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Effects of PKC activation on the meiotic maturation, fertilization and early embryonic development of mouse oocytes

He-Mei Quan[1,2], *Heng-Yu Fan*[1], *Xiao-Qian Meng*[1], *Li-Jun Huo*[1], *Da-Yuan Chen*[1], *Heide Schatten*[3], *Pei-Man Yang*[2] *and Qing-Yuan Sun*[1]

Institute of Zoology, Chinese Academy of Sciences, Beijing; Dalian Medical University, Dalian, People's Republic of China; and University of Missouri, Columbia, USA

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Summary

Protein kinase C (PKC) is a family of Ser/Thr protein kinase widely distributed in eukaryotes. There is evidence that PKC plays key roles in the meiotic maturation and activation of mammalian oocytes. However, the mechanism of PKC's actions and the PKC isoforms responsible for these actions are poorly understood. In this study, we reveal in mouse eggs and early embryos: (1) the effects of PKC on the meiotic and mitotic cell cycle progression during oocyte maturation, egg activation and embryonic cleavages; (2) the functional importance of classical PKC subclasses in these processes; and (3) the subcellular localization of the PKCα isoform during development from GV stage oocytes to the blastocyst stage embryos. The results indicate that the PKC activator phorbol 12-myristate 13-acetate (PMA) inhibits the meiotic resumption of cumulus-free mouse oocytes by a mechanism dependent not only on classical PKC activity but also on other PKC isoforms. PKC activation after germinal vesicle breakdown leads to the inhibition of mitogen-activated protein kinase phosphorylation and the arrest of cell cycle at MI stage. The second polar body emission and the cleavages of early embryos are blocked after prolonged PKC activation. The subcellular localization of PKCα isoform in mouse oocytes and embryos is developmental-stage associated. All these results suggest that PKC has multiple functional roles in the cell cycle progression of mouse oocytes and embryos.

Keywords: PKC, Oocyte, Meiosis, Fertilization, Embryo

Introduction

In the ovary of adult mammals, the cell cycle of fully grown oocytes is arrested at the prophase of meiosis I, characterized by the existence of vascularized nucleus, which is termed germinal vesicle (GV). Oocyte maturation is triggered by a surge of pituitary gonadotropin at each sexual cycle *in vivo*, and can also be induced *in vitro* by artificially releasing the oocytes from the inhibitory follicular environment. Reinitiation of meiosis is manifested by germinal vesicle breakdown (GVBD) followed by chromosome condensation, spindle formation and the extrusion of the first polar body (PB1). Immediately thereafter, the second meiotic division commences and is arrested again at the metaphase of meiosis II (MII) until fertilization. Sperm entry stimulates intracytoplasmic calcium oscillations and releases the oocytes from MII arrest, followed by the completion of MII and pronucleus (PN) formation. Recent studies have shown that several protein kinases play vital roles in the regulation of cell cycle progression during oocyte meiotic maturation and fertilization (for reviews, see Sun *et al*., 1999*a*; Fan *et al*., 2002*a*, 2003*a*).

Protein kinase C (PKC) is a family of serine/threonine kinases that can be activated by Ca^{2+} and

All correspondence to: Qing-Yuan Sun, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China. Tel: +8610 6256 3923. Fax: +8610 6256 5689. e-mail: sunqy1@yahoo.com

[1] State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China.

[2] Department of Anatomy, Dalian Medical University, Dilian 116027, People's Republic of China.

[3] Department of Veterinary Pathobiology, University of Missouri – Columbia, Columbia, MO 65211, USA.

diacylglycerol (DAG). At present, at least 11 isoforms have been identified, which are divided into three subclasses based on differences in amino acid sequence and cofactor requirement. These subclasses include the classical PKCs (cPKCs) α, βI, βII and γ, the novel PKCs (nPKCs) δ, ε, η and θ and the atypical PKCs (aPKCs) ζ, λ and μ. The cPKCs are activated by Ca^{2+} and DAG, whereas the activation of nPKCs depends only on calcium. The activity of aPKCs is regulated by neither Ca^{2+} nor DAG but by some specific binding proteins (Moscat and Dia-Meco, 2000). PKCs are expressed in mouse (Luria *et al*., 2000), rat (Raz *et al*., 1998) and pig (Fan *et al*., 2002*b*) oocytes, and can affect the progression of meiosis at multiple stages. Our previous studies indicate that PKC activators inhibit the meiotic resumption and mitogen-activated protein kinase (MAPK) phosphorylation in isolated mouse (Sun *et al*., 1999*b*), rat (Lu *et al*., 2001) and pig (Fan *et al*., 2003*a*) oocytes, and that this effect can be abolished by PKC inhibitors. PKC might also participate in the meiosis I to meiosis II transition in mouse oocytes (Viveiros *et al*., 2001). Its activity increases with meiotic maturation and reaches the peak at late MI stage. It has been suggested that fertilization induces an immediate increase in intracellular free Ca^{2+} concentration, which subsequently activates PKCs (Xu *et al*., 1994). Activation of PKC has been shown to be sufficient to cause inactivation of maturation promoting factor (MPF) (Colonna *et al*., 1997; Gallicano *et al*., 1997), MAPK dephosphorylation (Sun *et al*., 1999*b*; Lu *et al*., 2002) and release from MII arrest in mouse and rat eggs.

However, the importance of distinct PKC isoforms or subclasses in these events is unclear. Recently, Luria *et al*. (2000) and Eliyahu *et al*. (2002) reported the translocation of cPKCs from cytoplasm to plasma membrane in mouse and rat eggs, respectively, after fertilization. We observed the same phenomenon in pig eggs, and further proved that the PKCα isoform is indispensable for PMA- or sperm-induced cortical granule exocytosis. It is not clear whether the effects of PKC activators on meiotic cell cycle progression are mediated solely by cPKCs or also by other subclasses.

The cell cycles of oocytes and early embryos are different from those of somatic cells. The effects of PKC activation on cell division and MAPK phosphorylation are inhibitory but, in cultured cell lines, activation of PKC stimulates MAPK phosphorylation and cell division (Lu *et al*., 2001). Cleavages of early embryos occur by mitosis, but they are faster than normal cell divisions and there is no G1 stage between two divisions. The effects of PKC activation on cleaving embryos have never been reported.

Although there is evidence that PKC is involved in the regulation of nuclear envelope assembly and spindle construction (Collas, 1999), the spatial association of PKC with these structures in oocytes and zygotes has scarcely been explored. Recently, we reported the concentration of cPKCs in the nucleus of pig oocytes and fertilized eggs (Fan *et al*., 2002*b*). In mouse oocytes, PKCδ was localized to the meiotic spindle during the MI–MII transition and then to the chromosomes at MII stage (Viveiros *et al*., 2001). However, it is not known whether cPKCs are concentrated to the cell division apparatus like other kinases involved in the meiotic cell cycle regulation, such as MAPK (Lee *et al*., 2000), Polo-like kinase (Plk) (Tong *et al*., 2002) or calmodulin-dependent protein kinase (CaMKII) (Johnson *et al*., 1998).

In this study, we reveal in mouse eggs and early embryos: (1) the effects of PKC on the meiotic and mitotic cell cycle progression during oocyte maturation, egg activation and embryonic cleavages; (2) the functional importance of classical PKC subclasses in these processes; and (3) the subcellular localization of PKCα isoform during development from GV stage oocytes to blastocyst stage embryos.

Materials and methods

Animals and oocyte collection

Animals used in this experiment are mice of Kunming strain, which is a randomly bred strain that is widely used in China. Sexually mature females (4–6 weeks old and approximately 25 g weight) from our laboratory colony received intraperitoneal injections of 10 international units (IU) of pregnant mare's serum gonadotropin in 0.1 ml of 0.9% NaCl for induction of follicular development. The mice were sacrificed 48 h later. All animal experimentations were conducted in conformance with the Guiding Principles for Research Involving Animals and Human Beings. Cumulus-free and GV-intact oocytes were released from the large antral follicles by puncturing with a needle in M2 (Sigma) medium with 60 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin.

MII-arrested eggs were obtained from mice of the same strain. Females were superovulated by intraperitoneal injection with 10 IU pregnant mare's serum gonadotropin and, 48 h later, they were injected with 10 IU human chorionic gonadotrophin (hCG). Mice were killed 15 h (for *in vitro* fertilization) or 20 h (for parthenogenetic activation) after hCG injection. The cumulus cell masses surrounding the eggs were removed by a brief exposure to 300 IU ml^{-1} hyaluronidase in M2 medium and repeated pippeting.

In vivo and *in vitro* fertilization

In vivo fertilized zygotes were collected 15 h after hCG injection from the oviduct ampullae of superovulated

females that had been mated with the same strain of males. After removing cumulus cells with 300 IU ml^{-1} hyaluronidase in M2 medium, zygotes with two PNs were cultured in M16 (Sigma) medium plus 2.5 mM taurine (pH 7.4), which is a modified medium that supports the *in vitro* development of mouse embryos form one-cell-stage to blastula, as reported previously (Tong *et al*., 2002).

In vitro fertilization was performed using 1×10^6 ml^{-1} motile cauda epididymal sperm, which had been previously capacitated in M16 medium with 2.5 mM taurine for 1 h. Eggs free of zona pellucida (ZP) were used to achieve a more synchronous timing of fertilization within each stage group and to minimize the lag period of sperm–egg interaction. ZP was removed by a short exposure of the eggs to the acidified Tyrode's medium (pH 2.5), followed by sufficient washing in M2 medium. The emission of the second polar body (PB2) and the formation of the PNs were observed with an inverted phase-contrast microscope.

Confocal microscopy

After removing the ZP in acidified Tyrode's medium, oocytes were fixed with 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 for 15 min at 37 °C, followed by blocking in 1% bovine serum albumin (BSA) for 1 h and incubation overnight at 4 °C with rabbit anti-mouse PKCα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in blocking solution. After three washes in PBS for 5 min each, the eggs were labelled with FITC-conjugated goat anti-rabbit IgG diluted 1:100. Nuclear status of oocytes was evaluated by staining with 10 μg ml^{-1} propidium iodide (PI) in PBS for 10 min. Following extensive washing, samples were mounted between a coverslip and a glass slide supported by four columns of a mixture of Vaseline and paraffin (9:1). The slides were sealed with nail polish. Nonspecific staining was determined by incubation without primary antibodies. Each experiment was repeated at least twice, with approximately 15 oocytes examined each time. Cells were observed under a Leica confocal laser scanning microscope (TCS-NT) on the same day.

The spindle organization was determined by incubating the eggs in 1:50 diluted FITC-conjugated anti-α-tubulin (Sigma) for 1 h after fixation and permeabilization as described above.

Electrophoresis and Western immunoblotting

Proteins from 50 oocytes per treatment were extracted with double-strength electrophoresis buffer. After being boiled for 4 min and centrifuged for 3 min at 15 000 rpm, the lysates were kept frozen at −20 °C until use. Proteins were separated on a 10% SDS-polyacrylamide gel for 2.5 h at 120 V and then transferred onto Gelman transfer membrane for 2.5 h at 200 mA. The membrane was blocked with 5% skimmed milk in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl) containing 0.1% Tween-20 (TBST) overnight at 4 °C and then incubated for 2 h at 37 °C with monoclonal mouse anti-phosphorylated-MAPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in TBST containing 5% skimmed milk (pH 7.4). After three washes of 10 min each in TBST, the membrane was incubated for 1 h at 37 °C with horseradish-peroxidase-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000. Finally, the membrane was washed three times in TBST for 10 min each and then processed using the ECL detection system (Amersham International, Amersham, UK).

For reprobing, the membrane was stripped of the bound antibodies in stripping buffer (100 mM Tris, 100 mM β-mercaptoethanol, 2% SDS, pH 6.7) for 30 min at 50–55 °C. After re-blocking, the membrane was reprobed with polyclonal rabbit anti-ERK2 antibody diluted 1:300, incubated with horseradish-peroxidase-labelled goat anti-rabbit IgG diluted 1:1000 and finally processed as described above.

Experimental designs

Experiment 1

To study the importance of PKC, especially cPKC subclasses, in the regulation of meiotic maturation, denuded and GV-intact mouse oocytes were cultured in M2 medium containing 16.2 nM PKC activator PMA, 500 nM PKC inhibitor calphostin C, 16.2 nM PMA + 500 nM calphostin C, or 16.2 nM PMA + 10 nM cPKC-specific inhibitor Go 6976. The GVBD rate was recorded 5 h after culture The subcellular localization of PKCα isoform during meiotic maturation were detected by confocal microscopy.

Experiment 2

To clarify the involvement of PKC in the events of meiotic maturation following GVBD, denuded mouse oocytes were cultured in drug-free M2 medium for 2 h to allow GVBD. Then, the oocytes that underwent GVBD were transferred into M2 medium containing 16.2 nM PMA and were further cultured for 12 h. The emission of PB1, the configuration of microtubules and the phosphorylation of MAPK in these oocytes were detected.

Experiment 3

The roles of PKC, especially cPKC subclasses, in the regulation of activation events after fertilization were evaluated. ZP-free mouse eggs were fertilized *in vitro* at the presence of 16.2 nM PMA, 500 nM calphostin

C or 10 nM Go 6976, and then PB2 emission and PN formation were recorded at 2 h and 8 h after insemination, respectively. The subcellular distribution of PKCα in mouse eggs following fertilization or 16.2 nM PMA treatment were also studied.

Experiment 4

The effect of PKC activation on embryonic cleavage and localization of PKCα in early embryos were investigated after *in vivo* fertilization. Mouse zygotes were collected from the oviducts 18 h after hCG injection and cultured in M16 medium plus 2.5 mM taurine. Some of the embryos were transferred into M16 medium containing 16.2 nM PMA at the one-cell, two-cell, four-cell or eight-cell stage. The further cleavage of these embryos was observed on the next day. PKCα was stained by immunofluorescence in embryos at various developmental stages.

Statistical analysis

All proportions from three repeated experiments were expressed as mean ± SEM and the number of oocytes observed was also recorded. The rates of GVBD, polar body (PB) emission or PN formation were subjected to arcsin transformation. The transformed data were analysed by ANOVA followed by the Student–Newman–Keuls test. Differences at $p < 0.05$ were considered to be statistically significant.

Results

Effects of PKC activation on meiotic maturation of mouse oocytes

As shown in Fig. 1, released from the follicles and cultured in drug-free M2 medium, 84.73% ($n = 131$) of the oocytes underwent GVBD in 5 h. But the GVBD (3.28%, $n = 305$) was significantly inhibited by PKC activator PMA at a concentration of 16.2 nM. Among oocytes cultured in medium containing 500 nM PKC inhibitor calphostin C, 83.09% ($n = 136$) underwent GVBD at 5 h of culture, with no statistically significant difference from the GVBD rate in drug-free medium. More than half of the oocytes (53.13%, $n = 96$) underwent GVBD at the presence of both PMA (16.2 nM) and calphostin C (500 nM), but the GVBD rate is only 13.70% ($n = 219$) in medium containing PMA (16.2 nM) and the cPKC-specific inhibitor Go 6976 (10 nM).

When mouse oocytes were cultured in medium containing 16.2 nM PMA shortly after GVBD, the emission rate of PB1 (10.58%, $n = 104$) was significantly inhibited compared with the control (70.91%, $n = 110$). Results from confocal microscopy indicated that the cell cycle of these oocytes was blocked at the metaphase of meiosis I (Fig. 2).

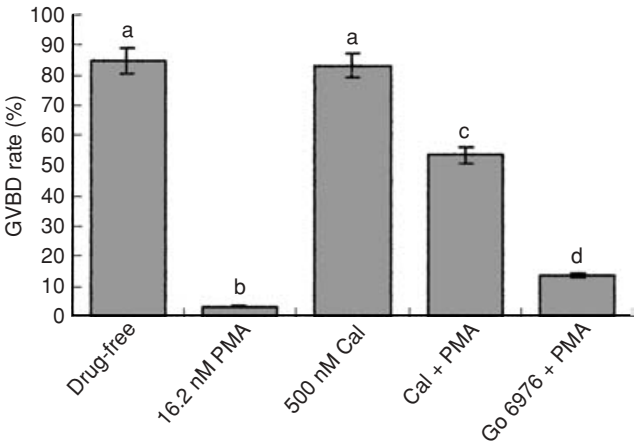

Figure 1 Effect of PKC activation and inhibition on spontaneous meiotic resumption of mouse oocytes. Cumulus-free and GV-intact oocytes were isolated from the ovaries and cultured for 5 hours each in drug-free M2 medium or M2 medium containing 16.2 nM PKC activator PMA, 500 nM PKC inhibitor calphostin C (Cal), 16.2 nM PMA + 500 nM calphostin C, or 16.2 nM PMA + 10 nM cPKC-specific inhibitor Go 6976. The nucleus status of oocytes was then observed using an inverted phase-contrast microscope. The value expressed by each bar represents the mean ± SD ($n = 3$). Different superscripts denote statistical difference at a level of significance of $p < 0.05$. The same statistical method was applied to subsequent figures.

As shown in Fig. 3, ERK at dephosphorylated state was detected by Western blot in oocytes at the GV stage (lane 1) and shortly after GVBD (lane 2). Phosphorylation of ERK was first detected 2 h after GVBD (lane 3). The phosphorylated state of ERK1/2 was maintained at 4 h (lane 4) and 8 h (lane 5) after GVBD. However, when the oocytes were transferred to medium containing 16.2 nM PMA shortly after GVBD,

Figure 2 Effects of PKC activation on cell cycle progression. Denuded mouse oocytes were allowed to undergo GVBD in M2 medium after 2 h of culture. The oocytes that underwent GVBD were transferred to medium containing 16.2 nM PMA. The cell cycle of these oocytes were blocked at MI stage with a normally formed spindle.

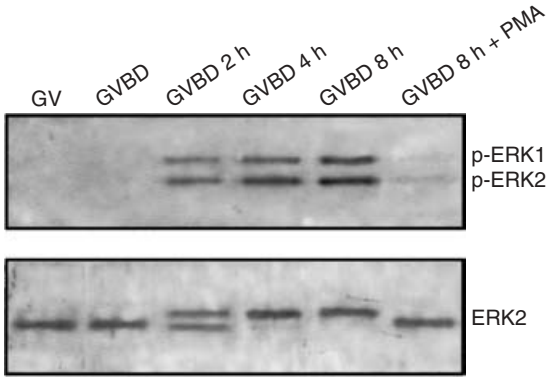

Figure 3 Detection of active ERK1/2 (top) and total ERK2 (bottom) by western blotting in mouse oocytes at GV stage (lane 1), shortly after GVBD (lane 2) and 2 h (lane 3), 4h (lane 4) and 8 h (lane 5) after GVBD. Some oocytes shortly after GVBD were transferred to medium containing 16.2 nM PMA and cultured for 8 h (lane 6). Each lane represented the total proteins from 50 oocytes.

ERK failed to be phosphorylated even at 8 h after culture (lane 6).

In oocytes at the GV stage, PKCα distributed evenly in the cytoplasm, with very weak staining in the germinal vesicle (Fig. 4A). At 2 h (Fig. 4B) or 4 h (Fig. 4C) after GVBD, a concentration of PKCα was detected around the condensed chromosomes. However, no special PKCα localization was observed after the formation of MI spindle (Fig. 4D).

Effects of PKC on egg activation

As shown in Fig. 5, a high *in vitro* fertilization rate was obtained in our experiment, with 92.23% ($n = 103$) of the eggs having released PB2 at 2 h after insemination, and 93.47% ($n = 92$) of them having formed two PNs at 6 h following fertilization. PMA at a concentration of 16.2 nM failed to activate the MII-arrested eggs at 15 h after hCG injection, and the PB2 emission rate (0, $n = 120$) and PN formation rate (3.33%, $n = 120$) were very low at 8 h after PMA treatment. ZP-free eggs could be fertilized at the presence of PMA, with a high PN formation rate (83.06%, $n = 124$). However, the PB2 emission was significantly inhibited by PMA: only 8.06% ($n = 124$) of the fertilized eggs had released their PB2 at 8 h following insemination. Both the PB2 emission rate (32.4%, $n = 108$) and the PN formation rate (38.89%, $n = 108$) after fertilization were decreased by the presence of calphostin C, compared with the control. However, the cPKC-specific inhibitor Go 6976 failed to block PB2 emission (90.32%, $n = 93$) and PN formation (92.47%, $n = 93$) after fertilization.

At 2 h after insemination, a concentration of PKCα could be detected around the incorporated sperm and at the cytoplasmic connection between the PB2 and the fertilized egg (Fig. 6A). After PN formation, commonly 6–8 h after insemination, strong staining of PKCα could be detected in both the male and female PN (Fig. 6B).

Effects of PKC on embryo development

As shown in Fig. 7, mouse zygotes fertilized *in vivo* have a high developmental potency in M16 medium plus 2.5 mM taurine. However, the embryonic cleavages from one-cell to two-cell (4.34%, $n = 161$), two-cell to four-cell (6.25%, $n = 128$), four-cell to eight-cell (6.25%, $n = 96$) and eight-cell to blastula (10%, $n = 90$) were significantly inhibited by the addition of 16.2 nM PMA in the culture medium. Confocal microscopy revealed that PKCα was concentrated in the nuclei of blastomeres in embryos at two-cell (Fig. 6C), four-cell (Fig. 6D), six- to eight-cell (Fig. 6E,F), morula (Fig. 6G) and blastocyst (Fig. 6H) stages. Prominent staining was detected in the whole nucleus except the nucleolus region at interphase.

Discussion

Mouse oocytes resume meiosis spontaneously when they are released from the inhibitory follicle

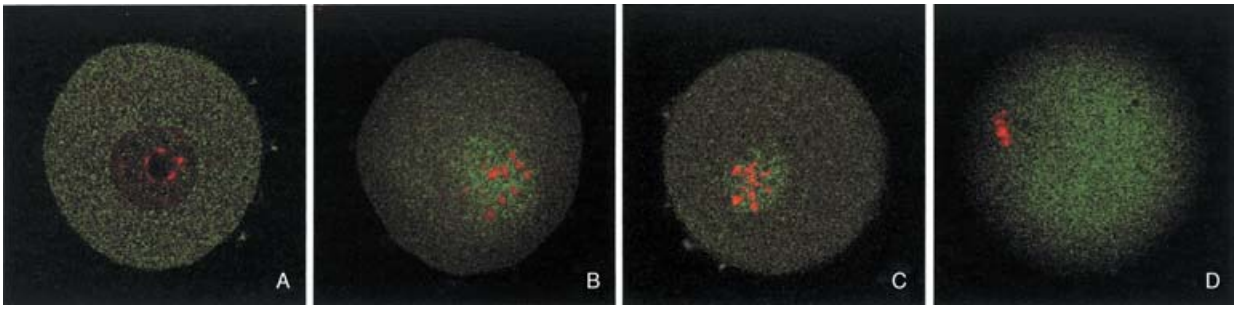

Figure 4 Subcellular localization of PKCα in mouse oocytes. This PKC isoform distributed evenly in the cytoplasm of GV oocytes, with weak staining in the nucleus (A). PKCα was concentrated at the condensed chromosomes at 2 h (B) or 4 h (C) after GVBD. No specific PKCα localization was detected in oocytes at MI stage (D). FITC staining of PKCα is shown in green, PI staining of chromatin is shown in red and the overlapping of green and red shows as orange.

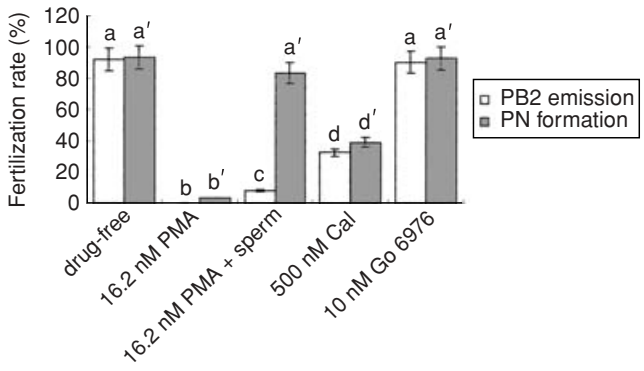

Figure 5 Effect of PKC activation and inhibition on PB2 emission and PN formation after fertilization *in vitro*. MII-arrested mouse eggs were fertilized in M16 medium containing 16.2 nM PMA, 500 nM calphostin C or 10 nM Go 6976, and PB2 emission and PN formation were observed at 2 h or 8 h, respectively, after insemination. As the positive control, some eggs were fertilized in drug-free medium. To exclude the possible induction of parthenogenetic activation by PMA at this concentration, some eggs were cultured in 16.2 nM PMA for 8 h and PB2 emission and PN formation were observed. Different superscripts (a or a′) denote statistical difference at a $p < 0.05$ level of significance in PB2 emission and PN formation, respectively.

environment. Although it is well recognized that the decrease of intracellular cAMP level is the initiating signal that leading to the spontaneous meiotic resumption (Cho *et al*., 1974; Schultz *et al*., 1983; Kim *et al*., 1995), its mechanism is still largely unknown. In our experiment, the meiotic resumption occurred normally at the presence of the PKC inhibitor calphostin C, suggesting that PKC activity is not necessary for the initiation of GVBD in denuded mouse oocytes. The PKC activator PMA significantly inhibited the GVBD in denuded oocytes and this effect could be partly overcome by the PKC inhibitor calphostin C. From these results, we deduced that the activation of PKC in denuded GV oocytes might inhibit the meiotic resumption. However, it is still not known whether this effect is mediated by classical PKC subclasses or by other PKC isoforms. We found in our experiment that the inhibitory effect of PMA on GVBD cannot be efficiently overcome by cPKC-specific inhibitor Go 6976, indicating that other PKC isoforms, such as members of new PKC or atypical PKC, might also be involved in the PMA-induced GVBD inhibition. We have reported before that the nPKCδ-isoform-specific inhibitor rottlerin failed to reverse the blocking effects of PMA on GVBD (Fan *et al*., 2003*b*). We could not exclude the possibility that other PKC isoforms perform key roles in PMA-induced GVBD inhibition, but it is more likely that PMA inhibits meiotic resumption by activating multiple PKC isoforms in oocytes and the effect of a single isoform can be substitute by other isoforms, so the inhibition of several PKC isoforms is not enough to overcome the effect of PMA.

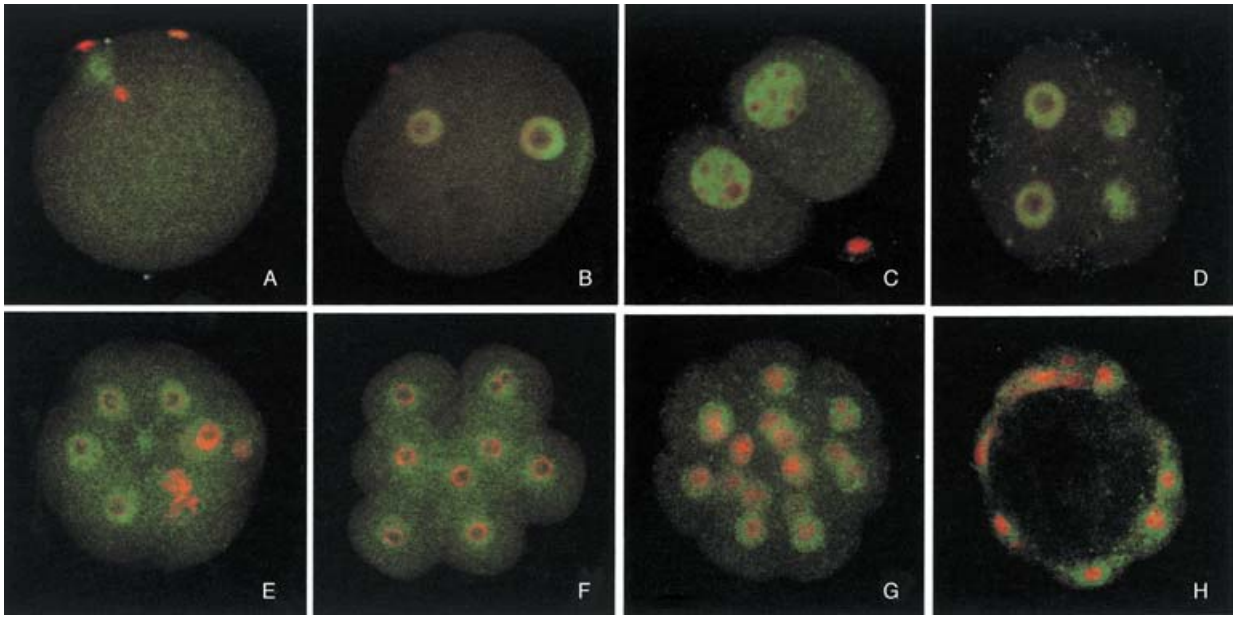

Figure 6 Distribution of PKCα in mouse preimplantation embryos. This PKC isoform can be detected at the connection between the extruding PB and the fertilized egg at 2 h after insemination (A). Prominent PKCα staining can also be detected in the male and female PNs at 6–8 h after fertilization (B). In mouse embryos at two-cell (C), four-cell (D), six- to eight-cell (E, F), morula (G) and blastocyst (H) stages, PKCα was localized to the nucleus except the nucleolus region at interphase.

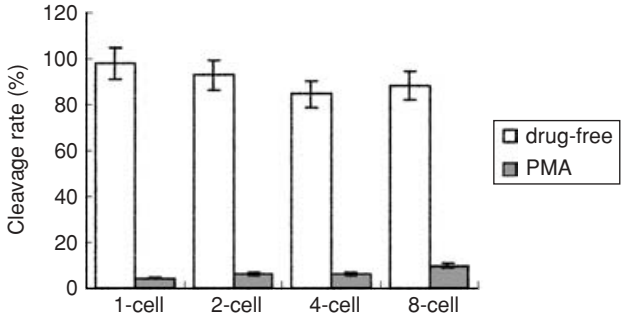

Figure 7 Effect of PKC activation on embryonic cleavages. Mouse *in vivo* fertilized embryos were collected at one-cell, two-cell, four-cell and eight-cell stages, and then cultured in M16 medium plus 2.5 mM taurine and 16.2 nM PMA. The cleavages were observed on the next day of culture. As the control, some embryos were cultured in drug-free medium for the same time.

In comparison to the roles of PKC in meiotic resumption, the involvement of this protein kinase in the subsequent events of meiotic maturation is largely unknown. Results from our lab have indicated that PMA prevented the phosphorylation of MAPK at the same time as inhibiting GVBD in mouse (Sun *et al*., 1999*b*) and rat (Lu *et al*., 2001) denuded oocytes. However, in rodent oocytes, MAPK is phosphorylated at ~2 h after GVBD. It is not clear whether the activation of PKC inhibits MAPK phosphorylation directly in the cytoplasm or simply prevents MAPK phosphorylation by inhibiting GVBD. The result in this experiment shows that, even in oocytes shortly after GVBD (in which MAPK has not been phosphorylated), PMA treatment might also result in the inhibition of MAPK activation. A recent report in pig oocytes indicated that the activation of MAPK in cytoplasm during meiotic resumption is GV independent (Sugiura *et al*., 2001). In combination, these results suggest that PKC activation is likely to inhibit MAPK phosphorylation in the cytoplasm independent of the presence of germinal vesicle in mouse oocytes.

As well as inhibiting MAPK activation, PMA treatment after GVBD also prevents the extrusion of PB1. The cell cycle of oocytes was blocked at the MI stage, as confirmed by the staining of microtubules. Morphologically normal spindle was formed in these oocytes, indicating that the cell cycle arrest is not due to the failure of spindle organization. A recent report indicates that PKC activity increases in MI and culminates at the MI stage (Viveiros *et al*., 2001). The inhibition of PKC activity at MI might facilitate the metaphase–anaphase transition. In oocytes from the LTXBO strain of mice, the meiosis was arrested at MI stage by an extraordinarily high PKC activity. Only when the PKC activity was inhibited artificially, could these oocytes pass the MI arrest (Viveiros *et al*., 2001). PKC activator PMA might lead to a long-term activation of PKC, which prevents the metaphase–anaphase transition and the PB1 emission.

The involvement of PKC in egg activation induced by sperm or parthenogenetic stimulation has been well documented. According to our results, PB2 emission and PN formation were prevented by the PKC inhibitor calphostin C, which is consistent with others' reports. However, some new phenomena were observed in this experiment. Traslocation of the cPKC isoforms α and βI were observed after fertilization or parthenogenetic activation of rodent (Luria *et al*., 2000; Eliyahu *et al*., 2002) and pig (Fan *et al*., 2002*b*) eggs, but we show here that inhibition of cPKC isoforms failed to block PB2 emission and PN formation after fertilization, suggesting that the functional roles of cPKCs might be substituted by other molecules, probably by other PKC subclasses. Although mouse eggs can be fertilized *in vitro* and form the male and female PNs at the presence of PMA, the emission of PB2 is almost totally inhibited. Using an optimized method of confocal microscopy developed by us recently (Fan *et al*., 2003*b*), we detected a concentration of PKCα at the cytoplasmic connection between the extruding PB2 and the fertilized egg. Thus, although PKC activity is necessary for the cortical granule exocytosis (Luria *et al*., 2000; Fan *et al*., 2002*b*; Eliyahu *et al*., 2002) and MPF inactivation (Collona *et al*., 1997) after fertilization, the continuous high PKC activity could inhibit PB2 emission as well as PB1 emission.

To study whether the mitotic cell cycles in early embryos can also be arrested by PKC activation, we added PMA to the culture medium of mouse preimplantation embryos at different developmental stages. The results showed that the cell cycles of embryos can always be arrested at the interphase from the one-cell to the eight-cell stage. It is the first evidence that PKC activation can inhibit not only the initiation of meiotic cell cycle in oocytes but also the progression of mitotic cell cycles in embryonic cells. Results from confocal microscopy revealed that PKCα isoform concentrated to both the male and female PNs after fertilization. And this PKC isoform was localized to the nucleus in the following embryo development. This pattern of subcellular distribution did not change after PMA treatment (data not shown). We also observed a concentration of the PKCα, βI and γ isoforms in the PN in pig fertilized eggs (Fan *et al*., 2002*b*). PKCs are regulators of cell proliferation and differentiation in various cell types (Wagner *et al*., 2001; Lin *et al*., 2001) and they exist in the nuclei of somatic cells (Garciaac *et al*., 2000). Thus, PKCα and γ might participate in the PN formation and chromatin reconstruction in pig eggs after fertilization. Another possibility is that they might participate in the initiation of DNA duplication or gene transcription, preparing for the first mitosis.

In conclusion, the PKC activator PMA inhibits the meiotic resumption of mouse oocytes by a mechanism dependent on not only classical PKC activity but also other PKC isoforms. PKC activation after GVBD leads to the inhibition of MAPK phosphorylation and the arrest of cell cycle at MI stage. PB2 emission and the cleavages of early embryos are blocked after prolonged PKC activation. The subcellular localization of PKCα isoform in mouse oocytes and embryos is developmental-stage associated. All these results suggest that PKC plays multiple functional roles in the cell cycle progression of mouse oocytes and embryos.

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