h. The oocytes were then removed from their follicles, and scored for the presence or absence of an intact prophase nucleus. %GVBD=the number of oocytes that had undergone breakdown of the nuclear envelope (germinal vesicle), divided by the number of oocytes that were counted, with oocytes from 4 experiments combined as a single group. The numbers in parentheses indicate the number of oocytes counted for each point. Results for all concentrations of LH were significantly different from the control without LH ($p=0.02$ for 0.01 μg/ml LH; $p<0.0001$ for 0.1 and 1.0 μg/ml; $p=0.002$ for 10 μg/ml; Fisher's exact test).

LH stimulation of meiotic resumption in mouse oocytes does not require a G_i family G protein in the oocyte

To test the hypothesis that LH causes the resumption of meiosis in prophase-arrested mouse oocytes by activating a G_i family G-protein, we injected antral follicle-enclosed oocytes with pertussis toxin (PTX), which inhibits the function of this family of G-proteins by ADP-ribosylation of the α subunit (see Gierschik, 1992; Fields and Casey, 1997). 1–3 h after injection of PTX (1.8 pg/oocyte), we applied 1 μg/ml LH to the follicles, and then cultured them for 3–4 h. At the end of this period, we removed the oocytes from their follicles to score for the presence or absence of a prophase nucleus.

The percent meiotic resumption was the same in the PTX-injected oocytes as in uninjected control oocytes that were exposed to LH in parallel (Fig. 2A). Similar results were obtained when the PTX-injected oocytes were exposed to 0.1 μg/ml LH; 7/7 had resumed meiosis when scored at 4 h after LH application. These results indicated that G_i activation is not required for LH action. PTX injection alone, without LH, did not stimulate meiotic resumption (Fig. 2A, column 2), demonstrating that the meiotic resumption observed in the presence of LH was not a consequence of the microinjection procedure.

We confirmed that PTX injection effectively inhibited G_i using 2 methods: (1) We injected the same PTX stock into starfish oocytes and showed that it inhibited meiotic resumption in response to the hormone 1-methyladenine (see Shilling et al., 1989; Chiba et al., 1992). 97% of uninjected starfish oocytes resumed meiosis in response to 1-methyladenine ($n=58$), compared with only 10% of oocytes that had been injected with PTX prior to applying the hormone ($n=36$). (2) We co-expressed in isolated mouse oocytes the muscarinic m2 acetylcholine receptor (which is G_i-linked; see Akam et al.,

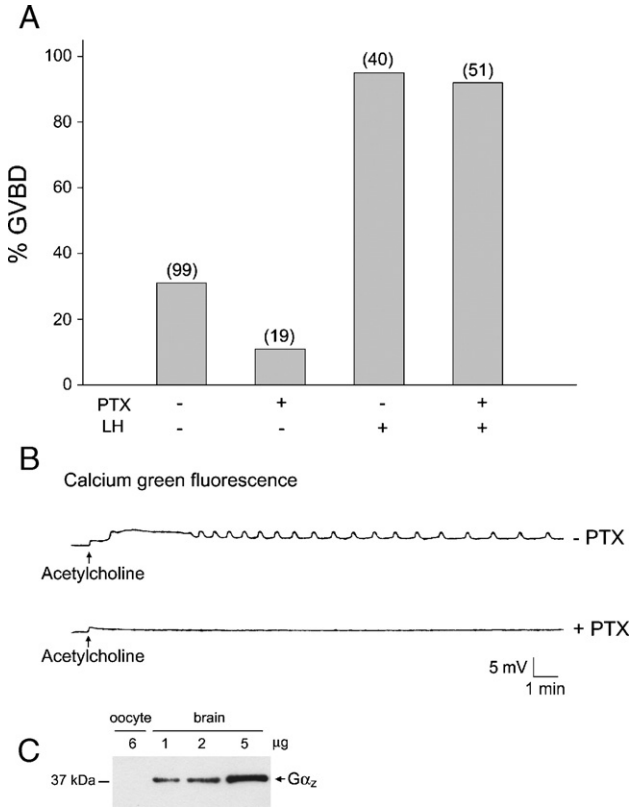


Fig. 2. A G_i-family G protein does not mediate LH stimulation of meiotic resumption. (A) PTX does not inhibit the LH response in follicle-enclosed oocytes. Follicle-enclosed oocytes were injected with PTX (1.8 pg), or left uninjected. 1–3 h later, they were exposed to 1 µg/ml LH, or control medium, for 3–4 h. The oocytes were then isolated to determine %GVBD. The numbers in parentheses indicate the number of oocytes counted for each point, combining the results of 9 experiments. Column 4 is not statistically different from column 3 ($p=0.7$) but is different from column 2 ($p<0.0001$); column 2 is not different from column 1 ($p=0.09$) (Fisher's exact test). When data for the %GVBD for column 2 were compared to the subset of data for column 1 that were collected on the same days as column 2, the %GVBD in column 1 (12%, $n=33$) was very close to that for column 2 (11%, $n=19$). (B) PTX is an effective inhibitor of G_i-signaling in the oocyte. Isolated oocytes expressing the m2 acetylcholine receptor and the chimeric G protein G_{qi} were injected with 17 µM calcium green dextran (upper trace), or 17 µM calcium green dextran+1.8 pg PTX (lower trace). Calcium green fluorescence was recorded before and after addition of 10 µM acetylcholine. The *x* axis shows time, and the *y* axis shows the voltage output of the photodiode amplifier, which is proportional to calcium green fluorescence. The initial deflection of the trace during acetylcholine addition is an artifact that is often seen when solution is pipetted into the droplet containing the oocyte (see Mehlmann et al., 1998). (C) Immunoblot showing the presence of $G\alpha_z$ protein in mouse brain (1, 2, or 5 µg) but not in mouse oocytes (6 µg=230 oocytes). Before the blot was stained for $G\alpha_z$, it was stained for total protein, using Ponceau S; this confirmed that the amount of protein transferred to the membrane from the 6-µg oocyte sample was comparable to the amount of protein from the 5-µg brain sample. The brain $G\alpha_z$ protein migrated with an electrophoretic mobility close to that of a 37-kDa molecular weight standard, close to its predicted molecular weight of 41 kDa; the slight discrepancy in size could be related to the use of a prestained molecular weight standard, which could have migrated more slowly than an unstained protein.

2001) and G_{qi} (which converts a G_i signal into a Ca^{2+} signal; see Conklin et al., 1993; Kalinowski et al., 2003). In 5/5 of these oocytes, acetylcholine application caused Ca^{2+} elevation (Fig. 2B). However, in oocytes that were also injected with 1.8 pg of PTX, acetylcholine application caused no Ca^{2+} rise ($n=5$) (Fig. 2B), or in one case caused a small and delayed rise.

The G_i family of G proteins includes eight α subunits that contain the C terminal cysteine that is ADP-ribosylated by PTX (α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, α_{t1}, and α_{t2} (transducin) and α_g (gustducin); see Gierschik, 1992; Fields and Casey, 1997). One other member of the G_i family, α_z, does not contain this cysteine and is therefore not inhibited by PTX (Fields and Casey, 1997). RNA encoding α_z is present in mouse oocytes (Williams et al., 1996), but α_z protein was not detected by immunoblotting of 6 µg of total oocyte protein (Fig. 2C). A strong α_z protein band was detected in 1 µg of brain lysate (Fig. 2C), indicating that if α_z protein is present in mouse oocytes, its abundance, normalized to total cell protein, is much less than in brain. Based on the intensity of the immunostaining of 1 µg of brain protein, α_z could almost certainly have been detected with as little as 0.3 µg of brain protein. Thus, the abundance of α_z protein in oocytes is <5% of that in brain, and so is unlikely to be required for meiotic resumption in response to LH. Together with the lack of effect of PTX, this finding supports the conclusion that LH causes mouse oocytes to resume meiosis by a pathway that is independent of a G_i family G protein. A previous study with PTX has shown that the function of a G_i family G protein is also not required for the subsequent progression of meiosis to formation of the first polar body (Jones and Schultz, 1990).

LH stimulation of meiotic resumption in mouse oocytes does not require an increase in Ca^{2+} in the oocyte

To determine if an increase in Ca^{2+} in the oocyte is required for meiotic resumption in response to LH, we examined the effect of preventing a rise in intracellular Ca^{2+}, using the Ca^{2+} buffer EGTA. Follicle-enclosed oocytes were injected with EGTA, at a cytoplasmic concentration of 10 mM; $CaCl_2$ was included in the solution to buffer free Ca^{2+} at ~30 nM. The follicles were then exposed to 1 µg/ml LH. 3–4 h later, the oocytes were removed from their follicles and scored for the presence or absence of a prophase nucleus. 10 mM EGTA did not inhibit the resumption of meiosis in response to LH (Fig. 3A). We also observed that at 18 h after isolation from the follicle, 8/9 of the EGTA-injected oocytes had formed a first polar body. This is consistent with a previous report showing that BAPTA-AM had only a minor inhibitory effect on formation of the first polar body (Tombes et al., 1992).

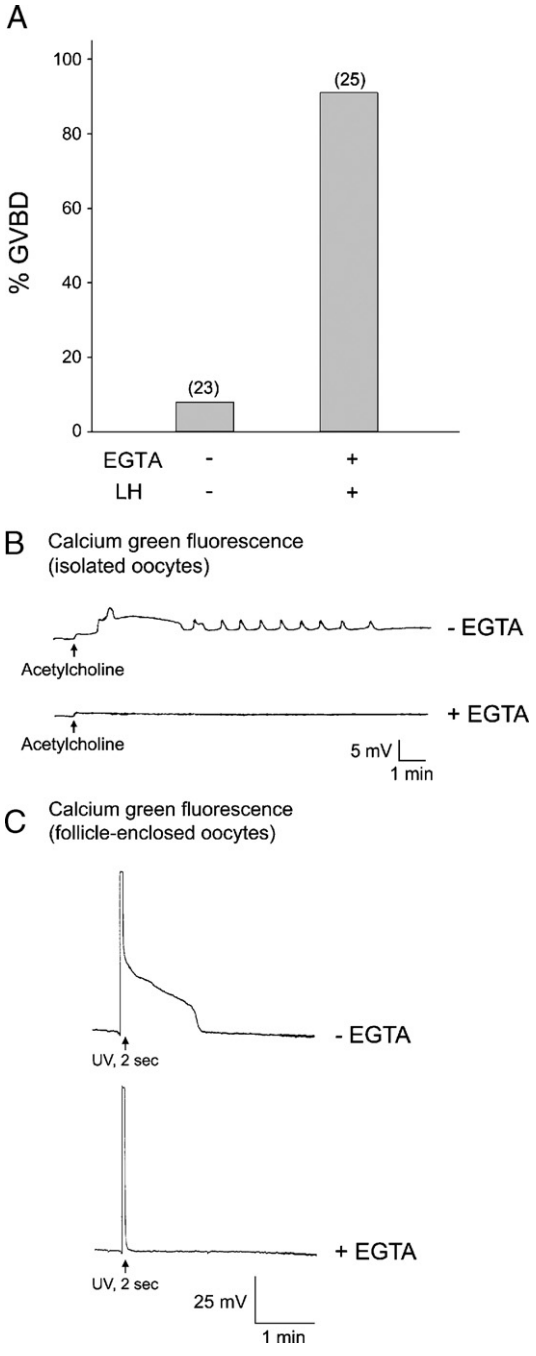
We confirmed that 10 mM EGTA effectively inhibited a rise in Ca^{2+} in the oocyte in two ways. First, we expressed the m1 acetylcholine receptor in isolated oocytes, and tested the effect of EGTA on the Ca^{2+} elevation seen in response to addition of acetylcholine (Williams et al., 1998). In 7/7 oocytes that were not injected with EGTA, application of acetylcholine caused an elevation of Ca^{2+}, consisting of an initial plateau of several minutes duration, followed by a series of oscillations lasting 10–30 s (Fig. 3B). However, in oocytes that had been injected 3–6 h previously with 10 mM of EGTA buffer, acetylcholine application caused no Ca^{2+} rise ($n=7$) (Fig. 3B). These control

experiments showed that 10 mM EGTA is an effective chelator of Ca^{2+} in isolated mouse oocytes, and that EGTA does not leak out through the oocyte plasma membrane over a several h period.

We next tested the possibility that in a follicle-enclosed oocyte, EGTA might leak out by way of the gap junctions with the surrounding cumulus cells. To do this, we injected antral follicle-enclosed oocytes with 10 mM EGTA buffer, 20 μM calcium green dextran, and 1 μM caged IP_3, incubated them for 3–4 h, and then uncaged the IP_3 by a 2-s exposure to 330± 40 nm light. In control oocytes without EGTA, uncaging of IP_3 caused a transient Ca^{2+} rise, but in the presence of EGTA, little or no Ca^{2+} elevation was seen in response to uncaging the IP_3 (Fig. 3C). Although it is possible that some EGTA left the oocyte under these conditions, enough remained in the oocyte to prevent a Ca^{2+} rise in response to IP_3. These experiments show that EGTA is an effective Ca^{2+} chelator, even in follicle-enclosed oocytes.

Our finding that injection of 10 mM EGTA does not inhibit the resumption of meiosis in response to LH indicates that a rise in oocyte Ca^{2+} is not required to mediate the LH signal. One caveat is that EGTA/Ca^{2+} binding has a time constant of $\sim 10^{-4}$ s (see below), so events lasting $<10^{-3}$ s (1 ms) would not be prevented by 10 mM EGTA. Such events would have to be 10,000 times shorter in duration than previously reported Ca^{2+} transients in mouse oocytes (see Introduction).

Further support for the conclusion that oocyte Ca^{2+} elevation does not transduce the LH signal was obtained by measuring calcium green fluorescence in follicle-enclosed oocytes during LH exposure. Fluorescence was measured at 1-s intervals. Although 4/4 oocytes had resumed meiosis after 3 h, Ca^{2+} transients were not detected during this time.



BAPTA toxicity

In an attempt to rule out a possible role for submillisecond Ca^{2+} transients in mediating the LH response, as well as to confirm our EGTA results, we considered the use of another Ca^{2+} chelator, BAPTA. For suppressing Ca^{2+}-dependent responses in cells, BAPTA is more effective, if the Ca^{2+}-dependent responses occur on a time scale of less than 1 ms. This is because the kinetics of Ca^{2+} binding are slower for EGTA than for BAPTA; at 10 mM, the time constant for Ca^{2+}/EGTA binding is ~67 μs, compared to ~0.17 μs for Ca^{2+}/BAPTA binding (Adler et al., 1991). Thus, BAPTA is more effective than EGTA for suppressing the submillisecond

Fig. 3. Elevation of oocyte Ca^{2+} does not mediate LH stimulation of meiotic resumption. (A) EGTA does not inhibit the LH response in follicle-enclosed oocytes. Follicle-enclosed oocytes were injected with EGTA buffer (10 mM EGTA in the cytoplasm, free Ca^{2+} ~30 nM), and exposed to 1 μg/ml LH for 3–4 h. The oocytes were then isolated to determine %GVBD. The numbers in parentheses indicate the number of oocytes counted for each point, combining the results of 3 experiments. Column 2 is statistically different from column 1 ($p<0.0001$) but is not different from column 3 of Fig. 2 ($p=0.6$) (Fisher's exact test). In a separate series of experiments, we found that injection of 10 mM of the EGTA buffer did not cause a significant increase in meiotic resumption (20% GVBD, $n=15$). (B) EGTA is an effective inhibitor of Ca^{2+} elevation in isolated mouse oocytes. Isolated oocytes expressing the m1 acetylcholine receptor were injected with 20 μM calcium green dextran (upper trace), or 20 μM calcium green dextran+10 mM of EGTA buffer (lower trace). Calcium green fluorescence was recorded before and after addition of 10 μM acetylcholine. (C) EGTA is an effective inhibitor of Ca^{2+} elevation in antral follicle-enclosed mouse oocytes. Antral follicle-enclosed oocytes were injected with 1 μM caged IP_3 and 20 μM calcium green 10 kDa–dextran, with or without 10 mM EGTA buffer. 3–4 h later, IP_3 was uncaged by exposing the follicle-enclosed oocytes to 330±40 nm light (UV), for a period of 2 s. In oocytes that had not been injected with EGTA, uncaging of IP_3 resulted in an increase in calcium green fluorescence ($n=6$ oocytes). In oocytes that had been injected with 10 mM EGTA buffer, 6/9 oocytes showed no detectable increase in calcium green fluorescence in response to uncaging IP_3, and 3 others showed a small increase (~10%, or in one case ~20%, of that seen without EGTA). Note that the measurement systems used for B and C differed, so the y axes cannot be directly compared.

processes leading to synaptic vesicle exocytosis, but for suppressing the slower Ca^{2+}-dependent event of synaptic facilitation, which occurs on a time scale of 100 ms, BAPTA is, for incompletely understood reasons, somewhat less effective (Rozov et al., 2001). In sea urchin eggs, EGTA and BAPTA are equally effective at suppressing the Ca^{2+} rise at fertilization, another example of a relatively slow process, with the rise to peak occurring over an ~30-s period (Swann et al., 1992). A disadvantage of BAPTA is that it is more hydrophobic than EGTA, and is thus less likely to remain free in the cytosol; after injection into muscle cells (Kurebayashi et al., 1993) or egg cells (Gillot and Whitaker, 1993), fluorescent BAPTA derivatives have been found to become compartmentalized, thus reducing their cytosolic concentration.

In initial experiments, we found that injection of 10 mM BAPTA into isolated oocytes that were held in meiotic arrest by including dbcAMP in the medium, caused the oocyte morphology to become abnormal (Fig. 4, upper panel). The shape of the oocytes became irregular, rather than spherical, and their diameters decreased somewhat. Granular inclusions that were larger than normal appeared in the cytoplasm. These structural changes in BAPTA-injected oocytes did not occur in oocytes that were injected with 10 mM EGTA (Fig. 4, lower panel). These observations are consistent with the toxicity that has been noted for frog oocytes that were injected with BAPTA at concentrations >1 mM (Sun and Machaca, 2004); BAPTA-induced changes in morphology and endoplasmic reticulum structure have also been seen in sea urchin eggs (L.A. Jaffe and M. Terasaki, unpublished results).

In parallel with the deleterious effects of 10 mM BAPTA on mouse oocyte morphology, we observed that 10 mM BAPTA delayed the spontaneous resumption of meiosis that occurs when dbcAMP is removed from isolated oocytes. Whereas 13/14 uninjected oocytes, and 5/7 oocytes injected with 10 mM EGTA, underwent nuclear envelope breakdown by 1 h after washout of dbcAMP, the BAPTA-injected oocytes resumed meiosis much more slowly (0/8 at 3 h, 4/8 at 5 h). Due to the structural changes associated with this concentration of BAPTA, the BAPTA-induced delay in meiotic resumption could not be attributed to Ca^{2+} chelation.

In an attempt to avoid the toxicity of 10 mM BAPTA, we reduced its concentration to 1 mM. However, at this concentration, BAPTA was insufficient to inhibit the Ca^{2+} elevation in response to m1 acetylcholine receptor stimulation ($n=3$ oocytes). Thus, we concluded that for mouse oocytes, the concentration of BAPTA needed for effective Ca^{2+} chelation was <10 times the concentration where toxicity was seen, and based on this, we decided against using BAPTA for investigating the role of Ca^{2+} in meiotic regulation.

We also considered the use of a form of BAPTA that was coupled to dextran, in order to prevent compartmentalization. For this purpose, we used the BAPTA derivative calcium green coupled to 3 kDa dextran (0.92 Ca green: 1 dextran). The maximum solubility of this compound in an aqueous buffer was found to be ~170 mg/ml (~50 mM). A 10% injection of this solution resulted in 17 mg/ml (5 mM) in the cytoplasm of follicle-enclosed oocytes. However, 5 oocytes that were injected with this solution all showed an abnormal wrinkled morphology, precluding its use for investigating the calcium dependence of LH action. The cause of the toxicity of calcium green dextran at this high concentration is unknown, but at 17 mg/ml, dextran alone could conceivably have a deleterious effect.

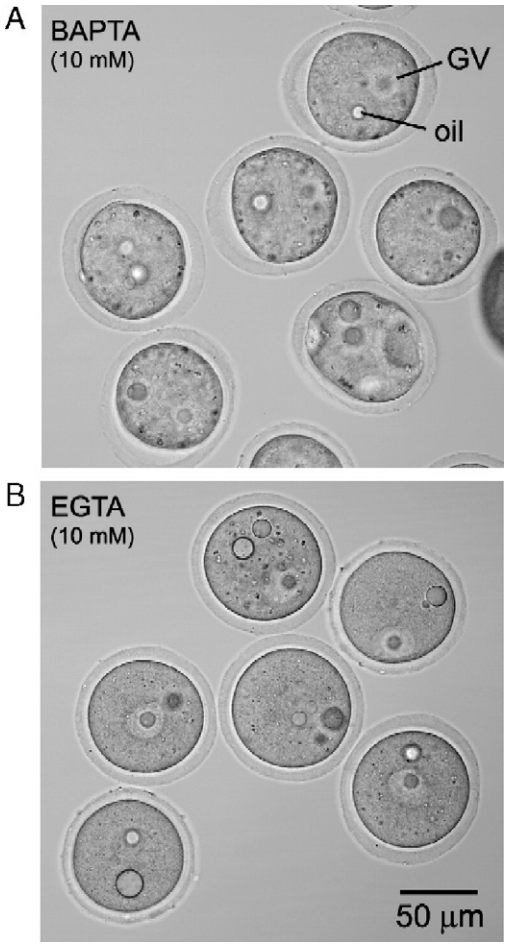


Fig. 4. Deleterious effect of BAPTA on oocyte structure. Mouse oocytes, which were held in meiotic arrest with 250 µM dbcAMP, were injected with BAPTA (upper panel) or EGTA (lower panel), at a cytoplasmic concentration of 10 mM (~30 nM free Ca^{2+}). The oocytes were photographed 3–4 h after injection.

These experiments indicate that of the available Ca^{2+} chelators, EGTA is optimal for intracellular use, at least in oocytes, unless submillisecond events are critical. Coupling BAPTA to a single sugar molecule, rather than a dextran polymer, might lower its membrane permeability, without an excessive increase in molecular size, but at present such compounds are not available.

Discussion

The concentration of cAMP, which controls the progression of meiosis from prophase to metaphase in the mouse oocyte, is regulated by an adenylate cyclase, AC3, that produces it, and a phosphodiesterase, PDE3A, that degrades it (Fig. 5). For a cell within a tissue, surrounding cells connected by gap junctions could also be a source or sink for cAMP (Bedner et al., 2006;

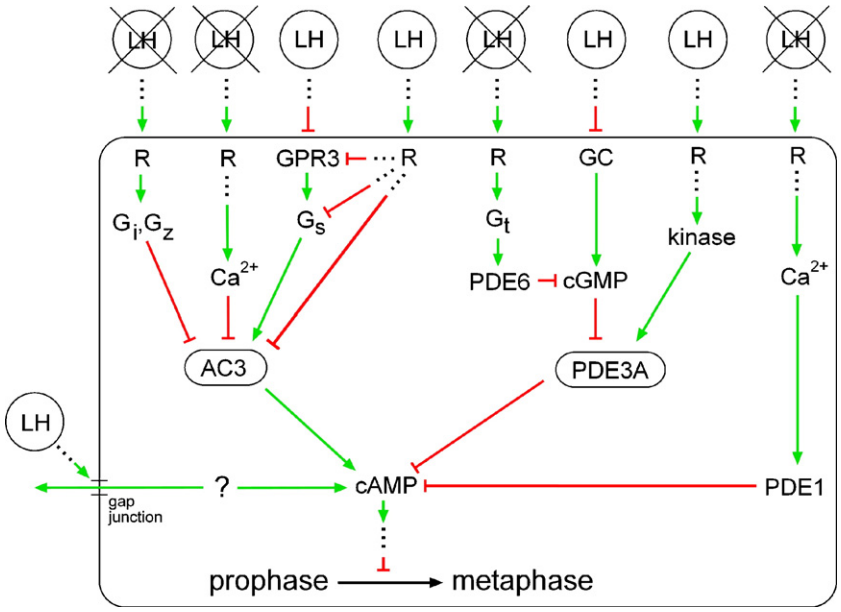


Fig. 5. Some of the possible pathways by which luteinizing hormone (LH) action on the somatic cells of the mammalian ovarian follicle could decrease cAMP in the oocyte, leading to the progression of meiosis. Of the pathways shown, experiments described in this paper argue against those marked with X's; others remain to be tested. R=receptor; GPR3=G protein receptor 3; AC3=adenylate cyclase 3; GC=guanylate cyclase; G_i, G_z, G_s, G_t=G protein isoforms; PDE1, PDE3A, PDE6=cyclic nucleotide phosphodiesterase isoforms. Green arrows indicate stimuli that increase the activity or amount of the indicated molecule; red lines indicate stimuli that decrease the activity or amount of the molecule. Pathways occurring in the somatic cells of the follicle, including signaling through the LH receptor, production of EGF receptor ligands, and the possible role of a steroid, are not shown (see Introduction and Discussion).

Fig. 5). However, in the case of the follicle-enclosed mouse oocyte, the permeability of these junctions to cAMP has not been definitively determined (see Introduction).

In this study, we investigated whether a G_i family G protein or Ca^{2+}, molecules that could directly or indirectly regulate adenylate cyclases or cAMP phosphodiesterases in the oocyte, are required in the pathway by which luteinizing hormone causes meiotic resumption. We tested these hypotheses by injecting follicle-enclosed mouse oocytes with pertussis toxin, which inhibits G_i, or with EGTA, which chelates Ca^{2+}, and determining if these agents inhibited meiotic resumption in response to LH. Our findings showed that although both PTX and EGTA were effective inhibitors of their respective targets in the oocyte, neither inhibited LH stimulation of the prophase-to-metaphase transition.

The LH that was used for these studies was ~98% pure (see Materials and methods), and thus the meiotic resumption observed in the presence of PTX and EGTA is very unlikely to be accounted for by a contaminant in the LH preparation. In particular, follicle stimulating hormone can also cause meiotic resumption in isolated follicles at concentrations similar to those required for LH action (Törnell et al., 1995), but the LH preparations that we used contained <0.06% FSH. Thus, a 1 μg/ml solution of LH contained <0.6 ng/ml FSH, a level that is well below that needed to cause meiotic resumption.

These results argue against some of the possible pathways by which LH action on the somatic cells of the ovarian follicle might lower oocyte cAMP (Fig. 5). In particular, they indicate that LH does not act by way of a receptor in the oocyte membrane that is linked to cAMP through a G_i family G protein or Ca^{2+}.

Other possibilities for how LH may reduce cAMP and reinitiate meiosis in the mouse oocyte

An LH-induced decrease in the permeability of gap junctions connecting the somatic cells and the oocyte has been proposed as a possible means by which LH action could lower oocyte cAMP, based on the idea that prior to LH action, cAMP could be entering the oocyte from the somatic cells. However, current evidence that cAMP generated within the oocyte is necessary to maintain prophase arrest favors the possibilities that LH regulates oocyte cAMP either by lowering the synthesis of cAMP, or by increasing its degradation (see Introduction). Thus, possible targets of LH action include inhibition of the GPR3/G_s pathway that constitutively activates adenylate cyclase, and/or stimulation of cAMP phosphodiesterase activity (Fig. 5). It is also possible that LH action could result in *increased* cAMP permeability of the gap junctions between the oocyte and the cumulus cells, such that cAMP produced in the oocyte would be depleted by efflux into the surrounding cumulus cells.

In frog oocytes, where a steroid (testosterone or progesterone) mediates the LH stimulation of meiotic resumption (Fortune, 1983), the steroid inhibits adenylate cyclase in the oocyte (see Ferrell, 1999). Progesterone also slows the rate of guanine nucleotide release from frog oocyte membranes, which is consistent with a decrease in G_s activity (Sadler and Maller, 1983). Conflicting results have been obtained as to whether a steroid, such as testosterone or FF-MAS, does or does not mediate LH stimulation of meiosis in mammalian oocytes (Tsafriri et al., 1972, 2005; Faerge et al., 2001; Jamnongjit et al., 2005). Whether LH decreases G_s and/or adenylate cyclase activity in mouse oocytes is unknown, but this could occur if

LH caused the synthesis of an inverse agonist that inhibited the constitutive activity of GPR3, analogous to the agouti-related protein that inhibits the constitutive activity of the melanocortin 4 receptor (Chai et al., 2003). LH could also inhibit the GPR3/G_s/adenylate cyclase system by way of increasing the amount or activity of proteins such as RGS2, GRK's, and β-arrestins, which can inhibit signaling by G protein-coupled receptors, G_s, and adenylate cyclase (Reiter and Lefkowitz, 2006; Roy et al., 2006; Zou et al., in press).

In frog oocytes, progesterone application does not produce a measurable increase in cAMP phosphodiesterase activity; however, insulin, which is also a promoter of the prophase-to-metaphase transition, does stimulate phosphodiesterase activity, and this effect is enhanced by progesterone (Sadler and Maller, 1987). Because testosterone and progesterone levels in frog ovarian follicles increase in response to LH (Fortune, 1983), and because the LH response is inhibited by inhibitors of progesterone synthesis (see Fortune, 1983), a steroid is considered to be a natural mediator of LH action on frog oocytes. Whether insulin is produced in response to LH is unknown. It is also unknown whether LH causes an increase in cAMP phosphodiesterase activity in frog oocytes.

In mammalian oocytes, however, there is evidence that LH increases PDE3A activity (Richard et al., 2001). One way that LH could increase PDE3A activity in the oocyte would be by lowering cGMP, because cGMP inhibits PDE3A (Törnell et al., 1990; Hambleton et al., 2005). Although cGMP levels in mammalian oocytes following LH stimulation have not been examined, cGMP inhibits cAMP phosphodiesterase activity in mouse oocyte extracts (Bornslaeger et al., 1984). In addition, the decrease in PDE3A activity that is seen after rat oocyte isolation (Richard et al., 2001) is accompanied by a decrease in oocyte cGMP (Törnell et al., 1990). Another possibility for how LH could lead to stimulation of oocyte cAMP phosphodiesterase activity is by way of a pathway involving phosphorylation and activation of oocyte PDE3A (Andersen et al., 1998, 2003; Elbatarny and Maurice, 2005) (see Fig. 5).

How extracellular stimuli control the progression of the cell cycle has been a long-standing question, and the mammalian ovarian follicle provides an exceptional opportunity to investigate this issue in the context of an intact tissue. In particular, as we have shown here, follicle-enclosed oocytes can be microinjected with membrane- and gap junction-impermeant inhibitors and indicators, and then exposed to LH, thus allowing direct tests of the function of signaling molecules in the oocyte, within the regulatory environment of the surrounding somatic cells. These experiments have provided definitive evidence against LH action requiring activation of a G_i family G protein, or elevation of Ca^{2+} in the oocyte, and have also defined some of the technical challenges of investigating single cell physiology within a complex biological tissue.

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