Regulation of diacylglycerol production and protein kinase C stimulation during sperm- and PLCζ-mediated mouse egg activation

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Background information. At fertilization in mammalian eggs, the sperm induces a series of Ca\(^{2+}\) oscillations via the production of inositol 1,4,5-trisphosphate. Increased inositol 1,4,5-trisphosphate production appears to be triggered by a sperm-derived PLCζ (phospholipase C-ζ) that enters the egg after gamete fusion. The specific phosphatidylinositol 4,5-bisphosphate hydrolytic activity of PLCζ implies that DAG (diacylglycerol) production, and hence PKC (protein kinase C) stimulation, also occurs during mammalian egg fertilization. Fertilization-mediated increase in PKC activity has been demonstrated; however, its precise role is unclear.

Results. We investigated PLCζ- and fertilization-mediated generation of DAG in mouse eggs by monitoring plasma-membrane translocation of a fluorescent DAG-specific reporter. Consistent plasma-membrane DAG formation at fertilization, or after injection of physiological concentrations of PLCζ, was barely detectable. However, when PLCζ is overexpressed in eggs, significant plasma-membrane DAG production occurs in concert with a series of unexpected secondary high-frequency Ca\(^{2+}\) oscillations. We show that these secondary Ca\(^{2+}\) oscillations can be mimicked in a variety of situations by the stimulation of PKC and that they can be prevented by PKC inhibition. The way PKC leads to secondary Ca\(^{2+}\) oscillations appears to involve Ca\(^{2+}\) influx and the loading of thapsigargin-sensitive Ca\(^{2+}\) stores.

Conclusions. Our results suggest that overproduction of DAG in PLCζ-injected eggs can lead to PKC-mediated Ca\(^{2+}\) influx and subsequent overloading of Ca\(^{2+}\) stores. These results suggest that DAG generation in the plasma membrane of fertilizing mouse eggs is minimized since it can perturb egg Ca\(^{2+}\) homoeostasis via excessive Ca\(^{2+}\) influx.

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Key words: Ca\(^{2+}\) store, diacylglycerol, egg activation, fertilization, protein kinase C, phospholipase C-ζ (PLCζ).

Abbreviations used: BAPTA, bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid; CHE, chelerythrine chloride; cps, counts per seconds; DAG, diacylglycerol; fura 2/AM, fura 2 acetoxymethyl ester; GFP, green florescent protein; hCG, human chorionic gonadotropin; CCD camera, charge-coupled-device camera; IVF, in vitro fertilization; ICCD camera, intensified CCD camera; PKC, protein kinase C; (m)PLC, (mouse) phospholipase C; PM/C ratio, plasma membrane/cytosol ratio.

Introduction

During fertilization in mammalian eggs, the sperm stimulates a series of intracellular Ca\(^{2+}\) oscillations that is responsible for activating the egg (Stricker 1999; Miyazaki and Ito, 2006). These Ca\(^{2+}\) oscillations stimulate calmodulin-dependent protein kinase II, which can trigger meiotic resumption and early development in mouse eggs (Madgwick et al.,...
The Ca\textsuperscript{2+} oscillations at fertilization are triggered by the production of inositol 1,4,5-trisphosphate, which causes release of Ca\textsuperscript{2+} from intracellular stores (Miyazaki et al., 1993; Lee et al., 2006). The sperm appears to trigger the increase in inositol 1,4,5-trisphosphate production at fertilization by introducing a sperm-specific PLC (phospholipase C) (PLC\textsubscript{ζ}) into the egg cytoplasm following sperm–egg fusion (Saunders et al., 2002; Knott et al., 2005; Lee et al., 2006; Miyazaki and Ito, 2006; Swann et al., 2006). Since PLC\textsubscript{ζ} hydrolyses [PIP\textsubscript{2} (phosphatidylinositol 4,5-bisphosphate)], it generates both inositol 1,4,5-trisphosphate and DAG (diacylglycerol) in eggs (Kouchi et al., 2004; Nomikos et al., 2005). While many studies have reported Ca\textsuperscript{2+} oscillations in PLC\textsubscript{ζ}-injected eggs, there have been no investigations of PLC\textsubscript{ζ}-stimulated generation of DAG.

The main signalling target for DAG in cells is PKC (protein kinase C) (Nishizuka, 1992). It has been shown that PKC activity increases at fertilization in mouse eggs (Gallicano et al., 1997; Tatone et al., 2003). Furthermore, mouse and rat eggs have been shown to express a number of different isoforms of PKC including the conventional α, β and γ isoforms that are activated by Ca\textsuperscript{2+} and DAG, the novel isoform δ, which is stimulated by DAG, and the atypical isoforms ζ and λ, which are not stimulated by either Ca\textsuperscript{2+} or DAG (Jones, 1998; Pauken and Capco, 2000; Baluch et al., 2004; Halet, 2004). The translocation of α, β and γ isoforms to the egg cortex has been demonstrated after sperm–egg interaction or after Ca\textsuperscript{2+} ionophore-induced egg activation (Luria et al., 2000; Halet et al., 2004). PKCδ and PKCζ are localized to the meiotic spindle and may play a role in spindle stability and progression through meiosis (Tatone et al., 2003; Viveiros et al., 2003; Baluch et al., 2004). A role for PKC at fertilization is suggested by the finding that the potent PKC activator, PMA, can stimulate the initial stages of second polar body emission and trigger a degree of cortical granule exocytosis in mouse eggs (Gallicano et al., 1997; Eliyahu and Shalgi, 2002). PKC may also regulate Ca\textsuperscript{2+} signals in eggs since some studies have shown that PMA causes small Ca\textsuperscript{2+} oscillations (Cuthbertson and Cobbold, 1985) and, more recently, it was shown that PMA can stimulate store-operated Ca\textsuperscript{2+} influx (Halet et al., 2004). However, the role of PKC activation at fertilization is unclear because while some inhibitors of PKC block events such as meiotic resumption, cortical granule exocytosis and transcription at the one-cell stage (Gallicano et al., 1997; Yu et al., 2007), other inhibitors are without any effect (Ducibella and Lefèvre, 1997).

The dynamic translocation of PKCs to the plasma membrane of fertilizing mouse eggs was previously measured using GFP (green florescent protein)-tagged PKCα or PKCγ (Halet et al., 2004). It was shown that these PKC isoforms translocate to the plasma membrane with similar kinetics to the sperm-induced Ca\textsuperscript{2+} increases (Halet et al., 2004). The translocation is transient and largely dependent on the amplitude of the initial Ca\textsuperscript{2+} increase, and was mainly driven by the Ca\textsuperscript{2+}-dependent C2 domain of these conventional PKC isoforms. Here, we investigate DAG production and PKC activation in fertilizing, and PLCζ-injected, mouse eggs using a fluorescent probe for DAG based on the tandem C1 domain of PKCδ (C1δ–GFP; Codazzi et al., 2001; Dries et al., 2007). We find that minimal DAG production occurs in the plasma membrane at fertilization, or after physiological levels of PLCζ injection, in mouse eggs. However, overproduction of DAG by high levels of PLCζ can lead to excessive Ca\textsuperscript{2+} influx and an unusual pattern of high-frequency Ca\textsuperscript{2+} oscillations.

**Results**

**C1δ–GFP detects plasma-membrane DAG in mouse eggs**

In a previous study, the C1 domain of conventional PKCα was used as a DAG biosensor in mouse eggs, but it failed to detect any DAG in the plasma membrane of fertilized eggs (Halet et al., 2004). We re-investigated DAG synthesis in fertilized eggs using tandem C1 domain of PKCδ (C1δ–GFP), which is reported to have an affinity for DAG binding two orders of magnitude higher than the binding affinity of the C1 domain from PKCα (Dries et al., 2007). Figure 1(A) shows that ionomycin treatment of unfertilized mouse eggs caused a large Ca\textsuperscript{2+} increase and a delayed translocation of C1δ–GFP to the plasma membrane, indicating DAG production. In contrast, the addition of 100 ng/ml PMA to eggs caused an immediate increase in C1δ–GFP plasma-membrane translocation without any increase in Ca\textsuperscript{2+}
The translocation of C12-δ–GFP, indicating DAG accumulation, was measured as the plasma-membrane fluorescence divided by the bulk cytoplasmic fluorescence (PM/C ratio) (dotted line, labelled ‘DAG’). The fluorescence of Fura Red was monitored to indicate changes in Ca^{2+} (solid line, labelled ‘Ca^{2+}’). Fura Red fluorescence decreases on Ca^{2+} binding, so we inverted the fluorescence signal of Fura Red (at 488 nm excitation) to represent Ca^{2+} levels. The slight drift up in the trace with time was due to some loss of dye from the egg. The insets show example images of eggs taken at the time indicated by the arrows. In (A), the egg was exposed to 2 μM ionomycin, which caused an immediate Ca^{2+} increase and a delayed C12-δ–GFP translocation from cytosol to the plasma membrane (n = 10). In (B), eggs treated with 100 ng/ml PMA showed an immediate C12-δ–GFP translocation without a Ca^{2+} increase (n = 11). In (C), a recording is shown for 1 of 2 eggs that showed a small C12-δ–GFP translocation during the initial Ca^{2+} increase at fertilization. In (D), a typical trace is shown from the remaining 12 eggs where there was no detectable increase in C12-δ–GFP translocation during Ca^{2+} oscillations at fertilization.

These results show that C12-δ–GFP can effectively report DAG production at the plasma membrane in mouse egg, which is presumably due to the activity of endogenous Ca^{2+}-sensitive PLCs (Halet et al., 2004). When we examined eggs at fertilization, we found that translocation of C12-δ–GFP to the plasma membrane was only just detectable in two eggs (Figure 1C), whereas in most of the eggs at fertilization (12/14) there was no evidence for translocation to the plasma membrane (Figure 1D). In the two eggs that showed evidence of C12-δ–GFP translocation, this was restricted only to the initial Ca^{2+} increase at fertilization and tended to be evident in a restricted portion of the cortex. C12-δ–GFP did not show any clear localization in the meiotic spindle either before or after fertilization. These results suggest that there is very little DAG production in the plasma membrane of fertilizing mouse eggs in comparison with that seen with ionomycin.

**High levels of PLCζ promote DAG synthesis in mouse eggs**

PLCζ triggers Ca^{2+} oscillations similar to those seen at fertilization, but unlike fertilization it can be expressed in eggs at a range of different concentrations that can be quantified with a luciferase tag (Nomikos et al., 2005; Yu et al., 2008). Figure 2 shows the patterns of Ca^{2+} oscillations triggered by injection of luciferase-tagged PLCζ RNA (PLCζ–luc) at various concentrations. Increasing levels of mouse PLCζ expression led to an increase in Ca^{2+} oscillation frequency. However, when expression was higher than...
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Figure 2 | The patterns of Ca\(^{2+}\) oscillations triggered by PLC\(\varepsilon\)-luc

Different concentrations of mPLC\(\varepsilon\)-luc RNA (0.05–1 \(\mu g/\mu l\)) were injected into mouse eggs. The oscillations in Ca\(^{2+}\) were indicated by changes in fluorescence intensity (in arbitrary units) of Oregon Green BAPTA. The relative expression levels of PLC\(\varepsilon\)-luc protein are indicated by luminescence in photon cps along the left-hand side of each fluorescence trace. The traces are representative and were selected from 150 eggs (in the range of 0.1–40 cps) and the expression level on the left-hand side is that determined for each sample egg (rounded to two significant figures). Low PLC\(\varepsilon\)-luc expression was associated with long-lasting low-frequency Ca\(^{2+}\) oscillations, whereas oscillations similar to fertilization were associated with expression levels of 1–5 cps. High levels of PLC\(\varepsilon\)-luc expression were associated with two distinct series of Ca\(^{2+}\) oscillations.

10 cps (counts per seconds), Ca\(^{2+}\) oscillations ceased prematurely, and the highest expression levels (20–40 cps) were associated with an unexpected secondary series of high-frequency Ca\(^{2+}\) oscillations that occurred with a delay of 3–4 h (Figure 2). When we examined C1\(\varepsilon\)δ–GFP translocation in PLC\(\varepsilon\)-injected eggs, we found that the initial series of Ca\(^{2+}\) oscillations was not associated with any evidence of translocation (Figure 3). However, with high PLC\(\varepsilon\) expression levels, and when secondary Ca\(^{2+}\) oscillations were initiated, C1\(\varepsilon\)δ–GFP translocation to the plasma membrane was clearly detectable in all eggs in synchrony with the initial Ca\(^{2+}\) transient of the secondary series. These results suggest that DAG production can occur in the plasma membrane of mouse eggs when PLC\(\varepsilon\) is overexpressed for inducing a secondary series of Ca\(^{2+}\) oscillations.

PKC activation and Ca\(^{2+}\) influx promote secondary Ca\(^{2+}\) oscillations

The premature cessation, and the subsequent generation of a delayed secondary series of Ca\(^{2+}\) oscillations, is a novel feature of high levels of PLC\(\varepsilon\) expression. Previous studies have suggested that pronuclear formation can regulate Ca\(^{2+}\) oscillations by sequestering PLC\(\varepsilon\) (Larman et al., 2004). However, pronuclear formation or dissolution did not play any role in the pattern of Ca\(^{2+}\) changes in eggs overexpressing PLC\(\varepsilon\), because the basic phenomena of premature cessation and secondary oscillations were still seen in eggs that had been enucleated (results not shown). The cessation of Ca\(^{2+}\) oscillations could occur because of inositol 1,4,5-trisphosphate receptor down-regulation, since this can be induced by injection of agents such as the inositol 1,4,5-trisphosphate receptor agonist, adenophostin, which causes a single series of Ca\(^{2+}\) oscillations (Brind et al., 2000;
DAG production during mouse egg activation

Figure 4 | Secondary Ca\(^{2+}\) oscillations triggered by PMA and raising extracellular Ca\(^{2+}\)

Eggs were injected with adenophostin (Ad), or with PLC\(_{\zeta}\) to cause low levels of expression (luciferase expression indicated in parentheses), or they were fertilized (IVF). In each case, a single series of Ca\(^{2+}\) oscillations was seen and then PMA (1 \(\mu\)g/ml, at the time indicated by the arrows) was added (in A–C). The addition of PMA led to another set of Ca\(^{2+}\) oscillations in most of the eggs (the number of eggs responding is indicated above each trace). In a similar set of experiments (D–F), the extracellular Ca\(^{2+}\) was increased to 10 mM after the cessation of the initial Ca\(^{2+}\) oscillations. In these cases, all the eggs responded to the raising of extracellular Ca\(^{2+}\) by showing a second set of Ca\(^{2+}\) oscillations (the numbers of eggs are indicated above each sample trace).

Jellerette et al., 2000). The occurrence of secondary Ca\(^{2+}\) oscillations, however, is not obviously explained by this down-regulation mechanism.

We then considered the possibility that DAG production and hence PKC stimulation could play a role in causing the secondary oscillations. Figure 4 shows eggs that had been injected either with adenophostin, or with PLC\(_{\zeta}\) (corresponding to low levels of expression), or else fertilized in vitro by sperm. With each of these stimuli, there is normally only a singular series of Ca\(^{2+}\) oscillations. However, when PMA was applied after the cessation of Ca\(^{2+}\) oscillations, we found that most of the eggs underwent an extra series of Ca\(^{2+}\) oscillations (Figures 4A–4C). These secondary series of Ca\(^{2+}\) oscillations were of high frequency and resembled the pattern of secondary Ca\(^{2+}\) oscillations seen with high PLC\(_{\zeta}\) expression.

Previous studies in mouse eggs have suggested that PMA can increase the rate of Ca\(^{2+}\) influx (Halet et al., 2004). If the above effects of PMA are mediated via Ca\(^{2+}\) influx, then it should be possible to stimulate secondary Ca\(^{2+}\) oscillations by increasing extracellular Ca\(^{2+}\) levels. Figure 4 shows eggs that had again been injected either with adenophostin, or with PLC\(_{\zeta}\) (low level of expression), or that had been fertilized in vitro. After the normal primary series of Ca\(^{2+}\) oscillations had ceased, raising extracellular Ca\(^{2+}\) to 10 mM led to a secondary series of Ca\(^{2+}\) oscillations that again resembled the high-frequency response observed with high PLC\(_{\zeta}\) expression (Figures 4D–4F). These results suggest that stimulation of PKC and subsequent Ca\(^{2+}\) influx can produce secondary Ca\(^{2+}\) oscillations in mouse eggs.

If PKC plays a role in triggering the secondary Ca\(^{2+}\) oscillations, we should be able to inhibit PLC\(_{\zeta}\)-induced secondary oscillations by blocking PKC activity. The PKC inhibitor, CHE (chelerythrine chloride), has previously been shown to be an effective inhibitor of PKC activity in mouse eggs (Ducibella and Lefevre, 1997; Gallicano et al., 1997). Figure 5 shows the effects of adding CHE to mouse eggs that were injected with PLC\(_{\zeta}\). We found that the occurrence of PLC\(_{\zeta}\)-induced secondary Ca\(^{2+}\) oscillations was virtually abolished by treatment with CHE.

Figure 5 | PLC\(_{\zeta}\)-luc induced Ca\(^{2+}\) oscillations in eggs treated with the PKC inhibitor CHE

In (A), two example traces are shown where eggs were expressing high relative levels of PLC\(_{\zeta}\)-luc expression (32 cps). The top traces show the usual pattern of initial and secondary Ca\(^{2+}\) oscillations, whereas the bottom trace shows that secondary Ca\(^{2+}\) oscillations did not occur in egg incubated with 0.8 \(\mu\)M CHE. In (B), the histogram summarizes data on eggs treated with CHE (open bars) or control eggs without CHE (filled bars). The expression levels are placed into groups according to the expression levels and the numbers of eggs for each group are indicated above columns bars. The asterisks indicate statistically significant differences between groups (\(P < 0.05\)).
CHE (0.8 μM). The results collected from almost 300 eggs showed that incubation with CHE reduced the occurrence of secondary Ca$^{2+}$ oscillations from 37.8% (8/23) to 10.6% (5/47), and 85.7% (12/14) to 16.7% (3/18) in the PLCζ expression ranges of 15–30 and 30–45 cps respectively. These results support the hypothesis that increased PKC activity plays a role in triggering secondary Ca$^{2+}$ oscillations in mouse eggs.

**PKC activation promotes Ca$^{2+}$ influx and overloading of the Ca$^{2+}$ stores**

Since PKC-driven Ca$^{2+}$ influx could cause the secondary Ca$^{2+}$ oscillations, we sought to assay Ca$^{2+}$ influx in eggs. The Mn$^{2+}$-induced quenching of fura 2 fluorescence has previously been used as a surrogate for monitoring the rate of Ca$^{2+}$ influx into mouse eggs (McGuiness et al., 1996; Mohri et al., 2001). To establish whether PKC activity stimulates bivalent cation influx, eggs were treated with 1 μg/ml PMA for 1 h before the addition of Mn$^{2+}$. The trace in Figure 6(A) shows the quenching of fura 2 and indicates that PMA significantly stimulated the Mn$^{2+}$ influx rate (4.93 ± 0.54 relative fluorescence units/s decrease, n = 25 compared with the control eggs; 1.5 ± 0.1 fluorescence units, n = 19, P < 0.01). In order to investigate whether Ca$^{2+}$ influx was related to the occurrence of secondary Ca$^{2+}$ oscillations, we used the same Mn$^{2+}$ quenching protocol at different time points in eggs injected with either adenophostin or high PLCζ concentrations. We compared overexpression of PLCζ with the effects of adenophostin injected, since adenophostin only causes a single set of Ca$^{2+}$ oscillations and it is not expected to cause significant DAG production since it generates moderate Ca$^{2+}$ increase compared with ionomycin (Brind et al., 2000; Jellerette et al., 2000). Eggs at 2 and 4 h after mPLCζ (mouse PLCζ) or adenophostin injection were loaded with fura 2 and then exposed to Mn$^{2+}$ for inducing fura 2 quenching (Figures 6B and 6C). We chose the 2 h time point because all of the eggs should be completing the primary oscillations phase, whereas at 4 h the PLCζ–luc injected eggs should be commencing the secondary Ca$^{2+}$ oscillations. At 2 h post-injection, the quenching rate (measured over 150 s) in both mPLCζ (2.7 ± 0.30, n = 9) and adenophostin A (2.7 ± 0.15, n = 21) injected eggs were similar, but higher than that in control eggs (1.8 ± 0.12, n = 23). However, at 4 h after injection, the quenching rate became higher (3.54 ± 0.39, n = 11) in mPLCζ–luc injected eggs, while it returned to control levels in adenophostin-injected eggs (2.1 ± 0.2, n = 15). These data support previous data on Ca$^{2+}$ influx in fertilizing mouse eggs (Halet et al., 2004) and suggest that high PLCζ expression differs from adenophostin in that there is a prolonged phase of increased Ca$^{2+}$ influx.

Increased Ca$^{2+}$ influx could promote intracellular Ca$^{2+}$ release via the overloading of Ca$^{2+}$ stores. To investigate this possibility, we measured the relative amount of Ca$^{2+}$ in the thapsigargin-releasable Ca$^{2+}$ store 4 h after injection of adenophostin, or PLCζ, when maximal differences might be expected. Thapsigargin and BAPTA [bis-(o-aminophenoxy)ethane-N,N,N,N’-tetra-acetic acid] were added to the Ca$^{2+}$-free medium containing the eggs to trigger Ca$^{2+}$ release that was detected with fura 2 dextran fluorescence at 380 nm wavelength [(Ft − F0)/Ft]. This phasic Ca$^{2+}$ increase is used as a measure of the size of the Ca$^{2+}$ store (El-Jouni et al., 2005). Figure 7 shows that 4 h after injection, the Ca$^{2+}$ increase elicited by thapsigargin was larger in eggs overexpressing PLCζ–luc compared with eggs injected with adenophostin (Figure 7A). The size of the Ca$^{2+}$ stores was assessed either by the amplitude of the thapsigargin-induced Ca$^{2+}$ increase or by the integral of the phasic Ca$^{2+}$ increase (area under the curve). With either metric, there was a significant increase in Ca$^{2+}$ store content in eggs overexpressing PLCζ–luc (Figures 7C and 7D). The increase in Ca$^{2+}$ store content was also present in un.injected eggs that had been treated with PMA for 1 h (Figure 7B). These results suggest that Ca$^{2+}$ stores are substantially more loaded than normal by overexpression of PLCζ and by PKC stimulation.

**Discussion**

Most studies on signalling at fertilization in mouse eggs have concentrated on inositol 1,4,5-trisphosphate-induced Ca$^{2+}$ release (Miyazaki et al., 1993; Kurakawa et al., 2004; Miyazaki and Ito, 2006). However, the involvement of PLCζ or any other PLCs will inevitably generate DAG and this has been less extensively studied for technical reasons. Here, we have used a GFP-linked probe with a high sensitivity and specificity for DAG. At fertilization, DAG synthesis in the plasma membrane was not consistently observed, and the signal detected...
Bivalent cation influx in mouse eggs in response to adenophostin, PLCζ and PMA

Bivalent cation influx was measured by the quenching of fura 2 fluorescence by Mn²⁺ (0.1 mM) that was added at the arrows for each sample trace. In each case, the dotted line represents a control (uninjected or untreated) egg that was treated at the same time in the same experiment with the numbers of eggs for each experiment indicated. In (A), Mn²⁺ was added to eggs after 1 h of PMA treatment. In (B), eggs were injected with 10 μM adenophostin A and monitored 2 and 4 h after injection. In (C), eggs were injected with PLCζ-luc and Mn²⁺ quenching was monitored for 2 and 4 h after injection. The straight lines indicate the slopes used for estimating the initial Mn²⁺ influx rate.

was very small compared with the DAG increase elicited by ionomycin. Moreover, the transient increase in plasma-membrane DAG occurred solely during the first Ca²⁺ transient, which is consistent with a previous report showing that conventional PKC translocation to the plasma membrane of fertilized eggs is driven essentially by the Ca²⁺-binding C2 domain (Halet et al., 2004). Our results suggest that DAG accumulation in the plasma membrane of fertilized eggs is both relatively modest and short-lived. Since a very large Ca²⁺ increase itself can be a stimulus for DAG production, some egg-derived plasma-membrane PLCs may be stimulated in eggs and this could be the PLCβ isoforms (Igirashi et al., 2007).
As with fertilization, the expression of physiological levels of PLCζ did not lead to any measurable increase in DAG in the plasma membrane. However, overexpression of PLCζ resulted in the appearance of a delayed, second series of high-frequency Ca²⁺ oscillations that was consistently associated with plasma-membrane DAG production. Since a gap of several hours separates the two series of oscillations, the eggs would have expressed a significant level of PLCζ by that time, which may contribute to the increased DAG synthesis as a result of Ca²⁺-induced PLCζ stimulation since PLCζ is highly sensitive to cytosolic Ca²⁺ (Kouchi et al., 2004; Nomikos et al., 2005). This may resemble the situation where inositol 1,4,5-trisphosphate was monitored in mouse eggs using a fluorescence indicator that could only detect inositol 1,4,5-trisphosphate after a period that allowed for increased PLCζ expression (Shirakawa et al., 2006).

The secondary Ca²⁺ oscillations we describe in the present study have not been previously reported, because earlier studies injected PLCζ RNA corresponding approximately to the amount contained in a single sperm (Saunders et al., 2002; Miayzaki and Ito, 2006). The overexpression of PLCζ is likely to lead to the down-regulation of inositol 1,4,5-trisphosphate receptors, which could then explain the early
cessation of oscillations (Brind et al., 2000; Jellerette et al., 2000). However, inositol 1,4,5-trisphosphate receptor down-regulation does not preclude further Ca\(^{2+}\) oscillations, because it has been shown that some sufficiently strong stimuli can still generate Ca\(^{2+}\) oscillations after 70–80% down-regulation of inositol 1,4,5-trisphosphate receptors (Malcuit et al., 2005). Our results suggest that PKC is important in providing the extra stimulus that re-stimulates Ca\(^{2+}\) oscillations, since PMA triggers a series of secondary Ca\(^{2+}\) oscillations in eggs that would otherwise only generate a single series of Ca\(^{2+}\) oscillations. Furthermore, the PKC inhibitor, CHE, significantly inhibits the occurrence of secondary Ca\(^{2+}\) oscillations in eggs injected with high concentrations of PLC\(\zeta\). This shows that PKC-stimulation is both necessary and sufficient to trigger the secondary Ca\(^{2+}\) release that occurs with high levels of PLC\(\zeta\).

It is remarkable that these dramatic effects of PKC can be inferred in the absence of any detectable DAG production in the plasma membrane of eggs during the time between the first and secondary Ca\(^{2+}\) oscillations. It should be noted that in astrocytes the same C1\(_2\)δ–GFP domain that we used in mouse eggs is very effective at reporting plasma-membrane DAG generation in response to metabotropic glutamate receptor-mediated stimulation that activates PLC\(\beta\) (Codazzi et al., 2001). Our inability to observe translocation of the C1\(_2\)δ–GFP domain at fertilization, or during the period leading up to the secondary Ca\(^{2+}\) oscillations caused by excessive PLC\(\zeta\) expression, is therefore unlikely to represent any deficiency in the affinity of the DAG probe. It is possible that any DAG increase is too gradual to be readily detected with a translocation-based indicator, or else DAG is localized differently in eggs compared with astrocytes.

Our results suggest that the mechanism by which PKC causes secondary Ca\(^{2+}\) oscillations appears to involve Ca\(^{2+}\) influx. We also found evidence for enhanced filling of Ca\(^{2+}\) stores after PKC stimulation or overexpression of PLC\(\zeta\). It is possible that Ca\(^{2+}\) influx and the subsequent overloading of Ca\(^{2+}\) stores could trigger the secondary Ca\(^{2+}\) oscillations, since the overloading of Ca\(^{2+}\) stores can lead to a ‘spontaneous’ release in sea-urchin egg homogenates (Galion et al., 1993). However, we cannot exclude the possibility that PKC increases could also alter the sensitivity of the inositol 1,4,5-trisphosphate receptor to inositol 1,4,5-trisphosphate such that Ca\(^{2+}\) release is enhanced (Vermassen et al., 2004). Whatever the mechanisms, our current findings imply that PKC overstimulation can have major negative effects on eggs when it causes an abnormal series of secondary Ca\(^{2+}\) oscillations. Similar to studies with human PLC\(\zeta\), we found that none of the eggs that expressed high levels of mouse PLC\(\zeta\) developed to blastocyst stages even though they formed pronuclei (Yu et al., 2008). This suggests that the stimulation of PKC in the plasma membrane has to be minimal at fertilization. Given the evidence that calmodulin-dependent protein kinase II can trigger meiotic resumption and early development, the role of PKC in egg activation has to be questioned (Jones 1998; Madgwick et al., 2005; Knott et al., 2006). It is possible that most of the increase in PKC activity that has been reported at fertilization is due to non-conventional and novel isoforms such as PKC\(\epsilon\) and PKCD, which are localized to the meiotic spindle in mature mouse eggs where they may play a permissive role in meiotic resumption (Tatone et al., 2003; Viveiros et al., 2003).

Materials and methods

Gamete collection, RNA preparation and microinjection

MF1 female mice were superovulated by intraperitoneal injection of 7.5 i.u. of PMSG (pregnant mare’s serum gonadotrophin; Folligon) followed 48 h later by 10 i.u. of hCG (human chorionic gonadotropin; Folligon) (Saunders et al., 2002; Larman et al., 2004). Eggs (13–16 h post-hCG) were released from the oviduct into warmed M2 medium (Sigma). Cumulus cells were removed by a brief exposure to hyaluronidase and the zona pellucida removed by exposure to acid Tyrode’s solution (Gibco). Oocytes were held in drops of M2 medium under paraffin oil in Falcon tissue culture dishes. Spermatozoa were expelled from cauda epididymis of male CBA/C57 mice into 1 ml of T6 medium containing 16 mg/ml BSA, and incubated under oil for 2–3 h at 37°C and 5% CO\(_2\) to capacitate. For IVF (in vitro fertilization) experiments, approx. 10 μl of sperm suspension was added to the dish containing the eggs. Complementary RNA encoding mouse PLC\(\zeta\) fused via the C-terminus to firefly luciferase (mPLC\(\zeta\)-luc), and PKC\(\delta\) tandem C1 domain, tagged with GFP (C1\(_3\)δ–GFP in pcDNA3.1), were prepared with an mMessage mMachine T7 Ultra kit (Ambion), followed by polyadenylation (Halet et al., 2004; Nomikos et al., 2005). Microinjection procedures were carried out as previously described (Saunders et al., 2002). Eggs were injected to 3–5% egg volume with a solution containing different concentrations of mPLC\(\zeta\)-luc cRNA (0.05–1 μg/μl) mixed with 1 mM (pipette concentration) Oregon Green BAPTA dextran (Molecular Probes, http://www.probes.com) and/or C1\(_3\)δ–GFP cRNA (∼0.5 μg/μl). Unless otherwise stated, media and reagents were purchased from Sigma (Poole, Dorset, U.K.).
Confocal imaging
At 2–3 h after C12δ–GFP cRNA injection, eggs were loaded with 10 μM Fura Red/AM (Fura Red acetoxymethyl ester) (Molecular Probes) for 20 min and subsequently the zona pellucida was removed by incubation in acidic Tyrode’s medium at 37°C. Zona-free eggs were placed in a chamber seated in a heated stage, and containing 1 ml BSA-free hKSOM medium. The changes in the distribution of GFP-tagged proteins and Ca2+ induced by mPLCδ luc or other reagents were monitored simultaneously at the equator of the cells using a confocal microscope (TCS SP5; Leica), under a ×20 (0.75 NA) lens. Excitation was provided by the 488-nm line of an argon laser and time series were acquired at a rate of 1 frame every 10 s. Confocal data were analysed as described previously (Halet et al., 2004), using ImageJ (http://rsb.info.nih.gov/ij/). In brief, regions of interest were drawn in the cytosol or around the plasma membrane, and changes in fluorescence intensity were measured during confocal time series. The value of the PM/C ratio (plasma membrane/cytosol ratio) was used as an index of membrane translocation.

Measurement of intracellular Ca2+ and luciferase expression
The imaging of Ca2+ oscillations induced by mPLCδ luc and the quantification of mPLCδ luc expression were carried out as previously described (Yu et al., 2008). Briefly, injected oocytes were placed in a chamber with hKSOM medium containing 100 μM luciferin, on the temperature-controlled stage of an inverted microscope. In some cases, injected eggs were zona-free and attached to the polylysine-coated glass bottom of the chamber in calcium-free hKSOM medium (Larman et al., 2004). Ca2+ oscillations were monitored by measuring the fluorescence of Oregon Green BAPTA dextran, and luciferase expression was monitored by the luminescence. These measurements were both carried out on the same sets of eggs using a Zeiss Axiovert S100 microscope with light from the stage directed towards a cooled ICCD camera (intensified CCD camera (charge-coupled device camera)). The microscope and ICCD camera were inside a custom-made dark box, and the data collection and analysis was carried out using software supplied with the camera, which was from Photek (http://www.photek.co.uk). In most experiments, the fluorescence was recorded first by exposing oocytes to excitation light (450–490 nm) and reducing the sensitivity of the ICCD camera to 10%, and then the luminescence was recorded by removing the excitation light and switching the ICCD camera to maximum sensitivity.

Ca2+ influx measurement
The Ca2+ influx rate in eggs at 2 or 4 h after injection of mPLCδ luc or adenophostin was estimated by using Mn2+ as a surrogate for Ca2+ and measuring Mn2+ quenching as described by McGuinness et al. (1996) and Mohri et al. (2001). Briefly, eggs were loaded with fura 2/AM (fura 2 acetoxymethyl ester; Molecular Probes; 1 μM in M2 medium) supplemented with 0.02% pluronic acid (Molecular Probes) at 37°C for 30 min. After the zona pellucida was removed, these eggs were transferred to BSA-free hKSOM medium on a coverslip that had been precoated with polylysine (1 mg/ml) in a chamber. The chamber was mounted on the microscope stage and kept at 37°C with a heated plate. Eggs were imaged using a CCD camera (CoolSNAP-HQ2) and collected using Image Q software both supplied by Photomet-
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Received 29 February 2008/11 April 2008; accepted 9 May 2008
Published as Immediate Publication 9 May 2008, doi:10.1042/BC20080033