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Translocation of the Classic Protein Kinase C Isoforms in Porcine Oocytes: Implications of Protein Kinase C Involvement in the Regulation of Nuclear Activity and Cortical Granule Exocytosis

Heng-Yu Fan,* Chao Tong,* Man-Yu Li,* Li Lian,* Da-Yuan Chen,* Heide Schatten,† and Qing-Yuan Sun*[,1]

**State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China; and †Department of Veterinary Pathobiology, University of Missouri—Columbia, Columbia, Missouri 65211*

Protein kinase C (PKC) is a family of Ser/Thr protein kinases categorized into three subfamilies: classical, novel, and atypical. The subcellular localization of classical PKCα, -βI, and -γ in the process of porcine oocyte maturation, fertilization, and parthenogenetic activation and their involvement in cortical granule (CG) exocytosis were investigated. The results of Western blot showed that PKCα, -βI, and -γ were expressed in the oocytes at the germinal vesicle (GV) and metaphase II (MII) stages. Confocal microscopy revealed that the three PKC isoforms were concentrated in the GV but evenly distributed in the cytoplasm of MII eggs. PKCα and -γ were translocated to the plasma membrane soon after sperm penetration. cPKCs migrated into the pronucleus in fertilized eggs. Following treatment with a PKC activator, phorbol 12-myristate 13-acetate (PMA), CGs were released and PKCα and -γ were translocated to the membrane. The CG exocytosis and PKC redistribution induced by PMA could be blocked by the PKC inhibitor staurosporine. Parthenogenetic stimulation with ionophore A23187 or electrical pulse also induced cPKC translocation and CG exocytosis. Eggs injected with PKCα isoform-specific antibody failed to undergo CG exocytosis after PMA treatment or fertilization. The results suggest that cPKCs, especially the α-isotype, regulate nuclear function and CG exocytosis in porcine eggs.
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Key Words: **kinases; signal transduction; oocyte development; *in vitro* fertilization.**

INTRODUCTION

Mammalian oocytes are arrested at the G2–M phase transition of the first meiotic division. Fully grown oocytes liberated from their follicles spontaneously reinitiate meiosis I *in vitro,* characterized by germinal vesicle breakdown (GVBD), chromatin condensation, spindle assembly, emission of the first polar body, and progression to the metaphase of the second meiotic division (MII), at which stage they undergo a second arrest until fertilization. In mammals, early fertilization events include a rise in intracellular calcium [1], which in turn stimulates the exocytosis of egg cortical granules (CGs) [2, 3, 4], the enzymes of which are involved in the zona pellucida (ZP) block to polyspermy.

The CGs, a specialized group of membrane-bound secretory granules composed of specialized enzymes and glycoproteins, are located mostly in the cortex of unpenetrated oocytes. Calcium oscillation leads to the release of CGs in mouse oocytes [5]. Intracellular calcium appears to be sufficient to induce CG exocytosis, since the calcium ionophore, A23187, stimulates CG release in murine and porcine eggs in a calcium-free medium [6, 7]. In mammalian eggs, the principal messenger responsible for the increase in intracellular calcium is inositol 1,4,5-trisphosphate (IP_3) [8], but the mechanisms by which the fertilization-associated elevation of calcium stimulates exocytosis is unknown.

Protein kinase C (PKC), a multigene family of serine/threonine kinase, is one of the transducers of the Ca^{2+} and IP_3 signal system. The PKC family comprises at least 11 different isoforms and they can be divided into three major groups: classical (cPKCα, -βI, -βII, and -γ), novel (nPKCδ, -ε, -η, -θ, and -μ), and atypical (aPKCι/-λ, and ξ) [9, 10]. Classical PKCs and novel PKCs translocate from the cytosol to membranous sites upon activation with diacyl glycerol (DAG), whereas cPKCs are dependent on Ca^{2+} for activation [11]. The activity of atypical PKCs is regulated by some binding proteins, instead of DAG or Ca^{2+} [12].

Expression of individual PKC isoforms is cell-type specific and developmentally regulated. Raz *et al.* [13] detected eight PKC isoforms in rat eggs. Recent studies have shown that PKC isoforms exist in mouse oocytes and include cPKCα, -βI, -βII [14], nPKCδ, and aPKCλ, and -ξ [15]. PKCs have been shown to participate in both cell-cycle regulation and CG exocytosis in rodent oocytes. Our recent work showed that PKC activators inhibit GVBD in denuded mouse and rat oocytes by preventing the phosphorylation of mitogen-activated

[1] To whom correspondence and reprint requests should be addressed. Fax: 8610-6256-5689. E-mail: sunqy1@yahoo.com.

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protein kinase (MAPK) [16, 17]. In addition, a recent report showed that PKC might participate in the meiosis I-to-meiosis II transition in mouse oocytes [18]. PKC activity increased with meiotic maturation and reached its peak at late MI stage. PKCδ was localized to the meiotic spindle during MI/MII transition and then to the chromosomes at MII [18]. Treatment of mouse and hamster eggs with activators of PKC can mimic certain events of egg activation, including CG exocytosis. Moreover, physiological activation of MII eggs by sperm-induced cortical granule exocytosis was associated with significant translocation of cPKCα and -βI, but not -βII, to the plasma membrane in mouse eggs [14]. However, accumulating information suggests that rodents may be atypical with regard to regulating mechanisms of oocyte maturation and fertilization. The mechanisms for fertilization in domestic animals such as pig and cattle may be more similar to those in low vertebrates than those in the mouse.

We previously [19] reported that PKC activators, 1-oleoyl-2-acetyl-glycerol (OAG) or phorbol 12-myristate 13-acetate (PMA), induced CG release in porcine eggs, and this effect is Ca^{2+}-independent and can be overcome by PKC inhibitors, indicating that the induction of PKC on CG release is downstream to [Ca^{2+}] rise. Our present study, by using Western blot, confocal microscopy and antibody microinjection, for the first time investigated in pig oocytes: (1) the existence of classical PKC isoforms, (2) their translocation during oocyte maturation and fertilization, and (3) their involvement in CG exocytosis induced by parthenogenetic stimulations or fertilization.

MATERIALS AND METHODS

In vitro maturation and fertilization of oocytes. Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 1.5 h in 0.9% NaCl solution containing 75 μg penicillin G/ml and 50 μg streptomycin/ml at 37°C. Oocytes were aspirated from antral follicles (~2–6 mm in diameter) with an 18-gauge needle fixed to a 20-ml disposable syringe. After washing three times with maturation medium (see below), oocytes possessing a compact cumulus and evenly granulated ooplasm were selected for maturation culture. The medium used for maturation culture was improved 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 sulfate, 10 lU/ml pregnant mare serum gonadotropin, 10 IU/ml hCG, and 10% (v/v) heat-inactivated fetal calf serum (FCS). A group of 45–50 oocytes was cultured in a 500- μl drop of maturation medium for 46 h at 38.5°C in an atmosphere of 5% CO_2 and saturated humidity.

After maturation culture, oocytes were freed of cumulus cells by treatment with 300 IU/ml hyaluronidase (Sigma) and repeated pipetting. The denuded eggs were then washed twice in TCM-199 and were used for either *in vitro* fertilization (IVF) or drug treatment. The IVF was carried out by the method reported previously [20]. Oocytes were inseminated in a 100-μl drop of modified Tris-buffered medium (mTBM) containing 0.4% bovine serum albumin (BSA) (A7888, Sigma) and 2.5 mM caffeine with freshly ejaculated spermatozoa (1 × 10^6 cells/ml) that were previously incubated for 2 h in the same medium. Six hours after insemination, eggs were removed from the fertilization drop and cultured in 500 μl North Carolina State University- (NCSU) 23 medium containing 4 mg/ml BSA (A8022, Sigma) for 12 h.

In another experiment, zona-free eggs and noncapacitated spermatozoa were used to study the relationship between PKC translocation and sperm binding. Zona pellucida was removed by a short exposure to acidified Tyrode's medium (pH 2.5). After washing in DPBS, spermatozoa were added to the fertilization drops. Eggs were collected for immunocytochemistry 30 min after sperm binding.

Electrical activation of eggs. The method used for egg activation by an electrical pulse was essentially the same as that reported by Onishi *et al.* [21]. Briefly, after washing three times in the electroportion medium [0.28 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) BSA], cumulus-free eggs were put in a fusion chamber. An 80 μs pulse at 120 V/mm DC was exerted to eggs. The eggs were then washed three times and cultured in NCSU-23 medium containing 0.04% BSA for 15 min before fixation.

Drug treatment of eggs. All the chemicals used in this experiment were purchased from Sigma except for those specially mentioned. Drugs were prepared as stock solutions by dissolution in dimethylsulfoxide (DMSO) and stored in a dark box at −20°C. The stock solutions were diluted with TCM-199 prior to use.

To study the relationship between cPKCs activation and CG exocytosis, MII eggs were treated with 1.62 μM PMA for 15 min and then the translocation of cPKCs and release of CGs were evaluated by confocal microscopy (see below). As the control, some eggs were treated with 1.62 μM 4 α-PDD, a biologically inactive phobal ester. In another experiment, eggs were pretreated with 2 μM staurosporine (an efficient PKC inhibitor) for 15 min and then incubated in 1.62 μM PMA +2 μM staurosporine for 15 min. The Ca^{2+} ionophore A23187 was used to elevate the intracellular [Ca^{2+}] and induce CG exocytosis at a concentration of 10 μM.

Microinjection of antibodies. To determine the role of each cPKC isoform in CG exocytosis, microinjection of antibodies against PKCα, -βI, and -γ was performed as described by Dai *et al.* [22]. An Eppendoff microinjector was used in this experiment. All microinjection into the cytoplasm was performed by using a beveled micropipette to minimize damage. A microinjection volume of about 7 pl per egg was used in all the experiments. Each experiment consisted of three separate replicate groups and approximately 50 eggs were injected altogether. Rabbit IgG was injected as control. After microinjection, eggs were washed three times with TCM-199 and recovered in the same medium for 1 h. Then the injected eggs were fertilized or treated with PMA as mentioned above.

SDS–PAGE and Western blot analysis. A total of 100 oocytes at the GV stage or MII stage were collected in SDS sample buffer and heated to 100°C for 4 min. After cooling on ice and centrifuging at 12,000*g* for 4 min. samples were frozen at −70°C until use. The total proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 4% stacking gel and a 10% separating gel for 2.5 h at 120 V and then electrophoretically transferred onto nitrocellulose membrane for 2 h at 200 mA at 4°C. After blocking for 1 h in TBST buffer [20 mM Tris, 137 mM NaCl, 0.1% Tween 20 (pH 7.4)] containing 1% low-fat milk, the membrane was incubated overnight at 4°C in TBST containing 1:300 polyclonal isotype-specific rabbit anti-mouse PKC antibodies (Santa Cruz Biotechnology, Santa Cruz, CN). After three washes of 10 min each in TBST, the membrane was incubated for 1 h at 37°C with horseradish-conjugated peroxidase labeled goat anti-rabbit IgG diluted 1:1000 in TBST. The membrane was washed three times in TBST and then processed using the enhanced chemiluminescence (ECL) detection system. Specificity was confirmed by preincubating the antibodies with their blocking peptide before immunoblotting. All experiments were repeated at least three times.

Confocal microscopy of cPKCs. After removing the zona pellucida 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 5 min (RT),

followed by blocking in 1% BSA for 1 h and incubation overnight at 4°C with rabbit anti-mouse PKCα, -βI, or -γ antibody diluted 1:100 in blocking solution. After three washes in PBS containing 0.1% BSA for 5 min each, the eggs were labeled with FITC-conjugated goat anti-rabbit IgG diluted 1:300. Nuclear status of oocytes was evaluated by staining with 10 μg/ml 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 three times and at least 15 oocytes were examined each time. Cells were observed under a Leica confocal laser scanning microscope (TCS-4D) on the same day. The translocation of cPKCs were determined by the intensity of fluorescence using Leica TCS-NT image-analysis software connected to the confocal microscope.

Labeling of cortical granules. After the removal of zona pellucida, fixation and permeabilization as described above, the eggs were washed three times in PBS containing 3 mg/ml BSA and 100 mM glycine (blocking solution) at 5-min intervals, followed by incubation in 100 μg/ml FITC-conjugated peanut agglutinin (FITC-PNA) in blocking solution for 1 h at RT in a dark box. Then the eggs were washed three times with PBS containing 0.01% Triton X-100, mounted, and observed as described above.

RESULTS

Identification of cPKC Isoforms during Oocyte Development

As shown in Fig. 1, PKCα, -βI, and -γ proteins were detected in GV oocytes and MII eggs through SDS–PAGE and Western blot analysis. An approximately 80-kDa band was labeled with PKC isoform-specific antibodies. The specificity of the antibody was demonstrated by its preabsorption with the specific blocking peptide, which completely abolished immunodetection of the 80-kDa protein band (data not shown).

In GV oocytes freshly released from the growing follicles, PKCα, -βI, and -γ were localized to the entire germinal vesicles except the nucleolus, with a very weak expression in the cytoplasm (Figs. 2A–2C). In these oocytes, a prominent nucleolus surrounded by chromatin can be observed in the GV. Less than 10% of oocytes failed to undergo GVBD after maturation culture for 44 h. In these oocytes, the chromatin lost its configuration of surrounding the nucleolus and formed a condensed network in the center of the GV. Accompanying this change, PKCα and -βI were concentrated to the condensed DNA (Fig. 3), while the distribution of PKCγ remain unchanged in the GV (data not shown). In MII eggs, PKCα, -βI, and -γ were distributed evenly in the ooplasm (Figs. 2D–2F). No staining was seen in the negative control experiment in which the cPKC antibodies were preincubated with their blocking peptide before immunoblotting (data not shown).

Migration of PKC Isoforms and Cortical Granule Exocytosis after Fertilization

Sperm penetration was first observed 3 h following insemination and was largely completed by ~5–6 h.

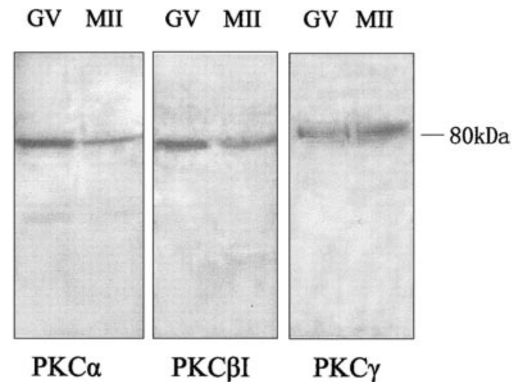


FIG. 1. Detection of cPKC isoforms in pig oocytes and eggs. Proteins were extracted with sample buffer, separated with SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted by PKC isoform-specific antibodies. Protein blots show the presence of PKCα, -βI, and -γ in GV oocytes and MII eggs. Each lane contains total protein extract from 100 oocytes or eggs.

One or two pronuclei (PN) could be seen 18 h after insemination. PKCα was translocated to the periphery of the fertilized eggs ~5–6 h following insemination, shortly after sperm penetration (Fig. 2G). However, PKCα could not be induced to translocate by only binding of noncapacitated sperm (Fig. 4). The distribution of PKCβI and -γ remained unchanged ~5–6 h after insemination (Figs. 2H and 2I). With PN formation, PKCα and -γ were concentrated in the PN except for the nucleolar region, with low levels of expression in the cytoplasm of the zygotes (Figs. 2J and 2L). The concentration of PKCβI in the PN is only slightly higher than in the cytoplasm (Fig. 2K).

In intact M II eggs, the FITC-PNA-labeled CGs formed a monolayer next to the plasma membrane (Fig. 5A). Following fertilization, the eggs penetrated by the sperm released their CGs, and large fluorescent clusters were observed around the eggs (Figs. 5B and 5C).

Effects of Drug Treatment and Electric Activation on Translocation of cPKC Isoforms and Cortical Granule Exocytosis

After exposing MII eggs to 1.62 μM PMA for 15 min, PKCα and -γ translocated to the periphery of the cell (Figs. 6A and 6C), but the even distribution of PKCβI remained unchanged (Fig. 6B). Following PMA treatment, all eggs released their CGs completely or partially (Fig. 6D). Some of the PMA-treated eggs were removed and cultured in TCM-199 for an additional 2 h. PKCα and -γ redistributed to the cytoplasm in these eggs (data not shown). The biologically inactive PMA analog 4α-PDD could neither induce the PKC translocation nor the CG exocytosis at the same concentration of PMA (Figs. 5E–5H). After preincubation

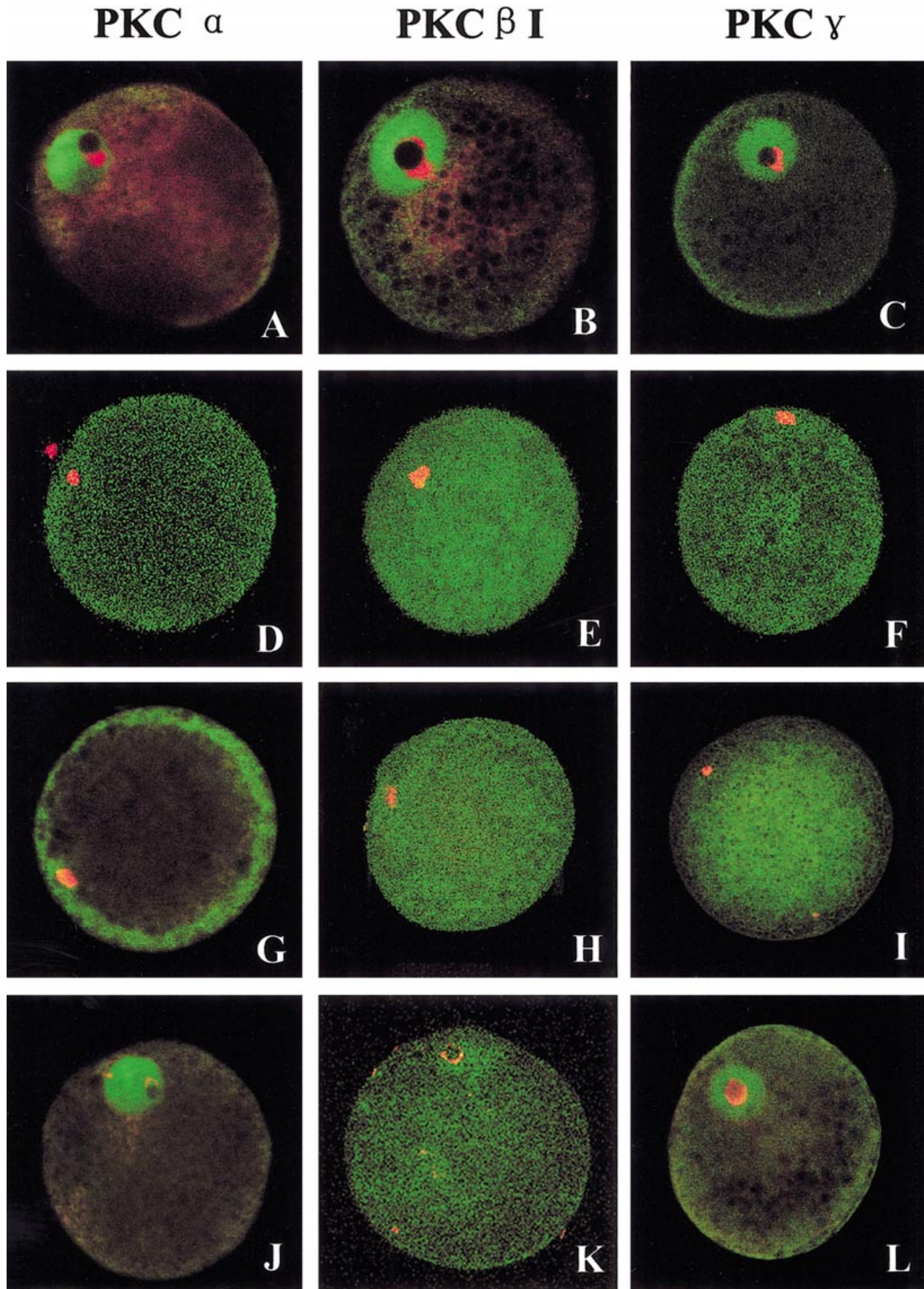
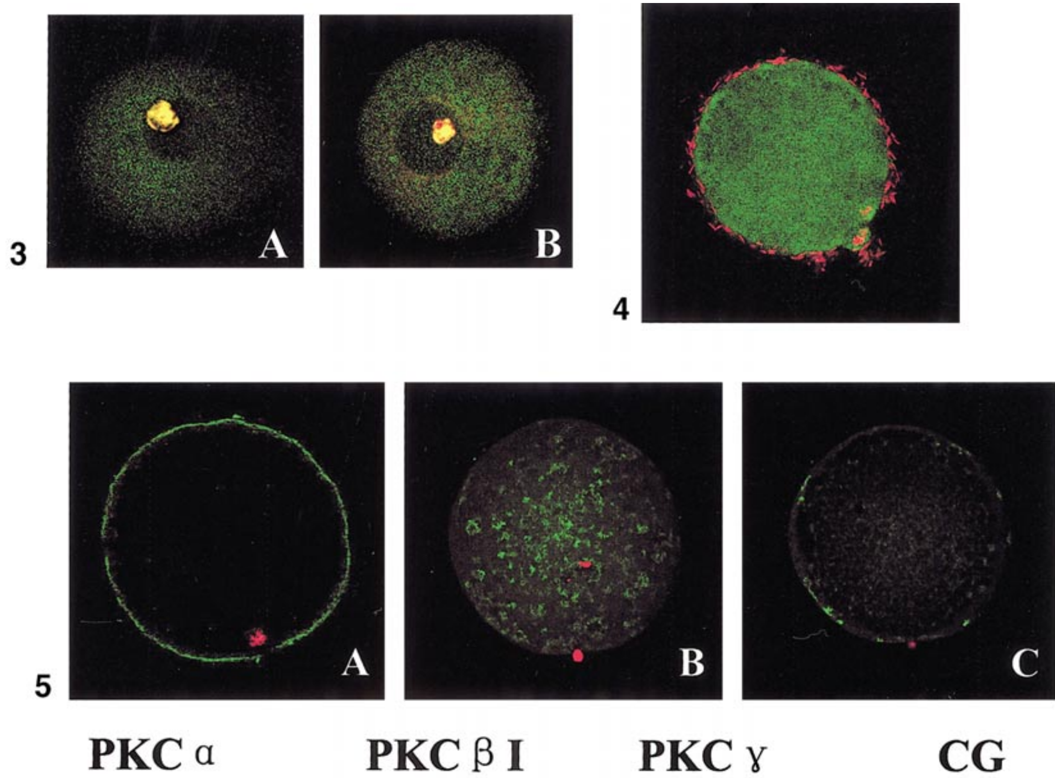


FIG. 2. The localization of cPKC isoforms during oocyte maturation and fertilization. (Green) PKC; (red) chromatin; (yellow) overlapping of green and red. PKCα (A), -βI (B), and -γ (C) were localized to the GV of the oocytes, with a weak expression in the cytoplasm. PKCα, -βI, and -γ were detected in the entire GV except the nucleolus. In MII eggs, PKCα (D), -βI (E), and -γ (F) were distributed evenly in the ooplasm. PKCα translocated to the periphery of the fertilized eggs 5–6 h following insemination (G), while the distribution of PKCβI (H) and -γ (I) remained unchanged. With pronucleus (PN) formation, PKCα (J) and -γ (L) were concentrated in the PN except for the nucleolar region, with low levels of expression in the cytoplasm. The concentration of PKCβI (K) in the PN is only a little higher than in the cytoplasm.



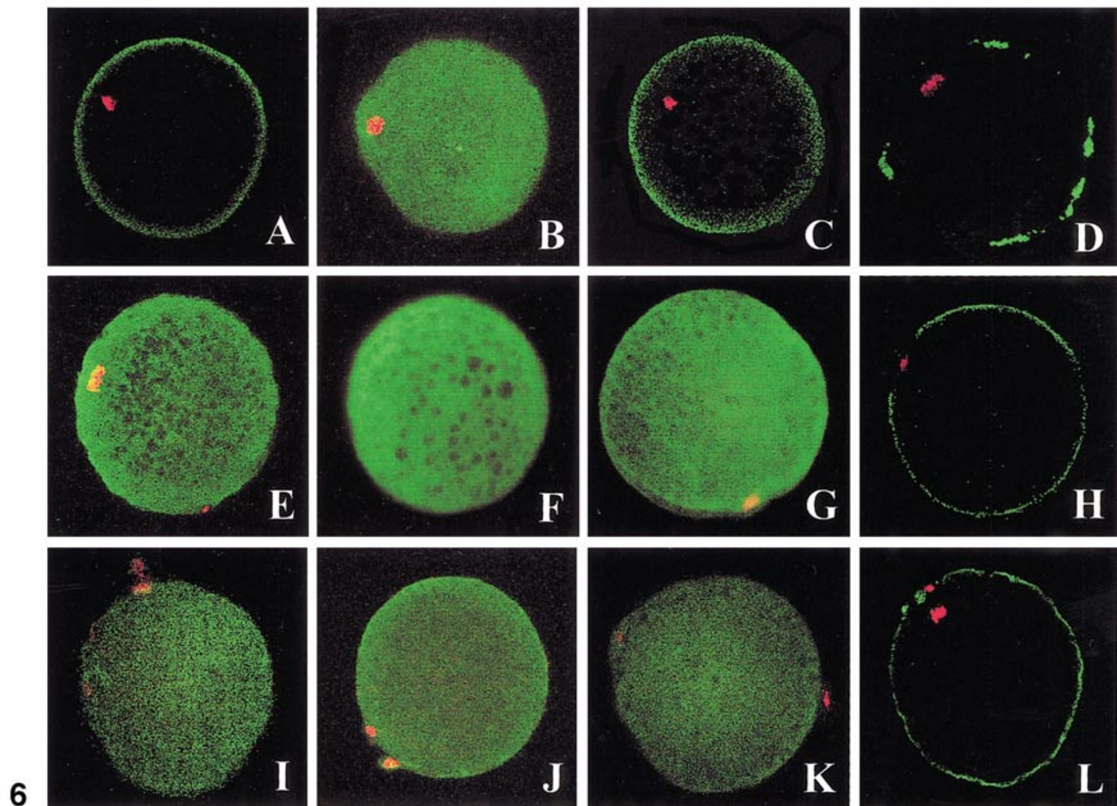


FIG. 3. Localization of PKCα (A) and -βI (B) in oocytes that failed to undergo GVBD 44 h after maturation culture. Both isoforms were localized to the condensed chromatin in the nucleus.

FIG. 4. Zona-free eggs were inseminated with incapacitated spermatozoa for 30 min, and PKCα was detected with confocal microscopy. Though the spermatozoa bound to the egg membrane, PKCα translocation was not induced.

with the PKC inhibitor staurosporine, both the PKC isoform translocation and the CG release induced by PMA were blocked (Figs. 6I–6L).

Since the intracellular Ca^{2+} elevation is necessary for the release of CGs, we treated the eggs with the calcium ionophore A23187 for 30 min as reported by Wang *et al.* [7]. The CGs were released as expected, and at the same time, PKCα evidently translocated to the membrane. Some of the PKCβI and -γ migrated to the periphery of the cells, resulting in higher fluorescent labeling of the outer cytoplasm than the central cytoplasm (Figs. 7A–7D). Eggs stimulated by a single electrical pulse also released their CGs. PKCα, -βI, and -γ were localized to the cortex of the eggs 30 min after stimulation, while there was still some PKCγ that remained at the central part of the cells (Figs. 7A–7D).

Cortical Granule Exocytosis after Fertilization or PMA Treatment of Eggs with Microinjected PKC Antibodies

All of the eggs (30/30 examined) injected with rabbit IgG were induced to release their CGs by PMA treatment, suggesting that little damage was inflicted during microinjection. None of the eggs (34/34) injected with PKCα antibody release their CGs after PMA exposure (Fig. 8A). Most eggs injected with PKCβI (27/33) or -γ (24/34) antibodies released their CGs after PMA treatment (Figs. 8B and 8C). Some eggs injected with PKCγ antibody showed an interesting pattern of CG release: Instead of release at the surface of the cells, most CG contents aggregated beneath the plasma membrane. There were also some unreleased CGs remaining at the cortical region (Fig. 8D).

The effects of PKCα on CG release during fertilization were further studied. CG release was observed in 22.73% (10/44) of the eggs injected with PKCα antibody 6 h after insemination (Fig. 8E), whereas 71.15% (37/52) of the oocytes injected with rabbit IgG release their CGs at the same time (Fig. 8F). Twenty hours after insemination, all of the eggs with PN formation released their CGs, both in the experimental group (38/38, Fig. 8G) and in the control group (46/46, Fig. 8H).

DISCUSSION

Protein kinase C isozymes are distributed differentially throughout the cell, and numerous targeting proteins have been described [23]. In secretory cells, PKC is involved in granule exocytosis and may act by a hypothetical protein [24, 25].

The expression of cPKCα, -βI, and -γ proteins in pig oocytes and eggs was detected through Western blot analysis. The profiles of PKC isozymes were previously investigated in mouse and rat eggs. The presence of eight PKC isoforms (cPKCα, -βI, and -γ; nPKCδ, -ε, and -μ; and aPKCξ and -λ) was shown in rat eggs by Western blot [13]. cPKCα, -βI, and -βII; nPKCδ; and aPKCλ and -ξ existed in mouse eggs [14, 15]. Our results, together with these reports, show that cPKCα and -βI are present in all mammalian eggs examined. Furthermore, we reported for the first time the expression of cPKCγ isoform in mammalian oocytes and eggs. This may reflect the different PKC expression patterns between pigs and rodents, since the expression of cPKCγ has never been detected in rodent oocytes.

In our experiments, cPKC isoforms were found concentrated in the nucleus at the GV stage and dispersed in the MII eggs. The functions of PKCs in the GV are unknown at present, but their compartmentalization during oocyte development may be physiologically important. In mammalian species, immature oocytes obtained from follicles can be penetrated by spermatozoa *in vitro,* but they fail to release their CGs after sperm penetration. We previously [7] reported that the CGs dispersed in the ooplasm at GV stage and migrated to the cortex at the MII stage in pig oocytes. The confinement of PKCs in the GV and the absence of CGs in the cortex may be responsible for the depletion of CGs release in immature oocytes. Accompanying nuclear maturation many cytoplasmic changes, termed cytoplasm maturation, occur. Although cytoplasm maturation is also considered important for normal fertilization and development, the detailed mechanisms are still unclear. The migration of PKCs from the GV to the ooplasm may be one aspect of cytoplasmic maturation.

In our experiments, PKCα and -γ were found to concentrate into the pronuclei after fertilization, suggesting that they may have functions other than the induction of CG exocytosis. It has been shown that PKC activity after fertilization is required for remodeling of the mouse egg into the zygote. In eggs pretreated with PKC pseudosubstrate, pronuclear formation was significantly inhibited [26]. PKCs are regulators of cell proliferation and differentiation in

FIG. 5. Labeling of CGs in MII eggs and zygotes. (Green) CGs; (red) chromatin. In the intact MII eggs, the FITC-PNA-labeled CGs formed a monolayer next to the plasma membrane (A). Following fertilization, the eggs penetrated by the sperm released their CGs. (B) View from the cell surface. (C) Equatorial region.

FIG. 6. Effects of drug treatment on PKC translocation and CG exocytosis. After exposing MII eggs to 1.62 μM PMA for 15 min, PKCα (A) and -γ (C) translocated to the periphery of the cell, but the even distribution of PKCβI (B) remained unchanged. The eggs treated with PMA released their CGs (D). The biologically inactive PMA analog 4 α-PDD could induce neither the translocation of three cPKC isoforms (E, F, and G) nor the CG release (H). PKC inhibitor staurosporine blocked both the translocation of cPKCα (I) and -γ (K) and the CG release (L) induced by PMA. The distribution of cPKCβI remained unchanged after staurosporine (J) treatment.

various cell types [27, 28], and they were found to exist in the nuclei of somatic cells [29]. Thus PKCα and -γ may participate in the PN formation and chromatin reconstruction in pig eggs after fertilization. Another possibility is that they may participate in the initiation of DNA duplication or gene transcription, preparing for the first mitosis.

At the time shortly after sperm penetration (5–6 h after insemination) into zona-intact eggs, PKCα translocated from the cytoplasm to the submembraneous cortex, indicating that it was activated by the stimulation of sperm penetration. However, acrosome intact, noncapacitated sperm binding to the egg membrane did not induce the translocation of PKCα. The acrosome reaction is normally induced by the zona pellucida. In our experiments, when the spermatozoa were washed in DPBS and added to the drops with ZP-free eggs, the sperm bound to the egg surface, but they did not induce PKC translocation. This revealed that the physical binding of sperm to the egg membrane is not enough to activate PKCs. The exposure of certain protein domains on the sperm surface may be necessary for the signaling of PKC activation. There was evidence that a crude extract isolated from boar sperm induced Ca^{2+} release and triggered subsequent early and late activation events upon injection into matured porcine oocytes [30]. Thus, sperm might trigger the Ca^{2+} release and PKC activation in oocytes by introducing a soluble factor into the ooplasm after gamete fusion. Because sperm is the natural inducer of CG exocytosis, PKCα may mediate the signal, resulting in CG release during fertilization.

PMA induced the CG exocytosis and membrane translocation of cPKCα and -γ, but not the βI-isoform. These effects were blocked in the presence of staurosporine, a potent inhibitor of PKC, suggesting that the activation of PKCs is necessary for their translocation and CG release. Calcium ionophore treatment and electrical stimulation were also used in the present study because it has been reported that these treatments could mimic sperm penetration in inducing CG exocytosis [31, 32]. Three cPKC isoforms were translocated to the periphery of the cell after A23187 treatment or electrical stimulation, with the release of CGs. The migration of PKCα was more remarkable than that of PKCβI or -γ, indicating that PKCα is more sensitive to these treatments compared to the other two isoforms. A23187 and electrical pulse could induce CG exocytosis and parthenogenetic activation of pig eggs by releasing the free Ca^{2+} from intracellular stores. We showed here that the translocation of cPKCs occurred with these treatments, and we propose that cPKCs, especially PKCα, may be the mediator of CG exocytosis induced by Ca^{2+} elevation. However, it should be noted that the PKC migration patterns in parthenogenetic stimulation are different from that of normal fertilization. As mentioned above, only PKCα was translocated from the cytoplasm to the membrane at fertilization, while all three cPKC isoforms migrated to the periphery of the cells during parthenogenetic activation. This may be due to the different pattern of Ca^{2+} release in these processes. There is a repetitive calcium release following fertilization termed Ca^{2+} oscillation, compared to a single Ca^{2+} elevation after A32187 treatment or electrical stimulation. PKCβI and -γ may be activated by a prolonged high level of Ca^{2+} rather than the Ca^{2+} oscillation.

Microinjection of cPKC isoform-specific antibodies indicated that PKCα is indispensable for the PMA-induced CG exocytosis, suggesting its crucial roles in the cortical reaction during normal fertilization. However, we should analyze this result with caution, since phorbol ester does not completely mimic CG release in fertilized eggs. TPA treatment results in an atypical pattern of CG release in which there is a greater net loss of CGs in the equatorial region of the egg than in the region opposite the spindle. PKC inhibitor bisindolylmaleimide or chelerytherine blocks the CG release induced by TPA, but not that induced by fertilization [33]. Therefore, whether the physiological induction of CG release can be blocked by the injection of PKCα-specific antibody was further studied. The results indicated that PKCα antibody injection does inhibit the CG exocytosis during a short time after fertilization. However, the CG release could not be blocked perpetually and completely by PKCα antibody injection. All of the injected eggs released their CGs after PN formation, and there were still 22.73% (10/44) of the injected eggs release their CGs 6 h after insemination. These facts suggested that PKCα performs important roles in both fertilization- and PMA-induced CG xocytosis, but redundant mechanisms of CG exocytosis independent of PKCα may also exist in the fertilization of pig eggs. In the case of PKCγ antibody injection, some abnormal patterns of CG release were observed. It is not known if this abnormality is due to the pharmacological effects of PMA treatment. In future research, isoform-specific inhibitors or pseudosubstrates need to be used to test the results obtained with antibody injection.

In conclusion, we for the first time reveal that the classic PKC isoforms PKCα, -βI, and -γ exist in pig oocytes. Their subcellular distribution is differently regulated during oocyte maturation, fertilization, and parthenogenetic activation. Cortical granule exocytosis induced by phorbol ester, ionophore A23187, or electrical pulse occurs with the mobilization of cPKC isoforms. Intracytoplasmic injection of isoform-specific antibodies reveals that PKCα, instead of the βI- and γ-isotypes, may be necessary for fertilization- or PMA-induced CG release. This is the first evidence that a single PKC isoform plays a crucial role in the events at fertilization. Future studies will identify the downstream mediator of PKCα, leading to CG exocytosis

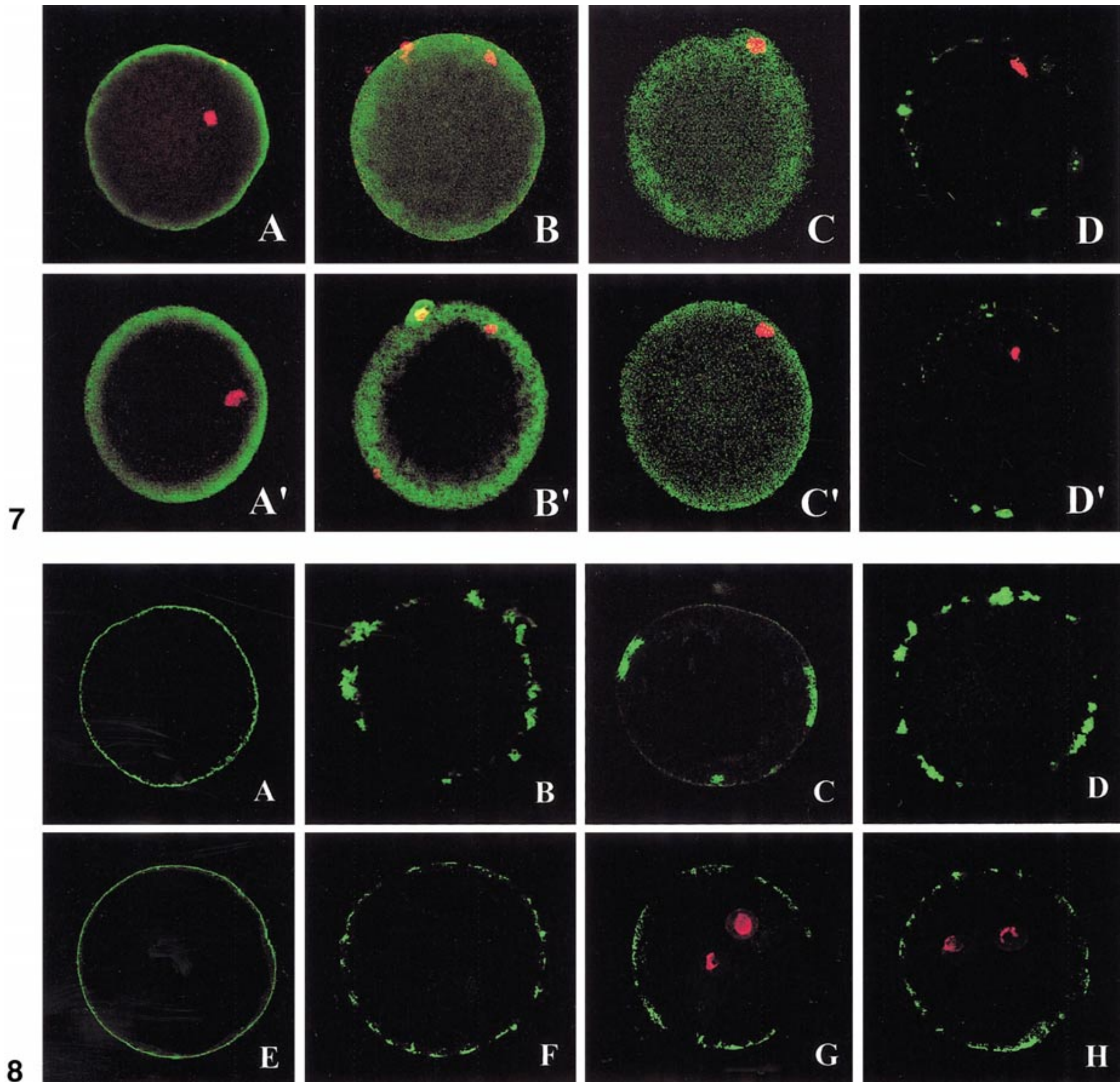


FIG. 7. Three cPKC isoforms translocated to the cortical region after the eggs were stimulated with A23187 (A, B, and C) and electrical pulse (A′, B′, and C′). At the same time, CG exocytosis was observed (D and D′).

FIG. 8. Effects of PKC isoform-specific antibody injection on PMA- or fertilization-induced CG release. Eggs injected with PKCα antibody could not release CGs after PMA treatment (A); eggs injected with PKCβI (B) or -γ (C) antibodies were observed to release their CGs after PMA treatment. In some eggs injected with PKCγ antibody CG contents aggregated beneath the plasma membrane. There were also some unreleased CGs remaining at the cortical region. Eggs injected with rabbit IgG can be induced to release their CGs by PMA treatment (D). CG exocytosis was also blocked in eggs injected with PKCα antibody (E), but not those injected with rabbit IgG (F) 6 h after insemination. CG release was observed in the fertilized eggs injected with PKCα (G) antibody or rabbit IgG (H) 20 h after fertilization.

and roles of cPKC isoforms in meiotic maturation orchestrating with other protein kinases.

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