Sperm-induced calcium oscillations at fertilisation in ascidians are controlled by cyclin B1-dependent kinase activity

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SUMMARY

The generation of calcium oscillations at fertilisation and during mitosis appears to be controlled by the cell cycle machinery. For example, the calcium oscillations in oocytes and embryos occur during metaphase and terminate upon entry into interphase. Here we report the manipulation of sperm-triggered calcium oscillations by cyclin-dependent kinase (CDK) activity, the major component of maturation/ M phase promoting factor (MPF). To control the CDK activity we microinjected mRNAs encoding full-length GFP-tagged cyclin B1 or a truncated and therefore stabilised form of cyclin B1 (Δ90) into unfertilised oocytes. In the presence of full-length cyclin B1, the calcium oscillations terminate when cyclin B1 levels fall along with the concomitant fall in the associated CDK activity. In addition, when the CDK activity is elevated indefinitely

with Δ90 cyclin B1, the calcium oscillations also continue indefinitely. Finally, in oocytes that contain low mitogen-activated protein (MAP) kinase activity and elevated CDK activity, the sperm-triggered calcium oscillations are again prolonged. We conclude that the CDK activity of the ascidian oocyte can be regarded as a positive regulator of sperm-triggered calcium oscillations, a finding that may apply to other oocytes that display sperm-triggered calcium oscillations at fertilisation. Furthermore, these findings may have a bearing upon the mitotic calcium signals of early embryos.

Key words: Calcium, Cyclin B1-CDK, Fertilisation, MAP kinase, Oscillation

INTRODUCTION

Cytosolic calcium oscillations are displayed by a diverse array of cell types and control an equally diverse array of cellular processes (Berridge and Dupont, 1994) that include fertilisation (Cuthbertson et al., 1981; Swann and Ozil, 1994) and mitosis (Groigno and Whitaker, 1998). At fertilisation these calcium oscillations stimulate release from meiotic arrest (at MI or MII), completion of meiosis and further embryonic development (Ozil, 1990; Bos-Mikich et al., 1997; Lawrence et al., 1998; McDougall and Sardet, 1995), and during mitosis are involved in the completion of anaphase (Groigno and Whitaker, 1998).

Oocytes that display sperm-triggered calcium oscillations are all released from meiotic metaphase arrest by sperm. These include some molluscan (Deguchi and Osanai, 1994), nemertean (Stricker, 1996), annelid (Eckberg and Miller, 1995), arthropod (Goudeau and Goudeau, 1996), ascidian (Speksnijder et al., 1989) and mammalian (Cuthbertson et al., 1981) oocytes. Single, or monotonic, calcium increases are triggered when a spermatozoon fertilises an oocyte arrested either at the germinal vesicle (GV) stage (e.g. some molluscan oocytes: *Mactra chinensis*, Deguchi and Osanai, 1994) or at the G_1 stage (e.g. sea urchin: Steinhardt et al., 1977). However, polyspermy can trigger calcium oscillations in GV-arrested

mouse oocytes (Mehlmann and Kline, 1994). When meiotic metaphase is prolonged with microtubule depolymerising drugs the sperm-triggered calcium oscillations are also prolonged (Jones et al., 1995). These data, coupled with the observation that sperm-triggered calcium oscillations occur during meiotic metaphase, led to the suggestion that meiotic metaphase regulates the calcium oscillations triggered by the sperm (see reviews by Whitaker, 1996; Jones, 1998). In addition to the sperm-triggered calcium oscillations, calcium increases also occur during the early mitotic divisions of sea urchin (Groigno and Whitaker, 1998) and mouse (Kono et al., 1995) embryos. In mouse embryos these mitotic calcium oscillations are thought to be generated through residual sperm factor activity (Kono et al., 1996). Three main ideas have been put forward to explain how sperm-triggered calcium oscillations might be regulated during meiotic metaphase.

One idea is that a sperm-derived calcium oscillogen (sperm factor) is sequestered by the nucleus or perinuclear material (Kono et al., 1996). For example, the sperm-triggered calcium oscillations in the mouse oocyte terminate just before pronuclear formation (Jones et al., 1995). Transfer of nuclei from two cell embryos to unfertilised eggs triggers calcium oscillations (Kono et al., 1996). Another idea is based on the finding that the calcium signalling system desensitises at the end of meiosis when the calcium oscillations terminate (Jones

et al., 1995; Kono et al., 1996). This is likely because the predominant form of the inositol 1,4,5-trisphosphate (InsP$_3$) receptor, type I, becomes degraded towards the end of meiosis when the calcium oscillations terminate (Parrington et al., 1998). Finally, in ascidians, both these ideas would be consistent with the final termination of the sperm-triggered calcium oscillations. However, they would not explain the gap phase, where the calcium oscillations pause even though the pronuclei are absent and the calcium signalling system remains sensitive (McDougall and Levasseur, 1998). In addition to sequestration and desensitisation, another idea is that it is the kinase activities themselves that establish the metaphase state and that directly control the generation of the calcium signals (McDougall and Levasseur, 1998).

The metaphase (meiotic and mitotic) state is characterised by the activity of two kinases: these are MPF (M phase/ maturation-promoting factor) and mitogen-activated protein (MAP) kinase. Progression of the meiotic cell cycle is controlled by the activity of MPF (Masui and Markert, 1971; Newport and Kirschner, 1984; Murray and Kirschner, 1989). During meiosis MPF is stabilised by an activity termed cytostatic factor (CSF) (Masui, 1974; see Masui, 1991 for a review), which is equivalent to the Mos/MAP kinase pathway (Colledge et al., 1994; Hashimoto et al., 1994; Verlhac 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). Two MAP kinase isozymes are active during meiosis in mammalian oocytes: these are extracellular signal-regulated protein kinase (ERK1 and ERK2) (Verlhac et al., 1994). MAP kinase is now thought to be active during mitosis (Philipova and Whitaker, 1998; Chiri et al., 1998; Shapiro et al., 1998). During metaphase (meiotic or mitotic) either cyclin B1-CDK1 or MAP kinase activity is capable of maintaining the metaphase state, which is normally characterised by condensed chromosomes and the absence of a nuclear envelope (Verlhac et al., 1994; Moos et al., 1996).

MPF is composed principally of a regulatory cyclin B subunit (Evans et al., 1983; Lohka et al., 1988; reviewed by Pines, 1996) and cyclin-dependent kinase (CDK1: Nurse and Thuriaux, 1980; Gautier et al., 1988; Labbe et al., 1989). Cyclin B1-CDK1 associated histone H1 kinase activity is a measure of the principal component of MPF. Activation of cyclin B1-CDK is dependent upon cyclin binding and a series of phosphorylation dephosphorylation reactions (see Morgan, 1997 for a review). The inactivation of cyclin B1-CDK is triggered by the proteolytic destruction of ubiquitin-tagged cyclin B via the cyclosome/APC (anaphase promoting complex) (Sudakin et al., 1995: King et al., 1995; Irninger et al., 1995). Destruction of cyclin B is dependent on the N-terminal 90 amino acids (Murray et al., 1989), which contain a sequence of nine amino acids (in position 42-50 in human cyclin B1) termed the destruction box that confers the ability to become ubiquitinated (Glotzer et al., 1991). A number of N-terminal lysine residues lying between the destruction box and up to the amino acid in position 90 subsequently become ubiquitinated (King et al., 1996; Yamano et al., 1998). Deletion of the first 90 amino acids from cyclin B (Δ90) thus renders the protein resistant to ubiquitination and destruction, but it can still interact with CDK1 and phosphorylate histone H1 (Murray et al., 1989; Glotzer et al., 1991).

It has been difficult to determine which of the two candidate kinases, MAP kinase or cyclin B1-CDK, are involved in controlling either the sperm-triggered calcium oscillations or the mitotic calcium signals, for the following reasons. Firstly, during fertilisation in the mouse oocyte correlative evidence would suggest that MAP kinase is a more likely candidate for involvement in the generation of calcium oscillations. This is because the calcium oscillations terminate just before pronuclear envelope formation (Jones et al., 1995), when the MAP kinase activity falls to low levels (Verlhac et al., 1994). Secondly, during mitosis it is difficult to determine whether B1-CDK1 or MAP kinase activity is involved in the generation of the mitotic calcium signals. This is because MAP kinase is now thought to become elevated during mitosis in sea urchin embryos (Philipova and Whitaker, 1998; Chiri et al., 1998) and somatic cells (Shapiro et al., 1998). However, MAP kinase activity is thought to be low in mouse oocytes during mitosis (Verlhac et al., 1994). Finally, our experiments in ascidian oocytes suggest that cyclin B1-CDK is the most likely candidate for controlling the calcium oscillations (McDougall and Levasseur, 1998). This is because sperm trigger two series of calcium oscillations in metaphase I-arrested ascidian oocytes, which trigger meiotic exit (McDougall and Sardet, 1995; Yoshida et al., 1998), and which terminate when the egg exits meiotic metaphase (Speksnijder et al., 1989). Each series of calcium oscillations correlates with elevated MPF activity, which is low during a gap phase equivalent to the time when the first polar body is extruded (McDougall and Levasseur, 1998). However, these data are all correlative. Neither the cyclin B1-CDK1 nor the MAP kinase activities have been manipulated directly in any system to resolve this issue.

We set out to manipulate the cyclin B1-CDK activity of the oocyte during fertilisation specifically and reversibly. To achieve this we chose two tools, mRNAs encoding full-length cyclin B1 and Δ90 cyclin B1, both tagged C-terminally with green fluorescent protein (GFP) to allow estimation of their intracellular protein concentration. Exit from metaphase arrest is initiated at fertilisation by a sperm-triggered increase in the internal concentration of free cytosolic calcium ions ($[Ca^{2+}]_i$) (Whitaker and Swann, 1993). The $[Ca^{2+}]_i$ increase reinitiates embryonic development by inactivating first the cyclin B1-CDK activity, through destruction of cyclin B, then CSF (Lorca et al., 1993). With these tools we have been able to manipulate the cyclin B1-CDK activity following the sperm-triggered calcium increases at fertilisation.

MATERIALS AND METHODS

Biological material

Specimens of the tunicate *Ascidiella aspersa* were collected locally on the North East coast and kept in the laboratory in a tank of sea water at 10°C. Oocytes and sperm were harvested by removing the outer tunic and then puncturing first the gonoduct followed by the sperm duct, taking care not to allow contamination of the oocytes with the sperm. Sperm were stored dry at 4°C, and activated 20 minutes prior to use by addition of chorionated oocytes in natural sea water and incubation at room temperature (this increases the rate of fertilisation; Sardet et al., 1989). Oocytes were immediately transferred to medium size Petri dishes containing 4.5 ml of filter-sterilised (0.2 μM pore size, Millipore) natural sea water. The eggs were first treated with 0.1% trypsin for 45-60 minutes, then

additionally sheared to remove the chorions. Following dechorionation, they were transferred to gelatin/formaldehyde (each 0.1%)-coated Petri dishes and kept on a cooled copper plate at 18°C until required (the formaldehyde was previously removed by air drying followed by washing with sea water). Any glass or plasticware subsequently used that came into contact with the oocytes was also treated with the gelatin/formaldehyde mixture.

Cyclin B1::GFP fusion constructs

The cyclin B1::GFP plasmid used (a gift from Jonathan Pines, Wellcome/CRC Institute, Cambridge, UK) consists of full-length human cyclin B1 fused via a 5-amino-acid linker (AGAQF) to the second amino acid residue of mmGFP, the mutations of which have been described (Zernicka-Goetz et al., 1996, 1997). This fusion had been cloned into a vector, pRN3 (Lemaire et al., 1995), specifically designed to produce mRNA transcripts with maximal stability (conferred by the presence of 5′ globin UTR (untranslated region) upstream and both 3′UTR and a poly(A)-encoding tract downstream of the gene to be transcribed). Truncated cyclin B1 (Δ90) was derived from full-length cyclin B1::GFP by a simple PCR approach. Primers for the region immediately downstream of the first 90 amino acids of cyclin B1 and to the 3′ end of mmGFP were used to amplify product using the full-length construct as target. Following suitable restriction digestion this product was subcloned into the pRN3 transcription vector (a gift from J. B. Gurdon, Wellcome/CRC Institute, Cambridge, UK).

mRNA was produced from both 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 oocytes (as outlined below) to approximately 5% of total oocyte volume (approximately equivalent to 100 pg of mRNA).

Microinjection and fluorescent measurement of calcium, GFP and DNA

Oocytes were introduced into a wedge based on the design of Keihart (1982). The microinjection technique has been described previously (McDougall and Levasseur, 1998). 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 specimen was epi-illuminated by the light from a Xenon lamp (Opti Quip, 150 W) that was band-pass-filtered through 350 nm, 365 nm, 380 nm and 488 nm filters to epi-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) to direct the light to the specimen and then to the collecting device. The emitted light was collected using a cooled (−10°C) charge-coupled device (CCD) camera (MicroMax, Sony Interline chip, 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. Calcium/GFP measurements were carried out using the 10× or the 20× objective lenses. For calibration of the GFP intensity we used the 20× lens. The 60× lens was used for monitoring the chromatin and the oocyte morphology. To visualise the DNA, oocytes were bathed for 15 minutes in the vital DNA dye Hoechst 33342 (10 µg/ml, Sigma) then returned to natural sea water.

Estimation of translation product concentration

Injecting known amounts of purified recombinant enhanced GFP (EGFP, Clontech), to produce a calibration curve of known amounts of EGFP injected against resulting green fluorescence permitted the estimation of protein product concentration. Since EGFP is known to have the same spectral properties as mmGFP (J. Pines, personal communication), amounts of mmGFP produced as a result of in vivo translation could easily be estimated by referring to the calibration curve. Since mmGFP is translated as a result of downstream fusion to both cyclin B1 and Δ90, the concentration of these proteins within

injected oocytes could be directly extrapolated from e[EGFP] values.

Immunoblotting

Proteins were separated by standard SDS-polyacrylamide gel electrophoresis on 7.5% gels and transferred to a PVDF membrane (BioRad) using a Mini Trans-Blot electrophoretic transfer apparatus (BioRad) according to the manufacturers' instructions.

Transfer was performed in double-strength buffer (50 mM Tris, 384 mM glycine, 20% v/v methanol, pH 8.3), which maximises transfer of proteins within the 40,000-200,000 kDa size range. Membrane blocking and antibody incubations were done in 2% BSA/3% non-fat milk powder in TBS (25 mM Tris, 150 mM NaCl, pH 8.0). Monoclonal anti-GFP antibody (Clontech) was used at a dilution of 1:1000, while polyclonal anti-cyclin B1 (Insight Biotechnology Ltd) was diluted 1:500. Washes were performed in TBS/Tween (TBS + 0.05% Tween 20), and bands were visualised using the ECL chemiluminescence system (Amersham) according to the manufacturers' protocol.

Histone H1 and MBP kinase assays

The histone H1 and MBP (myelin basic protein) kinase assays have been described in detail previously (McDougall and Levasseur, 1998) and were based on the methods described by Verlhac et al. (1994). Generally only three oocytes were taken for each sample, due to limitations in oocyte availability when assaying oocytes that had previously been injected with mRNA, but this number still gave an adequate signal in the final analysis. It is not yet feasible to perform an immunoprecipitation reaction with only 30-40 injected oocytes to determine whether it is CDK1 or CDK2 that interacts with the exogenous cyclin B1.

Treatment with pharmacological inhibitors roscovitine and U0126

Roscovitine (Calbiochem) was dissolved in DMSO (dimethyl sulphoxide) to give a stock concentration of 50 mM and subsequently dissolved in sea water; its effective concentration was determined by assaying for polar body extrusion. Concentrations of 200 µM resulted in polar body extrusion in 30 minutes. Oocytes were therefore treated with 200 µM roscovitine, injected with Fura-2 and fertilised once they displayed a polar body.

The MAP kinase inhibitor U0126 (Promega) was dissolved in DMSO to a concentration of 10 mM. Batches of eggs were treated with a range of concentrations of U0126 for 20 minutes and the MBP kinase activity measured as outlined above. Once we established that 10 µM U0126 resulted in maximal inhibition of MBP kinase activity, we treated unfertilised eggs with 10 µM U0126 then fertilised them 20 minutes later. We removed samples of these U0126-treated oocytes to measure the MBP kinase activity at 10 minute intervals after fertilisation and compared the MBP kinase activity to control untreated oocytes. Finally, we treated oocytes that contained the Δ90 cyclin B1-GFP fusion protein and Fura-2 with 10 µM U0126 for 20 minutes before adding sperm. We then measured the calcium oscillations triggered by sperm at fertilisation. The inhibitor was made up freshly before each use due to its' relative instability in DMSO (manufacturer states a 50% loss of activity within 1 week even when stored at –20°C).

RESULTS

Messenger RNA injected into unfertilised metaphase I-arrested ascidian oocytes is faithfully translated into protein product

Messenger RNAs (mRNAs) coding for either full-length cyclin B1 or Δ90 cyclin B1 (hereafter referred to as cyclin B1 and

Δ90, respectively) fused to the N terminus of mmGFP were synthesised by in vitro transcription, microinjected into *A. aspersa* oocytes and incubated at 19°C for at least 3 hours to allow sufficient accumulation of translation product (as indicated by the level of green fluorescence produced by the fused GFP). Following incubation and harvesting, translation products were analysed by western blotting (generally 30-40 oocytes were used for each sample lane) and probed with either anti-cyclin B1 (human) or anti-GFP antibodies. The approximate expected sizes for the two translation products are 97 kDa for full-length cyclinB1::mmGFP and 85 kDa for Δ90::mmGFP (Fig. 1).

When a western blot was probed with anti-cyclin B1 antibody, a discrete band of the expected size was obtained for oocytes injected with the full-length fusion mRNA. However, no bands were obtained in either the control lane (uninjected oocytes), as expected, or in the Δ90:mmGFP-injected sample lane (Fig. 1C). This is possibly because, although the antibody used was raised against full-length cyclinB1, the reactive epitope(s) may only lie in the N-terminal part of the protein, which in the case of Δ90 has been deleted.

This problem was resolved by probing a similar western blot with anti-GFP antibody. Again no signal was observed in the control lane (mRNA encoding GFP alone), proving that the antibody does not crossreact with any native ascidian oocyte proteins in this size range and that the high molecular mass reactive GFP epitope is produced only in cyclin containing

Fig. 1. To determine whether the injected mRNA to full-length and truncated cyclin B1-GFP was faithfully translated in the oocyte we probed western blots with anti-cyclin B1 and anti-GFP antibodies. (A) Schematic map of full-length human cyclin B1 coupled C-terminally to green fluorescent protein (mmGFP). The areas containing the destruction box (DB), the cyclin box and GFP are shown. (B) Schematic map of Δ90 cyclin B1 coupled C-terminally to mmGFP. Note the area containing the destruction box has been deleted whilst the area containing the cyclin box remains present. (C) Western blot analysis of 30 oocytes previously injected with mRNA coding for cyclin B1 or Δ90. Anti-cyclin B1 crossreacts with a protein of the expected molecular mass in cyclin B1 mRNA-injected oocytes, but not in control oocytes. This antibody does not crossreact with Δ90 cyclin B1, presumably because the reactive epitopes lie in the N-terminal region of cyclin B1. (D) Western blot analysis of 30 oocytes previously injected with mRNA encoding cyclin B1 or Δ90 cyclin B1. In Δ90 mRNA-injected oocytes a protein of the expected molecular mass is present. In cyclin B1 mRNA-injected oocytes a larger protein of the expected molecular mass is present. In control oocytes (mRNA encoding GFP alone) no cross reactivity is present.
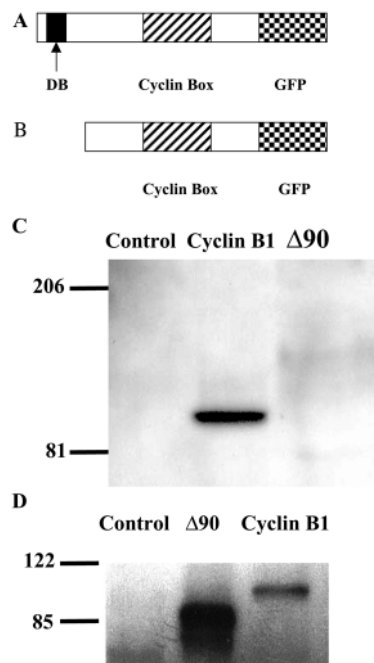
oocytes. Bands of the expected size were obtained for both the B1:GFP- and Δ90-injected samples, confirming that translation of the injected mRNAs was giving rise to the expected protein products (Fig. 1D).

Injection of cyclin B1 mRNA results in a prolonged period of cyclin B1-CDK activation

In order to determine cyclin B1-CDK levels in mRNA-injected oocytes, batches of 30-40 oocytes were first injected with cyclin B1 mRNA, harvested and incubated for at least 3 hours. Activated sperm were then added and, provided the oocytes fertilised synchronously, samples were taken at regular time intervals and used to perform histone H1 (a substrate of cyclin B1-CDK) kinase assays. Suitable non-injected controls were always included for comparison.

For oocytes injected with full-length cyclin B1, it can be seen that active cyclin B1-CDK levels remain elevated at 5 and 10 minutes post-fertilisation (Fig. 2), during which period the controls demonstrate their characteristic fall in cyclin B1-CDK activity and the Ca^{2+} oscillations are known to pause (*n*=4). Subsequent to this, the injected oocytes show a gradual decline in cyclin B1-CDK activity (*n*=4). We attribute this to the destructible nature of the exogenous full-length cyclin B1 and maintained activity of the APC/26S proteasome complex (Sudakin et al., 1995; King et al., 1995; Irninger et al., 1995; Kawahara and Yokasawa, 1994). Conversely, cyclin B1-CDK levels in control oocytes rise again sharply at 15 minutes, falling at 30 minutes coinciding with normal exit from meiosis; the histone H1 kinase activity only start to rise again as the first mitotic division occurs.

Injection of Δ90 mRNA results in persistently elevated levels of activated cyclin B1-CDK

A somewhat different situation is observed for oocytes injected with Δ90 mRNA (Fig. 3). Here the active cyclin B1-CDK levels remain elevated for the duration of observation (*n*=4).
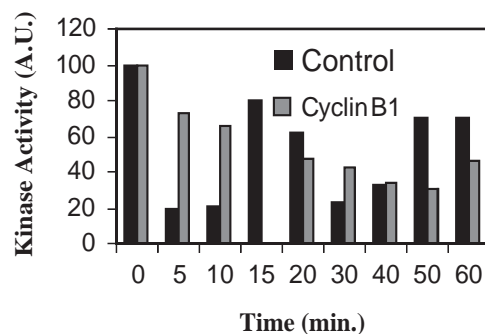

Fig. 2. The histone H1 kinase activity of control uninjected oocytes and oocytes injected with cyclin B1 mRNA. In control oocytes fertilisation triggers the H1 kinase activity to fall to low levels 5 minutes after fertilisation. The H1 kinase activity is low for a further 5 minutes before increasing to elevated levels 15 minutes after fertilisation. The H1 kinase activity finally declines to low levels 30 minutes after fertilisation when the oocyte exits meiosis. In cyclin B1 mRNA-injected oocytes the H1 kinase activity falls steadily to low levels over a longer period of time. In particular, in cyclin B1 containing oocytes the H1 kinase activity is elevated 5 and 10 minutes after fertilisation when it is low in control oocytes (*n*=4). A.U., arbitrary units.
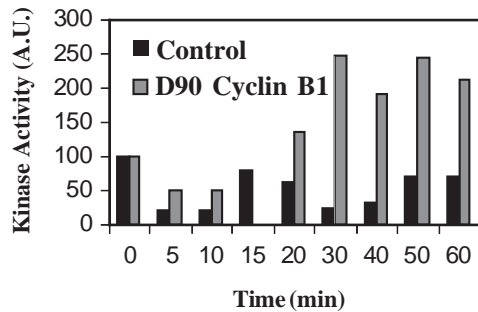
Fig. 3. The H1 kinase activity in control oocytes falls 5 minutes after fertilisation to low levels and stays low for a further 5 minutes. The H1 kinase activity increases and falls to low levels again, this time 30 minutes after fertilisation. The H1 kinase activity in Δ90 cyclin B1 mRNA-injected oocytes does not decline to low levels 5-10 minutes after fertilisation (but does decline). After a period of 20-30 minutes the H1 kinase activity begins to increase and reaches levels approximately double that found in unfertilised oocytes and remains at these levels throughout the remainder of the experiment (*n*=4). A.U., arbitrary units.

As was observed for cyclin B1 mRNA injected oocytes, levels remain elevated at 5 and 10 minutes (see above, Fig. 2), the period noted for displaying a decline in MPF in control oocytes. It should be noted that the cyclin B1-CDK activity does fall by 50%. We attribute this to the time it takes for Δ90 cyclin B1 molecules to form a kinase complex with their free CDK partners. The cyclin B1-CDK activity of full-length cyclin B1 also falls slightly during this period, to 60-70% the level found in unfertilised oocytes. The slightly higher level of kinase activity may be due to the higher levels of full-length cyclin B1 in the oocytes (compare Figs 5Bii and 6Bii). However, 20 minutes after fertilisation, Δ90-injected oocytes then proceed to show a marked rise in active cyclin B1-CDK. We conclude that this is due to the non-destructible nature of the Δ90 cyclin. Indeed, far from being destroyed, it continues to accumulate following fertilisation, and in so doing is capable of giving rise to approximately twofold higher levels of active cyclin B1-CDK.

Sustained elevation of cyclin B1-CDK activity prevents polar body extrusion and chromatin decondensation

We have previously reported (McDougall and Levasseur, 1998), in accordance with a result in another species of ascidian (Russo et al, 1996), that MPF activity falls each time a polar body is extruded. Here we show that maintaining elevated levels of active cyclin B1-CDK by injection of cyclin B1 mRNA results in a delay to extrusion of the first polar body of approximately 20 minutes (Fig. 4A). As we have shown above (Fig. 2), the presence of exogenous cyclin B1 results in a prolonged period of cyclin B1-CDK activation, which declines as the cyclin B1 is destroyed via the APC/26S proteasome. We conclude that in the oocyte pictured in Fig. 4A, reduction in cyclin B1-CDK activity to a level permissible for polar body extrusion was achieved after 26 minutes. We also observed decondensation of the chromatin, which we conclude is another indication of decreased cyclin B1-CDK activity at this time (Fig. 4B). In one oocyte the female pronucleus (♀) starts to decondense approximately 28 minutes
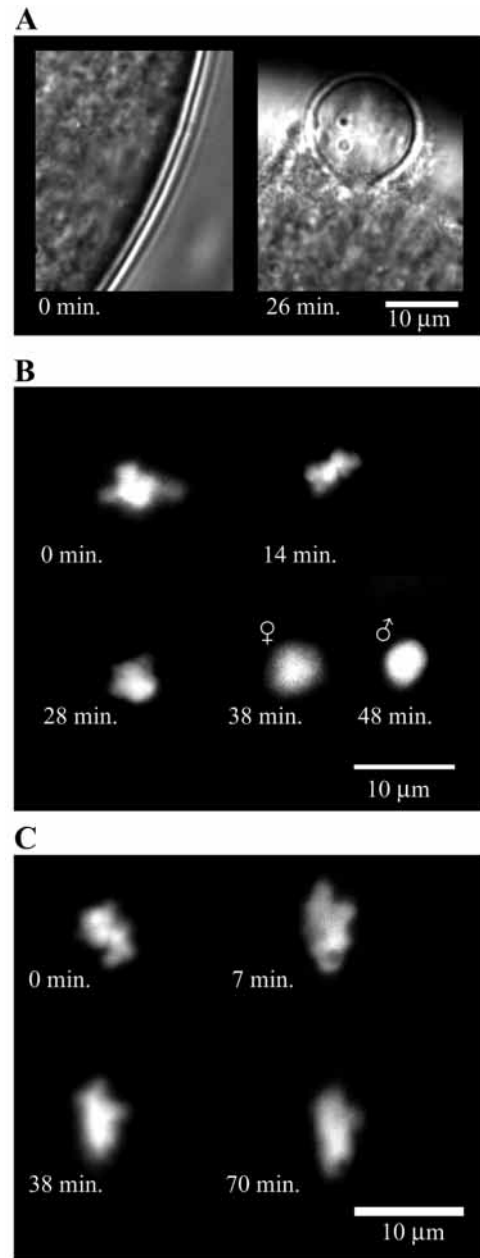

Fig. 4. The morphology of the fertilised oocyte after injection of either cyclin B1 or Δ90 cyclin B1 mRNA. (A) We monitored polar body formation in cyclin B1 mRNA-injected oocytes. Polar body formation was either completely abolished or substantially delayed. The example shows a polar body extruded 26 minutes after fertilisation (the first polar body is normally emitted 7-8 minutes after fertilisation). (B) Oocytes were injected with cyclin B1 mRNA and left for 3 hours. The oocyte chromatin was then stained with the vital DNA-specific dye Hoechst 33342 (10 μg/ml for 15 minutes). Following this the oocyte was fertilised and the changes in chromatin conformation recorded. In all oocytes the chromatin appeared to evolve with time towards an interphase-like state. In one out of five oocytes the chromatin decondensed at the time it would in control oocytes (e.g. 26-30 minutes after fertilisation), otherwise decondensation occurred at later times. In the oocytes pictured the chromatin is fully decondensed 38 (♀) and 48 (♂) minutes after fertilisation. (C) In Δ90 mRNA-injected oocytes the chromatin shows no visible indication of evolving with time towards a more decondensed form. These oocytes never display polar bodies (*n*=17).

after fertilisation and appears fully decondensed 38 minutes after fertilisation, while in another oocyte the male pronucleus (♂) appeared decondensed at around 48 minutes (Fig. 4B). Oocytes injected with cyclin B1 mRNA did not always manage to extrude a first polar body. This we attributed to levels of cyclin B1 translated: the more cyclin B1 translated (as indicated by intensity of green fluorescence), the longer the period became before extrusion occurred, and in some cases it was not seen at all within the period of observation (normally 60 minutes).

Oocytes injected with Δ90 on the other hand consistently failed to extrude a polar body (not shown, *n*=17), and no decondensation of the chromatin occurred during a 70 minute observation (Fig. 4C). Our conclusion is that this is due to maintenance of consistently high levels of active cyclin B1-CDK throughout this period (Fig. 3). Contrasting the appearance of the chromatin in cyclin B1 mRNA versus Δ90 mRNA-injected oocytes (Fig. 4B,C) further supports our conclusion that decondensation has occurred in the former, where pronuclei containing decondensed chromatin clearly form. No such changes can be observed in the Δ90 oocytes, consistent with the notion that here the chromatin remains condensed and pronuclei absent for the entire period of observation.

Calcium oscillations continue as long as cyclin B1-CDK activity remains elevated

We have previously shown (McDougall and Levasseur, 1998) that upon fertilisation, the oocytes of the ascidian *A. aspersa* display two phases of Ca^{2+} oscillations separated by a gap, during which the Ca^{2+} level is low and the first polar body is extruded. This gap phase also coincides with a period of low MPF activity. MPF activity rises again as the second phase of oscillations start and is finally inactivated, at which point the

A
(i)

(ii)

B
(i)

(ii)

C

Fig. 5. The sperm-triggered calcium oscillations do not pause during the gap phase and finally stop when the cyclin B1 levels fall. (Ai) GFP containing-oocytes were injected with Fura-2 dextran to give an intracellular concentration of approximately 20 μM. The oocytes were then inseminated 30 minutes later and the pattern of calcium oscillations recorded. Sperm trigger the normal pattern of calcium oscillations: a distinctive gap is evident after the initial calcium signal followed by a train of calcium increases (*n*=8). (Aii) The fluorescence from GFP was simultaneously measured in these oocytes (*n*=8). (Bi) The calcium oscillations in cyclin B1 mRNA-injected oocytes were measured through the use of Fura-2 dextran (20 μM intracellular concentration). In the presence of cyclin B1 the gap phase is clearly absent (*n*=18). The oscillation frequency is elevated during the gap phase (this is not always the case) and is within normal limits during the time that the second phase calcium oscillations would normally appear. (Bii) The cyclin B1 levels begin to fall following fertilisation. The initial drop in fluorescence is because the calcium signal triggers a cortical contraction and the oocytes sometimes move. The cyclin B1 levels begin to fall about 3 minutes after fertilisation and reach low levels about 25 minutes after fertilisation. (C) The entire duration of the calcium oscillations was plotted against the estimated concentration of cyclin B1-GFP in the unfertilised oocyte. For increased accuracy we used those data accumulated using the 20×/0.75 n.a. lens to calibrate the GFP levels (*n*=7). The curve represents the straight line that best fits these data (least-squares linear regression). These data show a significant linear relationship between the initial cyclin B1 levels and the duration of the calcium oscillations (*r*=0.94, *P*=0.0014).
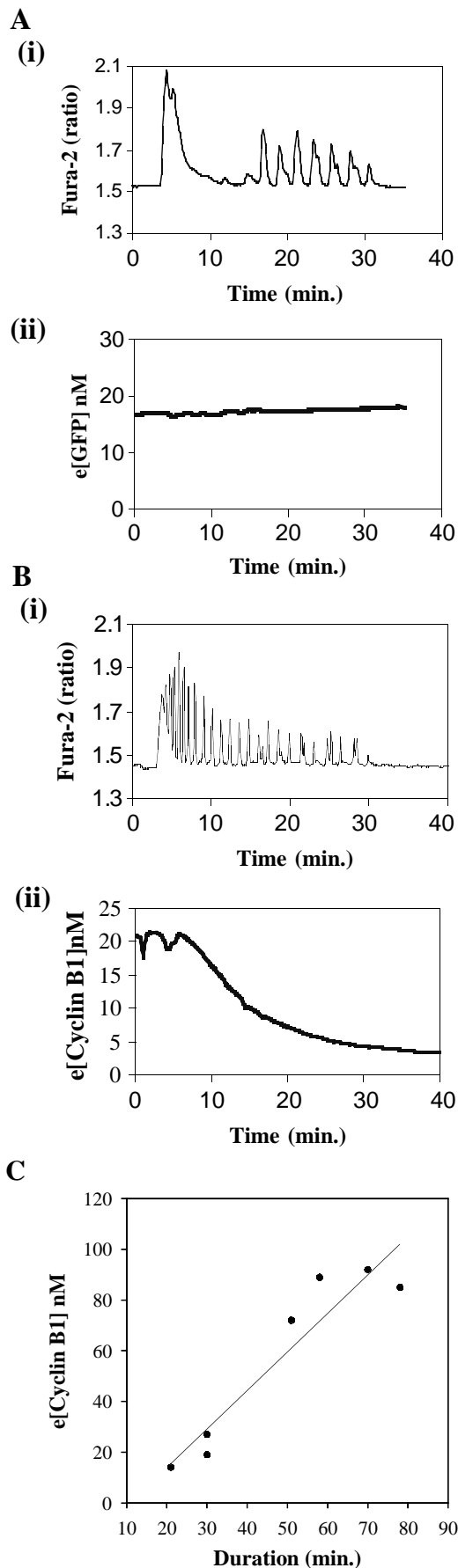
Ca^{2+} oscillations cease and the second polar body is extruded marking exit from meiosis.

Injection of mRNA coding for mmGFP alone produced a detectable green fluorescent signal within approximately 2 hours. Using the calibration system outlined above (see Materials and Methods) we calculated that the mmGFP was at a concentration of approximately 20 nM after 3 hours. At this point Fura-2 dextran was injected, activated sperm added and changes in intracellular Ca^{2+} recorded at fertilisation. In this way it was confirmed that the presence of translated mmGFP protein had no deleterious effects, as a normal pattern of calcium oscillations was always observed (n=8) for oocytes treated this way (Figs 5A, 6A). Accumulation of green fluorescent signal was also further confirmation of in vivo translation of injected mRNA, while the estimation of GFP protein concentration was used to quantify amounts of protein expressed from GFP fusion mRNAs.

Injection of cyclin B1 mRNA, shown to maintain transiently elevated levels of activated cyclin B1-CDK (Fig. 2), resulted in abolition of the gap in calcium oscillations (n=18) and elongation of the period over which the oscillations continue (Fig. 5B,C). This latter effect is entirely dose dependent: the higher the concentration of exogenous cyclin B1 (as inferred by levels of green fluorescence) within the oocyte, the longer the oscillations are seen to continue. This was shown by plotting oscillation periods against the estimated cyclin B1 concentration (e[cyclin B1], n=7) to produce a correlation coefficient (r) of 0.943 and a P value of 0.0014, obtained using linear regression analysis (Fig. 5C). This indicates that there is a significant linear correlation between the starting cyclin B1 concentration and the duration of the Ca^{2+} oscillations.

When GFP is coupled to the C terminus of full-length cyclin B1, the fluorescence from GFP is lost during the metaphase-anaphase transition (Clute and Pines, 1999), presumably through destruction of both cyclin B1 and GFP. The level of GFP fluorescence should therefore also serve as an indicator of destruction of the cyclinB1::GFP fusion protein via the APC/26S proteasome pathway (Sudakin et al., 1995; King et al., 1995; Irninger et al., 1995; Kawahara and Yokasawa,

Fig. 6. The sperm-triggered calcium oscillations do not pause during the gap phase and continue indefinitely in the presence of Δ90 cyclin B1 (n=17). (Ai) GFP mRNA-injected oocytes were injected with Fura-2 dextran to give an intracellular concentration of approximately 20 µM. Approximately 30 minutes later they were fertilised. These oocytes again displayed a normal pattern of calcium signals at fertilisation: two phases of calcium oscillations that lasted about 25 minutes in total separated by a 5-10 minute gap (n=8). (Aii) The GFP levels in these oocytes began to increase following fertilisation. (Bi) Δ90 cyclin B1 mRNA-injected oocytes were injected with Fura-2 dextran (20 µM cytosolic concentration) and inseminated 30 minutes later. Sperm triggered a series of calcium oscillations that did not pause during the gap phase. In addition to this, the calcium oscillations did not terminate during the entire 1 hour period of observation (n=17). The oscillation frequency sometimes declined 30-40 minutes after fertilisation. (Bii) The Δ90 fluorescence increased following fertilisation. (Ci) An oocyte injected with Fura-2 dextran (10-20 µM) was then fertilised. Two phases of calcium oscillations were clearly present. (Cii) An oocyte from the same batch was treated with 200 µM roscovitine for 30 minutes, was injected with Fura-2 dextran (10-20 µM) then inseminated, when it displayed one large polar body. The second phase of calcium oscillations was clearly absent (4/4 oocytes displayed this pattern).
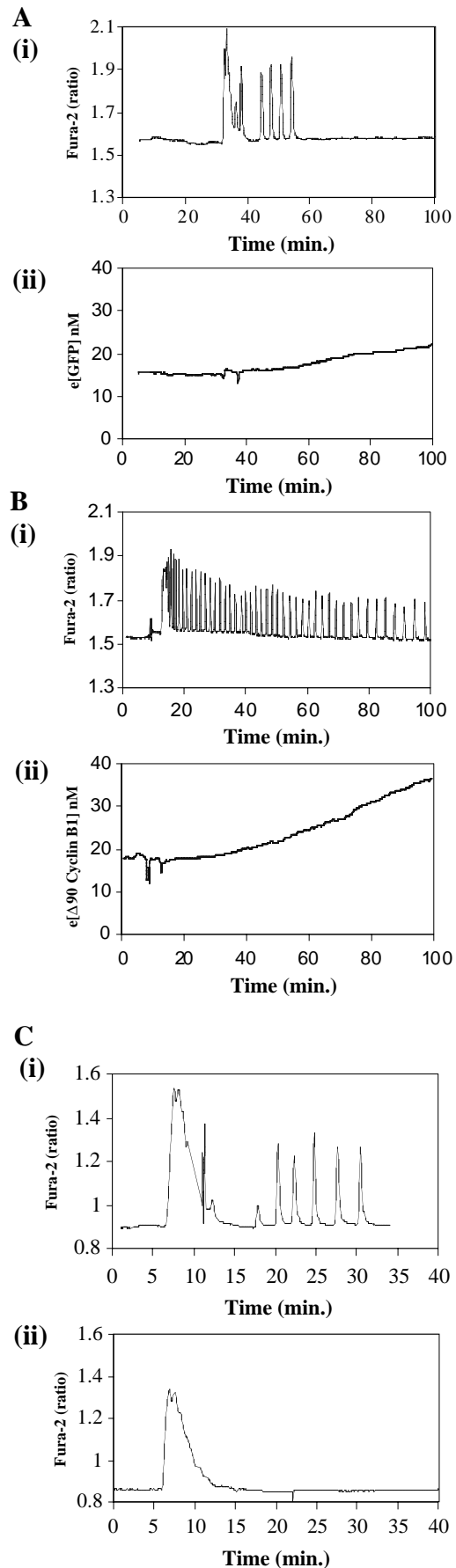
1994). Approximately 3 minutes after fertilisation, GFP fluorescence begins to decline quite sharply (Fig. 5B), clearly implying that the fusion protein is being targeted for destruction from this point onwards. This correlates temporally with the situation in normal ascidian oocytes, where the first phase of cyclin B1-CDK inactivation occurs approximately 5 minutes after fertilisation. Although fluorescence continues to decline, calcium oscillations continue, albeit at declining frequency and intensity, until green fluorescence has effectively reached basal levels. At this point, with exogenous cyclin B1 exhausted, the calcium oscillations stop.

Oocytes injected with $\Delta 90$ behave differently again (Fig. 6B). Oocytes from the same animal injected with GFP mRNA again gave a normal pattern of calcium oscillations when fertilised (Fig. 6A). Fertilised oocytes injected with $\Delta 90$ not only failed to produce a gap in Ca^{2+} oscillations, but also then continued to oscillate indefinitely (Fig. 6B), or as long as observation was continued, which in one case was for 4 hours ($n=17$). Lack of destruction was indicated by the fact that green fluorescence did not start to decline post-fertilisation (indeed it actually continued to increase slowly during the period of observation; Fig. 6B). This implies that levels of exogenous $\Delta 90$ remained high, and in so doing were able to maintain the persistently elevated levels of activated cyclin B1-CDK observed above (Fig. 3) in $\Delta 90$ mRNA-injected oocytes.

Oocytes were bathed in various concentrations of roscovitine to inhibit the cyclin B-dependent kinase activity (Meijer, 1996). We found that 200 μM roscovitine for 30 minutes resulted in polar body extrusion (the polar bodies were larger than normal). Fertilisation of these oocytes resulted in one calcium signal ($n=4$, Fig. 6C). The second phase of calcium oscillations were absent (4/4, Fig. 6C).

Inhibition of MAP kinase has no effect on $\Delta 90$ prolonged Ca^{2+} oscillations

In order to investigate the role, if any, played by MAP kinase in the control of the sperm-triggered calcium oscillations we used the specific MAP kinase kinase (MKK) inhibitor U0126 (Favata et al., 1998).

Firstly, we established a dose-response curve for the inhibitor, and in so doing were able to use the lowest concentration of U0126 that maximally inhibited MAP kinase activity. This was found to be 10 μM (Fig. 7A), a figure that agreed well with previous findings (Favata et al., 1998). We fertilised control oocytes in the presence of 10 μM U0126. Sperm triggered two phases of calcium oscillations, as they did in control oocytes (data not shown), and the HH1 kinase activity was present during the second phase of calcium oscillations as in control oocytes (data not shown). However, it could be argued that by inhibiting MAP kinase activity the MPF activity could also decline due to the CSF activity associated with MAP kinase (see Masui, 1991, for a review). Indeed, when oocytes are left for longer time periods in U0126 a pronucleus forms (data not shown). These oocytes do not display the second phase of calcium oscillations upon fertilisation (data not shown).

To be sure that cyclin B1-CDK activity was not indirectly affected by U0126, we treated oocytes containing $\Delta 90$ cyclin B1 with U0126 and fertilised them. Oocytes were injected with $\Delta 90$ and following the standard incubation period were subsequently divided into two batches. One batch was further
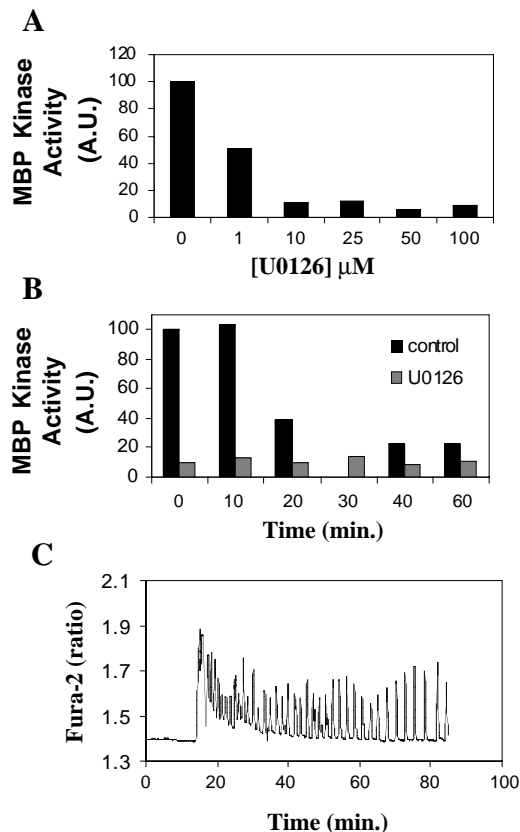
A

B

C

Fig. 7. The sperm-triggered calcium oscillations continue in the absence of MAP kinase if cyclin B1-CDK remains active. (A) The myelin basic protein (MBP) kinase activity of the unfertilised oocytes was measured in groups of oocytes treated with an MKK (mitogen-activated protein kinase kinase) inhibitor U0126 for 20 minutes. This dose response shows that 10 μM U0126 is effective as an inhibitor of MKK and leads to the loss of MAP kinase activity. (B) $\Delta 90$ cyclin B1 mRNA-injected oocytes were treated with 10 μM U0126 and fertilised. The MBP kinase activity was measured following fertilisation in U0126 treated oocytes and compared with control $\Delta 90$ containing oocytes. The MBP kinase activity in U0126-treated oocytes is clearly inhibited to low levels. (C) The calcium oscillations were measured in oocytes from the same batch as used for measuring the MBP kinase activity. As usual Fura-2 dextran was injected into oocytes to give an intracellular concentration of approximately 20 μM. Next the oocytes were bathed in 10 μM U0126 and fertilised 20 minutes later. The data clearly show that the calcium oscillation pattern is not altered by the presence of U0126 ($n=9$). A.U., arbitrary units.

injected with Fura-2 dextran and then fertilised after 20 minutes incubation in the presence of 10 μM U0126. The resulting Ca^{2+} measurements were essentially as previously observed for $\Delta 90$-injected oocytes (Fig. 7C), i.e. the oocytes showed a prolonged pattern of oscillations. However, higher concentrations of U0126 (100 μM) did inhibit the calcium oscillations: this effect may be non-specific since the MBP kinase activity is maximally inhibited by 10 μM U0126. In order to show that MBP kinase activity was low in the presence of U0126 in the batch of oocytes in which the calcium oscillations were measured, the second batch, also containing $\Delta 90$, was further split into two groups. One was fertilised and used to produce time points for a standard MBP kinase assay.

The other was first incubated for 20 minutes with 10 µM U0126, and then also fertilised to provide time points for a MBP kinase assay. The data clearly show that MBP kinase activity is reduced approximately tenfold by 10 µM U0126 compared with control untreated oocytes (Fig. 7B). From these data we conclude that MBP kinase has no effect on the prolonged Ca^{2+} oscillations seen in oocytes with persistently elevated cyclin B1-CDK levels (*n*=9).

DISCUSSION

Cyclin B1-CDK controls the generation of sperm-triggered calcium oscillations

We injected mRNAs encoding either full-length cyclin B1 or Δ90 in order to manipulate the cyclin B1-CDK activity. Once satisfied that these two tools did permit the manipulation of the oocyte cyclin B1-CDK activity, we measured the sperm-triggered calcium oscillations in either cyclin B1- or Δ90-containing oocytes. In oocytes containing cyclin B1, the calcium oscillations terminated when the cyclin B1 levels declined (Fig. 5). Furthermore, the calcium oscillations do not pause during the gap phase as they do in control oocytes (Fig. 5). These data further support the idea that the calcium signalling system is not desensitised during the gap phase as a consequence of depletion of intracellular calcium stores following the large initial calcium wave (McDougall and Levasseur, 1998). In addition, we noticed a clear correlation between the starting concentration of cyclin B1 and the duration of the calcium oscillations (Fig. 5). Furthermore, in Δ90 mRNA-injected oocytes the calcium oscillations continued indefinitely (Fig. 6). Finally, the second phase of calcium oscillations can be inhibited by 200 µM roscovitine (Fig. 6C), an inhibitor of cyclin B1-CDK activity (Meijer, 1996). These data therefore strongly imply that the cyclin B1-CDK activity of the oocyte controls sperm-triggered calcium oscillations. Sperm trigger an increase in InsP3 levels at fertilisation in sea urchin eggs (Ciapa and Whitaker, 1986). We can therefore speculate that sperm may also trigger an increase in InsP3 levels during the second phase of calcium oscillations in the tunicate egg; if this is the case then cyclin B1-CDK might control the generation of InsP3.

MAP kinase is not involved in generating the calcium oscillations at fertilisation

To determine whether MAP kinase affects the pattern of calcium oscillations triggered by sperm, we inhibited the upstream activators of ERK1 and ERK2, MKK1 and MKK2 pharmacologically with U0126 (Favata et al., 1998) in those oocytes containing Δ90 and measured the calcium oscillations. In *A. aspersa* we previously showed that the MAP kinase species behaved like rat p42 MAP kinase (ERK2) in an 'in-gel kinase' assay (McDougall and Levasseur, 1998). Even in the absence of MAP kinase activity, when the oocytes contain Δ90 cyclin B1 we found that the calcium oscillations are prolonged (Fig. 7). In addition, fertilisation of control oocytes treated with U0126 results in the generation of two phases of calcium oscillations (data not shown). Elevated H1 kinase activity accompanies the second phase oscillations, as would be expected if cyclin B1-CDK activity controls the generation of calcium oscillations (data not shown). Furthermore, incubating oocytes in 10 µM U0126 for 120 minutes results in pronuclear formation, indicating, as expected, that the MPF activity eventually declines when the MAP kinase activity is lost; when such oocytes are fertilised they only give one or two calcium signals (data not shown). This is presumably because they have no MPF activity. These data therefore suggest that the cyclin B1-CDK activity of the oocyte controls the generation of periodic calcium signals.

Sperm-triggered calcium oscillations in other species

In the ascidian, the second phase of calcium oscillations occurs when two criteria are met: sperm have entered the egg and cyclin B1-CDK is active. In other words, the sperm-triggered calcium oscillations occur during meiotic metaphase II. In other species sperm-triggered calcium oscillations are also displayed during meiosis (see Sardet et al., 1998, for a review). We would therefore predict that those oocytes that are fertilised either before entry into meiotic metaphase (at the GV stage) or after exit from meiosis (at the G_1 stage) would not display calcium oscillations. Some molluscs are fertilised at the GV stage, e.g. *Mactra chinensis* and some echinoderms at the G_1 stage, e.g. sea urchins. In both these species of oocyte sperm trigger only one calcium signal (Deguchi and Osanai, 1994; Steinhardt et al., 1977). In addition, since meiotic exit is rapid (about 10 minutes following fertilisation) in those oocytes where sperm initiate only one calcium signal during meiosis (*Xenopus* and *Medaka*), we would not expect to see calcium oscillations since the cyclin B1-CDK activity would fall rapidly.

The only apparent exception is given by mammalian oocytes. The calcium oscillations at fertilisation in the mouse oocyte clearly correlate with pronuclear reformation and not polar body extrusion (Jones et al., 1995). Pronuclear formation occurs when the MAP kinase activity declines and polar body formation when the MPF activity declines (Verlhac et al., 1994). How then can we reconcile these data with our observations in the ascidian? In rabbit oocytes a single calcium stimulation initiates MPF inactivation, but multiple stimuli are necessary to maintain the kinase inactive (Collas et al., 1995). Enough cyclin B1 must therefore be available even after inactivation of the MPF activity to reform active MPF. It would be useful to have a measure of MPF activity in single fertilised oocytes since there is the possibility that the MPF activity will oscillate, falling immediately after a calcium signal. Such oscillating levels of MPF activity could easily be missed when several oocytes are used to measure bulk MPF activity.

Although we would argue that cyclin B1-CDK is involved in controlling sperm-triggered calcium oscillations, we do not find that cyclin B1-CDK is involved in generating the first calcium signal (Fig. 6C). Also, in those oocytes that are fertilised either before entry into meiotic metaphase (during the GV stage: *Mactra chinensis*) or after exit from meiosis (during G_1: sea urchin), the cyclin B1-CDK activity is low yet they display a monotonic calcium signal (although mammalian oocytes are the exception here too). We would therefore suggest that the mechanism responsible for generating the calcium signal is already primed before delivery to the egg, and that it is the cyclin B1-CDK activity that reactivates it.

The mechanisms of fertilisation

Our model does not differentiate between the two hypotheses of egg activation, the receptor hypothesis (Jaffe et al., 1988; review by Evans and Kopf, 1998) and the sperm factor hypothesis (Swann, 1990; reviewed by Fissore et al., 1999). The discovery that the calcium-releasing second messenger InsP$_3$ increases at fertilisation is a central starting point of fertilisation (Ciapa and Whitaker, 1986). The receptor hypothesis takes as the starting point that sperm trigger the calcium signal in the egg through interaction between factors on the sperm and egg surfaces. This interaction is thought to lead to the activation of an egg phospholipase C (PLC) and the generation of InsP$_3$ in the egg. However, the molecular identity of the interacting ligands remains unknown. The sperm factor hypothesis takes as the starting point sperm-egg fusion, and the subsequent delivery of a sperm factor into the egg cytosol (Swann, 1990). Sperm cytosolic extracts faithfully mimic the pattern of calcium oscillations triggered by sperm at fertilisation in mammals (Swann, 1990), nemerteans (Stricker, 1997) and ascidians (Kyozuka et al., 1998). Such a factor has been proposed to be a high molecular mass protein (Parrington et al., 1996; Wu et al., 1998). All attempts to determine the identity of such a molecule have so far proved unsuccessful, although PLC (Jones et al., 1998) and a truncated form of the c-kit tyrosine kinase present in spermatozoa remain ongoing candidates (Sette et al., 1998).

Whatever mechanism operates at fertilisation, we suggest that cyclin B1-CDK activity is required to sustain periodic calcium increases in ascidian oocytes. This mechanism could also be involved in the generation of the mitotic calcium signals of early embryos (Groigno and Whitaker, 1998; Kono et al., 1996).

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