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Activation of Protein Kinase C Induces Mitogen-Activated Protein Kinase Dephosphorylation and Pronucleus Formation in Rat Oocytes[1]

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ABSTRACT

Mammalian oocytes are arrested at metaphase of the second meiotic division (MII) before fertilization. When oocytes are stimulated by spermatozoa, they exit MII stage and complete meiosis. It has been suggested that an immediate increase in intracellular free calcium concentration and inactivation of maturation promoting factor (MPF) are required for oocyte activation. However, the underlying mechanism is still unclear. In the present study, we investigated the role of protein kinase C (PKC) and mitogen-activated protein (MAP) kinase, and their interplay in rat oocyte activation. We found that MAP kinase became dephosphorylated in correlation with pronucleus formation after fertilization. Protein kinase C activators, phorbol 12-myriatate 13-acetate (PMA) and 1,2-dioctanoyl-*rac*-glycerol (diC8), triggered dephosphorylation of MAP kinase and pronucleus formation in a dose-dependent and time-dependent manner. Dephosphorylation of MAP kinase was also correlated with pronucleus formation when oocytes were treated with PKC activators. Effects of PKC activators were abolished by the PKC inhibitors, calphostin C and staurosporine, as well as a protein phosphatase blocker, okadaic acid (OA). These results suggest that PKC activation may cause rat oocyte pronucleus formation via MAP kinase dephosphorylation, which is probably mediated by OA-sensitive protein phosphatases. We also provide evidence supporting the involvement of such a process in fertilization.

in vitro fertilization, kinases, meiosis, phosphatases, signal transduction

INTRODUCTION

Mammalian oocytes are arrested at metaphase of the second meiotic division (MII), before fertilization. Stimulation by spermatozoa will initiate resumption of meiosis. Oocytes undergo Ca^{2+} oscillations, cortical granule exocytosis, second polar body extrusion, protein synthesis and modification, chromosome decondensation, and pronucleus formation. It is believed that MII arrest is maintained by a c-Mos protooncogene product and maturation promoting factor (MPF) [1, 2]. Studies have shown that loss of MPF activity is necessary for meiosis resumption [3–5]. However, the mechanism responsible for MPF inactivation when spermatozoon entry occurs is not clear. It has been suggested that stimulation by spermatozoa results in an immediate increase in intracellular free calcium concentration [6], which subsequently activates protein kinase C (PKC) [7–9]. Activated PKC has been shown as sufficient to cause MPF inactivation and meiosis resumption in mouse oocytes [10, 11]. On the contrary, it has been reported that activation of PKC does not play a major role in meiosis resumption in oocytes of mice, rats, and hamsters [12–14]. Our previous study has also shown that PKC activators induce cortical granule exocytosis, but not resumption of the cell cycle in porcine oocytes [15]. These results suggest that the roles of PKC in oocyte activation may be species specific.

Besides PKC, tyrosyl-phosphorylated proteins have been suggested to be involved in regulating rat oocyte activation [16]. Mitogen-activated protein (MAP) kinase, also known as extracellular signal-regulated kinase, is a serine/threonine protein kinase that requires threonine and tyrosine phosphorylation in order to become activated [17]. MAP kinase is phosphorylated and activated by MAP kinase kinase (MEK), which is activated by a c-Mos protooncogene product in oocytes [18–21]. MAP kinase has been found to participate in pronuclear envelope assembly and disassembly during fertilization in mouse oocytes [22, 23]. Our previous studies have also indicated that inactivation of MAP kinase may be required for pronucleus formation in oocytes of mice, pigs, and humans after fertilization [24–27]. However, there are no data to show whether MAP kinase is involved in rat oocyte activation. The objectives of this study were to clarify the functional importance of PKC and MAP kinase, as well as their cross-talk, in rat oocyte activation. Our previous studies have suggested that PKC activation and subsequent MAP kinase inactivation are required for pronucleus formation in mouse oocytes [28]. In this study, we hypothesized that PKC activation may cause pronucleus formation via MAP kinase dephosphorylation, and that such a process is involved in fertilization in rat oocytes.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) except those noted. Stock solutions of phorbol 12-myriatate 13-acetate (PMA; 1.62 mM), 4α-phorbol 12,13-didecanoate (4α-PDD, 1.62 mM), 1,2-dioctanoyl-*rac*-glycerol (diC8, 100 mM), calphostin C (0.5 mM), and okadaic acid (OA, 0.2 mM) were prepared with dimethyl sulfoxide. All stock solutions were frozen at −20°C. They were diluted with medium before use. The basal medium for oocyte activation and fertilization in this study was Dulbecco modified Eagle medium (DMEM) that contains 20 mg/ml BSA.

Preparation of Oocytes and Spermatozoa

Twenty-five-day-old female and 3-mo-old male Sprague-Dawley rats were purchased from the Laboratory Animal Core, Institute of Genetics, The Chinese Academy of Sciences. Animals were housed in a temperature

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controlled room with proper dark-light cycles, were fed a regular diet, and were under the care of the Laboratory Animal Unit, State Key Laboratory of Reproductive Biology, Institute of Zoology, The Chinese Academy of Sciences. Following 5 days of acclimation, superovulation was induced by injecting eCG (Sigma, 15 IU per rat) s.c. 48 h before an i.p. injection of hCG (Sigma, 15 IU per rat). Animals were killed by cervical dislocation 16 h after hCG injection. Oviducts were dissected away, and cumulus-enclosed MII oocytes were recovered from them. Cumulus cells were removed by a brief exposure to 450 IU/ml of hyaluronidase (type 1-s, Sigma), and zona pellucidae were removed with acid Tyrode solution (pH 2.5; Sigma).

On the day that oocytes were collected, the males were also killed by cervical dislocation. The cauda epididymides were dissected away and minced slightly into 2 ml of DMEM (Gibco Invitrogen Co., Grand Island, NY) that had been warmed to 37°C, and then placed in a 5% CO_2:air incubator for 10 min at 37°C to allow spermatozoa to swim up. We collected the top sperm suspension, which showed vigorous, progressive motility, and which contained about 1.5×10^7 spermatozoa/ml. For capacitation, the sperm suspension in the concentrated form was kept in a 5% CO_2:air incubator for 3 h. The capacitated sperm suspension was diluted at 1:10 before they were allowed to inseminate MII oocytes.

Experimental Design

Experiment 1. To investigate the change of MAP kinase phosphorylation during fertilization in vitro, cumulus cell-free and zona pellucida-free MII oocytes were inseminated with spermatozoa, which had been previously capacitated for 3 h. The unbound spermatozoa were washed away 2 h after insemination. The oocytes were transferred to fresh medium (DMEM + 20 mg/ml BSA) and cultured for another 6 h. Oocytes for detecting MAP kinase were collected 2, 4, 6, and 8 h after insemination. Meanwhile, pronucleus formation was evaluated with an inverted phase-contrast microscope. Metaphase II oocytes were incubated in DMEM plus 20 mg/ml BSA for 8 h in the absence of spermatozoa, and those fresh-collected MII oocytes were used as controls.

Experiment 2. To study the role of PKC in meiosis resumption and dephosphorylation of MAP kinase, cumulus-free MII oocytes were incubated for 8 h in media with different concentrations of PMA, a pharmacological activator of PKC. Oocytes cultured in drug-free medium and those treated with 4α-PDD, a biologically inactive phorbol ester, were adopted as controls. The proportion of oocytes with pronuclei and MAP kinase dephosphorylation were evaluated 8 h after treatment. The same experiment was performed using a physiological PKC activator, diC8. To test whether PKC-induced dephosphorylation of MAP kinase is temporarily correlated with pronucleus formation, MII oocytes were treated for 8 h with 1.62 nM PMA or 100 μM diC8. Pronucleus formation and MAP kinase dephosphorylation were evaluated at 0, 2, 4, 6, and 8 h of incubation.

Experiment 3. To test how long treatment by PKC activators was needed to achieve pronucleus formation and MAP kinase dephosphorylation, MII oocytes were treated with 1.62 nM PMA or 100 μM diC8 for 10, 15, 30, and 60 min. After washing, they were transferred to a drug-free medium and cultured for up to 8 h. Pronucleus formation and MAP kinase dephosphorylation were detected at 8 h of incubation. Metaphase II oocytes treated with 1.62 nM PMA or 100 μM diC8 for 8 h were adopted as controls.

Experiment 4. In order to confirm that MAP kinase dephosphorylation and pronucleus formation induced by PMA or diC8 are mediated by activating PKC, MII oocytes were pretreated with 1.62 nM PMA for 1 h. After washing, they were transferred to a medium containing 1 μM calphostin C (activated for 10 min by light) or 30 nM staurosporine for 8 h. As a control, MII oocytes were pretreated with 1.62 nM PMA for 1 h. After washing, they were transferred to a drug-free medium for 8 h. Metaphase II oocytes treated with PKC inhibitors alone for 8 h were also designated as controls. After 8 h of treatment the proportion of oocytes with pronuclei and MAP kinase activity was evaluated. A similar experiment was conducted using diC8 instead of PMA.

Experiment 5. To elucidate the possible involvement of OA-sensitive protein phosphatases in the regulation of MAP kinase dephosphorylation caused by PKC activation, MII oocytes were treated with 1.62 nM PMA for 1 h, then transferred to a medium containing 2 μM OA, and cultured for 8 h. As a control, oocytes were transferred to a drug-free medium and cultured for 8 h after PMA treatment. Metaphase II oocytes cultured in a medium with OA alone for 8 h were also adopted as controls. After treatment, pronucleus formation and MAP kinase dephosphorylation were judged. A similar experiment was performed using diC8 instead of PMA.

Experiment 6. In order to clarify whether PKC activation and MAP inactivation are involved in fertilization, MII oocytes were inseminated in a medium containing PKC inhibitor (1 μM calphostin C or 30 nM staurosporine). Pronucleus formation and MAP kinase dephosphorylation were detected 8 h after insemination. As controls, MII oocytes were inseminated in a medium without PKC modulators.

Western Blot Analysis

A total of 30 denuded oocytes in each treatment were extracted with SDS electrophoresis sample buffer and heated to 100°C for 5 min. After cooling on ice, samples were frozen at −80°C until use. Proteins were separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. After blocking overnight at 4°C with 5% skim milk in TBST (10 mM Tris, 150 mM NaCl, and 0.1% Tween-20; pH 7.4), the membrane was incubated for 1 h at room temperature with rabbit polyclonal anti-Active MAPK antibody (Promega, Madison, WI; 1:1000 dilution). Following washes, blots were incubated for 1 h at room temperature with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig) G (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:5000 dilution). After washing, the membrane was processed using the enhanced chemiluminiscence (ECL) detection system (Amersham Life Science Ltd., Little Chalfont, England). For reprobing, the bound antibodies were removed in stripping buffer (62.5 mM Tris pH 6.7, 100 mM β-mercaptoethanol, and 2% [w/v] SDS) for 30 min in a 55°C water bath with occasional agitation. MAP kinase expression was detected with rabbit polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500 dilution) and HRP-conjugated goat anti-rabbit IgG (1:5000 dilution). Complete stripping of the blots was verified by exposing the membrane to the ECL detection system before immunoblotting with anti-ERK2 antibody. All antibodies were diluted in 0.5% skim milk in TBST. Image processing was conducted with Photoshop 5.0 software (Adobe Systems, San Jose, CA).

Data Analysis

All data were statistically analyzed using χ^2 analysis. Differences at $P < 0.05$ were considered significant. Oocytes displaying signs of degeneration as indicated by dark cytoplasm or fragmented cytoplasm were excluded from analysis.

RESULTS

Dephosphorylation of MAP Kinase During In Vitro Fertilization in Rat Oocytes

Because penetration and fertilization rates are very low using conventional in vitro fertilization (IVF) in rat oocytes, in this study, in order to increase the fertilization rate, cumulus cells and zona pellucida were removed from MII oocytes, and spermatozoa were capacitated for 3 h before fertilization. In this IVF system, 30.9% (121 out of 392) and 54.8% (215 out of 392) of oocytes formed 2 pronuclei 6 and 8 h after insemination, respectively. Pronucleus formation was not observed within 4 h after insemination. After 8 h of insemination, 4.8% (19 out of 392) of oocytes had more than 2 pronuclei (polyspermic fertilization), 4.3% (17 out of 392) had 1 pronucleus (parthenogenetically activated by aging), 34.7% (136 out of 392) still remained at MII stage, and 1.3% (5 out of 392) showed degeneration. Correspondingly, MAP kinase existed in a phosphorylated form within 4 h after insemination and was partially dephosphorylated at 6 h after insemination. Little phosphorylated MAP kinase was detected 8 h after insemination (Fig. 1, top panel). Expression of MAP kinase remained stable during the metaphase-interphase transition (Fig. 1, bottom panel). Pronucleus formation was observed in 5.0% (3 out of 60) of MII oocytes that had been incubated for 8 h in medium without spermatozoa. Mitogen-activated protein kinase remained phosphorylated in these treated group oocytes.

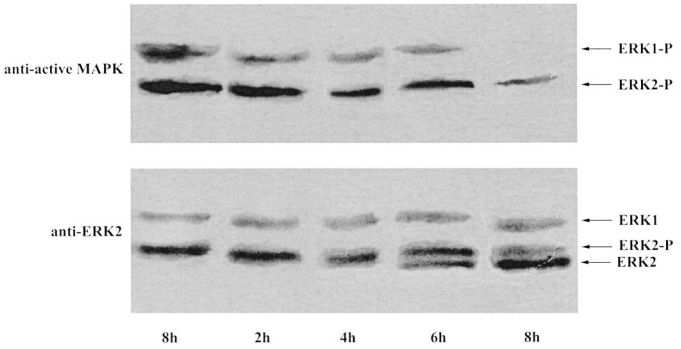

FIG. 1. Changes in MAP kinase expression and phosphorylation during in vitro fertilization of rat oocytes. Lane 1: control MII oocytes incubated for 8 h in the absence of spermatozoa; lanes 2–5: oocytes collected at 2, 4, 6, and 8 h after insemination. A total of 30 oocytes were loaded into each lane. The blot was incubated with a polyclonal anti-Active MAPK antibody to show phosphorylated (active) MAP kinase (top panel). The same blot was stripped and reprobed with a polyclonal anti-ERK2 antibody to show MAP kinase expression (bottom panel). The same process was applied to Figures 3, 5, and 7.

PMA and diC8 Induced MAP Kinase Dephosphorylation and Pronucleus Formation in a Dose-Dependent Manner

As shown in Figure 2A, the PKC activator PMA induced pronucleus formation in rat oocytes in a dose-dependent manner. The effective dosage was 0.0162 nM, causing 41.3% of MII oocytes to form pronuclei. Its maximal activating dosage was 1.62 nM, which induced 81.1% of oocytes to form pronuclei. Similar results were obtained when the physiological PKC activator diC8 was used instead of PMA. The effective dosage of diC8 was 25 μM, which triggered 30.8% of MII oocytes to form pronuclei, and its maximal dosage was 100 μM, which resulted in activation of 88.5% of oocytes. Both PMA and diC8 dephosphorylated MAP kinase in correlation with pronucleus formation. Dephosphorylation of MAP kinase occurred when about 30%–40% of oocytes were activated by PKC activators. MAP protein kinase was almost completely inactivated when more than 80% of oocytes formed pronuclei (Fig. 3A, top panel). Neither PMA nor diC8 had any effect on MAP kinase expression (Fig. 3A, bottom panel). To confirm whether PKC-induced dephosphorylation of MAP kinase is temporarily correlated with pronucleus formation, MII oocytes were treated with 1.62 nM PMA or 100 μM diC8 for 8 h. We found that MAP kinase remained phosphorylated in the first 2 h, when there was no pronucleus formation. MAP kinase activity decreased to half at 4 h of treatment, when approximately half the oocytes had pronuclei, and decreased further to a negligible level at 8 h of treatment when about 85% of oocytes formed pronuclei (data not shown).

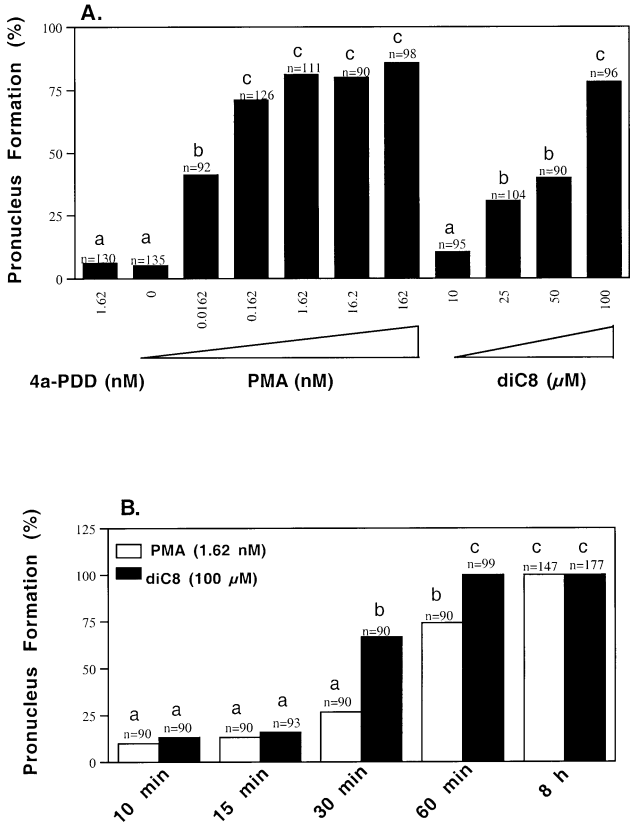

FIG. 2. Effects of PKC activators on pronucleus formation of rat oocytes. **A**) MII oocytes were treated with different concentrations of PMA and diC8 for 8 h, and then pronucleus formation was evaluated. MII oocytes treated with 4α-PDD or drug-free medium for 8 h were designated as controls. **B**) MII oocytes were treated with 1.62 nM PMA or 100 μM diC8 for 10, 15, 30, and 60 min, and after washing were transferred to a drug-free medium and cultured for 8 h. Pronucleus formation was judged after 8 h of culture. MII oocytes cultured in PMA or diC8 for 8 h were adopted as controls.

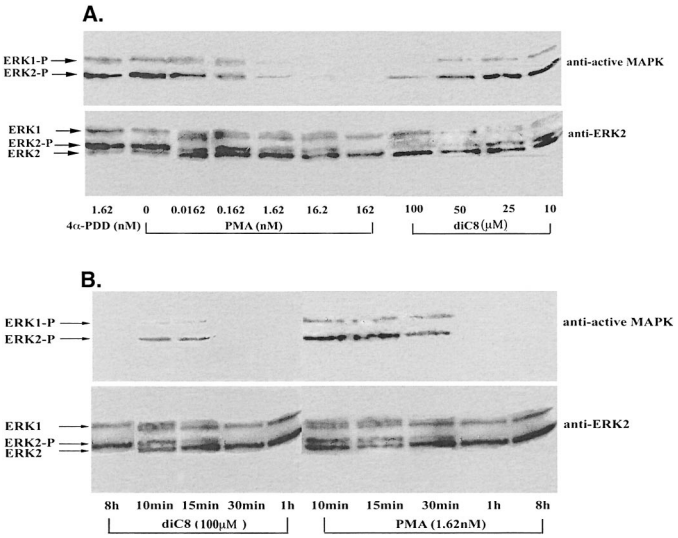

FIG. 3. Effects of PKC activators on MAP kinase dephosphorylation. **A**) Lane 1: MII oocytes treated with 1.62 nM 4α-PDD for 8 h; lane 2: MII oocytes cultured in drug-free medium for 8 h; lanes 3–7: MII oocytes treated with different concentrations of PMA for 8 h; lanes 8–11: MII oocytes treated with different concentrations of diC8 for 8 h. Samples for immunoblotting were collected after 8 h of treatment. **B**) MII oocytes were treated with 1.62 nM PMA or 100 μM diC8 for 10, 15, 30, or 60 min, and then transferred to a drug-free medium and incubated for 8 h. Samples for immunoblotting were collected at 8 h after treatment. MII oocytes treated with 1.62 nM PMA or 100 μM diC8 for 8 h were designated as controls.

Effects of Treatment Time of PMA and diC8 on MAP Kinase Dephosphorylation and Pronucleus Formation

As shown in Figure 2B, pronuclei were formed in 74.2% of oocytes treated for 1 h with 1.62 nM PMA. Treatment for 30 min with 100 μM diC8 induced 66.7% of oocytes to form pronuclei. In other words, 1 h of treatment with 1.62 nM PMA or 30 min of treatment with 100 μM diC8 was required to achieve pronucleus formation. The kinetics

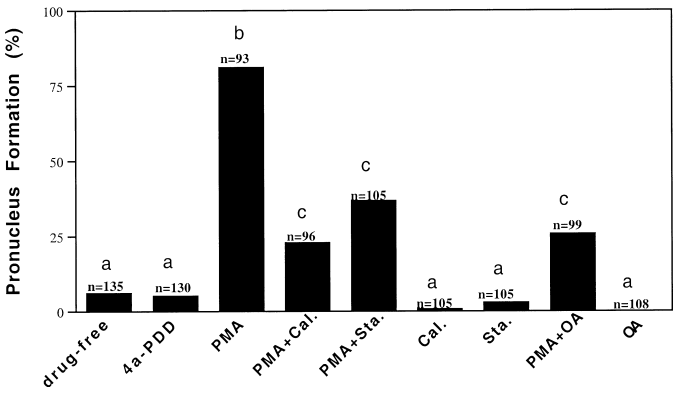

FIG. 4. Effects of PKC activation and protein phosphatases on pronucleus formation. MII oocytes were treated with PMA (1.62 nM) for 1 h, and then transferred to a medium containing calphostin C (1 μM), staurosporine (30 nM), or OA (2 μM) and cultured for 8 h. MII oocytes pretreated with PMA (1.62 nM) for 1 h and then transferred to a drug-free medium and cultured for 8 h were designated as controls. MII oocytes treated with calphostin C, staurosporine, OA, 4α-PDD alone, or drug-free medium for 8 h also served as controls.

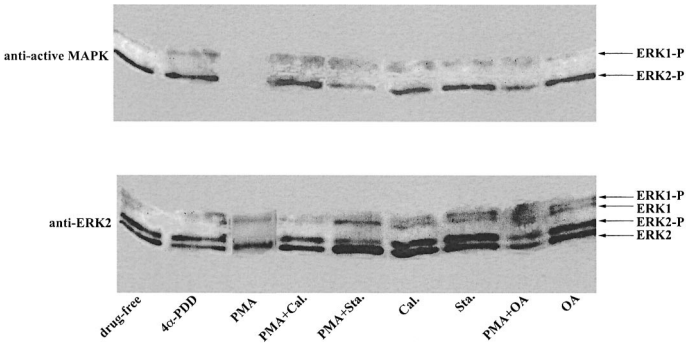

FIG. 5. Changes in MAP kinase phosphorylation induced by PKC modulators and protein phosphatase inhibitor. Lanes 1 and 2: controls (MII oocytes cultured in drug-free medium or treated with 1.62 nM 4α-PDD for 8 h); lane 3: MII oocytes pretreated with 1.62 nM PMA for 1 h, then transferred to a drug-free medium and cultured for 8 h; lanes 4, 5, and 8: MII oocytes pretreated with 1.62 nM PMA for 1 h and then transferred to a medium containing 1 μM calphostin C, 30 nM staurosporine, or 2 μM OA, and cultured for 8 h; lanes 6, 7, and 9: MII oocytes treated with 1 μM calphostin C, 30 nM staurosporine, or 2 μM OA alone, respectively, for 8 h. All samples for detecting MAP kinase were collected at 8 h of treatment.

of MAP kinase dephosphorylation were well correlated with interphase entry. At least 1 h of treatment with 1.62 nM PMA was required to completely dephosphorylate MAP kinase. Treatment of oocytes with 100 μM diC8 for less than 30 min did not dephosphorylate MAP kinase (Fig. 3B, top panel). Expression of MAP kinase did not change during treatments (Fig. 3B, bottom panel).

Effects of PKC Activators on MAP Kinase Dephosphorylation and Pronucleus Formation Were Reversed by PKC Inhibitors

To test whether MAP kinase inactivation and pronucleus formation induced by PMA were mediated by PKC activation, MII oocytes were pretreated with 1.62 nM PMA for 1 h, and then transferred to a medium containing 1 μM calphostin C or 30 nM staurosporine, and cultured for 8 h. We found that these media reversed pronucleus formation induced by PMA. The less-specific PKC inhibitor, staurosporine, was less effective in reversing the effect of PKC activator on oocyte activation than the specific PKC inhibitor, calphostin C (Fig. 4). It is interesting that dephosphorylation of MAP kinase by the treatment of PMA was also blocked by calphostin C and staurosporine. Pronucleus formation in oocytes was consistent with MAP kinase dephosphorylation in each treatment. PKC inhibitors alone neither activated oocytes nor dephosphorylated MAP kinase (Fig. 5, top panel). Similar results were achieved when diC8 was used instead of PMA (data not shown).

Effects of PKC Activators on MAP Kinase Dephosphorylation and Pronucleus Formation Were Abrogated by OA

To investigate the possible involvement of OA-sensitive protein phosphases in the regulation of MAP kinase dephosphorylation caused by PKC activation, MII oocytes were treated with 1.62 nM PMA for 1 h, then transferred to a medium containing 2 μM OA, and incubated for 8 h. Both pronucleus formation and MAP kinase dephosphorylation induced by PMA were decreased by OA (Figs. 4 and 5). Similar results were obtained when diC8 was used instead of PMA (data not shown).

Effects of PKC Inhibitors on Fertilization In Vitro

In IVF, pronucleus formation was significantly decreased when calphostin C or staurosporine were added (Fig. 6). Correspondingly, MAP kinase phosphorylation was increased by adding these PKC inhibitors (Fig. 7). The degree of MAP kinase inactivation was consistent with the proportion of oocytes with pronuclei.

DISCUSSION

Oocyte activation and fertilization is a complex process. PKC has been suggested to activate mouse oocytes [7–11, 29]. However, a negligible effect by PKC in fertilization and parthenogenetic activation also have been reported in oocytes of various species [12–15]. Recently, different isoenzymes of PKC have been found in mouse and rat oocytes [30–32]. In this study, we found that the PKC activators, PMA and diC8, induced pronucleus formation in a dose-dependent and time-dependent manner, suggesting that activation of PKC may be required for rat oocyte activation. Our previous study has shown that a high concentration of the protein kinase inhibitor staurosporine (2 μM) parthe-

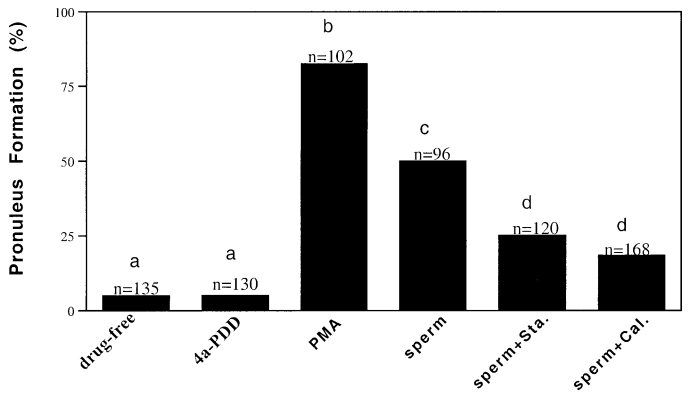

FIG. 6. Effects of PKC modulators on pronucleus formation during in vitro fertilization. MII oocytes were inseminated by spermatozoa alone or in the presence of 30 nM staurosporine or 1 μM calphostin C for 8 h. MII oocytes cultured in a drug-free medium or treated with 1.62 nM 4α-PDD or 1.62 nM PMA for 8 h were used as controls.

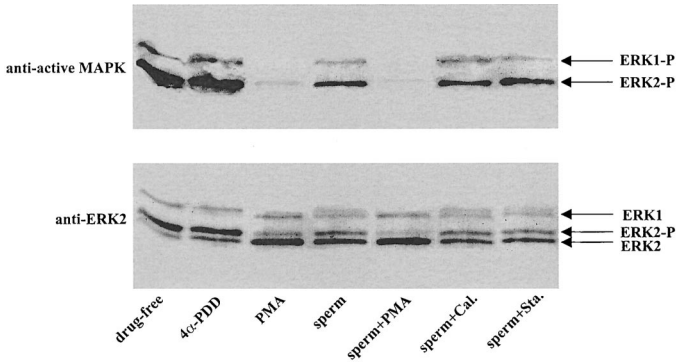

FIG. 7. Effects of PKC modulators on MAP kinase activity during in vitro fertilization. Lanes 1–3: controls (MII oocytes cultured in a drug-free medium or treated with 1.62 nM 4α-PDD or 1.62 nM PMA for 8 h); lane 4: MII oocytes inseminated by spermatozoa and cultured for 8 h; lanes 5–7: MII oocytes inseminated by spermatozoa for 8 h in the presence of 1.62 nM PMA, 30 nM staurosporine, or 1 μM calphostin C, respectively. Samples for detecting MAP kinase were collected at 8 h.

nogenetically activated mouse oocytes and caused pronuclear formation without emitting the second polar body. However, low-dosage staurosporine (30 nM), which normally inhibited PKC, did not show any effect on oocyte activation [24]. In this study we found that 30 nM staurosporine blocked the effect of PMA and diC8 on pronucleus formation. In order to confirm this result, a specific PKC inhibitor, calphostin C at 1 μM, was used instead of 30 nM staurosporine. We found that the effects of PKC activators were also abolished by this concentration of calphostin C. Staurosporine at 30 nM and calphostin C at 1 μM alone affected neither oocyte viability nor oocyte activation. These results suggest that PMA and diC8 trigger oocyte activation by activating PKC. In this study we found that both PKC inhibitors attenuated pronucleus formation during fertilization, indicating that PKC activation may be involved in fertilization.

MAP kinase exists in a phosphorylated form in mammalian MII oocytes [33–38]. Inactivation of MAP kinase causes oocytes to undergo spontaneous parthenogenesis rather than arrest at MII stage [39, 40]. MAP kinase has been suggested as being involved in pronuclear envelope assembly and disassembly [22, 23]. Our previous studies have also shown that pronucleus formation was correlated with MAP kinase dephosphorylation in oocytes of mice, pigs, and humans [24–27]. In the present study, in order to investigate whether fertilization triggers MAP kinase dephosphorylation, and if so, whether inactivation of MAP kinase is correlated with pronucleus formation in rat oocytes, cumulus-free and zona pellucida-free MII oocytes were inseminated with spermatozoa that had been capacitated for 3 h. The reason we chose zona pellucida-free instead of regular oocytes is that zona pellucida-enclosed oocytes are difficult to fertilize in our IVF system. Removing the zona pellucida increases the rate of polyspermic fertilization. This problem was solved by increasing the time for sperm capacitation, but long-time capacitation led to a lower fertilization rate. Three-hour capacitation reduced the rate of polyspermic fertilization to less than 5% and resulted in enough normal fertilized oocytes (more than 50%) for study. In this IVF system, we found that MAP kinase remained active 4 h after insemination, and was partially dephosphorylated at 6 h after insemination, when some oocytes formed pronuclei. MAP kinase was almost completely inactivated at 8 h, when most of the oocytes had pronuclei. These data suggest that, similar to other species, fertilization causes MAP kinase dephosphorylation, and that dephosphorylation of MAP kinase may be required for pronucleus formation in rat oocytes.

Because both PKC activation and MAP kinase inactivation are correlated with pronucleus formation during fertilization, the question is whether MAP kinase activity is regulated by PKC. Our previous studies have shown that MAP kinase activity is down-regulated by PKC activation during oocyte maturation and activation in mice as well as during oocyte maturation in rats [28, 41]. In this study, we found that MAP kinase was dephosphorylated by both PMA and diC8 in a dose-dependent and time-dependent manner. Also, MAP kinase dephosphorylation was consistent with pronucleus formation when oocytes were treated with PKC activators. This effect of PKC activators was overcome by PKC inhibitors. These data suggest that PKC activation may induce pronucleus formation via a cascade that dephosphorylates MAP kinase in rat oocytes. Importantly, in IVF, PKC inhibitors attenuated MAP kinase dephosphorylation and pronucleus formation was triggered by spermatozoa, which suggests that PKC activation and the resulting MAP kinase inactivation may be required for pronucleus formation during fertilization. Thus the question is: How does PKC activation induce dephosphorylation of MAP kinase? Active MAP kinase and its activator MEK are dephosphorylated and inactivated by both threonine/tyrosine-specific and tyrosine-specific protein phosphatases in mammalian cells [42]. Serine/threonine protein phosphatase (PP-2A) has also been suggested to dephosphorylate and inactivate MEK and MAP kinase [43–45]. Our recent studies have shown that PP2A is present in mouse MII oocytes [46] and that protein phosphatases are pivotal regulators of MAP kinase phosphorylation in pig oocytes [47]. Inhibition of protein phosphatases may directly cause activation of MAP kinase or some other molecules upstream of MAP kinase in the cascade, such as MEK [47]. In this study, we found that the effects of PKC activation on MAP kinase dephosphorylation was abrogated by OA, a protein phosphatase-1 and PP2A inhibitor, indicating that PKC may activate PP2A, resulting in dephosphorylation of MAP kinase.

In summary, PKC activation may cause pronucleus formation via MAP kinase dephosphorylation, which is probably mediated by OA-sensitive protein phosphatases in rat oocytes, and such a process may be involved in fertilization.

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