Exploring the mechanism of action of the sperm-triggered calcium-wave pacemaker in ascidian zygotes

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Summary

In ascidians, as in mammals, sperm trigger repetitive Ca^{2+}-waves that originate from cortical pacemakers situated in the vegetal hemisphere of the zygotes. In ascidians, a vegetal protrusion termed the contraction pole (CP) acts as the Ca^{2+}-wave pacemaker, but the mechanism that underlies the generation of a Ca^{2+}-wave pacemaker is not known. Here, we tested four hypotheses to determine which factors at the CP are involved in setting the pace of the ascidian Ca^{2+}-wave pacemaker: (1) localized Ca^{2+} influx; (2) accumulation of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2]; (3) accumulation of cortical endoplasmic reticulum (cER); and (4) enrichment of the sperm activating factor. We developed a method of dynamically monitoring the location of the CP during fertilization using a plekstrin homology (PH) domain from phospholipase Cδ1 coupled to green fluorescent protein (GFP) that binds PtdIns(4,5)P_2. We found that eggs in Ca^{2+}-free sea water displayed Ca^{2+} waves that originated from the CP, showing that enhanced CP Ca^{2+} influx does not determine the origin of the pacemaker. Also, disruption of the PH::GFP-labelled CP once it had formed did not dislodge the Ca^{2+}-wave pacemaker from that site. Next, when we prevented the accumulation of cER at the CP, all of the Ca^{2+} waves came from the site of sperm-egg fusion and the frequency of Ca^{2+} oscillations was unaltered. These data show that local Ca^{2+} influx, the accumulation of PtdIns(4,5)P_2 and cER at the CP are not required for Ca^{2+}-wave pacemaker function and instead suggest that a factor associated with the sperm determines the site of the Ca^{2+}-wave pacemaker. Finally, when we injected ascidian sperm extract into the centre of unfertilized ascidian eggs that had been treated with microfilament- and microtubule-disrupting drugs, all the Ca^{2+} waves still originated from near the plasma membrane, showing that the sperm factor does not require an intact cortex if it is enriched near the plasma membrane (PM). We suggest that the Ca^{2+}-releasing sperm factor might be tethered near or on the PM and that following the cortical contraction, it is translocated to the vegetal CP, thus making that site act as a Ca^{2+}-wave pacemaker.

Key words: Egg, Fertilization, Calcium, Pacemaker

Introduction

Sperm trigger a Ca^{2+} increase at fertilization that is responsible for activating virtually every species of egg tested to date (reviewed by Stricker, 1999). In eggs from different organisms, a repetitive Ca^{2+} increase is often required to trigger embryonic development [mammals (Swann and Ozil, 1994); nemerteans (Stricker, 1996); ascidians (McDougall and Sardet, 1995)]. Repetitive Ca^{2+} increases during fertilization usually occur as waves that come from vegetal Ca^{2+}-wave pacemakers (reviewed by Dumollard et al., 2002). The first sperm-induced Ca^{2+}-wave pacemaker to be described was that of a marine chordate (the ascidian *Phallusia mammillata*) (Speksnijder, 1992). Similar Ca^{2+}-wave pacemakers are now known to exist in the fertilized eggs of nemertean worms (Stricker, 1996) and in mammals (Kline et al., 1999; Deguchi et al., 2000). It is still unknown how these Ca^{2+}-wave pacemakers operate and also whether similar mechanisms operate between species.

The large transparent eggs of ascidians still offer one of the clearest examples in the animal kingdom of a Ca^{2+}-wave pacemaker (Speksnijder, 1992; McDougall and Sardet, 1995). This pacemaker forms minutes after fertilization because of a sperm-induced Ca^{2+} wave that triggers a cortical contraction, culminating in the formation of a surface protrusion in the vegetal hemisphere termed the contraction pole (CP) (Roegiers et al., 1995). During this cortical contraction, most of the cortical endoplasmic reticulum (cER) is accumulated in a 2-6 μm thick domain located adjacent to the plasma membrane (PM) at the CP (Speksnijder et al., 1993). Once formed, the CP (and, in particular, the domain of cER that accumulates there) is the site of origin of all the repetitive Ca^{2+} waves (McDougall and Sardet, 1995) (Fig. 1). However, in eggs

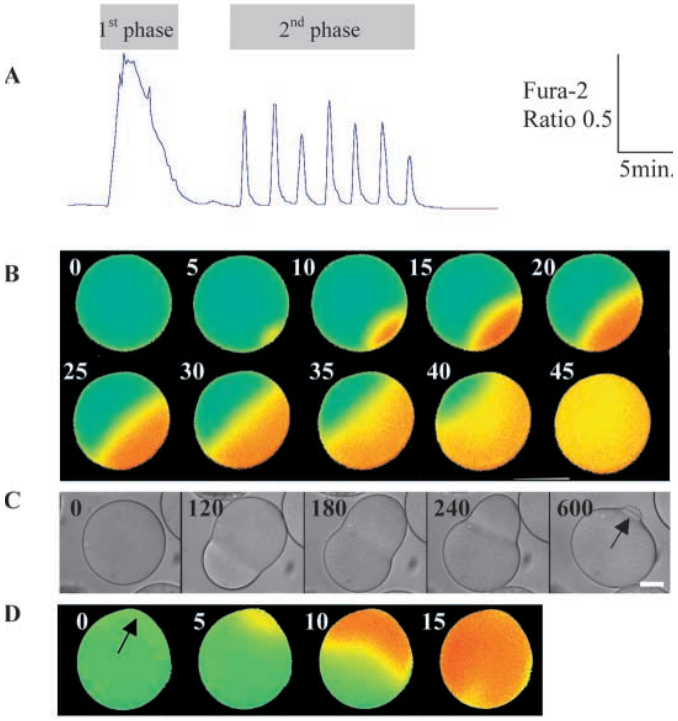

Fig. 1. Sperm-triggered Ca^{2+} waves in *A. aspersa*. Dechorionated eggs were injected with Fura-2-dextran (10-20 μM final concentration) and fertilized with preactivated sperm. As noted in several other species and previously also in *A. aspersa*, two phases of Ca^{2+} oscillations are observed, separated by a brief gap. (A) The temporal pattern of all Ca^{2+} oscillations is displayed. (B) The spatial pattern of the first Ca^{2+} wave is displayed. Time in seconds. (C) This Ca^{2+} wave is followed within 30-40 seconds by a visible cortical contraction that produces a surface protrusion termed the contraction pole (CP; arrow). Bar, 50 μm. Time in seconds. (D) All of the second-phase Ca^{2+} waves originate from the CP (arrow). As noted previously, these waves are faster than the initial Ca^{2+} wave and propagate more cortically (McDougall and Sardet, 1995). Time in seconds. Temperature 19°C, *n*=34 replicates.

injected with the Ca^{2+}-releasing second messenger inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3], a CP forms but no repetitive Ca^{2+} waves are generated (McDougall and Levasseur, 1998). Clearly, sperm are required to generate the signal that initiates all the repetitive Ca^{2+} waves and the CP acts as a Ca^{2+}-wave pacemaker for an as yet unknown reason.

A sperm-borne Ca^{2+} releasing factor probably activates mammalian (Swann, 1990), nemertean (Stricker, 1997), ascidian (Kyozuka et al., 1998) and perhaps most eggs following sperm-egg fusion. The best candidate so far for the role of 'sperm factor' is a novel sperm-specific phospolipase C, PLCζ, identified initially in mouse (Saunders et al., 2002) then human (Cox et al., 2002) sperm that triggers Ca^{2+} oscillations when expressed in mouse eggs (Saunders et al., 2002; Cox et al., 2002). However, in invertebrates egg PLCγ is thought to be involved; dual SH2-domain peptides from PLCγ1 inhibit fertilization in starfish (Carroll et al., 1999), sea urchin (Shearer et al., 1999) and ascidian (Runft and Jaffe, 2000) eggs but not in mammalian eggs (Mehlmann et al., 1998). Consistent with a role for PLCs in the ascidian, all of the repetitive Ca^{2+} waves in the ascidian egg following fertilization rely on the Ins(1,4,5)P_3 receptor ($InsP_3R$) (McDougall and Sardet, 1995; Yoshida et al., 1998).

Two hypotheses have been proposed to explain $InsP_3R$-based pacemakers. Pacemakers are controlled by either sensitization of $InsP_3Rs$, as in HeLa cells (Thomas et al., 2000) or accumulation of $InsP_3Rs$, as in smooth-muscle cells (Hirose and Iino, 1994). However, in fertilized ascidian eggs, the CP is not the most sensitive part of the egg to Ins(1,4,5)P_3 (Dumollard and Sardet, 2001). These data indicate that $InsP_3Rs$ are probably not accumulated or sensitized sufficiently at the CP to affect the Ca^{2+} release properties. Based on these data it has been suggested that the CP acts as a Ca^{2+}-wave pacemaker because $InsP_3$ is preferentially produced in that region of the egg (Dumollard and Sardet, 2001). These data suggest that factors other than the $InsP_3R$ are accumulated at the CP, and that it is these factors that earmark the site as the Ca^{2+}-wave pacemaker.

Here, we have tested four components of the ascidian Ca^{2+}-wave pacemaker to determine which component(s) is required for Ca^{2+}-wave pacemaker activity: enhanced Ca^{2+} influx at the CP, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] accumulation at the CP, cER accumulation and sperm factor enrichment at the CP. We have found that: (1) in the absence of external Ca^{2+} the CP remains the wave Ca^{2+} pacemaker; (2) the accumulation of PtdIns(4,5)P_2 at the CP is not required for Ca^{2+}-wave pacemaker activity; (3) in the absence of a cER accumulation the Ca^{2+}-wave pacemaker in the ascidian egg correlates with the site of sperm-egg fusion; and finally (4) injection of ascidian sperm extract (aSE) into eggs that were prevented from forming a CP and any ER accumulations still resulted in repetitive Ca^{2+} waves that originated at the cortex, suggesting that the sperm factor might be localized cortically. We therefore propose that the Ca^{2+}-releasing factor introduced into the egg either by sperm at fertilization or upon injection aSE becomes enriched at the cortical CP thus making that site act as a Ca^{2+}-wave pacemaker.

Materials and Methods

Biological material

Ascidiella aspersa were collected from Blyth South Harbour, and kept in holding tanks containing natural sea water (NSW) for 1-2 weeks at 10°C. Eggs and sperm were harvested by first removing the outer tunic of the animal and then puncturing the gonoduct. The eggs were then collected by pipetting and transferred to a Petri dish containing filtered sea water (0.2 μm pore size, Millipore). Next, sperm were collected from the same animal using a pipette and transferred to 1.5 ml plastic tubes and stored at 4°C. To remove the outer chorion of the eggs, the chorionated eggs were treated for 45-60 minutes at 18-19°C with 0.1% trypsin (Sigma), then pipetted gently to shear the digested chorion from the eggs. Following dechorionation, the eggs were transferred to Petri dishes that had been previously coated with gelatin (0.1% gelatin/formaldehyde – the formaldehyde had been previously removed by air drying, followed by washing with tap water then sea water). All glass and plastic ware that came into contact with the eggs had also been coated with gelatin, thus preventing the eggs from sticking to those surfaces.

PH::GFP fusion construct

The plasmid encoding the PH domain (a gift from T. Meyer) was amplified by PCR and subcloned into pRN3-GFP, a specialized vector suitable for in vitro transcription of messenger RNA (see Levasseur and McDougall, 2000). mRNA encoding the PH::GFP construct was

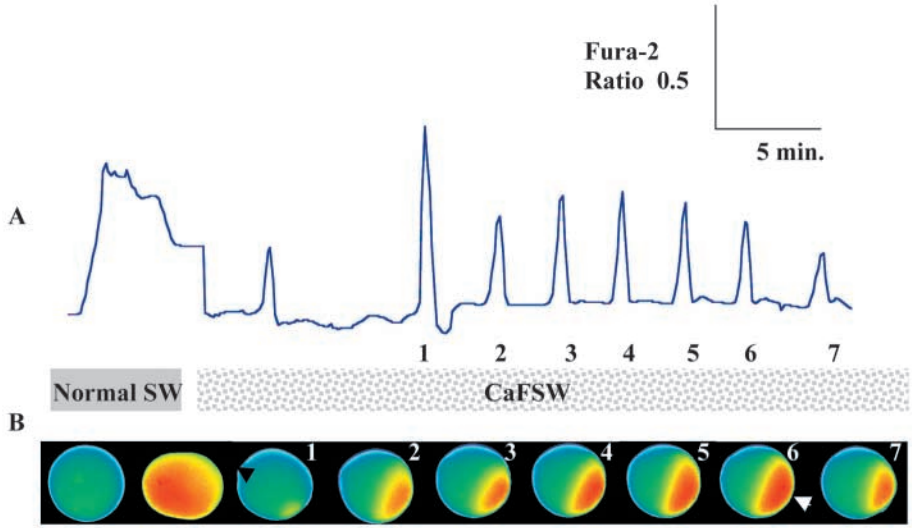

Fig. 2. Ca^{2+} influx during the second phase of Ca^{2+} waves. Fura-2-dextran-injected eggs were fertilized in natural (normal) sea water (SW) (shaded box). Following the first Ca^{2+} wave, individual eggs were then removed from the wedge and transferred to a large excess of Ca^{2+}-free sea water (CaFSW: stippled box; approx. 5 µl into 2 ml) supplemented with 2 mM EGTA. Once the eggs had sunk to the bottom of the CaFSW imaging was restarted. (A) The temporal pattern of Ca^{2+} oscillations is displayed for an egg transferred to CaFSW. The transfer to CaFSW took ~1 minute. Following transfer to CaFSW, the second phase of Ca^{2+} oscillations were present (7 waves in total in this particular egg). (B) The origin of each of the 7 waves is displayed. The white arrowhead indicates the location of the CP and the black arrowhead the location of the pb1. Temperature 19°C, *n*=5 replicates, 3 animals.

synthesized using the mMESSAGE mMACHINE kit (Ambion), resuspended in nuclease-free water and stored in 1 µl aliquots at –20°C until required.

The negative control was a non-PtdIns(4,5)P_2-binding variant of the PH::GFP in which the lysine residues at positions 30 and 32 have both been replaced with asparagine by site-directed mutagenesis [using the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega)]. This was used to transcribe mRNA in exactly the same way.

The PH::GFP coding region was cloned into the pCal-n vector (Stratagene) and transformed into BL21Gold cells (Stratagene). The PH::GFP chimeric protein was isolated using the pCAL-n Affinity® Protein Expression System (Stratagene) according to the manufacturer's instructions. The elution buffer was exchanged by concentrating the protein using Microcon® 50MWCO filters (Amicon) and dilution with ten volumes of injection buffer (0.5 M KCl, 20 mM PIPES pH 7.2, 100 µM EGTA). These concentration/dilution cycles were repeated twice before adjusting the final concentration to 25 mg ml^{-1}. Aliquots were stored at –80°C until use.

Microinjection and fluorescence measurement of Ca^{2+} and GFP

Eggs were introduced into a wedge based on the design of Kiehart (Kiehart, 1982). The microinjection system used has been described previously. Briefly, the injection system consisted of a Narisige three-way hydraulic micromanipulator and compressed air injection system (IM300, Narisige) (McDougall and Levasseur, 1998). Intracellular Ca^{2+} levels were measured using Fura-2-dextran (10 kDa, Molecular Probes), microinjected into eggs to give a final intracellular concentration of 10-20 µM. The mRNA encoding PH::GFP was injected to produce the protein product. Fura-2 and GFP were excited with the appropriate light using a 75 W xenon bulb as the light source. To change the wavelength of excitation appropriate filters (350 nm, 365 nm, 380 nm and 488 nm) housed in a filter wheel (Sutter) were selected using MetaFluor software (Universal Imaging). A triple-pass dichroic filter (Chroma, set 61000) was used to reflect the excited light to the specimen and to pass the appropriate emitted light to a cooled CCD camera (MicroMax, Princeton Instruments). We used an Olympus IX70, fitted with 10×/0.3 NA, 20×/0.75 NA and 60×/1.2 NA water immersion lens for all these experiments.

Ascidian sperm extracts were prepared as previously described (McDougall et al., 2000).

Treatment with pharmacological agents

Cytochalasin B (Sigma) was dissolved in distilled water at 1 mg ml^{-1} and diluted before use to 2 µg ml^{-1}. Nocodazole (Sigma) was dissolved in DMSO to 1 mM and diluted prior to use to 2 µM. To visualize the DNA configuration, eggs were bathed in sea water containing 10 µg ml^{-1} Hoechst 33342 for 10-20 minutes then excited at 365 nm.

Results

Localized Ca^{2+} entry to the CP

The typical spatiotemporal pattern of Ca^{2+} increase following fertilization of *Ascidiella aspersa* eggs is shown in Fig.1. Following the initial Ca^{2+} increase (sometimes displaying sinusoidal oscillations, see Fig. 5), a second series of baseline Ca^{2+} spikes was observed (Fig. 1). The first Ca^{2+} wave (Fig. 1B) triggers a cortical contraction of the egg that culminates in formation of a surface protrusion termed the contraction pole (CP) (Roegiers et al., 1995) (Fig. 1C). Following extrusion of the first polar body (pb1), all second-phase Ca^{2+} waves came from the CP and (as previously noted) these waves are faster than the initial wave (McDougall and Sardet, 1995) (Fig. 1D).

To determine whether localized Ca^{2+} influx at the CP was required for the CP to act as a Ca^{2+}-wave pacemaker, eggs were fertilized in natural sea water (NSW) and then transferred to nominally Ca^{2+}-free sea water supplemented with 2 mM EGTA. In all the eggs examined (*n*=5) all the repetitive Ca^{2+} waves originated from the CP (the origins of which can be seen in Fig. 2B) even in the absence of external Ca^{2+}.

PtdIns(4,5)P_2 localisation at the vegetal pacemaker

Here we examined whether the specific association of the PH domain of PLC δ-1 with PtdIns(4,5)P_2 could be used to visualize the cellular distribution of PtdIns(4,5)P_2 in an intact

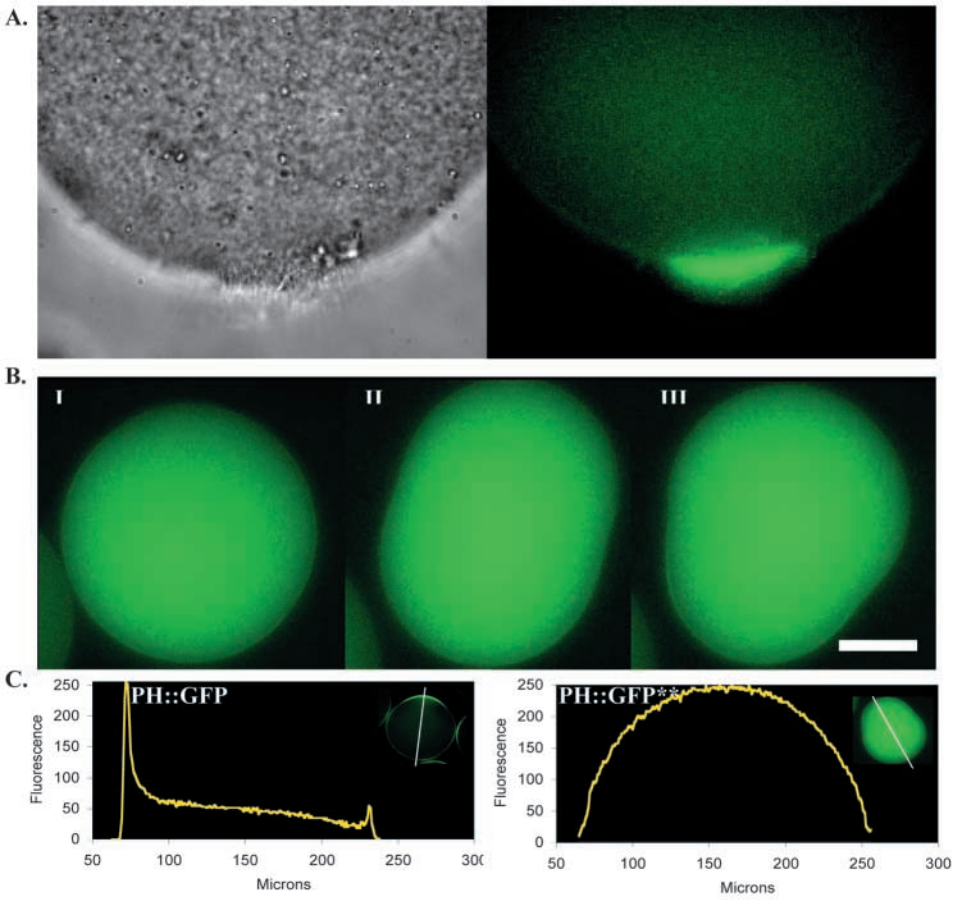

Fig. 3. PtdIns(4,5)P_2 accumulation at the CP. Unfertilized dechorionated eggs were injected with mRNA encoding either PH::GFP or a mutated form PH::GFP** whose PH domain does not recognise PtdIns(4,5)P_2. Eggs were given sufficient time to produce translated protein products (~5 hours). (A) Brightfield and fluorescence images are displayed following fertilization showing the accumulation of microvilli at the CP and the enrichment of PH::GFP at the same region of the fertilized egg. Temperature 19°C, *n*=7 replicates, 3 animals. (B) PH::GFP** did not localize to the PM before or during fertilization and had a more cytoplasmic distribution. This is seen in the three images showing an unfertilized egg (I), an egg 3 minutes after fertilization (II) and 4 minutes after fertilization, when the CP has formed (III). Bar, 50 μm. Temperature 19°C, *n*=5 replicates, 3 animals. (C) Line-scan analysis of eggs injected with PH::GFP or the mutant PH::GFP**. PH::GFP is clearly accumulated at the CP following fertilization, while some PH::GFP also remains accumulated at the PM. PH::GFP**, however, is not accumulated at the plasma membrane and displays a cytoplasmic distribution.

ascidian egg during fertilization. To achieve this, a fusion construct between the PH domain of PLC δ-1 and GFP was made (PH::GFP). Either the mRNA encoding this fusion construct or purified PH::GFP protein were microinjected into unfertilized eggs of *A. aspersa*, where, after sufficient time for expression or translocation, its localization could be observed.

When expressed in the egg, the PH::GFP construct was predominately localized to the CP, where the microvilli are also located (Fig. 3A). Some PH::GFP was still localized on the rest of the PM at this time (Fig. 3C). To examine whether this domain displayed specific binding to PtdIns(4,5)P_2 and could therefore serve as a suitable marker for PtdIns(4,5)P_2 localization, site-directed mutagenesis was performed to replace the basic amino acid in the PtdIns-interacting residues of the PH domain (Lys 30/Asn and Lys 32/Asn). This mutant version of the PH::GFP fusion construct (PH::GFP**) was uniformly distributed when expressed in the eggs and showed no PM labelling before or during fertilization (Fig. 3B,C, *n*=5).

Post-acquisition line-scan analysis was performed to compare image intensity in fertilized eggs expressing PH::GFP or PH::GFP**. For each egg, a line intensity profile was produced (schematically represented on the eggs, Fig. 3C, insets). Eggs expressing PH::GFP revealed a large increase in fluorescence at the CP region shortly after fertilization (Fig. 3B,C). By contrast, however, the PH::GFP** was uniformly distributed both before and during fertilization (Fig. 3C). Next, we wanted to determine whether the accumulation of PtdIns(4,5)P_2 played a role, if any, during the generation of the periodic Ca^{2+} waves from that site.

Pharmacological removal of microvilli from the CP

To examine whether this accumulation of PtdIns(4,5)P_2 at the CP was due to an increased density of microvilli at the CP or to some other localization mechanism, and to determine the role this accumulation of microvilli and PtdIns(4,5)P_2 played during the generation of Ca^{2+} waves, fertilized eggs were treated with the fungal alkaloid cytochalasin B to disrupt the actin cytoskeleton and so disrupt microvilli. Eggs were previously injected with Fura-2-dextran (10-20 μM) and then injected with mRNA encoding PH::GFP and fertilized. Following fertilization and the accumulation of PtdIns(4,5)P_2 at the CP, eggs were treated with 2 μg ml^{-1} cytochalasin B. Within 1 minute of adding cytochalasin B, the eggs rounded up, the microvilli disappeared and the PH::GFP became more uniform around the PM (Fig. 4, compare II and V). The accumulated PtdIns(4,5)P_2 at the CP therefore reflected an accumulation of microvilli. In addition, the Ca^{2+} waves still continued even though the PtdIns(4,5)P_2 and microvilli were confirmed to be absent.

To determine precisely the location of the Ca^{2+}-wave pacemaker in the absence of a CP, eggs were labelled with Nile Blue beads to mark the location of CP before addition of cytochalasin B (Fig. 4I, IV, VII). The pb1 also acts as an indicator of the position of the CP because it often lies opposite to the CP (Fig. 4I). Following the addition of cytochalasin B to fertilized eggs, the CP was no longer present and the second phase of Ca^{2+} waves still originated from a vegetal site (Fig. 4IV) close to where the tuft of microvilli and the CP previously resided (Fig. 4I). In addition, the site of the Ca^{2+}-wave

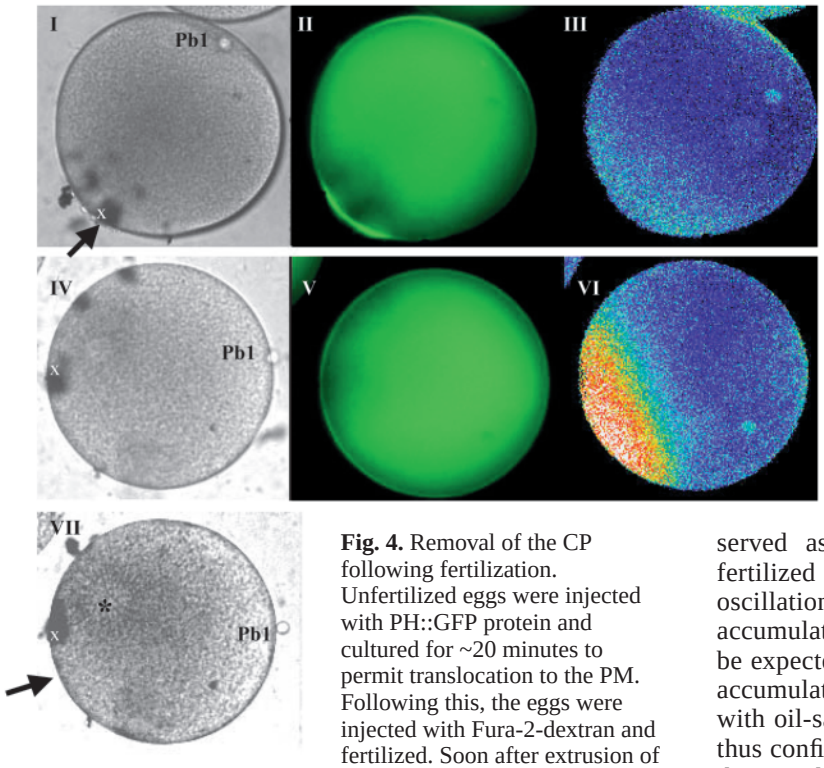

Fig. 4. Removal of the CP following fertilization. Unfertilized eggs were injected with PH::GFP protein and cultured for ~20 minutes to permit translocation to the PM. Following this, the eggs were injected with Fura-2-dextran and fertilized. Soon after extrusion of the first polar body pb1 (I), and once the CP was clearly visible (II) and the second series of Ca^{2+} waves had begun to originate from the CP (III), the eggs were treated with 2 μg ml^{-1} cytochalasin B. Rounded-up eggs (IV) reflect the loss of the actin basket; microvilli are also lost from the CP as is the accumulation of PH::GFP (V). Even though the CP has been lost and PtdIns(4,5)P_2 is now redistributed, the Ca^{2+} waves still originate from the site where the CP previously resided (VI). To confirm that the site where the Ca^{2+} waves originate is identical to the site where the CP previously resided, we attached Nile Blue beads to the surface of the egg before fertilization [see arrow and bead (x) in I]. Following fertilization, the bead is located on the border of the CP (I); after the pharmacological removal of the CP the bead borders the site where the repetitive Ca^{2+} waves originated (IV, x). Finally, we noted the position of the sperm aster following removal of the CP (VII, marked *). The sperm aster is not near the site of origin of the Ca^{2+} waves (VII, arrow). Temperature 19°C, *n*=5 replicates, 3 animals.

pacemaker did not colocalize with the position of the sperm aster, as shown by the clear separation between the position of the sperm aster (Fig. 4VII) and the Ca^{2+}-wave pacemaker (Fig. 4VI). From these data, we concluded that neither the CP protrusion nor the accumulation of PtdIns(4,5)P_2 was required for the functioning of the Ca^{2+}-wave pacemaker because all the second-phase Ca^{2+} waves still originated from the CP protrusion area, even when the CP and localized PtdIns(4,5)P_2 were no longer present.

Accumulation of cER

During contraction of the actomyosin basket, the cER becomes concentrated rapidly at the CP (Speksnijder et al., 1993) and it has been suggested that this cER domain plays a role in the determination of the CP as a Ca^{2+}-wave pacemaker (Speksnijder et al., 1993). Before formation of the CP and its associated Ca^{2+}-wave pacemaker activity, ER first accumulates around the growing sperm aster and it has been shown that this accumulation of ER correlates with the location of the mobile Ca^{2+}-wave pacemaker (Dumollard and Sardet, 2001), which is equivalent to the first phase of sinusoidal Ca^{2+} waves that are sometimes seen during fertilization in *A. aspersa* (compare Figs 1 and 5). However, recent evidence casts doubt on ER accumulation having a role in Ca^{2+}-wave pacemakers, because these ER accumulations are not the part of the egg that is most sensitive to $InsP_3$ (Dumollard and Sardet, 2001). By preventing ER accumulation at the CP (absence of the cortical contraction served as a positive control for cytochalasin B) and/or at the sperm aster (absence of sperm aster and pb1 extrusion served as positive controls for nocodazole), eggs were fertilized to determine whether the frequency of Ca^{2+} oscillations would be reduced by the absence of an accumulation of ER at the CP and/or sperm aster (as would be expected if these sites act as pacemakers because of their accumulation of ER). Some eggs were previously injected with oil-saturated dialcylcarbocyanine (DiI) to label the ER, thus confirming that ER accumulations were abolished when the cytoskeleton was disrupted (data not shown).

Eggs treated with nocodazole alone displayed both series of Ca^{2+} oscillations (Fig. 5Ai, *n*=8). The microtubule-based sperm aster did not form and the pbs were not extruded. These eggs displayed the actomyosin-dependent cortical contraction, and the second series of Ca^{2+} waves displayed a frequency not significantly different from control fertilizations (Table 1).

Eggs treated with cytochalasin B alone also displayed both phases of Ca^{2+} oscillations (Fig. 5Aii, *n*=9). The actomyosin-dependent cortical contraction and pb extrusion were both inhibited. However, the formation of the microtubule-based sperm aster was unaffected. These eggs displayed a frequency of second-phase Ca^{2+} oscillations not significantly different from control fertilizations (Table 1).

When eggs were treated with both nocodazole and cytochalasin B to prevent both forms of ER accumulation, the fertilized eggs still displayed two series of Ca^{2+} waves that all originated from the point of sperm fusion (Fig. 5B, *n*=18). To determine more precisely the site of sperm-egg fusion, eggs were bathed in the vital DNA dye Hoechst 33342 and a mixture of cytochalasin B (2 μg ml^{-1}) and nocodazole (2 μM) then injected with Ca^{2+}-Green-1-dextran (10-20 μM) and fertilized. Hoechst 33342 staining revealed that the position of the sperm nucleus corresponded to the initiation site of both phases of Ca^{2+} waves when both ER accumulation at the CP and sperm aster formation were prevented (Fig. 5B). Finally, the frequency of Ca^{2+} spikes during the second phase in eggs treated with the cytoskeleton inhibitors was similar to those in untreated eggs (controls) (Table 1). These data indicate that the accumulation of cER at the CP (or around the sperm nucleus) is not required for the generation of the second series of Ca^{2+} waves and has no effect on the frequency of the second phase of Ca^{2+} waves. We did find, however, that the rate of Ca^{2+}-wave propagation was slower in eggs treated with cytochalasin B (data not shown).

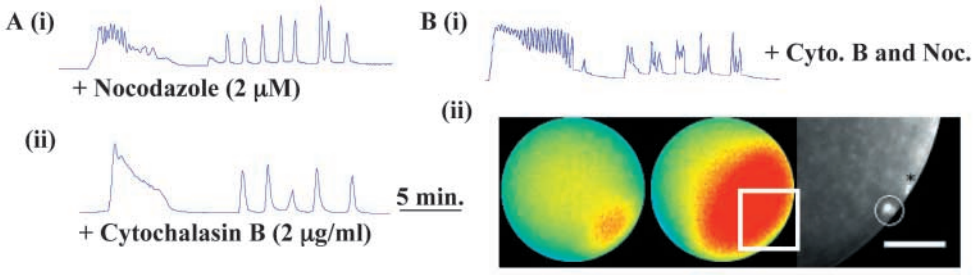

Fig. 5. Preventing ER accumulation during fertilization. Unfertilized eggs were injected with Ca^{2+}-Green-1-dextran and treated with 2 μg ml^{-1} cytochalasin B or 2 μM nocodazole or a combination of both, and fertilized. (Ai) The pattern of Ca^{2+} oscillations in an egg treated with nocadazole alone; *n*=8. (Aii) The pattern of Ca^{2+} oscillations in an egg treated with cytochalasin B alone; *n*=9. (Bi) The pattern of Ca^{2+} oscillations in an egg treated with a combination of cytochalasin B (2 μg ml^{-1}) and nocodazole (2 μM). (Bii) All of the Ca^{2+} waves originated at the same site in the egg (boxed). This boxed area is shown enlarged and contains the round sperm with condensed DNA (circled area). The black asterisk shows the elongated form of non-incorporated sperm DNA, which remains attached to the outer surface of the egg. Temperature 19°C, *n*=18 replicates, 5 animals.

Localization of ascidian sperm factor during fertilization

It has been shown in mouse and ascidian eggs that the cortex is the site of the initial Ca^{2+} release when sperm extract (SE) is injected centrally [mouse (Oda et al., 1999); ascidian (Kyozuka et al., 1998)] and that the sensitivity to SE and $InsP_3$ is higher in the cortex of mouse oocytes (Oda et al., 1999). In the ascidian, the CP is not the region of the egg that is most sensitive to $InsP_3$ (Dumollard and Sardet, 2001), even though all of the second-phase Ca^{2+} waves originate there when aSE is injected centrally (McDougall et al., 2000). These data suggest that aSE accumulates near or on the PM at the CP following the cortical contraction.

We were therefore interested in determining whether the cortex plays a role in anchoring the factor present in aSE near the PM. Eggs were injected with Ca^{2+}-Green-1-dextran (10-20 μM) and soaked in cytochalasin B (2 μg ml^{-1}) and nocodazole (2 μM) for approximately 30 minutes. After this, aSE was injected into the centre of the egg (0.1-0.5% cytoplasmic volume, ~3-20 mg ml^{-1} injected by pipette) (Fig. 6). After a lag time of approximately 2 minutes, the initial Ca^{2+} transient originated from a site on the cortex instead of the deeper cytoplasmic injection site (Fig. 6). All the Ca^{2+} waves originated from the near the PM (Fig. 6, 6/6 eggs).

Discussion

We have examined how the sperm-induced Ca^{2+}-wave pacemaker in the ascidian egg functions and conclude that the pacemaker probably contains an accumulation of the sperm factor that generates all the repetitive Ca^{2+} waves. In addition, we find that the accumulated domain of cER, Ca^{2+} influx (following pb1) and PtdIns(4,5)P_2 enrichment at the CP are not required for Ca^{2+}-wave pacemaker activity. Finally, we demonstrate that injecting aSE into eggs treated with microfilament- and microtubule-disrupting drugs triggers repetitive Ca^{2+} waves that originate near the PM, and also that all the repetitive Ca^{2+} waves originate at the site of sperm-egg fusion in fertilized eggs prevented from forming a CP.

Mechanism of Ca^{2+}-wave pacemaker operation

There are two phases of Ca^{2+} waves in the ascidian. The first is between fertilization and pb1 extrusion (~0-7 minutes). We discounted Ca^{2+} influx as having a role in setting the placement of the Ca^{2+}-wave pacemaker. However, because external Ca^{2+} was present during sperm-egg fusion, it is possible that Ca^{2+} influx during that time somehow sets the site of the Ca^{2+}-wave pacemaker. The regulation and pattern of Ca^{2+} oscillations are the same as normal fertilization following injection of aSE (Kyozuka et al., 1998; Runft and Jaffe, 2000; McDougall et al., 2000) where no sperm PM is present. It therefore seems unlikely that external Ca^{2+} influx through sperm-borne Ca^{2+} channels results in the establishment of the Ca^{2+}-wave pacemaker because the pacemaker is also established following injection of aSE. The simplest interpretation of our data is that a factor(s) introduced into the egg with the sperm stays near the cortex. Following introduction into the egg, this factor stimulates local production of $InsP_3$, which in turn spreads across the egg, causing the first Ca^{2+} increase. This Ca^{2+} increase in turn stimulates a cortical contraction, dragging cortical components towards the vegetal hemisphere of the egg (Roegiers et al., 1995). We hypothesize that the sperm factor is also dragged cortically. It is interesting that, before the stable Ca^{2+}-wave pacemaker forms at the CP, a mobile Ca^{2+}-wave pacemaker is present (McDougall and Sardet, 1995). This mobile Ca^{2+}-wave pacemaker correlates with an accumulation of ER around the sperm aster (Dumollard and Sardet, 2001). Here, we define the accumulation or aggregation of ER as the assembly of ER microdomains (~2 μm diameter) into larger structures (several μm diameter). We hypothesize that the mobile Ca^{2+}-wave pacemaker represents

Table 1. Average number of Ca^{2+} peaks during the second phase in oocytes treated with cytoskeleton inhibitors compared with untreated oocytes (controls)

	Nocodazole	Cytochalasin B	Nocodazole and cytochalasin B	Control
Average no. of Ca^{2+} peaks during the second phase	6.0 (*n*=8)	4.9 (*n*=9)	3.9 (*n*=18)	5.0 (*n*=34)

Unpaired *t*-test of oocytes treated with the cytoskeleton inhibitors and untreated oocytes showed no significant difference between the number of Ca^{2+} spikes during the second phase of Ca^{2+} oscillations ($P>0.05$).

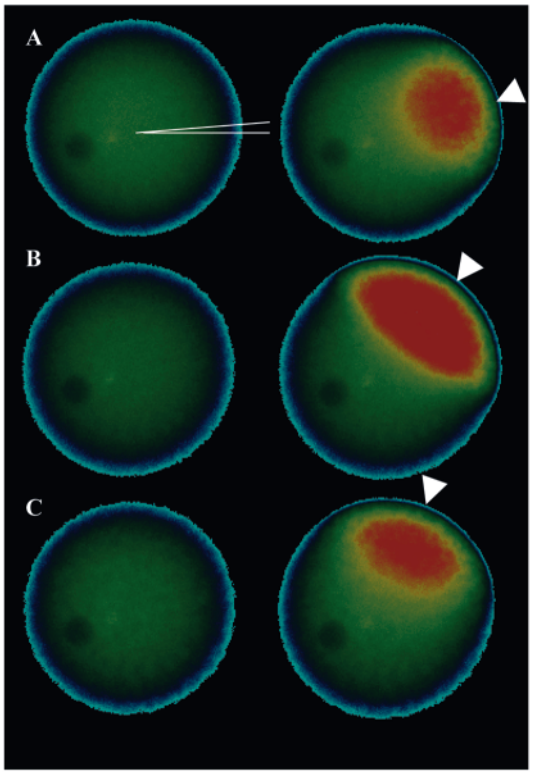

Fig. 6. Ca^{2+}-Green-1-dextran was injected into unfertilized eggs. Subsequently, eggs were treated with cytochalasin B and nocodazole for 30 minutes and then injected with aSE. Thereafter, all Ca^{2+} waves originated at the cortex of the egg (A,B,C). Arrowheads mark the origin of three separate Ca^{2+} waves within the same egg. Temperature 14°C, *n*=6 replicates, 3 animals.

the movement of the ascidian sperm factor from the site of sperm-egg fusion towards the vegetal hemisphere of the egg.

The second phase is between pb1 extrusion and pb2 extrusion (~7-25 minutes after fertilization). Following formation of the CP, all Ca^{2+} waves come from that site. We hypothesize that the CP contains an accumulation of the sperm factor (perhaps not all but an enrichment of it) for the following reasons. When the cortical contraction is abolished, all second-phase Ca^{2+} waves come from the site of sperm-egg fusion. This shows that the factor probably remains near the site of sperm-egg fusion, even though the sperm factor might be a soluble protein. Also, when the CP protrusion was removed pharmacologically, all the Ca^{2+} waves still came from the site previously occupied by the CP rather than the site of sperm-egg fusion. We do not know whether and, if so, how, the sperm factor is anchored to or near the PM but it might be through the association with membrane lipid rafts. It has been shown that lipid rafts function as a signalling scaffold where several cell signalling molecules, including PLCs and Src kinases accumulate (reviewed in Zajchowski and Robbins, 2002) and recent reports have shown that the disruption of these domains perturbs the fertilization signalling pathway in sea urchin and *Xenopus* eggs (Belton et al., 2001; Sato et al., 2002).

The next problem is how the factor reaches to the CP, because the sperm nucleus clearly borders the CP but is not near its centre (Fig. 4VII). One possibility is that it is accumulated in the CP owing to the cortical contraction; if this is the case, it probably remains near the PM. For example, beads attached to the surface of the egg, near the site of sperm-egg fusion, move further into the CP than the underlying sperm nucleus (Roegiers et al., 1995). This is probably because the sperm nucleus lies on the cytoplasmic side of the actomyosin basket and therefore experiences more viscous drag than PM proteins tethered to the actomyosin basket. Finally, the frequency of Ca^{2+} oscillations is not significantly affected by preventing formation of the CP. This shows that the local accumulation of cER, PtdIns(4,5)P_2 and other factors, such as egg PLCs, at the CP are not required, and that sufficient cER, PtdIns(4,5)P_2, PLCs and other components of the signalling cascade can be found around the egg periphery. The CP is therefore the Ca^{2+}-wave pacemaker by default because by preventing its formation, Ca^{2+} waves are still generated at the correct frequency, albeit from the site of sperm-egg fusion. Cytochalasin B treatment does appear to reduce the frequency of Ca^{2+} oscillations in another species of ascidian (Dumollard and Sardet, 2001). However, because the object of that work was to show that three Ca^{2+}-wave pacemakers can co-exist in fertilized ascidian zygotes, no statistical analysis was provided concerning the frequency of oscillations. It is therefore impossible to know unequivocally whether cytochalasin treatment leads to a significant reduction in oscillation frequency (Dumollard and Sardet, 2001). It is clear from our present and previous experiments that the range of oscillation frequency is large (McDougall et al., 2000). One of the last aspects of the vegetal Ca^{2+}-wave pacemaker that has not been tested is whether it provides a cue to polarity and thus whether it has significance for later developmental events.

Mechanisms of egg activation

There is evidence that, in the mammalian egg, the sperm-specific PLCζ can induce Ca^{2+} oscillations that mimic fertilization (Saunders et al., 2002). In other organisms, such as echinoderms, it has often been reported that PLCγ and Src-like tyrosine kinases are involved in generating the Ca^{2+} increase at fertilization (Carroll et al., 1999; Shearer et al., 1999; Giusti et al., 2003). In ascidians, there is some evidence that PLCγ and Src-like tyrosine kinases are likewise involved in triggering the first Ca^{2+} increase at fertilization (Runft and Jaffe, 2000). However, PLCζ has not yet been identified in a non-mammalian system.

In invertebrates, both PLCγ and forms of Src have been proposed to be involved in the mechanism of egg activation. These proteins are accumulated to the PM when activated (reviewed in Rebecchi and Pentyala, 2000; Featherstone, 1997), so the ascidian sperm factor might be tethered to the membrane by one of these proteins. Alternatively, the ascidian sperm factor might be a form of PLCζ that remains to be identified. As for localisation near the PM, it is not known yet how PLCζ is localized in mammalian eggs following expression of PLCζ copyRNA or fertilization. One final point to make about localization is the nuclear localisation of mammalian sperm factor following the formation of pronuclei (Marangos et al., 2003). If the ascidian sperm factor were located in a pronucleus following fertilization, the male pronucleus would make the ideal choice because it is closest to the CP. However, because the Ca^{2+} oscillations stop before

the pronuclei form (McDougall and Levasseur, 1998), it is clear that the Ca^{2+} waves generated by the ascidian sperm can be interrupted in a way that does not require pronucleus formation. The Ca^{2+} oscillations in the ascidian egg correlate well with the activity of cyclin-dependent kinase 1 (Cdk1) activity, pause when Cdk1 activity falls and finally stop when Cdk1 activity falls a second time, a few minutes before formation of the pronuclei (McDougall and Levasseur, 1998). Indeed, Ca^{2+} oscillations can be maintained indefinitely by preventing Cdk1 inactivation (Levasseur and McDougall, 2000).

Finally, we wondered whether ER integrity might play a role in the pattern of Ca^{2+} oscillations observed, but found that the ER remains continuous in both control and cytochalasin-treated eggs during the period that the Ca^{2+} oscillations normally pause (data not shown). To resolve the localisation and regulation of the sperm factor fully will clearly require its identification.

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