

# PKC Regulation of Gametogenesis and Early Development

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## 1. Introduction

The protein kinase C (PKC) family is comprised of 11 isotypes and many can exist in a single cell simultaneously. There are three general categories of PKC based on their cofactor requirements for activation: The conventional, novel, and atypical PKCs. The first category, the conventional PKC isotypes, are PKC $\alpha$ , - $\beta$ I, - $\beta$ II, and - $\gamma$  and are activated by diacylglycerol (DAG), and negatively charged phospholipids in a calcium-dependent manner. The second category, the novel PKCs, are composed of PKC $\delta$ , - $\epsilon$ , - $\theta$ , and - $\eta$  and require negatively charged phospholipids and DAG, but act in a calcium-independent manner. Finally, the atypical PKCs are human  $\iota$ /mouse  $\lambda$  and PKC $\zeta$  and only require negatively charged phospholipids for activation. Each PKC isotype phosphorylates serine/threonine residues on protein substrates. There are several known substrates for PKC and they include MARCKS proteins, RACK proteins, Dynamin, EGFR, MEK5, the two subunits of MPF, p34<sup>cdc2</sup> and cyclin B, among others (Yu et al., 2004; Steinberg 2008; Meier et al., 2009).

Many PKC isotypes have overlapping substrate specificity. Since many isotypes function in different cellular locations and have different cofactor requirements for activation they provide for differential function during various cellular events. Germ cells (i.e. eggs and sperm) are highly specialized cells that eventually give rise to a new organism through several developmental transitions, these include: a) The oocyte must become mature as a fertilization-competent egg arrested in meiotic metaphase II of the cell cycle; b) Post fertilization events follow a series of time-dependent changes both at the structural and biochemical level that convert the egg to the zygote, and c) The zygote to the embryo through pivotal developmental transitions. Disruption of these time-dependent changes, likely results in spontaneous abortion or abnormal development. Equally important is the maturation process the sperm undergoes. Sperm cells require first that capacitation and later the acrosome reaction occur before becoming fertilization-competent. If these gametes interact without first undergoing these initial changes, the orchestration of fertilization will not take place. Indeed the egg should be thought of as a cell programmed for death, but rescued by the key developmental transition of fertilization. This chapter examines the involvement of PKC isotypes in oocytes and eggs. The involvement of PKC in sperm development is next considered - followed by examining the involvement of PKC in fertilization and development of the early embryo. The concluding section addresses methods for the study of PKC in eggs/embryos.

## 2. Oocytes and eggs

With the exception of M-phase at which point the spindle apparatus reorganizes from the nucleus, the cell can be thought of as partitioned into nuclear and cytoplasmic compartments. As such, the nuclear envelope provides a physical barrier permitting access of different signaling molecules to these two compartments. At the onset of M-phase the nuclear envelope is disassembled and, accompanied by the disassembly of the interphase array of microtubules and replacement with the meiotic spindle apparatus. Despite the absence of the nuclear envelope in partitioning the cell, differential enzyme substrate specificity can continue to afford unique enzyme action at different cellular locations. Thus, in a sense, it can be thought that the nuclear-cytoplasmic partition remains.

In mammals, oocytes are arrested in the diplotene state of the meiotic cell cycle within the ovary awaiting maturity. At this point in the cell's development the oocyte nucleus, also known as the germinal vesicle, is still present. Rupture of the follicles trigger resumption of meiosis and there is a release of oocytes each surrounded by a few layers of cumulus cells. During this time the oocyte is released from arrest at the diplotene state and progresses to the next cell cycle arrest point, meiotic metaphase II. Briefly, in mammals cumulus cells are responsible for physical support of the developing gamete and maintenance of a so-called microenvironment as reviewed elsewhere (Huang & Wells, 2010).

The presence of cumulus cells surrounding the oocyte can cause problems during experimental analysis, most notably when agonists or antagonists of signal transduction pathways are applied because they are applied externally. When applied in the short term these pharmacological agents necessarily act first on the cumulus cells surrounding the oocyte and then later on the cumulus-enclosed oocyte. Under these conditions the exact concentration of the pharmacological agent within the oocyte is unlikely to be consistent since the membrane-permeate, pharmacological agent must first interact with the "sponge-like" cumulus cells that surround the oocyte before diffusing into the oocyte. In addition, many pharmacological agents are foreign to cellular metabolism and thus any paracrine communication between not only cumulus cells, but also the cumulus cell and the oocyte may be altered. Thus, for investigations employing agents applied to oocytes, it is best to first remove the cumulus cells prior to application of a PKC agonist or antagonist. However, when the natural, unaltered distribution of PKC or some other endogenous component is to be studied, the experiment is benefited by the retention of the cumulus cells, since that is the natural state (Downs et al., 2006).

One such study (Avazeri et al., 2004) shows that all of the conventional PKC isotypes, except for PKC $\gamma$ , undergo a change from a cytoplasmic to nuclear localization just prior to germinal vesicle breakdown. The investigators also injected antibodies directed against each of the previously mentioned PKC isotypes to block their biological activity. When injected into the nucleus, blocking PKC $\alpha$  and  $\beta$ II had the greatest effect on inhibition of germinal vesicle breakdown, followed by a lesser effect of antibodies to PKC $\beta$ I and finally antibodies to PKC $\gamma$  had no effect. Other studies examined a single PKC isotype and confirm the presence of PKC $\alpha$  (Quan et al., 2003), and PKC $\beta$ I (Denys et al., 2007) in the nucleus prior to germinal vesicle breakdown. One study (Luria et al, 2000) examined PKC $\alpha$ ,  $\beta$ I,  $\beta$ II and found all three isotypes present in the cytoplasm of the germinal vesicle

stage oocyte which is in agreement with the above studies at the early germinal vesicle stage (Avazeri et al., 2004). The focus of the study by Luria and colleagues (2000) was to apply PMA as an agonist to activate PKCs. This treatment caused resumption of the meiotic cell cycle and consequent disruption of the germinal vesicle. Through biochemical techniques it was shown that PKC $\delta$  is localized in the germinal vesicle as well as the cytoplasm when PKC activity is elevated. Later, during meiosis I, PKC $\delta$  was shown to be associated with the spindle apparatus (Viveiros et al., 2001, 2003), and that its active form, p-PKC $\delta$ , was enriched at the spindle poles and colocalized with both pericentrin and  $\gamma$ -tubulin (Ma et al., 2008).

The fertilization-competent egg arrested at meiotic metaphase II features notable differences in PKC isotypes and their individual localization. Total antibody, that is, antibody that detects both the phosphorylated and unphosphorylated form of the kinase, directed against PKC $\alpha$ ,  $-\gamma$ ,  $-\delta$  and  $-\zeta$  were found not only in the cytoplasm, but also enriched on the spindle apparatus of the fertilization-competent egg (Baluch et al., 2004). The tightness of this PKC binding was assessed by detergent extraction, a process which removes the soluble components of the cell and retains the egg's cytoskeleton and associated proteins. Total PKC $\zeta$  remained behind in the cytoskeleton after the detergent extraction process; the closeness of this binding was confirmed by FRET analysis between  $\alpha$ -tubulin of the meiotic spindle and the PKC isotypes. FRET analysis demonstrated a close molecular proximity not only between  $\alpha$ -tubulin and PKC $\zeta$ , but also between  $\alpha$ -tubulin and PKC $\delta$  (Baluch et al., 2004, Baluch & Capco, 2008). Other investigations have shown that PKC $\beta$  exists in the cytoplasm of the fertilization-competent egg (Raz et al., 1998). In mouse eggs, p-PKC $\zeta$  is enriched at the ends of the acentrosomal spindle, whereas total PKC $\zeta$  decorates the length of the spindle apparatus.

Upon fertilization PKC $\alpha$ ,  $-\beta$ , and  $-\gamma$  localize to the plasma membrane (Raz et al., 1998; Baluch et al., 2004, Halet et al., 2004). Here these conventional PKCs are poised to become active, as they are in a prime location to interact with their co-factors (i.e. DAG and phospholipids) for activation. Intriguingly, total PKC $\zeta$  and the phosphorylated form of PKC $\zeta$  (p-PKC $\zeta$ ) remain associated with the spindle apparatus, from the arrest at meiotic metaphase II through the anaphase II transition. This result suggested that p-PKC $\zeta$  may play a role in its association with the spindle. In fact, inhibition of p-PKC $\zeta$  with a specific, membrane-permeate, peptide inhibitor caused inactivation of the isotype and appeared to result in a disruption of the spindle apparatus. Inhibitors that would block most other active isotypes of PKC, but not p-PKC $\zeta$ , did not result in spindle disassembly.

Evidence exists that also suggests a role for p-PKC $\delta$  in spindle stability (Ma et al., 2008) as others have noted p-PKC $\delta$  is enriched in the same region of the spindle pole as p-PKC $\zeta$  in the metaphase egg. A targeted knockdown of PKC $\delta$  expression with siRNA disrupts the spindle apparatus and also decreases the expression of pericentrin. However, these data leave open the possibility that the knockdown may have several effects on the egg's spindle apparatus (Ma et al., 2008), as the reduction of pericentrin would provide no sites for p-PKC $\zeta$  to bind at the poles. This alternative interpretation could explain the disruption of the spindle since p-PKC $\zeta$  activity is required for spindle stability as previously described (Baluch et al., 2004, 2008). Moreover, both immunopurification and FRET analysis at the spindle poles demonstrates that total and p-PKC $\zeta$  are in close molecular proximity with  $\gamma$ -

tubulin, Par6, and ser9-GSK3 $\beta$  (Baluch & Capco, 2008). The Par6/PKC $\zeta$ /GSK3 $\beta$  pathway may have a role in microtubule stability (Baluch & Capco, 2008). When GSK3 $\beta$  is active, microtubule networks can be destabilized (Zhou & Snider, 2005). However, when GSK3 $\beta$  is rendered inactive by phosphorylation on serine 9, spindle microtubules are stabilized. Ser9 p-GSK3 $\beta$  and p-PKC $\zeta$  are localized to the spindle poles and kinetochore regions of the mouse egg and FRET analysis demonstrates their close molecular proximity (Baluch & Capco, 2008). A knockdown of the PKC $\delta$  gene in mice using gene targeting strategy shows that mice are viable and fertile, and thus suggests no deleterious effects on the spindle apparatus (Leitges et al., 2001). This may suggest that cells have a “plan-B” to protect the spindle in the event of a complete gene knockout. However, the aim of that study was to investigate vein arteriosclerosis and therefore the spindle was not specifically examined, so it remains unclear if the PKC $\delta$  gene knockout triggered any other signaling events to occur regarding the spindle.

### 3. Sperm

Using a combination of biochemical and immunocytochemical methods, Rotem and coworkers (1990) demonstrated that cPKCs are present in sperm in the equatorial segment, the principal piece of the tail and occasionally visualized in the centriole region. Application of staurosporine, 2-amino-4-octadecene-1,3-diol (sphingosine), or 1-(5-isoquinoylinylsulfonyl)-2-methylpiperazine (H7) all older, concentration-dependent PKC inhibitors blocked sperm motility, while application of a phorbol ester, phorbol 12-myristate 13-acetate (PMA) which activates PKC, increased motility.

For sperm to become fertilization competent two separate events must first take place that is, capacitation and subsequently the acrosome reaction. Capacitation is a series of physiological changes in the sperm that typically occur as the sperm traverses the female reproductive tract to reach the egg. During capacitation, there is an efflux of cholesterol from the sperm plasma membrane followed by an increase in the level of intracellular-free calcium, and cAMP levels increase to activate PKA (Breitbart, 2003). Capacitation also induces a rise in free-calcium that itself could lower the requirement for PKC activation even in the absence of increased levels of DAG. However, the calcium-dependent isotype of PLC (PLC $\gamma$ ) is activated by calcium which could also generate DAG by hydrolysis of PIP $_2$ , leading to the activation of many of the PKC isotypes (Breitbart, 2003) at the time of capacitation.

Capacitation is accompanied by assembly of an actin network between the outer acrosomal membrane and the overlying sperm plasma membrane. PKC itself or in concert with PLC $\gamma$  and/or phospholipase D could mediate the formation of this network as both have been shown to be associated with the underlying actin network in this area (Breitbart 2002; Cohen et al., 2004). In order for the acrosome reaction to take place this cortical actin network must subsequently disassemble. Evidence that the disassembly of the actin network is necessary for completion of the acrosome comes from the application of an actin-stabilizing agent such as phalloidin which was shown to inhibit the acrosome reaction (Spungin et al., 1995). The opening of calcium channels is thought to be mediated by the binding of sperm to ligands in the zona pellucida and initiates the acrosome reaction (Breitbart 2002). This calcium influx induced by the zona pellucida could

activate PLC which in turn can produce DAG, all of which are required for PKC activation. Studies on human sperm (Rotem et al., 1992) demonstrated that capacitated sperm could be induced to undergo the acrosome reaction in the absence of intracellular-free calcium via the non-specific PKC agonist, PMA. Both PKC $\alpha$  and  $\beta$ II were found in a sharp band at the equatorial segment, whereas PKC $\beta$ I and PKC $\epsilon$  were found at the principal piece of the tail (Rotem et al., 1992).

The acrosome reaction is a secretory event in the sperm where the large secretory vesicle effectively a large lysosomal vesicle of the sperm head, the acrosome, vesiculates to release digestive enzymes. This permits the fertilization-competent sperm to burrow its way between cumulus cells to reach the egg zona pellucida. Here, through the use of digestive enzymes and other proteins released from the acrosome, the sperm is able to penetrate the zona pellucida to fuse with the egg plasma membrane. The zona pellucida is a glycoprotein-rich network exterior to the plasma membrane of the egg. The acrosome reaction is dependent on extracellular-free calcium (Yanagimachi & Usui, 1974; Florman et al., 1989); however, utilizing PMA to activate PKC induced the acrosome reaction independent of extracellular-free calcium (Rotem et al. 1992). PMA is not part of normal cellular metabolism and therefore the possibility exists that the calcium-independent acrosome reaction was an artifact. The notion, however, that PKC is involved in the acrosome reaction is supported by application of a diacylglycerol, a natural PKC agonist that also induced the acrosome reaction in the absence of extracellular-free calcium. Moreover, it was speculated that the increase in intracellular-free calcium required during the earlier capacitation event supplied sufficient calcium for the PKC-induced acrosome reaction while inhibitors to PKC blocked this response (O'Toole et al., 1996).

Although progesterone secreting cumulus cells surrounding the mammalian egg have been shown to trigger the acrosome reaction (Garcia & Meizel, 1999), PKC inhibitors block the acrosome in human sperm (O'Toole et al., 1996). In addition, some have shown that components of the egg zona pellucida are responsible for the acrosome reaction, but similarly the older drug inhibitor of PKC, Staurosporine, blocks the acrosome reaction (Liu and Baker, 1997).

It has also been reported that PKC is essential for the maintenance of sea urchin sperm motility, although other kinases such as PKA and tyrosine kinase are present, but not key for motility (White et al., 2007). Intriguingly, this article also reported the presence of PKM, the nonmembrane-bound catalytic subunit of PKC in the sea urchin sperm. PKC is cleaved to form PKM which has different substrate specificity than PKC. Since PKM is not membrane-bound it phosphorylates substrates in the sperm interior. PKM also has been reported in mammalian eggs and has been shown to phosphorylate cytoskeletal substrates in the egg interior as discussed in the fertilization section (Gallicano & Capco 1995). Although activation of PKC in sperm seems to result in similar developmental fates in a diversity of animals, it is not to be considered a universal activator since PKC activation in fowl sperm blocks the acrosome reaction (Ashizawa et al., 2006). Some level of PKC activity also has been implicated in retaining the proper volume in sperm as it traverses fluids of different osmolarity during its passage from the testis to the oviduct (Petrunkina et al., 2007).

### 4. Fertilization

The process of fertilization induces a number of structural and biochemical changes within the egg as the single sperm cell penetrates the egg to subsequently form the zygote. This pivotal developmental transition appears to be dependent on a series of signal transduction events initiated by sperm penetration (Figure 1). Increasing evidence suggest that phospholipase C (PLC)  $\zeta$ , supplied by the sperm, initiates this developmental transition within the egg (Saunders et al., 2002; Knott et al., 2005; Lee et al., 2006; Swann et al., 2006; Yu et al., 2008). Once PLC $\zeta$  is within the egg, it then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to produce inositol triphosphate (IP<sub>3</sub>) and DAG. IP<sub>3</sub> subsequently diffuses and binds to IP<sub>3</sub> receptors to initiate a rise in calcium from sequestered stores in the egg, which potentially initiates calcium oscillations through a calcium-induced activation of PLC or PKC.

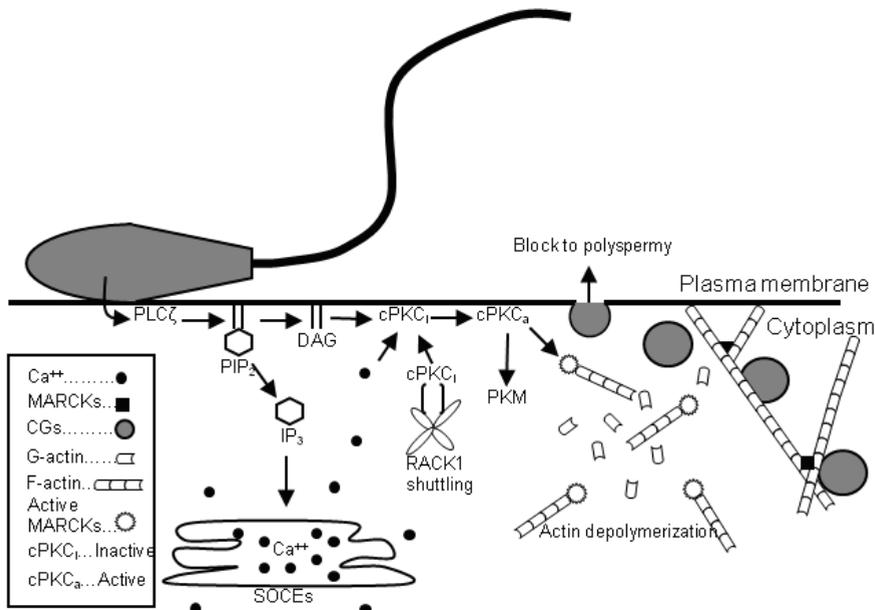


Fig. 1. Diagrammatic representation of PKC signaling events that may be involved in cortical granule exocytosis, the block to polyspermy, and subsequently fertilization.

Intracellular-free calcium along with DAG are essential cofactors for the activation of conventional PKCs. Translocation of the conventional PKC isotypes to the cell cortex indicates activation of the kinase and one study (Halet et al., 2004) has provided strong evidence that the cPKC, PKC $\alpha$ , is translocated to the egg plasma membrane after every calcium oscillation, and functions to induce calcium transients as part of a positive feedback loop that regulates store-operated calcium entry (SOCE). Others have shown the translocation of PKCs to the egg plasma membrane resulting from egg activation (Gallicano et al., 1995; Raz et al., 1998, Baluch et al., 2004; Carbone & Tatone, 2009) however, the investigation by Halet et al. (2004) clearly provided a link to calcium oscillations and shows

that PKC has a role in the positive feedback loop that generates the calcium oscillations through SOCE.

After recruitment to the egg plasma membrane, perhaps in part by the proposed shuttling action of RACK1 (Haberman et al., 2010), activated PKCs are poised to phosphorylate MARCKS proteins. MARCKS proteins are substrates for PKC, and MARCKS have the ability to crosslink actin filaments into a network at the cell cortex (Eliyahu et al., 2005, 2006). However, once MARCKS are phosphorylated by PKC the actin network can then disassemble (Tsaadon et al., 2008). Disassembly of the actin network at the egg cortex could release cortical granules from the cortical actin network and allow them to reach the plasma membrane to exocytose (Tsaadon et al., 2008). Once cPKCs are activated, as part of their down regulation, the kinase is cleaved between its membrane-binding domain and catalytic subunit to form PKM (Jaken 1990; Hashimoto et al., 1991). PKM remains active in the cytosol and its altered folding provides for altered substrate specificities. Gallicano et al. (1995) demonstrated that PKM which forms as a consequence of fertilization acts to phosphorylate a specialized intermediate filament network known as cytoskeletal sheets (reviewed by Capco 2001). In addition, through a series of experiments it was also shown to remodel the cytoskeletal interior (Gallicano et al., 1995). The authors first determined that it was indeed PKC acting downstream of calcium responsible for the changes in the cytoskeletal interior by experimentally activating PKC while clamping calcium to low levels. Subsequently the distribution of PKC was mapped over time with the PKC reporter dye Rim-1 and PKC was found at the egg cortex indicating it was activated (Gallicano et al., 1995). Furthermore, it was noticed during a biochemical assay that utilized a monoclonal antibody to the catalytic subunit of PKC $\alpha$  that there was a change in the molecular weight of PKC from 80 to 50 kDa and that the kinase shifted compartments from the detergent-soluble fraction to the detergent-resistant, sheet-enriched fraction indicative of the formation of PKM. A further biochemical assay utilizing inhibitors to PKC, myosin light-chain kinase, and tyrosine kinase could not block the phosphorylation of exogenous substrate while an inhibitor to PKC/PKM blocked phosphorylation (Gallicano et al., 1995). Taken together these results suggest that the membrane-bound PKC downstream of calcium was cleaved forming PKM and that PKM bound to and phosphorylated the internal cytoskeletal elements. For these reasons the cPKCs were proposed to have a chronometer function that acts on the cell spatially and temporally. Through the action of PKC at the membrane the egg first blocks polyspermy by inducing cortical granule exocytosis and later, following each calcium oscillation, acts on substrates within the egg interior through the action of PKM as the egg remodels to form the cytoskeletal framework appropriate for the zygote.

There has been debate as to whether or not PKC acts alone to stimulate cortical granule exocytosis. Some reports suggest that another calcium-dependent kinase known as calcium/calmodulin-dependent protein kinase II (CaMKII) may be involved (Abbott & Ducibella, 2001; Abbott et al., 2001; Madgwick et al., 2005). In a series of experiments, Knott et al. (2006) injected constitutively active CaMKII $\alpha$  into mouse eggs and it was reported that CaMKII was responsible for meiotic resumption and cortical granule exocytosis. Both PKC and CaMKII have several different isotypes, and the different isotypes have different substrate specificities. The  $\delta$  subunit of CaMKII was shown to be effective in the resumption of mouse eggs (Johnson et al., 1998), while others have shown cell cycle resumption to be dependent on the  $\gamma$  subunit (Change et al., 2009; Backs et al., 2010). A plausible alternative

explanation is upon injection the constitutively active CaMKII $\alpha$  (Knott et al., 2006) could immediately phosphorylate substrates in the cytoplasm and alter the sequence of events leading to activation. As many others have noted, since the cortical granule block to polyspermy is key for continued development, redundant mechanisms regulating this important event are likely to be in place.

## 5. The early embryo

Various studies can demonstrate that the zygote and preimplantation embryo display a change in cellular localization of several PKC isotypes during postfertilization development. Both PKC  $\zeta$  and  $-\delta$ , which were enriched on the spindle apparatus prior to fertilization later appear enriched within the pronuclei at six hours postfertilization, while PKC $\alpha$  and  $-\gamma$  are absent from the pronuclei (Viveros et al. 2003; Baluch et al., 2004). By the two-cell stage PKC $\alpha$ ,  $\gamma$ ,  $\delta$ , and  $\zeta$  are enriched in the nuclei (Viveros et al. 2003; Baluch et al., 2004; Dehghani and Hahnel, 2005). Reports from Pauken and Capco (1999, 2000) have shown a marked reduction in the nuclear localization of these isotypes after the two-cell stage with the exception that PKC $\zeta$  lines the nuclear periphery at the four-cell stage. However, these isotypes appear in unique locations poised to presumably interact during subsequent developmental transitions: At the time of compaction PKC $\alpha$ ,  $\gamma$ ,  $\delta$ , and  $\mu$  line the cell-cell boundaries to differing extents, and these isotypes are considerably absent from the nucleus (Pauken & Capco, 1999, 2000). During the late eight-cell stage just prior to compaction the  $\zeta$  isotype is greatly enriched in the nuclei (Pauken & Capco, 2000). Moreover, when PKC was experimentally activated by the natural agonist (DiC8 a diacylglycerol and natural activator of PKC) it induces PKC $\alpha$  to localize at internal cell-cell boundaries. Then  $\beta$ -catenin becomes phosphorylated and accumulates at these internal cell-cell boundaries as the blastomeres begin to flatten out on each other during the process of embryonic compaction (Pauken & Capco, 1999). In addition, it was shown that immediately before compaction begins  $\beta$ -catenin becomes part of the detergent-resistant cytoskeleton at intercellular boundaries indicative of its association with the adherens junctions that are responsible for adhering and subsequently flattening of the blastomeres later during compaction (Pauken & Capco, 1999). Although PKC $\beta$ I and  $-\beta$ II were not investigated in the aforementioned study, others (Dehghani and Hahnel, 2005) have shown that PKC $\beta$ I accumulates in the nuclei of embryos at the four-cell stage and later during the postcompaction stage, while PKC $\beta$ II appears to become uniformly distributed throughout the cytoplasm and nuclei from the four-cell stage until the blastocyst stage.

PKC $\delta$  and  $\epsilon$  are reported to transiently enrich in nuclei of the four-cell embryo (Dehghani and Hahnel, 2005). In this study investigators blocked both PKC $\delta$  and  $\epsilon$  with peptides that interfere with adapter sites to block movement of these isotypes, which subsequently altered transcription. This suggests that the location of the isotype may be involved in its activity and that both isotypes have a role in transcription at the four-cell stage.

## 6. Consideration of experimental procedures & methods

### 6.1 Pharmacological agents & PKC

To determine the role of a single type of kinase investigators often employ various pharmacological agents (Table 1).

Common name	Also known as	Target kinase(s)	IC <sub>50</sub>
<b>Inhibitors</b>	N/A	PKA	7 nM
Staurosporine		CaMK MLCK PKC PKG	20 nM 1.3 nM 700 pM 8.5 nM
Chelerythrine Chloride	N/A	PKC	660 nM
Bisindolylmaleimide	BIM	PKC GSK-3 GSK-3β PKA	10 nM 360 nM 170 nM 2 μM
Rottlerin	Mallotoxin	PKCδ/θ PKCα/β/γ PKCε/η/ζ CaMKIII	3-6 μM 30-40 μM 80-100 μM 5 μM
H-7	1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride, Isoquinoline-5-sulfonic 2-methyl-1-piperazine dihydrochloride	PKA PKC	Determine empirically
Myristoylated PKCζ Pseudosubstrate	N/A	PKCζ	Determine empirically
Myristoylated PKCθ Pseudosubstrate	N/A	PKCθ	Determine empirically
Myristoylated PKCη Pseudosubstrate	N/A	PKCη	Determine empirically
<b>Activators</b>			Determine empirically
PMA	TPA, phorbol 12-myristate 13-acetate	PKC	Determine empirically
OAG	1-(cis-9-Octadecenoyl)-2-acetyl-sn-glycerol, 2-Acetyl-1-oleoyl-sn-glycerol	PKC	Determine empirically
DiC8	1,2-Dioctanoyl- <i>sn</i> -glycerol	PKC PKC	Determine empirically

Table 1. A large variety of agonists/antagonists exist for the experimental study of PKC. Some PKC inhibitors have the ability to interact with multiple kinases since the concentration of the drug increases as it accumulates within the cell over time.

While their use is essential, it is important to understand their limitations and design experiments containing the proper controls when conclusions are drawn from their uses. Ideally, a log-fold dose response curve should be conducted prior to experimentation to determine the lowest working concentration to activate/inhibit a single type of kinase of interest. The lowest working concentration should be used in subsequent experiments as many agonists/antagonists have the ability to interact with multiple kinases as the cell

continues to absorb the membrane-permeate pharmacological agent over time. Examples include the PKC inhibitors 3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (BIM, also referred to as Gö 6850, or GF 10923X), Staurosporin, and Rottlerin, among others. An alternative to pharmacological inhibitors of PKC are membrane-permeant peptide inhibitors, which exist for some of the PKC isotypes and other kinases and have a greater degree of specificity since their peptide sequence is designed to block the active site of the specific kinase. In addition to membrane-permeant peptide inhibitors, it would be wise to employ two structurally and chemically distinct inhibitors to reduce the possibility of misinterpretation of experimental data (Stricker et al., 2010).

It also is wise to adopt natural agonists such as a diacylglycerol (DAG); for instance OAG or DiC8 can be routinely used in parallel when pharmacological agents are employed (Gallicano et al., 1995; Pauken & Capco, 1999; Eliyahu & Shalgi, 2002; Halet et al., 2004). In addition, it is useful to confirm the activity of the PKC isotype. A rapid method to determine whether a kinase is active is to employ an antibody specific to the phosphorylated/active form of the isotype during immunocytochemical or western blot analysis.

Some molecular strategies at the DNA or RNA level have been developed to remove or knockdown a specific PKC isotype. Knockout mice have been developed for PKC $\alpha$ , PKC $\beta$ , PKC $\gamma$ , PKC $\delta$ , PKC $\epsilon$ , PKC $\theta$ , and PKC $\zeta$  and the effects of gene knockout have been studied (Abeliovich et al., 1993; Leitges et al., 1996, 2001a, 2001b, 2002; Sun et al., 2000; Meier et al., 2007). Care should be taken when interpreting experimental data from these models as PKC operates within many other signaling pathways.

## 6.2 Introduction to the handling and procurement of mouse eggs

There exist several technical challenges concerning the procurement and manipulation of mammalian eggs/embryos. For starters, the mouse egg is about 80  $\mu\text{m}$  in diameter. This small size requires the use of a finely pulled, flame-polished Pasteur pipette attached to a pipetting apparatus; a 1 mL syringe attached to a 24" piece of flexible tubing through which suction can be created for aspiration or expulsion. Under optimal conditions, only 20-40 eggs can be obtained from a single female, and consequently any loss of sample during handling can significantly decrease the amount of material for study. In order to obtain 20-40 eggs/embryos female mice are superovulated by an injection regimen of gonadotropins. An outbred mouse strain CD-1 (Charles River Laboratories) is routinely used in this laboratory and maintained under a 14-hour light/ 10-hour dark schedule and given *ad libitum* access to food and water.

### 6.2.1 Procedure for the procurement of cumulus-free eggs

1. Pregnant mare serum gonadotropin (PMSG) administration is accomplished by intraperitoneally (i.p.) injection of 5.0 IU PMSG in a 0.1 mL sterile volume of 100 mM Phosphate-buffered saline (PBS) at approximated 3:00 p.m.
2. Approximately 48 hours later, inject sterile human chorionic gonadotropin (5 IU).
3. Approximately 15 hours post-hCG injection, females are euthanized according to the established IUCUC protocol for the given institution.
4. Oviducts are removed by gently cutting away the mesometrium while holding the uterine horn near the oviduct. Remove the fat that allows the ovary to adhere to the

abdominal viscera. Cut between the oviduct and ovary followed by a cut at the top of the uterine horn to remove the oviduct. Place the oviduct in a Petri dish with pre-equilibrated FHM-BSA. All subsequent steps are conducted under a dissection microscope equipped with a stage warmer set to 37°C and fiber-optic lighting. Locate the cumulus mass. Removal of the cumulus mass is accomplished by carefully immobilizing one side of the oviduct with Dumont no. 5 forceps while gently slicing the oviduct on the opposite side with a clean hypodermic needle. If the cumulus mass does not immediately “pop” out of the oviduct, gently tease it out with the blunt side of the needle. Alternatively, a 26 or 30G needle coupled to a syringe filled with medium can be inserted in the ostium and flushed with excess medium to release the cumulus mass from the opposite end of the oviduct.

5. Collect cumulus masses with aid from a fire polished unpulled, 5” glass Pasteur pipette and place in the pre-equilibrated medium FHM-BSA (Millipore, product number MR-024-D) containing 300 µg/mL hyaluronidase and observe to determine when cumulus cells are released.
6. Once denuded, immediately transfer eggs through several drops (approximately 1 mL) of FHM-BSA to wash away any remaining hyaluronidase. Proceed immediately to the next step in the experiment (e.g. treatment with peptide inhibitors or pharmacological agents, immobilization for cytological fixation, or production of a lysate for a biochemical kinase assay, etc.) as spontaneous activation will begin at approximately 9 A.M.

The above procedure details the procurement of cumulus-free mouse eggs for experimental purposes. However, this protocol can be modified to collect fertilized eggs and embryos. To produce fertilized eggs and embryos a male is placed with a female for copulation after hCG injection. The following morning the female is checked for a white copulation (vaginal) plug to indicate that copulation occurred. The day at which the plug is detected is 0.5 days post coitum (dpc), since fertilization is assumed to occur at midnight. Two-cell embryos can be collected at noon 1.5 dpc and four-cell embryos approximately 8-12 hours later. Eight-cell embryos are collected on the morning of 2.5 dpc, while morulas are collected in the evening.

It is most efficient to flush the oviducts with approximately 0.1 mL of FHM-BSA since, beginning at the two-cell stage and beyond, the cumulus cells sloughed off which makes them difficult to locate.

### 6.3 Detergent extraction

The cytoskeleton is the cellular framework composed of actin filaments, intermediate filaments, microtubules and associated protein that regularly reorganize in response to stimuli. Each filament has a subset of signaling proteins responsible for cytoskeletal reorganization. During meiosis, the egg undergoes a remodeling of the cytoskeleton in preparation of the developmental transition of fertilization. If one is interested in studying the cytoskeleton and its associated proteins at a morphological and biochemical level a technique known as detergent extraction can be employed.

Detergent extraction removes the soluble components of the cell through the use of a non-ionic detergent in an intracellular buffer (ICB) that mimics the intracellular milieu. As a

result of this process approximately one-third of the egg's total protein is extracted into a cell fraction referred to as the soluble fraction. Two-thirds remains insoluble and constitutes the detergent-resistant, cytoskeletal fraction. This latter fraction (i.e. the cytoskeletal fraction) contains all three filament systems and those proteins that are tightly bound to each cytoskeletal framework. For eggs and embryos the detergent extraction medium is ICB (Aggeler et al., 1983; Webster and McGaughy, 1990) made 1% with Tween-20 and 200  $\mu\text{g}/\text{mL}$  with the protease inhibitor 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). ICB is composed of 100 mM KCl, 5 mM  $\text{MgCl}_2$ , 3 mM EGTA, 20 mM HEPES, (pH 6.8). Tween-20 is the non-ionic detergent used for eggs and embryos as Triton X-100 was shown to remove selective peptides from and destabilize the cytoskeletal network in mammalian eggs and embryos (Gallicano et al., 1991, 1994).

How long does it take to completely detergent extract cells? The answer to this question is dependent on the cell type the investigator employs and should be determined empirically. To determine the proper extraction time the investigator should detergent extract the cells and take aliquots of the medium at increasing time intervals. The protein containing medium can then be analyzed to determine protein concentration and the plateau at which the proteins are no longer rapidly released from cells. The time that corresponds to the establishment of a plateau should then be used in replicate experiments as the extraction end-point and clearly indicated in the methods section of the manuscript.

### 6.3.1 Detergent extraction procedure for eggs/embryos

1. Eggs/embryos are washed through 3 drops of PBS and placed in detergent extraction medium (ICB made 1% with Tween-20 and 200  $\mu\text{g}/\text{mL}$  AEBSF).
2. Prepare the soluble-fraction and detergent-resistant cytoskeleton as in the detergent extraction section. For biochemical analysis (i.e. gel electrophoresis), eggs/embryos can be transferred into SDS-sample buffer executing caution not to transfer excess fluid with the cytoskeleton. The detergent-soluble fraction is isolated in a large volume and needs to be concentrated; this is done by precipitating the soluble-fraction in ice-cold 95% ethanol and subsequently incubating it at  $-20^\circ\text{C}$  overnight. Centrifuge the precipitated soluble fraction into a pellet and decant the supernatant. The pellet containing the soluble-fraction can then be solubilized by the addition of SDS-sample buffer.
3. The fixative is largely dependent on the type of microscopic analysis being conducted. The detergent-resistant cytoskeleton of eggs/embryos is processed for observation by light or electron microscopy. For instance, if immunocytochemistry is the form of analysis, the detergent-extracted specimen is cytologically fixed by the addition of 0.05% glutaraldehyde 2% formaldehyde made in ICB for 5 minutes at room temperature and then transferred to 2% formaldehyde made in ICB for 25 minutes at room temperature. Any remaining free aldehyde groups are inactivated, and sticky sites are mitigated by transferring eggs/embryos through the blocking solution ICB-BSA (ICB made 1% with bovine serum albumin). This treatment allows the specimen to be transferred through antibody-containing solutions to view specific proteins.

### 6.4 Conventional and embedment-free electron microscopy

Conventional, resin-embedded transmission electron microscopy has been utilized by investigators as a powerful tool to interrogate the ultrastructural changes that accompany

fertilization (Bement and Capco, 1989; 1991; Gallicano et al., 1991, 1994, 1995). However, mammalian eggs and embryos do not afford high contrast images in resin-embedded, ultrathin sections (silver to gold interference patterns corresponding to 60-70 nm). To mitigate the inherent low-contrast of eggs and embryos the authors routinely employ 0.1% tannic acid during glutaraldehyde fixation. Tannic acid acts as a mordant allowing  $\text{OsO}_4$  to more effectively bind. Alternatively, *en block* staining in aqueous 2% uranyl acetate prior to dehydration can be used to further improve contrast of the biological material. The aforementioned steps produce marked electron density for resin-embedded ultrathin sections and provide detail unattainable at the level of the light microscope.

Further detail and three-dimensional information is afforded by removing the embedding medium entirely. This requires employing embedding medium that can be removed without damaging the specimen. By implementing the embedment medium, diethylene glycol disterate (DGD, also referred to as Pentament), Capco and coworkers (1984) refined a procedure for the complete removal of the DGD in HeLa and Madin-Darby Canine Kidney cultured cells for its use in transmission and scanning electron microscopy. Removing embedment not only provides high resolution images, but significantly increased electron density in biological material due to the absence of a resin, and also allowed for the use of thick sections (200 nm sections and greater corresponding to purple interference) at the level of the electron microscope. Gallicano and coworkers exploited the use of DGD in detergent extracted mammalian eggs and for the first time novel cytoskeletal elements known as "sheets" were characterized in mammalian eggs (Gallicano et al., 1991).

#### **6.4.1 Procedure for embedment-free electron microscopy for use with eggs/embryos**

1. Before obtaining eggs/embryos coverslips are prepared by cleaning no. 1 coverglass in a beaker containing acetone by sonication for 3x for 5 minutes each followed by sonication in distilled water for 3x for 5 minutes each. Allow coverslips to dry by placing them on Whatman no. 1 filter paper in a Petri dish protected from dust. Poly-L-lysine (10% w/v) is spread evenly over the surface of the coverslips and placed on a 37°C slide warmer until dry.
2. Obtain eggs/embryos (see Procurement section) and wash through 3 drops of PBS at 37°C.
3. In a glass Petri dish, eggs/embryos are added to prepared coverslips immersed in an excess volume of PBS. The specimens should adhere immediately. If specimens fail to adhere, they are reaspirated washed again and added to a different prepared coverslip. Care should be exercised during manipulation as poly-L-lysine will clog the tip of the micropipette in the event that the tip touches the poly-L-lysine and this is typically followed by a significant loss of sample. Using a disposable, fire polished, 5" glass Pasteur pipette, remove three-fourths total volume of PBS and slowly add copious amounts of extraction buffer. The density of the extraction buffer will displace the PBS. Repeat the controlled addition of extraction buffer 1x. Allow specimens to extract.
4. Remove three-fourths total volume of the extraction buffer and add fresh extraction buffer containing 2% (v/v) glutaraldehyde and 1% formaldehyde for 30 minutes at room temperature.
5. Remove three-fourths total volume of the fixation medium for 3x 15 minutes with 100 mM sodium cacodylate.

6. Post fixation occurs by removal of three-fourths total volume of sodium cacodylate wash buffer and the addition of fresh 1% (v/v) OsO<sub>4</sub> in sodium cacodylate at 4°C in the dark for 1 hour. Upon completion of secondary fixation specimens are washed 3x for 15 minutes in sodium cacodylate containing no OsO<sub>4</sub>.
7. Specimens are then dehydrated through a series of increasing ethanol concentrations starting with 10% ethanol. Remove three-fourths volume of sodium cacodylate wash buffer and add 10% ethanol. Repeat 1x and allow 10-15 minutes time for the specimen to equilibrate. This procedure should be repeated for 30, 50, 70, 90, and 100% ethanol. Exchange 100% ethanol 3x for 20 minutes each with 1:1 100% ethanol: 100% *n*-butyl alcohol. After the third change allow the specimens to equilibrate for 30 minutes. Remove three-fourths volume 1:1 100% ethanol: 100% *n*-butyl alcohol, and replace with 100% *n*-butyl alcohol 3x for 20 minutes each change. After the third change allow the specimens to equilibrate for 30 minutes. Place the Petri dish in a 70°C oven for 15 minutes. Pour off the *n*-butyl alcohol and immediately add prewarmed 1:1 100% *n*-butyl alcohol: 100% DGD. Repeat this 3x for 30 minutes each. Pour off 1:1 100% *n*-butyl alcohol: 100% DGD and add fresh DGD 3x for 30 minutes each.
8. Preheat the flat embedding mold (Ted Pella, Inc., Redding CA; product 10505) in an oven. Add molten DGD to each block of a Teflon-coated flat-embedding mold under a heat lamp. Carefully remove eggs/embryos from the poly-L-lysine with the blunt end of a prewarmed needle. Using a prewarmed, fire-polished pulled pipette, immediately transfer eggs/embryos to the embedding mold and allow the wax to solidify at room temperature. It is necessary to add additional DGD as the block begins to cool and contract. Remove blocks carefully once solidified, as they tend to stick if left for extended periods of time.
9. Trim the block face to produce a square. Blocks are sectioned on an ultramicrotome with a knife angle adjusted to 10° (Capco et al., 1993). 200 nm sections are collected on formvar-coated, carbon-stabilized grids precoated with poly-L-lysine and subsequently dried overnight in a vacuum desiccator.
10. Removal of the wax is accomplished by placing the formvar-coated, carbon-stabilized grids containing thick sections into excess 100% *n*-butyl alcohol for 1 hour at room temperature in a glass Petri dish. After 1 hour gently swirl the dish containing 100% *n*-butyl alcohol and let sit for an additional 15 minutes. Remove the grids and place into a fresh Petri dish containing the transition fluid 1:1 100% ethanol: 100% *n*-butyl alcohol for 15 minutes. Remove the grids and place into 100% ethanol for 1 hour. After 1 hour, carefully swirl the 100% ethanol and let sit for an additional 15 minutes.
11. Dry the specimens through the CO<sub>2</sub> critical point and immediately view the sample with a conventional transmission electron microscope with an accelerating voltage of 60 kV.

## 7. Conclusion

The 11 isotype family of PKC has been identified within both the sperm and egg during key developmental transitions such as gametogenesis, fertilization, and early development, and this suggests that the PKC family may take on multiple, essential tasks. Differential regulation of individual isotypes can occur in a number of ways, most notably through different cofactor requirements for activation of individual isotypes, differential substrate specificity of individual isotypes, and localization or enrichment of individual isotypes.

These mechanisms likely serve to impart distinction among isotypes regarding the function of the kinase.

Elucidation of the possible involvement of PKC during each developmental transition will be expedited by the use of antibodies and inhibitors specific to individual isotypes. For instance, the use of a pan- (i.e. total) antibody directed against all of the PKC isotypes has been previously studied in great detail in this area, and is therefore counterproductive for use in a manuscript. In addition, care should be employed with the use of older, concentration-dependent PKC inhibitors as the interpretation of the results obtained by the use of these inhibitors can be difficult as these inhibitors can interact with multiple kinases. Furthermore, investigations focusing on localization or enrichment of specific PKC isotypes will likely reveal signaling-mediated changes during developmental transitions. These studies may expose the possible redundancy of specific isotypes, i.e. are there backup mechanisms in place in the event of a complete dysfunction of an individual isotype? Lastly, few have studied the potential role of PKM, the catalytic subunit of cPKCs that was shown to interact in the egg cytoskeletal interior. Studies directed towards PKM may reveal time-dependent changes in the egg interior that have long been ignored.

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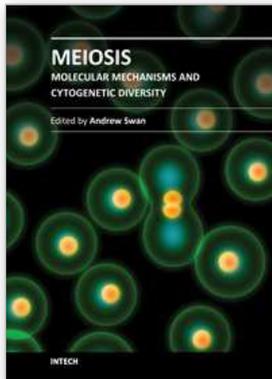
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## **Meiosis - Molecular Mechanisms and Cytogenetic Diversity**

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Meiosis, the process of forming gametes in preparation for sexual reproduction, has long been a focus of intense study. Meiosis has been studied at the cytological, genetic, molecular and cellular levels. Studies in model systems have revealed common underlying mechanisms while in parallel, studies in diverse organisms have revealed the incredible variation in meiotic mechanisms. This book brings together many of the diverse strands of investigation into this fascinating and challenging field of biology.

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