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## Sperm-triggered calcium oscillations during meiosis in ascidian oocytes first pause, restart, then stop: correlations with cell cycle kinase activity

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### SUMMARY

We have investigated the relationship between the sperm-triggered  $\text{Ca}^{2+}$  oscillations and the activities of two cell cycle kinases (MPF activity and MAP kinase activity) at fertilisation of *Ascidella aspersa* oocytes. Maturation Promoting Factor (MPF) activity is elevated in the metaphase I (MI)-arrested unfertilised oocyte (as measured by phosphorylation of exogenous histone H1) and falls 5 minutes after fertilisation to remain at low levels for 5 minutes. The first polar body (pb1) is extruded when the MPF activity is low. The MPF activity is elevated again 15 minutes after fertilisation and finally becomes inactivated 25 minutes after fertilisation when the pb2 is extruded. MAP kinase activity increases from an initially elevated level to reach maximal activity 10 minutes after fertilisation and subsequently falls to reach low levels 25 minutes after fertilisation. Sperm trigger a series of  $\text{Ca}^{2+}$  oscillations that pause for 5 minutes while only the MPF activity is low and are present when both MPF and MAP kinase activity are elevated.

We next attempted to determine whether the second phase of calcium oscillations is required to reactivate the MPF activity that precedes extrusion of the second polar body. To do this, we triggered a monotonic  $\text{Ca}^{2+}$  signal. This leads to the inactivation of MPF followed by MPF reactivation. The MPF activity then remains elevated for an extended period of time. During this period, the chromatin remains condensed and a metaphase II (MII) spindle forms. Fertilisation of these MII oocytes triggers extrusion of pb2 in 7 minutes. Interestingly, the second phase of  $\text{Ca}^{2+}$  oscillations is completely absent when MII oocytes are fertilised. Thus, in both MI and MII oocytes, the sperm-triggered  $\text{Ca}^{2+}$  oscillations follow the MPF activity.

Finally we discuss our finding that the  $\text{Ca}^{2+}$  release system remains sensitive during the metaphase-like state (including the period when the  $\text{Ca}^{2+}$  oscillations pause).

Key words: Ascidian, Oocyte, Calcium, MAP kinase, MPF, Meiosis

### INTRODUCTION

Sperm-triggered calcium ( $\text{Ca}^{2+}$ ) oscillations occur during meiosis in many species of oocyte: mammal, ascidian, starfish, mollusc, nemertean and annelid (Cuthbertson and Cobbold, 1985; Speksnijder et al., 1989; Stricker, 1995, 1996; Deguchi et al., 1996; Eckberg and Miller, 1995). In both mammalian and ascidian oocytes,  $\text{Ca}^{2+}$  oscillations are the trigger that initiates embryonic development (Miyazaki et al., 1993; Swann and Ozil, 1994; McDougall and Sardet, 1995). The culmination of evidence suggests that there are at least two factors that trigger and maintain these  $\text{Ca}^{2+}$  oscillations. Sperm trigger these  $\text{Ca}^{2+}$  oscillations but they are maintained by the metaphase-like cytoplasm of the oocyte.

The identity and mode of action of the sperm factor that triggers these  $\text{Ca}^{2+}$  oscillations is not known with certainty. There are however three hypotheses. These are the  $\text{Ca}^{2+}$  bomb hypothesis (Jaffe, 1991), the receptor hypothesis (Jaffe, 1990) and the soluble sperm factor hypothesis (Whitaker and Swann, 1993). The observation that sperm cytosolic extracts faithfully

mimic the sperm-triggered  $\text{Ca}^{2+}$  oscillations argues in favour of a soluble sperm factor model of fertilisation (Swann, 1990).

Likewise the exact nature of the maternal factor that maintains the sperm-triggered  $\text{Ca}^{2+}$  oscillations is not known with certainty. It is known, however, that the  $\text{Ca}^{2+}$  oscillations in mouse oocytes are maintained during the meiotic metaphase-like state and terminate as the fertilised oocyte enters interphase (Jones et al., 1995). In addition, when the metaphase-like state is prolonged artificially the sperm-triggered  $\text{Ca}^{2+}$  oscillations are also prolonged for as long as the metaphase-like state persists (Jones et al., 1995).

The metaphase state is established by the kinase activity of maturation/mitosis promoting factor (MPF) and is characterised by nuclear envelope breakdown, chromatin condensation and the formation of a mitotic spindle (reviewed by Doree and Galas, 1994). MPF is composed of a kinase (Cdk1: Cell division kinase 1) and a regulatory cyclin B subunit (Nurse and Thuriaux, 1980; Lohka et al., 1988; Gautier et al., 1988; Dunphy et al., 1988; Evans et al., 1983). Metaphase ends when several proteins, including cyclin B, are targeted via ubiquitination for proteolytic destruction by the anaphase

promoting complex (APC) (Murray et al., 1989; King et al., 1995; Irninger et al., 1995; Shirayama et al., 1998).

The meiotic cell cycle of oocytes is different from the mitotic cell cycles in that the metaphase-like organisation of the microtubules and chromosomes does not follow precisely variations in MPF activity as it does in mitosis (Verlhac et al., 1994; reviewed by Murray, 1998). Instead, the metaphase-like organisation of the chromatin, the microtubules and the nuclear envelope follows variations in mitogen-activated protein (MAP) kinase activity (Verlhac et al., 1994; Moos et al., 1996). MAP kinase is the family name for a number of Ser/Thr protein kinases (Cobb et al., 1991; Payne et al., 1991; Seger et al., 1991). During meiotic maturation in clam (Shibuya et al., 1992), *Xenopus* (Ferrel et al., 1991) and mouse (Verlhac et al., 1994) MAP kinase is stimulated. With the exception of clam, where the MAP kinase activity is only transient, MAP kinase activity persists during the completion of meiosis (Ferrel et al., 1991; Verlhac et al., 1994). These persistently elevated MAP kinase levels are responsible for the CSF (cytostatic factor) activity that arrests mammalian oocytes at metaphase II (MII) by reducing the rate of cyclin B destruction (Colledge et al., 1994; Hashimoto et al., 1994; Verlhac et al., 1996). In *Xenopus* oocytes, the sperm-triggered  $Ca^{2+}$  signal overrides the CSF activity of the MAP kinase pathway and initiates cyclin B destruction followed by the loss of the upstream activator of MAP kinase Mos (Lorca et al., 1993). It is not yet known how CSF or the sperm-triggered  $Ca^{2+}$  signals interact with the APC to affect cyclin B ubiquitination and cyclin B destruction. However,  $Ca^{2+}$  signals have been shown to increase the activity of the 26 S proteasome that degrades ubiquitinated cyclin B at two points during the meiotic cell cycle in fertilised ascidian oocytes (Kawahara and Yokasawa, 1994).

Fertilisation of metaphase I-arrested (MI) ascidian oocytes triggers two phases of  $Ca^{2+}$  oscillations that accompany meiosis (Speknsnijder et al., 1989). Recently it has been shown that the second phase of  $Ca^{2+}$  oscillations in ascidian oocytes is required to reactivate the MPF activity that precedes extrusion of the second polar body (Russo et al., 1996). However, the finding that a monotonic  $Ca^{2+}$  signal leads to the formation of a MII spindle suggests that the MPF activity rises again even in the absence of further  $Ca^{2+}$  signals (Sensui and Morisawa, 1996). To resolve this issue, we have directly measured the MPF activity in oocytes that are activated by triggering a monotonic  $Ca^{2+}$  signal to determine whether subsequent  $Ca^{2+}$  signals are required to reactivate MPF during meiosis. In addition to this, we have tested the hypothesis that the sperm-triggered  $Ca^{2+}$  oscillations are regulated by either MAP kinase or MPF activity during meiosis.

## MATERIALS AND METHODS

### Biological material

The tunicate *Ascidia aspersa* was collected locally from North East England. The animals were kept at 10°C in the laboratory. Oocytes and sperm were obtained by removing the animals from their outer tunic and puncturing first the gonoduct then the sperm duct. The oocytes were transferred to 4.5 ml Millipore-filtered natural sea water at 19°C and the sperm were stored dry at 4°C. All further experiments were performed with this sea water. The oocytes were partially dechorionated by incubating them with 0.1–0.2% trypsin at room temperature for 30–45 minutes. Following incubation with trypsin, the oocytes were aspirated gently once or twice to remove their chorion mechanically. Once the chorion was removed the oocytes were kept

in 1% gelatin/formaldehyde-coated Petri dishes. All further glass and plastic ware that the oocytes come into contact with was coated with this mixture of gelatin/formaldehyde.

### Visualisation of chromatin and microtubule dynamics

Oocytes were bathed in the vital DNA dye Hoechst 33342 (10 µg/ml) (Sigma) for 10–15 minutes then returned to sea water. The microtubules were visualised by microinjection of Rhodamine-labelled tubulin (Cytoskeleton). The chromatin and microtubules were visualised using a Leica confocal microscope (40×/n.a. 1.0 oil) and by using an epifluorescence microscope (Olympus IX70, 60×/n.a. 1.2 water objective).

### Microinjection and intracellular calcium visualisation

Oocytes were transferred to a wedge based on the design by Keihart (1982). Micropipettes were pulled on a Kopf 720 puller from GC100-T10 glass (Clarke Electromedical). The micropipettes were advanced towards the oocytes using a Narishige hydraulic three-way micromanipulator. The micropipettes were tip filled then inserted into the oocytes. The oocytes were pressure injected using a Narishige IM-300.

Intracellular  $Ca^{2+}$  levels were recorded by microinjecting fura 2 dextran (10 kDa; Molecular Probes) to give an intracellular concentration of between 10 and 20 µM. About 30 minutes after injection, the oocytes were observed with an epifluorescence Olympus IX70 microscope (40×/n.a. 0.9 air objective). The exciting light passed through a filter wheel (Newcastle Photonics) housing 340 nm and 380 nm band-pass filters and a 450 nm dichroic before reaching the injected oocytes. The emitted light collected by the objective lens was filtered through a 510 nm band-pass filter and entered a photomultiplier housed on the microscope (9124 A, Thorn EMI). The analogue signal was digitised and counted using a photon-counting card (Newcastle Photonics).

### MBP kinase and Histone H1 kinase measurements

The MBP (Myelin Basic Protein) kinase activity and histone H1 kinase activity were assayed as described previously (Verlhac et al., 1994). Samples of 5 oocytes were collected by first washing the oocytes through 1 M glycine three times to remove the sea water (this does not alter meiotic progression). For one series of experiments, the oocytes were first microinjected with  $InsP_3$  then harvested in the same manner. The oocytes were then removed in a volume of 2 µl and transferred to 8 µl reaction buffer (25 mM Hepes, 80 mM β-glycerophosphate, 5 mM EGTA, 10 mM  $MgCl_2$ , 1 mM DTT, 10 µg/ml leupeptin/pepstatin/aprotinin, 0.2 mM AEBSEF, 1 mM benzamide, 100 µM  $NaVO_4$ , 5 mM NaF, pH 7.2). At this point, the oocytes were snap-frozen in liquid nitrogen. After defrosting the samples on ice, which is sufficient to lyse the dechorionated oocytes, 2 µl 6× reaction mixture was added to the lid of each Eppendorph (0.9 mg/ml myelin basic protein (Sigma) or histone H1 (Sigma type III from calf thymus), 0.6 mM ATP, 0.5 mCi/ml [ $^{32}P$ ]ATP, 60 µM cAMP-dependent protein kinase inhibitor in 1× Reaction Buffer at pH 7.2). The reaction was started synchronously by spinning the Eppendorphs. They were then transferred to a water bath at 30°C for 10 minutes. Following this, the reaction was stopped synchronously by adding 2× sample buffer to the lid of each Eppendorph and spinning in a microfuge. The samples were then heated to 95°C for 3 minutes and resolved on 15% polyacrylamide gels. The resolved proteins on the gel were placed in a Phosphorimager (Fujix; 1500 Bas Reader) and the incorporation of [ $^{32}P$ ] measured quantitatively. The in gel kinase assay was based on the method of Shibuya and colleagues (1992). Briefly, 30 oocytes were collected at each time point in sample buffer and subjected to 15% PAGE. The kinase reaction, renaturation and washing steps were carried out as previously described (Shibuya et al., 1992). The incorporated radioactivity was measured using a Phosphorimager. These experiments were carried out in duplicate where one gel was prepared without the substrate myelin basic protein present. As a positive control and as a marker of molecular weight, we dissolved 2

$\mu$ l active p42 rat ERK2 (Upstate Biotechnology) in sample buffer and ran this alongside the ascidian oocyte samples.

## RESULTS

### MPF and MAP kinase activity during fertilisation

The histone H1 kinase activity is elevated in the unfertilised oocyte and falls each time a polar body is extruded. This is in agreement with previous data from a different species of ascidian (Russo et al., 1996). 5 minutes after fertilisation, the histone H1 kinase activity is low and remains at these low levels for a minimum period of 5 minutes (Fig. 1A). The histone H1 kinase activity is elevated again 15 minutes after fertilisation and finally falls to low levels 25 minutes after fertilisation (Fig. 1A).

The MBP kinase activity is also elevated in the unfertilised oocyte (Fig. 1B). Unlike the histone H1 kinase activity, the MBP kinase activity increases and reaches peak levels 10 minutes after fertilisation (Fig. 1B). The MBP kinase activity subsequently declines and reaches low levels 25 minutes after fertilisation (Fig. 1B). To determine the identity of the MBP kinase, we performed an 'in gel' kinase assay. The MBP kinase activity migrates at around 42 kDa (Fig. 1C). The autophosphorylation was less than 5% of the signal. The overall pattern of activity matches the pattern obtained in cell lysates (Fig. 1C).

### Chromatin and microtubule behaviour during fertilisation

The state of the chromatin correlates with the activity of either MPF or MAP kinase: it remains in a metaphase-like configuration during the entire period of meiosis after fertilisation (Fig. 2A). The chromatin finally decondenses at the end of meiosis 25-30 minutes after fertilisation (Fig. 2A). The decondensed chromatin constrained by the pronuclear

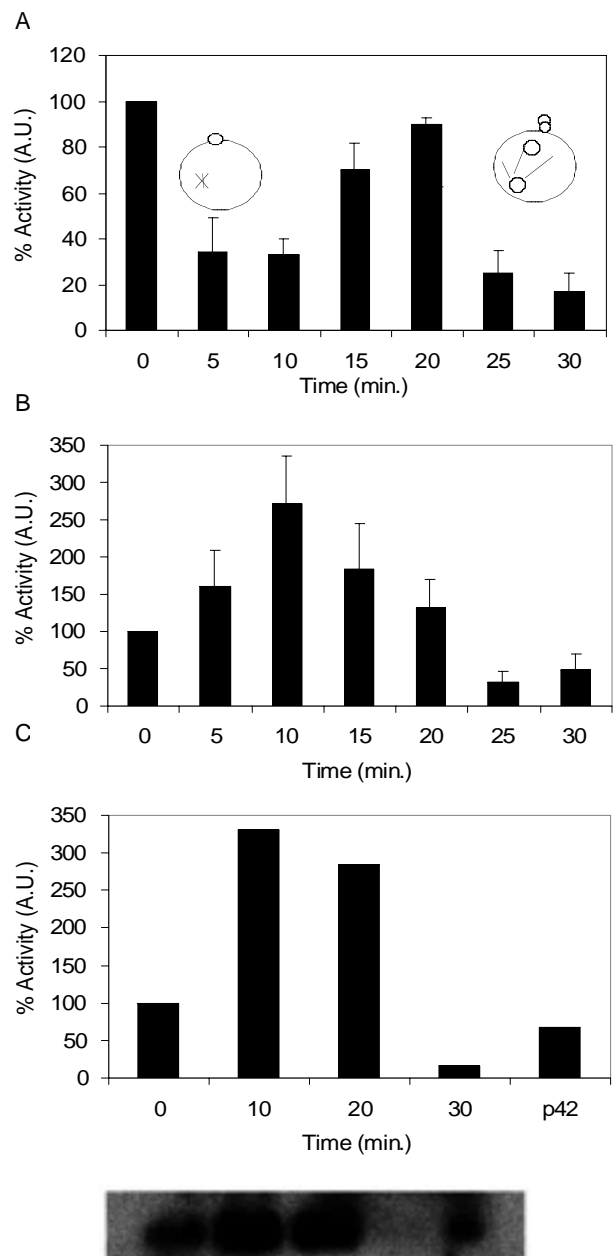
membrane is visible below the strongly stained polar bodies 30 minutes after fertilisation (Fig. 2A).

Likewise, the state of the microtubules correlates with the activity of either MPF or MAP kinase: they remain in a metaphase-like configuration throughout the entire period of meiosis following fertilisation (Fig. 2B). Extrusion of both polar bodies is depicted (Fig. 2B). These events correlate to the times that MPF activity is low. When the first polar body is being extruded, the sperm aster becomes visible but does not reach an interphase size (Fig. 2B). The sperm aster increases dramatically in size minutes after extrusion of the second polar body (Fig. 2B).

### Fertilisation of metaphase I-arrested oocytes triggers two series of calcium oscillations

Sperm trigger two series of  $Ca^{2+}$  oscillations in *A. aspersa* oocytes that correlate to the period when MPF activity is elevated.

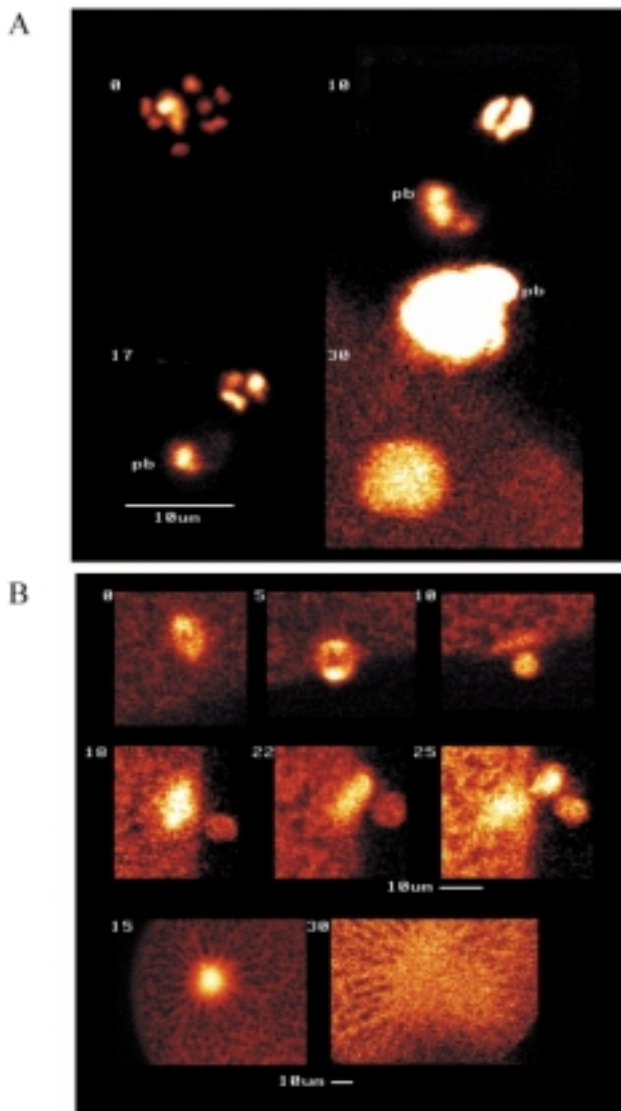
**Fig. 1.** Histone H1 kinase and MAP kinase activity following fertilisation. (A) Kinetics of Histone H1 kinase activity during the transition from metaphase I through to anaphase II and meiotic exit. Samples of 5 oocytes per experimental time point were collected and the kinase activity determined at 5 minute intervals following fertilisation. The histone H1 kinase activity in the unfertilised oocyte is standardised to 100% and all subsequent values are with reference to that (Mean  $\pm$  s.e.m.,  $n=6$ ; Temperature 19°C). The schematic representations show the state of the oocyte at two different times. (i) 7 minutes after fertilisation – the polar body and sperm aster are shown and (ii) 30 minutes after fertilisation – both polar bodies and pronuclei together with the large sperm aster are shown. (B) Kinetics of the MBP kinase activity following fertilisation. The MBP kinase activity was measured from 5 oocytes as detailed in the Materials and Methods. The MBP kinase activity is standardised so that the level found in the unfertilised oocyte equals 100%. All subsequent values are with reference to that. The MBP kinase activity increases 5 minutes after fertilisation and reaches peak levels 10 minutes after fertilisation (Mean  $\pm$  s.e.m.,  $n=6$ ; Temp. = 19°C). (C) In gel kinase assay. To confirm the identity of MBP kinase, we performed an in gel kinase assay. Samples of 30 unfertilised and fertilised oocytes were loaded in each lane. As a control, we loaded a constitutively active MAP kinase (rat p42ERK2). We also performed the same experiment without adding MBP to the gel. The autophosphorylation was less than 5% of the signal for all bands (data not shown). Only one band appears to be phosphorylated in each experimental lane. This phosphorylated band migrates at the same molecular weight as p42ERK2. The raw data is depicted at the bottom.



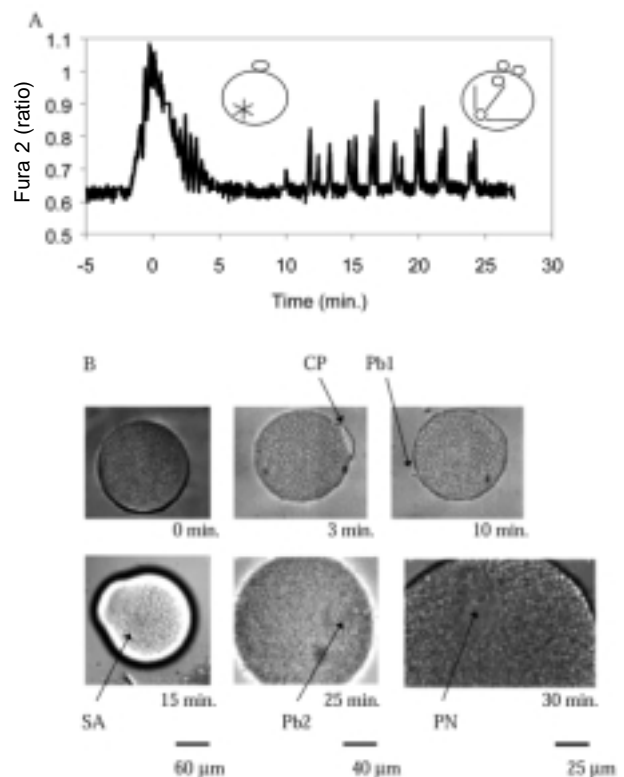
The first series of  $\text{Ca}^{2+}$  oscillations lasts for approximately 5 minutes and terminates 2-3 minutes before extrusion of the first polar body (Fig. 3A). After the first series of  $\text{Ca}^{2+}$  transients normally a clear gap phase of between 4 and 6 minutes ensues

(Fig. 3A). After the gap a second series of  $\text{Ca}^{2+}$  oscillations begins that lasts for between 10 and 15 minutes (Fig. 3A).

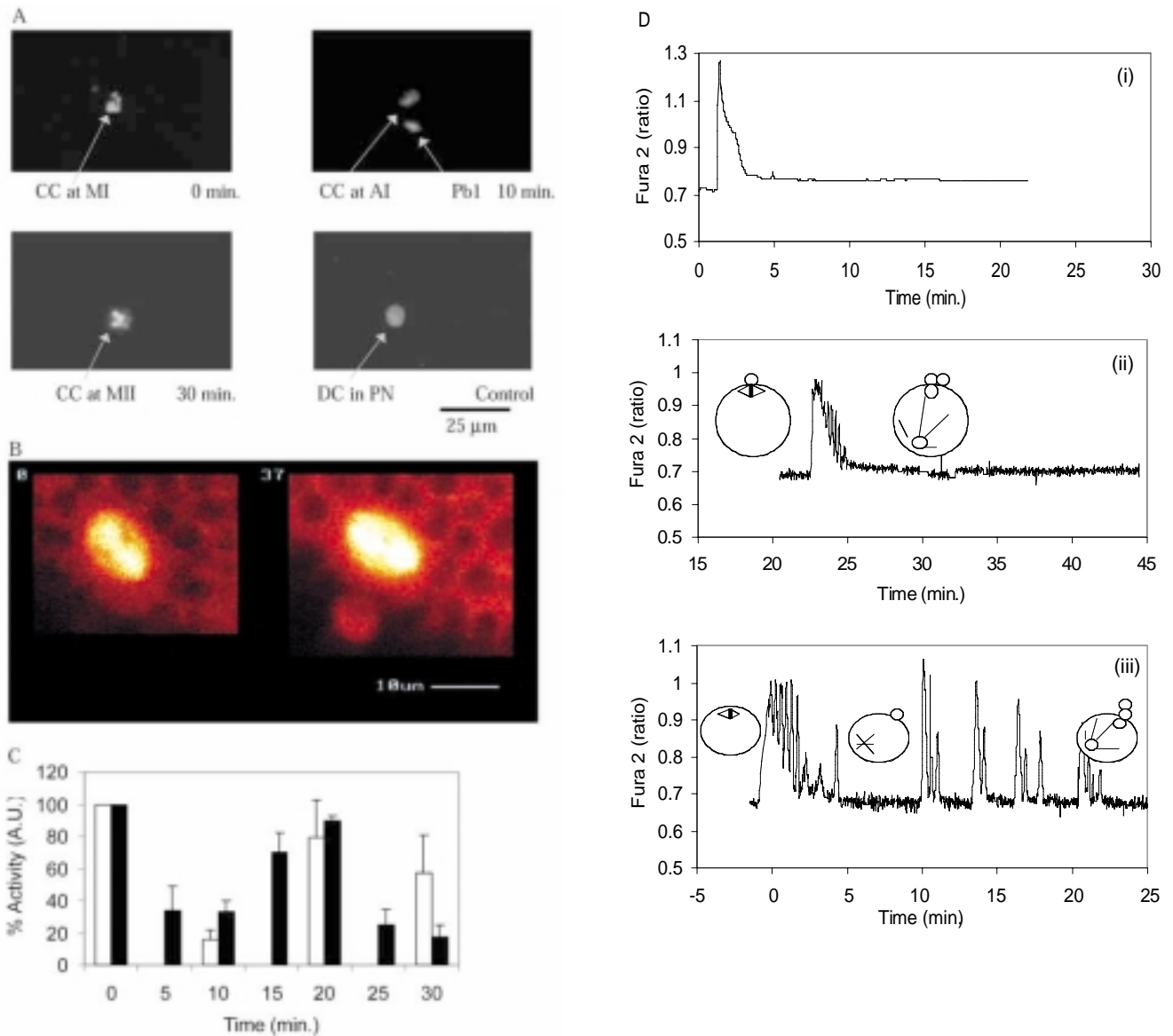
Ascidians display three easily recognisable characteristics that we have used to achieve cell cycle synchrony. The peak of the first  $\text{Ca}^{2+}$  transient triggers the first visible morphological event – the cortical contraction. The cortical contraction begins when the first  $\text{Ca}^{2+}$  signal has peaked in *Phallusia mammillata* oocytes (Roegiers et al., 1995). This is also the case in *A. aspersa* oocytes. For this reason, this time point is taken as zero and is subsequently referred to as the point of fertilisation (Fig. 3A). In this way, we have synchronised the  $\text{Ca}^{2+}$  data and the kinase data with the initiation of the cortical contraction. We have used the two other morphological characteristics to check cell cycle synchrony. These are extrusion of the first polar body (7-9 minutes postfertilisation) and extrusion of the second polar



**Fig. 2.** Chromatin and microtubule dynamics after fertilisation. (A) Unfertilised oocytes were bathed in 10  $\mu\text{g}/\text{ml}$  Hoechst 33342 for 10-15 minutes then fertilised. The chromatin was visualised using a Leica confocal microscope. The chromatin is condensed in the unfertilised MI-arrested oocyte. The chromatin remains condensed for the entire period of meiosis, even when the MPF activity is low 10 minutes after fertilisation. The chromatin finally decondenses 30 minutes after fertilisation. The polar bodies appear around 7 minutes and 25 minutes after fertilisation and are indicated on the relevant images (pb). The scale bar represents 10  $\mu\text{m}$ . (B) Unfertilised oocytes were microinjected with rhodamine tubulin and fertilised 30 minutes later. The confocal images show the meiotic spindle and the sperm aster during fertilisation. The first row of 3 images show extrusion of the first polar body. The second row of 3 images show extrusion of the second polar body. Both these rows of images are on the same scale. The third row of images show the sperm aster 15 minutes and 30 minutes after fertilisation. Both these images are on the same scale.



**Fig. 3.** The sperm-triggered calcium oscillations at fertilisation are associated with morphological events in the oocyte. (A) Unfertilised oocytes were microinjected with dextran-conjugated Fura 2 (10 kDa) to give an intracellular concentration of between 10 and 20  $\mu\text{M}$  Fura 2. The oocytes were fertilised 30 minutes after microinjection. Fertilisation triggers two series of  $\text{Ca}^{2+}$  oscillations separated by a gap. The schematic representations show the state of the oocyte at 2 different time points. (i) 7 minutes after fertilisation – the polar body and sperm aster are shown; (ii) 30 minutes after fertilisation – both polar bodies and pronuclei together with the large sperm aster are shown. (B) Bright-field images of oocytes during fertilisation. Sperm trigger a visible contraction of the oocyte that culminates in the formation of a contraction pole (CP) 2-3 minutes after the contraction has begun. By 7 minutes after fertilisation, the oocyte emits the first polar body (Pb1: shown here 10 minutes after fertilisation). By 15 minutes postfertilisation, the sperm aster (SA) is just visible. The second polar body (Pb2) is extruded 25 minutes after fertilisation and the male pronucleus (PN) becomes clearly visible 30 minutes postfertilisation.



**Fig. 4.** Fertilisation of metaphase-II ascidian oocytes. Unfertilised ascidian oocytes were microinjected with  $\text{InsP}_3$  ( $50 \mu\text{M}$  pipette concentration, 0.1–0.2% injected). This triggers the cortical contraction and extrusion of the first polar body. (A) Chromatin. Unfertilised oocytes incubated with  $10 \mu\text{g/ml}$  Hoechst 33342 for 15 minutes to label the chromatin then injected with  $\text{InsP}_3$ . These images were obtained using an epifluorescence microscope. The chromatin is condensed (CC) in the unfertilised MI-arrested ascidian oocyte and again remains in a condensed state following extrusion of the first polar body at anaphase I (CC at AI) when the MPF activity is low. The lower of the two stained objects represents the first polar body 10 minutes postfertilisation. The chromatin remains condensed 30 minutes after fertilisation and oocyte remains in metaphase II (CC at MII). The decondensed chromatin (DC) within the male pronucleus (PN) in a control oocyte 30 minutes after fertilisation is shown. (B) Meiotic spindle. Unfertilised oocytes were injected with Rhodamine tubulin then  $\text{InsP}_3$ . The first polar body is extruded. A second metaphase spindle forms horizontal to the plasma membrane below the first polar body. The MII spindle remains in a metaphase-like state 37 minutes after injection of  $\text{InsP}_3$ . (C) MPF activity in  $\text{InsP}_3$ -injected oocytes. The black bars represent the control MPF activity. The white bars represent the MPF activity following microinjection of  $\text{InsP}_3$  ( $50 \mu\text{M}$  pipette concentration, 0.1–0.2% injected). The MPF activity first falls and reaches low levels 10 minutes after injection. The MPF activity then reaches maximal levels 20 minutes after injection and remains elevated for a further 10 minutes (Mean  $\pm$  s.e.m.,  $n=3$ ). (D) Fertilisation of MII oocytes. (i) Unfertilised oocytes were injected with Fura 2 ( $10$ – $20 \mu\text{M}$  in the oocyte) about 20 minutes before injection of  $\text{InsP}_3$ . Two 0.1% injections of  $\text{InsP}_3$  at a pipette concentration of  $50 \mu\text{M}$  triggers a monotonic  $\text{Ca}^{2+}$  signal as shown. (ii) Unfertilised oocytes were microinjected with a mixture of  $\text{InsP}_3$  and Fura 2 ( $50 \mu\text{M}$   $\text{InsP}_3$  and  $5 \text{ mM}$  Fura 2) and fertilised 20–30 minutes later following extrusion of the first polar body (hence the x axis begins at 15 minutes). The sperm-triggered  $\text{Ca}^{2+}$  signal was recorded from 5 separate oocytes that came from 5 different animals. The sperm-triggered  $\text{Ca}^{2+}$  signals are displayed in an oocyte that fertilised 23 minutes after injection of  $\text{InsP}_3$ . The schematics show the morphology of the oocyte at the time of fertilisation and shortly after fertilisation. The first polar body was present before fertilisation and the second polar body formed around 7 minutes after fertilisation ( $n=5$ ). (iii) This trace shows the sperm-triggered  $\text{Ca}^{2+}$  signals in a control MI-arrested oocyte from the same batch. The schematics show the morphology of the oocyte before, during and after fertilisation. The microtubules, polar bodies and pronuclei are illustrated.

body (24–28 minutes postfertilisation (Fig. 3B)). A few minutes after this time, the male pronucleus becomes discernible near the centre of the enlarging sperm aster (Fig. 3B).

It is therefore possible to directly check cell cycle synchrony by observing these three morphological indicators and thus compare the pattern of sperm-triggered  $\text{Ca}^{2+}$  oscillations to the activities of both MPF and MAP kinase with a high degree of fidelity.

### A single $\text{Ca}^{2+}$ signal triggers formation of a MII spindle with the concomitant reactivation of MPF

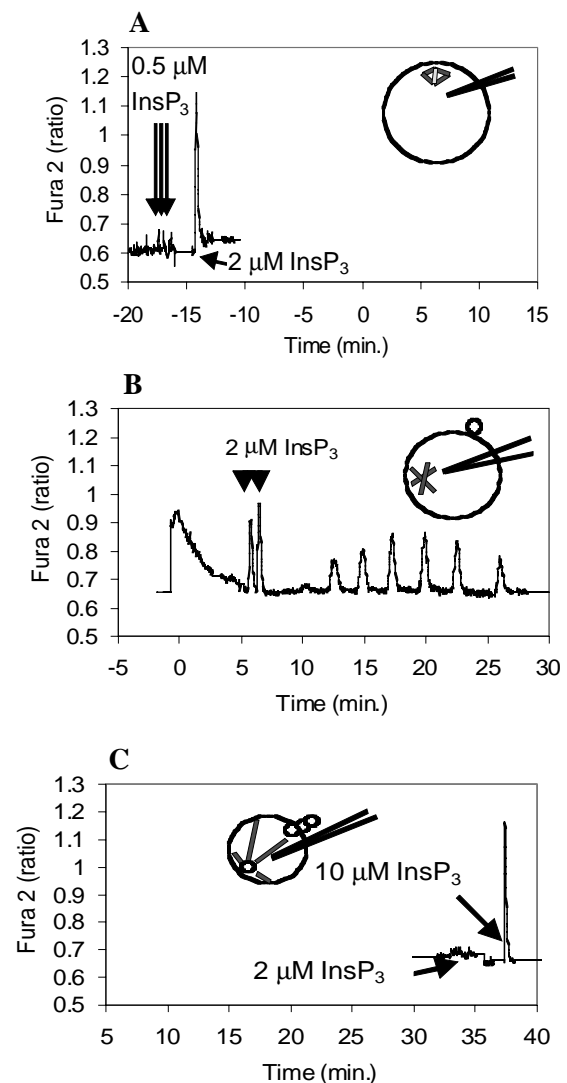
The second series of  $\text{Ca}^{2+}$  signals are proposed to reactivate the MPF activity that accompanies formation of the MII spindle (Russo et al., 1996). However, the observation that a single  $\text{Ca}^{2+}$  signal can result in the formation of a MII spindle suggests that further  $\text{Ca}^{2+}$  signals are not necessary to form a MII spindle (Sensui and Morisawa, 1996). In addition, it is not known whether a single  $\text{Ca}^{2+}$  signal results in the reactivation of MPF. For this reason, we have directly measured the MPF activity following a single  $\text{Ca}^{2+}$  signal triggered by microinjection of the  $\text{Ca}^{2+}$ -releasing second messenger  $\text{InsP}_3$ . This has the same effect as microinjection of  $\text{Ca}^{2+}$  buffers that transiently raise intracellular  $\text{Ca}^{2+}$  to around  $1 \mu\text{M}$  (Sensui and Morisawa, 1996). Microinjection of inositol (1,4,5)-trisphosphate ( $\text{InsP}_3$ ) at pipette concentrations that ranged from 25 to 200  $\mu\text{M}$  consistently activated the oocytes and triggered extrusion of the first polar body, but did not trigger extrusion of the second polar body ( $n=47$ ). Following extrusion of the first polar body, the chromatin remains condensed in a metaphase-like state 30

**Fig. 5.** The sensitivity of the  $\text{Ca}^{2+}$  release system to  $\text{InsP}_3$ . To determine the sensitivity of the  $\text{Ca}^{2+}$  release system  $\text{InsP}_3$  was microinjected before, during and after fertilisation. Unfertilised ascidian oocytes were microinjected with Fura 2 to give an intracellular concentration between 10–20  $\mu\text{M}$ . They were then injected with  $\text{InsP}_3$  at pipette concentrations that ranged between 0.5 and 10  $\mu\text{M}$ . The volume injected in all cases was between 0.1 and 0.2% oocyte volume. (A) Before fertilisation. To determine the sensitivity of the  $\text{Ca}^{2+}$  signalling system before fertilisation, unfertilised oocytes were microinjected with  $\text{InsP}_3$  at different pipette concentrations. Pipette concentrations of 0.5  $\mu\text{M}$  do not trigger significant  $\text{Ca}^{2+}$  signals. Pipette concentrations of 2  $\mu\text{M}$   $\text{InsP}_3$  do trigger significant  $\text{Ca}^{2+}$  signals. The schematic indicates the morphology of the oocyte at the time of microinjection. The negative time scale is chosen to highlight the fact that these oocytes were microinjected before fertilisation. (B) During fertilisation when the first polar body is extruded. To determine the sensitivity of the  $\text{Ca}^{2+}$  release system during meiosis, we microinjected  $\text{InsP}_3$  approximately 6–7 minutes after fertilisation when the polar body is being extruded. Threshold pipette concentrations of 2  $\mu\text{M}$   $\text{InsP}_3$  trigger significant  $\text{Ca}^{2+}$  signals 6–7 minutes after fertilisation. The schematic indicates the morphologies of the oocytes at the times of microinjection i.e., the first polar body is being extruded. (C) After fertilisation when the second polar body has been extruded and the pronuclei have reformed. To determine the sensitivity of the  $\text{Ca}^{2+}$  release system to  $\text{InsP}_3$  following exit from meiosis.  $\text{InsP}_3$  was microinjected 30–40 minutes after fertilisation. Pipette concentrations of 2  $\mu\text{M}$   $\text{InsP}_3$  do not trigger a significant  $\text{Ca}^{2+}$  transient whilst pipette concentrations of 10  $\mu\text{M}$   $\text{InsP}_3$  do trigger a significant  $\text{Ca}^{2+}$  transient. The time scale indicates the fact that these oocytes had exited meiosis. The schematic represents the morphology of the oocyte at this time. Both polar bodies are present, both pronuclei have formed and the astral microtubules are long and interphasic.

minutes after injection of  $\text{InsP}_3$  when it would normally be decondensed following fertilisation (Fig. 4A). The microtubules also remain in a metaphase-like state 37 minutes after injection: a horizontal MII spindle forms adjacent to the plasma membrane (Fig. 4B). The MPF activity falls following injection of  $\text{InsP}_3$  and the first polar body is extruded (Fig. 4C). The MPF activity then increases to elevated levels 20 minutes after microinjection (Fig. 4C). Interestingly, the MPF remains at elevated levels 30 minutes after microinjection when the MPF activity in control oocytes has fallen (Fig. 4C). These data show that MPF will reactivate following a single  $\text{Ca}^{2+}$  signal. In addition, they reveal that the MPF activity remains at elevated levels for a prolonged period of time. These oocytes seem to be arrested at MII.

### Fertilisation of metaphase II-arrested oocytes triggers only one phase of calcium oscillations

Sperm-triggered  $\text{Ca}^{2+}$  oscillations are maintained by the metaphase-like cytoplasm of the oocyte (Jones et al., 1995). In ascidians, these  $\text{Ca}^{2+}$  oscillations are temporally associated with MPF activity (Figs 1A, 3A). We therefore tested whether we could experimentally modulate the sperm-triggered  $\text{Ca}^{2+}$



oscillations by modulating the MPF activity. To do this, we exploited the finding that MII-arrested oocytes can be fertilised. To create MII oocytes, we microinjected InsP<sub>3</sub> (50 µM pipette concentration, 0.1-0.2% injected). This triggers a monotonic Ca<sup>2+</sup> signal (Fig. 4Di).

Fertilisation of these MII-arrested oocytes triggered only one series of Ca<sup>2+</sup> oscillations followed by extrusion of the second polar body (Fig. 4Dii). Fertilisation of control oocytes from the same batch displayed the two phases of Ca<sup>2+</sup> oscillations that normally accompany fertilisation (Fig. 4Diii). These data indicate that the sperm-triggered Ca<sup>2+</sup> oscillations can be entrained by the oocyte cytoplasm and again reveal that the sperm-triggered Ca<sup>2+</sup> oscillations correlate temporally with elevated MPF activity.

### The calcium signalling system remains sensitive during the entire period of meiosis following fertilisation

We were next interested to determine the nature of the mechanism responsible for inducing the pause that separates the two series of Ca<sup>2+</sup> oscillations. During this period, the first polar body is extruded. Two possibilities are either that the oocyte calcium release system becomes desensitised or that the signal generating the Ca<sup>2+</sup> oscillations becomes desensitised or depleted (in the case of the substrate). The results indicate that the Ca<sup>2+</sup> signalling system is not desensitised in mid meiosis when the Ca<sup>2+</sup> oscillations pause. The threshold pipette concentration of InsP<sub>3</sub> that triggers a Ca<sup>2+</sup> signal is 2 µM (Fig. 5A). InsP<sub>3</sub> at pipette concentrations of 2 µM also triggered a significant Ca<sup>2+</sup> release 7-9 minutes after fertilisation (Fig. 5B). These data suggest that the Ca<sup>2+</sup> oscillations are likely to pause because the signal generating them becomes inactive or the substrate depleted during the period when the first polar body is extruded.

In addition to this the Ca<sup>2+</sup> signalling system does eventually become desensitised at the end of meiosis when the Ca<sup>2+</sup> oscillations stop. Higher concentrations of 10 µM InsP<sub>3</sub> are required to trigger a Ca<sup>2+</sup> signal following exit from meiosis (Fig. 5C).

## DISCUSSION

### Fertilisation triggers one series of calcium oscillations that are associated with meiosis in the mammalian oocyte

The sperm-triggered Ca<sup>2+</sup> oscillations during fertilisation of mouse oocytes correlate temporally with the metaphase-like state and terminate just before pronuclear formation (Jones et al., 1995). This metaphase-like state is maintained by MAP kinase activity and not MPF activity (Verlhac et al., 1994). These observations would suggest that the MAP kinase-induced metaphase-like state entrains the sperm-triggered Ca<sup>2+</sup> oscillations. In addition, pronuclei from fertilised mouse oocytes are capable of triggering Ca<sup>2+</sup> oscillations when microinjected into unfertilised mouse oocytes (Kono et al., 1995). If the soluble sperm factor hypothesis is the correct view of how sperm trigger Ca<sup>2+</sup> oscillations at fertilisation (Whitaker and Swann, 1993), these data suggest the possibility that this soluble factor is either sequestered in or is associated with the pronuclei. This may account for the termination of the sperm-triggered Ca<sup>2+</sup> oscillations when the oocyte exits

meiosis and pronuclei form. We have used the ascidian oocyte to determine how the sperm-triggered Ca<sup>2+</sup> oscillations are modulated during the meiotic cell cycle.

### The sperm-triggered calcium oscillations in ascidian oocytes correlate with MPF activity

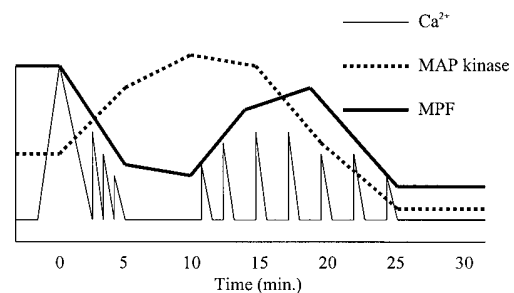
Ascidian oocytes, like the oocytes from numerous other species, display Ca<sup>2+</sup> oscillations at fertilisation (Sardet et al., 1998). Unlike mammalian oocytes, the Ca<sup>2+</sup> oscillations triggered at fertilisation of *A. aspersa* oocytes can be separated into two distinct series; the first series of Ca<sup>2+</sup> oscillations precede extrusion of the first polar body and the second series of Ca<sup>2+</sup> oscillations precede extrusion of the second polar body (Fig. 3A). The frequency of the Ca<sup>2+</sup> oscillations varies from batch to batch. For this reason, we will not discuss the obvious variation in spike frequency that sometimes distinguishes phase I Ca<sup>2+</sup> oscillations from phase II ones. The most obvious distinguishing feature is the gap.

The sperm-triggered Ca<sup>2+</sup> oscillations are present when the MPF activity is elevated and pause when the MPF activity is low (see model 1). However the sperm-triggered Ca<sup>2+</sup> oscillations do not seem to bear a temporal correlation with the MAP kinase activity which is elevated when the Ca<sup>2+</sup> oscillations pause and start again (see model 1). In addition to this, the Ca<sup>2+</sup> oscillations pause during interkinesis when the oocyte remains in a metaphase-like state: pronuclei are not present.

It is worth noting that the oocyte remains in a metaphase-like state during the period that the MPF activity is low – monitored by direct visualisation of the state of the chromatin, microtubules and pronuclei. These data add support to the idea that elevated MAP kinase activity maintains the metaphase-like configuration of the oocyte cytoplasm when the MPF activity is low during interkinesis (Verlhac et al., 1994). Interestingly, in addition to the chromatin and the microtubules, we find that the sensitivity of the Ca<sup>2+</sup> signalling system also correlates with the metaphase-like state (Fig. 5). It is not yet clear why this should be the case, but it is intriguing to note that the morphology of the endoplasmic reticulum (ER) changes dramatically during meiotic progression – large islands of ER form throughout the completion of meiosis (Speksnijder et al., 1993).

### A single Ca<sup>2+</sup> signal leads to the inactivation followed by the reactivation of MPF

By inhibiting the second phase of sperm-triggered Ca<sup>2+</sup> oscillations in *Ciona intestinalis* oocytes with the Ca<sup>2+</sup> chelator BAPTA, the reactivation of MPF that accompanies formation



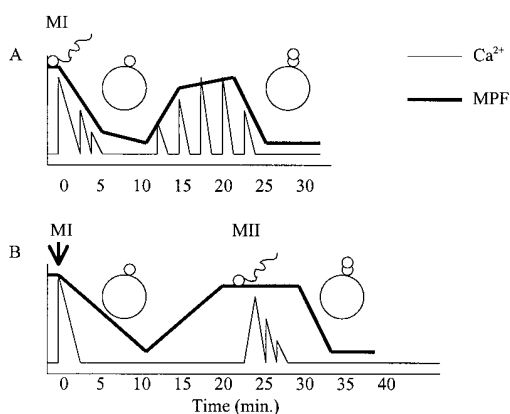
**Fig. 6.** Model 1. The MPF activity is indicated by the thick solid line and the MAP kinase by the dotted line. The Ca<sup>2+</sup> signals are indicated by the thin solid line. The time in minutes is shown on the x axis.

of the MII spindle is inhibited (Russo et al., 1996). These data imply that the second phase of  $\text{Ca}^{2+}$  oscillations is required to reactivate MPF prior to extrusion of the second polar body. However, a single increase in  $\text{Ca}^{2+}$  triggers extrusion of the first polar body followed by formation of a MII spindle (Sensui and Morisawa, 1996). These data suggest that the MPF activity initially decreases then increases even in the absence of further  $\text{Ca}^{2+}$  signals. We have triggered a single  $\text{Ca}^{2+}$  signal in order to determine whether MPF reactivates following extrusion of the first polar body. Our data show that a monotonic  $\text{Ca}^{2+}$  signal leads first to the inactivation then the reactivation of MPF activity (Fig. 4C). This is consistent with the finding that a monotonic  $\text{Ca}^{2+}$  signal leads to the formation of a MII spindle (Sensui and Morisawa, 1996). These data may be reconciled with the previous finding (Russo et al., 1996) when it is noted that high concentrations of BAPTA (circa 5 mM) have non-specific effects that are not associated with its  $\text{Ca}^{2+}$ -chelating ability (Pethig et al., 1989).

### Fertilisation of metaphase II-arrested ascidian oocytes triggers only one phase of calcium oscillations

To determine whether the metaphase cytoplasm of the oocyte can entrain the sperm-triggered  $\text{Ca}^{2+}$  oscillations, we compared the pattern of  $\text{Ca}^{2+}$  oscillations between two types of oocyte: those released from MI and those released from MII at fertilisation. If the  $\text{Ca}^{2+}$  oscillations are entrained by the cell cycle position, we would predict that the second phase of  $\text{Ca}^{2+}$  oscillations would be absent when MII-arrested oocytes are fertilised. Fertilisation of MII-arrested oocytes would not allow sufficient time to generate the second phase of  $\text{Ca}^{2+}$  oscillations during meiosis since the second polar body is extruded 7-9 minutes after fertilisation. Moreover, if the second phase of  $\text{Ca}^{2+}$  oscillations occurred they would be present during interphase.

Our data reveal that fertilisation of MII-arrested ascidian oocytes triggers the first phase of  $\text{Ca}^{2+}$  oscillations but not the second (Fig. 4Dii). These data are depicted in model 2 below.



**Fig. 7.** Model 2. The illustrated sperm indicate that fertilisation took place at MI in A and MII in B. The arrow in B indicates the  $\text{Ca}^{2+}$  signal generated by injection of  $\text{InsP}_3$ . The thick solid line represents the MPF activity and the  $\text{Ca}^{2+}$  levels are represented by the thin solid line. The schematics indicate oocyte morphology with respect to the number of polar bodies present. (A) This model represents the normal situation: fertilisation of MI-arrested oocytes. (B) This model represents the experimental situation: fertilisation of MII-arrested oocytes.

### The calcium signalling system remains sensitised even when the calcium oscillations pause and finally desensitises at the end of meiosis

Having established that the  $\text{Ca}^{2+}$  oscillations bear a close temporal correlation with the MPF activity, we tested two hypotheses. One, that the MPF activity sensitises the generation of the  $\text{Ca}^{2+}$  oscillations and two, that the MPF activity modulates the sensitivity of the  $\text{Ca}^{2+}$  signalling system. To distinguish between these two possibilities, we tested the sensitivity of the  $\text{Ca}^{2+}$  signalling system during the time that the  $\text{Ca}^{2+}$  oscillations pause. The second of these two possibilities seemed more likely since  $\text{Ca}^{2+}$  influx is required to sustain the second phase of  $\text{Ca}^{2+}$  oscillations in fertilised ascidian oocytes (Arnoult et al., 1996). We might therefore have predicted that the pause represents the time required to refill the depleted  $\text{Ca}^{2+}$  stores. Indeed  $\text{Ca}^{2+}$  store depletion has been demonstrated to desensitise the affinity of the  $\text{InsP}_3$  receptor for its agonist  $\text{InsP}_3$  (Iino and Endo, 1992; Nunn and Taylor, 1992). We therefore tested the sensitivity of the  $\text{Ca}^{2+}$ -releasing second messenger  $\text{InsP}_3$ . It should be noted that only the  $\text{InsP}_3$  receptor is involved in generating the second phase of  $\text{Ca}^{2+}$  oscillations in fertilised ascidian oocytes (McDougall and Sardet, 1995; Russo et al., 1996; Albrieux et al., 1997).

To determine whether the sperm-triggered  $\text{Ca}^{2+}$  oscillations pause because the  $\text{Ca}^{2+}$  signalling system becomes desensitised, we injected  $\text{InsP}_3$  into ascidian oocytes before, during and after meiosis. Our data reveal that the  $\text{Ca}^{2+}$  signalling system remains equally sensitive during the period that the sperm-triggered  $\text{Ca}^{2+}$  oscillations pause (Fig. 5B), and finally becomes desensitised when the  $\text{Ca}^{2+}$  oscillations have stopped and the oocyte exits meiosis (Fig. 5C).

These data indicate that the  $\text{Ca}^{2+}$  oscillations pause even when the  $\text{Ca}^{2+}$  release system remains sensitive. The most likely explanation of these data is that the metaphase cytoplasm (containing active MPF) of the oocyte sensitises either the paternally supplied trigger that initiates the  $\text{Ca}^{2+}$  oscillations or modulates the level of the substrate (phosphatidylinositol bisphosphate) that is cleaved to generate  $\text{InsP}_3$ . In addition to this, the  $\text{Ca}^{2+}$  signalling system is sensitised during the entire metaphase-like state. These two events together would help generate and sustain a  $\text{Ca}^{2+}$  oscillation during both meiotic metaphases in ascidian oocytes and so ensure rapid meiotic exit.

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