Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles

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Meiosis in mammalian oocytes is paused until luteinizing hormone (LH) activates receptors in the mural granulosa cells of the ovarian follicle. Prior work has established the central role of cyclic GMP (cGMP) from the granulosa cells in maintaining meiotic arrest, but it is not clear how binding of LH to receptors that are located up to 10 cell layers away from the oocyte lowers oocyte cGMP and restarts meiosis. Here, by visualizing intercellular trafficking of cGMP in real-time in live follicles from mice expressing a FRET sensor, we show that diffusion of cGMP through gap junctions is responsible not only for maintaining meiotic arrest, but also for rapid transmission of the signal that reinitiates meiosis from the follicle surface to the oocyte. Before LH exposure, the cGMP concentration throughout the follicle is at a uniformly high level of ∼2–4 μM. Then, within 1 min of LH application, cGMP begins to decrease in the peripheral granulosa cells. As a consequence, cGMP from the oocyte diffuses into the sink provided by the large granulosa cell volume, such that by 20 min the cGMP concentration in the follicle is uniformly low, ∼100 nM. The decrease in cGMP in the oocyte relieves the inhibition of the meiotic cell cycle. This direct demonstration that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions could provide a general mechanism for diverse cellular processes.

cyclic GMP | gap junctions | ovarian follicle | oocyte meiosis | luteinizing hormone

Meiosis in mammalian oocytes begins during embryonic development and then arrests in late prophase, for up to 50 y in women and for many months in mice. At the time of ovulation, luteinizing hormone (LH) acts on the granulosa cells of the follicle surrounding the oocyte to release the arrest and restart meiosis in preparation for fertilization (1–3). In the mouse preovulatory follicle, inhibition of meiotic progression is dependent upon the cyclic nucleotide cyclic GMP (cGMP), which diffuses from the granulosa cells into the oocyte through gap junctions that connect all cells of the follicle (4–6). The cGMP is produced by the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also known as guanylyl cyclase B), which is present in all of the granulosa cells, but not in the oocyte (7–11). In the oocyte, cGMP inhibits the degradation of another cyclic nucleotide, cAMP, which depends primarily on the phosphodiesterase PDE3A, an enzyme whose activity is antagonized by cGMP (4, 5). The resulting high level of cAMP, through a series of intermediate steps, inhibits meiotic progression (2, 3, 12).

LH signaling is initiated in the outer (mural) layers of granulosa cells; receptors for LH are absent in the oocyte and in the granulosa cells that directly surround it (the cumulus cells) (13–15). Ensuing events cause meiosis to resume by reducing cGMP in the oocyte (4, 5), but how LH receptor activation up to 10 cell layers away lowers oocyte cGMP is uncertain. LH signaling reduces gap junction permeability (16, 17), but meiosis can resume without the permeability decrease (17), arguing against gap junction closure as a primary mechanism for transmitting the signal. LH signaling also reduces cGMP in the follicle as a whole (4, 5, 18–20), suggesting that the decrease in oocyte cGMP is a consequence of the fall in cGMP in the large volume around the oocyte, to which it is connected by gap junctions (21).

Alternatively, recent work has suggested that the LH signal is transmitted to the oocyte by regulation of the release from the mural granulosa cells of peptides that diffuse through the extracellular space to the cumulus cells. In support of this concept, LH signaling decreases the ovarian content of the NPR2 agonist C-type natriuretic peptide, thus decreasing cGMP in the cumulus–oocyte complex (22); however, levels of this peptide decrease only after the decrease in oocyte cGMP (9, 20). LH signaling also increases the ovarian content of the EGF receptor ligands epiregulin and amphiregulin (23), and by a pathway that is not well understood, EGF receptor activation in isolated cumulus–oocyte complexes lowers their cGMP content (24). However, it is unknown if the increases in epiregulin and amphiregulin occur fast enough to cause the initial decrease in oocyte cGMP.

One missing link in understanding how LH lowers oocyte cGMP is precise information on the kinetics of the cGMP decreases in the oocyte and other regions of the follicle. Within 20 min after applying LH to isolated mouse follicles, the cGMP content of the whole follicle decreases from ∼2–4 μM to ∼100 nM

Significance

By imaging cyclic GMP (cGMP) in live ovarian follicles from mice, we show how luteinizing hormone signaling in the follicle periphery results in a rapid decrease in cGMP in the oocyte, thus reinitiating meiosis. Luteinizing hormone signaling lowers cGMP in the outer cells of the follicle, then cGMP in the oocyte decreases as a consequence of diffusion through gap junctions. These findings demonstrate directly that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions.

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(4, 19, 20) (Fig. S1*A*). This decrease occurs as a consequence of dephosphorylation and inactivation of the NPR2 guanylyl cyclase; activation of cGMP phosphodiesterases may also contribute (9, 11) (*Discussion*). By 1 h, cGMP in the oocyte decreases similarly, as measured with a fluorescent sensor of cGMP, cGi500, injected into the follicle-enclosed oocyte (4). However, the cGMP signals that occur in living follicles upon LH treatment have not been monitored except in the oocyte, and not before 1 h (4). Here we investigate how LH signaling causes the oocyte to resume meiosis by determining the spatiotemporal dynamics of the decrease in cGMP in the living follicle, and by examining whether gap junction permeability is needed for LH to lower cGMP in the cumulus–oocyte complex.

Results

The LH-Induced cGMP Decrease Occurs Sequentially in the Mural Granulosa Cells, Cumulus Cells, and Oocyte. Using antral follicles from mice that globally express the cGi500 sensor for cGMP (Fig. 1*A*) (25), we measured cGMP levels in mural granulosa, cumulus, and oocyte regions before and after addition of LH. Binding of cGMP to cGi500 decreases FRET between CFP and YFP, such that the CFP/YFP emission ratio measured after CFP excitation indicates cGMP concentration; the EC_{50} of cGi500 for cGMP is 500 nM (25–27), which is appropriate for detection of cGMP in the range of concentrations in mouse follicles before and after LH treatment. ELISA measurements of the cGMP content of follicles from cGi500-expressing mice showed an LH-induced decrease (Fig. S1*A*), and follicle-enclosed oocytes from these mice underwent nuclear envelope breakdown in response to LH with a normal time course (Fig. S1*B*). The cGi500-expressing follicles, which were spheres 320–400 μm in diameter when dissected, flattened to disks ∼200 μm in thickness after culture on an organotypic membrane. The follicles were imaged by confocal microscopy in a 200-μm-deep glass-bottomed chamber, with the focus at the oocyte equator, before and after perfusion of LH (Fig. 1 and Movie S1).

In the mural granulosa cells, cumulus cells, and oocyte, the CFP/YFP ratios before LH treatment were similar, indicating that the cGMP concentration was uniform in all parts of the follicle (Figs. 1*B*, *Left*, and 2 *A*–*D*). Similarly, at 20 min after LH treatment, the ratio values in the three compartments had decreased to the same plateau level (Figs. 1*B* and 2 *A* and *C*, and Fig. S2*A*), and remained at that level at 2 h (Fig. 2*D*). No change was seen with perfusion of control medium (Fig. 2*B* and Fig. S2*B*). However, the time for cGMP to decrease was greater in interior regions of the follicle (Figs. 1*B* and 2*A*, and Movie S1). In the mural granulosa cells, 10% of the decrease in CFP/YFP ratio had occurred at approximately 1 min after LH application, and 50% of the decrease had occurred at 2.8 ± 0.3 min (mean ± SEM, $n = 16$) (Fig. 2 *A*, *E*, and *F*). In the cumulus cells, 10% of the decrease had occurred at ∼5 min, and 50% of the decrease had occurred at 7.8 ± 0.4 min (Fig. 2 *A*, *E*, and *F*). In the oocyte, 10% of the decrease had occurred at ∼7 min, and 50% of the decrease had occurred at 9.9 ± 0.4 min (Fig. 2 *A*, *E*, and *F*). cGMP levels in the theca cells, located outside of the basal lamina of the follicle, remained at a constant low level before and after LH exposure (Fig. 1*B*).

The Rapid Decrease in cGMP in the Cumulus Cells Occurs via Outward Diffusion of cGMP Through Gap Junctions. To examine whether the LH-induced cGMP decrease in the cumulus cells occurs by diffusion through gap junctions, we preincubated follicles with carbenoxolone, which inhibits gap junction communication within the follicle (17). Carbenoxolone treatment itself had no effect on the cGMP level in the mural granulosa and cumulus cells (Fig. 3*B*). However, as previously reported (4), carbenoxolone treatment lowered cGMP in the oocyte, as a consequence of disconnecting it from the mural granulosa and cumulus cells where cGMP is produced (Fig. 3 *A* and *B*). Correspondingly, carbenoxolone treatment causes meiosis to resume (17).

With carbenoxolone present, LH caused cGMP to decrease in the mural granulosa cells, but not in the cumulus cells, during the initial 20 min (Fig. 3 *A* and *C*). Thus, only the cells in which LH

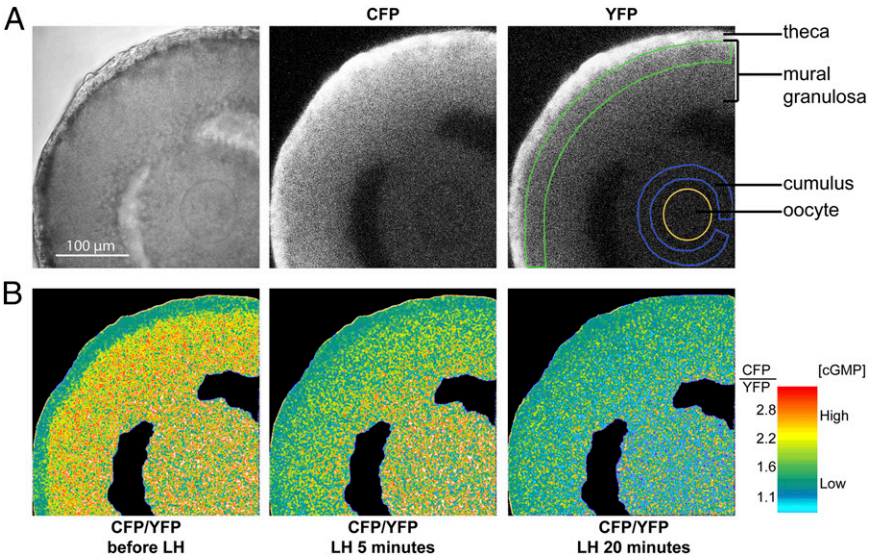

Fig. 1. LH receptor activation initiates an inwardly propagating cGMP decrease in the mouse ovarian follicle. (*A*) Isolated follicle expressing the cGi500 sensor for cGMP, showing a scanning transmission image (*Left*), CFP fluorescence (*Center*), and YFP fluorescence with the regions of measurement indicated (*Right*). (*B*) Images of the CFP/YFP ratio before LH perfusion, and at 5 and 20 min afterward, for the follicle shown in *A*. Before LH application, cGMP is at a uniformly high level throughout the follicle. cGMP in the surrounding theca cells is lower; the theca cells are not connected by gap junctions to the granulosa cells (17). After LH application, cGMP decreases first in the mural granulosa cells, then in the cumulus cells and oocyte, reaching a plateau at the same value in all regions. Movie S1 shows this time series. Based on ELISA measurements in wild-type follicles, the cGMP concentration before LH application is ∼4 μM, and the plateau value after LH is ∼100 nM (Fig. S1). No change in cGMP occurs in the theca cells.

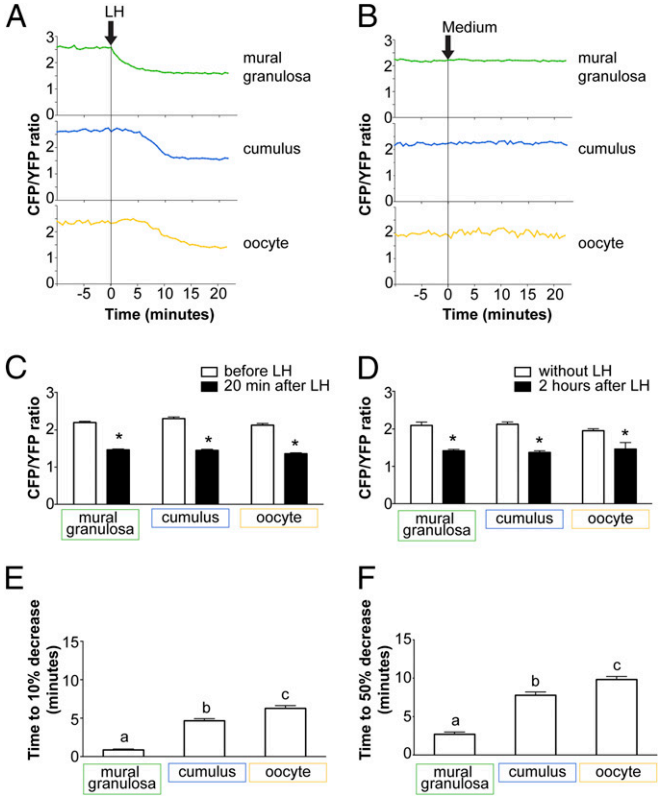

Fig. 2. Kinetics of the LH-induced cGMP decrease in mural granulosa, cumulus, and oocyte. (*A*) Time courses of the CFP/YFP ratios for the follicle shown in Fig. 1. For this and other graphs, ratios were calculated by dividing the mean CFP intensity in each region of interest, as shown in Fig. 1*A*, by the mean YFP intensity. (*B*) Recording from a follicle perfused with control medium without LH (representative of four experiments). (*C* and *D*) CFP/YFP ratios for mural granulosa cells, cumulus cells, and oocyte, before and at 20 min (*C*) or 2 h (*D*) after treatment with LH (16–20 follicles for each condition for *C*; 3–6 follicles for each condition for *D*). (*E* and *F*) Time to 10% or 50% of the decrease in CFP/YFP ratio in each region; results from 15 sets of measurements. Values that are indicated by an asterisk are significantly different from the control, and values not indicated by the same letter are significantly different from each other ($P < 0.05$); values indicate mean ± SEM.

receptors were present (the mural granulosa) showed a cGMP decrease. The lack of propagation of the cGMP decrease to the cumulus cells in the carbenoxolone-treated follicles indicates that after LH exposure, gap junction communication with the mural granulosa cells is needed for the rapid cGMP decrease to occur in the cumulus cells. However, by 2 h gap junction-independent signaling contributes to maintaining a low level of cGMP in the cumulus cells (Fig. 3*D*). This slower gap junction-independent cGMP decrease most likely results from EGF receptor ligands released from the mural granulosa cells acting on the cumulus cells (5, 19, 20), by an unknown pathway, and from a decrease in the NPR2 agonist C-type natriuretic peptide (9, 20, 22). Thus, different processes are responsible for the initial cGMP decrease in the cumulus cells, and for maintenance of low cGMP in the cumulus cells at later time points. Once cGMP in the mural granulosa and cumulus cells decreases, cGMP in the small volume of the oocyte (~0.2 nL) equilibrates with that in the large volume of the follicle (~20 nL), to which the oocyte is connected by gap junctions (17, 28).

Diffusion of cGMP Out of the Cumulus Cells Precedes the LH-Induced Decrease in Gap Junction Permeability. In response to LH, the permeability of the connexin-43 gap junctions between the mural granulosa cells and between the mural granulosa and cumulus cells decreases (17), raising the question of whether the permeability decrease occurs on a time scale that would interfere with the diffusion of cGMP from the cumulus–oocyte complex into the mural granulosa cells. The decrease in gap junction permeability occurs by 30–60 min after LH exposure, but earlier time points have not been examined (17). To investigate how rapidly the permeability decreases, we examined the rate of fluorescence redistribution after photobleaching of a small fluorescent tracer in the mural granulosa cells, as previously described (17). No decrease in redistribution rate was detected after a 10-min exposure to LH, indicating no decrease in permeability during this period (Fig. 4 *A* and *B*). Limitations of photobleaching deep in the follicle precluded similar measurements in the cumulus cells (17), but activation of LH receptors in the mural granulosa cells is unlikely to close gap junctions more rapidly in the cumulus cells than in the mural granulosa cells. Phosphorylation of key regulatory serines of connexin-43 that leads to the permeability decrease (29) was detectable by 10 min (Fig. 4*C*), but the resulting change in channel permeability did not occur until after 10 min (Fig. 4 *A* and *B*), after cGMP diffusion out of the cumulus cells is largely complete (Fig. 2*A*). Thus, cGMP diffusion out of the cumulus cells precedes the gap junction permeability decrease, such that diffusion would not be impeded.

Discussion

These results, obtained by imaging live ovarian follicles, show that diffusion of cGMP through gap junctions is responsible not only for maintaining meiotic arrest, but also for rapid transmission from the follicle surface to the oocyte of the hormonal signal that reinitiates meiosis. Before LH exposure, the cGMP

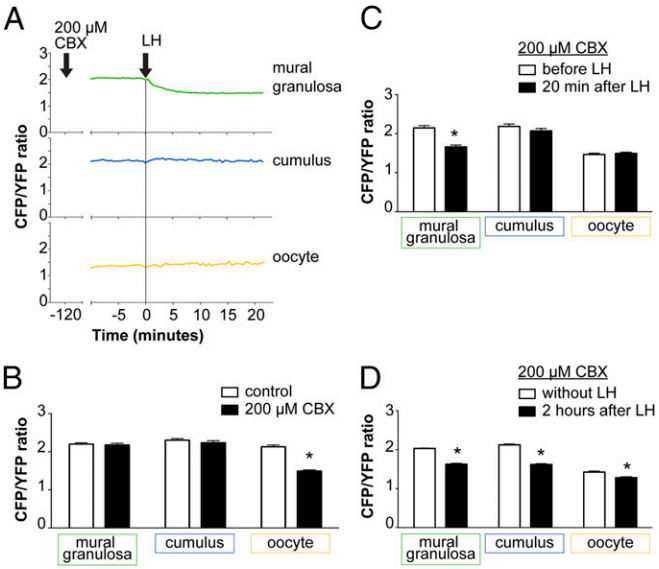

Fig. 3. The initial cGMP decrease in the cumulus cells requires gap junction communication. (*A*) A cGi500-expressing follicle was pretreated for 2 h with 200 μM carbenoxolone (CBX), and then perfused with LH. (*B*) CFP/YFP ratios for mural granulosa cells, cumulus, and oocyte, without LH exposure, for 10 follicles with CBX treatment; these are compared with the 20 control follicles from Fig. 2*C*. (*C* and *D*) CFP/YFP ratios for mural granulosa, cumulus cells, and oocytes, before and at 20 min (*C*) or 2 h (*D*) after treatment with LH, for follicles in the presence of CBX (7–15 follicles for each condition). Because CBX treatment lowers cGMP in the oocyte to approximately the same level attained after LH (*B*), LH treatment of follicles in the presence of CBX caused little further change in cGMP in the oocyte (*A*, *C*, and *D*). Values that are indicated by an asterisk are significantly different from the control ($P < 0.05$); values indicate mean ± SEM.

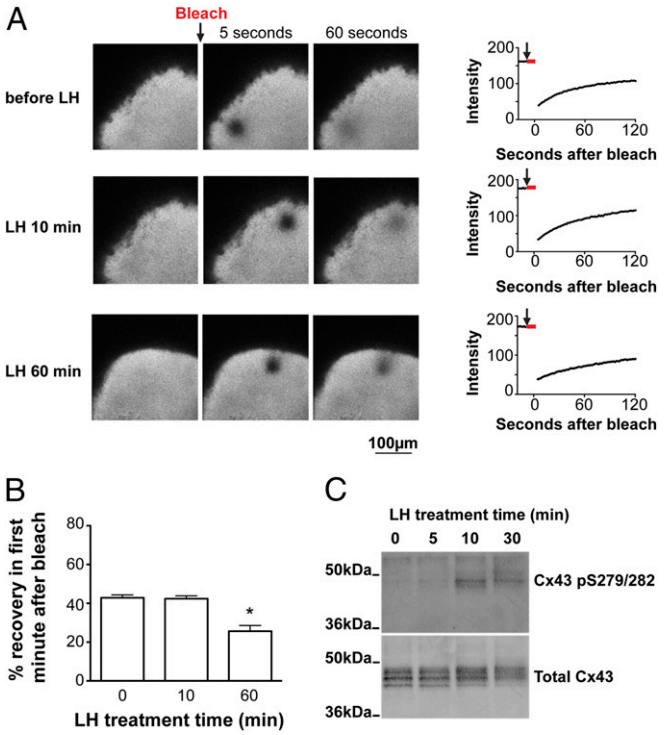

Fig. 4. Kinetics of the LH-induced decrease in gap junction permeability. (*A* and *B*) Fluorescence redistribution after photobleaching of Alexa-488 in the mural granulosa cells. (*A*) shows images of a follicle before photobleaching and at 5 and 60 s afterward, before (*Top*) or 10 min after LH perfusion (*Middle*). The bottom row shows a separate follicle that was photobleached 60 min after LH treatment. The graphs show the time courses of fluorescence recovery. (*B*) The percent recovery in the first minute after bleaching, for eight follicles before and after a 10-min LH treatment, and three follicles after a 60-min LH treatment. (*C*) Kinetics of Cx43 phosphorylation on serines 279 and 282 after applying LH to follicles. Similar results were obtained in another identical experiment. The value indicated by an asterisk is significantly different from the control ($P < 0.05$); values indicate mean ± SEM.

concentration throughout the follicle is at a uniformly high level of ~2–4 μM. Then within 1 min of LH application, cGMP begins to decrease in the mural granulosa cells. As a consequence, cGMP from the oocyte diffuses into the sink provided by the large granulosa cell volume, such that by 20 min the cGMP concentration in the follicle is uniformly low, ~100 nM. The decrease in cGMP in the oocyte relieves the inhibition of the meiotic cell cycle.

The signaling events leading to the rapid decrease in cGMP in the follicle begin with activation of G_s and other G proteins by the LH receptors in the mural granulosa cells (3) (Fig. 5). Through incompletely understood steps, this leads to dephosphorylation and inactivation of the NPR2 guanylyl cyclase, thus reducing the production of cGMP. Because cGMP phosphodiesterases are actively hydrolyzing cGMP in the granulosa cells (5), the decrease in the rate of cGMP production results in a lower equilibrium level of cGMP. In addition, there could be an increase in the activity of the cGMP phosphodiesterase PDE5, as indicated by evidence that PDE5 is phosphorylated (11) and evidence from studies of other cells that phosphorylation of PDE5 is associated with increased activity (30–32). G_s-mediated elevation of cAMP in the mural granulosa cells is very likely a step in this process, because in response to adenylyl cyclase activation by forskolin, cGMP in these cells decreases rapidly, reaching levels comparable to those seen with LH (Fig. S3). LH signaling also leads to activation of the EGF receptor (19, 20, 23), but our results do not indicate a role for EGF receptor activation in the initial decrease in cGMP (Fig. S4).

Previous studies have indicated that gap junction-mediated cyclic nucleotide diffusion can convey signals between pairs of cells (33–35), and the present findings are a direct demonstration that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions. Another example of rapid communication through a complex of cells via diffusion of a cyclic nucleotide through gap junctions occurs in immune cell signaling, where cGMP-AMP diffuses from a cell infected by a virus to neighboring cells, causing them to increase synthesis of interferons (35). With the development of mice expressing cyclic nucleotide sensors (25, 36), it should now be possible to investigate whether signals could be transmitted similarly in other processes in gap junction-coupled tissues where cyclic nucleotides are essential regulators. For example, both cGMP (37) and connexins (38) can suppress tumor growth, suggesting that cGMP diffusion from adjacent cells connected by gap junctions could suppress cell division, as it does in the ovarian follicle. As occurs during hormonal signaling in the ovary, signals that increase or decrease cGMP in the cells surrounding a tumor could, via gap junctions, affect its growth.

Methods

Imaging of cGi500 Fluorescence in Mouse Preovulatory Follicles. Antral follicles were dissected from 23- to 26-d-old transgenic mice [R26-CAG-cGi500(L1)] in which the cGi500 sensor for cGMP was introduced by targeted integration into the *Rosa26* locus and expressed under the control of the CAG promoter (25). All measurements of cGi500 fluorescence were done with heterozygous mice expressing one copy of the cGi500 transgene. All animal protocols were approved by the University of Connecticut Health Center Animal Care Committee. Based on Western blot immunodensity, the concentration of the cGi500 sensor in the follicles of heterozygous mice was ~20 μM (Fig. S5). Before use for imaging, the cGi500-expressing follicles were cultured for 24–30 h on organotypic membranes (Millipore; cat. no. PICMORG50), in the presence of follicle-stimulating hormone (17, 19). For some experiments, follicles were incubated with carbenoxolone (Tocris Bioscience).

Follicles were imaged while held between a plastic slide (ibidi; cat. no. 80161) and a glass coverslip; slides without adhesive were custom ordered from ibidi and assembled using silicon grease. The slide was constructed such that medium containing ovine LH (National Hormone and Peptide Program; 10 μg/mL) could be perfused through a 200-μm-deep channel holding the follicle. Temperature was maintained at 30–34 °C, by use of a warm air blower (Nevtek). Follicles were imaged using a Zeiss Pascal confocal system with a 40×/1.2 numerical aperture C-Apochromat objective with Immersol between the coverslip and objective (Carl Zeiss Microscopy). The excitation laser and emission filters were as previously described (4). The microscope was focused on the oocyte equator, with the confocal pinhole set for an ~14-μm optical section. The laser attenuation was adjusted to avoid saturation. Images were collected using 1.6-s scans at 30-s intervals, for 10 min before LH addition, and for 20 min afterward. Values for ratios "2 hours after LH" were obtained by incubating follicles on an organotypic membrane for 2 h, then placing them in an ibidi slide for measurement. Files were saved as 12-bit images.

Measurements of CFP and YFP emission intensities were from regions as shown in Fig. 1*A*; the mural granulosa region included the 25-μm-wide band just inside the basal lamina, and the cumulus region included the 15-μm-wide zone just outside of the oocyte. Oocyte intensities were measured from a circular region slightly smaller than the oocyte diameter. Measurements were corrected for autofluorescence and for spectral bleed-through of CFP into the YFP channel (4). Values for ratios "before LH" are averages for the 10 measurements before LH addition; values for ratios "20 minutes after LH" are averages of the 10 measurements between 15 and 20 min after LH perfusion through the ibidi slide. Ratios were calculated by dividing the mean CFP intensity in each region of interest by the mean YFP intensity. Data analysis was done using ImageJ and Excel software.

Ratio images shown in Fig. 1*B* and Movie S1 were made using Metamorph software (Molecular Devices) and ImageJ, masking the antral space and the space outside of the follicle. Ratios were calculated by binning measurements of CFP and YFP intensities over 16 pixel regions (2.5 × 2.5 μm), and then dividing the binned values.

Evaluation of Gap Junction Permeability and Connexin-43 Phosphorylation. To evaluate gap junction permeability by fluorescence redistribution after

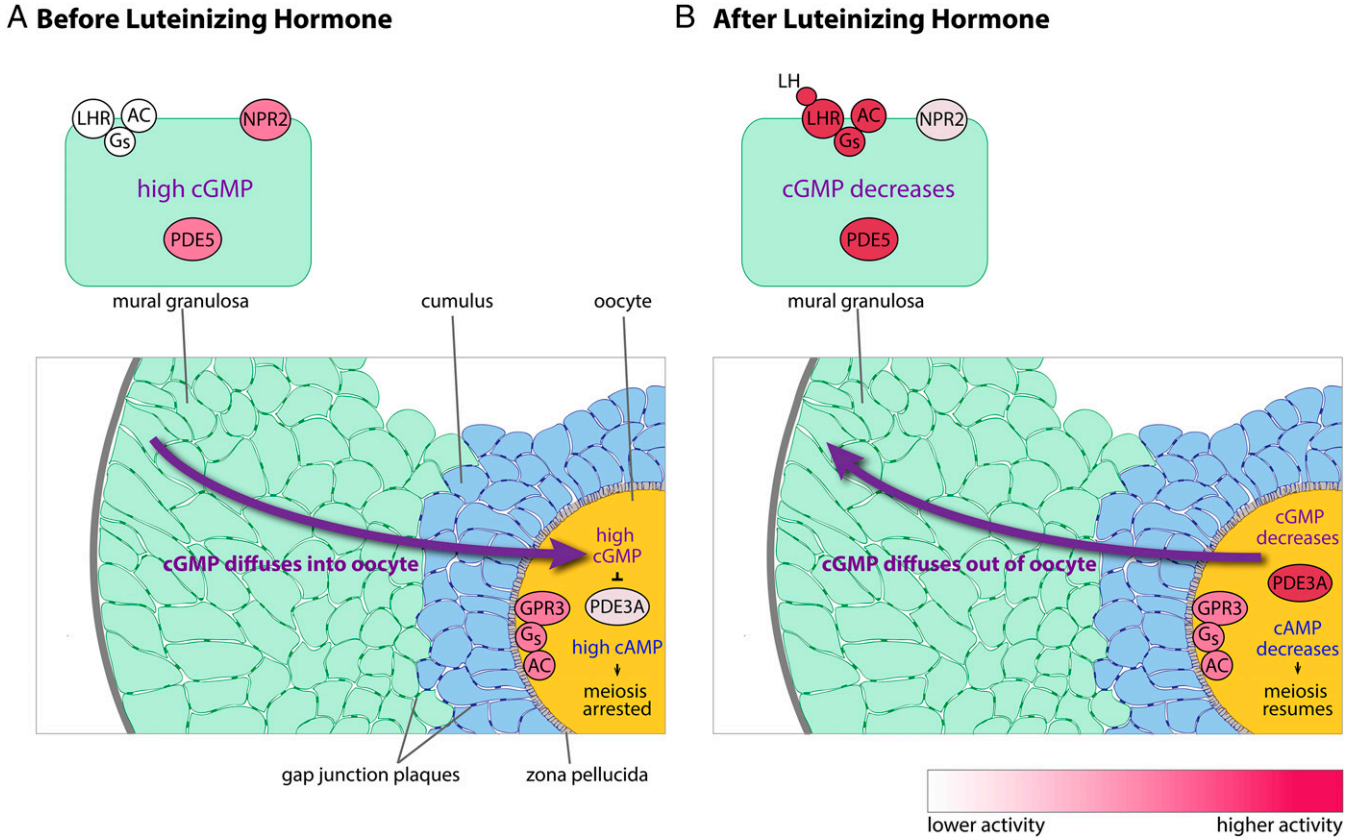

Fig. 5. Working model of how LH signaling rapidly decreases cGMP in the mural granulosa cells, and then via cGMP diffusion through gap junctions, decreases cGMP in the oocyte, leading to meiotic resumption. (*A*) Before LH exposure, cGMP concentrations are elevated throughout the follicle, because of a high rate of production of cGMP by the NPR2 guanylyl cyclase in the mural granulosa and cumulus cells. cGMP phosphodiesterases, including PDE5, degrade cGMP at a rate equal to its production, thus keeping the cGMP concentration at a constant level. Through gap junctions that connect all cells of the follicle, cGMP diffuses into the oocyte, where it inhibits the activity of PDE3A, maintaining cAMP at a level that inhibits meiotic resumption. The cAMP in the oocyte is produced by adenylyl cyclase 3 in the oocyte (39), and AC3 is kept active by the constitutive activity of the G_s-coupled receptor GPR3 (40). (*B*) When LH binds to its receptor in the mural granulosa cells, the activation of G_s and possibly other G proteins results in dephosphorylation of NPR2, which decreases its rate of production of cGMP. Activation of the LH receptor also increases phosphorylation of PDE5, and from studies of other cells, this should increase its rate of degradation of cGMP. Because of reduced NPR2 activity and increased cGMP phosphodiesterase activity, the concentration of cGMP in the mural granulosa cells decreases. Through the series of gap junctions that connects the oocyte to the large volume of the mural granulosa cells, cGMP in the oocyte diffuses down its concentration gradient, and the resulting decrease in oocyte cGMP relieves the inhibition of PDE3A in the oocyte, such that cAMP decreases. This model depicts only events occurring in the first 20 min after LH exposure. Subsequent events, including a decrease in gap junction permeability, an increase in EGF receptor ligands, and a decrease in C-type natriuretic peptide, also contribute to maintaining cGMP at the low level that triggers meiotic resumption. EGF receptor activation may also contribute to the early decrease in cGMP, although findings about this question are variable (Fig. S4). References and further discussion of this model are included in the text.

photobleaching, we loaded follicles with a fluorescent tracer by injecting follicle-enclosed oocytes with Alexa-488 (#A10436, Invitrogen; $M_r = 534$) and incubated the follicles on organotypic membranes for 3–4 h to allow the tracer to spread through gap junctions into the granulosa cells (17). Alexa-488 was used at a stock concentration of 2 mM, resulting in an initial concentration in the oocyte of 100 μM. Follicles were then placed in ibidi slides for FRAP analysis using a Zeiss Pascal confocal microscope, before and 10 min after perfusion of LH. For measurements at 60 min after LH exposure, follicles were exposed to LH before putting them in ibidi slides.

Using a 40×/1.2 NA objective and the 488 line of an Argon laser, we photobleached a 28 × 28-μm square in the mural granulosa cell layer, ∼20 μm below the follicle surface. The photobleaching was accomplished by using a zoom setting of 8. A 10-s laser exposure decreased the fluorescence intensity in the bleached region to ∼20% of the initial value. Postbleach images were collected with the same objective, but with the zoom setting reduced to 0.7 and the laser intensity reduced to 0.2% of that used for bleaching. The confocal pinhole was set for an ∼14-μm optical section, and a 505-nm long-pass filter was used to collect the emitted light; images were collected at 1.6-s intervals, and corrected for minor autofluorescence. These monitoring conditions did not significantly bleach the Alexa-488. To compare the time course of fluorescence redistribution with and without LH, we measured the change in Alexa-488 intensity in the bleached region during the first minute (between 5 and 60 s) after the end of the bleach.

To evaluate the time course of phosphorylation of connexin-43 after LH treatment, follicles were exposed to LH while positioned on an organotypic membrane, then washed in PBS and sonicated in Laemmli sample buffer containing protease and phosphatase inhibitors (17). For 5-min samples, the wash procedure was started at 3.5 min, and sample buffer was added at 5 min. For 10-min samples, the wash procedure was started at 8.5 min and sample buffer was added at 10 min. Western blots for phosphorylated and total connexin-43 were performed as previously described (17).

Statistics. Differences between multiple treatment conditions were analyzed by one- or two-way ANOVA followed by post hoc *t* tests with Bonferroni correction, using Prism software (GraphPad). Graph values that are indicated by an asterisk are significantly different from the control, and values not indicated by the same letter are significantly different from each other ($P < 0.05$); values indicate mean ± SEM.

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