Conventional PKCs regulate the temporal pattern of Ca\(^{2+}\) oscillations at fertilization in mouse eggs

Guillaume Halet,1 Richard Tunwell,1 Scott J. Parkinson,2 and John Carroll1

1Department of Physiology, University College London, London WC1E 6BT, England, UK
2Dana-Farber Cancer Institute, Boston, MA 02115

Introduction

A universal mechanism to activate signaling in eukaryotic cells is via increases in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). These Ca\(^{2+}\) signals often take the form of Ca\(^{2+}\) oscillations, the amplitude, frequency, and duration of which govern the nature of the cellular response (Berridge, 1993; Berridge et al., 2003). One means of increasing [Ca\(^{2+}\)]\(_i\), in cells is the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) by enzymes of the PLC family. PIP\(_2\) hydrolysis generates two products, the soluble Ca\(^{2+}\)-releasing second messenger inositol 1,4,5-trisphosphate (InsP\(_3\)) and the neutral lipid DAG, which plays a major role in the activation of PKC. Activation of this bifurcating signaling pathway occurs in many physiological situations in response to stimulation by hormones, growth factors, or neurotransmitters (Berger et al., 2003).

At fertilization, hydrolysis of PIP\(_2\) and generation of InsP\(_3\) by PLC activity triggers the Ca\(^{2+}\) signal necessary for egg activation and the initiation of embryonic development (Stricker, 1999). In mammalian eggs, fertilization is characterized by the generation of low frequency Ca\(^{2+}\) oscillations due to the opening of the InsP\(_3\)-sensitive Ca\(^{2+}\) release channels in the ER (Miyazaki et al., 1993). The mechanism underlying these Ca\(^{2+}\) oscillations has recently been suggested to require a novel sperm-borne PLC, PLC\(_\beta 1\) (Saunders et al., 2002). Fertilization is thus expected to activate a classical PIP\(_2\) hydrolysis pathway leading to the generation of InsP\(_3\) and DAG.

Fertilization-induced Ca\(^{2+}\) oscillations proceed for several hours and are the primary trigger for cortical granule exocytosis, exit from metaphase II arrest, and entry into the first mitotic division (Kline and Kline, 1992a; Xu et al., 1994). The number of Ca\(^{2+}\) oscillations has recently been proposed to differentially regulate these events (Ducibella et al., 2002). How the amplitude and frequency of these Ca\(^{2+}\) oscillations are decoded into specific activation events by the fertilized egg remains unclear. In somatic cells, PKC is a major downstream effector of Ca\(^{2+}\) signals, decoding Ca\(^{2+}\) oscillations into corresponding bursts of PKC substrate phosphorylation (Oancea and Meyer, 1998; Violin et al., 2003). In mammalian eggs, although biochemical assays have reported an increase in PKC activity as early as 10 min after insemination (Tatone et al., 2003), little is known about the kinetics and role(s) of PKC activation at fertilization.

PKCs form a large family of serine/threonine kinases involved in a multitude of cellular functions from cell...
growth and differentiation to secretion, gene expression, and regulation of other signaling pathways. 10 mammalian PKC isoforms have been described and classified into three major subfamilies, according to their structure and cofactor requirements (Mellor and Parker, 1998; Newton, 2001, 2003): conventional PKCs (cPKCs)—namely PKCα, βI, βII, and γ—are activated by negatively charged phospholipids and DAG in a Ca\(^{2+}\)-dependent manner; in contrast, novel PKCs (δ, ε, η, and θ) do not require Ca\(^{2+}\) for activation, but are regulated by anionic lipids and DAG, whereas atypical PKCs (ζ and ι/λ) require neither Ca\(^{2+}\) nor DAG for activation, but do require negatively charged phospholipids. Activation of PKCs requires the release of an autoinhibitory interaction between the NH\(_2\)-terminal pseudosubstrate motif and the COOH-terminal catalytic core (Oancea and Meyer, 1998; Newton, 2001). According to current models, activation of cPKCs involves the sequential binding of Ca\(^{2+}\) and DAG to their respective binding sites on the kinases, the C2 and C1 domains (Oancea and Meyer, 1998; Violin et al., 2003). The binding of Ca\(^{2+}\) ions to the C2 domain increases its affinity for phosphatidylinerine (Verdaguer et al., 1999; Staehelin et al., 2003) and results in the translocation of cPKCs to the plasma membrane, where DAG binding to the C1 domain provides maximal kinase activity. cPKC translocation to the plasma membrane is therefore regarded as a sign of cPKC activation (Oancea and Meyer, 1998; Newton, 2001; Violin et al., 2003).

Numerous PKC isotypes have been identified in mouse eggs at the mRNA or protein level, including cPKCs α and γ (Luria et al., 2000; Pauken and Capco, 2000; Tatone et al., 2003; Viveiros et al., 2003). Immunolocalization or staining with a fluorescently labeled PKC inhibitor have revealed the translocation of some PKC isoforms to the cortex of fertilized mammalian eggs (Gallicano et al., 1995, 1997; Luria et al., 2000; Eliyahu and Shalgi, 2002; Fan et al., 2002), raising the possibility that PKC could be a major downstream effector of Ca\(^{2+}\) oscillations at fertilization. However, these data were obtained in populations of eggs fixed some time after sperm addition, and they provided little information on the kinetics and regulation of PKC activation in living eggs.

In this work, we have imaged GFP fusion constructs of cPKCs and [Ca\(^{2+}\)], simultaneously in living mouse eggs to test the hypothesis that fertilization-induced Ca\(^{2+}\) oscillations are decoded by PKC. We show that fertilization-induced Ca\(^{2+}\) transients trigger the translocation of cPKCs to the egg membrane, and that this translocation is shaped by the frequency and amplitude of Ca\(^{2+}\) release. In addition, we provide evidence for a major role of cPKCs in the sustaining of long-lasting oscillations in fertilized eggs, via the regulation of store-operated Ca\(^{2+}\) influx.

**Results**

cPKCs undergo Ca\(^{2+}\)-dependent translocation to the plasma membrane of fertilizing eggs

Translocation from the cytosol to the plasma membrane is the hallmark of PKC activation in somatic cells. Here, we monitored the dynamics of fluorescently labeled cPKCs in living mouse eggs during fertilization-induced Ca\(^{2+}\) oscillations. Such constructs have been successfully used in somatic cells to study cPKC activation, and they were found to retain the catalytic and regulatory properties of the native protein (Sakai et al., 1997; Oancea and Meyer, 1998; Maesch et al., 2000; Schaefer et al., 2001; Tanimura et al., 2002). We have chosen to monitor PKCα and PKCγ, two isoforms naturally expressed in mouse eggs (Pauken and Capco, 2000). Confocal images acquired 3–4 h after cRNA injection indicated that EGFP-PKCα had a homogenous cytosolic distribution before fertilization-induced Ca\(^{2+}\) release (Fig. 1 A). Sperm fusion elicited in the egg the typical series of Ca\(^{2+}\) oscillations as described previously (Cuthbertson and Cobbold, 1985; Miyazaki et al., 1993; Jones et al., 1995; Deguchi et al., 2000). The first transient consisted of a rapid rise in [Ca\(^{2+}\)], to a plateau on which several fast Ca\(^{2+}\) oscillations—referred to as “Ca\(^{2+}\) spikes” in the following text—are superimposed (Fig. 1 A). The subsequent Ca\(^{2+}\) transients were shorter in duration, and their amplitude was similar to the plateau level of the first transient. As previously noticed (Cuthbertson and Cobbold, 1985), one or several Ca\(^{2+}\) spikes were frequently observed at the top of the following transients (see Fig. 5). EGFP-PKCα rapidly translocated from the cytosol to the plasma membrane during the first Ca\(^{2+}\) transient (Fig. 1 A; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1). Interestingly, translocation proceeded as a series of peaks matching the Ca\(^{2+}\) spikes and leading to an incremental accumulation of EGFP-PKCα at the membrane. After translocation reached its maximum, a large portion of the translocated kinase remained associated with the plasma membrane for the duration of the Ca\(^{2+}\) plateau. Termination of the Ca\(^{2+}\) transient was associated with a complete and rapid return of EGFP-PKCα to the cytosol. The subsequent Ca\(^{2+}\) transients were associated with translocations of lesser amplitude, corresponding approximately to the level seen at the end of the first transient. Close examination of the kinetics of the EGFP-PKCα and Ca\(^{2+}\) signals during the first transient indicated that translocation started only after the first Ca\(^{2+}\) spike was generated (Fig. 1 A), suggesting that translocation was a Ca\(^{2+}\)-driven process.

cPKCs are considered to be freely diffusible in quiescent cells, screening for potential binding partners in favor of a collisional coupling mechanism (Teruel and Meyer, 2000; Schaefer et al., 2001). A close examination of the confocal images revealed that during translocation, the subplasmalemmal cytosolic region was first depleted, before fluorescence decreased in the center of the egg (Fig. 1 B). This observation of a localized depletion zone in the vicinity of the plasma membrane is predicted by a diffusion-driven mode and can be considered as a direct evidence for a diffusion-limited translocation process (Schaefer et al., 2001). A similar Ca\(^{2+}\)-induced, diffusion-mediated translocation pattern was observed in eggs expressing PKCγ-GFP (Fig. 2 A, right). Together, these data demonstrate that cPKCs are rapidly activated at fertilization and their diffusion-driven translocation to the plasma membrane is controlled by the amplitude and frequency of Ca\(^{2+}\) signals.

**High amplitude Ca\(^{2+}\) spikes recruit cPKC C2 domain to the plasma membrane at fertilization**

The major membrane-binding module of cPKCs is the Ca\(^{2+}\)-dependent C2 domain, which gains high affinity for...
plasma membrane phosphatidylserine upon Ca2+ coordination (Verdaguer et al., 1999; Stahelin et al., 2003). To investigate the role of Ca2+ binding to the C2 domain during cPKC translocation at fertilization, we expressed the GFP-tagged C2 domain of PKCγ (C2-GFP; Oancea and Meyer, 1998) in mouse eggs, and followed its dy-
The C2 domain is recruited at the egg membrane by Ca\(^{2+}\) spikes. (A) Translocation of C2-GFP during the first fertilization Ca\(^{2+}\) transient. Confocal images (5 s apart) illustrate the first C2-GFP translocation pulse of the experiment displayed on the bottom left, where the corresponding time points are highlighted. Note that translocation occurs selectively during the Ca\(^{2+}\) spikes. The right panel displays two other experiments illustrating the different translocation patterns of PKC\(\gamma\)-GFP (black) and C2-GFP (red) during the first fertilization Ca\(^{2+}\) transient (Fura Red traces displayed underneath). (B) Experiment showing the repetitive translocation of C2-GFP during fertilization-induced Ca\(^{2+}\) transients. Note the higher amplitude of translocation during the first transient. These results are representative of at least six experiments. Bar, 10 \(\mu\)m.

During the first Ca\(^{2+}\) transient, C2-GFP translocated to the plasma membrane in an oscillatory manner, closely matching the pattern of the Ca\(^{2+}\) spikes generated at the top of the transient (Fig. 2 A, left). As observed for the full-length PKC\(\gamma\) and PKC\(\alpha\), each translocation pulse occurred subsequent to the corresponding Ca\(^{2+}\) spike, indicating that the increase in [Ca\(^{2+}\)]\(_i\) was the trigger for translocation (Fig. 2 A, left; Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1). Also, a subplasmalemmal depletion zone was observed during C2-GFP translocation, suggestive of a diffusion-mediated process (unpublished data). Translocations of smaller amplitude were observed during subsequent Ca\(^{2+}\) transients (Fig. 2 B). Interestingly, no translocation was observed during the rising phase or even during the plateau of the first Ca\(^{2+}\) transient, suggesting that a threshold [Ca\(^{2+}\)]\(_i\) is required to recruit C2-GFP at the membrane. The incremental translocation pattern described with the full-length PKC\(\alpha\) and PKC\(\gamma\) was never observed with C2-GFP, even when the Ca\(^{2+}\) spikes and C2-GFP translocation pulses occurred at high frequency (Fig. 2 A, right). This difference between full-length cPKCs and C2-GFP indicates that the frequency-dependent accumulation at the mem-
translocation, suggesting that the threshold \([\text{Ca}^{2+}]\), required to recruit the C2 domain at the membrane was in the 1–3-μM range.

**The DAG sensor C1-GFP fails to detect DAG accumulation at fertilization**

The differences in the translocation patterns of C2-GFP and the full-length cPKCs suggest that the latter establish additional interactions with the membrane after \(\text{Ca}^{2+}\)-induced translocation. One likely possibility is the binding to DAG that may be generated during sperm-induced PLC activation. To test whether cPKCs could detect coincident \(\text{Ca}^{2+}\) and DAG signals at fertilization, eggs were injected with a cRNA encoding a GFP-conjugated DAG-binding domain of PKCγ (C1-GFP), a construct that has previously been used to monitor DAG production after PLC activation in agonist-stimulated somatic cells (Oancea et al., 1998). C1-GFP distributed evenly in the cytosol of unfertilized eggs (Fig. 4 A). The PKC agonist PMA and the DAG analogue 1,2-dioctanoyl sn-glycerol (DiC8) successfully recruited C1-GFP to the plasma membrane of unfertilized eggs, demonstrating the ability of C1-GFP to bind DAG and phorbol esters (unpublished data). To establish that C1-GFP could detect PLC-induced DAG production, eggs were stimulated with the \(\text{Ca}^{2+}\) ionophore ionomycin to activate endogenous PLCs (Vármai and Balla, 1998). The rapid increase in \([\text{Ca}^{2+}]\), triggered by the ionophore was followed by DAG accumulation in the plasma membrane, as demonstrated by the translocation of C1-GFP (Fig. 4 A, left; Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200311023/DJC1). Evidence that DAG synthesis was a consequence of \(\text{Ca}^{2+}\)-induced activation of PLC is provided by the decrease in plasma membrane PIP2 staining in eggs expressing a PIP2-specific GFP-tagged pleckstrin homology domain (PH-GFP; Hallet et al., 2002) and exposed to ionomycin (Fig. 4 A, right; Video 4). In contrast, fertilization-induced \(\text{Ca}^{2+}\) oscillations were not associated with any detectable translocation of C1-GFP either to the plasma membrane or to any cytosolic compartment (Fig. 4 B). Addition of PMA at the end of the experiment induced the expected translocation of the probe to the egg membrane (Fig. 4 B). Thus, using C1-GFP as a DAG sensor, fertilization was not associated with a detectable DAG production, despite the generation of \(\text{Ca}^{2+}\) oscillations.

**Exogenous DAG can modulate cPKC translocation at fertilization**

In the absence of a detectable DAG signal, we asked whether DAG can influence the translocation of cPKCs at fertilization. To address this question, the membrane-permeable DAG analogue DiC8 was applied to eggs undergoing fertilization-induced \(\text{Ca}^{2+}\) oscillations. As illustrated in Fig. 5, the magnitude of PKCγ-GFP translocation was dramatically increased by up to ~30-fold after DiC8 addition, whereas the amplitude of the \(\text{Ca}^{2+}\) transients was unaffected. However, translocation remained a \(\text{Ca}^{2+}\)-regulated process, starting when \([\text{Ca}^{2+}]\), reached its peak, and finishing when \([\text{Ca}^{2+}]\), started to decrease, demonstrating

---

**Figure 3.** Cytosolic \([\text{Ca}^{2+}]\) reaches micromolar levels during the \(\text{Ca}^{2+}\) spikes. (A) Raw Mag-Fura-2 fluorescence trace showing the downward deflections corresponding to the fertilization \(\text{Ca}^{2+}\) transients. Ionomycin (iono) was added at the end of the experiment for calibration. Mag-Fura-2 fluorescence is expressed in arbitrary units. (B) \(\text{Ca}^{2+}\) spikes recorded during the first fertilization \(\text{Ca}^{2+}\) transient in another experiment and displayed with the corresponding calibrated \([\text{Ca}^{2+}]\), scale. Data are representative of seven experiments.

---

bran is not an intrinsic property of the C2 domain, and suggests that other domains of cPKC may be involved in retaining the kinase at the membrane.

\([\text{Ca}^{2+}]\), rises to micromolar levels during fertilization-induced \(\text{Ca}^{2+}\) spikes

Because of its high affinity for \(\text{Ca}^{2+}\) (in vitro Kd reported as 140 nM; Takahashi et al., 1999), it is likely that Fura Red becomes saturated under conditions where \([\text{Ca}^{2+}]\), exceeds micromolar levels, leading to an underestimation of the relative amplitudes of the \(\text{Ca}^{2+}\) transients and spikes. To estimate the maximal amplitude of the \(\text{Ca}^{2+}\) transients without worrying about indicator saturation, and to investigate the possibility that \(\text{Ca}^{2+}\) spikes reach high concentration critical for C2-GFP translocation, we monitored fertilization-induced \(\text{Ca}^{2+}\) oscillations with the low affinity \(\text{Ca}^{2+}\) indicator Mag-Fura-2 (Kd = 25 μM in vitro; Takahashi et al., 1999). The first fertilization \(\text{Ca}^{2+}\) transient appeared as a succession of 3–4 sharp \(\text{Ca}^{2+}\) spikes, whereas subsequent \(\text{Ca}^{2+}\) transients were detected as smaller, rather monophasic \(\text{Ca}^{2+}\) spikes (Fig. 3 A). Estimation of \([\text{Ca}^{2+}]\), from 420-nm fluorescence changes was done according to Ogden et al. (1995) using a Kd value of 25 μM, and after obtaining the \(F_{\text{max}}\) value with ionomycin (see Materials and methods). On average, \([\text{Ca}^{2+}]\), was found to rise up to 3.1 ± 0.5 μM \((n = 7)\) at the peak of the first \(\text{Ca}^{2+}\) spike of the first transient (Fig. 3 B). All subsequent transients were smaller, but transiently reached 1–2 μM. The \(\text{Ca}^{2+}\) plateau during the first transient was estimated to reach up to 1 μM. Overall, the micromolar \([\text{Ca}^{2+}]\), changes observed at fertilization exhibited a pattern very similar to the dynamics of C2-GFP
that DiC8 was not sufficient to retain the kinase at the membrane when [Ca\textsuperscript{2+}] is low. These observations suggest that, in the presence of Ca\textsuperscript{2+}, DiC8 has altered the kinetic parameters of PKC\textgreek{y}-GFP translocation, presumably by slowing down the dissociation from the membrane (Oancea and Meyer, 1998; Tanimura et al., 2002), resulting in a dramatic increase in the amplitude of translocation. In contrast, DiC8 had no effect on PKC\textgreek{y}-GFP localization in unfertilized eggs (unpublished data). These results are in agreement with the sequential model for cPKC activation, which states that DAG and Ca\textsuperscript{2+} are both required for full cPKC membrane affinity (Oancea and Meyer, 1998; Violin et al., 2003), and further, suggest that DAG generation may influence cPKC dynamics at fertilization.

**Activation of PKC regulates Ca\textsuperscript{2+} oscillations at fertilization**

To investigate the role of cPKCs in Ca\textsuperscript{2+} signaling at fertilization, we examined the effects of PKC\textgreek{y} overexpression on the pattern of Ca\textsuperscript{2+} oscillations. In control, buffer-injected eggs, the period of Ca\textsuperscript{2+} oscillations reached several minutes (~5 min when measured between transients 4 and 5; Table I), during which [Ca\textsuperscript{2+}], slowly increased up to a threshold level at which the next transient was generated (Fig. 6 A). This so-called Ca\textsuperscript{2+} pacemaker (Miyazaki et al., 1993), suggestive of a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism, was also observed in the absence of extracellular Ca\textsuperscript{2+} (Fig. 6 E), demonstrating that it resulted from intracellular Ca\textsuperscript{2+} release. In eggs overexpressing EGFP-PKCo, the duration of
the first transient was dramatically increased to >10 min (against 2–3 min in controls; Table I), due to a second phase of \([Ca^{2+}]_i\), increase that followed the first plateau (Fig. 6 B). It is noteworthy that the additional \(Ca^{2+}\) release occurred after the \(Ca^{2+}\) spikes were generated, i.e., after activation of cPKCs, according to our confocal data. The following oscillations proceeded at a frequency approximately threefold higher than in buffer-injected controls (Fig. 6 B; Table I). These results indicate that PKC\(\alpha\) overexpression has promoted \(Ca^{2+}\) oscillations at fertilization, revealing a change in the regulation of the \(Ca^{2+}\) release machinery.

An alternative means of overstimulating PKC is by the addition of the PKC agonist PMA, which induces the permanent translocation of cPKCs to the egg membrane (unpublished data). Addition of PMA during ongoing \(Ca^{2+}\) oscillations increased their frequency about threefold, but did not affect oscillation amplitude (Fig. 6 C). This effect of PMA on oscillation frequency could be reversed by adding 5 \(\mu\)M of the PKC inhibitor bisindolylmaleimide I (BIM; Toullec et al., 1991) to the medium (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1). When PMA was added before fertilization, the oscillation pattern was identical to EGFP-PKCA overexpression, i.e., a very long first transient and high oscillation frequency (Fig. 6 D; Table I). This similarity suggests that the effects of PMA on \(Ca^{2+}\) signaling are mediated by cPKCs, and further supports a role for cPKCs in modulating the dynamics of \(Ca^{2+}\) release at fertilization.

To examine the contribution of extracellular \(Ca^{2+}\) influx in the acceleration of \(Ca^{2+}\) oscillations after PKC activation, the effect of PMA at fertilization was examined after extracellular \(Ca^{2+}\) had been chelated. Eggs were first fertilized in a normal \(Ca^{2+}\)-containing medium (1.8 mM \([Ca^{2+}]_i\)) to trigger oscillations; then EGTA (3 mM) was added to the medium, resulting in a dramatic decrease in oscillation frequency and ultimately to the arrest of oscillations (Fig. 6 E). Addition of PMA did not stimulate \(Ca^{2+}\) release, nor did it increase the frequency of oscillations (Fig. 6 E), demonstrating that in the absence of extracellular \(Ca^{2+}\), PMA has no stimulatory effect on intracellular \(Ca^{2+}\) release. The effect of EGTA was overcome by raising extracellular \([Ca^{2+}]_i\) to 6 mM, as indicated by the resumption of \(Ca^{2+}\) oscillations (Fig. 6 E).

Inhibition of PKCs suppresses \(Ca^{2+}\) oscillations at fertilization

The data described above suggest that activation of PKC promotes \(Ca^{2+}\) oscillations at fertilization. To confirm this finding, we examined fertilization-induced \(Ca^{2+}\) transients in eggs treated with the PKC inhibitor BIM. In preliminary experiments, BIM was found to strongly inhibit second polar body emission in fertilized eggs (~70% inhibition; unpublished data), confirming an earlier report (Gallicano et al., 1997) and suggesting that PKC plays a major role in cell cycle resumption during egg activation. Gallicano et al. (1997) reported that polar body emission was also inhibited by a membrane-permeant inhibitory peptide mimicking PKC pseudosubstrate; however, we and others (Ducibella and LeFevre, 1997) found that this compound was toxic for the eggs at the concentration required to inhibit polar body emission.

Exposure to BIM did not affect the fertilizability of mouse eggs, but dramatically altered the duration of \(Ca^{2+}\) oscillations. Typically, the first transient was followed by 3–7 small, short-lived \(Ca^{2+}\) oscillations, ending with an aborted \(Ca^{2+}\) transient (Fig. 7 A). The overall duration of the \(Ca^{2+}\) oscillations in the presence of BIM never exceeded 45 min (against 3–4 h in controls). Interestingly, \(Ca^{2+}\) transients resumed after raising extracellular \([Ca^{2+}]_i\) to 6 mM (Fig. 7 A), suggesting that the inhibitory effect of BIM was not due to a failure of the \(Ca^{2+}\) release machinery, but rather to a deficit in the supply of \(Ca^{2+}\) to refill the stores. However, this recovery was transient, as oscillations stopped with an aborted \(Ca^{2+}\) transient after a few minutes (Fig. 7 A).

PKC regulates store-operated \(Ca^{2+}\) entry at fertilization

The effects of BIM suggest that PKC activity is required for long-lasting oscillations to proceed at fertilization, in a manner that requires extracellular \(Ca^{2+}\). In nonexcitable somatic cells, the maintenance of \(Ca^{2+}\) oscillations requires the re-

---

**Table 1. PKC stimulation alters the pattern of \(Ca^{2+}\) oscillations at fertilization**

|                | Control | EGFP-PKCA | PMA |
|----------------|---------|-----------|-----|
| Duration of the first transient (s) | 148.4 ± 7.8 | 682.2 ± 87.9 | 681.2 ± 29.0 |
| Period (s) | 302.8 ± 26.1 | 132.2 ± 21.7 | 112.5 ± 12.7 |

The duration of the first transient was measured between the upstroke and the falling phase of the transient at half-maximal amplitude. The period and fourth \(Ca^{2+}\) transients. EGFP-PKCA refers to experiments with eggs overexpressing this protein. PMA refers to experiments where the phorbol ester was already present in the medium before the start of fertilization-induced \(Ca^{2+}\) oscillations. *P* < 0.05.
plenishment of Ca\(^{2+}\) stores through the activation of the so-called store-operated (or capacitative) Ca\(^{2+}\) entry (SOCE) pathways (Putney, 1990; Berridge, 1995). Interestingly, the modulation of the rate of store refilling is considered to be one mechanism of setting oscillation frequency (Berridge, 1993). To investigate whether SOCE is under the regulation of PKC in mouse eggs, we performed an assay for SOCE using thapsigargin to deplete the Ca\(^{2+}\) stores and Ca\(^{2+}\) add-back to visualize SOCE (Kline and Kline, 1992b; McGuinness et al., 1996). In control eggs, SOCE appeared as a phasic increase in [Ca\(^{2+}\)]\(_i\) followed by a sustained plateau (Fig. 7 B). In the presence of BIM, the phasic response was unchanged, demonstrating that store depletion did activate SOCE; however, the sustained phase was strongly inhibited (Fig. 7 B). Occasionally (4/21 eggs), in the presence of BIM, SOCE exhibited oscillatory changes in [Ca\(^{2+}\)]\(_i\) before returning to baseline, suggestive of a feedback regulation of Ca\(^{2+}\) entry by Ca\(^{2+}\) itself (unpublished data). Conversely, when SOCE was elicited in the presence of PMA, the phasic increase in [Ca\(^{2+}\)]\(_i\) was rapidly followed by a chaotic and persistent increase in [Ca\(^{2+}\)]\(_i\) (Fig. 7 B) that frequently resulted in egg lysis, indicating a massive Ca\(^{2+}\) influx. These data demonstrate that PKC inhibition with BIM results in the inhibition of SOCE and the premature arrest of Ca\(^{2+}\) oscillations at fertilization, whereas PKC activation with PMA stimulates SOCE.

**Discussion**

To interpret the complex fertilization Ca\(^{2+}\) signal, the mammalian egg must possess molecular processors that can decode the amplitude and spatio-temporal pattern of Ca\(^{2+}\) transients into specific cellular responses. cPKCs have been proposed to play such a role in somatic cells (Oancea and Meyer, 1998). In the present work, we investigated the role of cPKCs in the decoding of Ca\(^{2+}\) oscillations at fertilization in living mouse eggs. Our data demonstrate that cPKCs are activated in a Ca\(^{2+}\)-dependent manner at fertilization, and that the activation is tuned to the frequency and amplitude of sperm-triggered Ca\(^{2+}\) transients. In addition, we provide evidence that one role for Ca\(^{2+}\)-driven cPKC activation is to promote Ca\(^{2+}\) entry via SOCE. Thus, cPKCs are a component of a positive feedback loop to ensure the persistent generation of Ca\(^{2+}\) transients at fertilization in mammals.

**Micromolar Ca\(^{2+}\) spikes trigger cPKC translocation to the egg membrane at fertilization**

One enigmatic feature of the fertilization Ca\(^{2+}\) signal in mammalian eggs is the presence of Ca\(^{2+}\) spikes on the top of the transients (Cutbertson and Cobbold, 1985; Jones et al., 1995; Deguchi et al., 2000). Our data reveal that these Ca\(^{2+}\) spikes—mounting up to 3 \(\mu\)M [Ca\(^{2+}\)] during the first transient—are the trigger for cPKC translocation to the egg membrane.
plasma membrane after sperm–egg fusion. The C2 domain of the kinase was found to act as an amplitude detector, driving translocation selectively when micromolar Ca\(^{2+}\) spikes are generated. Thus, the C2 domain behaved like a low affinity Ca\(^{2+}\) sensor, a property that was retained in the context of the full-length cPKCs.

Interestingly, cPKCs were also found to act as frequency detectors during these rapid Ca\(^{2+}\) spikes, as shown by the cumulative cPKC recruitment at the membrane, resulting in an increase in the amplitude of translocation. In addition, a residual pool of cPKCs remained associated with the membrane for the duration of the Ca\(^{2+}\) transients. These two features were not observed with the isolated C2 domain, suggesting that full-length cPKCs established additional interactions with the plasma membrane after translocation. This difference may be accounted for by interaction of full-length cPKCs with DAG, as suggested by the effect of DiC8 on cPKC dissociation from the membrane (Oancea and Meyer, 1998; Tanimura et al., 2002; present study). However, using C1-GFP, we could not detect the generation of DAG at fertilization. The reason for this may be that DAG production is limited and insufficient to trigger a detectable C1-GFP translocation. In support of this idea, we previously reported that plasma membrane PIP\(_2\) hydrolysis could not be detected at fertilization using PH-GFP, arguing for a rather low PIP\(_2\) turnover (Halet et al., 2002). An alternative possibility is that PIP\(_2\) itself could ensure maximal kinase recruitment and activation in the absence of DAG (Chauhan and Brockerhoff, 1988; Lee and Bell, 1991; Kochs et al., 1993; Pap et al., 1993; Corbalán-García et al., 2003). This possibility is consistent with the Ca\(^{2+}\)-dependent increase in plasma membrane PIP\(_2\) during fertilization-induced Ca\(^{2+}\) release (Halet et al., 2002). Finally, the interaction with specific anchoring proteins, such as the so-called receptors for activated C-kinase, may localize cPKCs in proximity to their substrates at the plasma membrane (Mochly-Rosen and Gordon, 1998). Further experimental evidence will be needed to explore the possibility of a regulation of cPKC activity by PIP\(_2\) or anchoring proteins in mouse eggs.

Because cPKCs seem to play a major role in sustaining Ca\(^{2+}\) oscillations (see below), their activation by high amplitude Ca\(^{2+}\) spikes early after sperm fusion may provide a checkpoint ensuring that oscillations will proceed only in eggs displaying Ca\(^{2+}\) transients with the correct amplitude. Considering that their role has never been investigated before, our observations are the first evidence that these Ca\(^{2+}\) spikes are required to activate Ca\(^{2+}\)-sensitive signaling proteins such as cPKCs. Other signaling proteins with a Ca\(^{2+}\)-dependent C2 domain may follow a similar activation pattern at fertilization, providing an interesting direction for future analyses examining Ca\(^{2+}\) signaling at fertilization.

**cPKCs support fertilization Ca\(^{2+}\) oscillations by regulating Ca\(^{2+}\) influx**

We have identified a previously unknown role for PKC at fertilization, that of providing a positive feedback on the generation of Ca\(^{2+}\) oscillations. We show that PKC\(_{\alpha}\) overexpression or PMA stimulation increases the frequency of Ca\(^{2+}\) oscillations while PMA stimulates SOCE, whereas PKC inhibition leads to a premature arrest of Ca\(^{2+}\) oscilla-
tions and the inhibition of SOCE. These data suggest that cPKCs may regulate SOCE, and consequently Ca^{2+} store refilling at fertilization.

Previous reports on mammalian eggs have attributed a role to PKC in the regulation of cortical granule exocytosis, resumption of the cell cycle, and second polar body formation at fertilization (Colonna and Tatone, 1993; Gallicano et al., 1997; Luria et al., 2000; Eliyahu and Shalgi, 2002; Fan et al., 2002). However, these reports did not consider the possibility that PKC activation/inhibition could alter the pattern of fertilization-induced Ca^{2+} release, the primary trigger for these activation events. In addition, Ducibella and LeFevre (1997) demonstrated that PKC inhibition with BIM did not alter cortical granule exocytosis nor cell cycle resumption at fertilization in mouse eggs, raising doubts on the actual involvement of PKC in these particular events. Interestingly, recent data suggest that the stimulatory action of phorbol esters on exocytosis may be mediated by members of the Munc13 family rather than PKCs (Rhee et al., 2002). On the other hand, the effect on the cell cycle may rely on the activation of PKC8, which has been found to bind to the meiotic spindle and chromosomes in mouse eggs (Tatone et al., 2003; Viveiros et al., 2003). Thus, our data reveal that one physiological role for cPKCs at fertilization is to control the pattern of Ca^{2+} signaling by regulating Ca^{2+} influx.

Mouse eggs possess a Ca^{2+} influx pathway activated by Ca^{2+} store depletion, which has been proposed to contribute to store refilling and underlie long-lasting oscillations at fertilization (Kline and Kline, 1992b; McGuinness et al., 1996). Although the molecular nature of this pathway has not yet been investigated, it is likely to correspond to a store-operated channel (SOC; Berridge et al., 2003), which are subjected to a negative feedback regulation by Ca^{2+} (red arrow). Micromolar increases in [Ca^{2+}]i trigger the translocation of cPKCs to the egg membrane (green arrow), where DAG may contribute to their activation and membrane anchoring. Activated cPKCs counteract the negative feedback by Ca^{2+} by phosphorylating SOCs or accessory proteins (blue arrow), thus maintaining SOC activation and promoting store refilling. Although not investigated in this work, we cannot exclude the possibility that cPKCs also regulate subplasmalemmal InsP_{3} receptors or InsP_{3} production by the sperm PLC.

In conclusion, our work reveals that cPKC activation at fertilization plays a crucial role in the regulation of the Ca^{2+} release machinery and the generation of long-lasting oscillations. This role for cPKCs at fertilization may have far-reaching effects because the pattern of Ca^{2+} oscillations dramatically affects activation events and developmental fate of the mouse embryo (Gordo et al., 2000; Ducibella et al., 2002). Further research to find the molecular identity of the SOC should help to elucidate whether it is a direct target of cPKCs, or whether an accessory protein is involved.

Materials and methods

Materials

Fura Red-AM, Fura-2-AM and Mag-Fura-2 were obtained from Molecular Probes, Inc. PMA, BIM, and thapsigargin were obtained from Calbiochem. DiC8 was obtained from Sigma-Aldrich. All these reagents were prepared as stock solutions in DMSO and were diluted in Hepes-buffered potassium simplex-optimized medium (H-KSOM) shortly before use.

Gamete collection and fertilization

Ovulated, metaphase II (MII)-arrested eggs were recovered from hormone-primed MF1 mice and stored in H-KSOM medium containing BSA, as described previously (Marangoz et al., 2003). Sperm from the epididymides of MF1 mice were released into T6 medium for capacitation, as described previously (Halet et al., 2002). In vitro fertilization was performed by adding 10–20 μl of the sperm suspension into the incubation chamber containing zona-free MII eggs in 1 ml H-KSOM. All experiments were conducted at 38 ± 0.5°C.
Expression of GFP fusion proteins in mouse eggs

Plasmids encoding PKCγ-GFP, C1-GFP, and C2-GFP were donated by Tobias Meyer (Stanford University, Stanford, CA). The constructs were provided into the pHiro vector, which contains an Sp6 promoter suitable for in vitro transcription (Oancea and Meyer, 1998). The EGFP-PKCa construct was previously described by Mostafavi-Pour et al. (2003), and was subcloned into pCNA3.1. The resulting construct was verified by sequence and restriction analysis. The plasmid encoding PH-GFP was provided by Tamas Balla (National Institutes of Health, Bethesda, MD). cRNAs encoding each of these constructs were made in vitro using the mMESSAGE mMACHINE™ kit (Ambion). The cRNAs were polyadenylated, purified, and micro-injected into mouse MII eggs as described previously (Halet et al., 2002).

Confocal imaging, [Ca2+]i imaging, and data analysis

2–3 h after cRNA injection, eggs were loaded with 10 μM Fura Red-AM (for 10 min) and freed of their zona pellucida by incubation in acidic Tyrode’s medium at 37°C. Zona-free eggs were transferred in an experimental chamber seated in a heated stage, and containing 1 ml H-KSOM medium without BSA. The changes in the distribution of GFP/EGFP-tagged proteins and [Ca2+]i at fertilization were monitored simultaneously at the equator of the cells using a confocal microscope (model LSM510; Carl Zeiss Micromaging, Inc.), using a 20× (0.75 NA) lens or a 40× (1.3 NA) oil immersion lens. Excitation was provided by the 488-nm line of an argon laser, with the laser power set at 1% of maximum. GFP/EGFP and Fura Red fluorescence were collected simultaneously using BP505-530 and LP560 emission filters respectively. Confocal time series were acquired at a rate of 1 frame every 5 s, and confocal settings (pin-hole size, detector gain) were the same in all experiments. Confocal data were analyzed using MetaMorph® (Universal Imaging Corp.) as previously published (Halet et al., 2002). In brief, regions of interest were drawn in the cytosol (C) or around the plasma membrane (PM), and changes in fluorescence intensity were measured during confocal time series. The value of the PM/C ratio was used as an index of membrane localization.

For ratiometric [Ca2+]i imaging, eggs were loaded with 2 μM Fura-2-AM (for 10 min) or 10 μM Fura Red-AM (for 10 min) 2–3 h after injection of cRNA encoding EGFP-PKCa or injection buffer. Eggs were then freed of their zona pellucida and were transferred in a similar heated chamber as used for confocal imaging. Eggs were observed with the 20× (0.75 NA) lens of an inverted microscope (Axiovert; Carl Zeiss Micromaging, Inc.) fitted with a cooled CCD camera (MicroMax; Princeton Instruments). Excitation wavelengths were adjusted to 340/380 nm (Fura-2) or 440/490 nm (Fura Red) using a monochromator (TILL Photonics). Camera shutter and monochromator settings were controlled using MetaFluor® (Universal Imaging Corp.). Emitted fluorescence was collected using a 520-nm long-pass filter (Fura Red) or a 600-nm long-pass filter (Fura-2). For measurements using Mag-Fura-2, eggs were injected with Mag-Fura-2 (1 mM in the injection pipette) at least 30 min before zona removal and recording of fertilization-induced Ca2+ transients. The estimated indicator concentration in the egg cytosol was in the range 20–50 μM, according to an injection volume equal to 2–5% of the egg volume. The indicator was excited at 420 nm (excitation range, 405–475 nm) using the monochromator, and fluorescence was collected every 5 s using a 520-nm long-pass filter. [Ca2+]i was calculated according to Ogden et al. (1995) using the equation established by Grynkiewicz et al. (1985): [Ca2+]i = Kd (F – Fmin)/Fmax – F, where Kd is the Mag-Fura-2 dissociation constant for Ca2+ binding, F is the experimentally measured fluorescence intensity, Fmax is the F value for the Ca2+-free indicator, and Fmin is the F value for the Ca2+-saturated indicator. Fmin was chosen as the fluorescence value immediately before the [Ca2+]i rise. Change in fluorescence was not affected by the fluorescence of the low affinity indicator (Ogden et al., 1995), whereas Fmax was obtained by adding ionomycin to saturate the dye with Ca2+. The Kd value was set at 250 nM (Takahashi et al., 1999). Fluorescence data from MetaMorph® or MetaFluor® analyses were exported to Microsoft Excel 2000 to generate line graphs.

SOCE assay

SOCE was monitored in zona-free mouse eggs loaded with Fura-2-AM. Ca2+ stores were first depleted with 10 μM thapsigargin in Ca2+-free H-KSOM. This treatment causes a transient rise in [Ca2+], that has been observed previously (Kline and Kline, 1992b; McGuinness et al., 1996). SOCE was visualized on the readout of Ca2+ in the extracellular medium at the final concentration of 1.8 mM.

Online supplemental material

Fig. 51 shows how the PKC inhibitor BIM reverses the stimulatory effect of PMA on the frequency of Ca2+ oscillations. Videos 1 and 2 show the membrane translocation of EGFP-PKCa and C2-GFP, respectively, during the first fertilization Ca2+ transient, as illustrated in Fig. 1 A and Fig. 2 A. Videos 3 and 4 show the translocation of C1-GFP and PH-GFP, respectively, when ionomycin is added to unfertilized eggs, as illustrated in Fig. 4 A. All supplemental material is available online at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1.

We thank Tobias Meyer and Tamas Balla for the gifts of plasmids. This work was supported by a Medical Research Council Career establishment grant to J. Carroll and by the Wellcome Trust.

Submitted: 5 November 2003
Accepted: 20 February 2004

References

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. Nature. 361: 315–325.

Berridge, M.J. 1995. Capacitative calcium entry. Biochem. J. 312:1–11.

Berridge, M.J., B.D. Boothman, and H.L. Rodericks. 2003. Calcium signalling: dynamics, homeostasis and remodeling. Nat. Rev. Mol. Cell Biol. 4:517–529.

Chauhan, V.P., and H. Brockerhoff. 1988. Phosphatidylinositol-4,5-bisphosphate may antedate diacylglycerol as activator of protein kinase C. Biochem. Biophys. Res. Commun. 155:18–23.

Colonna, R., and C. Tatone. 1993. Protein kinase C-dependent and independent events in mouse egg activation. Zygote. 2:124–256.

Corbalán-García, S., J. García-García, J.A. Rodríguez-Allaro, and J.C. Gómez-Fernández. 2003. A new phosphatidylinositol 4,5-bisphosphate-binding site located in the C2 domain of protein kinase Ca. J. Biol. Chem. 278: 4972–4980.

Cutthberth, K.S., and P.H. Cobbold. 1985. Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca2+. Nature. 316: 541–542.

Deguchi, R., H. Shikawa, S. Oda, T. Mohri, and S. Miyazaki. 2000. Spatiotemporal analysis of Ca2+ waves in relation to the sperm entry site and animal-vegetal axis during Ca2+ oscillations in fertilized mouse eggs. Dev. Biol. 218: 299–313.

Ducibella, T., and L. LeFevre. 1997. Study of protein kinase C antagonists on cortical granule exocytosis and cell-cycle resumption in fertilized mouse eggs. Mol. Reprod. Dev. 46:216–226.

Ducibella, T., D. Heneau, E. Angelichio, Z. Xu, R.M. Schulz, G.S. Kopf, R. Fissore, S. Madoux, and J.P. Ozil. 2002. Egg-to-embryo transition is driven by differential responses to Ca2+ oscillation number. Dev. Biol. 250:280–291.

Eliyahu, E., and R. Shalgi. 2002. A role for protein kinase C during rat egg activation. Biol. Reprod. 67:189–195.

Fan, H.Y., C. Tong, M.Y. Li, L. Lian, D.Y. Chen, H. Schatten, and Q.Y. Sun. 2002. Translocation of the classic protein kinase C isoforms in porcine oocytes: implications of protein kinase C involvement in the regulation of nuclear clear activity and cortical granule exocytosis. Exp. Cell Res. 277:183–191.

Gallicano, G.I., R.W. McGaughy, and D.G. Capco. 1995. Protein kinase C, the cytosolic counterpart of protein kinase C remodels the internal cytoskeleton of the mammalian egg during activation. Dev. Biol. 167:482–501.

Gallicano, G.I., R.W. McGaughy, and D.G. Capco. 1997. Activation of protein kinase C after fertilization is required for modeling the mouse egg into the zygote. Mol. Reprod. Dev. 46:587–601.

Gordo, A.C., H. Wu, C.L. He, and R.A. Fissore. 2000. Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of [Ca2+]i oscillations and oocyte age. Biol. Reprod. 62:1370–1379.

Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260: 3440–3450.

Halet, G., R. Tunwell, T. Balla, K. Swann, and J. Carroll. 2002. The dynamics of plasma membrane PtdIns(4,5)P2 at fertilization of mouse eggs. J. Cell Sci. 115:2139–2149.

Jones, K.T., J. Carroll, and D.G. Whittingham. 1995. Ionomycin, thapsigargin, ryanodine, and sperm induced Ca2+ release increase during meiotic maturation of mouse oocytes. J. Biol. Chem. 270:6671–6677.

Kline, D., and J.T. Kline. 1992a. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Dev. Biol. 149: 80–89.

Kline, D., and J.T. Kline. 1992b. Thapsigargin activates a calcium influx pathway.
in the unfertilized mouse egg and suppresses repetitive calcium transients in the fertilized egg. J. Biol. Chem. 267:17624–17630.

Kochs, G., R. Hummel, B. Fiebich, T.F. Sarre, D. Marme, and H. Hug. 1993. Activation of purified human protein kinase C α and β I isoenzymes in vitro by Ca\(^{2+}\), phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate. Biochim. J. 291:627–633.

Lee, M.H., and R.M. Bell. 1991. Mechanism of protein kinase C activation by phosphatidylinositol 4,5-bisphosphate. Biochemistry. 30:1041–1049.

Louzao, M.C., C.M.P. Ribeiro, G.S.J. Bird, and J.W. Putney, Jr. 1996. Cell type-specific modes of feedback regulation of capacitative calcium entry. J. Biol. Chem. 271:14807–14813.

Luria, A., T. Tennenbaum, Q.Y. Sun, S. Rubinstein, and H. Breitbart. 2000. Differential localization of conventional protein kinase C isoforms during mouse oocyte development. Biol. Reprod. 62:1564–1570.

Maasch, C., S. Wagner, C. Lindschau, G. Alexander, K. Buchner, M. Gollasch, M.A. Murer, and T. Meyer. 2001. Localization of the Ca\(^{2+}\) signaling pathway to the female pronucleus during early development. Biol. Reprod. 65:612–620.

Maasch, C., S. Wagner, C. Lindschau, G. Alexander, K. Buchner, M. Gollasch, M.A. Murer, and T. Meyer. 2001. Localization of the Ca\(^{2+}\) signaling pathway to the female pronucleus during early development. Biol. Reprod. 65:612–620.

Maasch, C., S. Wagner, C. Lindschau, G. Alexander, K. Buchner, M. Gollasch, M.A. Murer, and T. Meyer. 2001. Localization of the Ca\(^{2+}\) signaling pathway to the female pronucleus during early development. Biol. Reprod. 65:612–620.

Maasch, C., S. Wagner, C. Lindschau, G. Alexander, K. Buchner, M. Gollasch, M.A. Murer, and T. Meyer. 2001. Localization of the Ca\(^{2+}\) signaling pathway to the female pronucleus during early development. Biol. Reprod. 65:612–620.