

Translocation of phospho-protein kinase Cs implies their roles in meiotic-spindle organization, polar-body emission and nuclear activity in mouse eggs

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Abstract

The protein kinase Cs (PKCs) are a family of Ser/Thr protein kinases categorized into three subfamilies: classical, novel, and atypical. The phosphorylation of PKC in germ cells is not well defined. In this study, we described the subcellular localization of phospho-PKC in the process of mouse oocyte maturation, fertilization, and early embryonic mitosis. Confocal microscopy revealed that phospho-PKC (pan) was distributed abundantly in the nucleus at the germinal vesicle stage. After germinal vesicle breakdown, phospho-PKC was localized in the vicinity of the condensed chromosomes, distributed in the whole meiotic spindle, and concentrated at the spindle poles. After metaphase I, phospho-PKC was translocated gradually to the spindle mid-zone during emission of the first polar body. After sperm penetration and electrical activation, the distribution of phospho-PKC was moved from the spindle poles to the spindle mid-zone. After the extrusion of the second polar body (PB2) phospho-PKC was localized in the area between the oocyte and the PB2. In fertilized eggs, phospho-PKC was concentrated in the pronuclei except for the nucleolus. Phospho-PKC was dispersed after pronuclear envelope breakdown, but distributed on the entire spindle at mitotic metaphase. The results suggest that PKC activation may play important roles in regulating spindle organization and stabilization, polar-body extrusion, and nuclear activity during mouse oocyte meiosis, fertilization, and early embryonic mitosis.

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Introduction

Protein phosphorylation is considered one of the most versatile posttranslational modifications in eukaryotic cells. Protein kinases represent key molecules in a variety of signaling mechanisms capable of delivering information from the cell surface to the nucleus. One of the most important enzyme families is the protein kinase C (PKC) family. The PKC family consists of 11 different serine/threonine kinases that are subdivided into three groups based on sequence homology, as well as on activator and cofactor requirements. These groups include the conventional (PKC α , β I, β II, and γ), novel (PKC δ , ϵ , θ , μ , and η), and atypical (PKC λ / τ and ζ) isoforms (Mellor & Parker 1998, Viveiros *et al.* 2003). The conventional PKC isotypes are activated by Ca²⁺ and diacylglycerol. The novel PKCs are Ca²⁺-insensitive, but are still activated by diacylglycerol. The atypical PKCs are neither Ca²⁺-sensitive, nor do they respond to diacylglycerol (Mellor & Parker 1998, Ventura & Maioli 2001). Activated PKCs can translocate to the

nucleus where they phosphorylate a number of protein transcription regulators in a cell-cycle-dependent manner or in response to cell stimulation for exit from quiescence (G₀ → G₁ transition; Boulikas 1995).

In mammalian ovary, the oocyte is arrested at the diplotene stage of the first meiosis until sexual maturity. In each cycle, limited numbers of oocytes initiate meiosis, as indicated by the germinal vesicle (GV) breakdown (GVBD), and then they organize the meiotic spindle and extrude the first polar body, leading to the production of mature, fertilizable oocytes (Voronina & Wessel 2003). Fertilization initiates a rapid series of changes that restructure the egg into the zygote and initiate the program of early development. These changes are mediated by a series of cytoplasmic signal transduction events initiated by the rise in intracellular [Ca²⁺]_i. PKCs appear to have multiple functional roles in the cell-cycle progression during oocyte maturation (Downs *et al.* 2001, Quan *et al.* 2003). Stimulation of PKC is a sufficient and necessary event to

induce meiosis resumption during maturation of cumulus-enclosed mouse oocytes, but blocks meiosis resumption in cumulus-free oocytes (Sun *et al.* 1999, Downs *et al.* 2001, Quan *et al.* 2003). Prolonged activation of PKC arrests mouse oocytes at the first metaphase (MI) stage and blocks polar-body emission (Bornslaeger *et al.* 1986, LeFevre *et al.* 1992, Quan *et al.* 2003), while suppression of PKC promotes the onset of anaphase I (Viveiros *et al.* 2004). During fertilization, the mechanism by which a transient calcium burst triggers maturation promoting factor (MPF) inactivation involves a PKC-dependent pathway and activation of PKC is required for remodeling of the egg into the zygote (Colonna *et al.* 1997, Gallicano *et al.* 1997, Eliyahu & Shalgi 2002, Fan & Sun 2004).

Different PKC isoforms have been identified in mouse, rat and pig oocytes (Gangeswaran & Jones 1997, Raz *et al.* 1998, Fan *et al.* 2002). Conventional PKC α , β I, β II, and γ , novel PKC δ , and atypical PKC λ , μ , and ξ were found to exist in mouse oocytes and their subcellular localization was in a stage-dependent fashion during oocyte maturation and early development (Gangeswaran & Jones 1997, Luria *et al.* 2000, Page Pauken & Capco 2000). More recently, the specific function of PKC isoforms in oocyte was studied. It was found that the inhibition of GVBD of cumulus-free mouse oocytes was dependent on not only conventional PKC isoforms, but also other PKC isoforms (Quan *et al.* 2003). PKC δ was found to be associated with meiotic spindle and then with the chromosomes at the metaphase II (MII) stage in LTXBO strain mouse oocytes. PKC δ also participated in the regulatory mechanisms that delay the oocyte's entry into anaphase I. Its disruption promoted untimely entry into the interphase. Thus, loss of regulatory control over PKC activity during oocyte maturation disrupts the critical MI–MII transition, leading to a precocious exit from meiosis (Viveiros *et al.* 2001). PKC δ was also found to dephosphorylate after fertilization of mouse oocytes (Viveiros *et al.* 2003). However, the role of PKC phosphorylation in oocyte maturation and fertilization is largely unknown.

In this study, we characterized the subcellular distribution profile of phopho-PKC (pan) during the progression of mouse oocyte meiosis, egg activation, and early embryo cleavage with an antibody which recognizes phosphorylated isoforms of PKC α , β I, β II, γ , δ , ϵ , ζ , η , and θ using immunofluorescent confocal analysis.

Materials and Methods

Oocyte isolation and culture

All mice were bred and raised in the animal research colony of the State Key Laboratory of Reproductive Biology, Chinese Academy of Sciences, Beijing, People's Republic of China. GV-stage oocytes were recovered from ovaries of 4–6-week-old Kunming mice 48 h after the females were injected with 10 IU pregnant mare's serum gonadotropin (PMSG). Cumulus-free and GV-intact oocytes were

cultured in M2 medium at 37°C in an incubation chamber equilibrated with 5% CO₂/5% O₂/90% N₂. The oocytes were collected following 0, 2, 8, 10, and 12 h of culture for confocal microscopy. Progression to MII was indicated by the presence of the first polar body.

Electrical activation and fertilization of oocytes

Mice were treated with PMSG, followed by 10 IU human chorionic gonadotropin (hCG) approximately 48 h later. MII-stage eggs were recovered from the oviduct ampullae 15 h after hCG treatment and the surrounding cumulus cells were removed by a brief exposure to 300 IU/ml hyaluronidase (Sigma) in M2 medium. The denuded eggs were washed three times in electro-portion medium (0.25 M sorbic alcohol, 0.1 mM calcium acetate ((CH₃COO)₂Ca) 0.5 mM magnesium acetate ((CH₃COO)₂Mg and 0.5 mM Hepes) and were put in a fusion chamber, with two wires of 1 mm apart then a 10- μ s pulse at 180 v/mm was exerted. The eggs were washed three times and incubated in M16 medium at 37°C for an additional 6–8 h to evaluate pronucleus formation. A second group of MII eggs recovered from the oviduct was fertilized *in vitro*. The cauda epididymal spermatozoa were capacitated in M16 medium at 37°C in a humidified modular CO₂ incubation chamber for 1 h, then zona pellucida-free eggs were inseminated with capacitated spermatozoa (1×10^6 cells/ml) in a 50 μ l drop of M16 medium. The emission of the second polar body and the formation of the pronuclei were observed with an inverted microscope. The eggs were collected at 10 min, 30 min, 2 h, and 8 h after an electrical pulse and at 1, 2, and 8 h after insemination for confocal microscopy analysis.

For *in vivo* fertilization, the females were superovulated with 10 IU PMSG and 10 IU hCG, separated by an 48 h interval, and mated with the same strain of males. The zygotes were collected from the oviduct ampullae of superovulated females 16 h after hCG administration. After removing cumulus cells with 300 IU/ml hyaluronidase in M2 medium, zygotes were cultured in M16 medium at 37°C in a humidified atmosphere of 5% CO₂. The early embryos were collected at 6, 12, 18, and 24 h of culture for confocal microscopy analysis.

Immunofluorescence

A total of approximately 30 oocytes, eggs, or embryos in three replicates were collected at each time point of *in vitro* maturation, fertilization, and early embryo development. Evidently degenerated and untimely developed eggs were excluded from the analysis. The zona pellucida was removed by a short exposure of the oocytes, eggs, or early embryos to acid M2 medium (pH 2.5), and fixed with 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Cells were permeabilized with incubation buffer (0.5% Triton-100 in 20 mM Hepes, pH 7.4, 3 mM MgCl₂, 50 mM NaCl, 300 mM sucrose and 0.02% Na₃N) for 30 min at 37°C in an incubation chamber,

followed by blocking in 1% BSA for 1 h and incubation overnight at 4°C with polyclonal rabbit anti-phospho-PKC (pan) antibody (Cell Signaling Technology, Beverly, MA, USA; gamma Thr-514, catalog no. 9379S) diluted 1:100 in blocking solution. After three washes in PBS containing 0.1% BSA, 0.1% Tween-20, and 0.01% Triton-100 for 5 min each, the eggs were incubated with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:100 for 1 h. Following three washes, the nuclear status of oocytes was evaluated by staining with 10 µg/ml propidium iodide in PBS for 10 min. Oocytes/eggs that were labeled only with the second antibody were used as negative controls. 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. Each experiment was repeated three times. Cells were observed under a Leica confocal laser scanning microscope (TCS-4D).

Results

Subcellular localization of phospho-PKC during meiotic maturation

In GV oocytes freshly released from the follicles, phospho-PKC was localized to the entire GV except for the nucleolus (Fig. 1A). When GVBD just occurred, phospho-PKC

was distributed throughout the original GV area (Fig. 1B). Then, phospho-PKC condensed to numerous dots and was distributed around the condensed chromosomes (Fig. 1C); phospho-PKC staining moved to the opposite two sides of the chromosomes (Fig. 1D). It was distributed in the whole meiotic spindle but mainly localized to the meiotic-spindle poles at MI (Fig. 1E). During the transition from anaphase I to telophase I, following the separation of homologous chromosomes, phospho-PKC was translocated from the spindle poles to the midbody (Fig. 1F and G). Immediately after meiosis I, the oocytes entered meiosis II without interphase. The distribution pattern of phospho-PKC in MII oocytes was the same as that in MI oocytes (Fig. 1H). In oocytes that failed to undergo GVBD after maturation culture, phospho-PKC was still localized to the GV (Fig. 1I). In the negative control, oocytes that were treated only with the second antibody on staining were observed (results not shown).

Subcellular localization of phospho-PKC during electrical activation and *in vitro* fertilization of eggs

During *in vitro* fertilization, phospho-PKC was translocated from the spindle poles to the mid-zone (Fig. 2A and B). The eggs extruded their second polar body about 2 h after insemination, and phospho-PKC was localized between the separated oocyte and the second polar body (Fig. 2C). After formation of pronuclei 6–8 h following

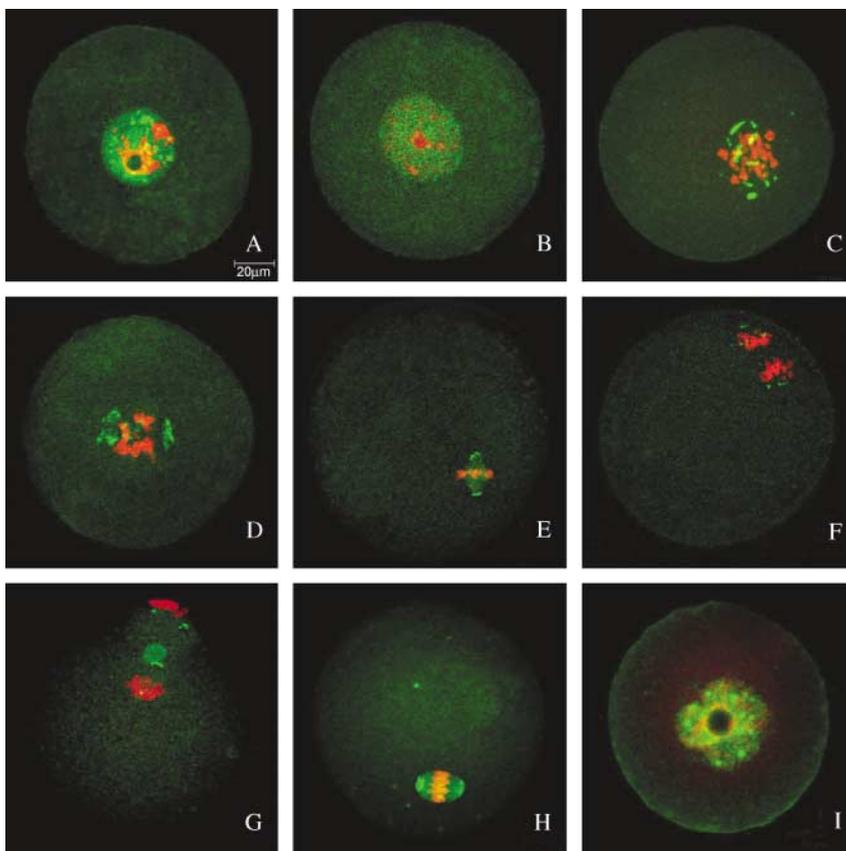


Figure 1 Immunofluorescent localization of phospho-PKC during meiotic maturation of mouse oocytes. Green, phospho-PKC; red, chromatin. During oocyte maturation, phospho-PKC gradually translocated from the GV area (A) to the condensed chromosomes (B, C), and finally moved to the organizing spindle poles (D, E). During the anaphase I–telophase I transition phospho-PKC was translocated from the middle poles to the middle plate of spindle (G). Phospho-PKC was present in the entire MII spindle, but mainly localized to the spindle poles at the MII stage (H). In the oocytes that failed to undergo GVBD after culture, phospho-PKC was still present in the GV (I).

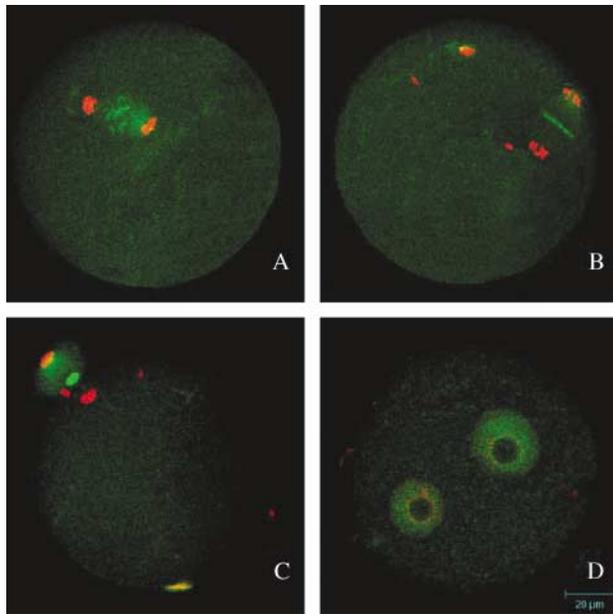


Figure 2 Subcellular localization of phospho-PKC during *in vitro* fertilization. During *in vitro* fertilization, phospho-PKC was translocated from the spindle poles (A) to the mid-zone at late anaphase (B) and telophase (C). In the pronuclear phase, phospho-PKC was distributed in the pronuclei except for the pronucleoli (D).

insemination, phospho-PKC was evenly localized to the pronuclei except for the pronucleoli (Fig. 2D). After electrical activation, phospho-PKC showed a similar distribution pattern to *in vitro*-fertilized eggs (Fig. 3).

Subcellular localization of phospho-PKC during early cleavage

In *in vivo*-fertilized eggs, phospho-PKC was concentrated in the pronuclei (Fig. 4A). When the zygotes underwent nuclear-envelope breakdown, phospho-PKC was distributed in the entire cytoplasm (Fig. 4B and C). At metaphase

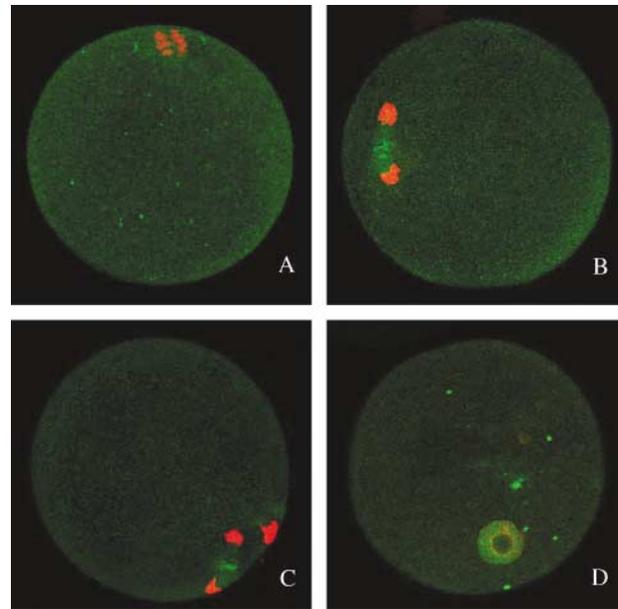


Figure 3 After electrical activation, phospho-PKC moved from the spindle poles at early anaphase (A) to the middle plate of the spindle at late anaphase (B), and finally concentrated to the midbody at telophase (C). Evident staining was observed in the pronuclei except for the nucleolus (D).

of the first mitosis, phospho-PKC was distributed in the whole mitotic spindle, with a more-concentrated localization at the spindle poles (Fig. 4D). In the two-cell stage, phospho-PKC showed a similar distribution pattern as in the one-cell stage (Fig. 4E and F).

Discussion

Anti-phospho-PKC (pan) detects endogenous levels of PKC α , β I, β II, γ , δ , ϵ , ζ , η , and θ that are phosphorylated at a C-terminal residue homologous to the residue Thr-514 of human PKC γ , as illustrated in the Cell Signaling

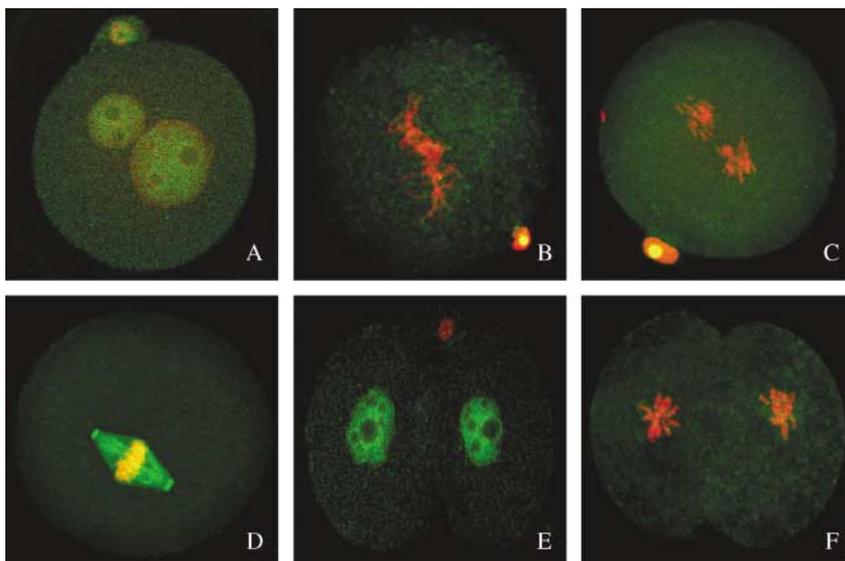


Figure 4 Subcellular localization of phospho-PKC during *in vivo* fertilization and early cleavage. In the pronuclear phase, phospho-PKC was localized to the pronuclei except in the nucleolus (A), but distributed evenly in the cytoplasm after nuclear-envelope breakdown (B, C). Phospho-PKC was distributed across the whole mitotic spindle and mainly localized to the spindle poles during the first cleavage (D). In the two-cell stage, phospho-PKC showed a similar distribution pattern as in first cleavage (E, F).

Technology product catalog. In the current study, by using this antibody, we characterized the localization of phospho-PKC, aiming to provide evidence for the involvement of PKC phosphorylation in various stages of mouse oocyte meiotic maturation, fertilization, and early mitotic division. It should be remembered that this antibody recognizes 9 of the 11 isoforms of PKC but that it does not detect PKC phosphorylated at other residues. In addition, this antibody is not isoform-specific, and thus the analysis of the exact role of specific PKC isoform is limited.

In mouse GV oocytes, PKC α , PKC β II, and RACK1 (receptor for activated C kinase 1) were uniformly distributed in the cytoplasm, while PKC β I was localized in the cytoplasm and in the plasma membrane as well (Luria *et al.* 2000). A recent study revealed diffuse expression of PKC δ throughout the cytoplasm and nucleus of GV-stage oocytes (Viveiros *et al.* 2003). In contrast, we found that phospho-PKC was concentrated in the entire GV except for the nucleolus. Previous reports by others and us indicated that PKC activation inhibited the meiotic resumption of cumulus-free mouse oocytes and this effect could be overcome by PKC inhibitors (LeFevre *et al.* 1992, Sun *et al.* 1999, Luria *et al.* 2000, Quan *et al.* 2003). Our present results, together with previous reports, provide evidence showing that PKC phosphorylation may be involved in GVBD regulation, but how PKC phosphorylation regulates GVBD needs further clarification.

Shortly after GVBD, phospho-PKC condensed and distributed around the condensed chromatin from which microtubules radiate. Phospho-PKC was distributed in the whole meiotic spindle and mainly localized to the meiotic-spindle poles at MI, but translocated to the spindle middle plate at the anaphase I–telophase I transition, following the separation of homologous chromosomes. Similar phospho-PKC translocation was also observed after sperm penetration or parthenogenetic activation of MII oocytes. By using the LTXBO mouse oocytes which are arrested at the MI stage, Viveiros *et al.* (2001) proposed a hypothesis that PKC participates in the meiosis I–meiosis II transition. Our previous study also showed that PKC activation after GVBD led to the inhibition of mitogen-activated protein kinase phosphorylation and arrested the cell cycle at the MI stage (Quan *et al.* 2003). It was reported recently that during meiotic maturation phosphorylated PKC δ was distinctly associated with the spindle apparatus during the first meiotic division, while it was dephosphorylated following completion of meiosis II after fertilization (Viveiros *et al.* 2003). By using normal oocytes isolated from small antral follicles, which have not yet developed the capacity to progress to MII, and also oocytes defective in their ability to exit MI, this group also reported that transient suppression of endogenous PKC activity by treatment with a PKC-specific inhibitor promoted the onset of anaphase I in a dose-dependent manner (Viveiros *et al.* 2004). Another recent report indicated that many isoforms of PKC were enriched around the meiotic spindle and that PKC ζ is involved in spindle

stability (Page Baluch *et al.* 2004). In different mitotic cells, various PKC isoforms were found to associate with the mitotic apparatus and colocalize with β -tubulin in spindle microtubules (Lehrich & Forrest 1994, Passalacqua *et al.* 1999, Battistella-Patterson *et al.* 2000, Chen *et al.* 2004). The striking presence of phosphorylated PKCs in meiotic-spindle poles and in the central portion of the elongating meiotic spindle as revealed in our study, together with previous reports, suggest a functional role for PKC phosphorylation in spindle organization and stabilization as well as cytokinesis during mouse oocyte meiosis.

PKC is activated in the nucleus during the G₂ phase of the cell cycle, where it is required for mitosis. The PKC α isoform translocated to the nucleus and is found not only associated with the nuclear envelope but mainly with the interchromatin domains (Zini *et al.* 1995). PKC β II, a mitotic lamin kinase (lamin is a PKC-binding protein), has been shown previously to translocate to the nucleus at the G₂/M stage and this was coupled to the generation of nuclear diacylglycerol (Sun *et al.* 1997, Deacon *et al.* 2002). In fertilized sea-urchin egg cytoplasmic extract, male pronuclear formation is initiated by the disassembly of the sperm nuclear lamina as a result of lamin phosphorylation by a cytosolic PKC (Collas *et al.* 1997). In our experiment, when the formation of pronucleus occurred, phospho-PKC was concentrated in the pronucleus except for the nucleolus region. Phospho-PKC had no special distribution pattern after nuclear-envelope breakdown and was localized on the entire metaphase spindle of a one-cell embryo. Phospho-PKC was also found to localize in the nucleus in early cleavage. The localization of phospho-PKC (pan) in pronuclei of fertilized eggs, nuclei of early mitotic embryo cells, and mitotic spindles suggest that PKC activation may regulate nuclear function and mitotic-spindle assembly in fertilized eggs and early embryos. Identifying potential target substrates and signal network for phospho-PKC will be key in helping to define its function(s) in mammalian early development.

Taken together, the unique distribution pattern of phosphorylated PKC implies possible roles of this family of kinases in regulating microtubule organization and stabilization, nuclear function and cytokinesis during mouse oocyte meiosis, fertilization, and early embryonic mitosis.

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