Cell cycle-dependent Ca^{2+} oscillations in mouse embryos are regulated by nuclear targeting of PLCζ

Mark G. Larman[1,2], Christopher M. Saunders[2], John Carroll[3], F. Anthony Lai[2] and Karl Swann[1,*]

[1]Department of Anatomy and Developmental Biology, University College London, London, WC1E 6BT, UK
[2]Cell Signalling Laboratory, Wales Heart Research Institute, University of Wales College of Medicine, Cardiff, CF14 4XN, UK
[3]Department of Physiology, University College London, London, WC1E 6BT, UK
*Author for correspondence (e-mail: k.swann@ucl.ac.uk)

Accepted 19 January 2004
Journal of Cell Science 117, 2513-2521 Published by The Company of Biologists 2004
doi:10.1242/jcs.01109

Summary

During the first cell cycle Ca^{2+} oscillations are regulated in a cell cycle-dependent manner, such that the oscillations are unique to M phase. How the Ca^{2+} oscillations are regulated with such cell cycle stage-dependency is unknown, despite their importance for egg activation and embryo development. We recently identified a novel, sperm-specific phospholipase C (PLCzeta; PLCζ) that triggers Ca^{2+} oscillations similar to those caused by sperm. We show that PLCζ-induced Ca^{2+} oscillations also occur exclusively during M phase. The cell cycle-dependency can be explained by PLCζ's localisation to the pronuclei, which depends specifically upon a nuclear localisation signal sequence. Preventing pronuclear localisation of PLCζ by mutation of the nuclear localisation signal, or by inhibiting pronuclear formation/import, can prolong Ca^{2+} oscillations or allow them to occur during interphase. These data suggest a novel mechanism for regulating a PLC through nuclear sequestration and may explain the cell cycle-dependent regulation of Ca^{2+} oscillations following fertilisation.

Key words: Fertilisation , Phospholipase C, Ca^{2+} oscillations, Cell cycle

Introduction

Increases in cytosolic Ca^{2+} concentrations regulate a vast range of cellular processes (Berridge et al., 2003). Control of cell cycle divisions by Ca^{2+} has been shown in a number of species (for reviews, see Stricker, 1999; Whitaker and Larman, 2001). Probably one of the most striking displays of increases in cytosolic Ca^{2+} is seen at mammalian fertilisation where the sperm triggers a long-lasting series of Ca^{2+} oscillations in the egg, which last for several hours and eventually terminate about the time of pronuclear formation when first interphase begins (reviewed by Carroll, 2001). These Ca^{2+} oscillations are both necessary and sufficient for egg activation at fertilisation (Kline and Kline, 1992; Miyazaki et al., 1993; Swann and Ozil, 1994) and later development (Ozil, 1990; Bos-Mikich et al., 1997; Lawerence et al., 1998; Ozil and Huneau, 2001). Generation of the Ca^{2+} transients is regulated in a cell cycle-dependent manner since no Ca^{2+} increases are observed during interphase, but oscillations do start again when the zygote enters first mitosis (Tombes et al., 1992; Kono et al., 1996; Day et al., 2000; Marangos et al., 2003). The stimulus for the mitotic Ca^{2+} oscillations appears to be related to some factor(s) from the sperm because they are seen in fertilised zygotes, but not in parthenogenetic embryos that have been artificially activated by strontium or ethanol (Kono et al., 1996).

The mechanism for generating these cell cycle-dependent Ca^{2+} signals has not been established (Carroll, 2001). One explanation is that the pronuclei may sequester a sperm-derived factor or cofactor in order to stop it triggering Ca^{2+} oscillations (Carroll, 2001). This idea is consistent with the ability of fertilised one-cell embryos, which contain a pronucleus, to activate metaphase II-arrested (MII) eggs when they are fused together (Zernicka-Goetz et al., 1995). Two further studies, using different approaches, have provided direct evidence for the sequestering role of the pronuclei in terminating the Ca^{2+} oscillations. Nuclear transplantation experiments involving the transfer of pronuclei from fertilised one-cell embryos to unfertilised mouse eggs demonstrated that Ca^{2+} oscillations followed subsequent pronuclear envelope breakdown in the meiotic metaphase cytoplasm (Kono et al., 1995). Secondly, blocking pronuclear formation or nuclear import was found to significantly prolong the fertilisation Ca^{2+} oscillations (Marangos et al., 2003). These data strongly support the idea that a factor responsible for controlling the Ca^{2+} oscillations may become localised to pronuclei during interphase. Thus, possible candidates for nuclear targeting include the Ca^{2+}-releasing factor, cofactor(s) and substrate (Marangos et al., 2003).

Cell cycle Ca^{2+} changes are also seen in embryos of other species, such as sea urchins (Whitaker and Patel, 1990; Whitaker and Larman, 2001). Biochemical assays in sea urchin embryos suggest that the cell cycle Ca^{2+} changes are due to endogenously generated increases in inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] production (Ciapa et al., 1994). This suggests that an endogenous phospholipase C (PLC) is being activated during the cell cycle but the mechanism and identity of the PLC(s) involved is unclear. We recently identified a mammalian sperm-specific PLC (PLCζ) (Cox et al., 2000; Saunders et al., 2002). Injecting PLCζ cRNA into mouse eggs, at a concentration that produces PLCζ protein in the same range as that found in a single sperm, triggers Ca^{2+}

oscillations during meiosis exit that are indistinguishable from those observed at fertilisation. Through immunodepletion, PLCζ appears to be the Ca^{2+}-releasing factor present in sperm (Saunders et al., 2002).

We investigated if the localisation of PLCζ can provide an explanation for cell cycle-dependency of the Ca^{2+} signals during the first division in mouse embryos. We first report that PLCζ-induced Ca^{2+} oscillations only occur during M-phase. By using an epitope-tagged PLCζ we then demonstrate that PLCζ localises to the pronuclei and that preventing nuclear sequestration by mutation of the PLCζ nuclear localisation signal (NLS) or inhibiting pronuclear formation/import prolongs the Ca^{2+} oscillations. We present a model of PLCζ nuclear localisation that offers a simple and novel explanation for the cell cycle-dependent Ca^{2+} oscillations during the first cell cycle.

Materials and Methods

Materials

All reagents used were from Sigma, unless stated otherwise. The fluorescently tagged nuclear localisation signal (FITC-BSA-NLS) was a kind gift from Mark Jackman (Wellcome/CRC, UK) and wheat germ agglutinin (WGA) from Calbiochem. Complementary RNA (cRNA) was synthesised from the open reading frame of mouse PLCζ and c-myc tagged-PLCζ (Saunders et al., 2002). Each batch of cRNA produced was checked for in vitro protein expression using a reticulocyte lysate system (Promega), as previously described (Saunders et al., 2002).

Preparation and handling of eggs and embryos

Female MF1 mice were superovulated by an injection of 5 IU of pregnant mare's serum gonadotropin (PMSG; Intervet). This was followed by an injection of human chorionic gonadotropin (HCG; Intervet) 48 hours later. Eggs were collected after a further 13.5-14.5 hours, as previously described (Lawrence et al., 1997) and maintained in 100 μl droplets of H-KSOM (HEPES-buffered potassium simplex optimized medium) (Summers et al., 2000) under mineral oil at 37°C. For experiments on MII-arrested eggs cRNA injections were carried out between 14.5 and 15.5 hours after HCG injection. To prepare parthenotes for interphase (G1), eggs were treated with 20 mM Sr^{2+} in Ca^{2+}-free H-KSOM (Kline and Kline, 1992). Interphase (G2) parthenotes were obtained by exposing eggs to H-KSOM containing 7% ethanol for 7 minutes at room temperature (Cuthbertson, 1983) 24 hours after HCG and culturing for a further 9-12 hours (Tang et al., 2000). All activations were carried out in H-KSOM containing 2 μM cytochalasin D to prevent second polar body extrusion, making the embryos diploid.

Microinjection and measurement of intracellular calcium changes

Eggs and embryos were injected as previously described (Marangos et al., 2003). cRNA solutions were diluted with an RNase-free injection buffer (0.5× PBS; Ambion). The volume injected was estimated from the diameter of cytoplasmic displacement caused by the bolus injection. Depending on the reagent, injection volumes ranged from 0.5-5% of the egg volume. The amount of PLCζ protein expressed after microinjection with a pipette concentration of 0.02 mg/ml cRNA (3-5% egg volume) initiates Ca^{2+} oscillations with a comparable amount of PLCζ that is present in a single sperm (Saunders et al., 2002). All experiments were carried out with 0.02 mg/ml, unless stated otherwise. In experiments that required a number of reagents to be introduced into the cell, to reduce possible damage caused by multiple injections, some reagents were co-injected (Marangos et al., 2003). Intracellular calcium changes were measured with Fura-dextran 10,000 M_r (Molecular Probes; pipette concentration 0.5 mM) made up in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4). Briefly, calcium measurements were carried out on CCD-based imaging systems using a Zeiss Axiovert 100 microscope with illumination from a monochromator (Photonics) controlled by MetaFluor v4.0 (Universal Imaging Corp.) (Marangos et al., 2003). Pseudo-simultaneous imaging of Ca^{2+} (2-4 μM Fura-dextran final concentration) and FITC-BSA-NLS (1-3 μM final concentration) was carried out using an excitation filter wheel (Sutter, UK; 350/380 nm for Fura-dextran and 490 nm for FITC-BSA-NLS), a 510 nm dichroic mirror and 520 nm long pass emission filter.

Immunostaining and confocal imaging

Eggs were microinjected with either 0.2 mg/ml PLCζ cRNA or c-Myc-PLCζ cRNA and cultured in H-KSOM medium containing 2 μM cytochalsin D. Once the embryos had formed pronuclei they were washed in PBS containing 4 mg/ml bovine serum albumin (BSA) for 5 minutes. Fixation was carried out by transferring the embryos into 4% paraformaldehyde (in PBS alone) for 15 minutes. Embryos were washed for a further 5 minutes before permeabilisation was achieved with 2% Triton X-100 (in PBS/BSA) for 10 minutes. The blocking step was carried out overnight in a solution of 50 mM glycine (in PBS/BSA) before the c-Myc primary antibody (9E10; Santa Cruz Biotechnology) was used at 0.4 ng/μl (in PBS/BSA) and applied for 16 hours. This was followed by another PBS/BSA wash and then the embryos were transferred into the secondary antibody solution (10 μg/ml) for 2 hours (goat anti-mouse Alexa Fluor® 488; Molecular Probes, USA).

Confocal microscopy was carried out on a Zeiss Axiovert 100TV microscope equipped with Biorad μRadiance. Using the 488 nm laser line of an Argon laser, the emission of Alexa Fluor® 488 was collected using a 520 nm (40 nm bandwidth) bandpass filter.

Generation of NLS mutant, Myc-PLCζ^{K377E}

Mutation of ^{377}Lys to ^{377}Glu within the putative PLCζ nuclear localisation signal sequence to produce Myc-PLCζ^{K377E} was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), as described previously (Saunders et al., 2002). The K377E mutant of Myc-PLCζ was generated using the primer pair 5′-gtcaagaaaaggaag**gag**aagatgaaaatagcc-3′ and 5′-ggctattttcatctt**ctc**c-ttccttttcttgac-3′, with the previously described Myc-PLCζ as template (Saunders et al., 2002). Incorporation of the mutated sequence in Myc-PLCζ^{K377E} was verified by nucleotide sequence analysis performed on an ABI Prism® 3100 Genetic Analyzer.

Results

PLCζ-induced Ca^{2+} oscillations are cell cycle-dependent

Expression of PLCζ protein in mouse eggs, through microinjection of PLCζ cRNA, produces a series of long lasting Ca^{2+} oscillations indistinguishable from those at fertilisation (Saunders et al., 2002). During the first cell cycle, sperm-triggered oscillations appear to be controlled with a cell cycle dependence (review by Carroll, 2001). To test if PLCζ-induced Ca^{2+} oscillations are also cell cycle dependent, mouse eggs were injected with PLCζ cRNA and Fura-dextran at three different stages of the first cell cycle. PLCζ injection into MII eggs triggers a series of long lasting Ca^{2+} oscillations that terminate as the embryo enters interphase (Fig. 1a). In contrast, eggs that had been Sr^{2+}-activated and allowed to form pronuclei (G1 interphase) did not show any increase in Ca^{2+}

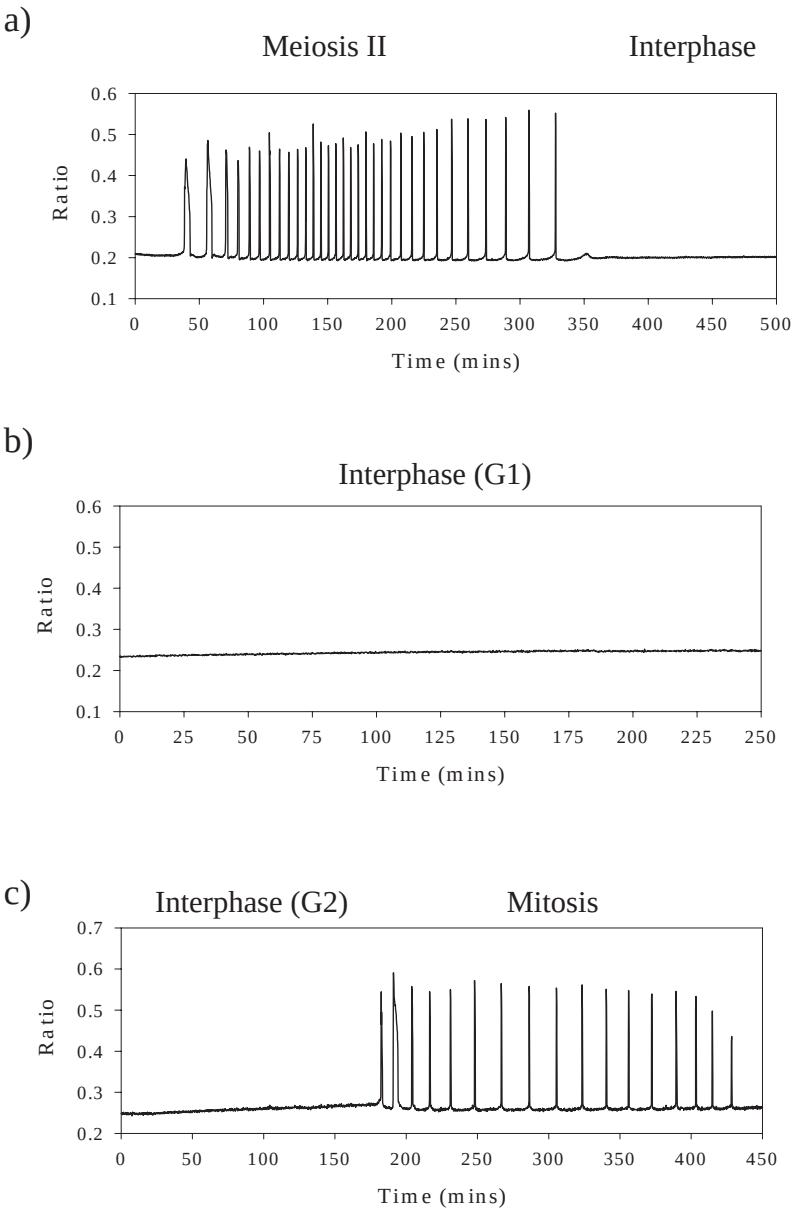

Fig. 1. PLCζ-induced Ca^{2+} oscillations are cell cycle-dependent. PLCζ cRNA was injected into MII-arrested mouse eggs and at two different stages of the first cell cycle following parthenogenetic activation: interphase G1 and late G2. Eggs were microinjected with Fura-dextran and PLCζ cRNA. Intracellular Ca^{2+} was monitored by changes in fluorescence excitation ratio. Time zero indicates approximately the point at which PLCζ was microinjected. (a) A series of Ca^{2+} oscillations occurs during MII that ceases as the egg enters interphase (*n*=20). (b) MII eggs were activated with Sr^{2+} in the presence of cytochalsin D. 2 hours after pronuclear formation the eggs were injected with Fura-dextran and PLCζ. No Ca^{2+} transients were detected in these parthenotes (*n*=20). (c) Parthenogentically activated eggs were cultured until interphase (G2), then 2-6 hours before NEB was due to occur embryos were injected with Fura-dextran and PLCζ cRNA (*n*=15). Ca^{2+} oscillations again occur.

following PLCζ injection, during the 4-hour observation period (Fig. 1b).

Following fertilisation no further Ca^{2+} transients are detected until mitosis (Kono et al., 1996; Day et al., 2000). This second phase of oscillations then lasts until nuclear envelope reformation (NER) in the daughter cells (Marangos et al., 2003). These Ca^{2+} oscillations are unique to eggs that have been fertilised or activated by sperm extract injection (Kono et al., 1996; Tang et al., 2000). Eggs activated through chemical-parthenogenetic activation do not display Ca^{2+} oscillations during mitosis (Kono et al., 1996). However, when ethanol-activated embryos were injected with PLCζ cRNA approximately 2-6 hours before nuclear envelope breakdown (NEB) or simply activated with PLCζ and then allowed to progress through the first cell division, they underwent a series of Ca^{2+} oscillations, similar to those produced by PLCζ in unfertilised eggs, which appeared to correlate with the duration of mitosis (Fig. 1c).

To determine exactly when mitosis entry and nuclear membrane reformation (NER) occurs, a probe for nuclear membrane integrity was required. Specific transport of proteins into the nucleus occurs via the recognition of a nuclear localisation signal (NLS) within the protein by the importin α/β heterodimer, which interacts with and then permits passage of the protein through the nuclear pore complex (NPC) (Gorlich, 1998). During G2 interphase embryos were injected with 1-3 μM FITC-labelled BSA that had been conjugated to a NLS sequence (FITC-BSA-NLS) (Jackman et al., 2002), in addition to Fura-dextran and PLCζ cRNA. Within 10-20 minutes the intact centred pronuclei were visible under fluorescence. Fig. 2a shows the pronuclei fluorescence of FITC-BSA-NLS. The time scale indicates how many minutes had elapsed after injection of PLCζ cRNA. The images of NEB with FITC-BSA-NLS and Ca^{2+} changes were acquired simultaneously. Measuring the decrease in fluorescence of each pronuclei and increases in Ca^{2+} clearly shows that one pronucleus begins to undergo NEB before the first increase in Ca^{2+} (Fig. 2b). We found this to be the case in all 12 experiments carried out, with the initial decrease in FITC-BSA-NLS fluorescence occurring 6.3±4.1 minutes before the peak of Ca^{2+}.

We then allowed the parthenogenetically activated embryos to complete mitosis to investigate if reformation of the nuclei and entry into the second interphase had a similar effect on PLCζ-induced Ca^{2+} oscillations to those triggered by sperm. As with NEB, FITC-BSA-NLS was used to monitor NER. Embryos were again injected during interphase (G2) with FITC-BSA-NLS, Fura-dextran and PLCζ. To limit the duration of exposure, fluorescence imaging was not carried out until the embryos had undergone NEB and were approaching cytokinesis. Ca^{2+} oscillations were detected in all embryos. Ca^{2+} transients continued through the progression of cytokinesis and it was common that the last Ca^{2+} increase detected occurred within just one of the daughter cells, suggesting the completion of cytokinesis. By simultaneously measuring the increase in FITC-BSA-NLS as NER occurred it was found that the first indication of NER was detected 14.3±4.3 minutes before the last Ca^{2+} spike

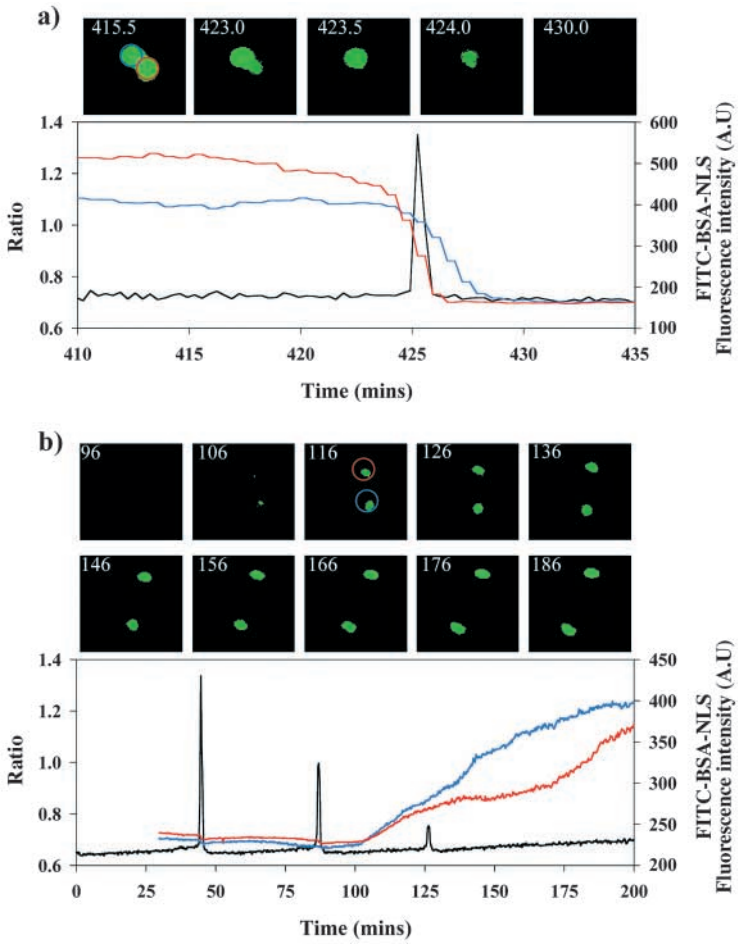

Fig. 2. PLCζ-induced Ca^{2+} oscillations at mitosis are controlled by nuclear membrane integrity. As in Fig. 1c, MII eggs were parthenogentically activated in the presence of cytochalsin D and cultured until interphase (G2). To monitor the exact timing of NEB and NER, embryos were injected (2-6 hours before NEB) with a fluorescent probe for nuclear membrane integrity (FITC-BSA-NLS) in addition to Fura-dextran and PLCζ cRNA. (a) Two pronuclei can be seen with FITC-BSA-NLS. At approximately 416 minutes (415.5) after injection of PLCζ cRNA the first pronucleus begins to break down. By 430 minutes both pronuclei have broken down and the FITC-BSA-NLS can no longer be detected. Measuring the decrease in nuclear FITC-BSA-NLS intensity of both pronuclei (red and blue lines) it was found that NEB of the first pronucleus occurred 6.3±4.1 minutes before the initial Ca^{2+} transient (n=12) that was indicated by the Fura-dextran fluorescence ratio (black line). (b) NER was also monitored with FITC-BSA-NLS. Measuring the accumulation of FITC-BSA-NLS into the reforming nuclei showed that the first indication of NER occurred just prior to the last Ca^{2+} transient (14.3±4.3 minutes). As in a, the red and blue lines represent fluorescence from the FITC-BSA-NLS regions and the black line is the Fura-dextran fluorescence ratio.

(Fig. 2bii). NER then proceeded over a period of about 1 hour, during which Ca^{2+} transients stopped.

Together these data show that PLCζ-induced Ca^{2+} oscillations during the first cell division have the same cell cycle-dependency as in fertilised and sperm extract-activated eggs. They also support the hypothesis that the precise correlation between the occurrence of Ca^{2+} oscillations and absence of the pronuclei is due to the free access of PLCζ to the embryo cytoplasm.

PLCζ localises to the pronuclei

If nuclear sequestration of PLCζ does control the Ca^{2+} oscillations it should be detectable in the pronuclei after PLCζ-induced egg activation. We first attempted to monitor PLCζ localisation by introducing a YFP-tag at the N terminus of PLCζ. When YFP-PLCζ was injected into eggs (pipette concentration up to 2 mg/ml) Ca^{2+} oscillations were seen, but no YFP fluorescence was detected using whole cell or confocal fluorescent-imaging techniques (data not shown). The lack of signal with this chimeric protein is consistent with the fact that the lower threshold of detection for EGFP above autofluorescence in mouse eggs is approximately 0.6 μM (Medvedev et al., 2002), whereas the amount of PLCζ that is required to generate Ca^{2+} oscillations is 0.3-6 nM. To investigate if PLCζ is indeed sequestered by the pronuclei, a more sensitive tagging method was required.

The localisation of PLCζ was successfully monitored using detection of immunofluorescence from a Myc-tagged PLCζ (Saunders et al., 2002). MII-arrested eggs were microinjected with Myc-PLCζ cRNA and cultured in H-KSOM medium containing the actin inhibitor cytochalasin D to create diploid embryos. After pronuclear formation, the embryos were fixed and then labelled using the c-Myc antibody and Alexa Fluor® 488-labelled secondary antibodies. As a control, some eggs were activated with untagged PLCζ (Fig. 3a). Fig. 3 shows brightfield and confocal anti-c-Myc immunostained images that were simultaneously acquired. The nucleoli can be seen as the circular unstained areas, marked with black arrows on the brightfield images. Surrounding the nucleoli is the nuclear membrane, although this is difficult to resolve. In contrast, all eggs activated with Myc-PLCζ possessed clearly fluorescent pronuclei. Representative confocal immunofluorescent images of eggs activated with Myc-PLCζ indicate the striking presence of PLCζ in the nucleoplasm of the pronuclei (Fig. 3b,c). Immunofluorescence of Myc-PLCζ-activated eggs cultured to the two-cell stage also showed nuclear localisation in each of the daughter cells after first mitosis (Fig. 3d). These data demonstrate that PLCζ localises to pronuclei in one-cell embryos and to the nuclei in two-cell embryos.

Pronuclear formation and PLCζ nuclear import are essential for terminating the Ca^{2+} oscillations

Sperm-triggered oscillations appear to be controlled by pronuclear formation (Marangos et al., 2003). To test if PLCζ-induced Ca^{2+} oscillations are also regulated by formation of the pronuclei, MII-arrested mouse eggs were injected with PLCζ cRNA, Fura-dextran and a lectin (wheat germ agglutinin; WGA) that blocks pronuclear formation (Davis, 1995). Parallel control eggs were injected with PLCζ cRNA and Fura-dextran only. PLCζ injection triggers a series of long-lasting Ca^{2+} oscillations that terminate at interphase (Fig. 4a).

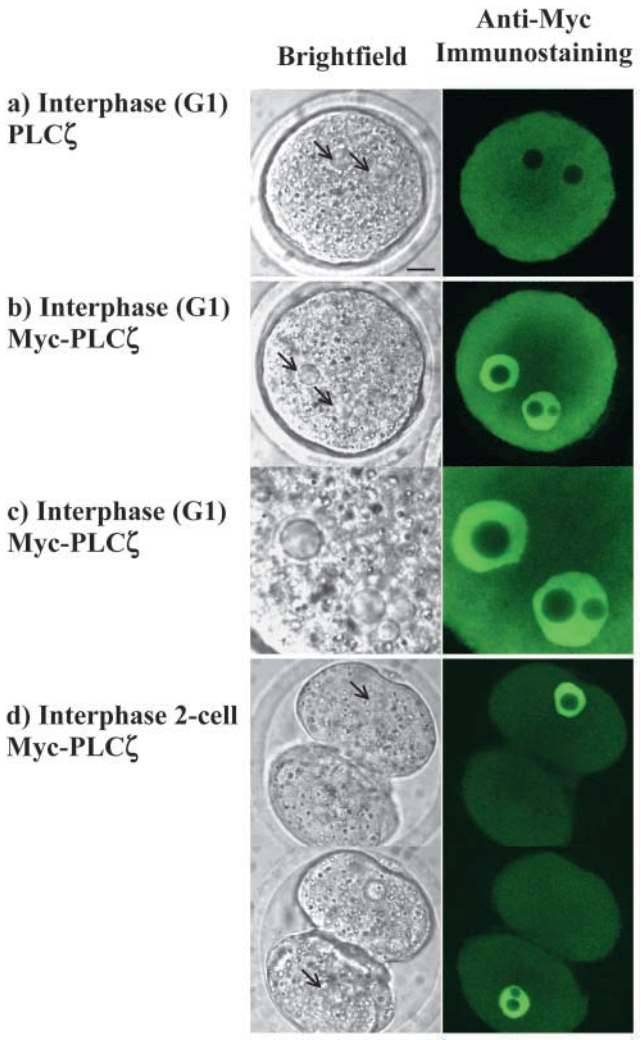

Fig. 3. PLCζ localises to the pronuclei. MII eggs were injected with PLCζ tagged with a c-Myc epitope and cultured in the presence of cytochalsin D until pronuclear formation, then they were fixed and stained using the c-Myc (9E10) monoclonal antibody and the anti-mouse Alexa Fluor® 488 secondary antibody. Control eggs were activated with untagged PLCζ. Confocal and brightfield images were simultaneously obtained. The black arrows in brightfield images indicate the nucleoli. Though not easily visible, the nuclear membrane surrounds the nucleolus. The control embryo shows non-specific binding. Eggs activated with Myc-PLCζ show a striking localisation to the nucleoplasm of each pronuclei. Scale bar: 10 μm.

In contrast to parallel control eggs, we found that WGA-treated eggs did not form proper expanded pronuclei, as previously reported (Vautier et al., 2001; Marangos et al., 2003). Significantly, WGA-mediated inhibition of pronuclear formation also prolonged the PLCζ-induced Ca^{2+} oscillations by about 6 hours (control: mean 444.2±142.0 minutes, n=14; WGA: mean 826.5±104.3 minutes, n=14; Fig. 4a,b).

The nuclear pore complex (NPC) offers the only route for nucleo-cytoplasmic exchange (Feldherr et al., 1984). Diffusion through the NPC is limited to proteins less than 40-60 kDa. Larger proteins traverse the nuclear membrane through an active-carrier mechanism that requires a nuclear localisation signal, which permits direct import into the nucleus via the importin pathway (Gorlich, 1998). Many nuclear proteins have been found to contain a cluster of basic amino acids that act as a NLS (Dingwall and Laskey, 1991). The amino acid sequence of mouse PLCζ contains a putative NLS (residues 374-379; KKRKRK) in between the X and Y catalytic domains (Saunders et al., 2002). If this NLS is functionally important in transporting PLCζ into the nuclei at pronuclear formation then mutating this region should prolong the Ca^{2+} oscillations.

To enable us to confirm that the mutation prevented nuclear localisation Myc-PLCζ was used. MII-arrested eggs were injected with the NLS mutant Myc-PLCζ^{K377E} cRNA and parallel control eggs were injected with Myc-PLCζ cRNA. Immunostaining of embryos injected with Myc-PLCζ^{K377E} confirmed that the NLS mutant prevented its nuclear import, whereas parallel control eggs injected with wild-type Myc-PLCζ showed the typical pronuclear localisation (Fig. 4cii and dii). Eggs injected with Myc-PLCζ^{K377E}-generated Ca^{2+} oscillations that were similar in form to those of control PLCζ-injected eggs, except that the Myc-PLCζ^{K377E}-induced oscillations lasted for about 6 hours longer (control: mean 344.4±87.5, n=20; K377E: 702.2±162.8, n=22; Fig. 4ci-di). These data, therefore, show that either mutation of the predicted NLS in the PLCζ gene or inhibition of pronuclear formation leads to a significantly prolonged series of Ca^{2+} oscillations.

To obtain further evidence that cytosolic PLCζ is transported into the pronuclei and it is this sequestration that ultimately dictates the Ca^{2+} oscillation cell cycle dependency, Myc-PLCζ was injected during G1 interphase (following Sr^{2+} activation) and prevented from entering the pronuclei by either injecting WGA or using the NLS mutant. WGA selectively blocks active nuclear import (Davis, 1995). Since PLCζ has a predicted molecular mass of 74 kDa (Saunders et al., 2002) it is larger than the ~50 kDa that can pass through the nuclear pore complex (NPC) via passive diffusion (Gorlich, 1998). As before no Ca^{2+} oscillations were observed after injection of PLCζ cRNA during interphase (Fig. 5ai). Fig. 5bi shows that blocking transport thorough the nuclear pores restored Ca^{2+} oscillations in interphase embryos injected with Myc-PLCζ cRNA. We also confirmed, by immunostaining for Myc-PLCζ, that WGA was blocking transport of PLCζ into the pronuclei. Fig. 5aii and bii shows that PLCζ is localised to the pronuclei when it is injected during interphase, but this localisation pattern is prevented by co-injection of WGA. Blocking the NPC would prevent most, if not all, nuclear import. Hence, to investigate if cytoplasmic retention of PLCζ alone is sufficient to resume Ca^{2+} oscillations during interphase Myc-PLCζ^{K377E} was injected together with Fura dextran. As before, no Ca^{2+} increases were observed in embryos injected with wild-type PLCζ. In contrast, mutating the NLS and preventing nuclear import of PLCζ, Ca^{2+} oscillations were observed that were comparable to those generated by blocking the NPC with lectin.

Discussion

Ca^{2+} oscillations generated by fertilisation and sperm extract injection have been shown to be cell cycle dependent, only occurring during M-phase (Jones et al., 1995; Kono et al., 1996; Day et al., 2000). Interestingly, no Ca^{2+} increases are observed during mitosis in eggs activated through chemical

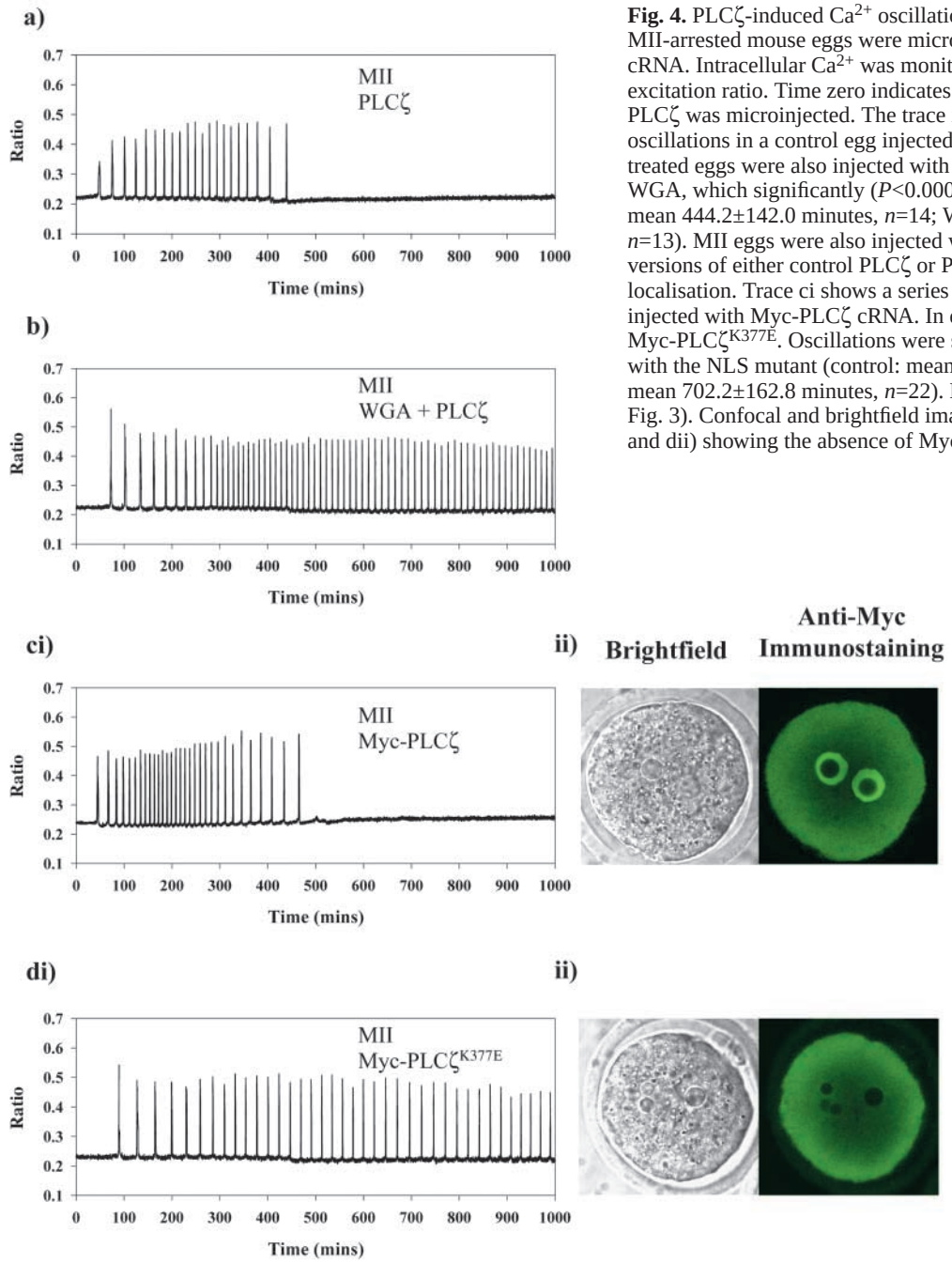

Fig. 4. PLCζ-induced Ca^{2+} oscillations are regulated by nuclear import. MII-arrested mouse eggs were microinjected with Fura-dextran and PLCζ cRNA. Intracellular Ca^{2+} was monitored by changes in fluorescence excitation ratio. Time zero indicates approximately the point at which PLCζ was microinjected. The trace in a shows a series of Ca^{2+} oscillations in a control egg injected with PLCζ cRNA. In b, parallel treated eggs were also injected with 10 mg/ml (pipette concentration) WGA, which significantly (P<0.0001) prolonged the oscillations (control: mean 444.2±142.0 minutes, n=14; WGA: mean 826.5±104.3 minutes, n=13). MII eggs were also injected with Fura-dextran and Myc-tagged versions of either control PLCζ or $PLC\zeta^{K377E}$ to monitor the nuclear localisation. Trace ci shows a series of Ca^{2+} oscillations in a control egg injected with Myc-PLCζ cRNA. In di, parallel eggs were injected with Myc-$PLC\zeta^{K377E}$. Oscillations were significantly (P<0.0001) prolonged with the NLS mutant (control: mean 335.9±93.2 minutes, n=20; K377E: mean 702.2±162.8 minutes, n=22). Embryos were fixed and stained (see Fig. 3). Confocal and brightfield images were simultaneously obtained (cii and dii) showing the absence of Myc-$PLC\zeta^{K377E}$ in the nucleoplasm.

parthenogenesis (Kono et al., 1996), which suggests that some factor supplied specifically by the sperm ultimately causes the mitotic Ca^{2+} signals in zygotes. A few key studies have led to the hypothesis that the cell cycle dependency of the Ca^{2+} oscillations may be due to nuclear sequestration of the Ca^{2+}-releasing factor delivered by the sperm (Kono et al., 1995; Zernicka-Goetz et al., 1995; Marangos et al., 2003)

We have recently shown that PLCζ is the protein responsible for the Ca^{2+}-releasing activity of sperm extracts (Saunders et al., 2002). PLCζ-induced Ca^{2+} oscillations also show cell cycle dependency during the first cell cycle. The current data suggest that the paternally supplied factor is PLCζ. Pronuclear envelope breakdown occurs just before the first mitotic Ca^{2+} transient in PLCζ-activated eggs. In addition, the reformation of nuclei coincides with cessation of PLCζ-induced Ca^{2+} transients during mitosis, suggesting that the mitotic Ca^{2+} transients are dependent on the release and sequestration of the Ca^{2+}-releasing factor. Consequently, one prediction is that PLCζ may localise to the nascent pronuclei in PLCζ-activated eggs. Our results indicate that PLCζ is indeed localised to the pronuclei at interphase following meiosis and mitosis. Furthermore, we show not only that nuclear sequestration plays a causal role in inhibiting the activity of PLCζ, but also that the molecular mechanism of sequestration is a predicted NLS present in the X-Y linker of PLCζ. This NLS appears to be a characteristic feature of PLCζ since, although different in precise sequence, a NLS is also predicted in the X-Y linker region of the human and monkey PLCζ (Cox et al., 2000).

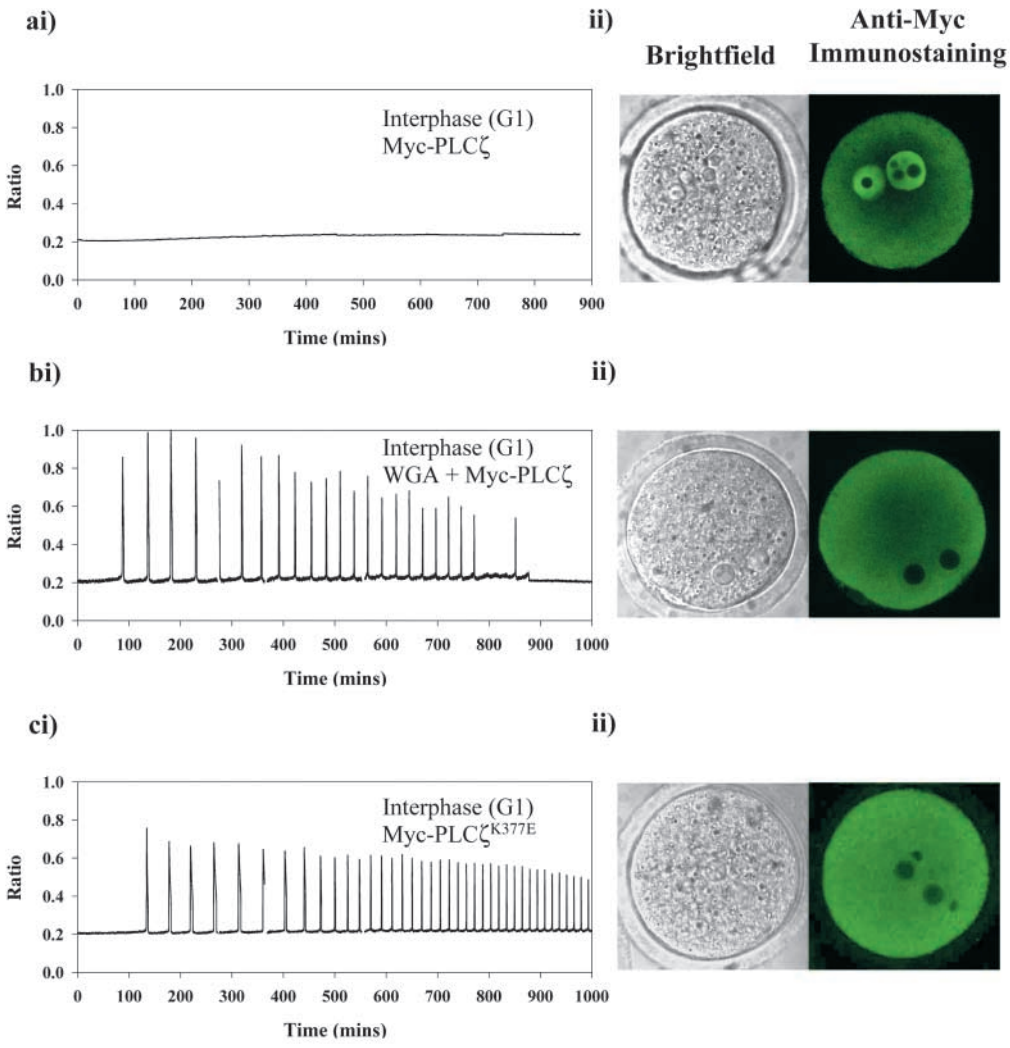

Fig. 5. Preventing PLCζ nuclear localisation permits Ca^{2+} oscillations during interphase. MII eggs were activated with Sr^{2+} in the presence of cytochalsin D. 2 hours after pronuclear formation the eggs were injected with Fura-dextran and Myc-PLCζ. (a) No Ca^{2+} transients were detected in these parthenotes (*n*=20). (b) Parallel parthenotes were also injected with WGA to block nuclear transport. In these embryos Myc-PLCζ triggered Ca^{2+} oscillations within 1 hour (*n*=20). Embryos were fixed and stained at the end of the experiment (as in Fig. 2) demonstrating that blocking nuclear pores with WGA, prevents import of PLCζ into the pronuclei (aii,bii). (c) Parthenotes were also injected with Myc-PLCζ^{K377E} (*n*=10). As with inhibiting nuclear import, preventing nuclear localisation of PLCζ through mutation of the NLS permitted Ca^{2+} oscillations during interphase. Such Ca^{2+} oscillations were not observed in parallel controls (*n*=10).

Our data shows that sequestration of PLCζ in the nuclear space is the causal link with inhibition of its activity in the Ins(1,4,5)P_3 production that leads to Ca^{2+} release. The reason for this is unclear, but it may be related to access to its relevant substrate. In contrast to most other PLC isoforms, PLCζ lacks a pleckstrin homology domain (Saunders et al., 2002) that targets plasma membrane phosphatidylinositol 4,5-bisphosphate (Ins(4,5)P_2) (Halet et al., 2002). At present the location of the Ins(4,5)P_2 substrate used by PLCζ in eggs is unknown, but it is possible that PLCζ may use Ins(4,5)P_2 associated with intracellular organelles (Rice et al., 2000). The way PLCζ is targeted within the cytoplasm might also explain why it is distinct from other mammalian PLCs and how this PLC activity in sperm extracts can hydrolyse Ins(4,5)P_2 in eggs and egg homogenates, whereas PLCs of the β, γ and δ class are inactive (Jones et al., 2000).

It should be noted that there are a number of other factors that have already been shown to affect the ability of mouse eggs and embryos to generate Ca^{2+} oscillations. For example, it has been shown that Ins(1,4,5)P_3 receptors are down-regulated after fertilisation, or sperm extract injection (Brind et al., 2000; Jellerette et al., 2000). After the start of Ca^{2+} oscillations at fertilisation there are also changes in the endoplasmic reticulum as seen by a decrease in cortical clusters (Kline et al., 1999; Fitzharris et al., 2003). Both of these factors may reduce the sensitivity of mouse zygotes to Ins(1,4,5)P_3-induced Ca^{2+} release. They may account for the finding that Ca^{2+} oscillations eventually terminate after injection of the NLS mutant PLCζ that does not localise to pronuclei. However, the changes in Ins(1,4,5)P_3 sensitivity of the zygote do not explain why Ca^{2+} oscillations normally cease around the time of pronuclear formation, nor do they explain why the oscillations return during the first mitosis. These changes in Ins(1,4,5)P_3 receptor sensitivity also fail to explain why Ca^{2+} oscillations during the first mitosis are only seen in fertilised zygotes and not in parthenogenetic embryos. Since this cell cycle dependency of Ca^{2+} release is a particular feature of a fertilised zygote, our current data also provides another specific example of PLCζ injection precisely mimicking the sperm from fertilisation through to the first mitotic division. They add further weight of evidence to the idea that PLCζ is the agent used by the sperm to trigger Ca^{2+} release in eggs at fertilisation (Saunders et al., 2002).

Our current data provides a simple molecular explanation for the cell cycle dependency of Ca^{2+} oscillations in early mouse embryos. In this model (Fig. 6) we suggest that the Ca^{2+}

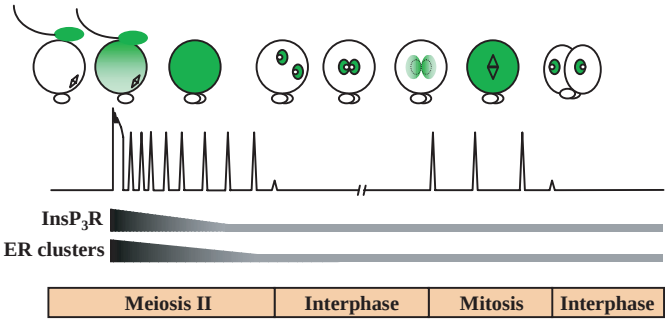

Fig. 6. Model for the regulation of Ca^{2+} oscillations during the first cell cycle. Model whereby PLCζ (green) is introduced from the sperm into the egg upon fusion. Long-lasting Ca^{2+} oscillations are maintained while PLCζ remains in the cytosol. During the initial phase of Ca^{2+} oscillations there is downregulation of InsP_3 receptors and a change in the endoplasmic reticulum (ER): a decrease in cortical clusters. Both of these changes may lead to a change in the early phase of Ca^{2+} oscillations. When the pronuclei form, indicating the start of interphase, PLCζ is transported into the nucleoplasm of the pronuclei terminating the Ca^{2+} oscillations. No further increases in Ca^{2+} are observed until the nuclear membranes breakdown upon mitosis entry, releasing PLCζ back into the cytosol. Again, Ca^{2+} oscillations are sustained until the nuclear membranes reform in the two daughter cells and PLCζ is imported into the nuclei.

oscillations at fertilisation are generated by the diffusion of sperm PLCζ into the egg cytoplasm after gamete fusion. As a result of pronuclear formation we propose that PLCζ is then transported into the newly formed pronuclei. This leads to the cessation of Ins(1,4,5)P_3 production and Ca^{2+} release in the interphase zygote cytoplasm. When pronuclear envelope breakdown occurs, as the zygote enters first mitosis, PLCζ is released into the cytoplasm and generates the mitotic Ca^{2+} oscillations that then cease when the two-cell stage nuclei form. As well as providing a potential explanation for the observed pattern of Ca^{2+} signals in mammalian zygotes, this localisation could represent a novel mechanism for the regulation of Ins(1,4,5)P_3 generation in cells.

This work was funded by a Wellcome Trust grant awarded to K.S. and by Strategic Infrastructure Fund (SIF) and Knowledge Exploitation Fund (KEF) grants to F.A.L. from University of Wales College of Medicine and Education and Learning for Wales, respectively.

References

Berridge, M. J., Bootman, M. D. and Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell. Biol.* **4**, 517-529.

Bos-Mikich, A., Whittingham, D. G. and Jones, K. T. (1997). Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Dev. Biol.* **182**, 172-179.

Brind, S., Swann, K. and Carroll, J. (2000). Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm and adenophostin A but not to increase in intracellular Ca^{2+} or egg activation. *Dev. Biol.* **223**, 251-265.

Carroll, J. (2001). The initiation and regulation of Ca^{2+} signalling at fertilisation in mammals. *Semin. Cell Dev. Biol.* **12**, 37-43.

Ciapa, B., Pesando, D., Wilding, M. and Whitaker, M. (1994). Cell-cycle calcium transients driven by cyclic changes in inositol trisphosphate levels. *Nature* **368**, 875-878.

Cox, L. J., Larman, M. G., Saunders, C. M., Hashimoto, K., Swann, K. and Lai, F. A. (2000). Sperm phospholipase Cζ from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. *Reproduction* **124**, 611-623.

Cuthbertson, K. S. (1983). Parthenogenetic activation of mouse oocytes in vitro with ethanol and benzyl alcohol. *J. Exp. Zool.* **226**, 311-314.

Davis, L. I. (1995). The nuclear pore complex. *Annu. Rev. Biochem.* **64**, 865-896.

Day, M. L., McGuinness, O. M., Berridge, M. J. and Johnson, M. H. (2000). Regulation of fertilisation-induced Ca^{2+} spiking in the mouse zygote. *Cell Calcium* **28**, 47-54.

Dingwall, C. and Laskey, R. A. (1991). Nuclear targeting sequences-a consensus? *Trends Biochem. Sci.* **16**, 478-481.

Feldherr, C. M., Kallenbach, E. and Schultz, N. (1984). Movement of karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* **99**, 2216-2222.

FitzHarris, D. G., Marangos, P. and Carroll, J. (2003). Cell cycle-dependent regulation of the structure of the endoplasmic reticulum and $InsP_3$-induced Ca^{2+} release in mouse oocytes and embryos. *Mol. Biol. Cell* **14**, 288-301.

Gorlich, D. (1998). Transport into and out of the cell nucleus. *EMBO J.* **17**, 2721-2727.

Halet, G., Tunwell, R., Balla, T., Swann, K. and Carroll, J. (2002). The dynamics of plasma membrane PtdIns(4,5)P_2 at fertilisation of mouse eggs. *J. Cell Sci.* **115**, 2139-2149.

Jackman, M., Kubota, Y., den Elzen, N., Hagting, A. and Pines, J. (2002). Cyclin A- and cyclin E-Cdk complexes shuttle between the nucleus and the cytoplasm. *Mol. Biol. Cell.* **13**, 1030-1045.

Jellerette, T., He, C. L., Wu, H., Parys, J. B. and Fissore, R. A. (2000). Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilisation or parthenogenetic activation. *Dev. Biol.* **223**, 238-250.

Jones, K. T., Carroll, J., Merriman, J. A., Whittingham, D. G. and Kono, T. (1995). Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development* **121**, 3259-3266.

Jones, K. T., Matsuda, M., Parrington, J., Katan, M. and Swann, K. (2000). Different Ca^{2+} releasing abilities of sperm extracts compared with tissue extracts and phospholipase C isoforms in sea urchin egg homogenate and mouse eggs. *Biochem. J.* **346**, 743-749.

Kline, D. and Kline, J. T. (1992). 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., Mehlmann, L., Fox, C. and Terasaki, M. (1999). The cortical endoplasmic reticulum (ER) of the mouse egg: localisation of ER clusters in relation to the generation of repetitive calcium waves. *Dev. Biol.* **215**, 431-442.

Kono, T., Carroll, J., Swann, K. and Whittingham, D. G. (1995). Nuclei from fertilised mouse embryos have calcium-releasing activity. *Development* **121**, 1123-1128.

Kono, T., Jones, K. T., Bos-Mikich, A., Whittingham, D. G. and Carroll, J. (1996). A cell cycle-associated change in Ca^{2+} releasing activity leads to the generation of Ca^{2+} transients in mouse embryos during the first mitotic division. *J. Cell Biol.* **132**, 915-923.

Lawrence, Y., Ozil, J. P. and Swann, K. (1998). The effects of a Ca^{2+} chelator and heavy-metal-ion chelators upon Ca^{2+} oscillations and activation at fertilisation in mouse eggs suggest a role for repetitive Ca^{2+} increases. *Biochem. J.* **335**, 335-342.

Lawrence, Y., Whitaker, M. and Swann, K. (1997). Sperm-egg fusion is the prelude to the initial Ca^{2+} increase at fertilisation in the mouse. *Development* **124**, 233-241.

Marangos, P., FitzHarris, G. and Carroll, J. (2003). Ca^{2+} oscillations at fertilisation in mammals are regulated by the formation of pronuclei. *Development* **130**, 1461-1472.

Medvedev, S. Y., Tokunaga, T., Schultz, R. M., Furukawa, T., Nagai, T., Yamaguchi, M., Hosoe, M., Yakovlev, A. F., Takahashi, S. and Izaike, Y. (2002). Quantitative analysis of gene expression in preimplantation mouse embryos using green fluorescent protein reporter. *Biol. Reprod.* **67**, 282-286.

Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. (1993). Essential role of the inositol 1, 4, 5-trisphosphate/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilisation of mammalian eggs. *Dev. Biol.* **158**, 62-78.

Ozil, J. P. (1990). The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* **109**, 117-127.

Ozil, J. P. and Huneau, D. (2001). Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development. *Development* **128**, 917-928.

Rice, A., Parrington, J., Jones, K. T. and Swann, K. (2000). Mammalian sperm contain a Ca^{2+} sensitive phospholipase C activity that can generate $InsP_3$ from PIP_2 associated with intracellular organelles. *Dev. Biol.* **227**, 125-135.

Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K. and Lai, F. A. (2002). PLCζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* **129**, 3533-3544.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilisation and egg activation in animals. *Dev. Biol.* **211**, 157-176.

Summers, M. C., McGinnis, L. K. M., Lawitts, J. A., Raffin, M. and Biggers, J. D. (2000). IVF of mouse ova in a simple optimized medium supplemented with amino acids. *Hum Reprod.*, **15**, 1791-1801.

Swann, K. and Ozil, J. P. (1994). Dynamics of the calcium signal that triggers mammalian egg activation. *Int. Rev. Cytol.* **152**, 183-222.

Tang, T. S., Dong, J. B., Huang, X. Y. and Sun, F. Z. (2000). Ca^{2+} oscillations induced by a cytosolic sperm protein factor are mediated by a maternal machinery that functions only once in mammalian eggs. *Development* **127**, 1141-1150.

Tombes, R. M., Simerly, C., Borisy, G. G. and Schatten, G. (1992). Meiosis, egg activation, and nuclear envelope breakdown are differentially reliant on Ca^{2+}, whereas germinal vesicle breakdown is Ca^{2+} independent in the mouse oocyte. *J. Cell Biol.* **117**, 799-811.

Vautier, D., Chesné, P., Cunha, C., Calado, A., Renard, J.-P. and Carmo-Fonseca, M. (2001). Transcription-dependent nucleo-cytoplasmic distribution of hnRNP A1 protein in early mouse embryos. *J. Cell Sci.* **114**, 1521-1531.

Whitaker, M. and Patel, R. (1990). Calcium and cell cycle control. *Development* **108**, 525-542.

Whitaker, M. and Larman, M. G. (2001). Calcium and mitosis. *Sem. Cell Dev. Biol.* **21**, 53-58.

Zernicka-Goetz, M., Ciemerych, M. A., Kubiak, J. Z., Tarkowski, A. K. and Maro, B. (1995). Cytostatic factor inactivation is induced by a calcium dependent mechanism present until the second cell cycle in fertilised but not in parthenogenetically activated mouse eggs. *J. Cell Sci.* **108**, 469-474.