MOLECULAR REPRODUCTION AND DEVELOPMENT 63:480–487 (2002)

Inhibitory Effects of cAMP and Protein Kinase C on Meiotic Maturation and MAP Kinase Phosphorylation in Porcine Oocytes

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ABSTRACT The regulation of MAP kinase phosphorylation by cAMP and protein kinase C (PKC) modulators during pig oocyte maturation was studied by Western immunoblotting. We showed that both forskolin and IBMX inhibited MAP kinase phosphorylation and meiosis resumption in a dose-dependent manner, and this inhibitory effect was overcome by the protein phosphatase inhibitor, okadaic acid. Pharmacological PKC activator phorbol myristate acetate or physiological PKC activator diC8 also delayed MAP kinase phosphorylation and meiosis resumption, and their effect was abrogated by PKC inhibitors, staurosporine, and calphostin C. The results suggest that meiotic resumption is inhibited by elevation of cAMP or delayed by activation of PKC probably via down-regulation of MAP kinase activation, which is mediated by protein phosphatase, during pig oocyte maturation. *Mol. Reprod. Dev. 63: 480–487, 2002.*

Key Words: protein kinase C; cAMP; MAP kinase; oocyte; pig; meiosis

INTRODUCTION

In mammals, oocyte meiotic maturation is arrested at the dictyte stage of the first meiosis, and oocytes resume meiosis, as indicated by germinal vesicle breakdown (GVBD), in response to the preovulatory gonadotropin surge. Meiotic resumption can also occur spontaneously as a consequence of releasing the oocytes from their follicular environment and culture in vitro. Numerous factors are involved in the regulation of meiosis resumption. It has been shown in recent years that a cascade of protein phosphorylation/dephosphorylation events, which are regulated by protein kinases and protein phosphatases (PPs), plays vital roles in the maintenance and release from meiotic arrest.

It has been well shown that oocyte meiotic resumption is associated with decreased concentration of intracellular cyclic adonosine monophosphate (cAMP) and resulted inactivation of cAMP-dependent protein kinase A (PKA) in various vertebrate species including the pig (Cho et al., 1974; Schultz et al., 1983; Kim and Menino, 1995). The presence of cAMP in the maturation medium inhibited GVBD and synchronized the GVs at a specific stage in pig oocytes (Funahashi et al., 1997). However, the mechanism by which cAMP/PKA signaling pathway prevents the meiotic resumption in oocytes remains unclear.

Another kinase that plays important roles in meiotic regulation in mammalian oocytes is protein kinase C (PKC), which is originally described as a family of serine/threonine kinases that can be activated by Ca^{2+} and diacylglycerol (DAG). PKC isoforms have recently been identified in mouse, rat, and pig oocytes (Gandeswaran and Jones, 1997; Luria et al., 2000; Downs et al., 2001; Fan et al., 2002a). Both inhibitory and stimulatory actions have been reported for the effects of PKC on mammalian oocyte maturation. Activation of PKC by pharmacological agents such as phorbol ester or DAG inhibits GVBD in mouse (Urner and Schorderet-Slatkine, 1984) and rat cumulus-free oocytes (Lu et al., 2001). However, a stimulatory effect of this kinase on mammalian oocyte maturation has been reported in mouse (Downs, 1995), rat (Aberdam and Dekel, 1985), and rabbit (Yoshimura et al., 1992). Coskun and Lin (1995) reported that phorbol myristate acetate (PMA), a tumor-promoting phorbol ester, enhanced the spontaneous maturation of pig oocytes. A recent report showed that PKC modulators affected pig oocyte maturation in a culture system containing hypoxanthine (Su et al., 1999).

Heng-Yu Fan and Man-Yu Li contributed equally to this work.

Grant sponsor: Special Funds for Major State Basic Research ("973") Project of China; Grant number: G1999055902; Grant sponsor: Knowledge Innovation Program of the Chinese Academy of Sciences; Grant number: KSCX2-SW-303; Grant sponsor: National Natural Science Foundation of China.

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Received 8 April 2002; Accepted 11 July 2002
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.10194

Recently, the roles of mitogen-activated protein (MAP) kinase in oocyte maturation have received much attention (for review, see Sun et al., 1999a; Fan et al., 2002b). MAP kinase activity is dispensable for GVBD in mouse oocytes, since the activation of this kinase occurs nearly 2 hr after GVBD, and U0126, the inhibitor of MAP kinase kinase (MAPKK), does not inhibit GVBD. However, the ability of mouse ooplasm to phosphorylate MAP kinase is a prerequisite for meiotic resumption and MAP kinase activation is required for post-GVBD events (Sun et al., 1999b). In pig oocytes, MAP kinase activation begins around the time of GVBD (Inoue et al., 1995; Sun et al., 2001; Shimada and Terada, 2001; Li et al., 2002) and injection of active MAP kinase into the GV may cause early GVBD (Inoue et al., 1998).

Thus, it is evident that cAMP, PKC, and MAP kinase are key regulators of meiotic cell cycle progression in mammalian oocytes. To understand how cAMP, PKC, and MAP kinase interact with each other in regulating meiosis is an important topic for investigating the mechanisms underlying oocyte maturation. Our recent studies have shown that elevation in cAMP levels or activation of PKC down-regulates MAP kinase phosphorylation and inhibits meiosis resumption in rodent oocytes (Sun et al., 1999b,c; Lu et al., 2001), and these effects are overcome by the PPs inhibitor OA. However, accumulating information suggests that rodents may be atypical with regard to regulating mechanisms of oocyte maturation and fertilization. The present study aims to investigate the regulation of MAP kinase phosphorylation by cAMP or PKC and the subsequent effect on meiosis resumption in pig oocytes.

MATERIALS AND METHODS

Chemicals

Stock solutions of forskolin (50 mM), isobutylmethylxanthine (IBMX, 1 M), okadaic acid (OA, 0.2 mM), 1,2-dioctanoyl-rac-glycerol (diC8, 100 mM), phorbol 12-myristate 13-acetate (PMA, 1.62 mM), 4α-phorbol 12, 13-didecanoate (4α-PDD, 1.62 mM), calphostin C (Cal, 0.5 mM), and staurosporine (Stau, 2 mM) were prepared in dimethyl sulfoxide (DMSO), and stored frozen at −20°C until use. Prior to use, they were diluted with TCM-199. All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

In Vitro Oocyte Maturation

Oocytes were aspirated from antral follicles, 3–5 mm in diameter, of ovaries collected from slaughtered prepubertal gilts. After being washed three times with HEPES-buffered Tyrodes's lactate containing 0.1% polyvinyl alcohol (HTL-PVA), oocytes surrounded by compact cumulus cells were washed again with tissue culture medium (TCM)-199 (Gibco, Grand Island, NY) supplemented with 3.05 mM D-glucose, 2.92 mM calcium lactate, 0.91 mM sodium pyruvate, 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulphate, and 0.57 mM cysteine. Each group of 50 oocytes was cultured for 24 hr or 44 hr at 39°C in an atmosphere of 5% CO_2 in air in a 500-μl drop of TCM-199 containing 10 IU/ml eCG (Sigma) and 10 IU/ml hCG (Sigma), with or without drugs.

Experimental Design

Experiment 1. To determine the effects of cAMP elevation on pig oocyte maturation and MAP kinase phosphorylation, fully grown GV oocytes enclosed by cumulus cells were cultured in the maturation medium containing 0, 1, 3.125, 6.25, 12.5, 25, 50, or 100 μM forskolin, which is a stimulator of adenylate cyclase (Schorderet-Slatkine and Baulieu, 1982); or cultured in the maturation medium containing 0, 1, 5, or 10 mM IBMX, an inhibitor of cAMP phosphodiesterase (Bornslaeger et al., 1986), for 24 hr.

Experiment 2. The possible involvement of PPs in the regulation of meiotic arrest and MAP kinase phosphorylation inhibition caused by cAMP elevators was studied. GV oocytes were first treated with 100 μM forskolin or 5 mM IBMX for 15 min, and then transferred to medium containing one of these chemicals plus 2 μM OA, and cultured for an additional 24 hr.

Experiment 3. To address the role of PKC in pig oocyte maturation and MAP kinase phosphorylation, cumulus-enclosed GV oocytes were treated with different concentrations of PMA (a pharmacological PKC activator, 0.162, 1.62, 16.2, 162 nM) or diC8 (a physiological PKC activator, 10, 50, 500, 1,000 nM) for 24 hr. As controls, GV oocytes were cultured in drug-free medium or treated with 4α-PDD (162 nM) for 24 hr.

Experiment 4. To further study the effects of long-term PMA treatment on pig oocyte maturation, cumulus-enclosed GV oocytes were treated with 1.62, 16.2, 162, or 1,620 nM PMA for 44 hr. To clarify whether short-term treatment of oocytes with PMA results in the inhibition of GVBD and MAP kinase phosphorylation as reported in rodents, GV oocytes were treated with 1,620 nM PMA for 15 min and then transferred to a drug-free medium for further maturation culture of 44 hr.

Experiment 5. In order to confirm that PKC is actually involved in the effects of PMA and diC8 on GVBD and MAP kinase phosphorylation, GV oocytes were pre-treated with PKC inhibitor calphostin C (1 μM) or staurosporine (30 nM) for 15 min, and then treated with 16.2 nM PMA or 500 μM diC8 for 24 hr, in the presence of one of the PKC inhibitors mentioned.

In each experiment, cumulus cells were removed from the oocytes with 0.03% hyaluoronidase (Sigma) treatment and repeated pipetting. After being washed three times, oocytes were used for either Western immunoblotting of MAP kinase or orcein staining for nuclear status detection. All experiments were repeated at least three times and the nuclear statues of ~30 oocytes were examined each time.

Evaluation of Nuclear Status

Orcein staining was conducted as described by Sun et al. (2001) with minor modification. Denuded oocytes

were mounted on slides, fixed in acetic acid:ethanol (1:3 v/v) for at least 48 hr, stained with 1% orcein, and examined with a phase-contrast microscope.

Electrophoresis and Western Immonoblotting

Proteins from a total of 30 oocytes per treatment were extracted with double-strength electrophoresis buffer. After boiling for 4 min and centrifuging 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 hr at 120 V, and then transferred onto Gelman transfer membrane for 2.5 hr at 200 mA. The membrane was blocked with 5% skim milk in 20 mM Tris, 137 mM NaCl (TBS) containing 0.1% Tween-20 (TBST) overnight at 4°C, and then incubated for 2 hr at 37°C with monoclonal mouse anti-phosphorylated MAP kinase antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1,000 in TBST containing 5% skim milk (pH 7.4). After three washes, 10 min each, in TBST, the membrane was incubated for 1 hr at 37°C with HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.) diluted 1:5,000. Finally, the membrane was washed three times in TBST, 5 min each, and then processed by using the ECL detection system (Amersham International, Amersham, United Kingdom).

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, and then incubated with HRP-labeled goat anti-rabbit IgG diluted 1:5,000, and finally processed as described above.

Data Analysis

All percentages on GVBD from three repeated experiments were subjected to arcsin transformation. The relative MAP kinase activity after each treatment was determined by densitometric scan of the band. The values of relative MAP kinase activity and the transformed data of GVBD were analyzed by ANOVA followed by Student–Newman–Keuls test. Differences at $P < 0.05$ were considered to be statistically significant.

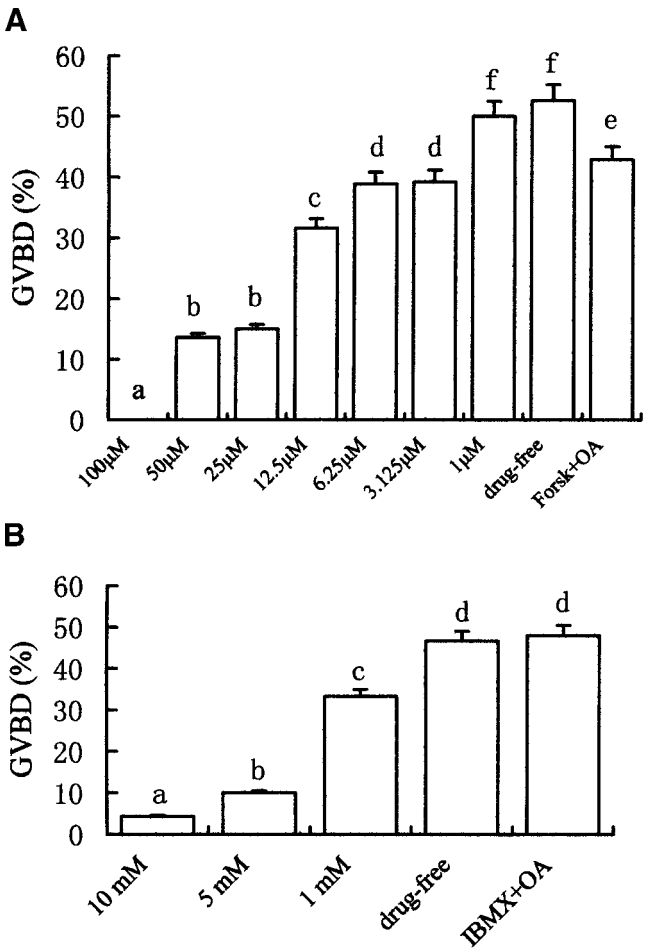

Fig. 1. Effects of cAMP elevators on GVBD in pig oocytes. The **top panel** (**A**) shows the effects of different dosages of forskolin on GVBD. The **bottom panel** (**B**) shows the effects of different concentrations of IBMX on GVBD. Cumulus-enclosed GV oocytes were treated with different concentrations of cAMP elevators (forskolin: 0, 1, 3.125, 6.25, 12.5, 25, 50, and 100 μM; IBMX: 0, 1, 5, or 10 mM) for 24 hr. As controls, oocytes at GV stage were cultured in drug-free medium for 24 hr. To determine the possible involvement of phosphatase in the effects of cAMP on GVBD, oocytes were treated with forskolin or IBMX at the presence of phosphatase inhibitor OA (2 μM) for 24 hr. The GVBD rate was shown as percentage (mean + SEM). Different superscripts denote statistical difference at a $P < 0.05$ level of significance. The same statistical method was applied to subsequent figures.

RESULTS

cAMP Elevation Inhibits MAP Kinase Phosphorylation and GVBD

As shown in Figure 1, GVBD was inhibited in a dose-dependent manner by both forskolin, an activator of adenylate cyclase, and IBMX, an inhibitor of phosphodiesterase, when observed at 24 hr of culture. With the increase in forskolin or IBMX concentration in the maturation medium, MAP kinase phosphorylation level was decreased (Figs. 2 and 3), while MAP kinase expression level was not changed. OA, a potent inhibitor of protein phosphotases 1 and 2A, overcame the inhibitory effect of forskolin (Figs. 1A and 4) or IBMX (Figs. 1B and 3) on GVBD and MAP kinase phosphorylation at a concentration of 2 μM.

Effects of PKC Activators on GVDB and MAP Kinase Activation

As shown in Figures 5A and 6, the addition of the phorbol ester, PMA, a pharmacological PKC activator, to the maturation medium inhibited resumption of meiotic maturation (GVBD) and MAP kinase phosphorylation in a dose-dependent manner. The effective concentration of PMA to inhibit GVBD was 0.162 nM (75% inhibition), with almost complete inhibition at the concentration of 162 nM. Whereas the biologically inactive phorbol ester, 4α-PDD, had no effect. The physiological PKC inhibitor, diC8, had similar effects on GVBD and MAP kinase phosphorylation as PMA (Figs. 5B and 7),

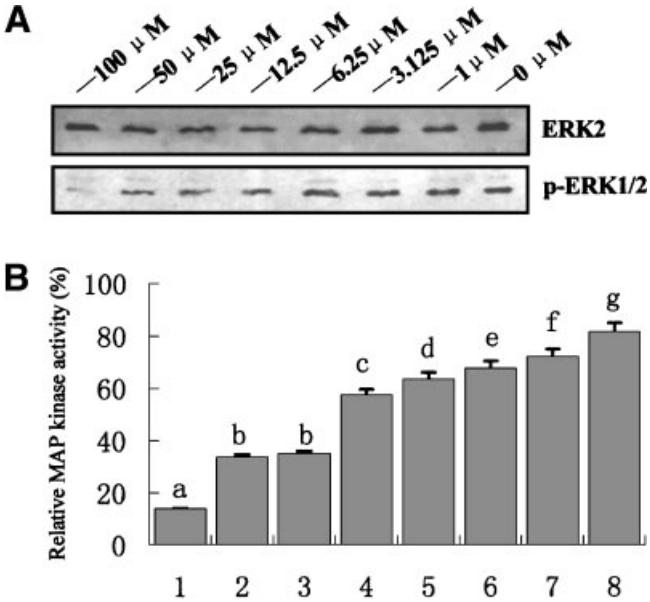

Fig. 2. Effects of forskolin on MAP kinase phosphorylation in pig oocytes. (**A**) Immunoblotting results. Samples for immunoblotting were collected after the oocytes were treated with different concentrations of forskolin (0, 1, 3.125, 6.25, 12.5, 25, 50, and 100 μM) for 24 hr. (**B**) Relative MAP kinase activity as determined by densitometric scans. The MAP kinase activity was normalized in each membrane by using the total amount of MAP kinase present in the upper sets of lanes to standardize the activity in the lower set of lanes. All data were expressed as mean + SEM. Different superscripts denote statistical difference at a $P < 0.05$ level of significance. The same treatment was applied to subsequent figures. MAP kinase was phosphorylated 24 hr after maturation culture in drug-free medium. Foskolin inhibits MAP kinase activation in a dose-dependent manner.

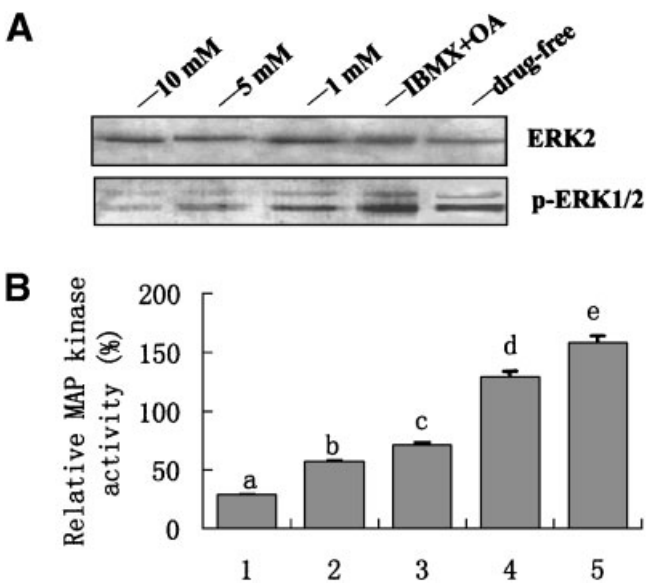

Fig. 3. Effects of IBMX on MAP kinase phosphorylation in pig oocytes. (**A**) Immunoblotting results. Samples were collected after treatment with IBMX of different concentrations (0, 1, 5, or 10 mM). Oocytes cultured in a drug-free medium for 24 hr were collected as control. Some oocytes were treated with 5 mM IBMX plus 2 μM OA for 24 hr. (**B**) Relative MAP kinase activity quantified by densitometric scans. IBMX inhibits MAP kinase activation in a dose-dependent manner, and this inhibitory effect can be overcome by OA.

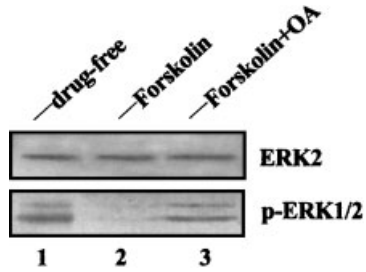

Fig. 4. The inhibition of GVBD and MAP kinase phosphorylation induced by forskolin is reversed by OA. (**Lane 1**) oocytes cultured in a drug-free medium for 24 hr as control; **lane 2**, in oocytes treated with 100 μM forskolin for 24 hr, MAP kinase activation was inhibited; and **lane 3**, MAP kinase was phosphorylated by treatment with 100 μM forskolin plus 2 μM OA.

with the effective concentration ranging from 10 μM (65% GVBD inhibition) up to 1 mM (95.8% GVBD inhibition). Inhibition of GVBD by both PKC activators was well correlated with inhibition of MAP kinase phosphorylation.

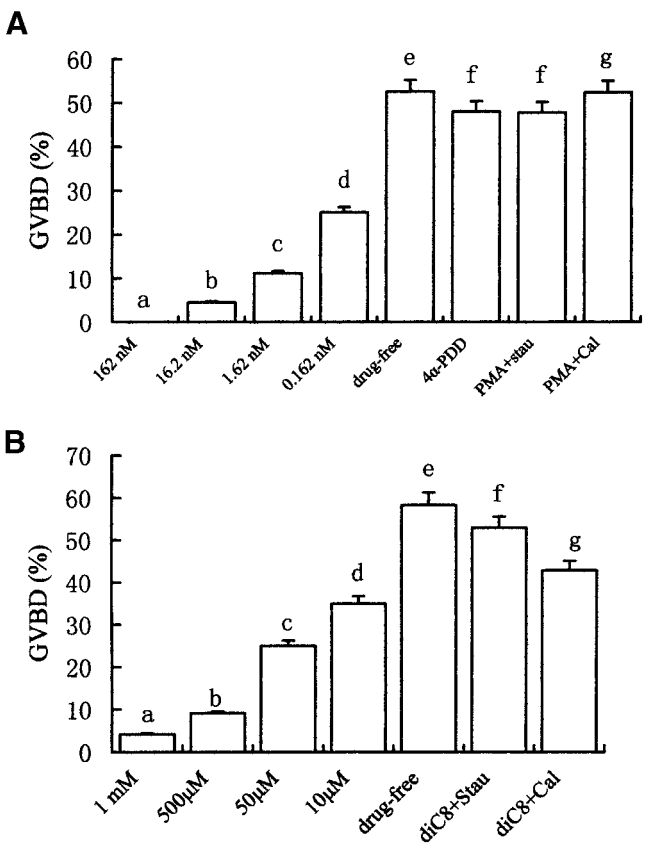

Fig. 5. Effect of PKC activators, PMA and diC8, on GVBD in pig oocytes is shown in the **upper panel** (**A**) and **lower panel** (**B**), respectively. PMA or diC8 inhibited GVBD in a dose-dependent manner after treatment of the oocytes for 24 hr, and their inhibitory effect on GVBD could be overcome in the presence of one of the PKC inhibitors, calphostin C (1 μM) or staurosporine (30 nM). Oocytes cultured in a drug-free medium or treated with biologically inactive phorbol ester 4α-PDD (162 nM) for 24 hr were used as control.

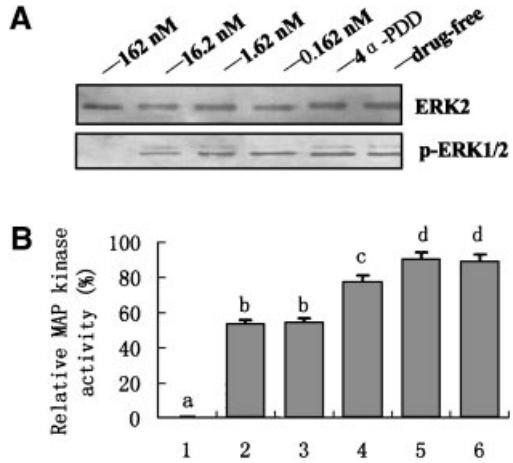

Fig. 6. Effect of PMA on MAP kinase phosphorylation in pig oocytes. (**A**) Immunoblotting results. Cumulus-enclosed oocytes at GV stage were subjected to different treatments for 24 hr and collected for immunoblotting. **Lanes 1–4**, oocytes treated with 162, 16.2, 1.62, and 0.162 nM PMA, respectively; **lane 5**, control, oocytes treated with 162 nM 4α-PDD; and **lane 6**, control, oocytes incubated in drug-free medium. (**B**) Relative MAP kinase activity changes as revealed by densitometry. MAP kinase activity was decreased with the increase of PMA concentration in the maturation medium. Biologically inactive phorbol ester 4α-PDD had no effect on MAP kinase phosphorylation.

PKC Inhibitors Abolish the Effects of PMA and diC8 on GVBD and MAP Kinase Phosphorylation

As shown in Figures 5 and 8, when oocytes were pretreated with the PKC inhibitor calphostin C or staurosporine, and then cultured in medium containing both the PKC activator, PMA or diC8, and one of these

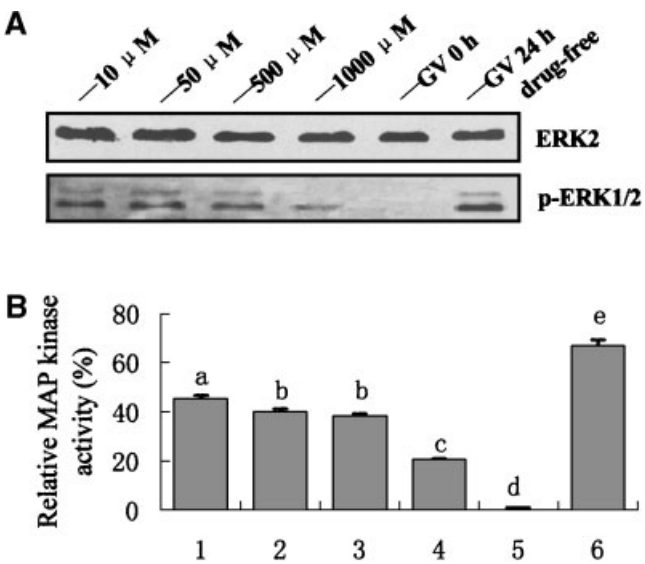

Fig. 7. Effect of diC8 on MAP kinase phosphorylation in pig oocytes. (**A**) Immunoblotting results. **Lanes 1–4**, Oocytes treated with 10, 50, 500, and 1,000 μM diC8 for 24 hr, respectively; **lane 5,** negative control, GV oocytes collected from the follicles; and **lane 6**, positive control, oocytes after 24 hr culture in a drug-free medium. (**B**) Results from densitometric examination of MAP kinase activity. DiC8 inhibited MAP kinase activation with increased concentrations.

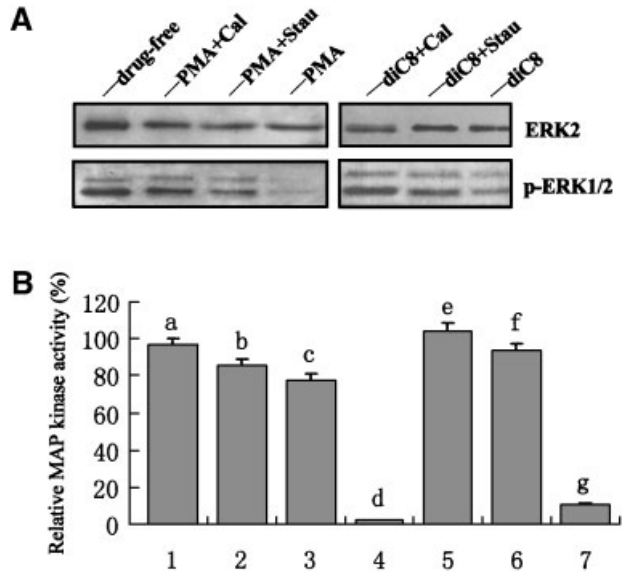

Fig. 8. Effects of PKC inhibitors on PMA- and diC8-induced inhibition of MAP kinase phosphorylation. (**A**) Immunoblotting results. Each lane represents samples after different treatment for 24 hr. **Lane 1**, oocytes cultured in drug-free medium as positive control; **lanes 2–4**, oocytes treated with PMA (162 nM) plus calphostin C (1 μM), PMA (162 nM) plus staurosporine (30 nM), and PMA (162 nM), respectively; **lanes 5–7**, oocytes treated with diC8 (500 μM) plus calphostin C (1 μM), diC8 (500 μM) plus staurosporine (30 nM), and diC8 (500 μM), respectively. (**B**) Relative MAP kinase activity determined by densitometric scans. The inhibitory effect of both PKC activators on MAP kinase activation could be abolished by PKC inhibitor, calphostin C or staurosporine.

PKC inhibitors, the inhibition of PKC activators on GVBD and MAP kinase phosphorylation could be partially overcome.

Effects of Long-Term and Short-Term Treatment of PMA on Meiotic Cell Cycle Progression and MAP Kinase Activation

As shown in Table 1, of oocytes cultured in maturation medium containing 1.62, 16.2, 162, or 1,620 nM PMA for 44 hr, most of them underwent GVBD after culture, but PMA at the concentration of 162 or 1,620 nM arrested the oocytes at M I stage at 44 hr, with only a few developed to M II stage. However, the MAP kinase phosphorylation was not impaired (Fig. 9). When oocytes were incubated with 1,620 nM PMA for a short term (15 min) and then transferred to maturation medium for further culture of 44 hr, neither GVBD nor MAP kinase phosphorylation was inhibited (Table 1 and Fig. 9).

DISSCUSSION

Although activation of MAP kinase is not required for meiotic resumption of rodent oocytes, existing evidence suggest the possible involvement of MAP kinase activation in the initiation of meiotic resumption in pig oocytes: (1) MAP kinase activation begins around the time of GVBD (Inoue et al., 1995; Sun et al., 2001; Shimada and Terada, 2001; Li et al., 2002); (2) both the GVBD and MAP kinase activation were inhibited in

TABLE 1. Meiotic Status of Pig Oocytes Treated with Different Concentrations of PMA or Different Time and Cultured for 44 hr

Concentrations of PMA (nM)	Nuclear status 44 hr after culture		
	GV	GVBD-M I	M II
1.62 (44 hr)	3.27% (2/61)	16.39% (10/61)	80.33% (49/61)
16.2 (44 hr)	5.95% (5/84)	15.48% (13/84)	52.38% (44/84)
162 (44 hr)	2.27% (2/88)	67.05% (59/88)	30.68% (27/88)
1,620 (44 hr)	2.63% (2/76)	82.89% (63/76)	14.47% (11/76)
1,620 (15 min)	— (0/54)	9.26% (5/54)	90.74% (49/54)
Drug-free control	4.26% (6/141)	9.93% (14/141)	85.82% (121/141)

cumulus-enclosed pig oocytes treated with MAPKK inhibitor U0126 (Fan et al., 2002c); (3) immunofluorescent staining showed that in porcine oocytes, active MAP kinase translocated to the condensed chromosomes when GVBD occurred and was aggregated to the spindle area as meiosis proceeded (Inoue et al., 1998); (4) MAP kinase activation did not occur when incompetent pig oocytes were cultured in vitro, and on the other hand, GVBD was accelerated when active MAP kinase was injected into the GV in some oocytes (Inoue et al., 1998); and furthermore, (5) both MAP kinase activation and GVBD were significantly accelerated by the protein phosphatase (PP) inhibitor OA (Sun et al., 2002).

Though the inhibitory roles of cAMP elevation and the resulting PKA activation on meiotic maturation in mammalian oocytes has been well proven, the mechanism is still unknown at present. The target molecules downstream of PKA activation are unclear. For the first time, we have proven that the MAP kinase, a key regulator of meiotic cell cycle progression in mammalian oocytes, is inhibited by the elevation of cAMP level in pig oocytes. It is possible that cAMP/PKA may inhibit GVBD via inhibition of MAP kinase activation, by increasing phosphorylation of one or more unidentified inhibitory proteins. It is well known that MOS, a 39 kDa Ser/Thr protein kinase, is required for MAP kinase activation in germ cells (for review, see Gebauer and Richter, 1996). It has been suggested that some unidentified protein phosphorylated by PKA directly or indirectly suppresses c-mos translation (Matten et al., 1994; Sagata, 1997), but how cAMP/PKA downregulates MOS synthesis and MAP kinase activation remains to be determined. In these experiments, we also observed that the inhibitory effects of cAMP elevation on GVBD and MAP kinase activation in porcine oocytes could be completely reversed by OA, a protein phosphatase inhibitor. The same results were obtained in rodents by us (Sun et al., 1999c; Lu et al., 2001). In our recent experiments, MAP kinase in porcine oocytes at GV stage could be activated as early as 5 min after treatment with OA, followed by significant acceleration of GVBD (Sun et al., 2002). All these facts inferred that the mechanism responsible for MAP kinase phosphorylation has already developed in GV pig oocytes, but it has been inhibited by a PPs-dependent pathway until the occurrence of GVBD. One possibility is that OA may stimulate GVBD via direct activation of MAP kinase. Another possibility is that OA stimulate GVBD in a manner unrelated to direct activation of MAP kinase. OA could have an effect down-stream of cAMP to bring about GVBD and MAP kinase activation could be activated simply as a consequence of GVBD. But a recent study showed that MAP kinase was activated normally when GVs were removed from the pig oocytes (Sugiura et al., 2001). Thus, elevation of cAMP, mediated by protein phosphatase, may cause inhibition of GVBD via inhibition of MAP kinase activation in pig oocytes.

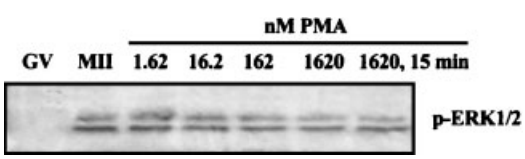

Fig. 9. Effects of long- and short-term treatment of PMA on the inhibition of MAP kinase phosphorylation. **Lane 1**, negative control, oocytes at CV stage obtained from follicles; **lane 2**, positive control, oocytes cultured for 44 hr in a drug-free medium; **lanes 3–6**, oocytes cultured in a medium containing 1.62, 16.2, 162, or 1,620 nM PMA for 44 hr; and **lane 7**, oocytes treated with 1,620 nM PMA for 15 min and transferred to a drug-free medium for 44 hr maturation culture.

PKC activators, PMA and diC8, could inhibit GVBD and down-regulate MAP kinase activity in a dose-dependent manner 24 hr after culture. These effects could be reversed by the addition of PKC inhibitors, calphostin C or staurosporine, to the maturation medium. These results indicate that the activation of PKC may be responsible for the PMA- or diC8-induced inhibition of GVBD and MAP kinase phosphorylation. Recently, we detected the expression of three isoforms of classical PKC, α, βI, and γ, in pig oocytes. These PKCs concentrated in the nucleus at GV stage and translocated to the cytoplasm after GVBD, suggesting their regulatory roles during pig oocyte maturation (Fan et al., 2002a). It is logical to conclude that PKC activators may inhibit GVBD via action upon MAP kinase or the upstream molecules that are required for MAP kinase phosphorylation. It is unknown if the PKC exerts its effects on GVBD and MAP kinase activation by the same mechanism as cAMP/PKA, or acts through a distinct pathway.

It has long been puzzling whether the activated PKA or PKC inhibits the molecules leading to MAP kinase phosphorylation directly in the cytoplasm, or through a nucleus-dependent mechanism. It seems that the

former may be the case. A recent report argued that the MAP kinase activation is independent of the germinal vesicle material during porcine oocyte maturation (Sugiura et al., 2001). We found that it is also the case in mouse oocytes, and the MAP kinase activation in enucleated mouse oocytes could also be inhibited by PKA or PKC activation (unpublished results). Further research needs to be performed to identify the components responsible for the inhibition of MAP kinase phosphorylation in the cytoplasm of mammalian oocytes.

One should be cautious when evaluating the effects of cAMP elevators or PKC activators on GVBD in mammalian oocytes, since the influence of cumulus cells is not negligible. It has been proposed that the contradictory actions of cAMP on oocyte maturation are due to compartmentalization of PKA isoforms and functions, such that type I PKA within the mouse oocyte suppresses GVBD, while type II PKA within the somatic compartment acts in a positive capacity to induce maturation (Downs and Hunzicker-Dunn, 1995). But until now, no report concerning the distribution and function of PKA isoforms in the pig cumulus–oocyte complex was published. PKC activators, PMA and 1-oleoyl-2-acetyl glycerol (OAG), stimulated GVBD in follicle-enclosed rat oocytes, but we found that both PMA and diC8 inhibit GVBD and MAP kinase phosphorylation in denuded rat oocytes (Lu et al., 2001; Tan et al., 2001). Recently, Downs et al. (2001) proposed in rodents, that direct PKC action on the oocyte inhibits GVBD while treatment of cumulus-enclosed or follicle-enclosed oocytes with PKC activators leads to stimulation of GVBD. But we reported here an inhibitory effect of PKC activation on GVBD in pig cumulus-enclosed oocytes at 24 hr of maturation culture. The reasons for the discrepant actions on oocyte maturation could be due to species variation in PKC isoform expression. It was reported until now, that cPKC α, βI, and βII (Luria et al., 2000), nPKC δ (Downs et al., 2001), aPKC λ and ξ (Gandeswaran and Jones, 1997) were detected in mouse eggs. Recently, we proved that the cPKC α and γ, instead of cPKC βI, were activated by PMA in pig oocytes (Fan et al., 2002a). It should be better to examine the effects of PKC activation in denuded pig oocytes, as compared to the results obtained in cumulus-enclosed oocytes. But there are technical obstacles, since the cumulus-free pig oocytes could not be cultured to maturation in vitro. Alternative approaches should be attempted to determine the roles of cumulus cells in response to PKC activators in future studies.

The effects of PMA in pig oocytes differ from those in rodent oocytes observed by us previously. In mouse and rat oocytes, PMA could inhibit GVBD and MAP kinase activation perpetually, and a short-term treatment of PMA worked well in inhibiting GVBD and MAP kinase phosphorylation (Sun et al., 1999b; Lu et al., 2001). However, in pig oocytes, GVBD was retarded, but not completely inhibited by PMA treatment. Though the GVBD was blocked by PMA 24 hr after maturation culture, it occurred several hours later (about 30 hr after culture). The diverse effects of PMA in mammalian oocytes may derive from the difference of maturation events between domestic animals and rodents. The mouse and rat oocytes resume meiosis promptly after release from their follicles. They undergo GVBD within 2–3 hr and no protein synthesis is necessary. Whereas bovine and porcine oocytes resume meiosis slower than rodents and certain protein synthesis during maturation culture is essential for GVBD (Kubelka et al., 1995). It is not known whether the accumulation of some molecules, such as MOS and cyclins, is important for cell cycle progression, leading to the breakdown of G2/M arrest induced by PKC activation in pig oocytes.

In our experiments, meiosis of pig oocytes was blocked at M I stage by long-term treatment with PMA, a pharmacological PKC activator. Recently, Viveiros et al. (2001) reported that PKC activity was high in M I stage in mouse oocytes, and the inactivation of PKC is necessary for the metaphase–anaphase transition of meiosis I. Our results infer that the same mechanisms may also exist in pigs, and the presence of PMA may sustain a high PKC activity in the oocytes, so as to inhibit the release from M I stage.

Taken together, the data suggest that: (1) elevation of intracellular cAMP level, through a protein phosphatase-dependent mechanism, may inhibit meiotic resumption via down-regulation of MAP kinase phosphorylation in cumulus-enclosed pig oocytes; (2) PKC activation delays MAP kinase activation and GVBD of cumulus-enclosed pig oocytes; and (3) PMA-treated oocytes can go through GVBD, but arrest at M I stage after longer maturation culture.

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