JCB Article

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 Carroll[1]

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

In mammalian eggs, sperm-induced Ca^{2+} oscillations at fertilization are the primary trigger for egg activation and initiation of embryonic development. Identifying the downstream effectors that decode this unique Ca^{2+} signal is essential to understand how the transition from egg to embryo is coordinated. Here, we investigated whether conventional PKCs (cPKCs) can decode Ca^{2+} oscillations at fertilization. By monitoring the dynamics of GFP-labeled PKCα and PKCγ in living mouse eggs, we demonstrate that cPKCs translocate to the egg membrane at fertilization following a pattern that is shaped by the amplitude, duration, and frequency of the Ca^{2+} transients. In addition, we show that cPKC translocation is driven by the C2 domain when Ca^{2+} concentration reaches 1–3 μM. Finally, we present evidence that one physiological function of activated cPKCs in fertilized eggs is to sustain long-lasting Ca^{2+} oscillations, presumably via the regulation of store-operated Ca^{2+} entry.

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 (Berridge 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, PLCζ (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

The online version of this article includes supplemental material.

Address correspondence to Guillaume Halet, Department of Physiology, University College London, Gower Street, London WC1E 6BT, England, UK. Tel.: 0207-679-3229. Fax: 0207-383-7005. email: g.halet@ucl.ac.uk; or John Carroll, Department of Physiology, University College London, Gower Street, London WC1E 6BT, England, UK. Tel.: 0207-679-3229. Fax: 0207-383-7005. email: j.carroll@ucl.ac.uk

Key words: calcium; PKC; oscillations; GFP; influx

Abbreviations used in this paper: BIM, bisindolylmaleimide I; cPKC, conventional protein kinase C; DiC8, 1,2-dioctanoyl *sn*-glycerol; $InsP_3$, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; SOC, store-operated channel; SOCE, store-operated calcium entry.

The Journal of Cell Biology, Volume 164, Number 7, March 29, 2004 1033–1044
http://www.jcb.org/cgi/doi/10.1083/jcb.200311023

The Journal of Cell Biology

growth and differentiation to secretion, gene expression, and regulation of other signaling pathways. 10 mammalian PKC isotypes 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 phosphatidylserine (Verdaguer et al., 1999; Stahelin 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 $[Ca^{2+}]_i$ 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 cPKC 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; Maasch et al., 2000; Schaefer et al., 2001; Tanimura et al., 2002). We have chosen to monitor PKCα and PKCγ, two isotypes 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 $[Ca^{2+}]_i$ 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 returned to the cytosol while $[Ca^{2+}]_i$ slowly decreased, but a pool of EGFP-PKCα 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

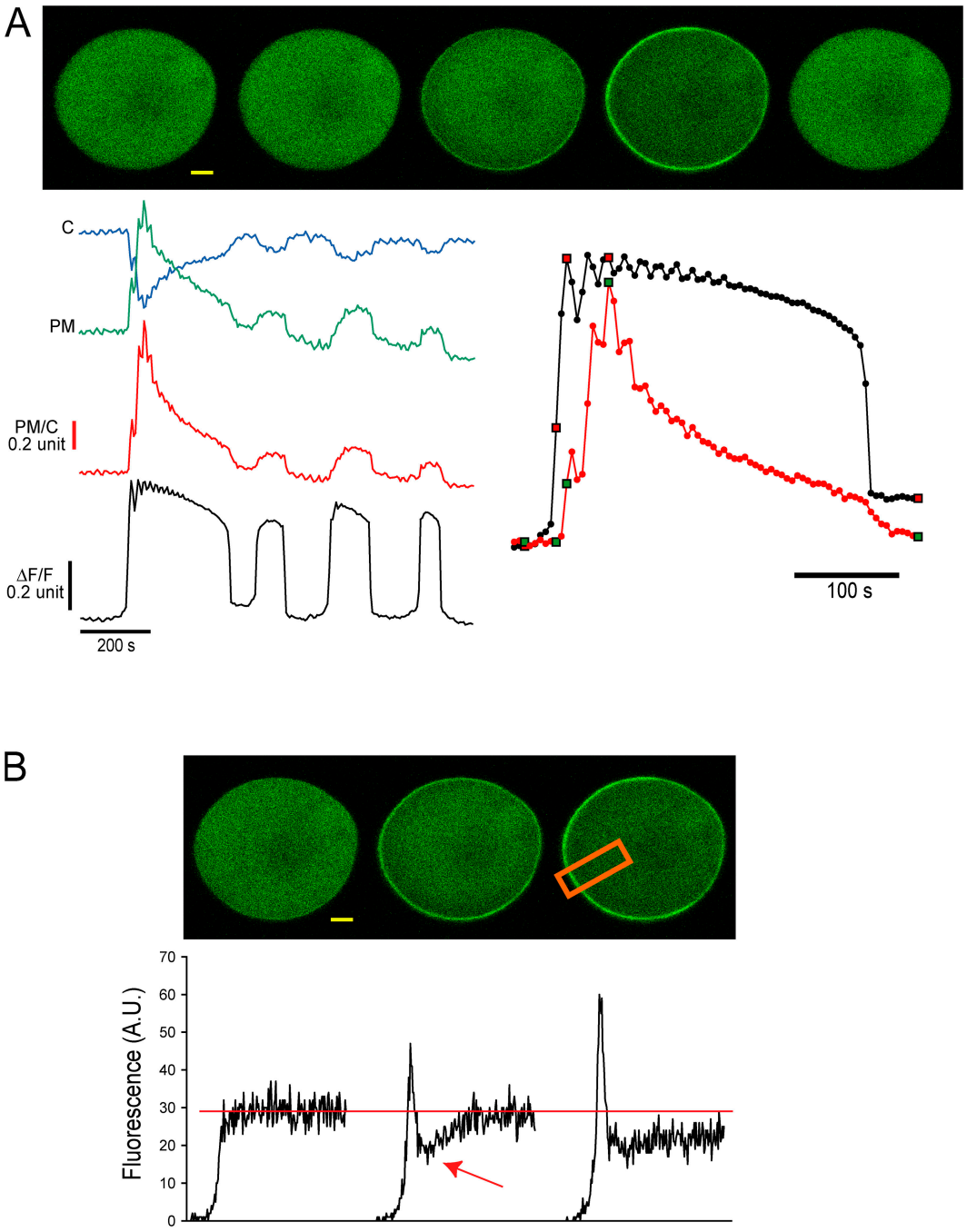

Figure 1. **PKCα translocates to the egg plasma membrane at fertilization.** (A) EGFP-PKCα repetitively translocates to the egg membrane during Ca^{2+} oscillations at fertilization. Confocal images show the translocation of EGFP-PKCα during the first Ca^{2+} transient of the experiment displayed below, and are taken from Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1). Left: EGFP-PKCα fluorescence was measured in the cytosol (C, blue) and at the plasma membrane level (PM, green) in every frame of the time series (1 frame every 5 s). The resulting net change in cPKC localization is expressed as the ratio PM/C and is shown in red. $[Ca^{2+}]_i$ fluctuations were monitored simultaneously with Fura Red and were expressed as ΔF/F (black). Right: The first Ca^{2+} transient and corresponding fluctuations in PM/C of the same recording are displayed on an expanded scale. Highlighted data points correspond to the five confocal frames displayed at the top. Note that the translocation (increase in PM/C, red) starts when the first Ca^{2+} spike is generated, and proceeds as increments phase-locked with the first Ca^{2+} spikes. (B) EGFP-PKCα translocation is a diffusion-mediated process. Three confocal frames are taken from the experiment shown in A. The graph represents the fluorescence intensity (in arbitrary units) measured in the region of interest (rectangle) during the process of translocation. The horizontal line indicates the cytosolic fluorescence level before translocation. Note the subplasmalemmal depletion zone (arrow), indicative of a diffusion-mediated translocation. These data are representative of seven experiments with eggs expressing EGFP-PKCα and 10 experiments with eggs expressing PKCγ-GFP. Bars, 10 μm.

plasma membrane phosphatidylserine upon Ca^{2+} coordination (Verdaguer et al., 1999; Stahelin et al., 2003). To investigate the role of Ca^{2+} 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-

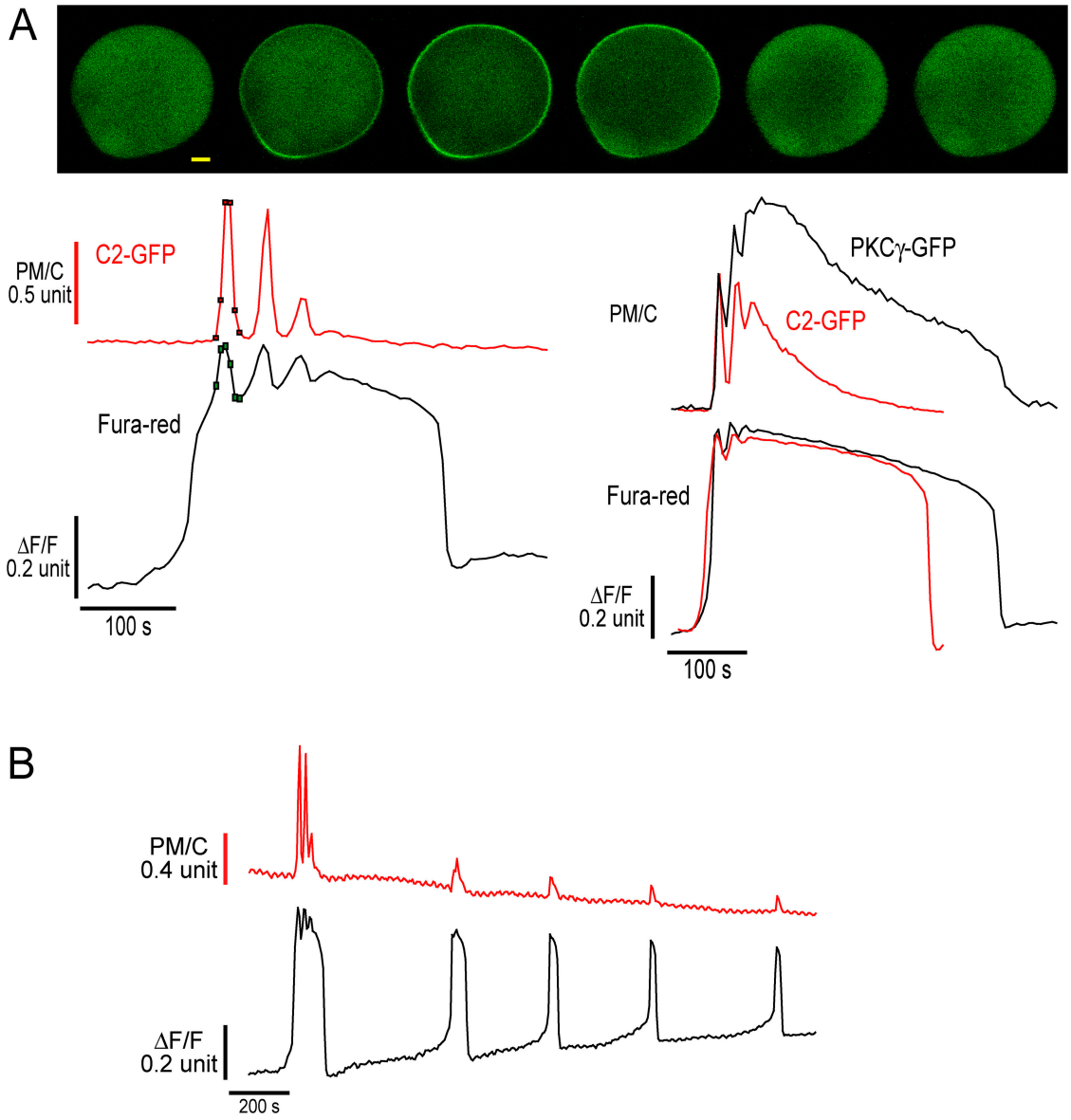

Figure 2. **The C2 domain is recruited at the egg membrane by Ca^{2+} spikes.** (A) Translocation of C2-GFP during the first fertilization Ca^{2+} transient. Confocal images (5 s apart) illustrate the first C2-GFP translocation pulse of the experiment displayed on the bottom left, where the corresponding 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 PKCγ-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 μm.

namics during fertilization-induced Ca^{2+} oscillations. 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 PKCγ and PKCα, 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 PKCα and PKCγ 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-

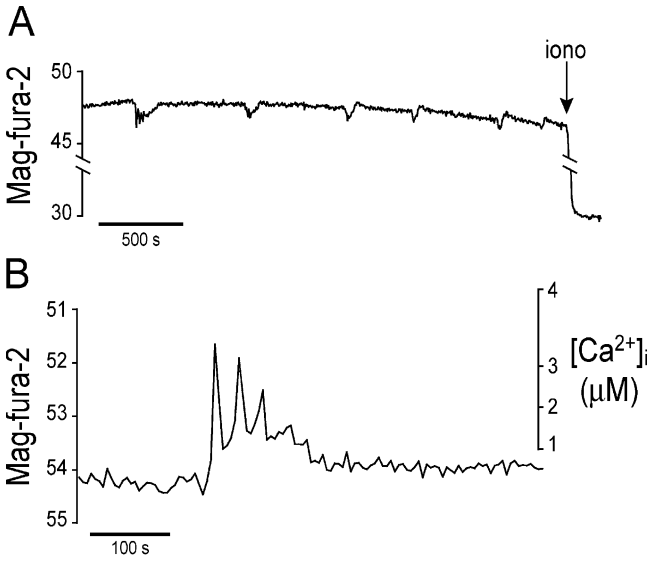

Figure 3. **Cytosolic $[Ca^{2+}]$ reaches micromolar levels during the Ca^{2+} spikes.** (A) Raw Mag-Fura-2 fluorescence trace showing the downward deflections corresponding to the fertilization Ca^{2+} transients. Ionomycin (iono) was added at the end of the experiment for calibration. Mag-Fura-2 fluorescence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

brane 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.

$[Ca^{2+}]_i$ rises to micromolar levels during fertilization-induced Ca^{2+} spikes

Because of its high affinity for Ca^{2+} (in vitro Kd reported as 140 nM; Takahashi et al., 1999), it is likely that Fura Red becomes saturated under conditions where $[Ca^{2+}]_i$ exceeds micromolar levels, leading to an underestimation of the relative amplitudes of the Ca^{2+} transients and spikes. To estimate the maximal amplitude of the Ca^{2+} transients without worrying about indicator saturation, and to investigate the possibility that Ca^{2+} spikes reach high concentration critical for C2-GFP translocation, we monitored fertilization-induced Ca^{2+} oscillations with the low affinity Ca^{2+} indicator Mag-Fura-2 (Kd = 25 μM in vitro; Takahashi et al., 1999). The first fertilization Ca^{2+} transient appeared as a succession of 3–4 sharp Ca^{2+} spikes, whereas subsequent Ca^{2+} transients were detected as smaller, rather monophasic Ca^{2+} spikes (Fig. 3 A). Estimation of $[Ca^{2+}]_i$ 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_{max} value with ionomycin (see Materials and methods). On average, $[Ca^{2+}]_i$ was found to rise up to 3.1 ± 0.5 μM (n = 7) at the peak of the first Ca^{2+} spike of the first transient (Fig. 3 B). All subsequent transients were smaller, but transiently reached 1–2 μM. The Ca^{2+} plateau during the first transient was estimated to reach up to 1 μM. Overall, the micromolar $[Ca^{2+}]_i$ changes observed at fertilization exhibited a pattern very similar to the dynamics of C2-GFP translocation, suggesting that the threshold $[Ca^{2+}]_i$ 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 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 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 and Meyer, 1998; 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 Ca^{2+} ionophore ionomycin to activate endogenous PLCs (Várnai and Balla, 1998). The rapid increase in $[Ca^{2+}]_i$ 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/DC1). Evidence that DAG synthesis was a consequence of Ca^{2+}-induced activation of PLC is provided by the decrease in plasma membrane PIP_2 staining in eggs expressing a PIP_2-specific GFP-tagged pleckstrin homology domain (PH-GFP; Halet et al., 2002) and exposed to ionomycin (Fig. 4 A, right; Video 4). In contrast, fertilization-induced 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 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 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 Ca^{2+} transients was unaffected. However, translocation remained a Ca^{2+}-regulated process, starting when $[Ca^{2+}]_i$ reached its peak, and finishing when $[Ca^{2+}]_i$ started to decrease, demonstrating

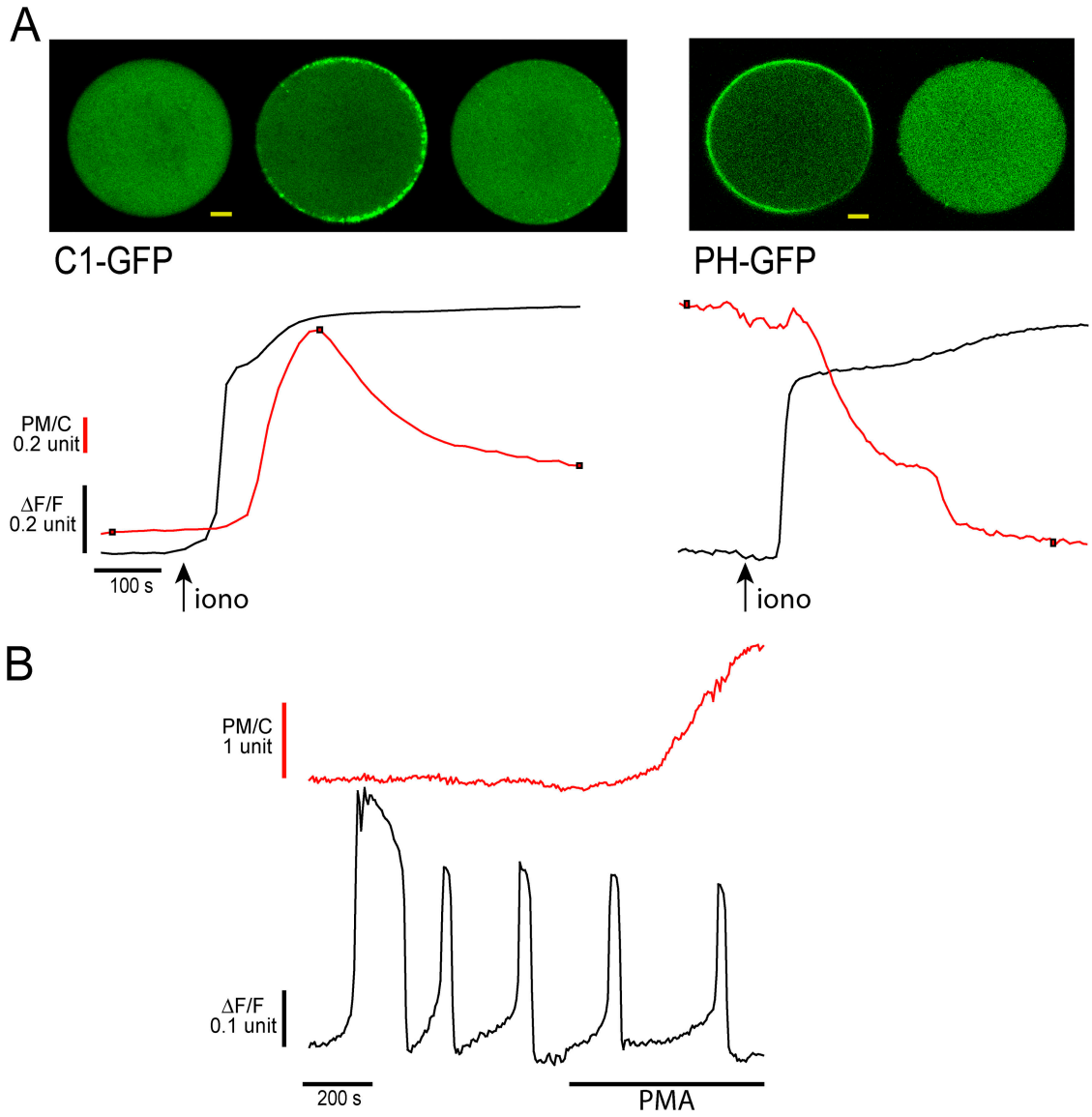

Figure 4. **DAG production remains undetectable at fertilization.** (A) Ionomycin-induced Ca^{2+} increase triggers PIP_2 hydrolysis and DAG production in unfertilized eggs. The Ca^{2+} increase triggered by ionomycin (iono; 1 μM final concentration) is rapidly followed by the translocation of C1-GFP to the egg membrane (left) and the translocation of PH-GFP to the cytosol (right), indicating DAG production and PIP_2 hydrolysis, respectively. Confocal frames displayed at the top correspond to the data points highlighted on the PM/C trace and are taken from Videos 3 and 4 (available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1). (B) DAG is not detected at fertilization. The localization of C1-GFP was monitored during fertilization-induced Ca^{2+} oscillations. No translocation was detected until PMA (10 ng/ml final concentration) was added to the medium after the third Ca^{2+} transient. All these experiments were repeated at least five times with similar results. Bar, 10 μm.

that DiC8 was not sufficient to retain the kinase at the membrane when $[Ca^{2+}]_i$ is low. These observations suggest that, in the presence of Ca^{2+}, DiC8 has altered the kinetic parameters of PKCγ-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 PKCγ-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^{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^{2+} oscillations at fertilization

To investigate the role of cPKCs in Ca^{2+} signaling at fertilization, we examined the effects of PKCα overexpression on the pattern of Ca^{2+} oscillations. In control, buffer-injected eggs, the period of Ca^{2+} oscillations reached several minutes (~5 min when measured between transients 4 and 5; Table I), during which $[Ca^{2+}]_i$ slowly increased up to a threshold level at which the next transient was generated (Fig. 6 A). This so-called Ca^{2+} pacemaker (Miyazaki et al., 1993), suggestive of a Ca^{2+}-induced Ca^{2+} release mechanism, was also observed in the absence of extracellular Ca^{2+} (Fig. 6 E), demonstrating that it resulted from intracellular Ca^{2+} release. In eggs overexpressing EGFP-PKCα, the duration of

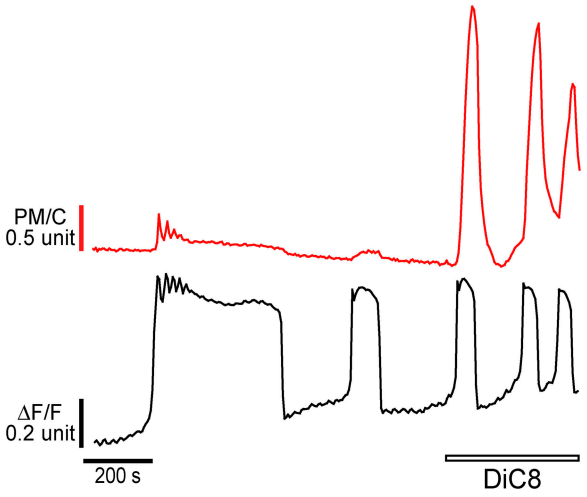

Figure 5. **Exogenous DAG increases the amplitude of PKCγ-GFP translocation at fertilization.** The DAG analogue DiC8 (100 μM final concentration) was added to the medium between two successive fertilization-induced Ca^{2+} oscillations (open bar). Note that the effect of DiC8 on translocation is apparent only during the Ca^{2+} transients. After two Ca^{2+} transients in the presence of DiC8 however, oscillation frequency was found to increase and PKCγ-GFP to accumulate at the membrane. Similar results were obtained in four experiments.

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 PKCα 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 μM 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-PKCα 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 $[Ca^{2+}]$) 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 $[Ca^{2+}]$ to 6 mM, as indicated by the resumption of Ca^{2+} oscillations (Fig. 6 E).

Table I. **PKC stimulation alters the pattern of Ca^{2+} oscillations at fertilization**

	Control	EGFP-PKCα	PMA
	n = 16	*n* = 9	*n* = 4
Duration of the first transient (s)	148.4 ± 7.8	682.2 ± 87.9[a]	681.2 ± 29.0[a]
Period (s)	302.8 ± 26.1	132.2 ± 21.7[a]	112.5 ± 12.7[a]

The duration of the first transient was measured between the upstroke and the falling phase of the transient at half-maximal amplitude. The period was measured between the third and fourth Ca^{2+} transients. EGFP-PKCα 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.
[a]$P < 0.05$.

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 $[Ca^{2+}]$ 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-

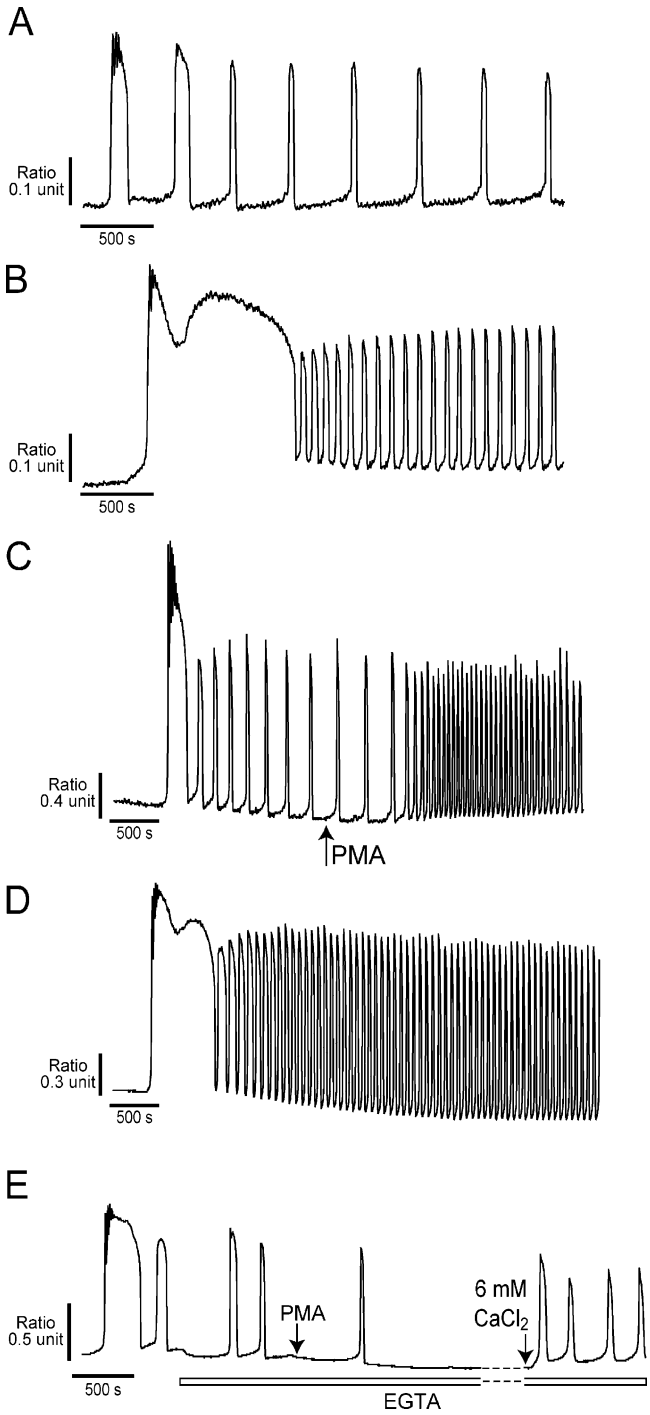

Figure 6. **PKC promotes Ca^{2+} oscillations at fertilization.** (A) Fertilization-induced Ca^{2+} oscillations recorded in an egg injected with injection buffer. (B) Fertilization-induced Ca^{2+} oscillations in an egg overexpressing EGFP-PKCα. Note the extra Ca^{2+} release during the first transient and the high oscillation frequency. (C) In this experiment, a fertilized egg was stimulated with PMA (10 ng/ml final concentration) during ongoing Ca^{2+} oscillations, resulting in an increase in oscillation frequency. The arrow indicates the time when PMA was added to the medium. (D) Fertilization-induced Ca^{2+} oscillations recorded in the continuous presence of 10 ng/ml PMA. (E) 3 mM EGTA (open bar) was added to the medium during ongoing fertilization-induced Ca^{2+} oscillations to chelate extracellular Ca^{2+}, resulting in a dramatic decrease in oscillation frequency, and ultimately in the arrest of the oscillations. Further addition of 10 ng/ml PMA did not stimulate Ca^{2+} transient generation. Increasing extracellular $[Ca^{2+}]$ to 6 mM (6 mM $CaCl_2$) 60 min after the last fertilization-induced Ca^{2+} transient was observed, triggered the resumption of the oscillations. Data in A and B were obtained using Fura Red-AM as the Ca^{2+} indicator; data in C–E were obtained with Fura-2-AM. All these data are representative of 5–12 similar experiments.

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+} transients in mammalian eggs is the presence of Ca^{2+} spikes on the top of the transients (Cuthbertson and Cobbold, 1985; Jones et al., 1995; Deguchi et al., 2000). Our data reveal that these Ca^{2+} spikes—mounting up to 3 μM $[Ca^{2+}]_i$ during the first transient—are the trigger for cPKC translocation to the egg

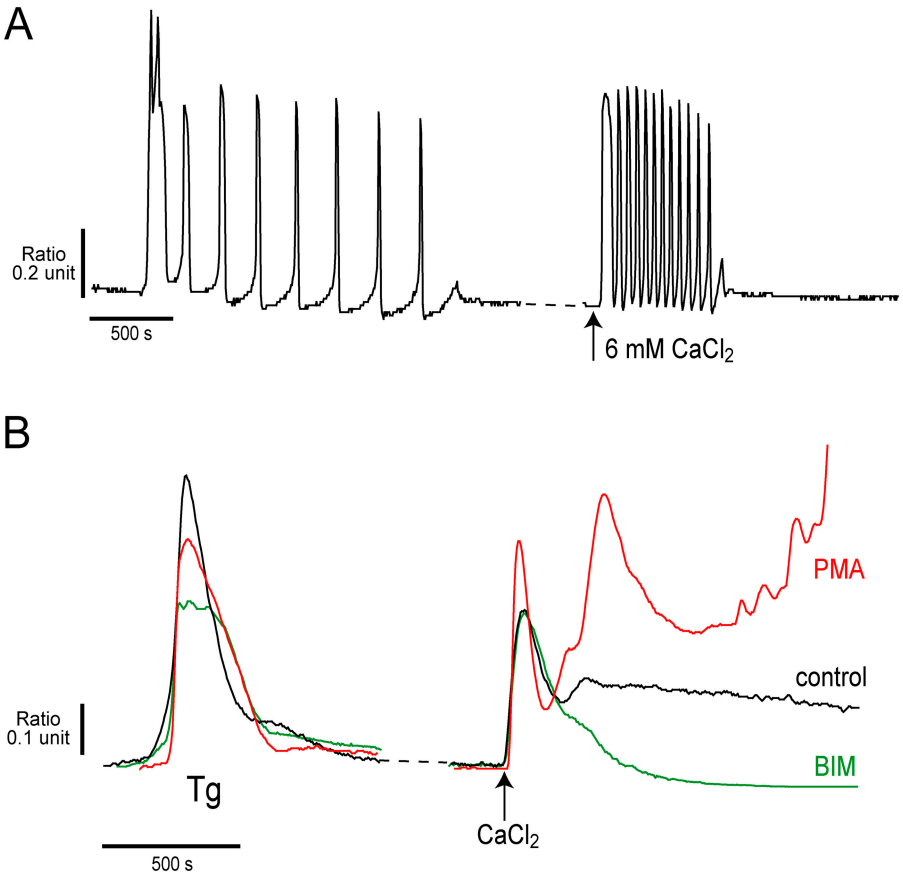

Figure 7. **PKC regulates SOCE in mouse eggs.** (A) PKC inhibition shortens fertilization-induced Ca^{2+} oscillations. After a 30-min incubation in the presence of 5 μM BIM, fertilization-triggered Ca^{2+} oscillations terminate prematurely with an aborted Ca^{2+} transient. Ca^{2+} oscillations transiently resumed after extracellular [Ca^{2+}] was raised to 6 mM (6 mM $CaCl_2$). This recording is representative of eight similar experiments. (B) SOCE is regulated by PKC activity. Unfertilized eggs were exposed to the Ca^{2+} pump inhibitor thapsigargin in a Ca^{2+}-free medium, to empty their Ca^{2+} stores. Application of thapsigargin resulted in a transient increase in $[Ca^{2+}]_i$ of variable amplitude, shown on the left (Tg). After 10–20 min, Ca^{2+} was readmitted into the medium ($CaCl_2$) at the final concentration of 1.8 mM in order to visualize SOCE. In control conditions (black), SOCE exhibited a phasic increase followed by a sustained plateau; the latter was absent or dramatically enhanced in eggs treated with 5 μM BIM (green) or exposed to 10 ng/ml PMA during the 20 min before $CaCl_2$ readmission (red). Recordings are representative of at least 13 similar experiments.

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 PKCα 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-

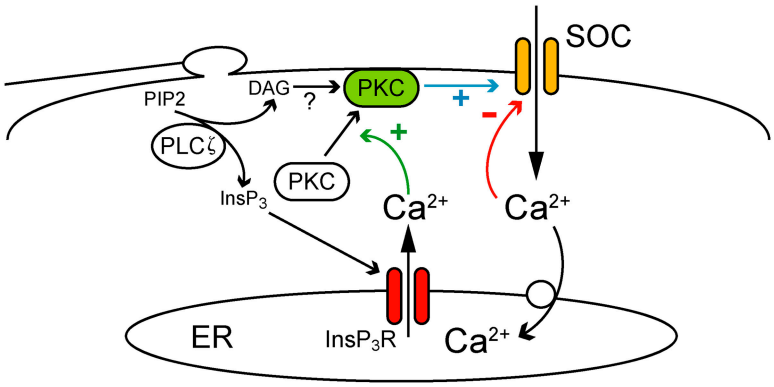

Figure 8. **The positive feedback loop of cPKCs in the egg Ca^{2+} oscillator.** This model illustrates how cPKCs may sustain the generation of long-lasting Ca^{2+} oscillations at fertilization. Sperm-derived PLCζ triggers $InsP_3$ production and Ca^{2+} release from the ER. Store depletion activates Ca^{2+}-permeant store-operated channels (SOC), 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 PLCζ.

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 PKCδ, 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). In our assay, Ca^{2+} influx activated by store depletion appeared as a phasic increase in $[Ca^{2+}]_i$, followed by a sustained plateau at an intermediate level. The sustained plateau probably reflects a new equilibrium that is maintained in part by negative feedback by Ca^{2+} on the SOCs, as has been suggested previously in somatic cells and *Xenopus* oocytes (Petersen and Berridge, 1994; Berridge, 1995; Louzao et al., 1996). Our finding that the plateau is dramatically increased by activating PKC and abolished by inhibiting PKC suggests that PKC may promote Ca^{2+} influx by counteracting the negative feedback on SOCs. We suggest that SOCs or some accessory proteins at the egg plasma membrane are substrates for cPKC phosphorylation, with the effect of counteracting the rapid feedback inhibition by Ca^{2+} (Fig. 8).

Our hypothesis that cPKCs promote SOCE predicts that an increase in the rate of Ca^{2+} influx should occur during each Ca^{2+} transient, when the kinases translocate to the plasma membrane. A former analysis by McGuinness and coworkers suggests that this is indeed the case: by monitoring SOC activation in mouse eggs with the manganese quench technique, the authors demonstrated that the rate of SOCE increases periodically during fertilization Ca^{2+} transients, suggesting that Ca^{2+} release potentiates Ca^{2+} influx (McGuinness et al., 1996). These observations are in agreement with the dynamics of cPKC translocation described in the present paper.

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 (Marangos et al., 2003). Sperm from the epididymis 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-PKCα construct was previously described by Mostafavi-Pour et al. (2003), and was subcloned into pcDNA3.1. The resulting construct was verified by sequencing 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, $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ at fertilization were monitored simultaneously at the equator of the cells using a confocal microscope (model LSM510; Carl Zeiss MicroImaging, 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 LP650 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 $[Ca^{2+}]_i$ 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-PKCα 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 MicroImaging, 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-2) or a 600-nm long-pass filter (Fura Red) every 5 s. For $[Ca^{2+}]_i$ 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 Ca^{2+} 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 (Ogden et al., 1995) using the monochromator, and fluorescence was collected every 5 s using a 520-nm long-pass filter. $[Ca^{2+}]_i$ was calculated according to Ogden et al. (1995) using the equation established by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = Kd\ (F - F_{min})/(F_{max} - F)$, where Kd is the Mag-Fura-2 dissociation constant for Ca^{2+} binding, F is the experimentally measured fluorescence intensity, F_{min} is the F value for the Ca^{2+}-free indicator, and F_{max} is the F value for the Ca^{2+}-saturated indicator. F_{min} was chosen as the fluorescence value immediately before the $[Ca^{2+}]_i$ change because resting $[Ca^{2+}]_i$ does not affect the fluorescence of the low affinity indicator (Ogden et al., 1995), whereas F_{max} was obtained by adding ionomycin to saturate the dye with Ca^{2+}. The Kd value was set at 25 μM (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. Ca^{2+} stores were first depleted with 10 μM thapsigargin in Ca^{2+}-free H-KSOM. This treatment causes a transient rise in $[Ca^{2+}]_i$ that has been observed previously (Kline and Kline, 1992b; McGuinness et al., 1996). SOCE was visualized on the readmission of $CaCl_2$ in the extracellular medium at the final concentration of 1.8 mM.

Online supplemental material

Fig. S1 shows how the PKC inhibitor BIM reverses the stimulatory effect of PMA on the frequency of Ca^{2+} oscillations. Videos 1 and 2 show the membrane translocation of EGFP-PKCα and C2-GFP, respectively, during the first fertilization Ca^{2+} 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

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