A novel mechanism controls the Ca^{2+} oscillations triggered by activation of ascidian eggs and has an absolute requirement for Cdk1 activity

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Summary

Fertilisation in ascidians triggers a series of periodic rises in cytosolic Ca^{2+} that are essential for release from metaphase I arrest and progression through meiosis II. These sperm-triggered Ca^{2+} oscillations are switched off at exit from meiosis II. Ascidian zygotes provided the first demonstration of the positive feedback loop whereby elevated Cdk1 activity maintained these Ca^{2+} oscillations. Since then it has been reported that Cdk1 sensitises the type I inositol trisphosphate [Ins(1,4,5)P_3] receptor in somatic cells, and that sperm-triggered Ca^{2+} oscillations in mouse zygotes stop because the forming pronuclei sequester phospholipase C zeta that was delivered to the egg by the fertilising sperm.

Here, using enucleation, we demonstrate in ascidian eggs that Ca^{2+} spiking stops at the correct time in the absence of pronuclei. Sequestration of sperm factor is therefore not involved in terminating Ca^{2+} spiking for these eggs. Instead we found that microinjection of the Cdk1 inhibitor p21 blocked Ca^{2+} spiking induced by ascidian sperm extract (ASE). However, such eggs were still capable of releasing Ca^{2+} in response to Ins(1,4,5)P_3 receptor agonists, indicating that ASE-triggered Ca^{2+} oscillations can stop even though the response to Ins(1,4,5)P_3 remained elevated. These data suggest that Cdk1 activity promotes Ins(1,4,5)P_3 production in the presence of the sperm factor, rather than sensitising the Ca^{2+} releasing machinery to Ins(1,4,5)P_3. These findings suggest a new link between this cell cycle kinase and the Ins(1,4,5)P_3 pathway.

Key words: Meiosis, Ca^{2+}, Fertilisation, Ascidian, Cdk1, Inositol trisphosphate

Introduction

The essential regulatory role played by intracellular Ca^{2+} in a wide variety of cellular events is well established (Berridge and Dupont, 1994). A good example is the rise in Ca^{2+} that occurs universally at fertilisation (Jaffe, 1980; Cuthbertson et al., 1981; Swann and Ozil, 1994). This rise may take the form of a single transient rise as seen in the jellyfish (Freeman and Ridgeway, 1993), some species of molluscs (Deguchi and Osani, 1994), sea urchins (Steinhardt et al., 1977) and frog (Busa and Nuccitelli, 1985), or as a series of transient rises or oscillations, as seen in ascidians (McDougall and Sardet, 1995; Runft and Jaffe, 2000), nemertean worms (Stricker, 1996) and mammals, e.g. mouse (Cuthbertson and Cobbold, 1985; Swann and Ozil, 1994) (reviewed by Whitaker, 2006). A correlation can be drawn between the number of Ca^{2+} rises and the stage of the meiotic cell cycle at which the eggs are arrested. As a rule, Ca^{2+} oscillations have only been observed in oocytes that are fertilised during meiotic metaphase (for a review, see Sardet et al., 1998). In these species, evidence suggests that full activation and exit from meiosis will only occur after a series of oscillations of sufficient magnitude and duration has taken place (Kline and Kline, 1992; Lawrence et al., 1998; Ozil et al., 2005).

Almost a century after Jacques Loeb suggested that "the spermatozoon carries a catalytic substance into the egg" (Loeb, 1901) it was demonstrated that sperm cytosol from hamsters contains a protein factor capable of triggering Ca^{2+} oscillations when microinjected into eggs (Swann, 1990). This sperm factor hypothesis was later confirmed in other mammalian species (Stice and Robl, 1990; Homa and Swann, 1994; Wu et al., 1997), nemerteans (Stricker, 1997) and ascidians (Kyozuka et al., 1998; McDougall et al., 2000; Runft and Jaffe, 2000). The sperm factor model is based upon sperm-egg fusion (allowing delivery of the sperm protein into the egg), and probably explained earlier observations in sea urchin eggs showing that egg activation could be blocked by preventing sperm-egg fusion; sperm-egg binding alone proving insufficient for activation (Shen and Steinhardt, 1984). Although the expectation was that a universal sperm factor would trigger the Ca^{2+} increase at fertilisation, this assumption was not supported with the identification of mammalian sperm factor in 2002 by Saunders et al. (Saunders et al., 2002). For example, the testes-specific isoform of the vertebrate sperm factor phospholipase C zeta (PLCζ) has not been found in sequenced genomes outside of the vertebrates (A.M. and K.T.J., unpublished observations). Rather there is evidence that the sperm factor in ascidians and echinoderms is most likely an activator of an Src family kinase (SFK) pathway, which at fertilisation triggers Ca^{2+} release by activation of egg-derived

PLCγ (Abassi et al., 2000; Runft et al., 2002; Giusti et al., 2003).

In both ascidians and mammals, sperm-triggered Ca^{2+} oscillations are controlled by the metaphase-like state of the zygote. Consequently it is important to determine how the egg switches off the Ca^{2+} oscillations when they have completed their task of activating the egg. In ascidians it was initially demonstrated that sperm-triggered Ca^{2+} oscillations are switched off at the precise moment that the fertilised egg exits meiosis (Speksnijder et al., 1989; McDougall and Levasseur, 1998). This correlation appears more than just a coincidence, not just in ascidians but also in mammals, since preventing meiotic exit in fertilised mouse eggs by inducing the spindle assembly checkpoint leads to persistent Ca^{2+} oscillations (Jones et al., 1995). The stimulatory effect of the metaphase-like state in maintaining Ca^{2+} oscillations was also demonstrated in fertilised ascidian zygotes injected with mRNA encoding a truncated form of cyclin B1 that prevents inactivation of Cdk1. The eggs were arrested in a metaphase-like state and Ca^{2+} oscillations persisted for as long as Cdk1 activity remained elevated (Levasseur and McDougall, 2000).

It has now been shown in somatic cells (a human T cell line) that the type I inositol trisphosphate receptor [Ins(1,4,5)P_3R] is phosphorylated on two sites by Cdk1, leading to an increase in inositol trisphosphate [Ins(1,4,5)P_3] binding (Malathi et al., 2003; Li et al., 2005). This observation may partly explain the Ca^{2+} rises that have been recorded during M phase in somatic cells (Ratan et al., 1986; Kao et al., 1990), during early embryogenesis (Groigno and Whitaker, 1998), and during meiotic M phase. However, how the production of Ins(1,4,5)P_3 is increased to trigger Ca^{2+} rises during M phase (meiotic or mitotic) is not known. One clue is that the mammalian sperm factor is active until it is sequestered by the pronuclei as the Ca^{2+} oscillations stop (Marangos et al., 2003; Larman et al., 2004). However, since PLCζ is testes-specific and confined to the vertebrates, there are likely other ways of regulating Ins(1,4,5)P_3 production during M phase (meiotic or mitotic). Here, using ascidians as a model system, we have explored the mechanism by which the Ca^{2+} signalling system is modulated during meiotic M phase. From a combined study of the specific inhibition of Cdk1 activity with p21, and flash photolysis of caged Ins(1,4,5)P_3 in activated eggs, we propose that Ins(1,4,5)P_3 production is inhibited when Cdk1 activity is abrogated. In addition, we suggest that the mechanism of regulating Ins(1,4,5)P_3 production during meiotic M phase is not dependent on pronucleus formation.

Results

Sperm-extract-triggered Ca^{2+} oscillations stop in the absence of pronuclei

We made small cytoplasts (Fig. 1A), into which ascidian sperm extract (ASE) was injected to test the hypothesis whether the fertilisation-associated Ca^{2+} oscillations stop as a result of pronuclear formation and sequestration of sperm factor into the pronuclei (Marangos et al., 2003; Larman et al., 2004). In whole eggs activated with ASE, Ca^{2+} oscillations propagate as waves (Kyozuka et al., 1998; McDougall et al., 2000). Similarly the Ca^{2+} oscillations were seen to propagate as waves in both large and small cytoplasts (Fig. 1B). The Ca^{2+}

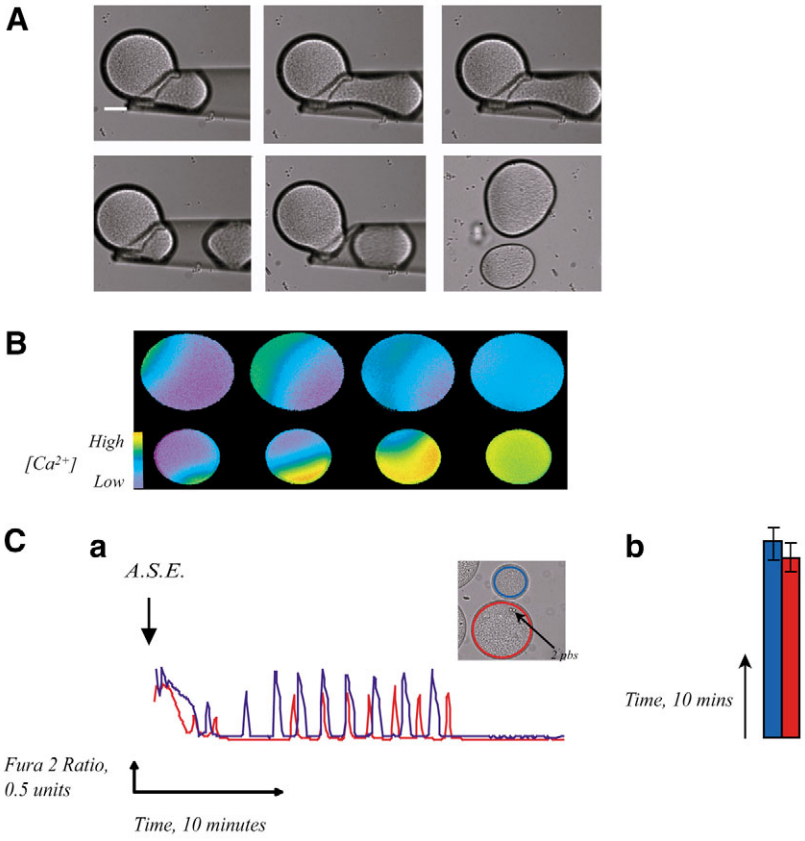

Fig. 1. ASE-triggered Ca^{2+} oscillations stop in the absence of pronuclei. (A) A series of images showing the procedure by which eggs were separated into small and large cytoplasts. See Materials and Methods for details. Bar, 50 μm. (B) Pseudocolour images showing that the Ca^{2+} oscillations progress as waves through the cytoplasm of both large and small cytoplasts. Low Ca^{2+} is indicated in magenta, higher levels in blue and highest by yellow, 10-second time lapse between images in upper panel, 5 seconds in the lower panel. (Ca) The ASE-induced Ca^{2+} oscillations stop in both cytoplasts simultaneously . Each cytoplast was injected with ASE within 30 seconds of each other. The traces from both cytoplasts show the oscillations stop in the smaller (blue line) at approximately the same time as the larger (red line) which extruded two polar bodies, evidence that this cytoplast contained the chromatin. (b) Mean time (± s.e.m.) at which the oscillations stop in cytoplasts with (red bar) and without (blue bar) chromatin (P=0.5, n=10, nine animals).

oscillations stopped in cytoplasts at the same time as they did in the (chromatin containing) karyoplast (Fig. 1C). In these experiments extrusion of polar bodies was taken as confirmation of the presence of chromatin since Hoechst staining was not used.

The above experiment suggests that in ascidian eggs, chromatin, and hence ultimately pronuclei, play little part in the cessation of Ca^{2+} oscillations. However, additional proof was needed that the termination of the Ca^{2+} oscillations following injection of ASE into cytoplasts was not a consequence of the small size of the cytoplast or the manipulation they had endured during their creation. We therefore made a second series of cytoplasts; eggs previously stained with Hoechst to reveal the location of the chromatin were enucleated leaving a very large cytoplast (Fig. 2A). We then injected ASE into these large cytoplasts and found that the Ca^{2+} oscillations again stopped at the same time as control, nucleated eggs (Fig. 2Ba). Furthermore, it was possible to induce long-lasting Ca^{2+} oscillations in these large cytoplasts by first injecting Δ90-cyclinB1::GFP protein to maintain high Cdk1 activity (Fig. 2Bb). This result suggested that the cytoplasts have a sufficiently intact Ca^{2+} homeostatic machinery to display long-lasting Ca^{2+} rises.

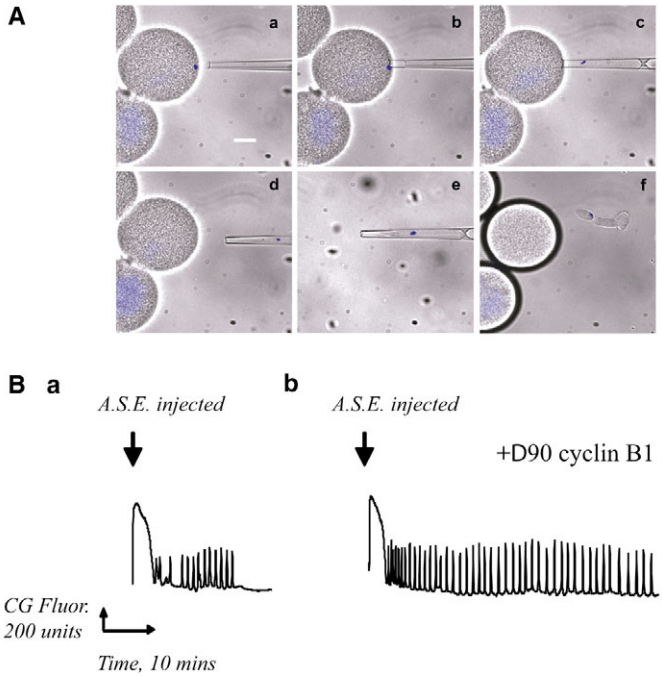

Fig. 2. ASE-triggered Ca^{2+} oscillations in enucleated eggs mimic those induced by sperm in eggs. (A) A second series of large enucleated cytoplasts was made. A large bore micropipette was advanced using a hydraulic manipulator to the edge of the egg where the chromatin was positioned (a). Suction was then applied by mouth aspirator such that a portion of the egg started to enter the pipette (b). Suction was stopped as soon as the chromatin had been removed (c), the pipette withdrawn from the egg (d,e), and the fragment expelled under gentle pressure to leave an enucleated egg and a small intact egg fragment containing the chromatin (f).Chromatin (in blue) was stained with Hoechst, and the images overlaid on a series of brightfield images. Bar, 50 μm. (Ba) ASE was injected into these large cytoplasts and the time at which the Ca^{2+} oscillations stopped [23.3±1.46 minutes (mean ± s.e.m.) n=9, three animals] was not significantly different from the mean of the times recorded for control, unmanipulated eggs (23.09±1.42 minutes, P=0.91). (b) Confirmation that these large cytoplasts could oscillate for an extended period of time. The cytoplast was first injected with Δ90 cyclin B1::GFP protein, and the Ca^{2+} oscillations persisted throughout the entire period they were observed (n=6, two animals).

Inhibiting Cdk1 activity inhibits the second series of Ca^{2+} oscillations

The above experiments appear to rule out a role for pronuclei in the termination of fertilisation-associated Ca^{2+} oscillations in ascidians, so we set out to investigate the role played by Cdk1 activity in regulating this process. In order to do this we used p21, a Cdk inhibitor (Xiong et al., 1993), which we have previously shown to be an effective inhibitor of Cdk1 activity and not to inhibit MAPK activity in ascidian eggs (Levasseur and McDougall, 2003).

Eggs injected with an inhibitory concentration of p21 (fused to GFP for quantification purposes) were then further injected with ASE. In these eggs the second phase Ca^{2+} oscillations were completely absent, whereas in control eggs injected only with ASE and imaged simultaneously the pattern of Ca^{2+} oscillations was normal (Fig. 3). This suggested a role for Cdk1 activity in generating second phase Ca^{2+} oscillations.

Prolonged Δ90 cyclin B1-mediated oscillations are terminated by the addition of p21

Finally, to obtain further evidence that Cdk1 activity is necessary for second phase Ca^{2+} oscillations, we set out to test the hypothesis that long-lasting Δ90-cyclin B1-mediated oscillations would stop if the Δ90-dependent Cdk1 activity was inhibited. Pairs of eggs were injected with Δ90 cyclin B1::GFP protein and Ca^{2+} oscillations were triggered by injection of ASE. Once it was clear that both eggs were giving prolonged oscillations, one egg was then injected with p21::GFP protein (the Δ90 cyclin B::GFP protein was present at such a concentration as to not interfere with quantification of p21). The oscillations stopped promptly, within 10 minutes, in the p21 injected egg (Fig. 4).

This, taken together with the previous data, suggests that Cdk1 activity is both necessary and sufficient for the generation of second phase Ca^{2+} oscillations. We next hypothesised that Cdk1 may regulate the oscillations in at least one of two ways; either by regulating Ins(1,4,5)P_3 generation, or by controlling the responsiveness of the Ca^{2+} releasing machinery, or possibly even both (see Discussion).

Low Cdk1 activity does not cause Ins(1,4,5)P_3 responsiveness to decline

We thus sought to test the hypothesis that Cdk1 activity is responsible for modulating the generation of Ins(1,4,5)P_3 that is necessary for the second phase Ca^{2+} oscillations. In order to do this we needed to eliminate the other possible mechanism, namely that Cdk1 regulates responsiveness of the Ca^{2+} releasing machinery. Thus we set out to test whether, by inhibiting Cdk1 activity, we could detect a decline in responsiveness to Ins(1,4,5)P_3. This was performed in two different ways.

Firstly we used the potent non-hydrolysable Ins(1,4,5)P_3

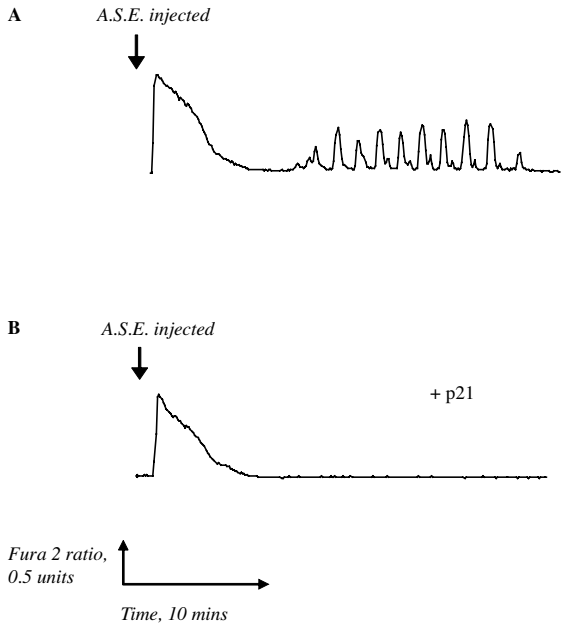

Fig. 3. Inhibition of Cdk1 activity inhibits the second phase of Ca^{2+} oscillations. (A) Normal pattern of Ca^{2+} oscillations after injection with ASE. (B) When p21::GFP protein was injected to inhibitory levels, injection of ASE resulted in only a single Ca^{2+} rise. Data in A and B are from eggs imaged simultaneously. Arrows indicate time of ASE injection and data is representative of every experiment (*n*=26, 16 animals).

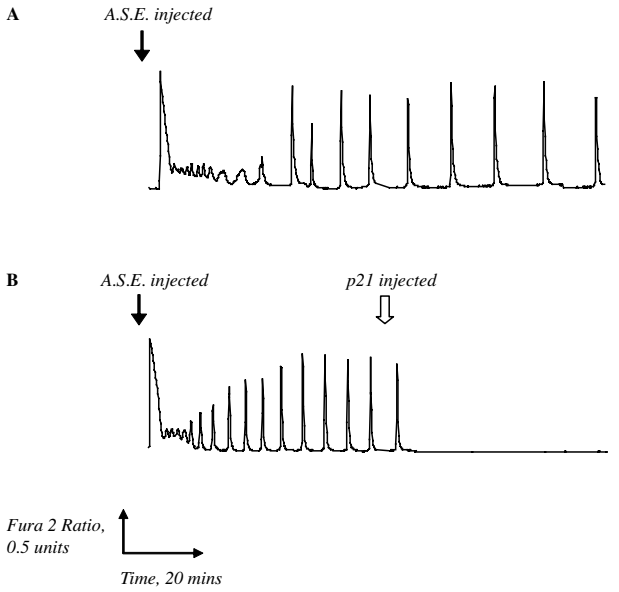

Fig. 4. Long lasting Δ90 cyclin B1 mediated oscillations stop rapidly when Cdk1 activity is inhibited. (A) Long lasting oscillations in an egg injected with Δ90 cyclin B1:: GFP protein and then ASE. (B) Rapid termination of oscillations when an egg previously injected with Δ90 cyclin B::GFP and ASE is then injected with p21::GFP protein. Black arrows indicate time of ASE injection, open arrow the time of p21::GFP injection. Data in A and B are from eggs imaged simultaneously. Time of termination was 8.33±0.58 minutes (mean ± s.e.m.) (*n*=8, four animals).

analogue, adenophostin A. The adenophostin A concentration was titrated to the lowest level needed to give repeated Ca^{2+} oscillations (Fig. 5A). This was found to be at a final egg concentration of approximately 0.25 μM. This gave one large initial Ca^{2+} rise followed by four or five oscillations. A final concentration of 0.1 μM gave the same large initial rise but only one or two very small oscillations, whereas concentrations up to 2 μM gave the same pattern of oscillations seen with 0.25 μM. Eggs were then injected with an inhibitory concentration of p21::GFP and activated by injection with ASE. Injection of adenophostin A to 0.25 μM during the period when the egg would normally have been producing second phase oscillations gave a large Ca^{2+} rise and the four or five oscillations seen during the titration experiment (Fig. 5Ba). Therefore, inhibition of Cdk1 activity did not result in a decline in responsiveness to Ins(1,4,5)P_3. As a control to show that this dose of adenophostin is not supramaximal, the same amount was injected into an egg after meiotic exit. This resulted in only a single Ca^{2+} rise with no subsequent oscillations (Fig. 5Bb), evidence that this dose of adenophostin is within the range that eggs naturally desensitise at meiotic exit.

Next, instead of adenophostin injection providing the exogenous source of agonist, we used caged gPtdIns(4,5)P_2, a slowly hydrolysable analogue of Ins(1,4,5)P_3, discrete doses of which could be delivered by ultraviolet (UV) uncaging. These experiments also allowed direct comparison of responsiveness between control eggs and eggs with low Cdk1 activity. Pairs of eggs were coinjected with caged gPtdIns(4,5)P_2 and the Ca^{2+} indicator Ca^{2+} Green (Molecular Probes Invitrogen), and then one egg was further injected with an inhibitory concentration of p21::HcRed protein (the red fusion variant was used so as not to interfere with the Ca^{2+} Green signal). Flashes of UV light of increasing duration were then applied to discover the threshold needed to give a measurable Ca^{2+} signal in the eggs (Fig. 6, flash no. 1). This was then the flash duration used throughout the rest of the experiment. Both eggs were then activated synchronously by injection of ASE, and once the second phase oscillations had commenced in the control egg, UV-light flashes were administered in between each control second phase oscillation. The result was that each flash elicited a response of similar (or even slightly larger) magnitude in the p21-injected egg compared to the control (Fig. 6, flashes 2-6). (We did note, however, that control eggs displayed a shoulder as the Ca^{2+} level approached basal levels whereas the p21-injected eggs did not display the shoulder.) This was taken as further evidence that lowering the Cdk1 activity does not result in a decline in responsiveness of the Ins(1,4,5)P_3R. As a control to show that this duration of flash was not releasing supramaximal amounts of gPtdIns(4,5)P_2, and thereby eliciting a false response, we gave two further flashes after the second phase oscillations had stopped in the control. These flashes caused no response in the now desensitised control egg (Fig. 6, flashes seven and eight), showing that the dose of gPtdIns(4,5)P_2 released was within the range of desensitisation that occurs at meiotic exit. A supramaximal dose would have caused a Ca^{2+} release in the control after meiotic exit.

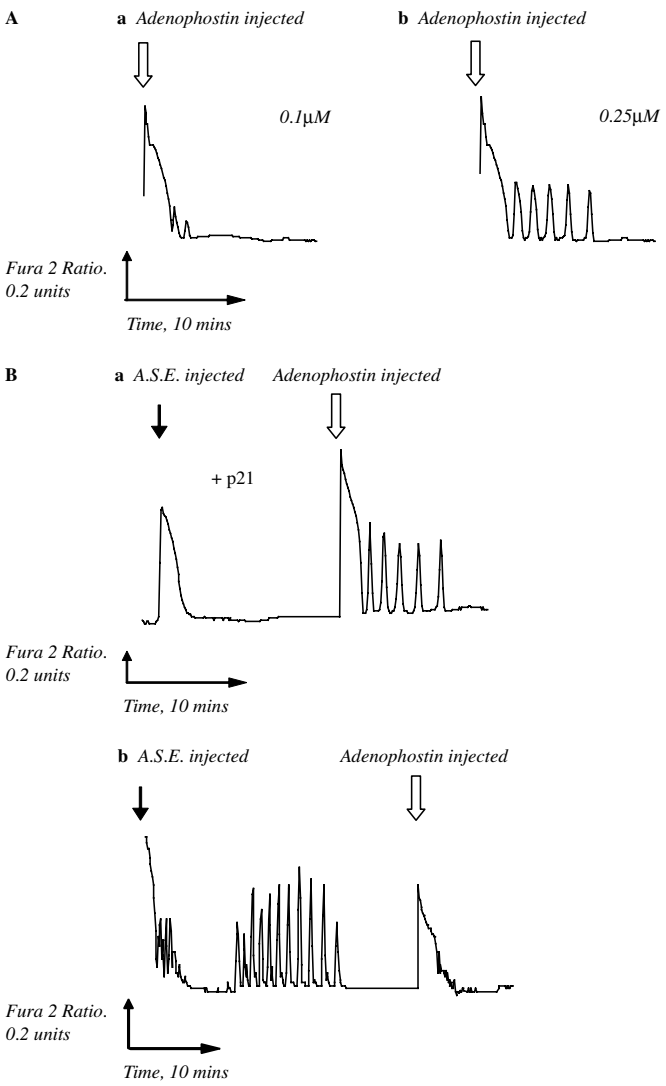

Fig. 5. Inhibition of Cdk1 activity does not decrease responsiveness to Ins(1,4,5)P_3. (A) Determination of the threshold concentration of the potent Ins(1,4,5)P_3 analogue adenophostin A required to give Ca^{2+} oscillations. (a) Injection of adenophostin A to a final concentration of approximately 0.1 μM gave a large Ca^{2+} rise and one (n=1, one animal) or two (n=2, two animals) small subsequent increases. (b) Injection of adenophostin A to a final concentration of approximately 0.25 μM gave a large initial Ca^{2+} rise that was always followed by at least four (n=2, two animals) or five (n=3, two animals) Ca^{2+} transients. Injection to higher final concentrations gave the same pattern of Ca^{2+} rises. 0.25 μM was thus used as the threshold concentration required to elicit oscillations. (Ba) Eggs remain responsive to this dose of adenophostin A when Cdk1 activity is low and they do not give second phase oscillations. Eggs were injected with p21::GFP protein, activated with ASE (black arrow) and then when an adjacent control egg (no p21) injected with ASE at approximately the same time was giving second phase oscillations, adenophostin A was injected (open arrow) into the p21-containing egg to a final concentration of 0.25 μM producing a large Ca^{2+} rise followed by several oscillations (four oscillations n=1, 5 oscillations, n=2, three animals). (Bb) Eggs that had exited meiosis did not respond to this dose of adenophostin A. Following injection of ASE (black arrow) and after allowing a full series of Ca^{2+} oscillations to proceed, as well as extrusion of both polar bodies, the egg was then injected with the same dose of adenophostin used in Bi (open arrow). In all cases this resulted in a single large rise in Ca^{2+} with no subsequent Ca^{2+} oscillations (n=6, three animals).

Discussion

The pronucleus plays no part in the temporal regulation of the sperm-triggered Ca^{2+} oscillations in the ascidian

In mammals elegant experiments indicate that sperm-triggered Ca^{2+} oscillations may be switched off by the sequestration of PLCζ into the pronuclei (Jones et al., 1995; Kono et al., 1995; Marangos et al., 2003; Larman et al., 2004). This model is unlikely to explain the pattern of the oscillations in ascidians, where the oscillations stop between meiosis I and meiosis II without formation of pronuclei (McDougall and Levasseur, 1998). However, since only the second phase of Ca^{2+} oscillations are inhibited by Cdk1 inhibition (Levasseur and McDougall, 2000), only the second phase of Ca^{2+} oscillations are associated with the metaphase-like state. Thus, the pause between the first and second phases of oscillations may be misleading. To understand how the sperm-triggered Ca^{2+} oscillations are controlled it is therefore important to focus only on the second phase of Ca^{2+} oscillations. Since these start during meiosis II and end minutes before formation of the pronucleus it is possible that they are switched off by a mechanism requiring pronuclear formation, as in mammals (Marangos et al., 2003; Larman et al., 2004).

We therefore decided to test whether the sequestration model is conserved in ascidian eggs, and to do this we developed a technique for producing two separate egg fragments from a single egg before activation; a karyoplast, which retains the nuclear material, and a cytoplast lacking the nuclear material and hence unable to form a pronucleus (Fig. 1A). We found that, as in whole eggs, the oscillations propagated as waves (Fig. 1B), and stopped at the expected time in both cytoplasts and karyoplasts (Fig. 1C).

A similar study in mouse eggs, in which one-cell embryos were bisected to produce nucleate and anucleate halves, showed oscillations stopped at approximately the same time in each half (Day et al., 2000). However, the difficulty in producing nucleate and anucleate halves during fertilisation without perturbing the ability of the cell to regulate intracellular Ca^{2+} has been raised (Marangos et al., 2003).

The bisection process we used (Fig. 1B) consisted of a simple aspiration technique which involved neither cytochalasin treatment nor segmentation by a fine needle. Nevertheless, we went on to develop an even more gentle bisection technique (Fig. 2A) whereby we were able to show that in large enucleated eggs the Ca^{2+} oscillations stopped at the expected time (Fig. 2Ba) and, moreover, that these cytoplasts were still capable of giving prolonged oscillations when Cdk1 activity remained elevated due to the presence of Δ90 cyclin B1 (Fig. 2Bb). These observations demonstrate that enucleated eggs behave indistinguishably from nucleated eggs in their physiological response to ASE.

We therefore conclude from these data that formation of pronuclei plays no essential role in the temporal regulation of the Ca^{2+} oscillations in ASE-activated ascidian eggs.

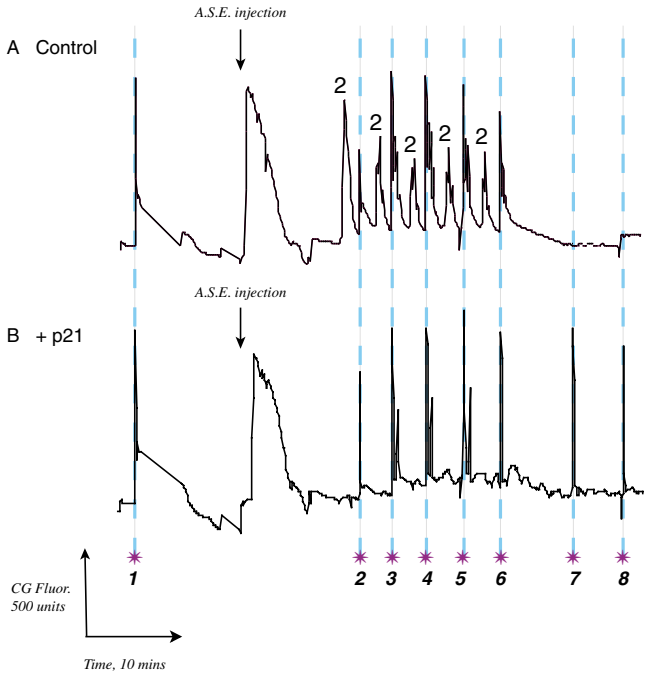

Fig. 6. Inhibition of Cdk1 activity does not decrease responsiveness to Ins(1,4,5)P_3. Pairs of eggs, A and B, were co-injected with 2 mM Ca^{2+} Green and 3.5 mM caged gPtdIns(4,5)P_2 [a slowly hydrolysable Ins(1,4,5)P_3 analogue] to give an intracellular concentration of approximately 20 μM Ca^{2+} Green and 35 μM gPtdIns(4,5)P_2, and Ca^{2+} increases were measured simultaneously. Flashes of UV light (purple stars) were administered in increasing duration until a significant rise in Ca^{2+} release was observed (flash number 1). This flash duration (750 milliseconds) was the duration used for all further flashes. (A) Control egg with no p21. (B) An egg injected with p21::HcRed protein (the red fluorescent variant was used to avoid interfering with the Ca^{2+} Green signal). Each egg was injected with sperm extract and once the control had started to give second phase oscillations, UV-light flashes (numbered 2-6) were delivered in between oscillations in the control (indicated by white 2 on black background symbols). The pale blue dashed lines emphasise synchronicity of each UV-light-flash-induced Ca^{2+} release. After the second phase oscillations had stopped in the control and a second polar body had been extruded, two further flashes (numbers 7 and 8) were given (n=7, six animals).

Cdk1 activity is both necessary and sufficient for the generation of second phase Ca^{2+} oscillations

We have previously shown that there is a close correlation between Cdk1 activity and sperm-triggered Ca^{2+} oscillations in the ascidian (McDougall and Levasseur, 1998), and that maintaining Cdk1 activity prolongs the oscillations, whereas the second phase oscillations are absent when Cdk1 activity is inhibited using the pharmacological inhibitor roscovitine (Levasseur and McDougall, 2000). However, although roscovitine has also been shown to abolish all but the first Ca^{2+} oscillation in fertilised mouse eggs (Deng and Shen, 2000), it has inhibitory effects on other Ca^{2+} channels (Yan et al., 2002), leading us to question the specificity of this pharmacological inhibitor.

Here instead of roscovitine we used the specific Cdk inhibitor p21, and showed that there were no second phase Ca^{2+} oscillations in eggs activated by ASE injection when Cdk1 activity is inhibited with p21 (Fig. 3). The results agree with those achieved using roscovitine (Levasseur and McDougall, 2000), in that the first Ca^{2+} rise is unaffected.

When p21 was injected into eggs where Cdk1 activity had been kept elevated with Δ90 cyclin B1, the prolonged oscillations rapidly ceased (Fig. 4). This corroborates our previous findings that Cdk1 activity positively regulates Ca^{2+} oscillations (Levasseur and McDougall, 2000). These data indicate that Cdk1 activity is both necessary and sufficient for second phase ASE-induced Ca^{2+} oscillations.

How does Cdk1 activity positively regulate the second phase Ca^{2+} oscillations?

Having provided evidence of the necessity for Cdk1 activity in the generation of second phase Ca^{2+} oscillations, we next addressed the mechanism by which Cdk1 controls these oscillations. We tested two possibilities; the first is that Cdk1 could regulate the responsiveness of the Ins(1,4,5)P_3Rs to Ins(1,4,5)P_3. The second is that Cdk1 activity modulates, directly or indirectly, Ins(1,4,5)P_3 production by a sperm-derived protein, which is not PLCζ, but more likely an SFK regulator in the ascidian (see below for our model).

The ability of eggs to respond to injection of adenophostin (Fig. 5) and uncaging of gPtdIns(4,5)P_2 (Fig. 6) when ASE-induced Ca^{2+} spiking was inhibited by p21 strongly suggests that inhibition of Cdk1 activity does not result in a decline in Ins(1,4,5)P_3 responsiveness, indicating that Cdk1 activity most likely plays a positive regulatory role in the generation of Ins(1,4,5)P_3.

Regulation of sperm-triggered Ca^{2+} oscillations in other species

In species other than the ascidian, one possibility is that Cdk1 activity regulates responsiveness of the Ins(1,4,5)P_3R. Evidence for this comes from somatic cells where the type I Ins(1,4,5)P_3R was demonstrated to be a substrate of Cdk1 and that the phosphorylation sites are conserved from human to *Drosophila* (Malathi et al., 2003), although interestingly this conservation does not extend to the ascidian (Fig. 7). However, in vertebrate eggs it is more likely that MAPK activity is involved in the control of sperm-triggered Ca^{2+} oscillations (Lee et al., 2006). These data are important in light of the finding that the response of the Ins(1,4,5)P_3R declines when the MAPK activity falls in activated ascidian (McDougall and Levasseur, 1998) and mouse eggs (Jellerette et al., 2004). However, there does not always appear to be a causal relationship between the termination of the Ca^{2+} oscillations and the fall in responsiveness of the Ins(1,4,5)P_3R. For example, the fall in responsiveness of the Ins(1,4,5)P_3R occurs after the oscillations terminate in the ascidian (Levasseur and McDougall, 2003), whereas the fall in responsiveness in the mouse occurs at the time the oscillations are switched off (Jellerette et al., 2004). It is more likely that the Ca^{2+} oscillations terminate because the production of Ins(1,4,5)P_3 is switched off as the zygote exits the metaphase-like state.

An elegant mechanism for switching off production of Ins(1,4,5)P_3 has been proposed in the mouse (Marangos et al., 2003). The sperm factor PLCζ has a bipartite nuclear localisation signal and when nuclear localisation signal (NLS)-truncated PLCζ cRNA is injected into unfertilised oocytes it

Ser421 Phosphorylation Site:

Human IP3R1	**M L K I G T S* P V K E D K**
Mouse IP3R1	**M L K I G T S* P V K E D K**
Xenopus IP3R1	**M L K I G T S* P V K E D K**
Drosophila IP3R1	**M S M V C C S* P I K E D K**
Ciona IP3R1	**M L K I G T C K Y K E D K**

Thr799 Phosphorylation Site:

Human IP3R1	**P Q E Q V T* P V K Y A R L**
Mouse IP3R1	**P Q E Q V T* P V K Y A R L**
Xenopus IP3R1	**P Q E Q V T* P V K Y A R L**
Drosophila IP3R1	**P Q E P V T* P V K Y A R L**
Ciona IP3R1	**P Q E P V I P V Q Y A R L**

Fig. 7. The ascidian (*Ciona*) type I Ins(1,4,5)P_3R (IP_3R1) sequence has diverged at the Cdk1 phosphorylation sites, which are conserved in *Drosophila*, *Xenopus* and human. Sequences are aligned, with differences outside the consensus S/T P motif shown in blue and differences in the consensus itself shown in red. Human mouse and *Xenopus* alignment is from Malathi et al. (Malathi et al., 2003). GenBank accession number for *Drosophila* is A43360, and the *Ciona* sequence was obtained from Nori Satoh's *Ciona* genome database, accession number ci0100150586.

triggers a series of Ca^{2+} oscillations that do not terminate (Larman et al., 2004), although this result was not seen in recent work in which point mutation in the NLS of PLCζ abolished pronuclear localisation but induced Ca^{2+} oscillations, which stopped prior to formation of the pronucleus (Kuroda et al., 2006). Further evidence for a role of the pronuclei comes from the finding that fertilised eggs injected with either WGA to prevent pronuclear formation, or with a dominant negative importin B to inhibit pronuclear localisation, display Ca^{2+} oscillations that persist after the time of pronuclear formation and the decline of both MAPK and Cdk1 activity (Marangos et al., 2003). Although there appears to be a growing body of evidence in support of the nuclear localisation hypothesis some discrepancies persist. First, zygotes that lack nuclei should not be able to switch off Ca^{2+} oscillations. However, one report indicates that the Ca^{2+} oscillations stop in nucleus-free cytoplasts at the same time as in control halves (Day et al., 2000). The bisection itself has been proposed as an explanation of the perturbation of the Ca^{2+} signalling machinery in these cytoplasts, but it has not yet been tested. Second, PLCζ should enter the nuclei before the Ca^{2+} oscillations stop. This is sometimes but not always the case: PLCζ was found to enter the nuclei both before and after the oscillations terminate (Yoda et al., 2004; Sone et al., 2005). However, even with these caveats, the observations that the Ca^{2+} oscillations are not stopped by blocking nuclear import of native proteins following fertilisation support the nuclear sequestration hypothesis in the mouse, although it is possible that other mechanisms, such as Ins(1,4,5)P_3R desensitisation, play some role.

Another role has been assigned for Cdk1 activity in nemertean worms, where it has been proposed to transform the endoplasmic reticulum (ER; the major cytosolic store of Ca^{2+}) into clusters, and consequently increases the capacity to produce normal Ca^{2+} oscillations, However, the ability to generate Ca^{2+} oscillations in the absence of ER clusters led the authors themselves to question a direct correlation between ER structure and oscillatory behaviour (Stricker and Smyth, 2003). Cdk1 has also been shown to be involved in regulating ER clustering in mouse eggs, but the same study also shows that there is no correlation between the time the clusters disperse and when the Ca^{2+} oscillations stop (Fitzharris et al., 2003). Although it is well known that there is ER-rich localisation in the ascidian egg (Speksnijder et al., 1993; Roegiers et al., 1995) we chose not to investigate a link between Cdk1 activity and ER structure as we report here that ascidian eggs show no decline in Ins(1,4,5)P_3 responsiveness when Cdk1 activity is inhibited. This would appear to rule out any significant disruption of ER structure due to low Cdk1 activity.

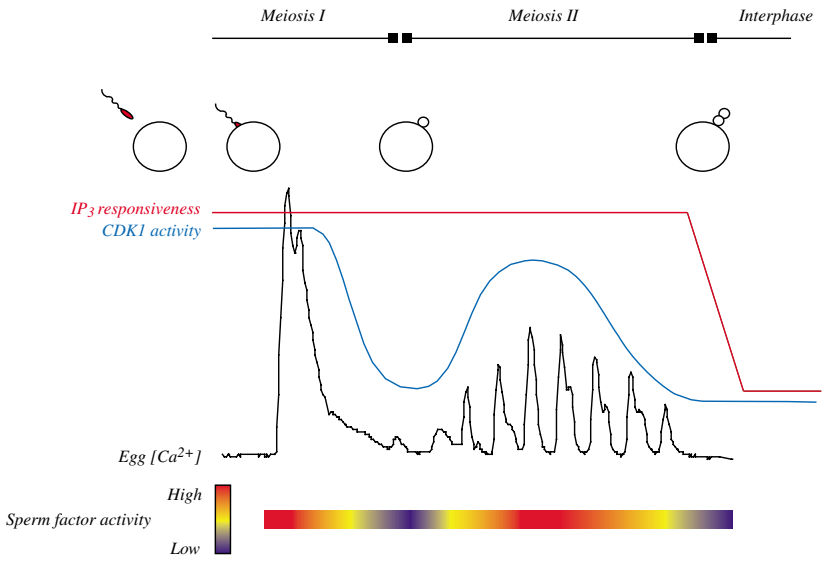

Fig. 8. The model we propose for control of the second phase Ca^{2+} oscillations by Cdk1 activity in the ascidan. (Top) Sperm-egg fusion and extrusion of first and second polar bodies are shown diagrammatically to indicate relative times of fertilisation, transition from meiosis I to meiosis II and meiotic exit into interphase. The sperm head, coloured in red, depicts the sperm arriving to deliver fully active sperm factor to the egg. (Bottom) A typical pattern of fertilisation-triggered Ca^{2+} oscillations is shown by the black line. Previously determined Cdk1 activity (McDougall and Levasseur, 1998) is indicated by the blue line, and Ins(1,4,5)P_3 responsiveness (Levasseur and McDougall, 2003) by the red line. Hypothetical sperm factor activity is indicated by the pseudocoloured bar, and correlates with Cdk1 activity.

A model for the regulation of sperm-triggered Ca^{2+} oscillations in the ascidian

We demonstrated in two different ways that in the absence of Cdk1 activity Ins(1,4,5)P_3 responsiveness does not decline, and therefore we conclude that Cdk1 controls the oscillations by positively regulating Ins(1,4,5)P_3 generation. This model is consistent with our previous observations that responsiveness does not decline when the oscillations pause and Cdk1 activity is low (McDougall and Levasseur, 1998), and also that responsiveness only declines *after* meiotic exit, when the oscillations have already stopped (Levasseur and McDougall, 2003). We have also shown here and previously (Levasseur and McDougall, 2000) that the first sperm-induced Ca^{2+} signal is Cdk1 independent. This is consistent with observations that there is a single Ca^{2+} rise in species that are fertilised when Cdk1 activity is low, i.e. either before or after meiotic entry (Stricker, 1999).

We therefore propose the following model (Fig. 8). Following fertilisation, the ascidian sperm factor is delivered into the egg with active Ca^{2+}-releasing activity, resulting in the first phase of Ca^{2+} oscillations. The sperm factor is then inactivated by an unknown egg-derived mechanism such that the Ca^{2+} oscillations pause, and it is the rise in Cdk1 activity following the metaphase-anaphase transition of meiosis I that restores the Ca^{2+}-releasing activity of the sperm factor, resulting in the second phase Ca^{2+} oscillations. The decline in Cdk1 activity prior to meiotic exit results in inactivation of the sperm factor and consequently the oscillations cease. We hypothesise that Cdk1 modulates sperm factor activity directly by phosphorylation. Indeed the closest match in the ascidian to SpSFK1, the SFK known to cause the fertilisation Ca^{2+} rise in the sea urchin (Giusti et al., 2003), has two consensus Cdk1 phosphorylation sites. Interestingly, these sites are not conserved in SpSFK1, but this is still consistent with our model, in that sea urchin eggs are arrested in interphase, and a single Ca^{2+} rise at fertilisation is sufficient for entry into first mitosis.

Finally, these findings may be of significance to how other non-PLCζ-derived Ca^{2+} signals are regulated in the mitotic cell cycle, for example, those that have been observed to occur at anaphase in sea urchin embryos (Groigno and Whitaker, 1998) and the G2-M transition in HeLa cells (Patel et al., 1999).

Materials and Methods

Biological material

Specimens of the two species of ascidian used in this study, *Ascidiella aspersa* and *Phallusia mammillata*, were collected on the North East coast of England and the Mediterranean coast of France, respectively. Animals were kept for several weeks in the laboratory in a tank of sea water at 10°C. Oocytes were harvested and prepared for microinjection and microscopy as described previously (Keihart, 1982).

Plasmid constructs and fusion protein purification

Construction and synthesis of mRNA from the Δ90 cyclin B1::GFP plasmid has been described previously (Levasseur and McDougall, 2000), as has the construction and purification of fusion protein from the p21::GFP plasmid (Levasseur and McDougall, 2003). Here we also made a red fluorescent p21 fusion, p21::HcRed. The plasmid containing HcRed, pHcRed 1-1, was purchased from BD Biosciences or Clontech, and the HcRed gene was then subcloned, in frame, next to p21 in the GST purification vector pGEX 6P-1 (Amersham Pharmacia Biotech) using a PCR-based approach. Fusion protein was then purified according to the kit protocol and the purified protein was buffer exchanged into PBS and concentrated to approximately 10 mg/ml using Microcon YM 30 spin concentrators (Millipore, UK). Δ90cyclin B::GFP fusion protein was also subcloned, expressed and purified in the same way to a final concentration of approximately 1 mg/ml. Aliquots were flash frozen in liquid nitrogen and stored at –70°C.

Microinjection and fluorescent measurement of Ca^{2+}, GFP, HcRED and DNA

Oocytes were mounted in a wedge based on the design of Keihart (Keihart, 1982) and microinjected as described previously (McDougall and Levasseur, 1998). Intracellular Ca^{2+} levels were measured by injecting either Fura 2 or Ca^{2+} Green to final concentrations of 20 and 50 μM respectively. Caged gPtdIns(4,5)P_2 (Calbiochem) was injected to a final concentration of 35 μM, a previously reported effective concentration (Dumollard and Sardet, 2001). p21 fusion proteins were injected to a final concentration equal to or greater than 1.6 μM, which we have previously shown to be the lowest concentration needed for effective inhibition of Cdk1 activity (Levasseur and McDougall, 2003). Concentration was determined by referring to a calibration curve of fluorescence against known amounts of injected recombinant EGFP (BD Biosciences or Clontech) or HcRED (purified by GST affinity chromatography as above), details of which we have previously reported (Levasseur and McDougall, 2000).

Imaging was performed using an Olympus IX70 microscope set up for epifluorescence. A xenon lamp (150 W; Opti Quip, Highland Mills, NY) provided the light source, which was band-pass filtered through 350 nm, 365 nm, 380 nm, 488 nm and 565 nm filters depending on the fluorophore or chromophore being excited. A triple pass dichroic/emission filter set (Chroma; set 6100 for use with DAPI, FITC and TRITC) directed light to the specimen and then to the collecting device. This was a charge-coupled device (CCD) camera (MicroMax, Sony Interline chip, Princeton Instruments, Trenton, NJ), operated at –10°C. Data collected was analysed by MetaFluor and MetaMorph software packages (Universal Imaging, Marlow, UK). Microinjection was done with the aid of a 10× objective lens, fluorescence imaging and Ca^{2+} measurement with a 20× objective, and chromatin imaging with a 60× water immersion objective. Oocytes were bathed in the vital dye Hoechst 33342 (Sigma) at 10 μg/ml in sea water, then returned to natural sea water prior to visualisation of the chromatin.

Flashes of UV light for uncaging were administered by exciting the specimen using the 365 nm filter.

Production of egg cytoplasts

We set out to partition eggs into fragments: karyoplasts, which contain the nuclear material, and cytoplasts which have none, and are therefore unable to form pronuclei.

Two methods were employed (see Figs 1 and 2). In the first, relatively large portions of the egg were drawn off with a wide bore glass micropipette using a tube aspirator assembly and suction pressure applied by mouth. This produced a large and small pair of egg fragments, one of which contained the chromatin (hereafter referred to as a karyoplast) and one which did not (a cytoplast). Both were used for subsequent experiments. In the second, a smaller bore micropipette was used and applied to the side of the egg where the chromatin was situated as determined using simultaneous brightfield and chromatin imaging. This small portion of the egg was then removed under suction pressure supplied by mouth to leave a very large cytoplast. The small karyoplast was discarded.

Egg activation

The experiments carried out required close synchrony of activation in eggs or cytoplasts. In order to achieve this, our chosen method of activation was sperm extract injection, use of which we justify in that we have shown it faithfully mimics all aspects of fertilisation in ascidian eggs (McDougall et al., 2000). Activation was achieved by microinjection of 1% egg volume of sperm extract, prepared as previously described (McDougall et al., 2000).

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