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Cell cycle-dependent repetitive Ca^{2+} waves induced by a cytosolic sperm extract in mature ascidian eggs mimic those observed at fertilization

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SUMMARY

Sperm-triggered Ca^{2+} oscillations occur throughout the animal kingdom. The mechanism sperm use to trigger Ca^{2+} oscillations at fertilization has not been resolved in any egg. The temporal, spatial and regulatory characteristics of the Ca^{2+} oscillations during fertilization in ascidians offer a unique advantage over other systems for determining the mechanism of fertilization. For example, sperm trigger two phases of Ca^{2+} oscillations that are all waves in ascidians. The first of these Ca^{2+} waves begins at the point of sperm-egg fusion while a second phase of Ca^{2+} waves originates at a vegetal protrusion termed the contraction pole. In addition, cyclin B1-dependent kinase activity provides a form of positive feedback, maintaining the second phase of Ca^{2+} waves during meiosis and thereby ensuring meiotic exit.

We therefore prepared cytosolic ascidian sperm extracts or MonoQ-fractionated ascidian sperm extracts from this urochordate to investigate if a Ca^{2+}-releasing sperm-borne factor was responsible for egg activation. Spatially, ascidian sperm extract induced repetitive Ca^{2+} waves that mimicked the spatial pattern displayed during fertilization: all the second-phase Ca^{2+} waves originated at a vegetal protrusion termed the contraction pole (thus mimicking fertilisation). We also demonstrated that ascidian sperm extract-induced Ca^{2+} oscillations were maintained when CDK activity was elevated and MAP kinase activity was low, as found previously for sperm-triggered Ca^{2+} oscillations. As would be predicted, large doses of ascidian sperm extract injected into prophase-stage oocytes, lacking CDK activity, failed to induce any Ca^{2+} release even though they responded to microinjection of the Ca^{2+}-releasing second messenger inositol 1,4,5-trisphosphate. Finally, since the Ca^{2+}-releasing activity from Mono-Q fractionated ascidian sperm extract eluted predominantly as one fraction, this may imply that one factor is responsible for the Ca^{2+}-releasing activity.

These data support a model of egg activation whereby the sperm introduces a Ca^{2+}-releasing cytosolic factor into the egg. We demonstrated that ascidian sperm contain a protein factor(s) that is regulated by the egg CDK activity and can trigger all the Ca^{2+} waves observed at fertilization with a spatial pattern that mimics those initiated by sperm.

Key words: Fertilization, Calcium, Sperm factor, Cell cycle, Ascidian

INTRODUCTION

Sperm activate eggs by triggering an increase in the cytosolic calcium ion concentration [Ca^{2+}] (reviewed by Fissore et al., 1999; Stricker, 1999). In many eggs, such as those from the nemertean worm *Cerabratulus lacteus* (Stricker, 1996), the polychaete worm *Chaetopterus* (Eckberg and Miller, 1995), several species of ascidian (Speksnijder et al., 1989a; Yoshida et al., 1998) and mammals (Cuthbertson et al., 1981; Miyazaki et al., 1986), the change in [Ca^{2+}] is not a single increase but a series of oscillations. It is likely that an enzyme of the phospholipase C (PLC) family is required for the full set of Ca^{2+} oscillations. In mammals this is based on the evidence that the Ca^{2+} oscillations are inhibited by U73122, a PLC inhibitor (Dupont et al., 1996). In addition, downregulation of the $InsP_3$ receptor number is consistent with $InsP_3$ production during fertilization in mouse eggs (Parrington et al., 1998). Finally, these data are consistent with the finding that a functionally blocking antibody to the type I $InsP_3$ receptor blocks Ca^{2+} oscillations in hamster (Miyazaki et al., 1992) and mouse eggs (Oda et al., 1999). It is similar in ascidians, where the same type I $InsP_3$ receptor antibody blocks the second phase of Ca^{2+} oscillations (Yoshida et al., 1998); however, the identity of the PLC and how it is activated are presently unresolved.

There are three main hypotheses of egg activation, termed the conduit, contact and content models (Jaffe, 1991). The conduit model suggests that sperm deliver Ca^{2+} channels to the egg, which result in egg activation through Ca^{2+} influx (Créton and Jaffe, 1995). However, mouse eggs display a

Ca^{2+} signal even when external [Ca^{2+}] is buffered to 13 nM (Jones et al., 1998b). In these experiments sperm were applied as a bolus some distance from the eggs, which were in a Ca^{2+}-free medium containing 100 μM EGTA to chelate additional Ca^{2+} added with the sperm. Although it is not known for certain whether the [Ca^{2+}] near the site of sperm-egg fusion was low at the moment of fertilization, the sperm bolus was deliberately applied far from the eggs by the experimenters to increase the likelihood that the external [Ca^{2+}] was low at the moment of sperm-egg fusion.

The contact model of egg activation suggests that sperm trigger the Ca^{2+} signal through a receptor-based mechanism, akin to hormone signalling. Initially, evidence from sea urchin eggs led to the development of the receptor hypothesis of egg activation where the sperm was proposed to activate PLCβ via a G-protein (Turner et al., 1986). More recent evidence in echinoderm eggs instead favours the involvement of PLCγ, since bovine PLCγ tandem SH2 domains block fertilization (Carroll et al., 1997, 1999; Shearer et al., 1999). However, bovine tandem SH2 domains do not block fertilization in mouse (Mehlmann et al., 1998) or *Xenopus* (Runft et al., 1999) eggs. In addition, inhibitory antibodies to Gq family G-proteins do not block fertilization in the eggs of either mouse (Williams et al., 1998) or *Xenopus* (Runft et al., 1999). It therefore remains unresolved how sperm activates the egg PLC activity via a cell surface receptor.

The content (or sperm factor) model of egg activation instead suggests that sperm introduce a cytosolic protein into the egg upon sperm-egg fusion that triggers the Ca^{2+} oscillations observed at fertilization (Swann, 1990; Wu et al., 1997). This is based on the observation that a sperm cytosolic extract containing an as yet unidentified protein factor triggers Ca^{2+} oscillations when injected into the homologous eggs of mammals (Swann, 1990; Wu et al., 1997; interspecies crosses are also effective), nemerteans (Stricker, 1997) and ascidians (Kyozuka et al., 1998). In mammals the most likely candidates are a truncated version of the c-kit receptor (Sette et al., 1997), which has been shown to be a PLC activator (Sette et al., 1998), or alternatively PLC itself (Jones et al., 1998a; Parrington et al., 1999). Consistent with the above hypothesis, in mouse eggs, fluorescent dextrans (Lawrence et al., 1997) and large molecular weight proteins (Jones et al., 1998b) can diffuse between gametes before any observed [Ca^{2+}] changes. Since bovine tandem SH2 domains are without effect during fertilization in mouse (Mehlmann et al., 1998), the PLC in mammalian sperm may already be active before entering the egg from the sperm.

Ascidians offer a stringent test of the sperm factor hypothesis since they combine cell cycle regulation (Levasseur and McDougall, 2000) and spatial control of wave site origin (Speksnijder, 1992). A dose of ascidian sperm extract (ASE) equivalent to 1.8 sperm clearly mimics the temporal pattern of Ca^{2+} oscillations triggered by sperm (Kyozuka et al., 1998), but it has not yet been tested whether the spatial pattern is also mimicked. The aim of the present study was to exploit these two aspects of fertilization in ascidians to stringently test the sperm factor hypothesis of egg activation. Our results confirm the sperm factor hypothesis and demonstrate that ascidian sperm extract (ASE) faithfully mimics sperm.

MATERIALS AND METHODS

Biological material

The tunicates (*Ascidiella aspersa*) were grown locally on ropes laid down at Blyth, North East England and kept in a holding tank for up to 2 weeks at 10°C. Animals that contained sperm were harvested by removing their outer tunic. Since ascidians are hermaphrodites, eggs were also collected from the same animal. For in vitro fertilization the sperm were immediately diluted in sea water (1- to 200-fold) that contained chorionated eggs and stored at 4°C (a further 100-fold sperm dilution was carried out prior to fertilization.) Chorionated *Ascidiella* eggs can be activated (circa 85%) with 5×10^3 sperm/ml. Chorionated eggs help to activate the sperm (Sardet et al., 1989). Eggs were next transferred to medium-sized Petri dishes containing filter-sterilised (0.2 μm pore size, Millipore) natural sea water. The eggs were then treated with 0.1% trypsin for 45-60 minutes then additionally sheared gently by passing them once through a Pasteur pipette to remove the chorions. Following dechorionation, the denuded eggs were transferred to gelatin/formaldehyde (0.1% each)-coated Petri dishes and kept on a cooled copper plate at 18°C until required. The formaldehyde was removed by air drying followed by washing with sea water. Any glass or plasticware subsequently used that came into contact with the eggs was also treated with the gelatin/formaldehyde mixture.

Preparation of ASE and fASE

Ascidian sperm extract was prepared by a simplified modification of that described previously (Kyozuka et al., 1998). Sperm were collected from between 10-200 animals and subsequent steps were all performed at 4°C. Sperm were pelleted at 5000 ***g*** and washed five times into ten volumes of Ca^{2+}-free sea water, before a final wash in 0.2 M phosphate buffer, pH 7.4. The supernatant was aspirated from the final sperm pellet, which was snap-frozen in liquid nitrogen. The sperm pellet was resuspended in 180 mM KCl, 100 μM EGTA, 30 mM BES, pH 7.1, and homogenised on ice using a hand-held homogeniser (Ultra-Turrax, T8) before ultracentrifugation at 100,000 ***g*** for 1 hour. For microinjection into eggs the extract was concentrated on 30,000 molecular weight ultrafiltration membranes (Centricons, Amicon, UK) to give a final protein concentration of 7.8 mg/ml. Samples of sperm extracts were frozen in liquid nitrogen and stored at −80°C. In control experiments ASE was treated with a volume excess of trypsin-agarose beads for at least 30 minutes (Sigma) and injected into unfertilized ascidian eggs.

Chromatography of sperm extracts was performed on a BioRad Biologic system at 4°C with a 1 ml MonoQ column (Pharmacia). The MonoQ column was loaded with 5 ml of sperm extract prepared as described above and desalted into 20 mM diethylamine-HCl, pH 8.5, using PD-10 columns (Pharmacia) and the protein eluted with increasing salt up to 1 M NaCl at 1 ml/minute. Several fractions were tested for activity. Protein concentration of the active fraction was 0.29 mg/ml.

Microinjection and fluorescence measurement of Ca^{2+} levels and condensation status of the DNA

Eggs were introduced into a wedge, based on the design of Keihart (1982). The microinjection technique has been described previously (McDougall and Levasseur, 1998). Briefly, microinjection was performed using a Narisige IM300 injection device coupled to a three-way hydraulic micromanipulator (Narisige). Injection pipettes were pulled using a Kopf vertical puller (Model 100C) and filament-free capillaries (GC100 T10, Clarke Electromedical). Intracellular Ca^{2+} levels were recorded by microinjection of Fura-2 dextran (10 kDa, Molecular Probes) to give a final intracellular concentration of approximately 20 μM. The condensation status of the DNA was determined by bathing the eggs in Hoechst 33342 (5 μg/ml) for 15 minutes. mRNA was produced from the Δ90 cyclin B1-GFP constructs using the T3 mMESSAGE mMACHINE kit (Ambion Inc.),

dissolved in nuclease-free water to a concentration of 1-1.5 μg/μl and microinjected into eggs to approximately 5% of total egg volume (approximately equivalent to 100 pg of mRNA). The excitation light source (Xenon lamp, Opti Quip, 150 W) passed through filters (350 nm, 365 nm, 380 nm and 488 nm) housed in a filter wheel (Sutter, Lambda 10-2) to illuminate the specimen and the chromophores employed. We used a triple pass dichroic/emission filter set (Chroma, set 61000: suitable for DAPI, FITC and TRITC) or a Fura-2 set (dichroic 430 nm, emission 510 nm) to direct the light to the specimen and then the collecting device. The emitted light was collected using a cooled (−10°C) charge-coupled device (CCD) camera (MicroMax, Princeton Ins.). We used an inverted Olympus microscope (IX70) fitted with a 10×/0.3 n.a. lens, a 20×/0.75 n.a. lens and a 60×/1.2 n.a. water immersion lens.

Treatment with pharmacological inhibitor U0126

The MAP kinase inhibitor U0126 (Promega) was dissolved in DMSO to a concentration of 10 mM. We measured the Ca^{2+} oscillations triggered by sperm at fertilization in eggs injected with Δ90 cyclin B1 mRNA and treated with U0126. The inhibitor was prepared freshly before each use due to its relative instability in DMSO.

RESULTS

Ascidian sperm extract (ASE) triggers two phases of Ca^{2+} oscillations and meiotic exit

Sperm trigger a predictable pattern of Ca^{2+} oscillations at fertilization in *A. aspersa* eggs that consists of two phases of Ca^{2+} oscillations separated by a gap phase (Table 1; Fig. 1A,B). Examples of the types of pattern we observe are illustrated in Fig. 1. Eggs from two different batches are shown. Either the first Ca^{2+} signal is a solitary oscillation (Fig. 1A) or there are a series Ca^{2+} oscillations associated with the first [Ca^{2+}] increase (Fig. 1B). Regardless of whether the first Ca^{2+} signal is solitary or is oscillatory, there follows a gap period when the Ca^{2+} oscillations pause (Fig. 1A,B). During this time the first polar body is extruded, the egg has an irregular shape and the MPF (maturation promoting factor) activity is low (McDougall and Levasseur, 1998). As the eggs regain a spherical shape the second phase of Ca^{2+} oscillations begins (Fig. 1A,B). Eggs display on average 2-3 Ca^{2+} signals during the first phase and 6 Ca^{2+} signals during the second phase (Table 1). The second phase of Ca^{2+} oscillations terminates seconds before extrusion of the second polar body (Speksnijder et al., 1989a; Fig. 1A,B). The egg then forms two pronuclei, the sperm aster enlarges and the egg changes shape yet again, flattening along the AV axis. These events all occur within a few minutes after extrusion of the second polar body in *A. aspersa* (McDougall and Levasseur, 1998).

Following microinjection of ASE it takes approximately 2 minutes before the [Ca^{2+}] first increases at a cortical site (n=8), presumably reflecting the time it takes the factor to diffuse to the cortex. This delay is reduced when extract is injected close to the cortex, as noted previously (Kyozuka et al., 1998), but is not further reduced in Δ90 cyclin B1

Table 1. Calcium oscillations during fertilisation in *A. aspersa* eggs

Number of oscillations	
First phase	Second phase
2 (range 1-4, n=28)	6 (range 3-14, n=28)

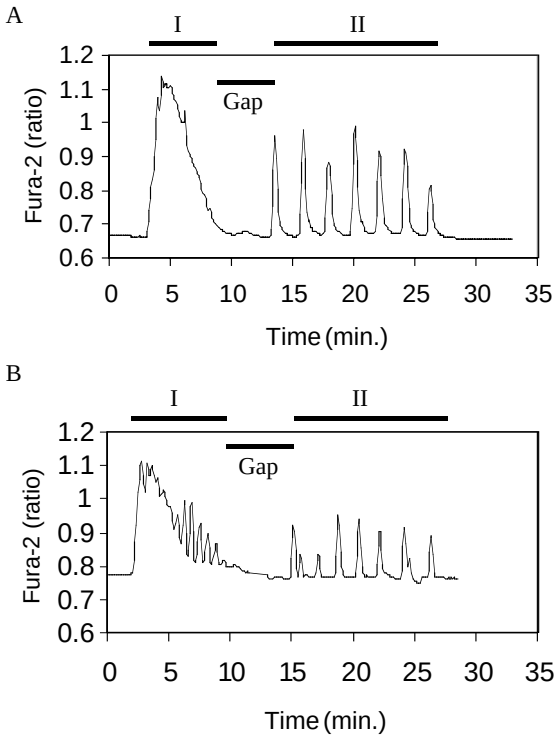

Fig. 1. (A,B) Examples of sperm-triggered Ca^{2+} oscillations at fertilization in different egg batches. (A) Unfertilized denuded ascidian eggs were injected with Fura-2 dextran to give an intracellular concentration of approximately 20 μM. The eggs were then fertilized and the Ca^{2+} oscillation pattern triggered by sperm recorded at 5 second intervals for a period of 35 minutes. (A) Sperm trigger two phases of Ca^{2+} oscillations (I and II) separated by a gap. (B) In addition to the pattern observed previously in A, there are a number of Ca^{2+} oscillations during the first phase (labelled I). Again there is a short gap phase before the second phase of Ca^{2+} oscillations are generated (n=28). Temperature, 19°C.

containing eggs (see later). Eggs injected with approximately 1.5-3 pl ASE (0.1-0.2% injection volumes) display two phases of Ca^{2+} oscillations separated by a gap phase (Fig. 2A, n=8; Kyozuka et al., 1998). Temporal patterns like these are hallmarks of fertilization in ascidians and so far no other stimuli are capable of triggering a pattern of Ca^{2+} oscillations that has these temporal characteristics. For example, adenophostin B (a nonmetabolisable agonist of the inositol trisphosphate receptor) triggers a single series of Ca^{2+} oscillations without a gap (Yoshida et al., 1998). ASE is also capable of triggering meiotic exit, e.g. both polar bodies are extruded (Kyozuka et al., 1998) and the female DNA decondenses (Fig. 2B) following injection of ASE.

ASE triggers the same spatial pattern of Ca^{2+} waves as sperm

Sperm normally fuse with the egg in the animal hemisphere, the greatest probability being around the animal pole (Speksnijder et al., 1989b). The first Ca^{2+} wave triggered by sperm initiates an actomyosin-driven cortical contraction (Sawada and Osanai, 1981; Roegiers et al., 1995). The second phase of Ca^{2+} waves originate at a vegetal protrusion termed the contraction pole (Speksnijder, 1992; McDougall and

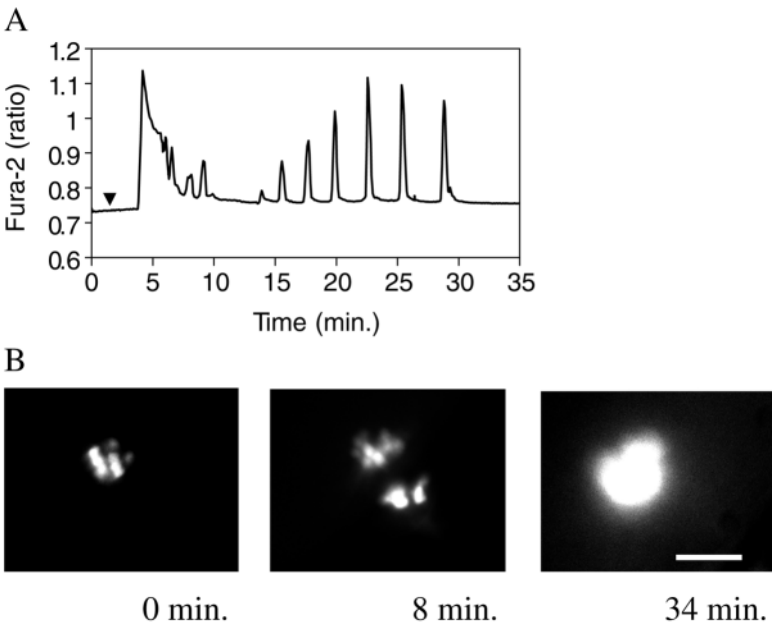

Fig. 2. Ascidian sperm extract (ASE) triggers two phases of Ca^{2+} oscillations when injected into unfertilised ascidian eggs. (A) Unfertilized eggs were first injected with Fura-2 dextran to give an intracellular concentration of approximately 20 μM. Approximately 2 minutes following injection of ASE (0.1-0.2%) (arrowhead) [Ca^{2+}] begins to increase. Two phases of Ca^{2+} oscillations separated by a gap are clearly present (*n*=8). This is in agreement with a previous publication (Kyozuka et al., 1998). (B) Eggs were bathed in the vital DNA dye Hoechst 33342 (5 μg/ml) for 15 minutes to monitor the status of the chromatin following injection of ASE. The DNA begins to undergo anaphase I at 8 minutes and decondenses 34 minutes following injection of ASE. Temperature, 19°C. Bar, 10 μm.

Sardet, 1995), which forms as a consequence of the cortical contraction in a 110° cone centred around the vegetal pole (Roegiers et al., 1995). We therefore tested whether these spatial aspects of the Ca^{2+} waves could be reproduced by injection of ASE.

To mimic these spatial aspects of fertilization, ASE was injected close to the animal pole of the unfertilised eggs so that the first Ca^{2+} wave would originate at that site (Fig. 3). The animal pole is easily identifiable since in the vicinity of metaphase I spindle cytoplasmic organelles are excluded, leaving a clear zone that is visible using differential interference contrast microscopy (Speksnijder et al., 1990). The injection pipette was inserted towards the animal pole from the vegetal pole. Injection of ASE triggered a [Ca^{2+}] rise that started first near the animal pole (large arrow in Fig. 3). This Ca^{2+} wave triggered a cortical contraction approximately 29 seconds following the first [Ca^{2+}] rise that resulted in the formation of a contraction pole in the vegetal hemisphere (Fig. 3). The egg rotates slightly due to the actomyosin contraction in a counter clockwise direction thus the contraction pole does not appear to form directly opposite the animal pole. Once the first [Ca^{2+}] increase has returned to basal values the egg enters the gap phase. The Ca^{2+} oscillations resume approximately 13 minutes following the first Ca^{2+} signal from the region of the egg where the contraction pole formed previously (Fig. 3). Three second-phase Ca^{2+} oscillations in this egg are displayed that all come from the same region of the egg (small arrow, Fig. 3).

Cyclin B1-dependent kinase activity maintains the Ca^{2+} oscillations triggered by ASE

Persistently elevated cyclin B1-dependent kinase (CDK) activity in ascidian eggs prolongs the duration of the Ca^{2+} oscillations triggered by the sperm (Levasseur and McDougall, 2000). We therefore tested whether ASE would also be controlled in a similar way by the CDK activity. Unfertilised ascidian eggs were injected with mRNA encoding an N-terminal 90-amino-acid-truncated form of human cyclin B1 termed Δ90 cyclin B1. Δ90 cyclin B1 is capable of binding to and activating CDK (Murray et al., 1989). However, Δ90 cyclin B1 escapes degradation by the ubiquitin/proteasome pathway because it does not contain the N-terminal 9-amino-acid motif known as the destruction box (Glotzer et al., 1991), nor the downstream lysine residues that become ubiquitinated. Unfertilised ascidian eggs appear to be translationally active since they can be activated with the protein synthesis inhibitor emetine (Abdelmajid et al., 1993). However, to confirm the translation of the injected mRNA we used a Δ90 cyclin B1-green fluorescent protein (GFP) fusion construct (GFP is fused to the C terminus of cyclin B1). mRNA-injected eggs displayed a green fluorescence approximately 3 hours after injection when excited at 488 nm due to the presence of the translated fusion protein product. The Δ90 cyclin B1-GFP translation product faithfully activates the oocyte histone H1 kinase activity, has a molecular mass equal to the fusion product and prevents polar body extrusion and decondensation of the egg DNA (Levasseur and McDougall, 2000).

Injection of ASE into eggs containing the Δ90 cyclin B1-GFP fusion protein resulted in one long series of Ca^{2+} oscillations that lasted for up to 1 hour (Fig. 4A). The sperm-triggered and the ASE-triggered Ca^{2+} oscillations normally last for approximately 25 minutes and terminate at the end of meiosis, marked by extrusion of the second polar body. These eggs containing Δ90 cyclin B1 did not extrude polar bodies (8/8) and, as had previously been shown, remained in a metaphase-like state (Levasseur and McDougall, 2000).

The repetitive Ca^{2+} waves in eggs that contained Δ90 cyclin B1 originated at the contraction pole. The ASE was injected near the centre of the egg at a random site relative to the animal pole. This triggered a Ca^{2+} wave that initiated again at the cortex (Fig. 4B), often opposite the site of injection, and not systematically at the site where the injection pipette entered the egg (this is always at 3 o'clock, see Fig. 3). This Ca^{2+} wave triggered a cortical contraction (not shown) and consequently the egg rotated (the eggs are immobilised in a wedge). Once

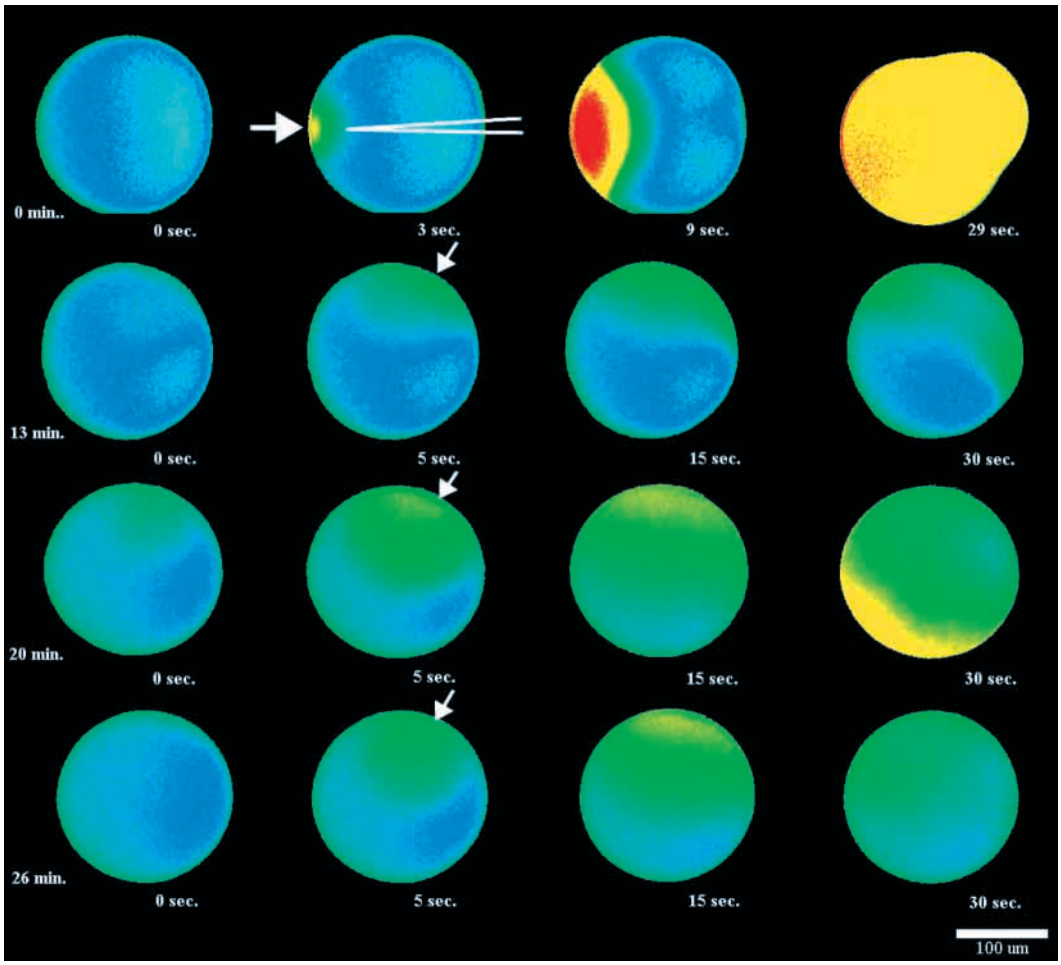

Fig. 3. ASE triggers a series of Ca^{2+} oscillations that mimic fertilization spatially. The pseudocolour images represent the ratio signal generated by the Fura-2 emission. Unfertilised denuded ascidian eggs contained approximately 20 μM Fura-2 dextran before injection of ASE. The figure shows a single egg at various times after injection. The animal pole was located at the point indicated by the large arrow. The injection pipette was introduced into the egg as indicated (although at this time the injection pipette had already been removed). Injection of ASE (0.1-0.2%) resulted in a rapid increase in Ca^{2+} levels near the site of injection (34 seconds following injection in this particular egg). About 29 seconds after the first $[Ca^{2+}]$ increase the cortical contraction is visible. Note the large bulge at 2 o'clock. The eggs are slightly wedged so occasionally rotate slightly. Once the contraction has ceased and the Ca^{2+} levels have returned to basal values the egg enters the gap phase. The $[Ca^{2+}]$ increases again 13 minutes after the first $[Ca^{2+}]$ increase. Three Ca^{2+} waves are displayed originating from the same site (small arrow). This site is far from the site of origin and was confirmed to be equivalent to the contraction pole area (n=8). Cooler colours represent basal Ca^{2+} levels (blue-green) and warmer colours (yellow to red) represent increasing Ca^{2+} levels. Temperature, 19°C.

the contraction had stopped the repetitive Ca^{2+} waves began. All subsequent Ca^{2+} waves initiated from the same pacemaker site of the egg (Fig. 4B). Analysis of the egg 1 hour after the injection of ASE revealed that the contraction pole was situated at the site where the repetitive Ca^{2+} waves were initiated (Fig. 4C). The contraction pole was not directly opposite the animal pole but was situated in the vegetal hemisphere of the egg (Fig. 4C).

ASE remains capable of triggering Ca^{2+} oscillations after chromatographic separation

Ascidian sperm extract (ASE) was prepared, fractionated and injected into unfertilised ascidian eggs in the same day. The sperm extract was prepared as detailed in the Materials and Methods section. Following a desalting step the ASE was applied to a MonoQ chromatography column and 13 fractions eluted using an increasing NaCl gradient (Fig. 5A). Fractions 8-13 were injected to determine which fraction was capable of triggering a $[Ca^{2+}]$ increase in the egg. The Ca^{2+}-induced egg deformation (see Fig. 3) was used initially as an indicator of increasing $[Ca^{2+}]$ in the egg. Table 2 displays the activity of the fractions in triggering a cortical contraction. Fraction 11 was the most potent with a delay of 40 seconds from injection to the first change in shape of the egg. Fraction 11 also triggered a rapid $[Ca^{2+}]$ increase that began approximately 10 seconds after injection (Fig. 5B). The activity predominantly fractionated as one peak, although there was residual activity remaining on the column (perhaps indicating nonspecific hydrophobic interactions). These data suggest that the active component in ascidian sperm may be a single protein factor and not multiple independent factors. The time from injection to the first detectable $[Ca^{2+}]$ increase was shorter than for ASE that had not been fractionated (approx. 2 minutes). This may indicate that the active component in fractionated ASE (fASE) is more concentrated than in ASE (the protein concentration in ASE was 7.8 mg/ml while in fASE it was 0.29 mg/ml). However, we have not yet determined the dose-response of ASE and fASE and therefore cannot yet determine the increase (if any) in specific activity. Regardless of the potency, two phases of Ca^{2+} oscillations are again stimulated by fASE (9/9). The number of Ca^{2+} oscillations triggered by fASE and by ASE is shown in Table 3. Small injections of fASE (approximately 0.1%) triggered an average of 8 second-phase Ca^{2+} oscillations and larger

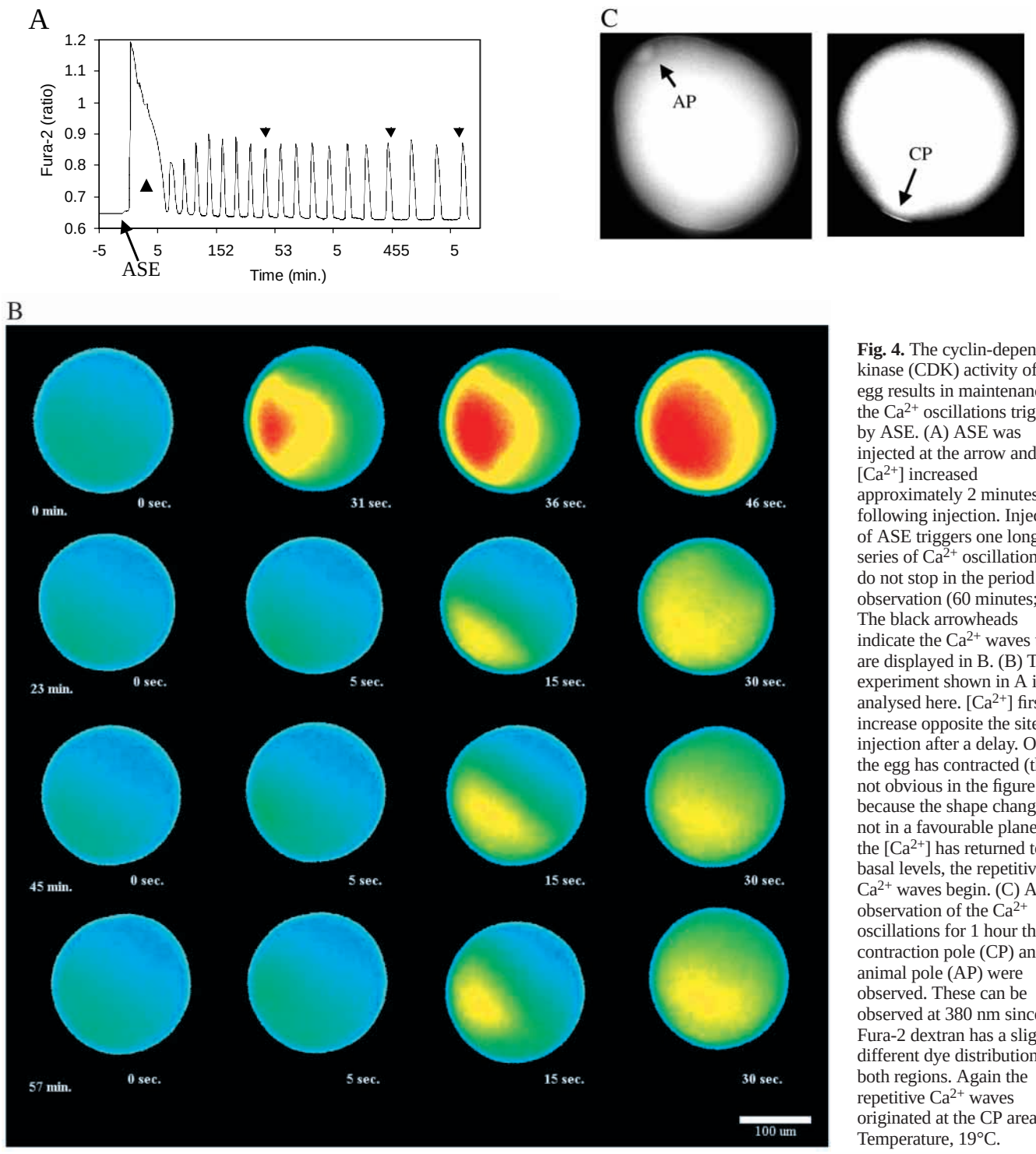

Fig. 4. The cyclin-dependent kinase (CDK) activity of the egg results in maintenance of the Ca^{2+} oscillations triggered by ASE. (A) ASE was injected at the arrow and the $[Ca^{2+}]$ increased approximately 2 minutes following injection. Injection of ASE triggers one long series of Ca^{2+} oscillations that do not stop in the period of observation (60 minutes; 8/8). The black arrowheads indicate the Ca^{2+} waves that are displayed in B. (B) The experiment shown in A is analysed here. $[Ca^{2+}]$ first increase opposite the site of injection after a delay. Once the egg has contracted (this is not obvious in the figure because the shape change was not in a favourable plane) and the $[Ca^{2+}]$ has returned to basal levels, the repetitive Ca^{2+} waves begin. (C) After observation of the Ca^{2+} oscillations for 1 hour the contraction pole (CP) and the animal pole (AP) were observed. These can be observed at 380 nm since the Fura-2 dextran has a slightly different dye distribution in both regions. Again the repetitive Ca^{2+} waves originated at the CP area. Temperature, 19°C.

Table 2. Microinjection of fractionated ascidian sperm extract into ascidian eggs

	Fraction number					
	8	9	10	11	12	13
Cortical contraction*	0/3	0/2	1/3	3/3	3/3	3/3
Approx. time from injection to contraction	NA	NA	6 minutes	40 seconds	1.3 minutes	2 minutes

Desalted ASE was applied to a MonoQ chromatography column and eluted via an NaCl gradient. Fractions obtained from the column were tested for their ability to trigger a $[Ca^{2+}]$ increase in ascidian eggs. See text for details.

*13 minutes was the standard time we waited to observe a cortical contraction.

injections of fASE (approximately 0.5%) triggered an average of 13 oscillations in the same period (Table 3). Finally, the ability of ASE to trigger Ca^{2+} oscillations was sensitive to trypsin beads (Table 3).

Table 3. Calcium oscillations triggered by ASE and fASE in *A. aspersa* eggs

	Number of oscillations	
	First phase	Second phase
ASE	2 (range 1-4, *n*=8)	8 (range 7-10, *n*=8)
Trypsin-treated ASE (approx. 0.5% ASE)	0 (*n*=3)*	0 (*n*=3)*
fASE		
Small injection (approx. 0.1%)	2 (range 1-3, *n*=4)	8 (range 6-9, *n*=4)
Large injection (approx. 0.5%)	4 (range 3-4, *n*=5)	13 (range 9-17, *n*=5)
GV oocytes (approx. 0.5% fASE)	0 (*n*=4)*	

*When the ASE or the fASE did not trigger calcium oscillations, we always performed a positive control in control metaphase I-arrested eggs from the same batch.
GV, germinal vesicle.

Regulation of fractionated ASE (fASE) by cell cycle kinases

The Ca^{2+} oscillations triggered by fASE are positively controlled by CDK activity in a way analogous to the sperm-triggered Ca^{2+} oscillations. Unfertilised eggs were injected with mRNA encoding Δ90 cyclin B1-GFP and left for 3 hours to accumulate green fluorescence. fASE injected into these eggs triggered a long-lasting series of Ca^{2+} oscillations that did not stop during a 60 minute observation (Fig. 6A, 4/4). This is similar to fertilization (Levasseur and McDougall, 2000) and ASE (Fig. 4A). Samples of eggs containing Δ90 cyclin B1-GFP were also bathed in an inhibitor of mitogen-activated protein (MAP) kinase kinase (MKK1/2) U0126 (Favata et al., 1998). This inhibitor at a concentration of 10 μM in sea water can effectively inhibit the myelin basic protein kinase activity associated with MAP kinase activity and does not affect the sperm-triggered Ca^{2+} oscillations in eggs containing Δ90 cyclin B1 (Levasseur and McDougall, 2000). Injection of fASE into eggs containing Δ90 cyclin B1-GFP bathed in U0126 triggered a long-lasting series of Ca^{2+} oscillations (Fig. 6B, 4/4). These data clearly indicate that fASE and sperm trigger Ca^{2+} oscillations that are maintained by the egg CDK activity.

Ascidian eggs can be collected at the prophase stage and as such should not have any cyclin B1-CDK activity. As

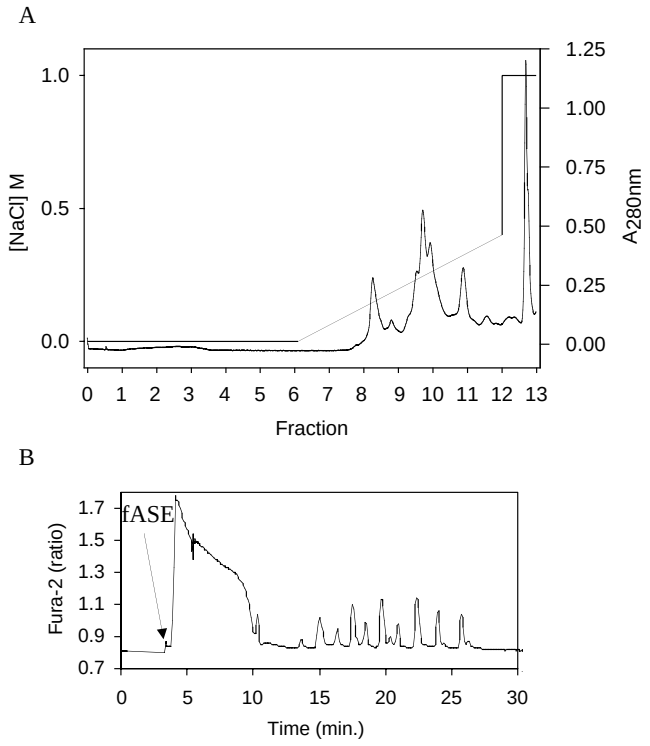

Fig. 5. Fractionation of ascidian sperm extract. (A) Ascidian sperm extract was desalted then applied to a MonoQ column at pH 8.5, eluted with an increasing gradient of NaCl and 2 ml fractions collected. Fractions 1-3 contain the material that did not bind to the column. (B) Injection of fraction 11 into an unfertilised ascidian egg (fASE at the arrow) containing approximately 20 μM Fura-2 dextran triggers two series of Ca^{2+} oscillations that terminate at the end of meiosis (9/9). The first Ca^{2+} signal is larger than that seen with ASE and is initiated sooner after injection, which may indicate a concentration of the ASE through fractionation.

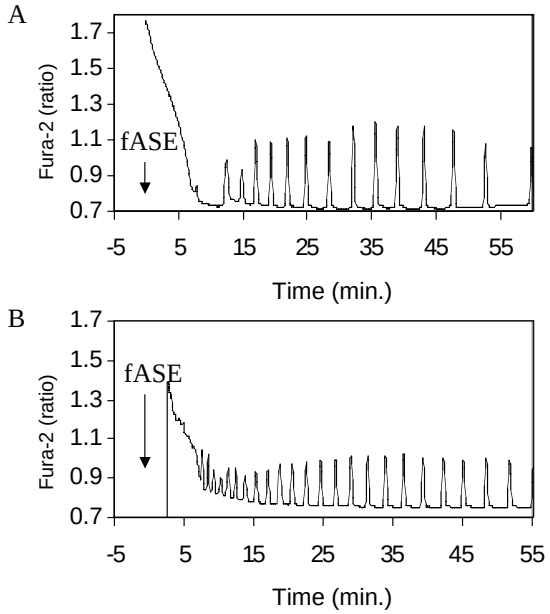

Fig. 6. Cyclin-dependent kinase (CDK) activity and not the MAP kinase activity controls the Ca^{2+} oscillations initiated by fASE. (A) Unfertilised ascidian eggs were injected with mRNA encoding Δ90 cyclin B1, left for 3 hours to produce Δ90 cyclin B1 protein (determined by fluorescence emission) and then injected with Fura-2 dextran (approximately 20 μM). fASE was then injected and the Ca^{2+} oscillations monitored. Injetion of fASE at the arrow triggers a series of Ca^{2+} oscillations that do not stop during a 60 minute observation period (4/4). (B) Batches of eggs containing Δ90 cyclin B1 and Fura 2-dextran were incubated in 10 μM U0126 for 20 minutes then injected with fASE at the arrow. The Ca^{2+} oscillations were monitored for a 60 minute period, and again the Ca^{2+} oscillations triggered by fASE did not stop during the period of observation (4/4). Temperature, 19°C.

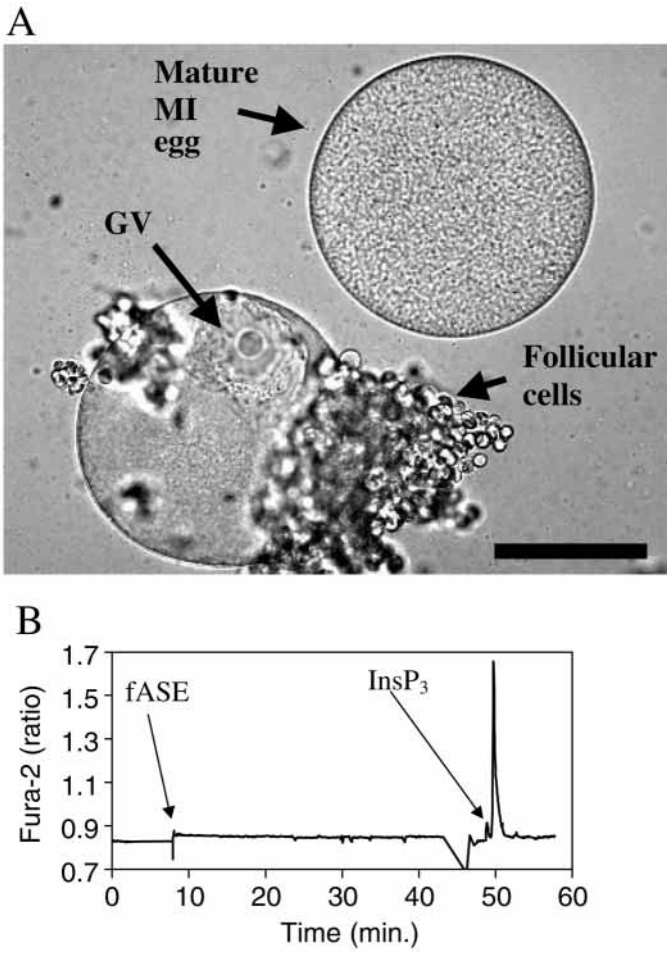

Fig. 7. fASE does not trigger Ca^{2+} release in prophase-stage oocytes. (A) A prophase-stage oocyte that was previously injected with fASE and then $InsP_3$, together with a mature egg. The nucleolus is clearly visible inside the large germinal vesicle (GV). There are some follicular cells attached to the prophase-stage oocyte. Bar, 100 μm. (B) Prophase-stage oocytes were microinjected with Fura-2 dextran (to give approximately 20 μM in the cytosol) and subsequently injected with a supramaximal dose of fASE (approx. 0.5%). This did not trigger any Ca^{2+} release in a 30 minute period of observation (4/4). Subsequent injection of $InsP_3$ did trigger a Ca^{2+} release (0.1% injection, 100 nM in the oocyte, 3/3). It was confirmed that fASE was capable of triggering Ca^{2+} oscillations in a mature egg from the same batch (data not shown). MI, metaphase I. Temperature, 19°C.

would be predicted by the above observations, injection of fASE into prophase-stage oocytes triggered no [Ca^{2+}] increases (Fig. 7, 4/4) even when the injection volume was large (0.5%), while smaller injections (0.1%) triggered Ca^{2+} oscillations in adjacent metaphase I eggs from the same batch (data not shown). In contrast to sperm extracts, the prophase-stage oocytes did release Ca^{2+} upon microinjection of the Ca^{2+}-releasing agonist inositol 1,4,5-trisphosphate (Fig. 7).

DISCUSSION

This study demonstrates that the Ca^{2+} oscillations triggered by ascidian sperm extract (ASE) in ascidian eggs mimics fertilization in three ways. (1) The ASE-triggered Ca^{2+} oscillations have the same temporal pattern as the Ca^{2+} oscillations at fertilization (confirming Kyozuka et al., 1998). (2) The Ca^{2+} oscillations triggered by ASE have the same spatial pattern as those triggered by sperm. (3) The Ca^{2+} oscillations triggered by ASE are controlled by the cyclin B1-dependent kinase (CDK) activity of the egg.

Ascidian sperm extract triggers the same spatial pattern of Ca^{2+} waves as sperm

Sperm trigger two phases of Ca^{2+} oscillations at fertilization that have a distinctive pattern in many species of ascidian (Speksnijder et al., 1989a; Russo et al., 1996; Kyozuka et al., 1998). The first Ca^{2+} wave originates at the point of sperm-egg fusion in the animal hemisphere (Speksnijder et al., 1989b) and the second phase of repetitive Ca^{2+} waves originate from a vegetal protrusion termed the contraction pole (Speksnijder, 1992). A cortical domain of endoplasmic reticulum 2-6 μm thick (Speksnijder et al., 1993) correlates with the site of this Ca^{2+} wave pacemaker (McDougall and Sardet, 1995). ASE injected randomly into the centre of an egg triggered a series of repetitive Ca^{2+} waves that began at the cortex (Fig. 3). This occurred after a delay of approximately 2 minutes, which presumably reflects the time it takes the Ca^{2+}-releasing factor to diffuse to the cortex, as noted previously (Kyozuka et al., 1998). The second phase of Ca^{2+} oscillations originated at the vegetal contraction pole. Even when the ASE was deliberately injected as far from the vegetal pole as possible the second phase of Ca^{2+} oscillations still originated at the vegetal contraction pole (Fig. 3). It is not known yet whether the ASE binds to cortical structures, but this could result in its concentration at the vegetal contraction pole during the first phase of ooplasmic segregation.

Fractionated ASE triggers two phases of Ca^{2+} oscillations

ASE was chromatographically separated on a MonoQ column and the collected fractions assayed for activity. Fractionated ASE (fASE) was assayed for activity by monitoring egg morphology following injection. An increase in the free cytosolic [Ca^{2+}] results in the contraction of an actomyosin basket (open at the animal pole) that triggers the first phase of ooplasmic segregation in ascidians (Sawada and Osanai, 1981). This actomyosin-driven cortical contraction is triggered approximately 30 seconds after the [Ca^{2+}] has first increased (Roegiers et al., 1995). Fraction 11 triggered a cortical contraction 40 seconds after injection while other fractions were either completely inactive or else had a longer delay (Table 2). Injection of fraction 11 into eggs that contained Fura-2 dextran resulted in an almost immediate [Ca^{2+}] rise (after only 10 seconds), which began at the cortex. The frequency of Ca^{2+} oscillations triggered by the fASE was dose-dependent. Larger injections of fASE triggered a higher oscillation frequency than smaller injections of fASE (Table 3). The smaller injections of fASE faithfully mimicked the sperm-triggered Ca^{2+} oscillation frequency (Table 3 and Fig. 5B). In all cases, the Ca^{2+} oscillations triggered by fASE stopped at the end of meiosis prior to formation of the second polar body (9/9).

ASE-triggered Ca^{2+} oscillations are controlled by the CDK activity of the egg

Eggs that contained elevated CDK activity and low MAP kinase activity displayed long-lasting Ca^{2+} oscillations when fertilized (Levasseur and McDougall, 2000). These data therefore led to the proposal that the CDK activity is a positive regulator of the sperm-triggered Ca^{2+} oscillations in ascidian eggs. Likewise, fractionated ASE triggered a long-lasting series of Ca^{2+} oscillations in eggs that contained elevated CDK activity and low MAP kinase activity (Figs 4A, 6A). These data are further evidence that the factor present in ascidian sperm faithfully mimics fertilization.

Sperm and sperm factor responses in oocytes, eggs and somatic cells

It seems clear from a number of studies that mammalian sperm extract can give Ca^{2+} oscillations independently of cell cycle kinase activity, e.g. DRG neurones (Currie et al., 1992), hepatocytes (Berrie et al., 1996), sea urchin homogenates (Galione et al., 1997), and prophase-stage immature mouse eggs (Wu et al., 1997). All of these would be predicted to have low MPF activity. Similarly, fertilization of prophase-stage mouse oocytes has been reported to give long-lasting Ca^{2+} oscillations (Mehlmann and Kline, 1994; Deng et al., 1998). However, it has also been suggested that sperm-induced Ca^{2+} oscillations at fertilization involve cell cycle components (Jones et al., 1995a). In keeping with this in mouse eggs, one study has shown that prophase-stage mouse oocytes do not display long-lasting Ca^{2+} oscillations at fertilization (Jones et al., 1995b). Similarly, in the nemertean worm high amplitude and sustained Ca^{2+} oscillations are normally lacking in fertilized prophase-arrested oocytes (Stricker et al., 1998). It remains to be tested whether the contradictory evidence in the mouse is due to the dose of sperm Ca^{2+}-releasing factor, since it is clear that immature mouse oocytes can become highly polyspermic (7-10 sperm; Mehlmann and Kline, 1994).

Given the conflicting observations that sperm and sperm extract can sometimes trigger long-lasting Ca^{2+} signals in immature oocytes, we tested whether ascidian sperm extract would trigger a Ca^{2+} signal(s) in immature prophase-stage ascidian oocytes. Our data revealed that ASE did not trigger a Ca^{2+} signal in prophase-stage ascidian oocytes even though the oocytes are responsive to $InsP_3$ (Fig. 7). Since even a large dose of fASE did not trigger a Ca^{2+} signal in immature ascidian oocytes it is unlikely that a single sperm would be able to do so.

It is not yet known how the ascidian oocyte cytoplasm matures to become capable of giving long-lasting Ca^{2+} oscillations, but it could involve the activation of MPF, since the Ca^{2+} oscillations triggered by ASE in ascidians are clearly maintained when CDK activity persist. We cannot yet rule out an effect of ER structure. In nemertean eggs, cortical ER clusters disperse at around the time the sperm-triggered Ca^{2+} oscillations stop and the egg exits meiosis (Stricker, 1998). ER restructuring into cortical clusters in-phase with the cell cycle also occurs during oocyte maturation in mammals (Shiraishi et al., 1995; Mehlmann et al., 1996). Recent evidence indicates that such ER clusters act as a vegetal Ca^{2+} wave pacemaker at fertilization in mouse eggs (Kline et al., 1999).

In conclusion, we have demonstrated that the ASE-triggered Ca^{2+} oscillations (1) have the same spatial characteristics as those at fertilization, and (2) are regulated by the cell cycle in the same way as those at fertilization. These observations are clearly strong evidence in favour of the sperm factor hypothesis of egg activation.

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