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Dynamic Changes of Connexin-43, Gap Junctional Protein, in Outer Layers of Cumulus Cells Are Regulated by PKC and PI 3-Kinase During Meiotic Resumption in Porcine Oocytes

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ABSTRACT

Mammalian oocytes are surrounded by numerous layers of cumulus cells, and the loss of gap junctional communication in the outer layers of cumulus cells induces meiotic resumption in oocytes. In this study, we investigated the dynamic changes in the gap junctional protein connexin-43 in cumulus cells during the meiotic resumption of porcine oocytes. The amount of connexin-43 in all layers of cumulus cells recovered from cumulus-oocyte complexes was increased after 4-h cultivation. However, at 12-h cultivation, the positive signal for connexin-43 immunoreactivity was markedly reduced in the outer layers of cumulus cells. When these reductions of connexin-43 were blocked by protein kinase C (PKC) or phosphatidylinositol (PI) 3-kinase inhibitor, networks of filamentous bivalents (i.e., advanced chromosomal status) were undetectable in the germinal vesicle of the oocyte. After 28-h cultivation, when the majority of oocytes were reaching the metaphase I (MI) stage, the connexin-43 in the inner layers of cumulus cells was phosphorylated, regardless of mitogen-activated protein (MAP) kinase activation. These results suggest that the initiation of meiotic resumption, namely, the formation of networks of filamentous bivalents in germinal vesicle, is associated with the reduction of gap junctional protein connexin-43 in the outer layers of cumulus cells via the PKC and/or PI 3-kinase pathway. Moreover, the connexin-43 in the inner layers of cumulus cells is phosphorylated during meiotic progression beyond the MI stage, regardless of MAP kinase activation in cumulus cells surrounding the oocyte.

cumulus cells, kinases, meiosis, ovum, signal transduction

INTRODUCTION

Mammalian follicular oocytes arrested at the diplotene stage of the first meiotic prophase are closely surrounded by cumulus or granulosa cells. Cumulus and granulosa cells synthesize some meiosis-inhibitory factors, including cyclic AMP (cAMP) transported into oocytes via numerous gap junctions, resulting in the suppression of meiotic resumption in oocytes [1–3]. The disruption of gap junctions within cumulus cells induces the meiotic resumption of rat and porcine oocytes due to blockage of the conduction of meiosis-inhibitory signals from the outer layers of cumulus cells to the oocytes [4–6].

Gap junctions, the specialized regions in opposite membranes between neighboring cells, are channels that pass low molecular weight substances such as cAMP and ions to enhance cellular interactions [7]. These channels are formed by hexametric structures consisting of connexin molecules (i.e., connexon) in numerous tissues [7]. Porcine ovarian follicles have been reported to express five members of the connexin gene family: connexin-26, connexin-30.3, connexin-32, connexin-43, and connexin-60 [8, 9]. The connexin-43 protein has numerous phosphorylated sites, and these phosphorylations play a key role in regulatory mechanisms governing the assembly of connexons into gap junctions in the plasma membrane and in gating the formed gap junction [10, 11]. In particular, the phosphorylation of serine on connexin-43 is stimulated by mitogen-activated protein (MAP) kinase (extracellular-regulated kinase [ERK]-1,2), closing gap junctional communication in rat liver cells [12, 13]. Recently, Hossain et al. [14] reported that in rat liver cells, the blockade of gap junctions due to the phosphorylation of connexin-43 was induced by the activation of MAP kinase downstream of either the phosphatidylinositol (PI) 3-kinase or the protein kinase C (PKC) pathway.

In our previous experiments [15, 16], we investigated the effects of PI 3-kinase-specific inhibitors (i.e., wortmannin and LY294002) on the resumption of meiosis in porcine follicular oocytes. The results of these experiments clearly showed that PI 3-kinase inhibitor suppressed the phosphorylation of connexin-43 in cumulus cells, with a higher proportion of oocytes arrested at the germinal vesicle (GV) stage. This shows that in porcine oocytes, meiotic resumption was associated with the phosphorylation of connexin-43 in cumulus cells. In rat follicular oocytes, connexin-43 has also been found to be phosphorylated through the PKC-dependent pathway during LH-induced meiotic resumption [17]. Thus, in follicle cells, the blockade of gap junctions might result from the phosphorylation of connexin-43 via the PKC or PI 3-kinase pathway in a way similar to that in rat liver cells as reported by Hossain et al. [14]. However, to our knowledge, no information is available describing the role of MAP kinase for the phosphorylation of connexin-43 in cumulus cells, and neither are details regarding the relationship between the phosphorylation of connexin-43 and meiotic resumption in oocytes.

In the present study, we elucidated the dynamic changes of connexin-43 in cumulus cells during meiotic resumption of porcine oocytes. The results revealed that connexin-43 was reduced in the outer layers of cumulus cells surrounding oocytes during the nuclear progression from the GVII to GVIII stage, followed by phosphorylation of connexin-43 in the inner layers of cumulus cells. Consistent with this, a higher proportion of oocytes reaching the metaphase I (MI) stage was noted. The synthesis of connexin-43 was down-regulated by the activation of PI 3-kinase or PKC in cumulus cells surrounding oocytes but was independent of the MAP kinase pathway.

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MATERIALS AND METHODS

Isolation and Culture of Porcine Cumulus-Oocyte Complexes and Denuded Oocytes

Porcine ovaries were collected from 5- to 7-mo-old prepubertal gilts at a local slaughterhouse and transported within 1.5 h to the laboratory in 0.85% (w/v) NaCl containing 0.1 mg/ml of kanamycin (Meiji Seika, Tokyo, Japan) at approximately 30°C. The surfaces of intact, healthy antral follicles of 3–8 mm in diameter were cut with a razor blade, and oocytes were collected by scraping the inner surface of the follicular walls using a surgical blade. The oocytes collected were placed in prewarmed PBS (pH 7.4) supplemented with 0.1% (w/v) polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, MO) and 0.1 mg/ml of kanamycin. Oocytes having evenly granulated cytoplasm with at least four layers of unexpanded cumulus oophorus cells were selected under a stereomicroscope and washed three times with maturation medium. The basic maturation medium was modified NCSU37 [18], containing 10% (v/v) fetal calf serum (Gibco BRL, Grand Island, NY), 0.6 μg/ml of pFSH (Sigma), 1.3 μg/ml of eLH (Sigma), 7 mM Taurine (Sigma), essential amino acids (Gibco), and nonessential amino acids (Gibco). Some cumulus-oocyte complexes (COCs) were mechanically denuded by pipetting in 0.02% (w/v) EDTA (Sigma)/PBS with Flame-Draw pipette tips, which had inner diameters slightly larger than the oocyte diameter. The remaining COCs and denuded oocytes (DOs) were cultured for various time periods in 100-μl drops of different maturation medium (~20 oocytes/drop) covered with mineral oil (Sigma) at 39°C in a humidified atmosphere of 5% CO_2 in air.

Treatment with Calphostin C, LY294002, or U0126

Calphostin C (Sigma) for the inhibition of PKC, LY294002 (Sigma) for the inhibition of PI 3-kinase, and U0126 (Promega, Madison, WI) for the inhibition of MAP kinase kinase (MEK) were dissolved in dimethyl sulfoxide (DMSO; Sigma) at 1×10^{-3} M, 5×10^{-2} M, or 2×10^{-2} M, respectively, and stored at −20°C. Each final concentration described below was obtained by dilution with the basic maturation medium. The COCs or DOs were cultured for 28 h in the medium supplemented with 1×10^{-6} M calphostin C, 5×10^{-5} M LY294002, or 2×10^{-5} M U0126, respectively. As a control, inhibitor-free medium was prepared by adding 0.098% (v/v) DMSO to the basic maturation medium. This concentration of DMSO does not affect porcine oocyte maturation [15].

Assessment of Nuclear Maturation

After incubation, the oocytes were freed from cumulus cells and then mounted on slides, fixed with acetic acid/ethanol (1:3 v/v) for 48 h, and stained with aceto-lacmoid before examination under a phase-contrast microscope (400×) for evaluation of their chromatin configurations. Oocytes in the GV stage were classified according to the method described by Motlik and Fulka [19] into four categories: GVI, characterized by a nucleus and chromatin in the form of a ring or horseshoe around the nucleus; GVII, characterized by a nucleus and chromatin as a ring or horseshoe around the nucleus but with some well-stained clumps localized around the nucleus; GVIII, characterized by a nucleus and irregular networks of filamentous bivalents in the whole area of the GV; and GVIV, characterized by no nucleus and irregular networks of filamentous bivalents in the whole area of the GV.

Extract Preparation

After being cultured for various time periods, COCs were separated into oocytes and cumulus cells. Either 10 oocytes or 1×10^4 cumulus cells isolated from the COCs and 10 DOs, respectively, were washed several times in PBS. These oocytes or cumulus cells were put into plastic tubes containing 5 μl of cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% [v/v] Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml of leupeptin, and 1 mM PMSF [Sigma]). Except for PMSF, drugs were purchased from New England Biolabs (Beverly, MA). After suspension of oocytes or cumulus cells, the samples were frozen in liquid nitrogen, and then the frozen samples were sonicated using an ultrasonic disruptor (UD-200; Tomy, Tokyo, Japan) fitted with CUP HORN (CH-0633; Tomy) three times for 25 sec each at 1°C. Cell extracts were frozen and stored at −80°C just before use.

Connexin-43 and MAP Kinase Detected by Immunoblotting Analysis

After cultivation of COCs, cumulus cells were separated from the COCs. The cumulus cells were lysed as described above, and the cumulus cell extract was diluted twofold with 2× Laemmli sample buffer [20]. After denaturing by boiling for 5 min, each 5 μl of protein sample, which contained 5×10^3 cumulus cells, was separated by SDS-PAGE on a 12.5% polyacrylamide gel (Pharmacia Biotech, Uppsala, Sweden), then transferred onto a polyvinylidene fluoride membrane (Amersham, Arlington Heights, IL) using the PhastTransfer system (Pharmacia Biotech). The membrane was blocked using SuperBlock blocking buffer (Pierce, Rockford, IL), then incubated with either mouse monoclonal anti-connexin 43 antibody (Chemicon International, Temecula, CA) at 1:2000 dilution or mouse monoclonal anti-MAP kinase antibody (Zymed, San Francisco, CA) at 1:1000 overnight at 4°C in 10% (v/v) SuperBlock blocking buffer in 0.1% (v/v) Tween 20-PBS (T-PBS). After three washes in T-PBS, the membranes were treated with horseradish-peroxidase-labeled anti-mouse IgG (1:7000; Amersham) in 10% (v/v) SuperBlock blocking buffer in T-PBS for 1 h at room temperature. After three washes of 10 min each with T-PBS, peroxidase activity was visualized using the ECL Plus Western blotting detection system (Amersham) according to the manufacturer's instructions. The intensity of the bands was analyzed using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). Each independent experiment was repeated three times.

Dephosphorylation of Connexin-43

Cumulus cells were separated from COCs immediately recovered from their follicles, and 2×10^4 cumulus cells were lysed using 5 μl of cell lysis buffer as described above, but in the absence of 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM Na_3VO_4. Four hundred units of lambda protein phosphatase (New England BioLabs) were preincubated at 30°C for 30 min in reaction buffer (50 mM Tris-HCl, 0.1 mM EDTA, 5 mM 1,4-dithiothreitol [DTT], 0.01% [v/v] Brij 35, and 2 mM $MnCl_2$ [pH 7.5]). Dephosphorylation was initiated by addition of 5 μl of cumulus cell extract (containing 2×10^4 cells) to 5 μl

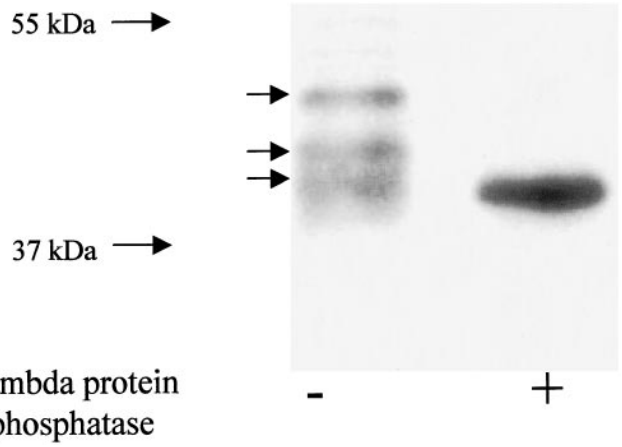

FIG. 1. Immunoblotting analysis of dephosphorylation of connexin-43 in porcine cumulus cells isolated from COCs immediately after collection from their follicles.

of lambda protein phosphatase-conjugated reaction buffer. After incubation for 30 min at 30°C, the samples were mixed with an equal volume of 2× Laemmli sample buffer and processed for immunoblotting analysis as described above. Thus, equal amounts of sample adjusted to 5×10^3 cells were added to each lane.

In Situ Immunofluorescence of Connexin-43

The COCs were fixed with 4% (w/v) paraformaldehyde-PBS (pH 7.4) at room temperature. After 30 min, they were rinsed three times with PBS and permeabilized with 0.5% (v/v) Triton X-100-PBS for 30 min at room temperature. The COCs were blocked by 5% (w/v) BSA (Sigma) and then incubated with connexin-43 monoclonal antibody (1:50) in 5% (w/v) BSA in T-PBS overnight at 4°C. The COCs were rinsed with T-PBS and incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:50 in 5% (w/v) BSA in T-PBS for 2 h at 38°C. After 2 h of incubation at 38°C, the COCs were rinsed with T-PBS to eliminate excess antibody. The COCs were next mounted on a slide with 10 μl of antifade solution (SlowFade Antifade Kit; Molecular Probes, Eugene, OR) to preserve the fluorescence, and then the COCs were observed by fluorescent microscopy (IMT-2; Olympus, Tokyo, Japan). At least 30 COCs incubated with connexin-43 antibody were observed at each time point of cultivation.

In Vitro MAP Kinase Assay

A p44/42 MAP kinase assay kit (New England BioLabs) was used for measuring MAP kinase activity. The methods for MAP kinase assay were based on those described by Anas et al. [21]. Briefly, after cultivation of COCs, cumulus cells were separated from the COCs and lysed according to the method described above. Next, 5 μl of cumulus cells extract (containing 1×10^4 cells) was mixed with 25 μl of kinase assay buffer A (25 mM Tris [pH 7.5], 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$), 0.1 mM ATP (Sigma), and 2 μg of Elk 1 fusion protein, then incubated for 30 min at 30°C. Except for ATP, drugs were purchased from New England Biolabs. The reaction was terminated by the addition of 10 μl of 4× Laemmli sample buffer, and after boiling for 5 min, the samples were subjected to 12.5% SDS-PAGE. The phosphorylation of Elk 1 fusion protein was detected by immunoblotting and chemiluminescence detection using anti-phospho-specific Elk 1 antibody. Each independent experiment was repeated four times.

In Vitro p34^{cdc2} Kinase Assay

The p34^{cdc2} kinase assay was performed using a MESACUP cdc2 kinase assay kit (code 5234; MBL, Nagoya, Japan) according to the method described by Shoujo et al. [22]. They showed that correlation coefficients for p34^{cdc2} kinase activity examined when using the MESACUP cdc2 kinase assay kit and histone H1 kinase activity measured by radioactive method were as high as 0.9961.

Briefly, after cultivation of COCs for various time periods, oocytes were separated from the COCs. The oocytes or DOs cultured for 28 h were lysed according to the method described above. Five microliters of oocyte extract (containing 10 oocytes) were mixed with 45 μl of kinase assay buffer B, which was composed of 25 mM Hepes buffer (pH 7.5; MBL), 10 mM $MgCl_2$ (MBL), 10% (v/v) MV peptide solution (SLYSSPGGAYC; MBL), and 0.1 mM ATP (Sigma), and the mixture was incubated for 30 min at 30°C. The reaction was terminated by the addition of 200 μl of PBS containing 50 mM EGTA (MBL). The phosphorylation of MV peptides was detected using ELISA analysis (MESACUP cdc2 kinase assay kit, code 5234; MBL). Data were expressed as the fold strength of p34^{cdc2} kinase activity in oocytes immediately after collection from

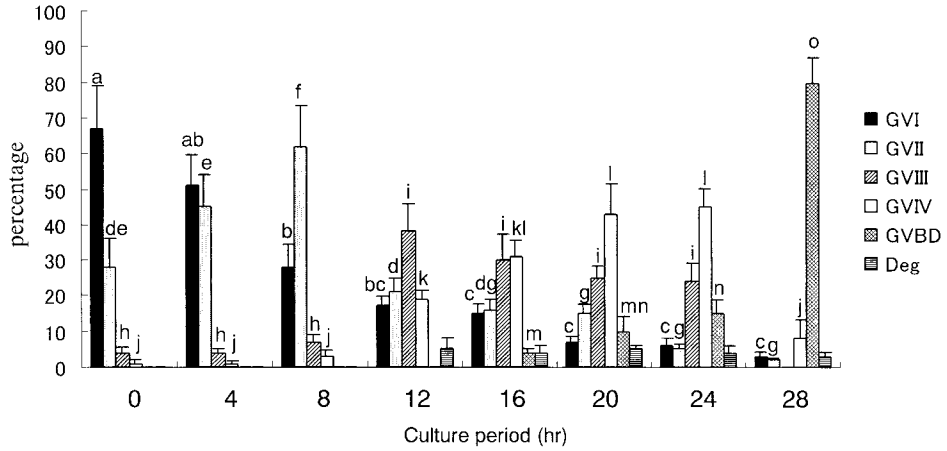

FIG. 2. Time-dependent change of nuclear maturation in the presence of FSH and LH. Data were obtained from four separate experiments. Columns with no common superscript are significantly different within the same nuclear status ($P < 0.01$).

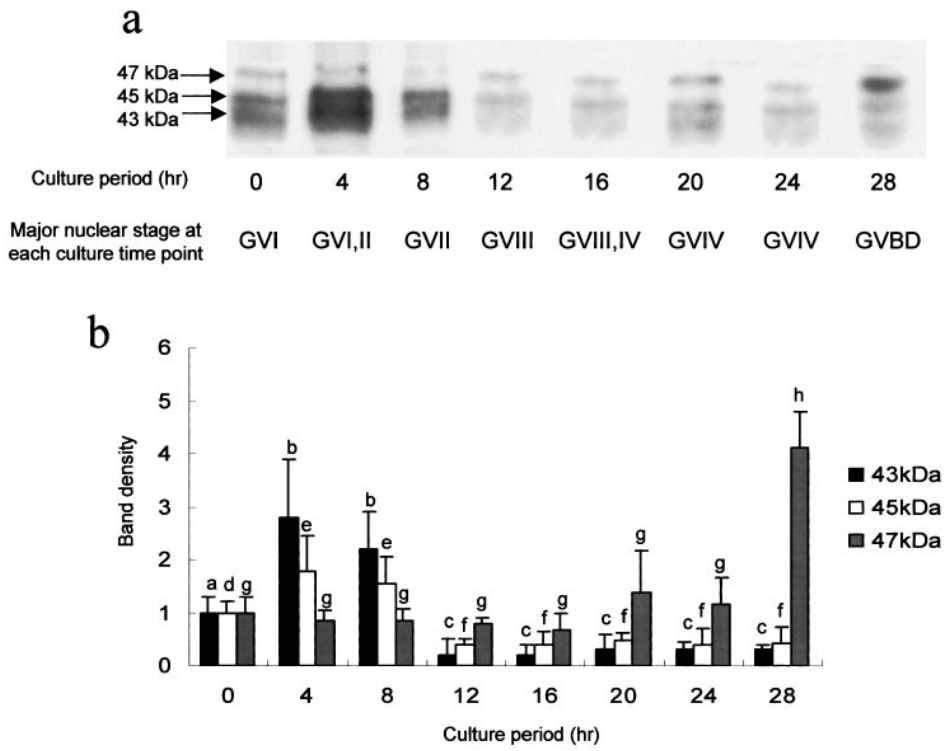

FIG. 3. Profiles of phosphorylation states of connexin-43 in cumulus cells from porcine COCs cultured in the presence of FSH and LH. **a**) Immunoblotting probed with anti-connexin-43 monoclonal antibody. **b**) Intensity of connexin-43 bands as determined by scanning densitometry. Data in each band are expressed as the fold strength of the intensity in cumulus cells immediately after collection from their follicles. Data were obtained from three separate experiments. Columns with no common superscript are significantly different within the bands migrated at the same levels ($P < 0.01$).

their follicles. Each independent experiment was repeated four times.

Statistical Analysis

Statistical analyses of all data from three or four replicates for comparison were performed by one-way ANOVA followed by Duncan's multiple ranges test using STATVIEW (Abacus Concepts, Berkeley, CA). All percentage data were subjected to arc-sine transformation before statistical analysis.

RESULTS

Connexin-43 Detected by Immunoblotting Analysis

Immunoblotting analysis of extracts of cumulus cells separated from COCs just after collection revealed that three bands of connexin-43 were detected between 43 and 47 kDa (Fig. 1). The combined treatment with lambda protein phosphatase and $MnCl_2$ increased the intensity of the lower molecular weight form of the protein and resulted in the disappearance of the two higher molecular weight forms. These observations show that the 43-kDa band is a nonphosphorylated band, whereas the 45- and 47-kDa bands are phosphorylated forms of this protein.

Time-Dependent Changes of Nuclear Maturation and Connexin-43 in Cumulus Cells from COCs During Meiotic Resumption

The majority of oocytes (67%) before cultivation were arrested at the GVI stage (Fig. 2). After 8 h of cultivation, significant reductions in GVI oocytes were noted, and the proportion of oocytes exhibiting the GVII stage were significantly increased. Further 4-h cultivation (i.e., total of 12 h) significantly promoted the meiotic progression to the GVIII and GVIV stage, in which networks of filamentous bivalents were detectable in the GV. When COCs were cultured for 28 h, 82% of oocytes underwent germinal vesicle breakdown (GVBD).

As shown in Figure 3, three strong bands situated at 43-, 45-, and 47-kDa on SDS-PAGE were detected in the lysates of cumulus cells isolated from COCs immediately after collection from their follicles. After 4- and 8-h cultivation of COCs, the staining intensities of faster (i.e., 43-kDa) and moderate (i.e., 45-kDa) migrating bands were significantly increased compared to those of cumulus cells separated from COCs immediately after collection. Using immunofluorescent analysis of COCs, positive signals for connexin-43 immunoreactivity existed in all layers of the cumulus cells, presumably including the oocyte surface (Fig. 4b′), and these signals were much more intense after 4-h cultivation (Fig. 4c′). However, after cultivation of 12 h and up to 28 h, when significant reductions in the intensity of 43- and 45-kDa connexin-43 bands were noted (Fig. 3), positive signal for connexin-43 immunoreactivity was detected in the inner layers of cumulus cells and oocytes but was markedly diminished in the outer layers of cumulus cells (Fig. 4d′). In contrast, no significant differences in the intensity of the phosphorylated form of 47-kDa connexin-43 were seen in cumulus cells from COCs cultured for up to 24 h, whereas the additional 4-h cultivation (i.e., total of 28 h) resulted in a significant rise in the intensity of this phosphorylated band (Fig. 3). Thus, the combined evidence from immunoblotting and immunofluorescent analyses of connexin-43 suggests that within the first 4 h of cultivation, a high amount of connexin-43 was synthesized in all layers

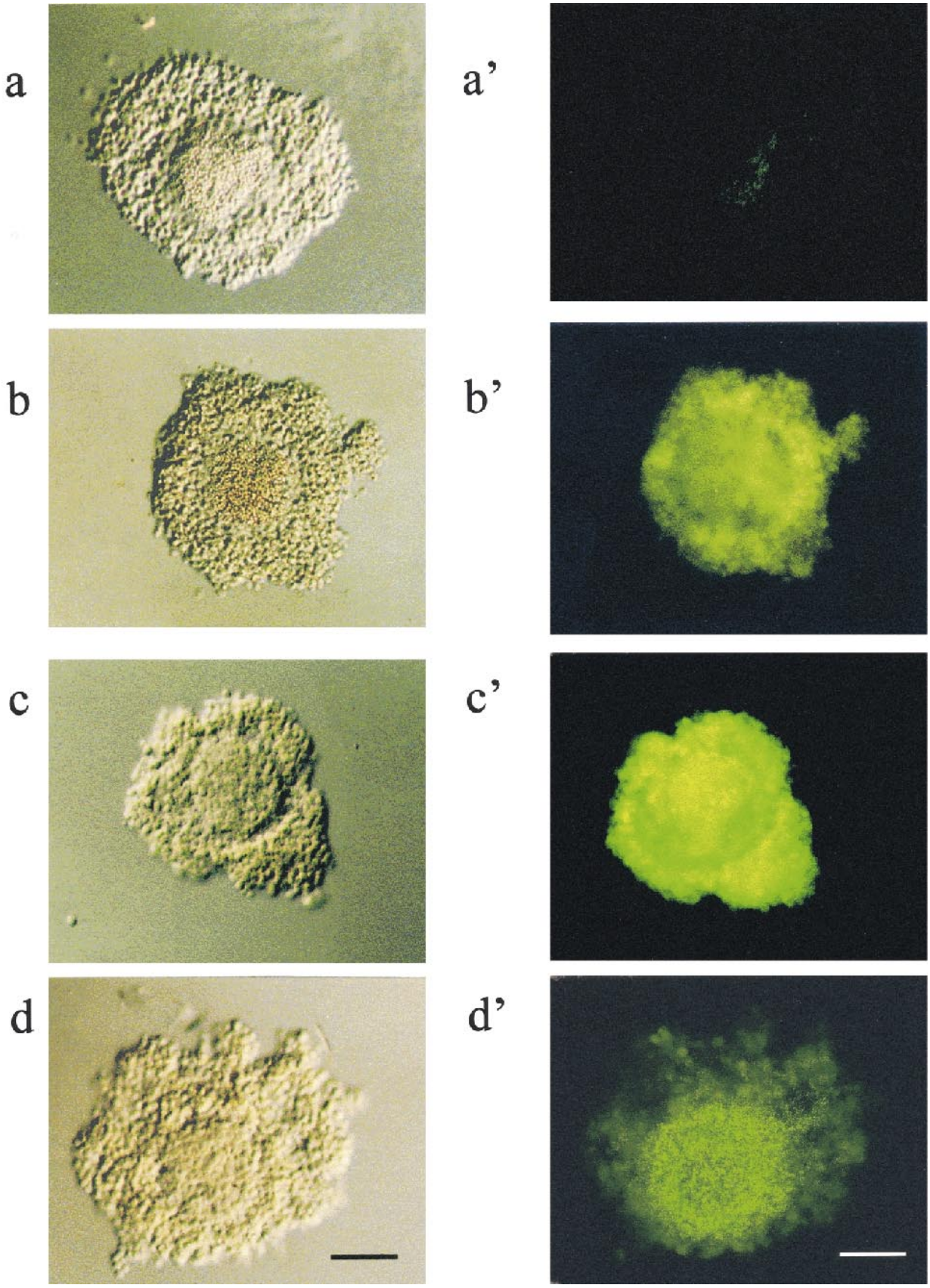

FIG. 4. Localization of connexin-43 in porcine COCs as detected by immunofluorescence using anti-connexin-43 monoclonal mouse IgG. **a**–**d**) Image obtained by differential interference microscopy. **a′**–**d′**) Image obtained by indirect immunofluorescence of anti-connexin-43 antibody. **a** and **a′**) Control samples in which no primary antibody was used. **b** and **b′**) COCs immediately after collection from their follicles. **c** and **c′**) COCs cultured for 4 h in the basic medium. **d** and **d′**) COCs cultured for 12 h in the basic medium. Bar = 100 μm.

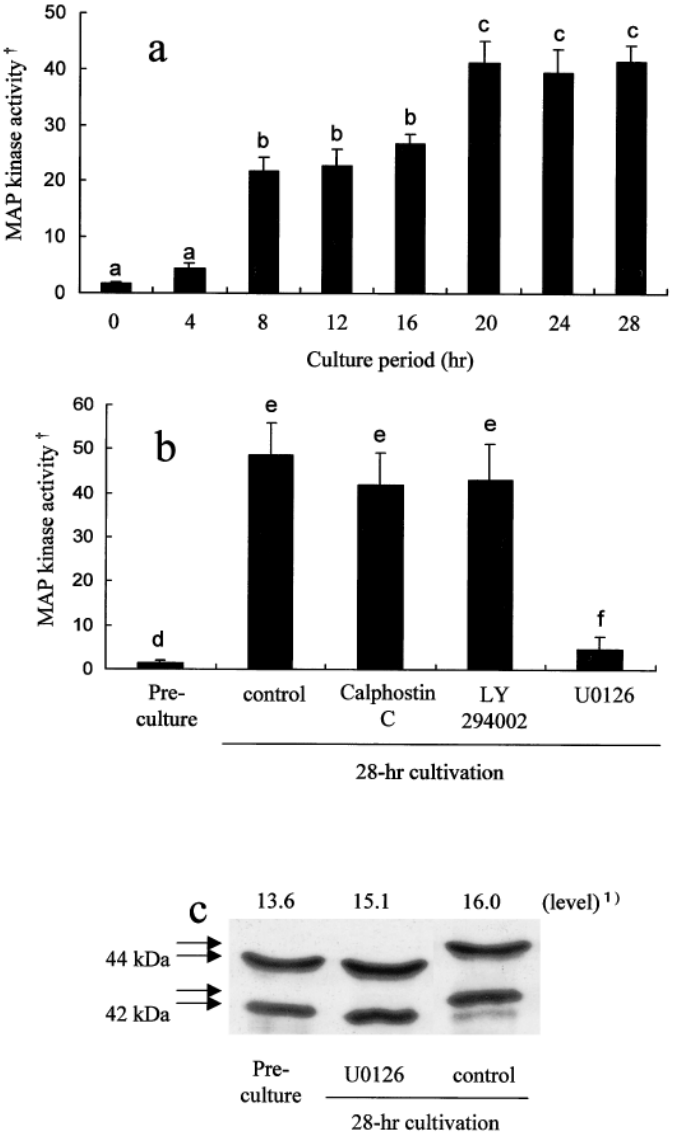

FIG. 5. Activities and profiles of MAP kinase in cumulus cells from COCs. **a**) Time-dependent changes of MAP kinase activity in cumulus cells from COCs during meiotic maturation. **b**) MAP kinase activities in cumulus cells from COCs cultured in LY294002 (PI 3-kinase inhibitor), calphostin C (PKC inhibitor), or U0126 (MEK inhibitor) for 28 h. **c**) Profiles of MAP kinase in cumulus cells separated from COCs cultured with or without U0126 (MEK inhibitor) analyzed by immunoblotting using anti-ERK-1,2 monoclonal antibody. Data for the MAP kinase assay were obtained from four separate experiments; data for immunoblotting using anti-ERK-1,2 monoclonal antibody were obtained from three experiments. Different alphabetical superscripts denote significant differences ($P < 0.01$). †Activity is expressed as fold MAP kinase activity in which positive control, 5-ng, active MAP kinase activity is defined as 100%. ¹Total amount of MAP kinase (ERK-1 + ERK-2) band as determined by scanning densitometry.

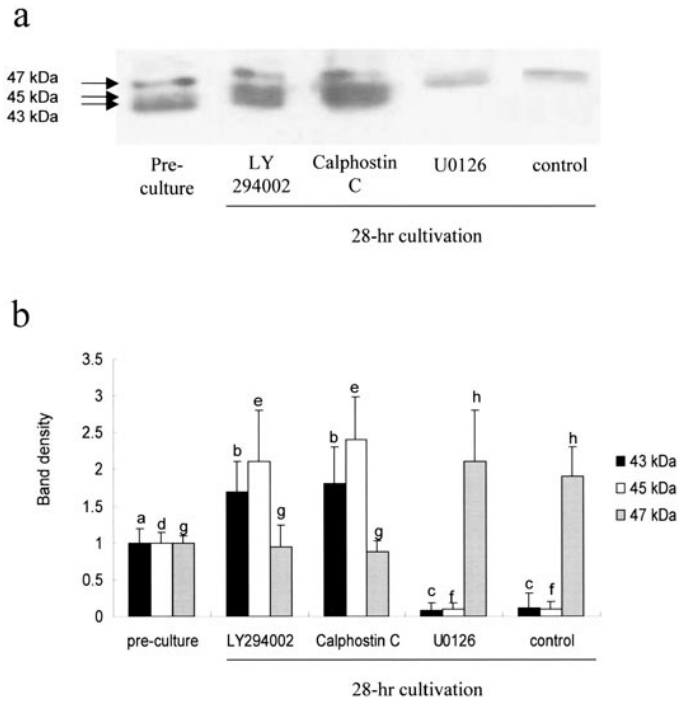

FIG. 6. Effects of LY294002 (PI 3-kinase inhibitor), calphostin C (PKC inhibitor), or U0126 (MEK inhibitor) on the phosphorylation of connexin-43 in cumulus cells from porcine COCs. **a**) Immunoblotting probed with anti-connexin-43 monoclonal antibody. **b**) Intensity of connexin-43 bands as determined by scanning densitometry. Data in each band are expressed as the fold strength of the intensity in cumulus cells immediately after collection from their follicles. Data were obtained from three separate experiments. Columns with no common superscript are significantly different within the bands migrated at the same levels ($P < 0.01$).

of the cumulus cells, whereas after 12 h, the connexin-43 disappeared in the outer layers of cumulus cells.

Time-Dependent Changes in MAP Kinase Activity of Cumulus Cells Surrounding Oocytes Around Meiotic Resumption

The aim of this experiment was to determine whether MAP kinase in cumulus cells is activated through either the PKC or PI 3-kinase cascade. Immunoblots of phosphorylated Elk 1 fusion protein were used as a measure of MAP kinase activity. The activity is expressed as fold MAP kinase activity in which positive control, 5 ng, active MAP kinase activity is defined as 100%. Time-dependent changes of MAP kinase activity in cumulus cells separated from COCs cultured for various time periods are shown in Figure 5a. The MAP kinase activity in cumulus cells was very low during the first 4-h cultivation of COCs, but it significantly increased at 8-h cultivation. At 20-h cultivation of COCs, the further activation of MAP kinase in cumulus cells was attained, and the higher kinase activity was sustained at up to 28-h cultivation.

Figure 5b presents the effects of calphostin C (PKC inhibitor), LY294002 (PI 3-kinase inhibitor), or U0126 (MEK inhibitor) on MAP kinase activity in cumulus cells separated from COCs cultured for 28 h. No significant differences between MAP kinase activities in cumulus cells in the presence and absence of either calphostin C or LY294002 were observed. However, this kinase activity in cumulus cells separated from COCs cultured in U0126 for 28 h was significantly lower than in the other three groups.

To investigate the mechanisms for suppression of MAP kinase activity by U0126, migrating forms of MAP kinase in cumulus cells separated form COCs were analyzed by immunoblotting using anti-ERK-1,2 monoclonal IgG. In cumulus cells immediately recovered from their follicles, two electronic migrations bands (42 and 44 kDa) were found (Fig. 5c). After COCs had been cultured for 28 h, the mobility shifts were observed in these bands (43 and 45 kDa; Fig. 5c). However, two bands of MAP kinase were situated at 42 and 44 kDa in cumulus cells separated from COCs cultured in the presence of U0126 for 28 h. In a

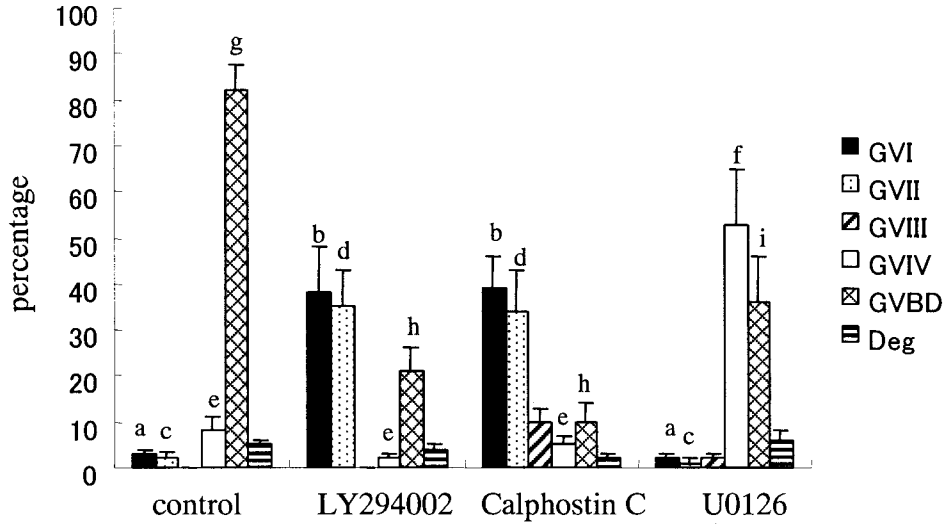

FIG. 7. Effects of LY294002 (PI 3-kinase inhibitor), calphostin C (PKC inhibitor), or U0126 (MEK inhibitor) on meiotic progression in porcine COCs cultured for 28 h. Data were obtained from four separate experiments. Different superscripts within the columns in each group denote significant differences ($P < 0.01$).

variety of cells, SDS-PAGE has shown that the inactive forms of MAP kinase migrate at 42 kDa (ERK-2) and 44 kDa (ERK-1) and active forms of ERK-2 and ERK-1 at 43 and 45 kDa, respectively. The total amount of MAP kinase (i.e., ERK-1 + ERK-2) in cumulus cells from COCs before cultivation was similar to that from COCs in the presence or absence of U0126 at 28-h cultivation. These results suggest that the activation of both ERK-1 and -2, which have already accumulated in cumulus cells, is dependent on MEK but is not dependent on PKC or PI 3-kinase during in vitro maturation of COCs.

Effects of LY294002, Calphostin C, or U0126 on Phosphorylation of Connexin-43 in Cumulus Cells Surrounding Oocytes

After 28-h cultivation, the relative intensities of 47-kDa connexin-43 in cumulus cells from COCs cultured either in the absence of any drugs (i.e., control) or in U0126 were significantly higher than those from COCs cultured in LY294002 and calphostin C (Fig. 6b). No significant difference between the intensity of the 47-kDa band in the U0126 treatment group and the control was observed. On the other hand, the intensity of the higher and moderate migration forms (i.e., 43 and 45 kDa) almost completely disappeared in cumulus cells separated from COCs cultured in control medium and in U0126 for 28 h (Fig. 6a). However, the addition of LY294002 and calphostin C to the culture medium produced a significant increase in the intensity of the 43- and 45-kDa bands compared to those of the cumulus cells in the control group or in the presence of U0126 after the COCs had been cultured for 28 h (Fig. 6b).

Effects of LY294002, Calphostin C, or U0126 on Meiotic Progression and p34^{cdc2} Kinase Activity in Oocytes with or Without Cumulus Cells

Treatments with LY294002 or calphostin C provided a significantly higher proportion of oocytes arrested at the GVI and GVII stages compared to those of the control and the U0126 treatment group after COCs had been cultured for 28 h (Fig. 7). The dramatically increased proportion of oocytes treated with U0126 was arrested at the GVIV stage, in which irregular networks of filamentous bivalents were detectable but the nucleolus disappeared. The progression of GVBD was significantly suppressed by the treatments with each kinase inhibitor compared to that in oocytes cultured in the absence of any drugs (i.e., control). The addition of LY294002 or calphostin C into the medium significantly decreased the proportion of oocytes exhibiting GVBD compared to that of U0126. Control oocytes cultured without any drug for 28 h had a significantly higher activity of p34^{cdc2} kinase than oocytes treated with each kinase inhibitor (Fig. 8). No difference in p34^{cdc2} kinase activity between oocytes cultured in any drug and those observed immediately after collection from their follicles was found (Fig. 8).

In DOs, GVBD and p34^{cdc2} kinase activity were not significantly suppressed by the addition of LY294002, calphostin C, or U0126, respectively, into medium (Fig. 9). These results show that these kinase inhibitors do not have any effects on oocytes but do have effects on cumulus cells surrounding oocytes.

DISCUSSION

The addition of FSH and LH into maturation medium produces a high level of cAMP in the cumulus cells surrounding sheep oocytes within 1 h of the initiation of maturation [23]. The elevation of intracellular cAMP increases connexin-43 mRNA and promotes gap junctional communication in the rat Morris hepatoma cell line [24]. Moreover, in human follicular cells, the gap junctions are up-regulated by cAMP [25]. In the present study, employing immunoblotting or in situ immunofluorescent analysis, the amount of connexin-43 in cumulus cells from porcine COCs cultured in the presence of FSH and LH was dramatically increased within 4 h of cultivation, and intensity staining for connexin-43 was observed in all layers of cumulus cells. Thus, it can be argued that in pigs, cAMP generated in cumulus cells stimulated by FSH and LH may enhance the synthesis of connexin-43 and, thereby, support the communication of cumulus cells or of cumulus cells and oocyte via gap junctional communication.

The loss of the cumulus-to-cumulus cell gap junction

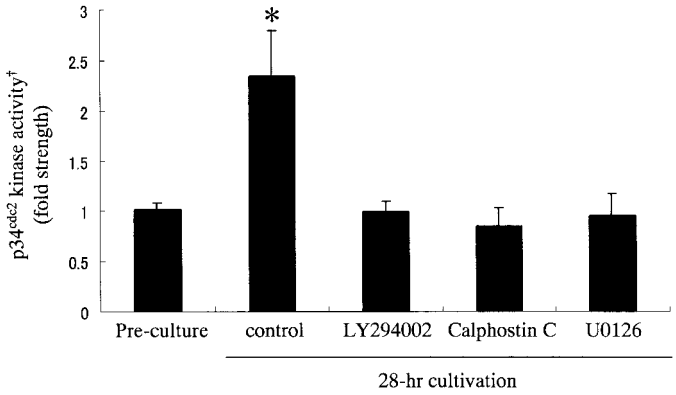

FIG. 8. Effects of LY294002 (PI 3-kinase inhibitor), calphostin C (PKC inhibitor), or U0126 (MEK inhibitor) on p34^{cdc2} kinase activity in porcine oocytes cultured for 28 h. *Significant difference from each treatment. †Data are expressed as the fold strength of p34^{cdc2} kinase activity in oocytes immediately after collection from their follicles; data were obtained from four separate experiments.

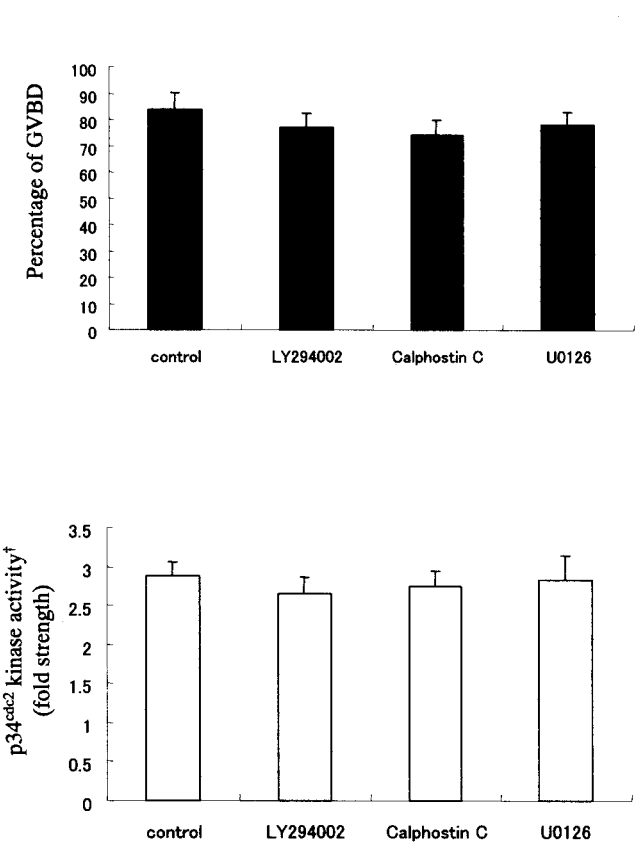

FIG. 9. Effects of LY294002 (PI 3-kinase inhibitor), calphostin C (PKC inhibitor), or U0126 (MEK inhibitor) on the incidence of GVBD or p34^{cdc2} kinase activity in porcine DOs cultured for 28 h. †Data are expressed as the fold strength of p34^{cdc2} kinase activity in oocytes immediately after collection from their follicles; data were obtained from four separate experiments.

preceding the induction of meiotic resumption has been reported in mouse COCs [26] and in rat COCs [4, 5]. In pigs, a significant positive correlation was seen between the proportion of GVBD oocytes and that of COCs exhibiting loss of the gap junctional communication within the outer layers of cumulus cells [6]. In the present study, to our knowledge, we are the first to demonstrate that the disruption of gap junctional communication in the outer layers of cumulus cells results from the reduction of connexin-43 in those layers, which is dependent on the activation of either the PKC or PI 3-kinase pathway but is independent of MAP kinase activation. At 12 h of cultivation, in which the total amount of connexin-43 was reduced, a majority of oocytes proceeded from the GVII to GVIII stage, in which were observed networks of filamentous bivalents in the GV. When this reduction of connexin-43 was blocked by either PKC or PI 3-kinase inhibitor, the proportion of oocytes arrested at the GVI or GVII stage was significantly elevated. Additionally, in DOs, PKC or PI 3-kinase inhibitors cannot affect both the formation of networks of filamentous bivalents in the GV and GVBD. Taken as a whole, the reduction of connexin-43 shut down the gap junctional communication in the outer layers of cumulus cells, leading to the formation of networks of filamentous bivalents in the oocyte.

In rat liver cells, the phosphorylation of connexin-43 is under the direct control of MAP kinase [13]. In the present study, MAP kinase in cumulus cells separated from COCs during meiotic maturation was activated through the MEK pathway independently of PKC and PI 3-kinase. However, MAP kinase activated in cumulus cells was not attributed to the phosphorylation of connexin-43. On the other hand, when MAP kinase activity in cumulus cells from COCs cultured with U0126 was completely inhibited, the activation of p34^{cdc2} kinase was not observed in these oocytes. Moreover, the majority of oocytes were arrested at the GVIV stage, in which networks of filamentous bivalents in the GV were visible. These findings indicate that MAP kinase in cumulus cells is not associated with the phosphorylation of connexin-43 and formation of networks of filamentous bivalents in the GV, but that it is needed for both the activation of p34^{cdc2} kinase and the progression of GVBD in porcine oocytes, because U0126 could not suppress p34^{cdc2} kinase activity and GVBD in DOs. Alternatively, it has been reported that MAP kinase plays a major role in protein synthesis and the transcription of mRNA [27–29], and that de novo mRNA synthesis within cumulus cells is required for gonadotropin-induced nuclear maturation of porcine COCs [30]. Thus, MAP kinase in cumulus cells may be an important factor in GVBD of porcine oocytes.

Evidence suggests that the gap junctional communication between cumulus cells and oocyte is closed during meiotic progression beyond the MI stage [6, 31, 32]. The present study showed that at 28 h of cultivation, when the fluorescence signals for the connexin-43 were seen only in the inner layers of cumulus cells, connexin-43 was phosphorylated. That phosphorylation of connexin-43 induces the gap junction blockade strongly suggests that gap junctional communication between cumulus cells and oocyte during meiotic progression beyond the MI stage is closed via phosphorylation of connexin-43, without any contribution of MAP kinase activation. Because whether PKC and PI 3-kinase directly induce the phosphorylation of connexin-43 has not been demonstrated in this study, further research is necessary to investigate the phosphorylation mechanisms of connexin-43 in cumulus cells.

In summary, during meiotic resumption, formation of networks of filamentous bivalents in the GV is initiated by the reduction of gap junctional protein connexin-43 in the outer layers of cumulus cells, via either the PKC or PI 3-kinase pathway, followed by activation of p34^{cdc2} kinase and GVBD in porcine oocytes, which are required for MAP kinase activity in cumulus cells due to the MEK pathway.

Moreover, connexin-43 in the inner layers of cumulus cells at the MI stage is phosphorylated independently of MAP kinase activation.

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