PKC signaling at fertilization in mammalian eggs

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Abstract

Protein kinase C (PKC) has been proposed to regulate major egg activation events during mammalian fertilization. Most of the evidence supporting this assumption has first been obtained using pharmacological activation and inhibition of the kinase, while egg activation was assessed by checking for exocytosis of the cortical granules, extrusion of the second polar body and formation of pronuclei. However, results have been inconclusive and sometimes contradictory regarding the exact role of PKC in regulating egg activation events. The PKC family is composed of various isotypes, which differ in their modular structures and regulatory properties. Hence the need to re-examine the roles of egg PKCs more specifically. Mammalian eggs express many PKC isotypes, the roles of which have been investigated using immunodetection, isotype-specific inhibition and, more recently, live imaging of fluorescent chimaeras. Here, I review the recent development of PKC research in mammalian fertilization and the evidence for a specific role for certain PKC isotypes in fertilization-induced egg activation.

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1. Introduction

Protein kinase C (PKC) is a ubiquitous serine/threonine kinase which plays a pivotal role in the regulation of numerous signaling pathways. Originally, PKC was identified as a Ca\(^{2+}\)-activated kinase regulated by anionic lipids and diacylglycerol (DAG) [1,2]. Accordingly, one paradigm for PKC activation is the hydrolysis of PtdIns(4,5)P\(_2\) by phospholipase C (PLC), which generates both a Ca\(^{2+}\) signal (via InsP\(_3\)-induced Ca\(^{2+}\) release) and DAG. The PKC family comprises several isotypes with different modular structures and cofactor requirements, which may underlie their different substrate specificities [1,2]. Conventional PKCs \(\alpha\), \(\beta I\), \(\beta II\) and \(\gamma\) (cPKC) possess a DAG-binding C1 domain and a Ca\(^{2+}\)-binding C2 domain and are thus coincidence detectors of Ca\(^{2+}\) and DAG signals [3]. Novel PKCs \(\delta\), \(\epsilon\), \(\eta\) and \(\theta\) (nPKC) lack a Ca\(^{2+}\)-dependent C2 domain, but possess a C1 domain which confers sensitivity to DAG. Atypical PKCs \(\zeta\) and \(\upsilon\lambda\) (aPKC) are both Ca\(^{2+}\)- and DAG-insensitive and their allosteric activators are still poorly characterized. However, phosphorylation may play a crucial role in their activation. Indeed, all PKC isotypes need to be phosphorylated at specific sites in the kinase domain to become stable, protease-resistant and catalytically competent [2,4]. This priming process involves both autophosphorylation and phosphorylation by an upstream kinase which in somatic cells has been identified primarily as PDK1 [2,4].

Fertilization in mammalian eggs is characterized by the generation of long-lasting Ca\(^{2+}\) oscillations which are believed to result from PtdIns(4,5)P\(_2\) hydrolysis and InsP\(_3\) production [5]. Therefore, DAG is believed to be generated at fertilization and to participate in PKC activation, though direct evidence to support this assumption is lacking. The fertilization-competent mammalian egg is arrested at metaphase of the second meiotic division (MII). Fertilization-induced Ca\(^{2+}\) oscillations trigger the resumption of meiosis and extrusion of the second polar body, so that the egg provides a haploid set of chromosomes to the one-cell embryo. They also trigger cortical granule exocytosis (CGE) and the zona-block to polyspermy, and ultimately the formation of pronuclei and entry into the first mitotic...
PKC signaling has been proposed to regulate these early and late fertilization events in mammalian eggs. Here I will review the experimental evidence supporting a role for PKC during mammalian fertilization, and I will focus on recent studies that have investigated the role of specific PKC isotypes and the spatiotemporal dynamics of PKC activation at fertilization.

1.1. PKC isotypes in mammalian eggs

Like most somatic cells, mammalian eggs are well equipped for PKC signaling and members of all three subfamilies of PKCs have been identified at the mRNA and/or protein levels in mammalian eggs [7–13]. For instance, mouse MII eggs express PKC \( \alpha \), \( \gamma \), \( \delta \), \( \zeta \), \( \lambda \), and possibly PKC \( \beta I \) and \( \beta II \) [7,9,11–13]. In contrast, only the transcripts for PKC \( \epsilon \) and \( \delta \) were detectable at this stage, but new messenger RNAs encoding other PKC isotypes are detected after fertilization, in the dividing embryo [7]. Fertilization and the early stages of embryonic development are therefore strongly reliant on the maternal pool of PKCs, which are most likely synthesized during oogenesis.

1.2. Evidence for PKC activation at fertilization

Activation of PKCs is associated with their translocation to the subcellular compartment where the kinases are needed. Thus, cPKCs activation is characterized by their translocation to the plasma membrane where the C1 and C2 domains interact with DAG and anionic lipids, respectively [3]. Monitoring the dynamic redistribution of PKCs in fertilized eggs could therefore indicate their site of action at fertilization. The first attempt was made by the group of David Capco, using Rim-1, a rhodamine-conjugated bisindolylmaleimide which binds to the ATP binding site of both inactive and active PKCs. The authors found that PKC staining accumulated at the plasma membrane and was enriched in the polar body area, in mouse eggs fixed 45 min after fertilization [14]. However, the PKC isotypes involved were not identified in this study. Because of their ability to act as Ca\(^{2+}\) sensors, cPKCs are very well suited for acting as effectors of fertilization-induced Ca\(^{2+}\) oscillations. Their distribution was investigated in fixed eggs using specific antibodies and immunodetection. Thus, plasma membrane translocation was reported for PKC\( \alpha \) and PKC\( \beta I \) in mouse eggs fixed 10 min after sperm addition [11]. Similar findings were reported for PKC\( \alpha \), \( \beta I \) and \( \beta II \) in fertilized rat eggs [15] and PKC\( \alpha \) in fertilized pig eggs [10].

PKC activation was also revealed biochemically in PKC activity assays using a known PKC substrate. Thus, an increase in PKC activity was detected 10 min after insemination of mouse eggs with sperm, and this activation persisted 20 min later when eggs started to enter anaphase [12]. Using the same methodology, Gallicano et al. [14] reported that PKC activity reached a peak within the first hour after fertilization. Interestingly, Tatone et al. [12] recently reported that PKC activation at fertilization was almost unaffected when Ca\(^{2+}\) elevations were buffered using Ca\(^{2+}\) chelators, suggesting that most of the PKC activity was supported by Ca\(^{2+}\)-independent PKC isotypes such as PKC\( \delta \). Although there is little doubt that cPKCs are rapidly activated at fertilization [16], the hypothesis of a Ca\(^{2+}\)-independent PKC activation pathway is challenging and recent studies support a role for Ca\(^{2+}\)-independent PKCs in fertilized eggs (see below).

1.3. The use of PMA as a PKC agonist to mimic fertilization events

The phorbol ester PMA (also called TPA) as well as synthetic DAGs have been extensively used to mimic some events of fertilization in mammalian eggs. The major event associated with PMA stimulation is PKC activation and translocation. We found that PMA could indeed trigger an irreversible translocation of cPKC to the plasma membrane of living mouse eggs (Fig. 1). Importantly, this was not accompanied by any significant increase in [Ca\(^{2+}\)], (data not shown). PMA and synthetic DAGs can trigger CGE and

Fig. 1. Recruitment of PKC\( \alpha \) at the plasma membrane by PMA. The left image shows the homogeneous distribution of EGFP-PKC\( \alpha \) in an unfertilized mouse egg, before PMA addition. The right image shows the same egg after a 5-min incubation with 10 ng/ml (=16.2 nM) PMA. Note that EGFP-PKC\( \alpha \) has virtually disappeared from the cytosol to become irreversibly membrane-bound in the presence of PMA.
partial modification of the zona pellucida in unfertilized eggs, without raising cytosolic Ca\(^{2+}\) (reviewed in Ref. [17]). In addition, PMA has been reported to trigger the exit from MII arrest and pronuclei formation, and to initiate polar body emission in hamster and mouse eggs [11,14,18–20]. Conversely, inhibition of PKC was found to reduce second polar body and pronuclei formation in fertilized eggs [14]. Collectively, these findings led many investigators to the conclusion that PKC was a major regulator of egg activation, acting downstream of the fertilization Ca\(^{2+}\) signal. However, these second polar body-like structures were eventually resorbed, revealing a failure to complete cytokinesis. Also, the pattern and extent of cortical granule release by PMA differ from what is observed after fertilization [21]. Finally, Ducibella and LeFevre [21] demonstrated that although PMA-induced CGE was inhibited by the PKC inhibitor bisindolylmaleimide, fertilization-induced CGE was not. Therefore, these studies did not clearly establish a role for PKC in egg activation (as discussed in Ref. [22]).

What then is the mechanism for PMA-induced cortical granule release? DAG- and PMA-binding C1 domains are found in a number of non-PKC proteins, including the Munc-13 family of scaffolding proteins, which have been found to mediate the stimulatory effect of PMA on synaptic vesicle release [23]. The role of PMA in triggering CGE should then be reevaluated in the light of these findings. There is still the possibility that cPKCs, which are indeed activated and recruited to the egg cortex during Ca\(^{2+}\) release (see below), modulate the kinetics and extent of CGE in synergy with Ca\(^{2+}\), for instance by promoting the recruitment of the granules to the sites of exocytosis or by sensitizing the fusion machinery to Ca\(^{2+}\).

1.4. Activation of cPKC, nPKC and aPKC at fertilization

The understanding of PKC signaling has been substantially improved by the development of chimaeric PKCs coupled to the green fluorescent protein, allowing visualization of PKC dynamics in living cells. We recently applied this strategy to mouse eggs expressing GFP-tagged PKC\(\alpha\) or PKC\(\gamma\) and GFP-tagged C1 and C2 domains [16]. Strikingly, the GFP-cPKCs were found to rapidly translocate from the cytosol to the plasma membrane during each fertilization-induced Ca\(^{2+}\) transient (Fig. 2). We found that translocation was driven essen-
tially by the C2 domain, when $[\text{Ca}^{2+}]$, reaches micromolar levels. Furthermore, we demonstrated for the first time that manipulating PKC activity could affect dramatically the pattern of $\text{Ca}^{2+}$ oscillations at fertilization. PMA or PKCo overexpression was shown to promote $\text{Ca}^{2+}$ oscillations, while bisindolylmaleimide was inhibitory. Finally, we presented evidence that cPKCs may control $\text{Ca}^{2+}$ oscillations, while bisindolylmaleimide was inhibitory.

Two groups recently investigated the subcellular localization of novel PKCo in fertilized mouse eggs, using specific antibodies and immunofluorescence. Tatone et al. [12] reported that PKCo was diffusely distributed in unfertilized eggs, but became associated with spindle microtubules after fertilization. A similar translocation to the spindle was observed in eggs loaded with a $\text{Ca}^{2+}$ chelator. In addition, PKCo was phosphorylated in MII eggs, but becomes dephosphorylated following fertilization. These data suggest that the activity and/or localization of the PKCo, corresponding to the C-terminal, catalytic fragment of the kinase [9]. Proteolysis is a mechanism for PKC down-regulation that may occur during persistent activation of PKC [2]. Cleavage in the hinge region that links the catalytic and regulatory domains releases an active PKC catalytic fragment that can phosphorylate targets in the cytosol. This fragment, often referred to as PKM, was identified in activated hamster eggs where it is thought to phosphorylate cytoskeletal proteins that mediate the reorganization of intermediate filaments [24]. The presence of a truncated PKCo in mouse eggs was also reported recently by Baluch et al. [13]. These studies suggest that PKCo may play a critical role in spindle dynamics and meiotic resumption after fertilization.

The role of atypical PKCz has been investigated recently in mouse eggs [13]. In unfertilized eggs, PKCz is tightly bound to spindle microtubules, and is enriched at the spindle poles. After activation with a $\text{Ca}^{2+}$ ionophore, PKCz remains associated with the spindle in eggs transiting through anaphase and telophase, and becomes enriched on midzone microtubules. Interestingly, in one-cell embryos at the pronuclear stage, PKCz is enriched in the egg cortex. Furthermore, using an antibody directed against the active (phosphorylated) form of the kinase, active PKCz was found essentially at the spindle poles of MII eggs, and also on midzone microtubules in activated eggs. Inactivation of this isotype with an inhibitory pseudosubstrate peptide caused a disruption of spindle microtubules and a misalignment of the chromosomes in the MII eggs. These findings strongly suggest that PKCz regulates spindle stability, possibly via the phosphorylation of components of the microtubule organizing centers and/or spindle-associated MAP kinases (as discussed in Ref. [13]). The activation mechanism of PKCz is still unknown, but may involve phosphorylation by an upstream kinase that remains to be identified.

1.5. The role of diacylglycerol

For maximal activation, cPKCs and nPKCs need to bind to DAG via their C1 domain [1–3]. Because of the difficulty to measure DAG at fertilization using traditional biochemical assays, we attempted to monitor DAG generation during mouse egg fertilization using the DAG probe C1-GFP [3]. However, we did not detect any plasma membrane translocation of the probe [16]. This is contrasting with the fact that cPKCs accumulate rapidly at the plasma membrane and may indicate that DAG generation is too small to trigger a significant translocation of the DAG probe (as discussed in Ref. [16]). However, for DAG to participate in cPKC activation at fertilization, one would expect DAG to be generated everywhere in the plasma membrane to match cPKC localization (see Fig. 2). I would like to suggest an alternative hypothesis, in which maximal cPKC activation is not reached via a coincident production of DAG, but rather via interaction with plasma membrane PtdIns(4,5)P2. Indeed, numerous studies have reported that, in vitro, PtdIns(4,5)P2 can replace DAG as a cofactor for maximal PKC activation, possibly by occupying the DAG binding site (see [16] and references therein). In this model, there is no need for PtdIns(4,5)P2 hydrolysis and DAG generation all over the plasma membrane. A better understanding of the role of DAG in PKC activation will need the elucidation of the spatiotemporal dynamics of PLC activation and PtdIns(4,5)P2 hydrolysis in fertilized eggs.

2. Conclusion

To answer the question asked by Jones [22] six years ago, the role of PKC at fertilization has not been overstated but rather undervalued. Indeed, recent investigations have provided evidence for the involvement of all three subfamilies of PKC during mammalian fertilization. cPKCs are essential for the generation of long-lasting $\text{Ca}^{2+}$ oscillations and, therefore, play a major regulatory role in all aspects of egg activation. PKCo and $\zeta$ have a more discrete subcellular localization and may regulate the dynamics and integrity of the meiotic spindle and cell-cycle progression. Further investigations are needed to identify the substrates of each PKC isotype. cPKCs are likely to phosphorylate membrane proteins involved in store-operated $\text{Ca}^{2+}$ entry, and possibly proteins from the exocytosis machinery. PKCo and $\zeta$ certainly phosphorylate proteins associated with the meiotic spindle, including tubulin itself.
For such a dynamic process as PKC translocation, the use of GFP-chimaeras has proven a more accurate approach than PKC immunodetection in fixed eggs. However, the latter technique has the advantage to detect endogenous PKCs. The generation of GFP constructs for nPKCs and αPKCs will certainly help in resolving the activation mechanisms and roles of these PKC isotypes. Also, expression of dominant-negative mutants or the use of the RNA interference strategy seems particularly suited to the investigation of PKC signaling in eggs. Conversely, the use of PMA as a PKC agonist must be considered with caution.

Finally, an interesting challenge will be to identify the upstream kinase(s) responsible for phosphorylating PKCs in mammalian eggs. This phosphorylation step is fundamental for PKC activity and may be under the control of specific signaling pathways during oocyte growth and maturation, and possibly during egg activation at fertilization.

References


