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Multiple Pathways Mediate Luteinizing Hormone Regulation of cGMP Signaling in the Mouse Ovarian Follicle[1]

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ABSTRACT

Luteinizing hormone (LH) regulation of the epidermal growth factor (EGF) network is critical for oocyte maturation and the ovulatory process. Recent studies have indicated that C-type natriuretic peptide (CNP) and its receptor natriuretic peptide receptor B (NPR2) play an important role in the control of meiotic arrest. Here, we investigated the involvement of the EGF network in the LH-dependent regulation of the CNP/NPR2 axis and cGMP accumulation. LH/hCG treatment causes a major decrease in both cGMP and the CNP precursor (natriuretic peptide precursor C [*Nppc*]) mRNA accumulation in vivo and in vitro. However, the cGMP downregulation precedes the decrease in *Nppc* mRNA by more than 1 h. Amphiregulin, an EGF-like factor, suppresses *Nppc* mRNA levels in cultured follicles to the same extent as LH, and this effect is completely prevented by the EGF receptor (EGFR) kinase inhibitor AG1478. However, the LH-dependent suppression of *Nppc* is insensitive to AG1478. Similarly, *Nppc* suppression by LH occurs in follicles from EGFR null mice. These findings document that EGFR signaling is sufficient to downregulate CNP, but is not necessary for LH action. When cGMP concentration in the follicle is measured, the short-term, but not long-term, LH effects on cGMP are prevented by AG1478, suggesting that ligand availability may be responsible for the late response. Human CG decreases the CNP-dependent cGMP synthesis in wild-type and EGFR knockdown cumulus-oocyte complexes. These findings demonstrate that redundant pathways are involved in the regulation of cGMP. EGFR-dependent events are involved in the short-term regulation of cGMP, whereas the long-term effects may involve regulation of the CNP.

cyclic-AMP, cyclic-GMP, epidermal growth factor, granulosa cells, luteinizing hormone (LH/LH receptor), natriuretic peptide precursor type C, oocyte maturation, oocyte meiotic arrest, ovary, signal transduction, preovulatory follicle

INTRODUCTION

For most of their development, including the phase of rapid growth, mammalian oocytes remain in a quiescent state that resembles a G2 phase of the cell cycle [1]. The maintenance of this state is due to the absence of key components required for cell cycle progression. When the follicle reaches the early antral stage, oocytes acquire the competence to progress through meiosis because they have assembled the necessary cell cycle machinery [2]. However, they remain in this suspended state because signals from the granulosa cell (GC) compartment preclude activation of this machinery required for the entry into M phase. The nature of these signals is now better understood, as it has been demonstrated that a complex interplay between cGMP and cAMP signaling maintains the oocyte meiotic arrest [3]. In the mouse follicle, cGMP synthesized in GCs diffuses to the oocyte through the large network of gap junction communications [4–6]. In the oocyte, cGMP inhibits the activity of the phosphodiesterase (PDE) 3A, thus allowing cAMP to accumulate [5–8]. Cyclic AMP in turn maintains the cAMP-dependent protein kinase (PK) A in an activated state that, through phosphorylation of key cell cycle components, prevents the activation of the cyclin-dependent kinase 1/cyclin B complex [7, 9].

Recently, genetic and pharmacological data have documented that cGMP levels sufficient to prevent oocyte maturation in the follicle are maintained through the activity of a paracrine loop that includes the guanylyl cyclase receptor natriuretic peptide receptor B (NPR2) and the ligand C-type natriuretic peptide (CNP) [10]. The natriuretic peptide precursor C (*Nppc*) is synthesized by mural GCs, processed, and released as mature CNP, which in turn activates the NPR2 receptor throughout the follicle in an autocrine or paracrine fashion [10]. In follicles deficient in either the receptor or the ligand, oocytes resume meiosis prematurely as soon as they reach the competence to mature at the early antral follicle stage [10–12].

Oocytes re-enter the meiotic cell cycle when the luteinizing hormone (LH) surge induces dramatic changes in GCs of the antral follicle necessary to signal oocyte maturation and, eventually, ovulation [1, 13]. LH acts by binding to a G protein-coupled receptor that signals through generation of cAMP [14]. Among the known LH effects is the decreased permeability of the gap junction that prevents equilibration of

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cGMP between the somatic/oocyte compartments [4, 15, 16]. Given the data showing that gap junctions are necessary for meiotic arrest, it is accepted that LH-dependent regulation of gap junction permeability is a factor in induction of maturation [4, 6, 17]. However, it has also been demonstrated that LH causes a dramatic decrease in cGMP in the follicle [5, 17]. The molecular steps by which LH signals this decrease are only partially known. A decrease in guanylyl cyclase activity is one possibility that has been recently supported by experimental data [18]. Contribution of increased cGMP degradation is another possible mechanism, even though the LH-dependent decrease in cGMP is detected when cGMP degradation is blocked with inhibitors of PDEs [5]. A decrease in CNP is an additional possibility that has been proposed [19, 20].

Many of the LH-induced changes in GCs at the time of ovulation are reproduced by epidermal growth factor (EGF)-like growth factors activation of the EGF receptor (EGFR). Amphiregulin (Areg), epiregulin (Ereg), and betacellulin are rapidly and transiently induced by LH in the somatic cells of preovulatory follicles (POFs) in mice [21], rats [22, 23], and humans [24]. These growth factors recapitulate many of the LH effects, including oocyte meiotic resumption, cumulus expansion, and activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK) signaling. The activation of these kinases in GCs and cumulus cells is necessary to induce gap junction closure [13, 15–17, 25]. Like LH, EGF-like growth factors also induce a decrease in cGMP in the follicle, but it is not clear whether these growth factors are indispensable mediators of the LH-dependent regulation of cGMP.

Taking advantage of genetic and pharmacological approaches, we have investigated the role of the EGF network in the regulation of cGMP levels and the CNP/NPR2 signaling in the follicle. We provide evidence that multiple redundant pathways are involved in the control of cGMP homeostasis in the follicle.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Rockford, IL) unless otherwise specified. Cyclic GMP concentrations in POFs were determined using the cGMP EIA kit (ENZO Life Sciences, Farmingdale, NY). A Natriuretic Peptide RIA kit used for CNP protein assay was from Calbiotech (Spring Valley, CA). Dulbecco PBS (DPBS) without calcium and magnesium salts was from the University of California, San Francisco, cell culture facility. Ultrapure water, Leibowitz L15 medium, minimum essential medium (MEM), MEM-α, medium 199 (M199), and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA). Recombinant LH (rLH) was from Serono International (Rockland, MA). Recombinant Areg was from R&D Systems, Inc. (Minneapolis, MN). Equine chorionic gonadotropin (eCG) and AG1478 were purchased from Calbiochem (San Diego, CA). GM6001 (Galardin) was from Biomol (ENZO Life Sciences, Farmingdale, NY). RNeasy Micro Kit was from QIAGEN Inc. (Valencia, CA), and AffinityScript QPCR cDNA Synthesis Kit was from Agilent Technologies, Inc. (Santa Clara, CA). SYBR GreenER qPCR SuperMix system was obtained from Invitrogen (Carlsbad, CA). ECL Western Blotting detection reagents were from Bio-Rad (Hercules, CA). Anti-EGFR antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-EGFR (Tyr1068) and RIPA buffer (10×) were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit IgG was from Amhersham (Pittsburg, PA). BCA protein assay kit and cocktail of EDTA-free protease inhibitors were from Pierce/Fisher Scientific (Pittsburgh, PA).

Animals and Hormone Treatments

All animal procedures were approved and followed the guidelines of the Institutional Animal Care and Use Committee at the University of California San Francisco. Mice used in this study were of mixed C57B16/129svj background. $Areg^{-/-}$ $Egfr^{+/+}$ and $Areg^{-/-}$ $Egfr^{wa2/wa2}$ mice were generated and genotyped as previously described [26]. A $Pde3a^{-/-}$ and $Pde3a^{+/-}$ colony, generated as previously described [27], was established and maintained through heterozygous breeding. Mice carrying the $Egfr^{fl}$ allele [28] and the $Egfr^{\Delta}$ null allele were generously provided by David W. Threadgill. The $Egfr^{fl}$ allele was detected by PCR using the lox3 sense and antisense primers described by Lee and Threadgill [28]. The delta-3 and delta-4 primers described by Lee and Threadgill [28] were used to detect the $Egfr^{\Delta}$ null allele. The *Cyp19-Cre* mice were from JoAnne S. Richards [29], and were genotyped by PCR using the primers shown in a previous article [16]. Mouse crosses were performed to generate $Egfr^{fl/fl}$*Cyp19-Cre*, $Egfr^{\Delta/fl}$*Cyp19-Cre*, and control littermates ($Egfr^{+/+}$, $Egfr^{fl/+}$, $Egfr^{fl/fl}$, $Egfr^{+/+}$*Cyp19-Cre and* $Egfr^{fl/+}$*Cyp19-Cre*). The cGMP measurements in heterozygous follicles for EGFR ($Egfr^{fl/+}$*Cyp19-Cre*) were not significantly different from wild type. The data from the two backgrounds were therefore combined.

Immature (21–24 days old) female mice were injected i.p. with 5 IU eCG to stimulate follicle development to the preovulatory stage. After 44–48 h, some animals were also injected i.p. with 5 IU hCG to induce differentiation of POFs, oocyte maturation, and ovulation. Ovaries were isolated at selected times after hormone priming and processed for quantitative RT-PCR, cGMP measurement, or CNP protein assay.

Culture of POFs

POFs were microdissected from eCG-primed mouse ovaries under a stereomicroscope, in Leibowitz L15 medium supplemented with 5% FBS, 100 U penicillin G, and 100 μg streptomycin. They were next transferred through MEM supplemented with 10% FBS, 100 U penicillin G, and 100 μg streptomycin sulfate three times, then cultured in 1 ml supplemented MEM under 95% O_2 and 5% CO_2. After equilibration, the POFs (5–30 POFs/group) were cultured for the indicated time with rLH. Some POFs (5–30 POFs/group) were cultured for 30 min in the presence or absence of AG1478 (500 nM), GM6001 (20 μM), or vehicle (dimethylsulfoxide) prior to stimulation with rLH (5 IU/ml) or Areg (100 nM). At the end of culture, POFs were washed in DPBS and homogenized for quantitative RT-PCR, cGMP, and Western blot measurement. No differences in responses were observed when 10 or 30 POFs were used. The ratio of in vitro germinal vesicle breakdown (GVBD) was assessed by collecting cultured follicles at specific times after rLH and placing them in Hepes-buffered MEM containing 3 mg/ml bovine serum albumin (BSA), 2 μM milrinone, 100 U penicillin G, and 100 μg streptomycin sulfate. POFs were punctured to release the cumulus-oocyte complexes (COCs), and oocytes were denuded of cumulus cells and evaluated for morphological evidence of GVBD under a stereomicroscope.

Culture of COCs

COCs were isolated by puncturing the ovaries from either unstimulated or hCG-stimulated mice with 25-gauge needles in the isolation medium, Hepes-buffered MEM supplemented with 2 μM milrinone, 100 U penicillin G, and 100 μg streptomycin sulfate. After isolation, COCs were washed in the final incubation medium and cultured for 1 h. The culture medium was MEM-α supplemented with 3% (wt/vol) BSA, 100 U penicillin G, and 100 μg streptomycin sulfate, with or without 10 nM CNP. Cultures were maintained in an atmosphere of 95% O_2 and 5% CO_2 at 37°C.

Culture of Human GCs

Luteinized human GCs were obtained from consenting patients undergoing in vitro fertilization treatment as previously described [24]. The purified human GCs were then resuspended in M199 media supplemented with 10% FBS and plated at approximately 30%–40% confluence on tissue culture plates and maintained in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 37°C in a modular incubation chamber (Billups Rothenberg, Del Mar, CA). After overnight culture, cells were washed in M199 and further cultured in M199 medium for an additional 24 h before any further treatments. For testing the stimulation of cGMP production, GCs were treated with 10 nM of CNP for varying times up to 1 h with or without 10 mIU/ml of hCG preincubation. For cells preincubated with hCG, the cells were then washed twice in M199 to remove any residual hCG. After appropriate stimulation, the supernatant was removed and the cells harvested by lysing in 200 μl of 0.1 M HCl and homogenized. The cell lysate was clarified by 10-min centrifugation, with the supernatants used to assay cGMP and the cell pellets used to normalize for protein concentration, as determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

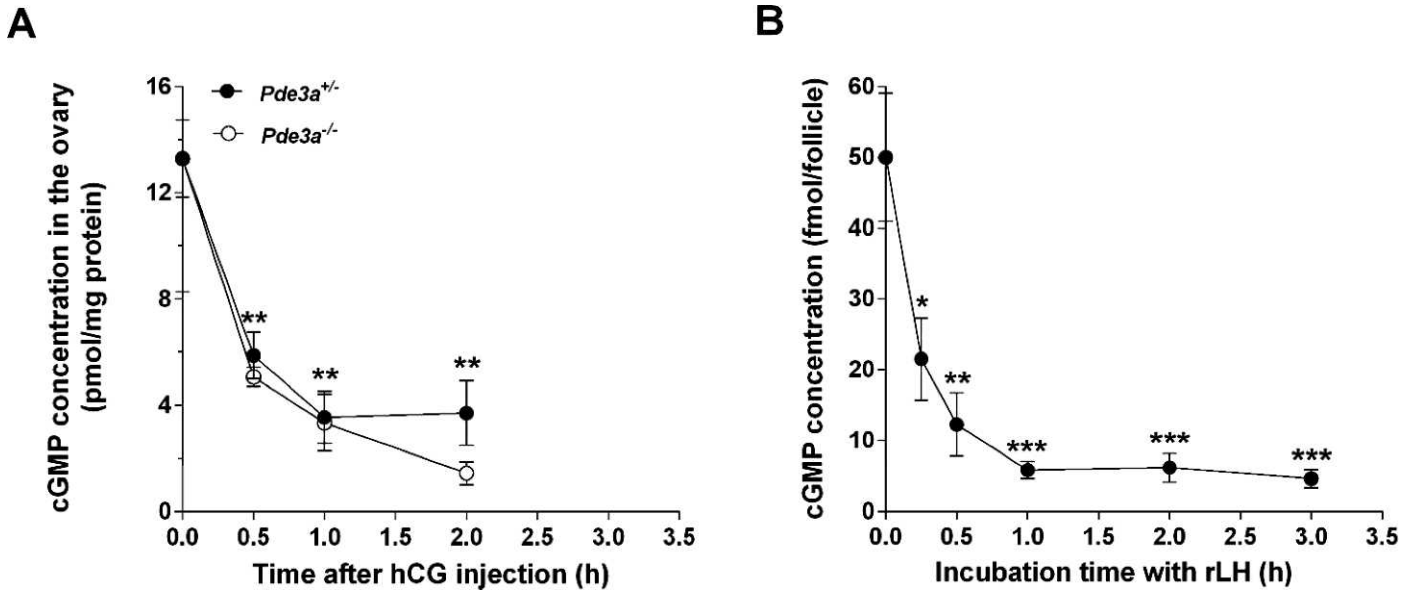

FIG. 1. Comparison of the rLH/hCG effects on cGMP levels in mouse ovary in vivo and cultured POFs in vitro. **A**) Comparison of the rLH/hCG effects on cGMP levels in *Pde3a*$^{-/-}$ and control ovaries. The 22- to 23-day-old mice were stimulated with eCG for 44–48 h, followed by a single hCG injection. Ovaries were collected at different times after the injection and immediately processed for cGMP measurement. The levels of cGMP were normalized by protein in the extract. Data are the mean ± SEM, n = 5. **B**) In vitro time course of cGMP levels in isolated follicles after rLH stimulation. POFs were isolated from ovaries stimulated with eCG for 44–48 h, and then stimulated in vitro with rLH (5 IU/ml) for the times indicated in the abscissa. Pools of 5–20 POFs were used for each measurement. Data are the mean ± SEM for follicles from three to five mice at each time point (n = 10). Data were analyzed using an unpaired *t*-test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

RNA Isolation and Quantitative RT-PCR

Mouse ovaries or cultured follicles were collected in 350 μl of RNeasy lysis buffer in microtubes and homogenized on ice using disposable pellet pestles. The cells were stored at −80°C until being analyzed for mRNA expression. Total RNA was isolated from frozen samples using the RNeasy micro-RNA isolation kit, per the manufacturer's instructions. Reverse transcription and real-time PCR was then conducted to quantify the steady-state mRNA levels of *Nppc* and the housekeeping gene *Rpl19* (internal control) using an ABI 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA). PCR primers for *Nppc* and *Rpl19* were as follows: Nppc-F (5′-GGTCTGGGATGTTAGTGCAGCTA-3′), Nppc-R(5′-TAAAAGCCA CATTGCGTTGGA-3′); Rpl19-F(5′-CCGCTGCGGGAAAAAGAAG-3′) and Rpl19-R(5′-CAGCCCATCCTTGATCAGCTT-3′). The levels of *Nppc* mRNA were first normalized to the expression levels of *Rpl19* and then were presented as relative to a control group, the expression level of which was set at 1. Each experiment was repeated independently at least three times.

Measurement of cGMP Levels

Ovary isolation and culture of POFs, COCs, and human GCs were as described above. Samples were washed in DPBS, then transferred to microcentrifuge tubes. The DPBS was aspirated, 200 μl of 0.1 M HCl was added, and samples were homogenized on ice using disposable pellet pestles. Samples were frozen at −80°C until ready for processing, or, in some cases, samples were analyzed directly without freezing. The frozen samples were thawed and homogenized again on ice. The precipitate was removed by centrifugation and the supernatant transferred to a clean tube. An Elisa Kit was used for the cGMP measurement using the manufacturer's protocol. All standards and samples were assayed in duplicate.

CNP Protein Assay

Ovaries collected at selective times from eCG-primed mice or after hCG treatment were homogenized by a microcentrifuge tube-compatible dounce homogenizer in 200 μl of working buffer (IP buffer mixed with RIA buffer from the kit with one to one ratio). After a 4-min centrifugation at 1500 × *g*, supernatants were used for determination of CNP concentration by using the CNP RIA kit, and protein concentrations were quantified using a bicinchoninic acid protein assay kit.

Western Blotting

Cultured POFs were homogenized and lysed in ice-cold RIPA buffer containing a cocktail of EDTA-free protease inhibitors. After centrifugation at 14 000 × *g* for 5 min at 4°C, supernatants were collected and protein concentrations assayed using the BCA protein assay kit.

Protein samples were separated on 8% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked for 1 h in Tris-buffered saline plus 0.2% Tween20 (TBST) plus 5% nonfat dry milk, washed in TBST, and incubated for 2 h at room temperature or overnight at 4°C with the primary antibody diluted as follows: anti-phospho-EGFR (1:1000) in TBST + 5% BSA; anti-EGFR (1:200) in TBST + 0.2% nonfat dry milk. After incubation with the primary antibody, membranes were washed in TBST and then incubated for 1 h at room temperature with anti-rabbit IgG-HRP (1:5000). Specific signals were detected using ECL reagent and visualized by autoradiography.

Statistical Analysis

All experiments were performed at least three times, and the values were presented as the mean ± SEM or as 10th–90th percentile, as indicated in the figure captions. The proportional data were subjected to an Arcsine transformation, and significant differences between experimental and control groups were analyzed by Student *t*-test or one-way ANOVA where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Recombinant LH/hCG Induces a Decrease in Follicular cGMP In Vivo and In Vitro

Cyclic GMP levels in mouse ovaries from mice injected with hCG or POFs incubated with rLH were measured at different times after the hormonal treatment. In the in vivo model, cGMP concentration decreased significantly 30 min after hCG stimulation, and reached a minimum after 1 h both in *Pde3a*$^{-/-}$ and control ovaries (Fig. 1A). Similarly, in the in vitro POF culture system, a rapid decrease in cGMP was observed 15–30 min after rLH addition, and levels reached a minimum after 1-h rLH treatment (Fig. 1B). Thus, rLH/hCG stimulation caused a rapid decrease of cGMP levels both in

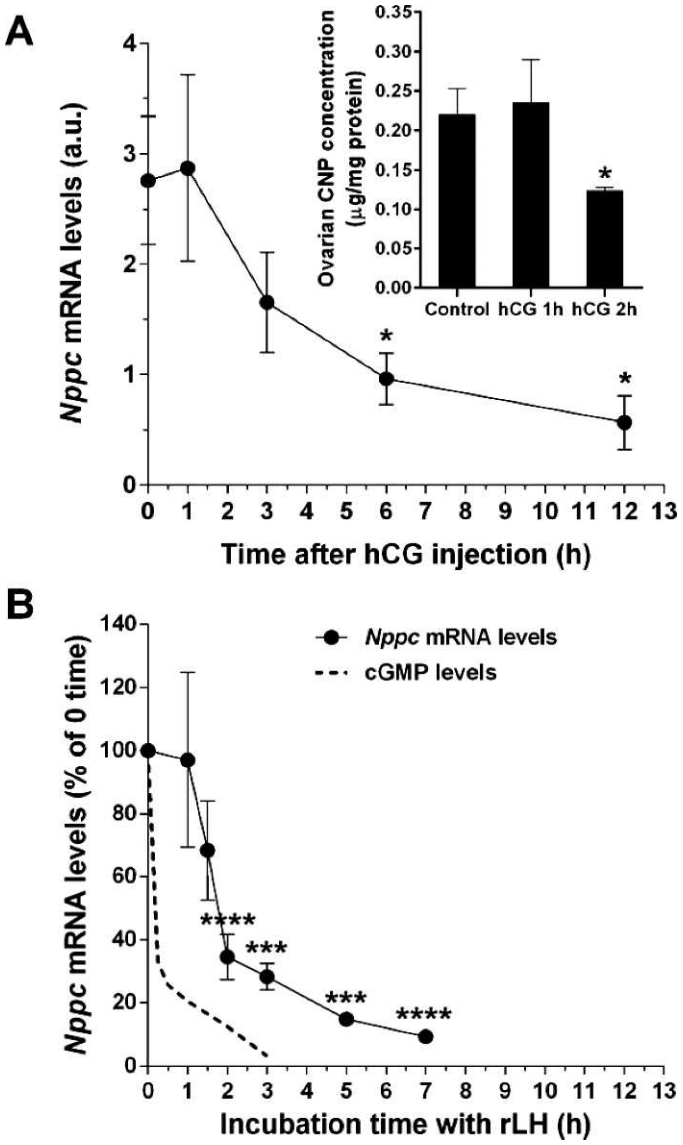

FIG. 2. *Nppc* mRNA levels in POFs after rLH/hCG treatment. **A**) *Nppc* mRNA in eCG-primed mouse ovaries treated with hCG. Ovaries were collected at the indicated times after hCG injection and processed by microarray analysis. Data are the mean ± SEM (n = 4). Inset: quantification of CNP protein content in ovaries following hCG stimulation. Data are the mean ± SEM (n = 5). **B**) *Nppc* mRNA content in follicles after rLH stimulation. POFs were isolated from mice primed with eCG for 44–48 h. A total of 3–10 POFs were used for each time point after rLH (5 IU/ml) treatment. Quantitative RT-PCR was performed on the RNA extracted at the end of the incubation. Data are the mean ± SEM (n = 4). Data were analyzed using an unpaired *t*-test; $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

vivo and in vitro, and demonstrates that this follicle culture system is an experimental model that temporally recapitulates the in vivo LH effects on cGMP. This decrease is dissociated from oocyte maturation, as shown in the *Pde3a*$^{-/-}$ mouse model where oocytes do not resume meiosis in spite of the gonadotropin stimulus, confirming that cGMP acts proximally to PDE3A.

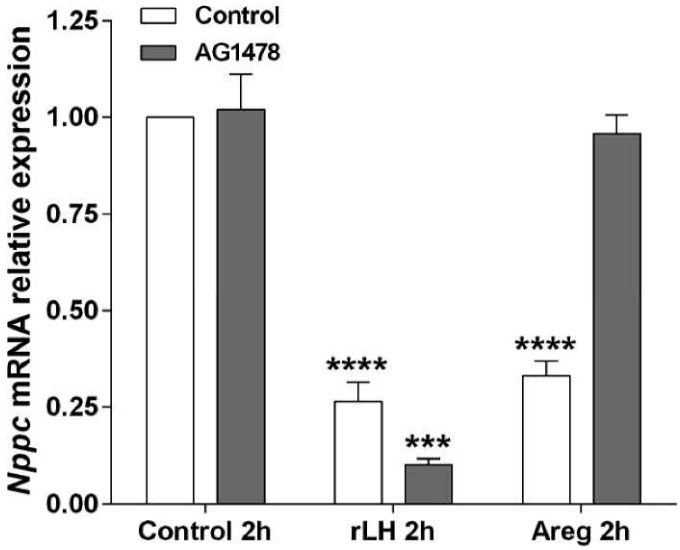

FIG. 3. Role of EGFR signaling on the LH-dependent suppression of *Nppc* mRNA. POFs were isolated from eCG-treated mice. They were then incubated in the absence or presence of rLH (5 IU/ml) or Areg (100 nM) for 2 h pretreated with or without AG1478 (500 nM) for 30 min. The *Nppc* mRNA levels were measured by quantitative RT-PCR after culture. Data are the mean ± SEM; the number of POFs analyzed (n) includes data from at least three experiments. Data were analyzed using an unpaired *t*-test; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Nppc *mRNA and Protein Levels in Cultured POFs after rLH Stimulation*

A decrease in the precursor of CNP has been reported in the mouse ovary after rLH/hCG administration [18, 19]. When the levels of *Nppc* mRNA were compared in the ovary in vivo and in follicles cultured in vitro, they decreased with comparable time courses (Fig. 2). The decrease in *Nppc* mRNA was associated with a decrease in the CNP protein in the ovary (Fig. 2A inset). However, the time course of *Nppc* mRNA decrease was considerably slower than the time course of cGMP decrease measured either in vivo or in vitro, lagging by more than 1 h (Fig. 2B). This finding suggests that availability of the ligand may contribute to the late, but not the early, phase of LH-dependent cGMP regulation.

Effect of EGF Signaling Pathway on Relative Levels of Nppc *mRNA in Cultured POFs*

Both the EGF network and CNP/NPR2 signaling are thought to be essential for oocyte maturation. To investigate the role of the EGF network in the regulation of *Nppc* expression, different hormones or growth factors were added to the cultured POFs for 2 h. LH significantly decreased *Nppc* mRNA levels by 2 h (Fig. 3). Consistent with this finding, those growth factors induced by LH at the time of ovulation recapitulate most LH effects; Areg also strongly suppressed the steady-state levels of *Nppc* mRNA (Fig. 3). The EGFR kinase inhibitor AG1478 completely blocked the Areg, but not the rLH, effect (Fig. 3).

To confirm the pharmacological data, we used several genetic models in which the EGF network is disrupted. Mutant mice with disruption of the EGF signaling at different steps (*Areg*$^{-/-}$ and *Areg*$^{-/-}$*Egfr*$^{wa2/wa2}$) and GC-specific knockout of the EGFR mice (conditional allele mice *Egfr*$^{fl/fl}$*Cyp19-Cre* and null allele mice *Egfr*$^{\Delta/fl}$*Cyp19-Cre*) were used for these experiments. POFs isolated from eCG-primed wild-type and

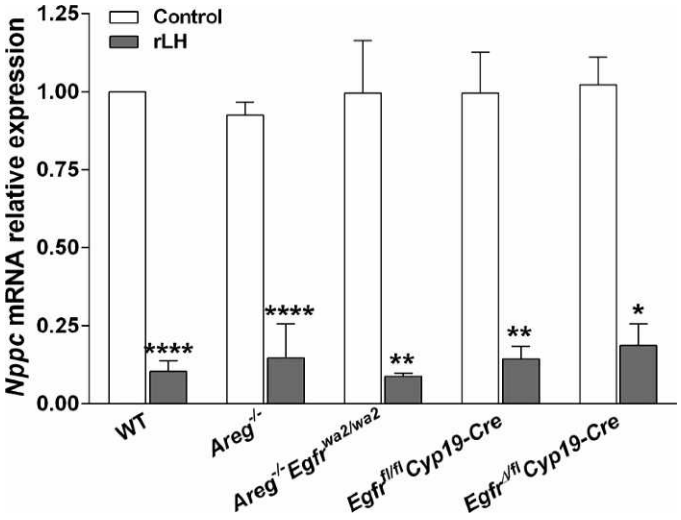

FIG. 4. Comparison of the rLH effects on *Nppc* mRNA in wild-type mice and in mice with different mutations in the EGFR signaling pathway. POFs were isolated from mice stimulated with eCG for 44–48 h and then incubated with rLH for 3 h. At the end of the incubation, mRNA was extracted from the follicles and *Nppc* mRNA levels were measured by quantitative RT-PCR. Data are the mean ± SEM (n = 5). Data were analyzed using an unpaired *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

mutant ovaries were cultured with or without rLH for 3 h. *Nppc* mRNA levels in these four different mutant mice clearly decreased after rLH as in wild-type mice (Fig. 4), confirming that activation of the EGF network is sufficient to mimic the LH effects, but not necessary for the LH regulation of *Nppc* mRNA expression.

Effect of the EGF Signaling Pathway on LH-Dependent Suppression of cGMP

Given the above findings that neither pharmacological inhibition nor the genetic inactivation of EGFR affects the LH-induced decrease in *Nppc* mRNA, we used these experimental conditions where the EGF network is inactivated to assess whether the reduction in the NPR2 ligand is sufficient to cause a decrease in cGMP levels in the follicle. EGFR inactivation may prevent signaling through several pathways downstream of LH, but not those involved in *Nppc* mRNA downregulation. Since complex mechanisms likely control cGMP with different time frames, we assessed the effect of AG1478 throughout the time course of LH action. Previous data using AG1478 had shown either complete inhibition of the LH-dependent cGMP decrease, or only partial effects [15, 16]. For these studies, follicles were incubated with 500 nM AG1478 for 30 min before the addition of the gonadotropin. This concentration of the inhibitor is sufficient to completely block the LH-induced EGFR autophosphorylation [30]. Under these experimental conditions, EGFR inhibition completely prevented the LH-dependent cGMP decrease at 15 and 30 min of incubation with rLH (Fig. 5A). A trend in cGMP decrease was detected 1 h after rLH; however, the decrease was not statistically significant (Fig. 5A). The decrease in cGMP became statistically significant only at 2 and 3 h of incubation (Fig. 5A). This experiment suggests that cGMP decrease becomes independent of EGFR signaling late after LH stimulation.

A possible explanation of the above finding is that AG1478 becomes progressively inactivated during the prolonged follicle incubation. To investigate this possibility, follicles were incubated with either LH or Areg for 2 h and the effect of AG1478 on cGMP levels measured. Again, AG1478 blocked the LH-dependent decrease in cGMP, although incompletely (Fig. 5B). Conversely, the kinase inhibitor completely prevented the Areg-dependent decrease in cGMP. This experiment therefore rules out the possibility that the time-dependent diminished effect of AG1478 is due to its inactivation during the incubation.

To further confirm the early EGFR-dependent effect, we pretreated POFs with a matrix metalloproteinase inhibitor GM6001. Metalloproteinases are required for shedding the EGF ligand, and we have shown that they also block the LH transactivation of EGFR in GCs. This inhibitor blocked the LH-dependent cGMP decrease completely (Fig. 5C).

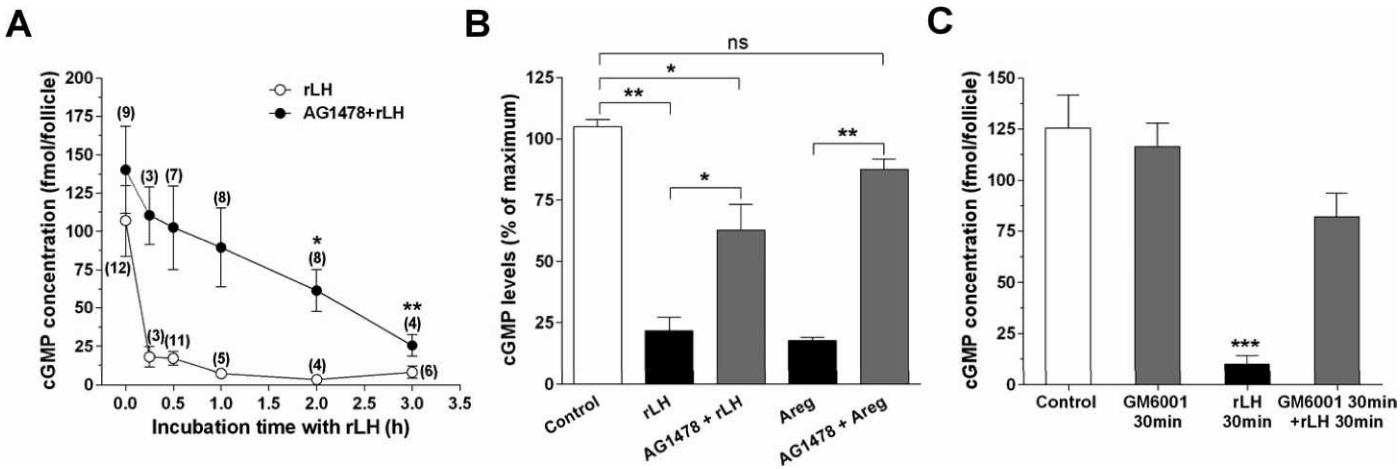

FIG. 5. Effect of pharmacological inhibition of EGFR on the LH-dependent decrease in cGMP in the ovarian follicle. **A**) Effect of AG1478 on cGMP levels in POFs cultured with rLH. POFs were incubated for different times with rLH in the absence or presence of 500 nM AG1478. At the end of the incubation, cGMP was measured as detailed in the *Materials and Methods*. Data are the mean ± SEM; the number of experiments is indicated above each time point. **B**) Effect of AG1478 on cGMP levels in POFs cultured with either rLH (5 IU/ml) or Areg (100 nM) for 2 h. Data are the mean ± SEM (n = 3). **C**) Effect of GM6001 on cGMP levels in POFs cultured with rLH for 30 min. Data are the mean ± SEM (n = 7). Data were analyzed using an unpaired *t*-test; ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

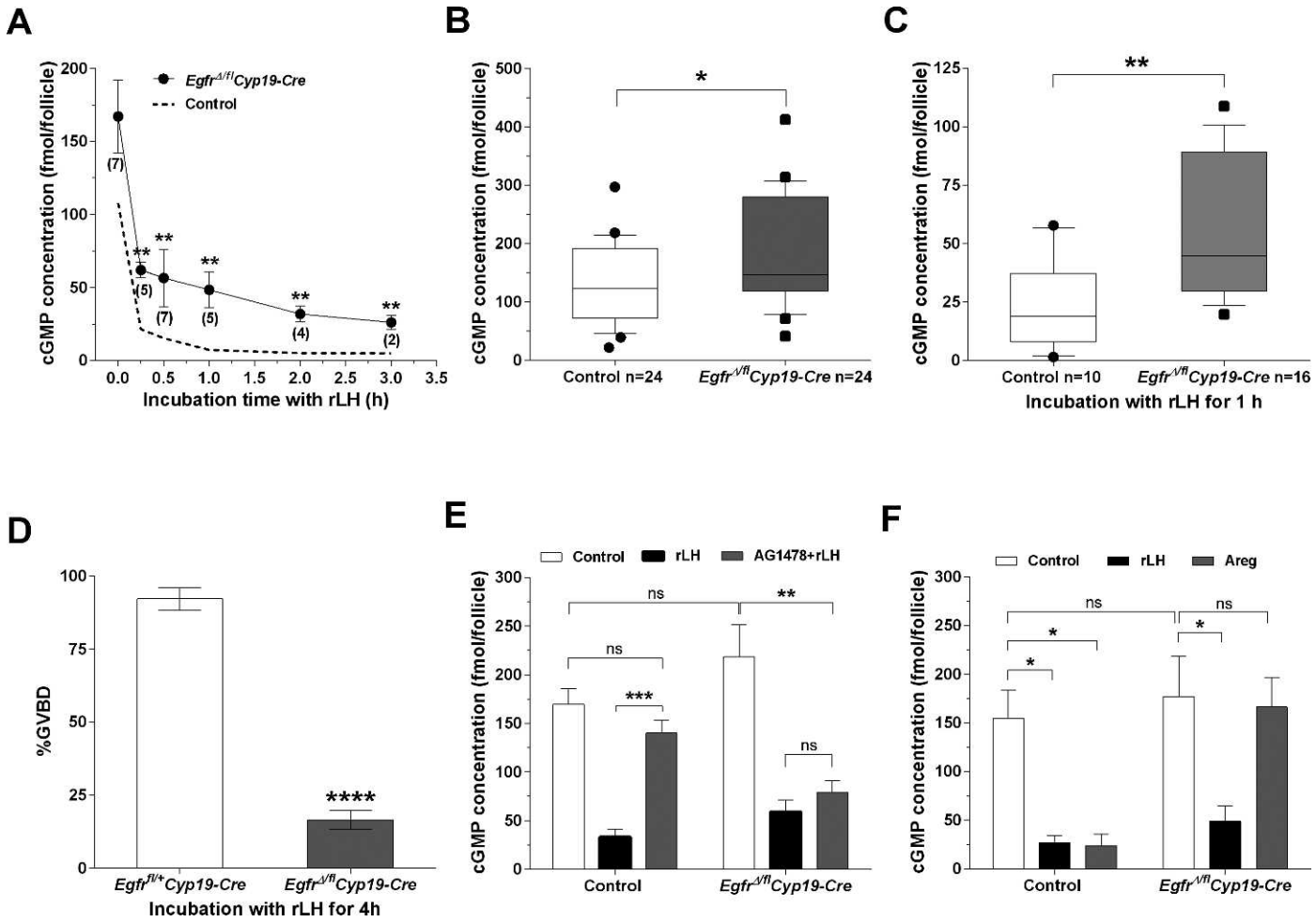

FIG. 6. Time course of cGMP levels in *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice after rLH treatment. **A**) POFs were isolated from *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice stimulated with eCG for 44–48 h. Each measurement used pools of 5–20 POFs. Data are the mean ± SEM from three to five mice at each time point, and the number of experiment is indicated above every time point. **B**) Summary of cGMP levels in the POFs isolated from eCG-primed *Egfr*$^{\Delta/fl}$*Cyp19-Cre* and control mice without treatment. Controls were *Egfr*$^{fl/+}$*Cyp19*$^{+/+}$, *Egfr*$^{fl/fl}$*Cyp19*$^{+/+}$, or *Egfr*$^{fl/+}$*Cyp19-Cre* mice from the same litter. Data are shown as 10th–90th percentile. **C**) Cyclic-GMP levels in the POFs isolated from eCG-primed *Egfr*$^{\Delta/fl}$*Cyp19-Cre* and control mice stimulated with rLH for 1 h. Data are shown as 10th–90th percentile. **D**) POFs isolated from *Egfr*$^{\Delta/fl}$*Cyp19-Cre* and control mice in the same litter were cultured with rLH for 4 h in vitro. The percentage of GVBD oocytes was assessed by light microscopy. Data are the mean ± SEM of the percentage for every mouse (n = 5). **E** and **F**) Cyclic-GMP levels were measured by EIA after rLH (5 IU), AG 1478 (500 nM), or Areg (100 nM) treatments for 1 h in POFs isolated from *Egfr*$^{\Delta/fl}$*Cyp19-Cre* and control mice from the same litter. Each point corresponds to measurement of pools of 5–20 POFs. Data are the mean ± SEM for follicles from two to three mice; the number of POFs analyzed (n) includes data from at least three experiments. Data were analyzed using an unpaired *t*-test; ns, not significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Cyclic GMP Levels after rLH Treatment in Egfr$^{\Delta/fl}$Cyp19-Cre *POFs*

The above data strongly suggest that the effect of LH on cGMP during the first hour of incubation is dependent on an active EGF network. To confirm this possibility, we investigated the time course of cGMP decrease after rLH stimulation in *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POFs (Fig. 6A). In this genetic background, the LH-induced decrease in cGMP in the follicle was disrupted, but not abolished (Fig. 6A). A significant increase in basal cGMP in the fully grown follicle's gonadotropin stimulation was observed (Fig. 6B). LH caused a decrease in cGMP during the first 15 min; however, levels did not continue to decrease in the following hour (Fig. 6A). Cumulative data from all the experiments performed show that the decrease in cGMP induced by LH in follicles where the EGF network was downregulated is about 25%–50% of control, whereas, in wild-type cells, it is decreased by more than 90% (Fig. 6C). Under these experimental conditions, the LH-dependent oocyte re-entry into the meiotic cell cycle is severely impaired. The percentage of oocytes in GVBD after rLH-treatment of *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POF with *Egfr*$^{fl/+}$*Cyp19-Cre* was decreased by 80% (Fig. 6D).

The following control experiments were performed to further understand the role of EGFR transactivation in the regulation of cGMP levels. To define whether AG1478 has off-target effects in the follicle, the compound inhibition was measured in follicles where EGFR is reduced by more than 80%, as in the *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice. AG1478 had no significant effect on this genetic model, even though the effect on wild-type controls continued to be clearly detectable (Fig. 6E). This experiment documents that the compound requires the presence of EGFR in order to have an effect, confirming that off-target effects are not responsible for blocking the decrease of cGMP at early time points. As a further control of the efficiency of EGFR inactivation, we assessed the effect of Areg on the EGFR knockdown model. Cyclic-GMP was no longer affected by the EGF-like growth factor on this genetic

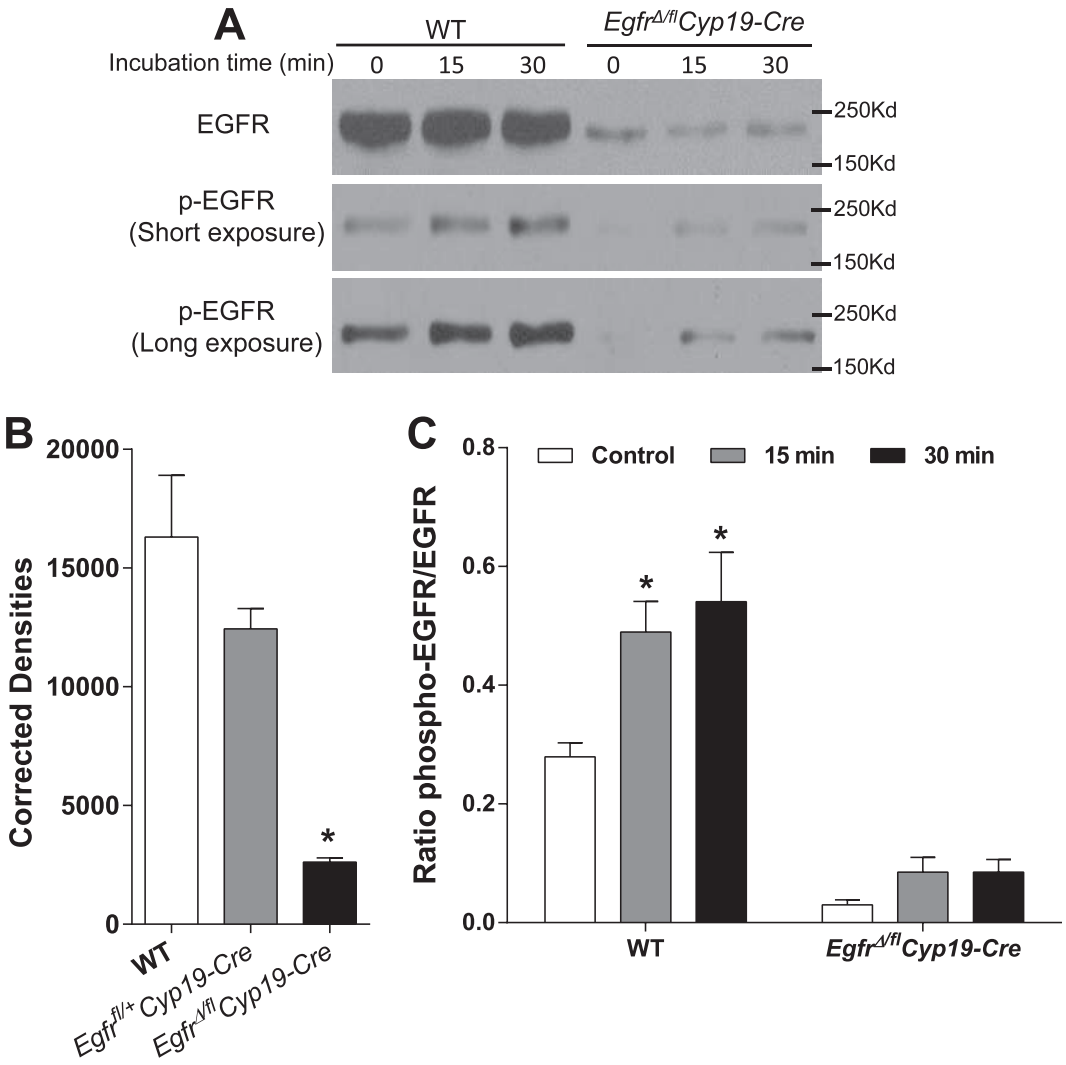

FIG. 7. Expression and phosphorylation of EGFR in wild-type and *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice. **A**) Representative blots are shown for total and phosphorylated EGFR in wild-type and *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POFs after treatment with rLH. **B**) Expression of total EGFR in wild-type, *Egfr*$^{fl/+}$*Cyp19-Cre*, and *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POFs. Data are the mean ± SEM (n = 4). **C**) LH-induced EGFR phosphorylation during the first 30 min of incubation. LH-stimulated EGFR phosphorylation levels were examined by Western blot in cell lysates of POFs incubated with rLH for 15 and 30 min. The ratio of phosphorylated EGFR to total EGFR was normalized to control. Results are expressed as the mean ± SEM of four separate experiments. Data were analyzed using an unpaired *t*-test; $*P < 0.05$.

background, confirming that EGFR function is greatly reduced in this model (Fig. 6F).

Expression and Activation of EGFR in Wild-Type and Egfr$^{\Delta/fl}$Cyp19-Cre *Mice*

In *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POFs, rLH still caused a partial decrease in cGMP during the first 15 min of incubation. To define whether residual LH-dependent transactivation of EGFR is detectable in this genetic model, we investigated the EGFR phosphorylation at early time points. The expression of the EGFR is decreased by 80%–85% in the *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POFs (Fig. 7, A and B), confirming our previous measurements [30]. The LH-induced EGFR autophosphorylation is significantly increased as early as 15 min after rLH stimulation (Fig. 7, A and C), and some transactivation is still present in the *Egfr*$^{\Delta/fl}$*Cyp19-Cre* POFs. This early phosphorylation of EGFR further supports the view that cGMP decrease takes place at a time when the EGFR is activated, and that EGFR signaling is not completely inactivated in the genetic model used.

Effect of LH/hCG on CNP-Dependent cGMP Production

The above data document complex regulations of cGMP levels in the follicle. It has been proposed that hCG-dependent regulation of NPR2 guanylyl cyclase activity is responsible at least in part for the decrease in cGMP in the follicle [18]. We therefore investigated whether LH/hCG pretreatment affects the GC sensitivity to CNP. To this aim, COCs were isolated from control and hCG-treated mice 1 h after injection. The COCs were then incubated with or without CNP, and cGMP levels measured after an in vitro incubation for 1 h. When COCs from vehicle-injected mice were used, addition of 10 nM CNP significantly increased cGMP content in COCs derived from wild-type and *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice. When COCs from hCG-primed mice were used, a significant decrease in cGMP accumulation was observed in both genotypes (Fig. 8A). A similar effect of hCG preincubation on the CNP-dependent cGMP accumulation was observed in a different model where human mural GCs were exposed to the gonadotropin in vitro (Fig. 8B).

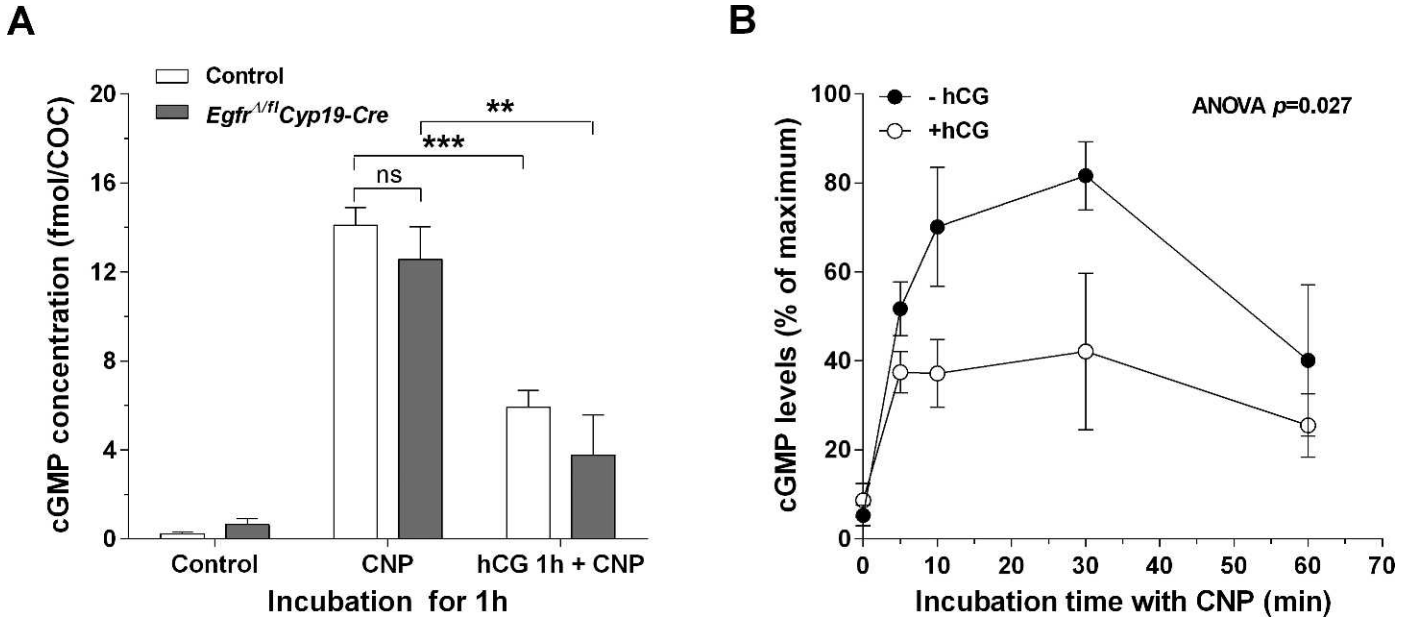

FIG. 8. LH/hCG signaling rapidly desensitizes the effects of CNP stimulation in mice and humans. **A**) COCs were isolated from mice eCG-primed or hCG-injected 1 h after eCG stimulation, which were cultured in medium with or without (control) 10 nM CNP for 1 h, as indicated. COCs were collected at the end of culture, and the levels of cGMP were measured. Bars show the mean ± SEM of at least three independent experiments. Data were analyzed using an unpaired *t*-test; ns, not significant. $^{**}P < 0.01$; $^{***}P < 0.001$. **B**) Luteinized human mural GCs were pretreated for 1 h with hCG at a physiologically relevant concentration of 10 mIU/ml, rapidly washed twice in M199 to remove any residual hCG, and then treated with 10 nM CNP. At the end of the indicated culture times, cGMP production was quantitated. Data are mean ± SEM of independent experiments (n = 5). Data were analyzed using one-way ANOVA.

DISCUSSION

The release of LH by the pituitary leads to dramatic and long-lasting changes in the ovarian follicles that are essential for ovulation of a fertilizable egg [13]. Gonadotropin binding to the LH receptor causes activation of stimulatory G protein and adenylyl cyclase followed by an increase in cAMP [14], as well as activation of Gq11, which leads to activation of phospholipase C and Ca^{2+} release [31–33]. The accumulation of these intracellular messengers in turn activates numerous downstream protein kinases, including PKA, MAPK, p38, AKT, and PKC, directly or indirectly, leading to complex branching pathways and altered gene expression by GCs [17, 34–37]. One of the downstream pathways that have recently emerged as critical for signaling oocyte re-entry into the cell cycle is cGMP. LH causes a rapid and profound decrease in cGMP in the follicle, a signal likely involved in oocyte meiotic resumption [5, 6, 38]. Previous reports from our and other laboratories demonstrated that the EGF network is essential for efficient propagation of the LH signal, activation of oocyte maturation, and ovulation [16, 21, 22, 25, 26, 30, 36, 39, 40]. Here, we provide evidence that EGFR transactivation mediates the LH effects on cGMP, but that additional EGFR-independent pathways cooperate to induce the overall decrease in cGMP.

The time course of the cGMP decay induced by LH is complex. A very rapid initial reduction in cGMP to 25% of control is observed within 15 min of LH/hCG in vivo and in vitro stimulation. In the following 1–2 h, cGMP continues to decrease to less than 5% of control. Complex regulations must be involved, because simple exponential decay equations do not adequately describe the cGMP time course. The decrease is independent of whether the oocyte re-enters the cell cycle, as documented by the unaffected cGMP decay in the *Pde3a*$^{-/-}$ mice. Several mechanisms have been implicated in the cGMP decline over the span of these 3 h. LH effects on cGMP synthesis via NPR2 desensitization [18], cGMP degradation via PDE activation [5], as well as LH effects on the level of the NPR2 may mediate the decline in cGMP levels [18]. LH stimulation also causes a decrease in *Nppc* mRNA and CNP protein [19], findings that we can reproduce in our study. However, the time course of *Nppc* mRNA and protein decreases is considerably slower than the time course of cGMP decrease. Significantly, we show here that the LH effect on mRNA encoding the CNP ligand occurs even if EGFR is inhibited either pharmacologically or genetically. In addition to documenting that some LH effects do not require EGFR signaling, these experiments provide a model where the effect of *Nppc* mRNA decrease could be monitored without the concomitant effects dependent on EGFR activation. We have used this experimental strategy to define whether the *Nppc* decrease is necessary and sufficient to decrease cGMP and to signal oocyte maturation. The data generated indicate that the decrease in cGMP during the latter period of LH action may be dependent on loss of the ligand. However, this effect on cGMP by itself is not sufficient to signal oocyte maturation.

The initial decrease in cGMP that occurs in the follicle is completely prevented by blocking the EGFR kinase with AG1478, which indicates that LH transactivation of EGFR is required for this first phase. We exclude an off-target effect of AG1478, because no effect on cGMP levels could be detected when EGFR was downregulated. Moreover, EGF-like growth factors produce an effect similar to LH, confirming that activation of EGFR is a signal sufficient to reduce cGMP. Here, we also provide evidence that EGFR phosphorylation is detectable at 15 and 30 min of stimulation by LH, further confirming that the decrease in cGMP coincides with the activation of EGFR. However, genetic inactivation of the EGFR reduces, but does not prevent, the LH-dependent cGMP decrease during the initial 30 min. We show that the reduction in cGMP in follicles from *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice reaches an

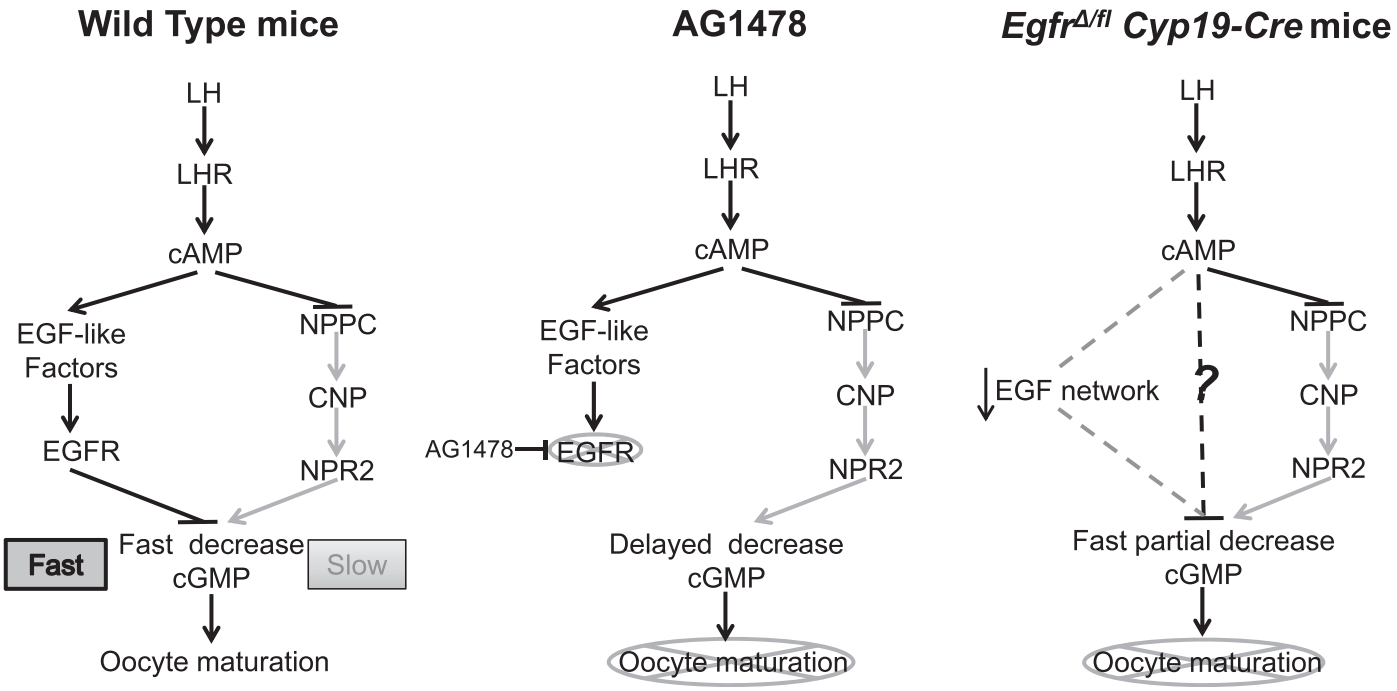

FIG. 9. Models of EGF network and CNP/NPR2 signaling in the regulation of LH-induced ovulation. In the wild-type mice, LH activates its receptor on GCs of POFs, leading to stimulation of cAMP-dependent signal cascades, which induces the expression of EGF-like factors and suppresses the CNP/NPR2 signaling. Activation of EGF network is involved at early time points (fast, 15–30 min), while CNP decrease may be involved at later time points (2–4 h). These two pathways act together or in parallel to induce meiotic resumption. In the presence of AG1478, CNP availability is primarily responsible for the reduction in cGMP. In the *Egfr*$^{\Delta/fl}$*Cyp19-Cre* mice, either the residual EGFR signaling or another pathway is responsible for the early decrease in cGMP.

average of 50 fmol/follicle, in contrast to less than 10 fmol/follicle measured in wild type. This incomplete effect may be due to the fact that the genetic model we used does not completely inactivate EGFR, given our finding that 10%–20% of the receptors are still expressed in *Egfr*$^{\Delta/fl}$*Cyp19-Cre* follicles [16] and some EGFR phosphorylation is still present. However, AG1478 is inactive in this genetic background. Thus, pathways dependent on the LH receptor activation, but independent of EGFR, should also be considered. This redundant pathway would be active when signaling through EGFR is chronically (*Egfr*$^{\Delta/fl}$*Cyp19-Cre*), but not acutely (AG1478), inactivated. Of note, this time-sensitive effect of AG1478 could explain the variable effects on cGMP reported by different laboratories, as different incubation times were used [15, 16, 41].

We envision that LH causes a rapid decrease in cGMP through LH-dependent EGFR transactivation. When this pathway is disrupted, another yet to be defined mechanism is sufficient to cause a rapid, but partial, decrease in cGMP. Of note, LH is unable to induce oocyte maturation in *Egfr*$^{\Delta/fl}$*Cyp19-Cre* follicles, indicating that this secondary pathway is insufficient by itself to signal oocyte re-entry into the meiotic cell cycle. We theorize that the bulk of cGMP that we measured is derived from mural GCs; therefore, we infer that incomplete gap junction closure [16] is associated with partial cGMP decrease in the granulosa compartment. These two disruptions prevent cGMP levels decreasing to below the threshold, which is necessary to signal meiotic re-entry.

A decrease in sensitivity to CNP was detected both in mural GCs exposed to hCG in vitro and in cumulus cells derived from mice injected with hCG. In the in vitro model, incubation of GCs with hCG for 1 h causes a detectable accumulation of Areg, whereas Areg production by COCs could not be measured. Therefore, we cannot determine whether the reduced sensitivity to CNP is mediated by Areg release. However, we show that the decrease in sensitivity of cumulus cells to CNP is induced by hCG even though the EGFR network has been disrupted. Again, this may be in part due to the incomplete inactivation of EGFR or due to activation of other pathways. A caveat of this conclusion is that the experimental protocol adopted involved in vivo exposure to hCG and in vitro stimulation with CNP for 1 h. These experimental settings may alter the contribution of different pathways to NPR2 desensitization.

The short-term effects of AG1478 are not compatible with the de novo synthesis of the EGF-like growth factors, as an increase in mRNA for Areg or Ereg is observed 1 h after LH stimulation [21, 30]. However, EGFR transactivation by LH is already detectable 15 min after stimulation. Thus, the early effects of AG1478 may be due to EGFR activation independent of de novo ligand production. This may be due to release of a pre-existing pool of growth factors via activation of an ecto protease, as suggested by the sensitivity to GM6001, or via an intracellular mechanism by which the EGFR kinase activity is being activated. The Src kinase, for instance, can phosphorylate and activate EGFR [42]. After 30–60 min, de novo production of EGF-like growth factors contributes to propagation of the LH effect throughout the follicle, as documented by the further increase in EGFR phosphorylation at 2 h [30].

The decreased efficacy of AG1478 after 1-h LH exposure and the decrease in *Nppc* even in the presence of this inhibitor is compatible with the hypothesis that reduction in the *Nppc* ligand is responsible for the decrease in cGMP in the follicle at later times after LH stimulation. This would be consistent with the pharmacological and genetic data showing that the LH effect on *Nppc* occurs independently of EGFR activation. It is also consistent with the slow decrease in *Nppc* mRNA. Measurements by Robinson et al. of CNP follow a time course similar to what we observe with the mRNA [18].

In summary, our data indicate that multiple pathways mediated the LH-dependent decrease in cGMP and that LH-dependent decrease in the CNP ligand may have an impact on cGMP during the late phase of LH action (Fig. 9). We propose that the cGMP decrease observed at 3 h in the presence of AG1478 or in the *Egfr*$^{\Delta/fl}$*Cyp19-Cre* follicles is due to decreased concentration of CNP. Since no oocyte maturation can be observed in either model, we suggest that the LH effect on the ligand cannot compensate for the loss of other signaling events necessary for the LH-dependent cGMP decrease. Our data support the view that oocyte maturation requires a rapid and profound decrease in cGMP, as well as closure of the gap junction to prevent its diffusion, and/or perhaps transfer of other signals. These critical events do not take place when EGFR signaling is disrupted.

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