

Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH

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Abstract

The meiotic cell cycle in mouse oocytes is arrested in prophase, and then restarted when LH acts on the surrounding granulosa cells. The granulosa cells keep meiosis arrested by providing a source of cGMP that diffuses into the oocyte through gap junctions, and LH restarts the cell cycle by closing the junctions and by decreasing granulosa cell cGMP, thus lowering oocyte cGMP. Epidermal growth factor receptor (EGFR) activation is an essential step in triggering LH-induced meiotic resumption, but its relationship to the cGMP decrease in the follicle is incompletely understood, and its possible function in causing gap junction closure has not been investigated. Here, we use EGFR agonists (epiregulin and amphiregulin) and an EGFR kinase inhibitor (AG1478) to study the function of the EGFR in the signaling pathways leading to the release of oocytes from prophase arrest. Our results indicate that the EGFR kinase contributes to LH-induced meiotic resumption in two different ways. First, it is required for gap junction closure. Second, it is required for an essential component of the decrease in follicle cGMP. Our data show that the EGFR kinase-dependent component of the cGMP decrease is required for LH-induced meiotic resumption, but they also indicate that an as yet unidentified pathway accounts for a large part of the cGMP decrease.

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Introduction

The mammalian ovarian follicle is comprised of multiple layers of granulosa cells enclosing a central oocyte, which is arrested in prophase of meiosis until LH acts on the outer granulosa cells to restart the cell cycle (Eppig *et al.* 2004, Jaffe & Norris 2010). In the mouse preovulatory follicle, all of the granulosa cells and the oocyte are interconnected by gap junctions (Norris *et al.* 2008), and the presence of the granulosa cells with open gap junctions is essential for maintaining the oocyte in meiotic prophase arrest. If the oocyte is removed from the follicle (Pincus & Enzmann 1935), or if the gap junctions are closed pharmacologically (Piontkewitz & Dekel 1993, Sela-Abramovich *et al.* 2006, Norris *et al.* 2008), the oocyte resumes meiosis.

The gap junctions provide a direct connection by which cGMP enters the oocyte from the granulosa cells, and in the oocyte, cGMP inhibits phosphodiesterase 3A, thus keeping cAMP high and maintaining meiotic arrest (Norris *et al.* 2009, Vaccari *et al.* 2009). cAMP is generated in the oocyte by a constitutively active G_s-linked receptor, GPR3 in mice or GPR12 in rats (Mehlmann *et al.* 2004, Hinckley *et al.* 2005, Ledent

et al. 2005, Mehlmann 2005). High cAMP in the oocyte maintains meiotic arrest at least in part by activating protein kinase A, which phosphorylates the phosphatase CDC25B; this results in sequestration of CDC25B in the cytoplasm, such that it cannot dephosphorylate and activate the kinase CDK1 in the nucleus, which directly controls the prophase-to-metaphase transition (Oh *et al.* 2010). CDK1 activity also depends on a regulatory cyclin B1 subunit, and by unknown means, high cAMP prevents the accumulation of cyclin B1 in the nucleus (Marangos & Carroll 2004, Holt *et al.* 2010).

LH receptor activation in the outer granulosa cells acts to reverse the inhibition of CDK1 in the oocyte by reducing the influx of cGMP from the granulosa cells into the oocyte. LH reduces cGMP influx both by causing the gap junctions to close (Sela-Abramovich *et al.* 2006, Norris *et al.* 2008) and by causing cGMP in the granulosa cells to decrease (Norris *et al.* 2009, Vaccari *et al.* 2009; see also Davis *et al.* 1986, Hubbard 1986, Törnell *et al.* 1991). Both of these LH actions have the consequence of lowering cGMP in the oocyte, thus lowering cAMP, activating CDK1, and restarting the meiotic cell cycle, which is characterized by the breakdown of the nuclear envelope between 2 and 4 h after LH exposure. LH acts

on a G-protein-coupled receptor (Jaffe & Norris 2010), but how this results in gap junction closure and the cGMP decrease in the mouse ovarian follicle is not well understood.

One important link between LH receptor activation in the outer granulosa cells and meiotic resumption in the oocyte is the rapid synthesis (Park *et al.* 2004) and proteolytic release from the granulosa cell surface (Ashkenazi *et al.* 2005, Andric & Ascoli 2008, Panigone *et al.* 2008) of the epidermal growth factor receptor (EGFR) ligands epiregulin and amphiregulin. EGFR kinase activity in the follicle increases by 30 min after LH application (Panigone *et al.* 2008). Treatment of isolated follicles with EGFR ligands causes meiosis to resume (Dekel & Sherizly 1985, Park *et al.* 2004, Ashkenazi *et al.* 2005), and AG1478, which inhibits EGFR kinase activity (Levitzki & Gazit 1995, Panigone *et al.* 2008), inhibits meiotic resumption in response to LH (Park *et al.* 2004, Ashkenazi *et al.* 2005). In addition, follicles from mice that express a mutated EGFR kinase with reduced activity, or that lack the EGFR ligands epiregulin or amphiregulin, show reduced meiotic resumption in response to LH (Hsieh *et al.* 2007). EGFRs are located on both mural granulosa and cumulus cells, but not on the oocyte (Downs *et al.* 1988, Park *et al.* 2004, Panigone *et al.* 2008, Su *et al.* 2010), supporting the concept that the release of epiregulin and amphiregulin from the outer granulosa cells, where the LH receptors are located, functions to propagate the LH signal into the follicle interior.

A previous study has shown that activation of the EGFR with amphiregulin or epiregulin lowers granulosa cell cGMP, and that the decrease in granulosa cell cGMP in response to LH can be reduced by inhibiting the EGFR kinase with AG1478 (Vaccari *et al.* 2009). Here, we further investigate the function of the EGFR kinase in causing the cGMP decrease, and also examine its function in causing gap junction closure. Our data indicate that while EGFR kinase activity is required for the LH-induced closure of gap junctions, an as yet unidentified pathway accounts for a large part of the cGMP decrease. However, the EGFR kinase-dependent component of the cGMP decrease is required for LH-induced meiotic resumption.

Results and Discussion

Magnitude and time course of the cGMP decrease in response to LH

Two previous studies have shown that cGMP in isolated mouse follicles decreases in response to LH, but the basal cGMP concentrations and the magnitudes of the decrease differed (Norris *et al.* 2009, Vaccari *et al.* 2009). Therefore, to establish a baseline for subsequent experiments, we determined the magnitude and time course of the cGMP decrease upon LH application for the experimental conditions used here. Measurements

were made using an ELISA method, and concentrations were calculated using an estimated value of 20 nl for the cellular volume of an isolated follicle (see Materials and Methods). Because the volume was not known exactly, the following concentrations should be considered approximations. The mean value for basal cGMP was $\sim 3 \mu\text{M}$, and this decreased to $\sim 500 \text{ nM}$ at 20 min after LH application; cGMP then continued to decrease, reaching $\sim 100 \text{ nM}$ at 1 h and $\sim 30 \text{ nM}$ at 5 h (Fig. 1).

The measurements reported here as well as those in our previous study (Norris *et al.* 2009) indicate that at 1 h after LH application, GMP has decreased to $\sim 4\%$ of the basal level, whereas another study (Vaccari *et al.* 2009) reported a smaller decrease to 39%. In addition, while we found that most of the cGMP decrease had occurred by 20 min after LH application, Vaccari *et al.* first detected the cGMP decrease at 1 h, with no change seen at 30 min. Interestingly, a study of rat granulosa cells in primary culture showed a decrease in cGMP as early as 3 min after applying LH (Davis *et al.* 1986).

Activation of the EGFR causes a decrease in follicle cGMP

To investigate to what extent the EGFR kinase contributes to the LH-induced decrease in follicle cGMP, we first measured cGMP in response to an EGFR ligand. The concentrations of epiregulin and amphiregulin proteins in the extracellular space of the follicle after LH exposure are unknown, so we used a concentration of epiregulin (100 nM) that has been previously found to maximally stimulate nuclear envelope breakdown when applied to isolated follicles (Park *et al.* 2004). We confirmed the previous finding that 100 nM epiregulin stimulates meiotic resumption to the same extent as LH (Fig. 2). Approximately 90% of oocytes underwent nuclear envelope breakdown by 4 h.

Some but not all of the cGMP decrease that occurred in response to LH also occurred when follicles were treated with epiregulin. The mean concentration of cGMP was $\sim 600 \text{ nM}$ following a 1 h treatment with 100 nM epiregulin versus $\sim 100 \text{ nM}$ cGMP in response to LH (Fig. 3A).

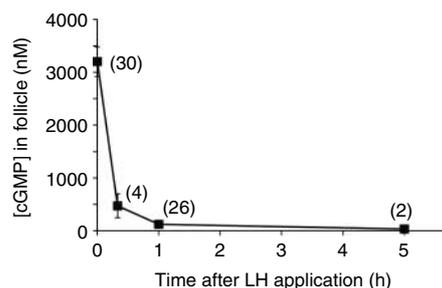


Figure 1 cGMP in mouse ovarian follicles at various times after applying LH. Bars indicate mean \pm s.e.m., and numbers in parentheses indicate the number of samples.

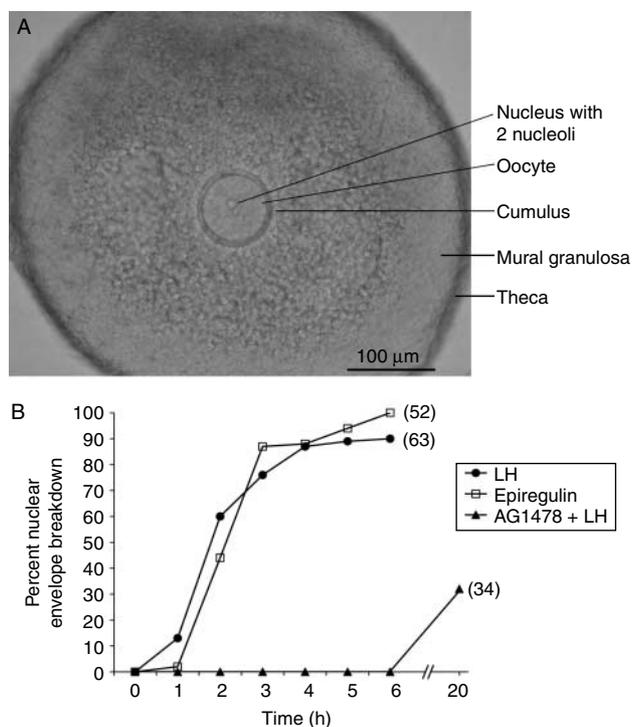


Figure 2 Observation of the time course of nuclear envelope breakdown in intact follicle-enclosed oocytes in response to an EGFR agonist (epiregulin), or to LH in the presence or absence of an EGFR kinase inhibitor (AG1478). (A) A mouse follicle on a Millicell membrane; the prophase-arrested nucleus and two nucleoli are visible. (B) Nuclear envelope breakdown at various times after treatment of follicles with LH (350 nM), epiregulin (100 nM), or AG1478 (500 nM) for 1 h followed by AG1478+LH. Numbers in parentheses indicate the number of follicles.

The EGFR kinase inhibitor AG1478 only partially inhibits the cGMP decrease in response to LH

To examine whether EGFR kinase activation is required for LH stimulation of the cGMP decrease, we used the selective EGFR kinase inhibitor AG1478. *In vitro*, AG1478 inhibits EGFR kinase activity with an IC_{50} of 3 nM (Levitzki & Gazit 1995). In follicles stimulated with LH, 500 nM AG1478 completely inhibits LH-induced EGFR phosphorylation on tyrosine 1068, one of several tyrosines that are phosphorylated in response to EGFR activation; this indicates that LH does not detectably increase EGFR activation under these conditions (Panigone *et al.* 2008).

As previously reported (Park *et al.* 2004, Ashkenazi *et al.* 2005), AG1478 had an inhibitory effect on nuclear envelope breakdown in response to LH. Under our conditions, 500 nM AG1478 completely inhibited nuclear envelope breakdown in response to LH when observed at 6 h, although ~30% of oocytes had resumed meiosis when observed at 20 h (Fig. 2B). The meiosis-inhibitory effect of AG1478 is on signaling in the granulosa cells, rather than the oocyte, since AG1478 does not inhibit spontaneous meiotic resumption in

isolated oocytes (Park *et al.* 2004, and Supplementary Figure 1, see section on supplementary data given at the end of this article).

At a concentration of 500 nM, AG1478 had only a partial inhibitory effect on the LH-induced cGMP decrease (Fig. 3A). At 1 h after applying LH in the presence of AG1478, cGMP had decreased to ~500 nM, corresponding to ~15% of the basal level. This was significantly different from the ~100 nM level attained at 1 h after LH

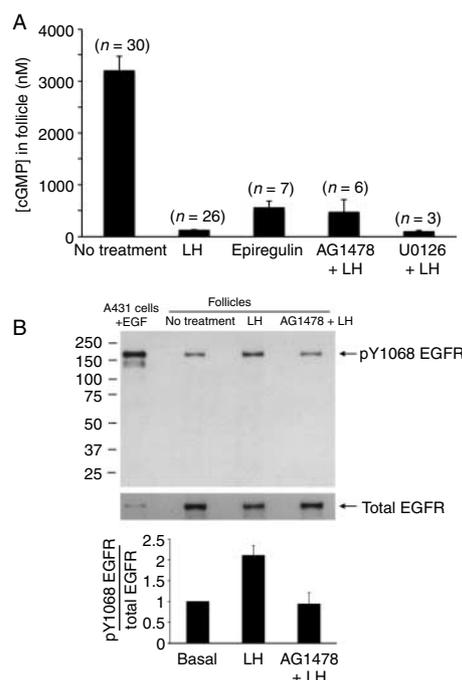


Figure 3 cGMP in follicles under experimental conditions affecting EGFR kinase or MAP kinase activity. (A) Measurement of follicle cGMP under the following conditions: no treatment (basal), 1 h LH (350 nM), 1 h epiregulin (100 nM), 1 h AG1478 (500 nM) followed by 1 h AG1478+LH, 1 h U0126 (10 μ M) followed by 1 h U0126+LH. Bars indicate mean \pm S.E.M., and numbers in parentheses indicate the number of samples. The cGMP concentration after 100 nM epiregulin treatment is significantly different from that for basal and LH conditions ($P < 0.0001$). Likewise, the cGMP concentration after AG1478+LH treatment is significantly different from that for basal ($P < 0.0001$) and LH ($P = 0.01$) conditions. The cGMP concentration after U0126+LH treatment is not significantly different from that after LH alone ($P = 0.7$), but is significantly different from the basal concentration ($P = 0.0004$). (B) Demonstration that in follicles stimulated with LH, treatment with 500 nM AG1478 as described above reduces EGFR phosphorylation on tyrosine 1068 to a level that is indistinguishable from the basal level without LH. Twenty micrograms of follicle lysates and 2 μ g of EGF-treated AG431 cells were used for immunoblotting with an antibody specific for pY1068 EGFR and then with an antibody specific for total EGFR. The graph shows the ratio of the intensities of the ~170 kDa band measured with the two antibodies, normalized to the no treatment condition (mean \pm S.D. for two independent experiments). Since the follicles were cultured for 24 h in the absence of EGFR ligands or serum prior to preparing the samples, the basal signal seen with the pY1068 antibody is probably not due to EGFR kinase activity; it could result from phosphorylation of the EGFR by other kinases or to cross reactivity of the antibody with non-phosphorylated EGFR.

in the absence of AG1478. However, the presence of AG1478 did not restore cGMP to the basal level of $\sim 3 \mu\text{M}$ measured in the absence of LH; the values for the basal cGMP concentration and that at 1 h after LH+500 nM AG1478 were significantly different (Fig. 3A).

The incomplete inhibition of the LH-induced cGMP decrease by AG1478 did not appear to be due to incomplete inhibition of the EGFR kinase activity, since 500 nM AG1478 prepared from the same stock solution as used for the cGMP measurements reduced LH-stimulated phosphorylation of the EGFR on tyrosine 1068 to a level that was indistinguishable from the basal level without LH (Fig. 3B). Thus, although epiregulin is sufficient to cause $\sim 80\%$ of the cGMP decrease in the follicle in response to LH, EGFR activity is not required for most of the LH-induced cGMP decrease. These findings support the concept that two separate and partially redundant mechanisms contribute to the decrease in cGMP in response to LH.

Inhibition of the activation of MAP kinase with U0126 has no inhibitory effect on the cGMP decrease in response to LH

One important consequence of EGFR kinase activation in the ovarian follicle is the activation of MAP kinase (Panigone *et al.* 2008). Therefore, we investigated the effect of inhibiting MAP kinase activation on the LH-induced cGMP decrease in the follicle. To do this, we used U0126, an inhibitor of the kinase (MAP kinase kinase, or MEK) that activates MAP kinase. U0126 at a concentration of $10 \mu\text{M}$, which has previously been demonstrated to fully inhibit MAP kinase activation in

response to LH (Norris *et al.* 2008), did not have any inhibitory effect on the cGMP decrease in response to LH (Fig. 3A). Thus, the cGMP decrease in response to LH, including its EGFR kinase-dependent component, occurs by a pathway other than MAP kinase activation. In contrast, $10 \mu\text{M}$ U0126 completely inhibits gap junction closure in response to LH (Norris *et al.* 2008).

Activation of the EGFR causes gap junction closure

To investigate whether gap junction closure in response to LH is mediated by activation of the EGFR kinase, we first compared gap junction permeability after applying epiregulin or LH. Gap junction permeability was assessed by injecting a fluorescent tracer into the oocyte, and 20 min later, measuring the ratio of fluorescence in the mural granulosa cells over that in the inner cumulus cells (Norris *et al.* 2008). Compared to the ratio in untreated follicles, 1 h treatments with LH and 100 nM epiregulin caused decreases to 9 and 32% of the basal level respectively (Fig. 4). Even when the concentration of EGFR agonist was increased 20 times by applying $1 \mu\text{M}$ epiregulin+ $1 \mu\text{M}$ amphiregulin, gap junction closure was less complete than that observed with LH application (Fig. 4), but it is unknown how well externally applied EGFR agonists penetrate throughout the follicle. Our results suggest that the stimulation of meiotic resumption by externally applied EGFR ligands (see Introduction and Fig. 2) is a consequence of partial closure of gap junctions and a partial decrease in granulosa cell cGMP, acting together to lower oocyte cGMP.

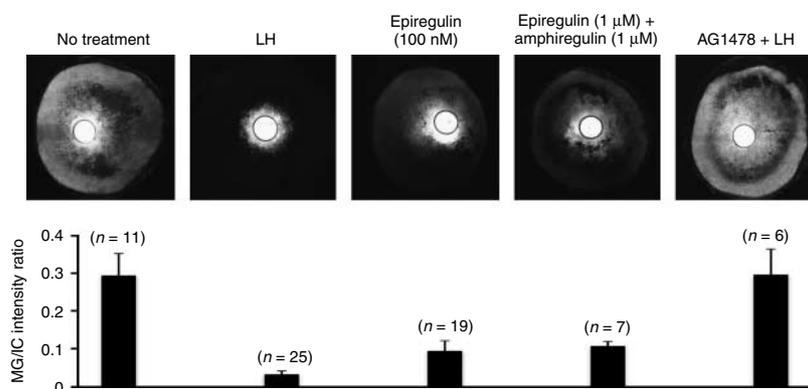


Figure 4 Gap junction communication in follicles under experimental conditions affecting EGFR kinase activity. Gap junction communication was assessed by injecting the oocyte with Alexa Fluor 350 and imaging the follicle 20 min later. (A) Images of follicles that were treated as follows prior to injection of the fluorescent tracer: no treatment (basal), 1 h LH (350 nM), 1 h epiregulin (100 nM), 1 h epiregulin (1 μM) + amphiregulin (1 μM), 1 h AG1478 (500 nM) followed by 1 h AG1478 + LH. (B) Ratios of fluorescence in the mural granulosa cells over that in the inner cumulus cells (MG/IC), at 20 min after injection, for each of the conditions indicated in (A). Bars indicate mean \pm S.E.M., and numbers in parentheses indicate the number of follicles. The MG/IC ratio after treatment with 100 nM epiregulin is significantly different from that after LH ($P=0.05$) and from the basal ratio ($P=0.005$). Likewise, the MG/IC ratio after treatment with 1 μM epiregulin + 1 μM amphiregulin is significantly different from that after LH ($P=0.0003$); the difference from the basal ratio is marginally significant ($P=0.06$). The MG/IC ratio for AG1478 + LH is significantly different from that for LH ($P<0.0001$), but not from the basal ratio ($P=1.0$).

The EGFR kinase inhibitor AG1478 inhibits gap junction closure in response to LH

We next examined the effect on LH-induced gap junction closure of inhibiting the EGFR kinase with AG1478. In the presence of 500 nM AG1478, gap junction permeability after LH application was indistinguishable from that in an untreated follicle (Fig. 4). It should be noted, however, that partial closure of gap junctions might not have been detected by the method used here. These results indicate that EGFR activation is an essential factor in causing gap junction closure in response to LH, but leave open the possibility that other signaling pathways contribute as well. The conclusion that EGFR activation is an important factor in causing gap junctions to close in response to LH is supported by a recent report that ovaries from mice with reduced EGFR activity show reduced phosphorylation of gap junction protein alpha 1 (GJA1, also known as connexin 43, CX43) in response to LH (Andric *et al.* 2010).

An alternative interpretation of these results is that non-specific effects of AG1478 could contribute to its inhibitory effect on gap junction closure (and on the cGMP decrease). An extensive characterization of the specificity of AG1478 has not been previously published, but information on this question was made available by the National Centre for Protein Kinase Profiling (MRC Protein Phosphorylation Unit, University of Dundee, Scotland, UK). The IC_{50} for AG1478 was $>1 \mu\text{M}$ for 101 out of the 110 kinases tested (see Supplementary Table 1, see section on supplementary data given at the end of this article). Although *in vivo* inhibitory concentrations cannot be predicted from the *in vitro* data, the *in vitro* inhibitory concentrations for all of these other kinases were much greater than that for the EGFR ($IC_{50}=3 \text{ nM}$, Levitzki & Gazit 1995). Importantly, the MAP kinases ERK1 and ERK2 were not inhibited by AG1478. Thus, although non-specific effects have not been absolutely excluded, the actions of 500 nM AG1478 on the LH-induced gap junction closure and follicle cGMP decrease are most likely due to inhibition of the EGFR kinase.

Comparison with previous studies

The results described here indicate that the EGFR kinase contributes to LH-induced meiotic resumption both by causing gap junction closure and by causing an essential component of the decrease in follicle cGMP. They also indicate that an as yet unidentified pathway accounts for a large part of the cGMP decrease. Although both this study and a previous study (Vaccari *et al.* 2009) support the conclusion that the EGFR kinase has a role in causing the cGMP decrease in response to LH, the two studies differ on the issue of whether EGFR activation is solely responsible. While we found that 500 nM AG1478 had only a partial inhibitory effect on the

cGMP decrease, the Vaccari *et al.* study reported that at 500 nM, AG1478 completely inhibited the cGMP decrease in response to LH.

The causes of the differences in some of the results reported here and by Vaccari *et al.* are unknown, but differences in the strain of mouse, the methods of follicle culture, and/or other methodological differences could be critical. One possible factor is that the mean value for basal cGMP reported here ($\sim 60 \text{ fmol/follicle}$) was somewhat greater than that in the Vaccari *et al.* study ($\sim 18 \text{ fmol/follicle}$), possibly due to the methods of sample preparation. While Vaccari *et al.* froze the follicles, and only after defrosting processed them to inactivate cGMP phosphodiesterases, we prepared samples from unfrozen follicles. In preliminary experiments in which we used frozen follicles, our measurements of basal cGMP were $\sim 10\%$ of the values obtained in subsequent experiments with follicles processed without prior freezing (Supplementary Figure 2, see section on supplementary data given at the end of this article). Possible loss of cGMP during sample processing could contribute to why Vaccari *et al.* observed complete inhibition of the LH-induced cGMP decrease by AG1478. If a non-EGFR kinase-dependent cGMP decrease occurred during sample preparation, it could mask a non-EGFR kinase-dependent component of the cGMP decrease that occurs in response to LH. Likewise, such an effect could contribute to the smaller percent decrease in follicle cGMP in response to LH reported by Vaccari *et al.* and to why an early decrease in cGMP was not detected. Other unknown factors may also be important.

Despite these differences, our data support the general conclusion that EGFR signaling is important for both pathways leading to meiotic resumption in response to LH. The physiological significance of the EGFR kinase-dependent component of the cGMP decrease is indicated by comparing the effects of U0126 and AG1478 on responses to LH. Both inhibitors prevent gap junction closure (Fig. 4, and Norris *et al.* 2008), but only AG1478 has an inhibitory effect on the cGMP decrease (Fig. 3A). Correspondingly, LH causes meiotic resumption in the presence of U0126 (Su *et al.* 2003, Norris *et al.* 2008), but not in the presence of AG1478 (see Introduction and Fig. 2). Thus, to decrease cGMP sufficiently to cause meiotic resumption, EGFR signaling is essential.

Unanswered questions

Activation of MAP kinase causes the closure of gap junctions in response to LH by phosphorylating GJA1 (Sela-Abramovich *et al.* 2005, Norris *et al.* 2008), and MAP kinase activation in the follicle in response to LH depends strongly, but not entirely, on EGFR kinase activity (Panigone *et al.* 2008). Likewise, GJA1 phosphorylation depends strongly on EGFR kinase activity (Andric *et al.* 2010). These results indicate that the EGFR

kinase acts to cause gap junction closure by initiating a signaling pathway that results in MAP kinase phosphorylation of GJA1. However, MAP kinase activation does not account for the cGMP decrease in response to LH-induced EGFR activation, since the LH-induced decrease in cGMP in the follicle occurs independently of MAP kinase. It remains to be determined how other consequences of EGFR kinase activation contribute to the cGMP decrease in the follicle (Fig. 5).

Our findings support the conclusion that pathways other than EGFR kinase activation also function to cause the LH-induced cGMP decrease in the follicle. In particular, while the LH-induced rise in cAMP in the mural granulosa cells leads to EGFR activation (Panigone *et al.* 2008), cAMP elevation may have other EGFR-independent effects. It also remains to be determined whether other EGFR-independent consequences of the

activation of G-proteins by LH are required for the cGMP decrease, and how these pathways lead to inhibition of a guanylyl cyclase and/or activation of a cGMP phosphodiesterase in the follicle (Fig. 5). Finally, much remains to be learned about how activation of the LH receptor causes the synthesis of epiregulin and amphiregulin precursors and the proteolytic cleavage of these peptides from the granulosa cell surface, leading to the activation of the EGFR (Fig. 5).

Materials and Methods

Hormones and reagents

FSH (ovine) and LH (ovine) were obtained from A F Parlow (National Hormone and Peptide Program, Torrance, CA, USA). Epiregulin and amphiregulin (mouse) were obtained from R&D Systems (Minneapolis, MN, USA). Alexa Fluor 350 was obtained from Invitrogen (#A10439). AG1478 and U0126 were obtained from EMD Chemicals (La Jolla, CA, USA) and were dissolved in DMSO at 10 and 100 mM respectively; aliquots were stored at -80°C .

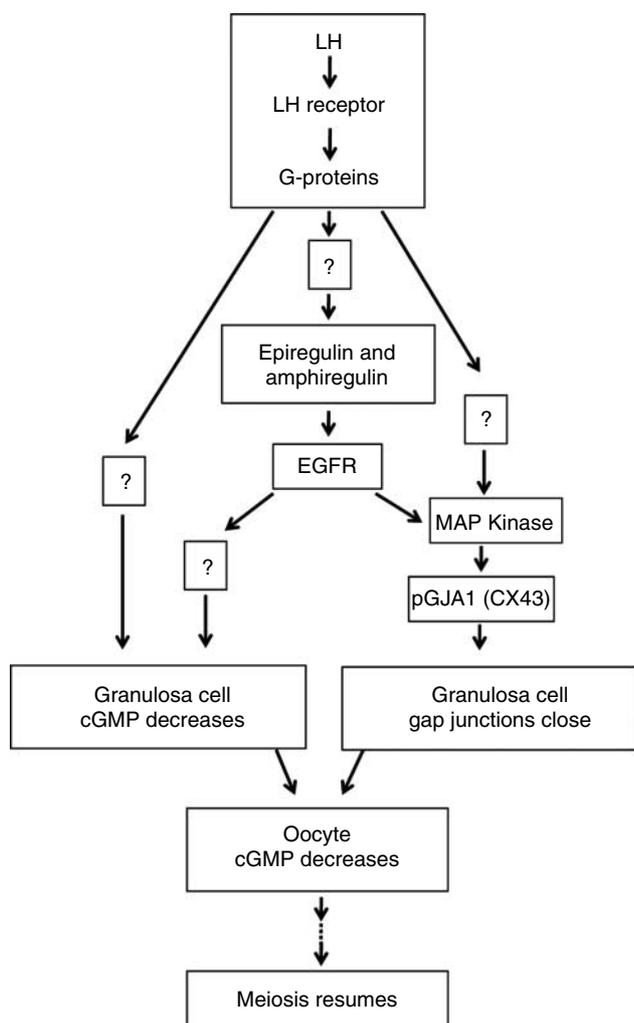
Mice and follicle culture

B6SJLF1 mice, 23–24 days old, were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). All procedures involving mice were approved by the University of Connecticut Health Center Animal Care Committee. The culture medium was as previously described (Norris *et al.* 2008), except that the MEM α (#41061029, Invitrogen) contained no phenol red, and contained 3 mg/ml BSA (MP Biomedicals, Solon, OH, USA) instead of fetal bovine serum. Antral follicles with diameters from ~ 320 to ~ 400 μm were dissected and placed on Millicell culture plates (PICMORG50, Millipore, Billerica, MA, USA) with 12–16 follicles per plate, at 37°C , with 5% CO_2 . The follicles were cultured for 24–32 h before use to allow the expression of LH receptors in response to the FSH in the medium, and to allow the follicles to flatten on the Millicell membranes, improving their optical clarity.

Under these conditions, we could easily see the oocyte nucleus with its one or two nucleoli through the follicle wall by observing the culture dish from the top with a $20\times/0.4$ NA long working distance objective (LD Achromplan #44 08 44; Carl Zeiss Inc., Thornwood, NY, USA; Fig. 2A). Follicles were checked at 24 h after dissection, and only those that were healthy and prophase-arrested were used for experiments. For determination of the time course of meiotic resumption, we excluded any experiment in which $<70\%$ of control follicles treated with LH had undergone nuclear envelope breakdown at 5 h, since this was considered to be an indication that unknown aspects of the culture conditions were not optimal on that day. LH was used at a concentration of 350 nM (10 $\mu\text{g}/\text{ml}$).

cGMP ELISA assays

cGMP in follicles was assayed using an ELISA kit (#CG200, Sigma–Aldrich), as described by Norris *et al.* (2009). To prepare the samples, 12–20 follicles on a Millicell membrane were



washed twice with calcium- and magnesium-free PBS (#10010-023, Invitrogen) and then transferred from the Millicell membrane to a microfuge tube. A volume of 100 μ l of 0.1 M HCl was added, and the follicles were lysed by applying \sim 30 pulses with a probe sonicator (model 60, Thermo Fisher Scientific, Waltham, MA, USA). In some cases, 1% Triton X-100 was added to the 0.1 M HCl in order to lyse the follicles more rapidly. The samples were then heated at 95 $^{\circ}$ C for 2 min and stored at -80° C until assayed.

The approximate cGMP concentration in the follicle was calculated based on an estimated volume of 20 nl for the cellular space of a follicle having an average diameter of 360 μ m (Norris *et al.* 2009). Of the 24 nl calculated for a sphere of this diameter, a few nanolitres are occupied by extracellular antral space. It should also be noted that mechanically dissected follicles have an \sim 10–20 μ m thick layer of adhering theca cells and debris that is not part of the follicle itself; this \sim 2–4 nl volume was disregarded. For these reasons, the absolute cGMP concentrations presented in the figures and text should be considered as approximate values.

Immunoblotting

Follicle samples for immunoblotting were prepared as previously described (Norris *et al.* 2008). A431 cells treated with EGF (#sc-2202; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as a positive control. Proteins were separated by SDS-PAGE (7.5% gel) and transferred to a PVDF membrane. Blots were probed with an antibody specific for pY1068 EGFR (#3777; Cell Signaling Technology, Beverly, MA, USA; 1:1000 dilution in Tris-buffered saline with 0.1% Tween-20 and 5% BSA) and processed for development with ECL-Plus (GE Healthcare, Piscataway NJ, USA). The blots were stripped with Restore western blot stripping buffer (Thermo Fisher Scientific) and probed with an antibody for total EGFR (#06-847, Millipore; 10 ng/ml in Tris-buffered saline with 0.1% Tween-20 and 5% BSA). Films were scanned, and the intensity of the \sim 170 kDa band was measured using ImageJ software (<http://rsbweb.nih.gov/ij/>). The use of a different antibody against pY1068 EGFR (#2234; Cell Signaling Technology) showed an effect on the \sim 170 kDa band that was similar to that shown in Fig. 3B.

Gap junction measurements

Gap junction permeability was assessed by microinjecting the follicle-enclosed oocyte with 2.5 mM of a 326 kDa fluorescent tracer, Alexa Fluor 350, and imaging the distribution of fluorescence in the follicle at 20 min after injection, using two-photon microscopy (Norris *et al.* 2008). The ratio of fluorescence in the mural granulosa cells over that in the inner cumulus cells was used as a measure of gap junction communication (Norris *et al.* 2008).

Statistical analyses

Statistical analyses were performed using the Mann–Whitney test function of InStat 3 for Macintosh (GraphPad Software, Inc., La Jolla, CA, USA).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0288>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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