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Gap junction -mediated cAMP movement between oocytes and somatic cells

Guankun Mao[1], Junxia Li[1], Fenghua Bian[1], Yingying Han[1], Meng Guo[1], Baoshan Xu[1], Meijia Zhang[1], Guoliang Xia[1]

[1]State Key Laboratory for Agrobiotechnology, College of Biological Science, China Agricultural University, Beijing 100193, China

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1. ABSTRACT

Cyclic AMP (cAMP) plays a critical role in oocyte meiotic maturation. However, the source of cAMP surge prior to maturation and the direction of gap junction-dependent cAMP movement are unclear. In this study, inhibition of gap junctional communication (GJC) using carbenoxolone (3.5 h) induced meiotic resumption in ~90% of follicle-enclosed oocytes (FEOs). The concentration of cAMP in a single oocyte was higher than that in a single cumulus cell, suggesting that the movement of cAMP proceeds from the oocyte to cumulus cells under passive diffusion. The mRNAs of adenylyl cyclases and the corresponding proteins were mainly detected in oocytes. Persistent or transient incubation with forskolin induced meiotic resumption in FEOs. The maturation induced by persistent forskolin treatment was inhibited by carbenoxolone. However, carbenoxolone had no effect on the maturation of FEOs transiently treated with forskolin or persistently treated with follicle-stimulating hormone. Oocyte maturation was inhibited by sequential treatment with carbenoxolone followed by forskolin. The carbenoxolone-induced maturation was accompanied by a cAMP surge, increased PDE3A and MAPK activation, and decreased levels of cGMP and cAMP-dependent PKA I activation.

2. INTRODUCTION

Meiosis in mammalian oocytes begins in the fetal ovary and enters a prolonged period of quiescence in late prophase at around the time of birth. Oocytes are arrested at the germinal vesicle (GV) stage of the first meiotic division via meiosis-arresting factors such as cyclic AMP (cAMP), until a surge of luteinizing hormone (LH) from the pituitary releases the arrest (1-2). However, meiotic resumption can also occur spontaneously when the fully grown oocytes are released from their follicular environment (3).

cAMP is an important signaling molecule that regulates meiotic arrest. A high cAMP level in the oocyte is essential to maintain prophase arrest (4-5) and prevents spontaneous maturation (6-7). It also inhibits luteinizing hormone (LH)- or follicle-stimulating hormone (FSH)-induced oocyte maturation (4). A cAMP surge in follicle-enclosed oocytes (FEOs) or cumulus-enclosed oocytes (CEOs), however, leads to meiotic resumption (8-9). Although the intracellular cAMP concentration can be maintained by its synthesis and hydrolyzation (10), cAMP transport between mammalian oocytes and the surrounding cumulus cells (CCs) can also occur via gap junctions. As such, the source of intraoocyte inhibitory cAMP is still a

matter of controversy and two major views have emerged. According to the first view, self-generation of cAMP in the oocyte occurs because of a constitutively activated heterotrimeric G protein (Gs)-linked receptor (GPR3, GPR6, or GPR12) that activates a membrane-bound Gs protein to stimulate oocyte adenylyl cyclase (AC) and cAMP production (11-14). The second view suggests that the surrounding granulosa cells (GCs) generate and transfer cAMP to the oocyte via gap junctions to maintain meiotic arrest (15-16). LH-induced oocyte maturation requires interruption of the gap junctional communication (GJC) network between the oocyte and the surrounding somatic cells (15, 17-18), which inhibits the supply of cAMP from the GCs to the oocyte, decreases intraoocyte cAMP accumulation, and results in meiotic resumption. However, this view does not explain the transient cAMP surge in oocytes following inhibition of GJC.

Hydrolyzation and degradation of cAMP in mammalian oocytes is primarily mediated by type III phosphodiesterase (PDE3) (19). There are two cyclic guanosine 3′,5′-monophosphate (cGMP)-inhibited, cAMP-PDE3 isoenzymes (PDE3A and PDE3B) (20-22), but only PDE3A is oocyte-specific and regulates meiotic resumption in rodent oocytes (23-24). The most convincing evidence that PDE3A regulates meiosis is that *pde3a* knockout female mice are sterile because oocytes are blocked at the G2–M transition (23).

Recent studies have demonstrated that mural GCs express natriuretic peptide precursor type C (NPPC) mRNA, whereas CCs express the NPPC receptor NPR2 mRNA, which is a guanylyl cyclase that produces cGMP (25-27). However, neither NPPC mRNA nor its receptor NPR2 mRNA can be detected in oocytes (27). Most Graafian follicles in NPPC or NPR2 mutant mice resume meiosis precociously (27), and GC-derived cGMP is essential to maintain meiotic arrest by inhibiting PDE3A in fully grown oocytes (27-29).

Gap junctions are essential for the transmission of both cAMP- and cGMP-mediated signaling between GCs and oocytes (16, 30). An extensive GJC network, comprising gap junctions composed of connexin hexamers, exists within each follicular cellular compartment (31). On/off GJC can regulate the levels of cAMP and cGMP in the oocyte to control meiotic arrest or reinitiation (28, 32-34).

cAMP maintains meiotic arrest in oocytes by activating the cAMP-dependent protein kinase A (PKA) (35-37). PKA is a ubiquitous serine/threonine kinase that phosphorylates mitogen-activated protein kinase (MAPK), which regulates cyclin-dependent protein kinases (38-39). PKA holoenzymes are tetramers composed of two regulatory subunits (RI or RII) and two inactive catalytic subunits (40). In oocytes, the most abundant regulatory subunit is RI (37). The localization of RI to the spindle during oocyte meiotic maturation suggests that it is involved in meiotic regulation (41-42). Activation of type I PKA can prevent FSH-induced oocyte maturation (37). Downregulation of RIα can initiate meiosis in oocytes, suggesting that RI may be the key regulator of PKA activity during meiotic arrest and maturation (42). Type I PKA also inhibits the phosphorylation of ERK1/2, which are components of the MAPK pathway, in NG108-15 cells (39). ERK1 and 2 are activated during oocyte maturation (18, 32, 43-45).

In the present study, the effect of LH on GJC was mimicked using CBX in the ovarian follicles. We investigated the cellular movement of cAMP and cGMP through the GJC network between the oocyte and the surrounding somatic cells during meiotic arrest and resumption. More specifically, we demonstrate that cAMP is transported from the oocyte to the somatic cells prior to the LH surge. Furthermore, our findings implicate the cGMP-PDE3A-cAMP-PKA I-MAPK pathway in mouse FEO maturation.

3. MATERIALS AND METHODS

3.1. Experimental animals

FEOs were recovered from immature 21–23-day-old Kunming white female mice (outbreed strain). All experiments were conducted with the approval of the Animal Care Committee of China Agricultural University (CAU). Mice were provided with water and chow *ad libitum* and housed in air-conditioned rooms illuminated for 12 h per day.

3.2. Reagents and antibodies

All reagents and chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise indicated. CBX (3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate), FSH, and forskolin were prepared in phosphate-buffered saline (PBS; stored at -20°C) and diluted to the indicated concentrations with culture medium prior to treatment. U0126, cilostamide, rolipram, isobutylmethylxanthine (IBMX), 8-AHA-cAMP, and 8-cl-cAMP were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO (<0.1%) had no effect on oocyte maturation (data not shown). Polyclonal goat anti-MAPK antibody, monoclonal mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, polyclonal rabbit anti-AC2, -AC3, and -AC9 antibodies, and the respective horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

3.3. Isolation and culture of ovarian follicles

Recovered ovaries were placed in L15 medium containing 0.1% bovine serum albumin (BSA), and large antral follicles (300–400 μm) were separated under a stereomicroscope using sterile 27-gauge needles (46). FEOs of equal size without apparent signs of necrosis were collected and incubated in DMEM (GIBCO, Gaithersburg, MD) supplemented with 0.1% lyophilized crystallized BSA, 5 μg/mL insulin, 2 ng/mL sodium selenite, 2 μg/mL transferrin, 0.23 mM sodium pyruvate, 2 mM glutamine, 75 mg/mL potassium penicillin G, and 50 mg/mL streptomycin sulfate. Incubations were performed at 37°C with 5% CO_2 in the presence or absence of treatment. Following the culture period, oocytes were mechanically

Table 1. Sequence of PCR primers used for QRT-PCR

Primer	Forward	Reverse
AC1	GTGGTGGCTGCCTCGCACTT	AGCAGGGCATTGGCACCGAG
AC2	GAAGTGATGGAGGAAGAGGTGAAG	CAGACTAGGGATGCGGTTACGAG
AC3	ACACGCTCACAAACATCAACAACC	CTCCAATGACGCCAGCCAGAAC
AC4	ACACTCACTCCTTGGTGCCTCTT	TCCTCTTGTCTATCCTTCCTGCCTAC
AC5	GGCGGCAACCAGGTGTCAAAGG	CCCAGAAACTCGTCCACTTCATCCT
AC6	TTGGCTTTGGGTCTGCTTCTTG	ATTGCCTCCCGTGCCCTGACTT
AC7	TTCGTGAGATGGGTGTCTTACTGT	CTGGAGGTTCCTTGGTGACTGCT
AC8	AGGTTTGTCGTCCTGGAAATGAT	GGATGTAGATGCGGTGGAACTGAT
AC9	CTTCTCTGGAGCATCTATTTCGC	CAGCTTGGTGAAAGTAAACAGGA

Abbreviations: AC, adenylyl cyclase

denuded and assessed for maturation. Oocyte maturation was scored based on the morphological identification of GV (meiotic arrest), GVBD (meiotic resumption), and PB1 (the first meiotic maturation). The percentage of oocytes with GVBD (% GVBD; including PB1) per total number of oocytes was calculated as a morphological marker for reinitiation of meiosis.

3.4. Determination of cAMP and cGMP levels in oocytes

Ovarian follicles cultured as described above were incubated with or without CBX (150 μM, the lowest effective dose; 0.5–3 h). The follicles were then placed in DMEM containing IBMX (200 μM). COCs were retrieved in the presence of IBMX, and the oocytes were stripped and washed in PBS before being suspended in HCl (0.1 N; 10 min). At least 50 FEOs were collected for use in the cAMP assay, and 250 FEOs for the cGMP assay. For cAMP measurements in CCs, at least 50 CEOs were collected. All samples were stored at -80^{o}C until use. Prior to cAMP and cGMP measurements, samples were thawed and centrifuged (12,000 × *g*; 15 min), and the supernatants were collected and dried in an oven at 60°C. cAMP content in these oocytes was measured using a cAMP radioimmunity kit (Beckman Coulter, Inc., Fullerton, CA), and cGMP immunoassay was performed using cGMP-EIA kit (Cayman Chemicals, Ann Arbor, MI).

3.5. Real-time Polymerase Chain Reaction (real-time PCR)

Total RNA was isolated from the somatic cells and oocytes of FEOs and purified using the RNeasy micro-RNA Isolation Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. Reverse transcription was performed using the QuantiTek reverse-transcription system (Qiagen), and real-time PCR was then conducted (ABI 7500 real-time PCR instrument; Applied Biosystems, Foster City, CA). Results were normalized to ribosomal protein L19 (Rpl19) expression using the $2^{-\Delta\Delta Ct}$ method (47). The AC primer sequences are listed in Table 1. To avoid false-positive signals, dissociation-curve analyses were performed after the amplification and the sizes of the PCR products were confirmed by agarose gel electrophoresis. PCR products were purified and sequenced to verify sequence identity. The reactions were conducted at least in duplicate, and each experiment was independently repeated three times.

3.6. PDE activity assay

Denuded oocytes of FEOs were collected and flash-frozen in liquid nitrogen before homogenization in isotonic buffer [10 mM sodium phosphate buffer, pH 7.2 (prepared from Na_2HPO_4 and NaH_2PO_4), 50 mM NaF, 150 mM NaCl, 2 mM EDTA, 5 mM β-mercaptoethanol, 30 mM sodium pyrophosphate, 3 mM benzamidine, 5 μg/mL leupeptin, 20 μg/mL pepstatin, 2 mM PMSF, and 1 mM microcystin]. The homogenates were centrifuged (4°C; 30 min; 14,000 rpm) to obtain a soluble fraction. PDE activity was measured using 0.5 μM cAMP as the substrate, according to the method described by Thompson *et al*. (48). Samples were assayed at 34°C in 200 μL (final volume) of assay solution [40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mg/mL BSA, and 0.5 μM cAMP and 25 nM [^{3}H] cAMP (~0.1 × 10^6 cpm/tube, 20 Ci/mmol); Amersham Biosciences, Piscataway, NJ]. To determine the contribution of oocyte-specific PDE (PDE3A) to the overall PDE activity of denuded oocytes, which was also due to the activity of PDE4 mainly expressed in the CCs/GCs, the specific PDE4 inhibitor rolipram (10 μM) was added to the incubation mixture.

3.7. Western blot analysis

Whole cell lysates of follicular cells were prepared by incubation with 2× electrophoresis sample buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate for 20 min on ice and stored at -80°C. Prior to use, whole cell lysates were boiled for 5 min, cooled on ice, and centrifuged at 12,000 × *g* for 5 min. The proteins were resolved by 10% SDS-PAGE and detected by western blotting. Nitrocellulose membranes (Amersham Pharmacia Biotech, Braunschweig, Germany) were blocked (5% nonfat milk) and incubated overnight with the corresponding antibodies. Proteins were detected using the SuperSignal West Pico (enhanced chemiluminescence) detection system (Thermo Scientific, Waltham, MA).

3.8. Statistical analysis

Results are presented as the mean (SEM) of at least three independent experiments. Differences between groups were analyzed using Student's *t*-test, two-way ANOVA, and subsequently by *post hoc* test. Statistical significance was defined as $P < 0.05$.

4. RESULTS

4.1. The kinetics of CBX-induced meiotic resumption in FEOs during continuous or transient incubation

To determine the effects of GJC disruption on the meiotic capacity of oocytes, FEOs were treated with CBX (150 μM; 3.5 h) and the reinitiation of meiosis was determined based on changes in % GVBD. As demonstrated in Figure 1, CBX did not induce meiotic

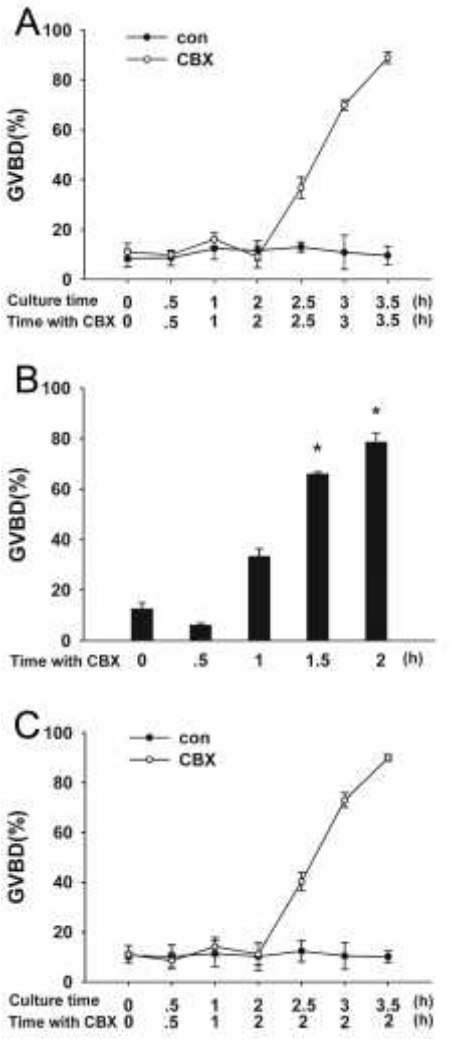

Figure 1. The kinetics of CBX-induced mouse oocyte meiotic resumption [continuous and transient (2 hours) stimulation]. A) FEOs were cultured in medium supplemented with CBX (150 µM). At various time points (0, 0.5, 1, 2, 2.5, 3, and 3.5 hours), CEOs were released and the oocytes were monitored for maturation. B) FEOs were transiently cultured in medium supplemented with CBX (150 µM) for the indicated times (0, 0.5, 1, 1.5, and 2 hours) and subsequently transferred to CBX-free medium for a total incubation time of 3.5 hours. After the culture period (3.5 hours), CEOs were recovered and the oocytes were monitored for maturation (*P < 0.05, compared to control). C) FEOs were preincubated with CBX for 2 hours and then transferred to CBX-free medium. At various incubation times (0, 0.5, 1, 2, 2.5, 3, and 3.5 hours), the CEOs were recovered and the oocytes were monitored for maturation. FEOs, follicle-enclosed oocytes; CBX, carbenoxolone; CEOs, cumulus-enclosed oocytes. Results are expressed as the mean ± SEM (n = 3 replicate experiments).

resumption in FEOs during the first 2 h, as almost all oocytes were arrested at the GV stage. However, from 2.5 to 3.5 h, CBX treatment induced oocyte meiotic resumption (36.75 ± 4.25% to 88.95 ± 2.25% GVBD), which did not occur in the control group (Figure 1A). To determine the time point at which oocytes become irreversibly committed to resuming meiosis, FEOs were transiently treated with CBX for various durations and subsequently cultured in CBX-free medium. Transient exposure of FEOs to CBX for 2 h followed by incubation in CBX-free medium for 1.5 h induced meiotic resumption in oocytes, similar to the levels seen in FEOs treated continuously with CBX. However, transient exposure to CBX for durations shorter than 2 h followed by culture in CBX-free medium resulted in decreased responses (Figure 1B). In addition, in the transiently (2 h) incubated groups, the kinetics of CBX-induced meiosis were the same as that in the continuously incubated groups (Figure 1C).

4.2. CBX induces a short transient rise in intraoocyte cAMP levels

Evidence suggests that in ovarian follicles, cAMP is mainly synthesized in somatic cells and transferred to the oocyte via gap junctions (16, 33, 49-50). However, recent studies suggest that the oocytes can independently generate cAMP (10, 13, 51-52). If intraoocyte cAMP is indeed supplied by somatic cells, inhibition of GJC would be expected to reduce cAMP concentrations within the oocyte. To investigate this possibility, we measured the cAMP concentrations in FEOs incubated with or without CBX. Under control conditions, FEOs contained an average of 1.35 ± 0.06 fmol cAMP in a single oocyte (Figure 2A) and 10.9 ± 0.44 fmol in CCs (Figure 2B). Notably, the addition of CBX to the incubation medium induced a surge in intraoocyte cAMP concentration followed by a decrease.

4.3. Oocytes produce more cAMP than GCs

In an FEO during meiotic arrest before the LH surge *in vivo*, the concentration of cAMP in the CC layer is approximately 10-fold higher than in the oocyte. Evidence suggests that the intraoocyte cAMP concentration is dependent in part on cAMP movement between follicular somatic cells and the oocyte via gap junctions. However, the source of the CBX-induced transient cAMP surge in oocytes remains unclear. To examine the possible source of cAMP responsible for the intraoocyte cAMP surge, the cAMP concentrations of an individual oocyte and CC were calculated. CCs were dissociated from FEOs by trypsin digestion (0.25%, room temperature; Amresco, Solon, OH) and total cell counts were obtained using a hemocytometer. As such, we determined that each oocyte is enclosed by ~3000–4000 CCs, and the diameters of a CC and oocyte were measured to be 9–12 µm and 55–65 µm, respectively. With these values, and those obtained above, the concentrations of cAMP in a single CC and oocyte were determined (Table 2); a higher concentration of cAMP was detected in a single oocyte than in a single somatic cell.

4.4. Differential expression of ACs in the oocyte and somatic cells of the mouse ovary

AC synthesizes cAMP from ATP. Given that oocyte cAMP is crucial for maintaining meiotic arrest, the expression of various AC isoforms in mouse somatic cells and oocytes was examined. The mRNA content of all the transmembrane isoforms of AC (isoforms AC1-9) was significantly higher in oocytes than in CCs and GCs+CCs, as determined by real-time PCR (Figure 2C). The AC isoforms with the highest expression levels in the oocytes

Table 2. Calculation of the concentration of cAMP in single oocyte and single somatic cell

Project	Oocyte	Cumulus cell
Concentration of cAMP per CEO	1.35 fmol ± 0.06	10.9 fmol ± 0.44
Diameter	55-65 μm	9-12 μm
Volume	6.97×10^5 μm^3-11.50×10^5 μm^3	3.05×10^3 μm^3-7.24×10^3 μm^3
Cells per CEO	1	3000-4000
cAMP concentration per CEO	1.17×10^{-6} fmol/μm^3-2.01×10^{-6} fmol/μm^3	0.37×10^{-6} fmol/μm^3-0.89×10^{-6} fmol/μm^3

Abbreviations: CEO, cumulus-enclosed oocytes

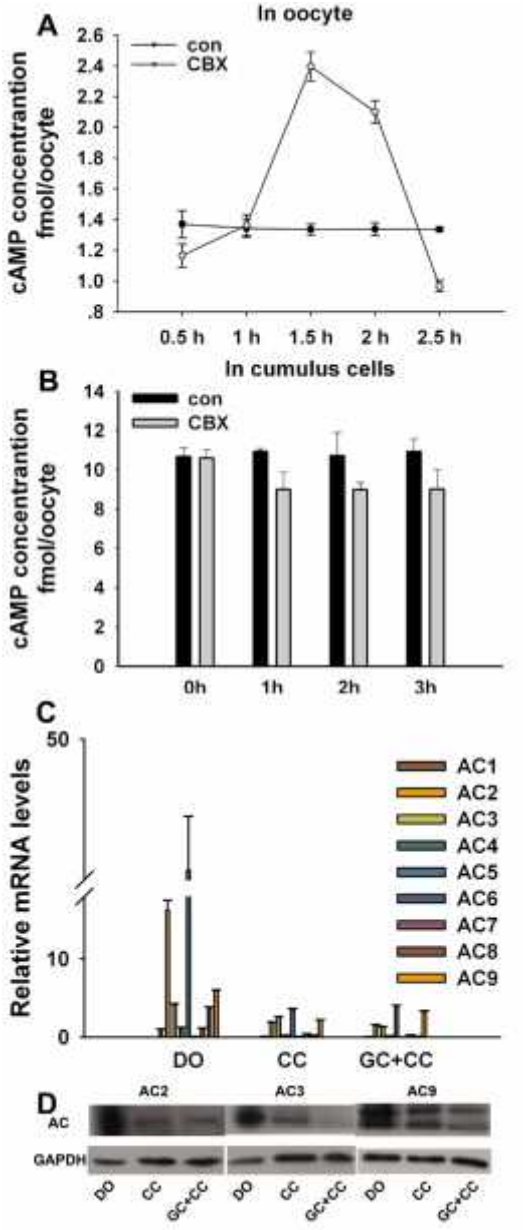

Figure 2. cAMP concentrations in FEOs incubated with or without CBX and the expression pattern of AC genes and proteins in FEOs. A) FEOs were incubated in medium supplemented with or without CBX (150 μM). Oocytes were isolated at different time points (0.5, 1, 1.5, 2, and 2.5 hours) in the presence of IBMX (2 mM), and the cAMP concentration was measured. B) cAMP concentration in cumulus cells in the control groups. C and D) Expression of adenylyl cyclase isoforms in mouse FEOs. mRNAs and protein content of ACs were detected by quantitative RT-PCR (AC1-9) and Western blot (AC2, -3, and -9). cDNA and protein extracts were obtained from isolated oocytes (DO), cumulus cells (CCs), and both granulosa cells (GCs) and CCs. Results are expressed as the mean ± SEM (n = 3 replicate experiments).

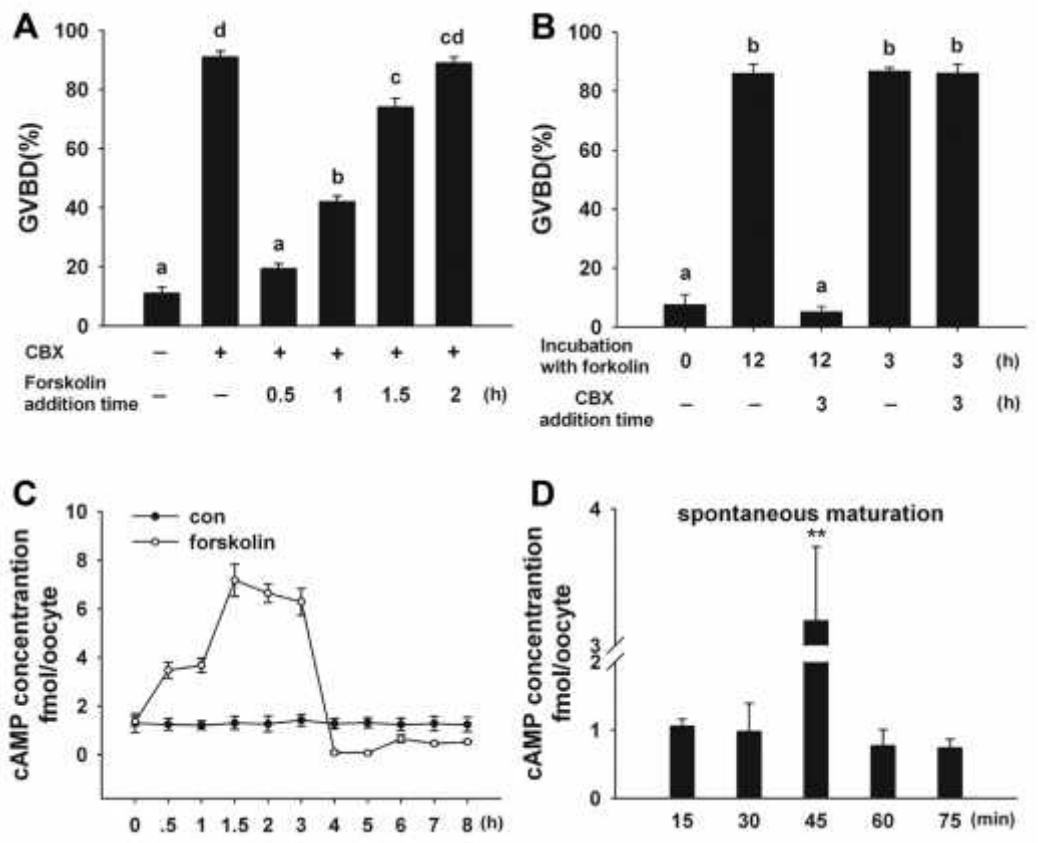

Figure 3. Effect of CBX and forskolin on oocyte meiotic resumption and intraoocyte cAMP concentration in forskolin-induced or spontaneous maturation. A) FEOs were cultured in medium containing 150 μM CBX for a total of 3.5 hours. At different time points (0.5, 1, 1.5, and 2 hours), 10 μM forskolin was added. The percentage of oocytes that showed GVBD after culture (3.5 hours) was measured. Results are expressed as the mean ± SEM (n = 3 replicate experiments). B) FEOs were preincubated with 10 μM forskolin for 3 hours, and the FEOs were quickly separated into four groups. Two groups were continuously incubated in medium containing 10 μM forskolin, and 150 μM CBX was added to one of these two groups. The other two groups were transferred to fresh medium without forskolin, and 150 μM CBX was added to one of these groups. The percentage of oocytes at GVBD after culture (12 hours) was measured. Bars with the different letters are significantly different ($P < 0.05$). Groups with a common letter are not significantly different. C) FEOs were incubated in medium in the presence or absence (con) of forskolin (10 μM). At different time points (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 hours), oocytes were isolated in the presence of IBMX (2 mM), and intraoocyte cAMP concentrations were measured. D) Denuded oocytes were incubated in DMEM and collected in the presence of IBMX (2 mM) at different time points (0, 15, 30, 45, 60, and 75 minutes). Intraoocyte cAMP concentrations were measured. Asterisks denote statistical significance by two-way ANOVA, followed by Bonferroni post-test, where ** is $P < 0.01$. Results are expressed as the mean ± SEM (n = 3 replicate experiments).

included AC2, AC3, AC5, AC8, and AC9; AC2, AC3, AC5, and AC9 exhibited the highest expression in somatic cells. Furthermore, the protein content of AC2, AC3, and AC9 was higher in oocytes than in somatic cells, as determined by western blotting (Figure 2D). These results suggest that oocytes may independently generate cAMP.

4.5. FEO maturation is inhibited by GJC blockade in the presence of forskolin but not FSH

Forskolin is an activator of ACs and can promote oocyte meiotic resumption. Both continuous and transient (2–3 h) treatment with forskolin induced activation of ACs in the oocyte and somatic cells and induced meiotic resumption in FEOs cultured for 12 h (Figure 3B). As shown in Figure 3A, forskolin (10 μM) completely inhibited the maturation of FEOs preincubated with CBX for 30 min, as indicated by the % GVBD (19.35 ± 1.65%), and partly inhibited the maturation of FEOs preincubated with CBX for 1 h (42.00 ± 2.00%). However, forskolin did not affect CBX-induced oocyte meiotic resumption if the CBX pretreatment duration was greater than 1 h (1.5 h; 74.00 ± 3.00). Next, we investigated the precise time point at which forskolin induced the rise in intraoocyte cAMP levels. Intraoocyte cAMP levels were highest after 1.5 h of forskolin treatment, an effect that persisted for up to 3 h (Figure 3C). Furthermore, as demonstrated in Figure 3B, FEOs were first preincubated with forskolin for 3 h to activate ACs and subsequently treated with CBX in medium with or without forskolin. CBX inhibited forskolin-induced oocyte maturation in the group continuously incubated with forskolin until the end of the culture period (12 h), whereas it had no effect on the transient (3 h) exposure to forskolin at the end of the culture period (12 h). These results suggest that cAMP flow from the oocyte into GCs is involved in oocyte maturation.

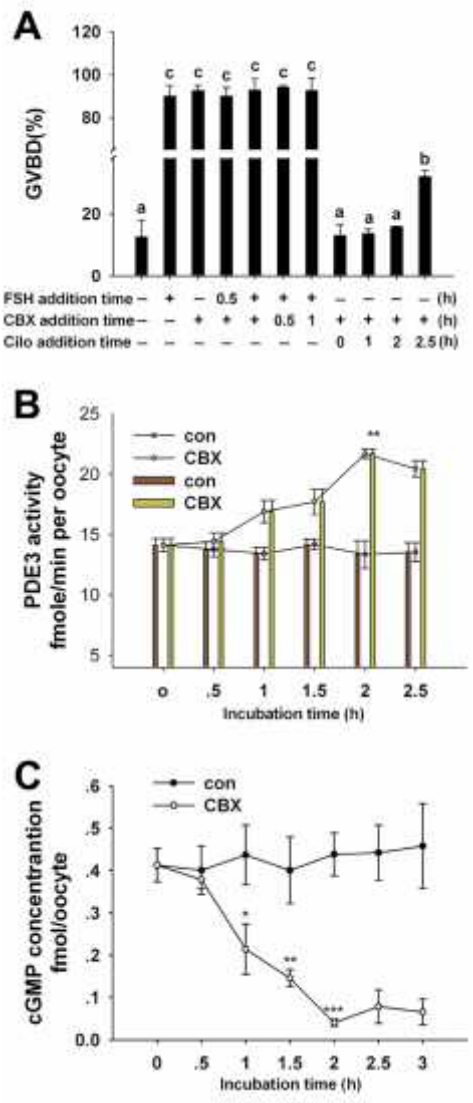

Figure 4. Effect of CBX, FSH, and PDE3A on oocyte meiotic resumption and CBX-induced changes in PDE3A activity and cGMP concentrations in FEOs. A) FEOs were incubated in medium with or without FSH (50 IU/L) for a total of 7 hours or in the presence or absence of CBX (150 µM) for a total of 3.5 hours. At different time points (0, 0.5, 1, 2, and 2.5 hours), CBX, FSH, or cilostamide (5 µM; cilo) was added. The percentage of oocytes at GVBD after culture (7 hours with FSH or 3.5 hours with CBX) was measured. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different. Results are expressed as the mean ± SEM (n = 3 replicate experiments). B) FEOs were incubated in medium supplemented with or without CBX (150 µM) for the indicated times (0, 0.5, 1, 1.5, 2, and 2.5 hours) and analyzed for PDE3 activity. C) In total, 250 FEOs were incubated in medium with or without (CTL) CBX (150 µM). Oocytes were isolated at various time points (0, 0.5, 1, 1.5, 2, 2.5, and 3 hours) in the presence of IBMX (2 mM). Cell extracts were analyzed for cGMP by ELISA with acetylated samples. Asterisks denote statistical significance by two-way ANOVA, followed by Bonferroni post-test, where *** is $P < 0.001$, ** is $P < 0.01$, and * is $P < 0.05$. Results are expressed as the mean ± SEM (n = 3 replicate experiments).

Figure 4A shows that both 50 IU/L FSH and 150 µM CBX induced FEO maturation (90.00 ± 5.00% and 92.50 ± 2.50%, respectively). Notably, CBX had no effect on FSH-induced oocyte maturation, in contrast to its effect on forskolin-induced oocyte maturation.

4.6. Changes in cAMP concentration during spontaneous oocyte maturation

To determine if the intraoocyte cAMP surge also occurred during spontaneous oocyte maturation, denuded oocytes were collected and cultured for 15–75 min in DMEM, and at the time of collection, 0.2 mM IBMX was added to the medium, after which the cAMP concentration was measured. As expected, a cAMP surge was detected in oocytes after 45 min in culture (Figure 3D). These results provide further evidence that oocytes can autonomously produce cAMP, which can be transferred to somatic cells via GJC.

4.7. CBX increases cAMP-PDE3A activity and decreases cGMP concentrations in oocytes

To address the potential involvement of PDE in the CBX-induced intraoocyte cAMP surge and the subsequent decrease in cAMP concentration, we exposed FEOs to cilostamide (a specific inhibitor of type 3 PDE) for various durations in the presence of CBX. Cilostamide significantly inhibited meiotic resumption, especially when added before 2.5 h (Figure 4A). Furthermore, because our results indicated that CBX induced a decrease in intraoocyte cAMP at 2.5 h of treatment (Figure 2A), we examined whether this effect was a result of PDE-dependent cAMP degradation. FEOs were exposed to CBX and the activity of an oocyte-specific PDE, PDE3A, was measured. As seen in Figure 4B, cAMP-PDE3A activity was slightly upregulated by CBX at 1.5 h and reached a maximum after 2 h.

cGMP is produced in follicle cells through a pathway involving the activation of NPR2, a guanylyl cyclase. cGMP inhibits PDE3A activity and cAMP hydrolysis and promotes mitotic arrest. To examine how CBX upregulates PDE3A activity, we measured the concentrations of cGMP in oocytes of FEOs incubated with or without CBX. As shown in Figure 4C, treatment of FEOs with CBX significantly decreased the intraoocyte cGMP concentration. CBX induced a decrease in intraoocyte cGMP content after 1 h of treatment with a maximal effect at 2 h.

4.8. The protein kinase A (PKA) I pathway plays a role in CBX-induced meiotic resumption

The cAMP/PKA signaling pathway plays an important role in maintaining meiotic arrest (33, 37). Intracellular cAMP maintains PKA in an active/dissociated state, which in turn leads to the phosphorylation of downstream substrates (37). In mouse oocytes, PKA I is the predominant subtype. To determine the involvement of intraoocyte PKA I in mouse oocyte maturation, CBX-treated FEOs were also treated with the PKA I-specific activator 8-AHA-cAMP (0–200 µM). As shown in Figure 5A, 8-AHA-cAMP inhibited CBX-induced FEO maturation in a dose-dependent manner.

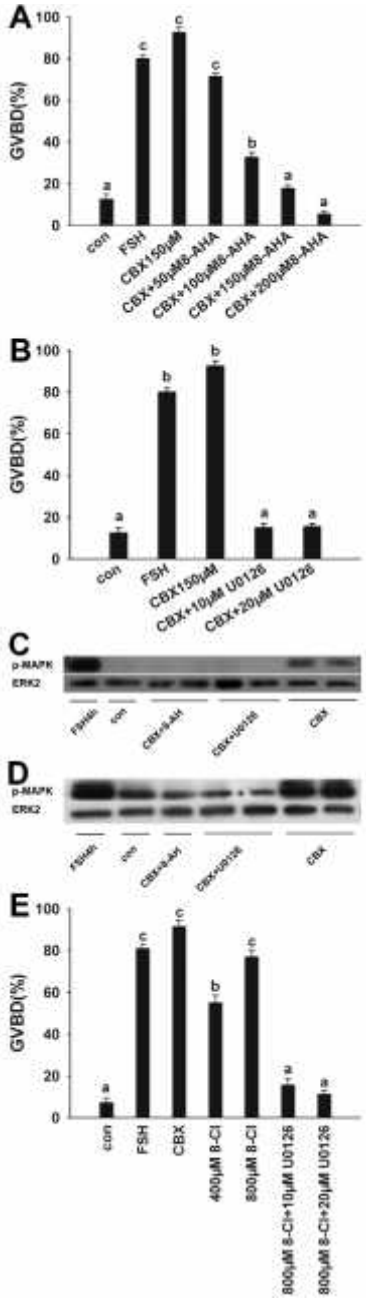

Figure 5. Effect of 8-AHA-cAMP and U0126 on FEO maturation and the relationship of the PKAI and MAPK pathways. A) FEOs were incubated in medium supplemented with FSH (50 IU/L) for 7 hours, followed by CBX (150 μM) and/or 8-AHA-cAMP (250 μM) for 7 hours. Follicles were then punctured after 7 hours and oocytes were scored for signs of meiotic resumption (GVBD). Results are expressed as the mean ± SEM (n = 3 replicate experiments). B) FEOs were incubated in medium supplemented with FSH (50 IU/L) for 7 hours, followed by CBX (150 μM) and/or U0126 (10 or 20 μM) for 3.5 hours. Follicles were then punctured, and oocytes were scored for signs of meiotic resumption (GVBD). Results are expressed as the mean ± SEM (n = 3 replicate experiments). C and D) Ovarian follicles were separated into five groups: control (con), adding FSH for 4 hours, CBX+8-AHA-cAMP (CBX+8-AHA), CBX+U0126, and CBX alone. Excluding the control and FSH groups, the other three groups were further divided into two separate treatment groups. The first group involved incubation with CBX for the entire culture period (2 hours and 15 minutes) (C). Follicles in the other treatment group were incubated with CBX for 2 hours and then washed and further incubated in CBX-free medium for an additional 15 minutes (D). After incubation, the oocytes were isolated and phospho-MAPK and total MAPK were detected by Western blotting. E) FEOs were cultured in medium supplemented with 8-cl-cAMP (400 or 800 μM) for 12 hours before GVBD scoring. In two groups of FEOs incubated with 8-cl-cAMP (800 μM), FEOs were first pre-cultured for 30 minutes with 10 or 20 μM of U0126, followed by the addition of 8-cl-cAMP. The percentage of oocytes at GVBD after culture (12 hours) was detected. Bars with different letters are significantly different ($P < 0.05$). Groups with a common letter are not significantly different. Results are expressed as the mean ± SEM (n = 3 replicate experiments).

4.9. CBX-induced FEO maturation is also dependent on MAPK activation in oocytes

Previous studies in both mice and rats have shown that activation of MAPK in the ovarian follicle mediates LH-induced meiotic resumption (32, 44). Moreover, MAPK is involved in the LH-induced disruption of GJC within the ovarian follicle (17, 32). Thus, we examined whether CBX-induced FEO maturation was dependent on MAPK activation. As shown in Figure 5C, MAPK activation was induced in FEOs treated with CBX for 2 h. We also examined whether follicles at 2.25 h exhibited MAPK activation after preincubation with CBX for 2 h followed by 15-min incubation in CBX-free medium, at which time maturation was initiated (Figure 1A). As shown in Figure 5D, MAPK activation was detected under these conditions. Moreover, a known inhibitor of the MAPK signaling pathway UO126 inhibited CBX-induced FEO maturation (Figure 5B).

4.10. The relationship between cAMP-PKA I and MAPK signaling during CBX-induced mouse FEO meiotic resumption

As shown in Figure 5C, 8-AHA-cAMP, the PKA I-specific activator, inhibited MAPK activation during CBX-induced oocyte maturation. In addition, 8-cl-cAMP, a specific inhibitor of PKA I, promoted meiotic resumption in a dose-dependent manner, which was inhibited by U0126 (79.2% GVBD versus 18.4% GVBD) (Figure 5E). Thus, we hypothesize that MAPK signaling is downstream of the PKA I pathway.

5. DISCUSSION

In the present study, we demonstrated that the concentration of cAMP in a single oocyte was higher than that in a single CC. As CBX-induced meiotic resumption in oocytes involves a transient surge in intraoocyte cAMP, we investigated whether cAMP can move from the oocyte to CCs via GJC.

LH is known to block GJC (0.5–2 h) transiently between the oocyte and somatic cells in mouse ovarian follicles—a process that promotes oocyte meiotic resumption (18). Moreover, interruption of GJC by CBX, a pharmacological agent that blocks gap junctions, promotes meiotic resumption in the FEOs of both rats and mice (18, 34). However, the precise mechanism by which GJC is involved in oocyte maturation is not known.

To address these questions, a mouse model of CBX-induced FEO maturation was used. The kinetics of CBX-induced meiotic resumption in oocytes was examined and FEO meiotic resumption was triggered after 2.5 h of incubation. GJC was disrupted by CBX within 20 min of incubation, as determined by a calcein-AM transfer assay (data not shown). Taken together, these results suggest that interruption of GJC within FEOs occurs before the oocytes mature to the GVBD stage. To determine the time point at which the FEOs became irreversibly committed to CBX-induced meiotic resumption, we transiently incubated FEOs with CBX for various durations followed by culture in CBX-free medium, and measured % GVBD. Incubation of FEOs in CBX for 2 h resulted in an irreversible commitment of the FEOs to resume meiosis. These results indicate that oocyte maturation mediated by transient CBX exposure may involve similar cellular processes as the LH surge (53). The activity of PDE3A, PKA I, and MAPK are all involved in CBX-induced maturation.

CBX imitated the LH response in the ovarian follicle. Therefore, we investigated the source of the cAMP surge during CBX-induced oocyte maturation and the direction of gap junction-mediated cAMP movement between somatic cells and ovarian follicular oocytes.

A cAMP surge was detected in both CBX-induced and spontaneous meiotic resumption in mouse FEOs. During mouse FEO meiotic resumption, intraoocyte cAMP concentrations were similar to the levels reported in rodent oocytes that resumed meiosis because of gonadotropin (LH or FSH) treatment (8-9).

The increase in intraoocyte cAMP concentration that occurred after CBX-induced GJC blockade supports the hypothesis that oocytes have the catalytic capacity to generate cAMP. Rodent oocytes express type 3 AC (AC3) (52), and the activation of AC using forskolin increases intraoocyte cAMP concentrations and inhibits meiotic maturation (4-5, 8, 54). Horner *et al.* also demonstrated that approximately 50% of oocytes resume meiosis in AC3-null mice (52). These results highlight the importance of intraoocyte cAMP synthesis in meiotic resumption (52). In the present study, we characterized the expression of primary AC subtypes in oocytes and somatic cells of mouse FEOs using real-time PCR and western blotting. AC expression (mRNA and protein) was higher in oocytes than in somatic cells, which suggests that oocytes have a higher capacity to synthesize cAMP than somatic cells. The higher cAMP concentrations in the oocyte suggest that cAMP flows from the oocyte to CCs through gap junctions based on a concentration gradient. CBX-induced gap junction blockade prevented the outflow of oocyte-synthesized cAMP into somatic cells, which resulted in intraoocyte cAMP accumulation. Indeed, the CBX-induced meiotic resumption (0.5-h preincubation with CBX) was inhibited by forskolin. In this case, cAMP synthesis was promoted via the activation of ACs by forskolin and the prevention of cAMP transport to somatic cells by CBX-induced GJC blockade, both of which are required for the maintenance of oocyte meiotic arrest. Forskolin requires about 1.5 h to elevate cAMP levels sufficiently in oocytes; treatment with forskolin after 1.5 h did not prevent FEO maturation, as the process of CBX-induced oocyte maturation may already have been initiated. Indeed, if FEOs were preincubated with forskolin for 3 h (which is sufficient to activate ACs), followed by the addition of CBX to the medium for the next 9 h in the presence of forskolin, meiotic resumption of FEOs was inhibited. Furthermore, when we removed forskolin after 3 h and added CBX, oocyte maturation was no longer interrupted by CBX. In both cases, forskolin induced activation of ACs and increased intraoocyte cAMP levels, promoting PDE3A activity in oocytes (55). When CBX was added in the presence of forskolin, intraoocyte cAMP was persistently generated by activated ACs and

cannot be transported to somatic cells. Thus, maturation was inhibited. When CBX was added after removing forskolin, intraoocyte cAMP was no longer generated and was degraded by activated PDE3A, therefore maturation was not inhibited. The PDE3A activity of oocytes may explain why a decrease in cAMP occurred at 4 h and why the FEO resumed meiosis when persistently incubated with forskolin alone.

These results suggest that if GJC is functional, forskolin-induced cAMP synthesis and accumulation in oocytes could decrease because of cAMP diffusing into somatic cells through gap junctions subsequent to PDE-mediated cAMP hydrolysis in somatic cells. However, if GJC was inhibited, cAMP flow from the oocyte into somatic cells would be blocked, resulting in high cAMP levels that would promote oocyte meiotic arrest. The hydrolytic activity of PDE3A in oocytes is elevated due to increased cAMP and decreased cGMP levels.

FSH receptor (FSHR) is expressed in somatic cells, but not in oocytes (56). Therefore, FSH-induced cAMP production occurs only in somatic cells through the activation of G protein (Gs)-linked FSHR. Interruption of GJC is essential to protect oocytes from the inflow of cAMP from somatic cells; thus, CBX had no effect on FSH-induced maturation. Movement of cAMP from the oocyte to CCs may be an essential event in the regulation of oocyte meiotic resumption. Further studies are required to determine how GJC between oocyte and somatic cells is regulated and how changes in cAMP affect forskolin- or FSH-induced FEO maturation.

cAMP is degraded by PDE-catalyzed hydrolysis (10, 23-24). Of the eleven tissue-specific PDE isoforms identified in mammals, the major isoform expressed in mouse oocytes is PDE3A (23-24). Our results demonstrated that PDE3A activity is enhanced prior to CBX-induced oocyte meiotic resumption. This differs from the results of Sela-Abramovich *et al.* (34) in rats, but this discrepancy may be species-specific. Moreover, by adding 5 μM cilostamide (an inhibitor of PDE3A) at different time points to the medium with CBX, we found that maturation of FEOs was regulated by PDE3 activity in oocytes *in vitro* because cilostamide inhibited meiotic resumption just before meiotic resumption was triggered at 2.5 h. Studies have shown that cGMP that flows from somatic cells into the oocyte maintains meiotic arrest by inhibiting PDE3A (27-29, 53, 57-58). Because the V_{max} and K_m of cGMP are lower than that for cAMP, cGMP becomes a competitive inhibitor in cAMP hydrolysis (20, 59-60). In cultured mouse follicles, cGMP levels significantly decreased 1.5 h after CBX treatment and prior to oocyte maturation, and reached nadir at 2 h. The decrease in intraoocyte cGMP at 1.5–2 h was accompanied by an increase in PDE3A activity, which could explain the decrease in intraoocyte cAMP levels after 1.5 h of CBX treatment.

Therefore, the balance between cAMP synthesis and hydrolysis by AC and PDE3A, respectively, may determine oocyte meiotic arrest or maturation. Meiotic maturation resumes only when cAMP hydrolysis and transportation from oocytes to somatic cells are faster than the cAMP synthesis in oocytes.

PKA I, the main PKA isoform expressed in oocytes, mediates meiotic arrest (37, 42). Studies have identified a link between cAMP and PKA based on the inhibition of the cdc2/cyclin B complex (MPF) (45, 61-64), which is a key regulator of the meiotic cycle. Previous studies have demonstrated that both LH-induced closure of GJC and LH-induced oocyte maturation are mediated by the activation of MAPK (17, 32, 58). In our study, we demonstrated a relationship between PKA I and MAPK in CBX-induced mouse FEO meiotic resumption. CBX-induced FEO maturation was inhibited by 8-AHA-cAMP (a specific activator of PKA I) and U0126 (a specific inhibitor of MAPK), whereas 8-cl-cAMP (a specific inhibitor of PKA I) promoted meiotic resumption, which was inhibited by U0126. CBX induced MAPK (described as ERK1/2) phosphorylation; however, the activation of MAPK was eliminated by 8-AHA-cAMP and U0126. These results show that PKA I inhibits ERK1/2 phosphorylation and activity, which is consistent with the results in NG108-15 cells (39), resulting in oocyte meiotic arrest. During meiotic arrest, cAMP, in the oocyte, activated cAMP-dependent PKA I, which inhibited the MAPK pathway. Interruption of GJC decreased intraoocyte cAMP levels leading to inactivation of PKA I, which induced the phosphorylation and activation of ERK1/2 before oocyte meiotic maturation.

In summary, we demonstrated that oocytes can autonomously synthesize cAMP and regulate the process of meiotic resumption by transporting cAMP to somatic cells through gap junctions. Moreover, the catalytic capacity of mouse oocytes to generate cAMP is sufficient to maintain meiotic arrest. We demonstrated that the effect of CBX on GJC blockade in the ovarian follicle, which is followed by a surge in cAMP and a decrease in intraoocyte cGMP concentrations, partially simulates meiotic resumption induced by LH. CBX-induced oocyte maturation involves both the cAMP/PKA I and MAPK pathways, in which cAMP/PKA I acts upstream of MAPK.

6. ACKNOWLEDGMENTS

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Abbreviations: FEO, follicle-enclosed oocyte; GJC, gap junctional commumication; CBX, carbenoxolone; GV, germinal vesicle; GVBD, GV breakdown; AC, adenylyl cyclases; PDE, phosphodiesterase; NPPA, natriuretic peptide precursor A; NPR1, Natriuretic Peptide Receptor 1; PKA I, the cAMP-dependent protein kinase A I; MAPK, Mitogen-Activated Protein Kinase; RIA, radioimmunoassay

Key Words: cAMP, cGMP, GJCs, PDE3A, PKA I, MAPK, follicle-enclosed oocyte, FEO, Maturation

Send correspondence to: Guoliang Xia, State Key Laboratories of Agro-biotechnology, College of Biological Sciences, China Agricultural University, Beijing 100094, PR China,Tel: 86-10-62733456, Fax: 86-10-62733456, E-mail: glxiachina@sohu.com