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Maturation Promoting Factor in ascidian oocytes is regulated by different intracellular signals at meiosis I and II

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SUMMARY

Using the fluorescent dye Calcium Green-dextran, we measured intracellular Ca^{2+} in oocytes of the ascidian *Ciona intestinalis* at fertilization and during progression through meiosis. The relative fluorescence intensity increased shortly after insemination in a single transient, the activation peak, and this was followed by several smaller oscillations that lasted for approximately 5 minutes (phase 1). The first polar body was extruded after the completion of the phase 1 transients, about 9 minutes after insemination, and then the intracellular calcium level remained at baseline for a period of 5 minutes (phase 2). At 14 minutes postinsemination a second series of oscillations was initiated that lasted 11 minutes (phase 3) and terminated at the time of second polar body extrusion. Phases 1 and 3 were inhibited by preloading oocytes with 5 mM heparin. Simultaneous measurements of membrane currents, in the whole-cell clamp configuration, showed that the 1-2 nA inward fertilization current correlated temporally with the activation peak, while a series of smaller oscillations of 0.1-0.3 nA amplitude were generated at the time of the phase 3 oscillations.

Biochemical characterization of Maturation Promoting Factor (MPF) in ascidian oocytes led to the identification of a Cdc2-like kinase activity. Using $p13^{suc1}$-sepharose as a reagent to precipitate the MPF complex, a 67 kDa (67×10^3 M_r) protein was identified as cyclin B. Histone H1 kinase activity was high at metaphase I and decreased within 5 minutes of insemination reaching a minimum level during phase 2, corresponding to telophase I. During phase 3, H1 kinase activity increased and then decayed again during telophase II. Oocytes preloaded with BAPTA and subsequently inseminated did not generate any calcium transients, nonetheless H1 kinase activity decreased 5 minutes after insemination, as in the controls, and remained low for at least 30 minutes. Injection of BAPTA during phase 2 suppressed the phase 3 calcium transients, and inhibited both the increase in H1 kinase activity normally encountered at metaphase II and second polar body extrusion.

Key words: MPF, calcium oscillation, meiosis, ascidian, oocyte, *Ciona intestinalis*

INTRODUCTION

In mammals and anurans, fully grown immature oocytes are arrested at first prophase (meiosis ENTRY) and resume the cell cycle in response to maternal hormones. The cell cycle is then arrested a second time at metaphase II and the oocyte remains in this state until activated by the fertilizing spermatozoon (Monroy 1965; Dale, 1983; Whitaker and Patel, 1990). M-phase or Maturation Promoting Factor (MPF), involved in the regulation of the meiotic cycle, is a heterodimer of at least two components: a catalytic subunit, the Cdc2 protein kinase, and a regulatory subunit, cyclin B (Hunt, 1989; Dorée, 1990; Norbury and Nurse, 1992; King et al., 1994; Nurse, 1994). In mammalian and frog metaphase II oocytes, MPF activity is maintained high by the activity of a cytostatic factor (CSF), whose catalytic subunit is the product of the c-*mos* proto-oncogene (Masui and Market, 1971; Sagata et al., 1989). In many deuterostomes, oocyte activation is accompanied by a large single calcium transient, generated from intracellular sources, that starts at the point of spermatozoon entry and traverses the oocyte in a wave to the antipode (Jaffe, 1985). This transient increase in calcium is thought to play a key role in the cell decision to progress through metaphase (Jaffe, 1985; Whitaker and Patel, 1990), by decreasing the activities of CSF and MPF (Meyerhof and Masui, 1977; Newport and Kirschner, 1984; Sagata et al., 1989; Watanabe et al., 1989); however, the interaction of this calcium signal with MPF, CSF and the other events leading to the completion of meiosis have not yet been resolved (Watanabe et al., 1991).

Meiotic progression in ascidian and some annelid oocytes appears to be regulated differently to that in vertebrate oocytes. Here, the signal for meiosis ENTRY is not known; however, the second meiotic block occurs earlier than in vertebrates with the oocytes arresting at metaphase I (Dale, 1983). Consequently, activation by the fertilizing spermatozoon drives the cell through two nuclear cycles without any detectable interphase and without DNA replication. Little is known about the kinetics of MPF in these oocytes. Despite this difference in

meiotic block, in both mammalian and ascidian oocytes, the large calcium wave is followed by a series of smaller repetitive waves (Cuthbertson and Cobbold, 1985; Speksnijder et al., 1989; McDougall and Sardet, 1995). In ascidian oocytes, the initial calcium wave is preceded by a 1-2 nA inward current across the plasma membrane (Brownlee and Dale, 1990), while in mammalian oocytes the calcium transients trigger hyperpolarizations in the plasma membrane by gating potassium channels (Miyazaki, 1988). Recently, it has been shown in oocytes of the ascidian *Phallusia mammillata*, that postactivation calcium waves are required to complete meiosis, and while the first calcium wave is initiated in the animal hemisphere, the latter waves are generated at the vegetal hemisphere (McDougall and Sardet, 1995).

The ascidian oocyte is an interesting model to study cell cycle regulation in meiosis. The cells are perfectly synchronized in metaphase I and fertilization induces rapid progression through meiosis. The purpose of the present study was to correlate intracellular calcium waves in *Ciona intestinalis* oocytes with MPF activity, plasma membrane currents and meiosis progression and, in particular, to compare meiosis I with meiosis II.

MATERIALS AND METHODS

Collection and fertilization of oocytes in vitro

Metaphase I-arrested oocytes were collected from the oviducts of the ascidian *Ciona intestinalis* from the Bay of Naples and washed several times in sea water. The chorion and follicle cells were removed manually using sharp steel needles or treatment with 0.1% trypsin as described (McDougall and Sardet, 1995). Dechorionated oocytes were kept in 0.1% gelatin-formaldehyde-coated Petri dishes to prevent lysis of nude oocytes (Sardet et al., 1989). Spermatozoa were collected with a fine Pasteur pipette and diluted 1,000-fold in sea water immediately before insemination.

Metabolic labeling of oocytes

Unfertilized oocytes obtained from a single adult animal were trypsin dechorionated and transferred in aliquots of 1.5 ml to 15 mm multidishes previously coated with gelatin-formaldehyde. After addition of a mixture of ^{35}S-labeled amino acids (Pro-mix, Amersham) to a final concentration of 330 μCi/ml for 70 minutes at 25°C, oocytes were fertilized and harvested at different times after sperm addition. The samples were briefly centrifuged to eliminate the excess of labeled mixture and the pellets were stored at –80°C for further analysis. Control experiments were performed as above without the addition of ^{35}S-amino acids.

p13^{suc1}-sepharose binding, electrophoresis and immunoblotting

Cell lysates were prepared from labeled and unlabeled oocytes at different times after fertilization by the addition of a lysis buffer (LB, 50 mM Tris-HCl, pH 7.4; 500 mM NaCl; 1% Nonidet P-40; 10 mM EDTA; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10% glycerol; 0.5 mM dithiothreitol) containing protease inhibitors (100 mg/l phenylmethylsulfonyl fluoride; 100 mg/l tosyl-phenyl-chloromethyl ketone; 1 mg/l leupeptin; 0.83 mg/l chymostatin; 10 mg/l soybean trypsin inhibitor; 1 mg/l pepstatin) and phosphatase inhibitors (20 mM 4-nitrophenyl phosphate; 1 mM sodium vanadate; 40 mM sodium fluoride; 1 mM sodium pyrophosphate). After determination of protein concentration (Bradford, 1976), equal amounts of total protein (usually 20 μg) were diluted to 0.5 ml in LB and incubated for 4 hours at 4°C with rocking in the presence of 30-40 μl of p13^{suc1}-sepharose (1:1 suspension in phosphate buffer saline solution, PBS) prepared as described (Brizuela et al., 1987). Finally, p13^{suc1}-sepharose complexes were washed twice in 0.5 ml LB and used either for immunoblotting or for kinase assays.

Immunoblotting was performed following standard procedures (Harlow and Lane, 1988). Briefly, total cell lysates or p13^{suc1}-sepharose complexes were added with 2× Laemmli sample buffer and heated for 5 minutes at 95°C before electrophoresis in 12% SDS-polyacrylamide gel. After blotting, the nitrocellulose membrane (Hybond-extra, Amersham) was incubated with a polyclonal antibody raised against the conserved peptide PSTAIRE included in the Cdk family of cell cycle regulated kinase (purchased from Santa Cruz Biotechnology) diluted 1:1,000 in PBS containing 0.1% Tween-20. As secondary antibodies, both goat anti-rabbit IgG alkaline phosphatase-conjugated (Sigma), or donkey anti-rabbit IgG peroxidase-conjugated (Amersham) were used. The detection of Cdc2-like protein was performed using a chemioluminescence method (ECL, Amersham), or a colorimetric assay for alkaline phosphatase (SIGMA-*FAST*, Sigma) following in both cases the manufacturer's instructions.

As molecular weight standards, we used the following: prestained markers from Biorad (phosphorylase B, 112 kDa; bovine serum albumin, 84 kDa; ovalbumin, 53.2 kDa; carbonic anhydrase, 34.9 kDa; soybean trypsin inhibitor, 28.7 kDa; lysozyme, 20.5 kDa) and low range markers from Pharmacia (phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; a-lactalbumin, 14.4 kDa).

Calcium measurements and microinjection

The fluorescent calcium indicator, Calcium Green dextran (M_r 10,000; Molecular Probes) was prepared as a stock solution in distilled water (100 mg/ml) and subsequently diluted to 5 mg/ml in intracellular-like solution (ICS) containing 10 mM Hepes pH 7.5; 200 mM K_2SO_4; 20 mM NaCl. The dye was injected into oocytes in the whole-cell clamp configuration as described previously (Brownlee and Dale, 1990), using a pressure injection system (Pico-spritzer, General Valve Corp.). To measure fluorescence intensity, a photomultiplier system (CRS-400, Biorad) in conjunction with an inverted microscope (Axiovert 135, Zeiss), was used. A 75W Xenon lamp with 490 nm excitation and 530 nm emission band pass filters were employed for the fluorescence measurements of the entire oocyte (140 μm diameter). Oocytes were exposed to the excitation beam for 200 μsec at 1 second intervals. Pharmacological agents were microinjected into oocytes in the whole-cell configuration, while a second electrode containing ICS was used to record whole-cell currents as described previously (DeFelice et al., 1986). BAPTA (Sigma) and heparin (ICN) were injected into oocytes in the whole-cell clamp configuration at pipette concentrations of 50 mM and 125 mM in ICS, respectively. The volume injected ranged from 1-10% of the oocyte volume.

MPF and MAP kinase assays

MPF kinase activity was determined by measuring the enzymatic activity of its catalytic subunit, Cdc2. The assay was performed as described previously (Marshak and Carroll, 1991; Marshak et al., 1991). Briefly, aliquots of 5-10 dechorionated oocytes were collected at different times after fertilization in a minimal volume of sea water and lysed in LB. The reaction mixture, in a final volume of 50 μl contained: Cdc2 buffer (50 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM EGTA); 50 μM [γ-^{32}P]ATP (1,000-2,000 cts/minute/pmol); 2.5 mM H1-derived peptide (H1-peptide; Beaudette et al., 1993) corresponding to residues 9-18 of human histone H1 (sequence: PKTPKKAKKL; purchased from Promega). The mixture was incubated at 30°C for 45 minutes and the reaction stopped by adding trichloroacetic acid to a final concentration of 10% (w/v) in the presence of bovine serum albumin (bsa) as carrier (Marshak et al., 1991). The radioactivity incorporated in the peptide substrate was determined using phosphocellulose paper (P81 chro-

matography paper, Whatman), or the Spinzyme units (Pierce) according to the manufacturer's instructions.

When p13^{suc1}-sepharose complexes were used as an enzymatic source, the beads were washed four times in 0.5 ml of Cdc2 kinase buffer before adding the reaction mixture described above. At the end of the incubation, the samples were centrifuged for 2 minutes, the supernatants were collected and the radioactivity incorporated in the peptide substrate measured. In some cases, after the kinase reaction, the p13^{suc1}-sepharose complexes were denatured by addition of electrophoresis sample buffer and analyzed for Cdc2.

MAP kinase was assayed using a kit from Amersham based on a peptide derived from EGF receptor and containing a highly specific MAP kinase phosphorylation site. The assays were performed following the manufacturer's instructions with some modifications. Briefly, 5 μl of cell lysate (corresponding to 10 μg of total proteins) were added to a reaction mixture containing 10 μl of substrate buffer and 15 μl of [γ-^{32}P]ATP (50 μM; 5,000 cts/minute/pmol). The reaction was incubated for 60 minutes at 30°C, and stopped by adding 10 μl of stop reagent. The amount of phosphorylated peptide was determined as described above for Cdc2 kinase assay.

Data presentation

The mean ± s.e.m. was calculated from a minimum of three independent experiments. Owing to half-life of [γ-^{32}P]ATP and the variable number of oocytes used in the assays, the kinase activity is expressed in arbitrary units (cts/minute×10^{-3}).

RESULTS

Characterization of MPF in *Ciona intestinalis* oocytes

In order to characterize the MPF complex in ascidian oocytes, we measured the kinase activity associated with Cdc2, the catalytic subunit of MPF, in a total cell lysate of unfertilized oocytes. To avoid possible cross-reactivity due to other kinases potentially able to phosphorylate histone H1, the classical Cdc2 substrate, we used the synthetic peptide H1-peptide described in the Materials and Methods. We also precipitated the MPF complex from the same cell lysate using p13^{suc1}-sepharose, a reagent known to bind preferentially to mitotic form(s) of Cdc2 (Dunphy et al., 1988), although it also has a low cross-reactivity with p42 MAP kinase (Shibuya et al., 1992), and we determined the kinase activity associated with the complex. The enzymatic activity towards H1-peptide is detectable in the total cell lysate as well as in the p13^{suc1}-sepharose precipitate (Fig. 1A). This confirms that the measured enzymatic activity was related to Cdc2 kinase, or at least to a Cdc2-like kinase. Similar results were obtained using as Cdc2 substrate the histone H1 (full protein) or a synthetic peptide derived from SV40 Large T antigen and containing the residue phosphorylated in vivo and in vitro by human Cdc2 (Marshak et al., 1991; data not reported). To further characterize the Cdc2-like kinase(s) present in ascidian oocytes, we determined the presence of the protein by immunoblotting. Since there are no antibodies to the specific subunits of MPF in the ascidian, we used several antisera raised to Cdc2 polypeptides from a variety of species. As shown in Fig. 1B, only the anti-PSTAIRE antibody recognized a protein of about 35 kDa on immunoblotting derived from a whole-cell lysate of ascidian oocytes (lane 2) and corresponding to the expected molecular weight of Cdc2. Anti-PSTAIRE antibody also recognized two other polypeptides of 53 kDa and 29 kDa, not present in the control experiment (lane 1). Immunoblotting after p13^{suc1}-sepharose precipitation of the cell lysate (Fig. 1C) showed only a band at 35 kDa, confirming the specificity of p13^{suc1}-sepharose for mitotic Cdc2. In the experiment in Fig. 1C, Cdc2-like protein is present as a double band probably corresponding to the different phosphorylation state(s) of the molecule as reported for Cdc2 in many different organisms (reviewed in Nurse, 1990; Clarke and Karsenti,

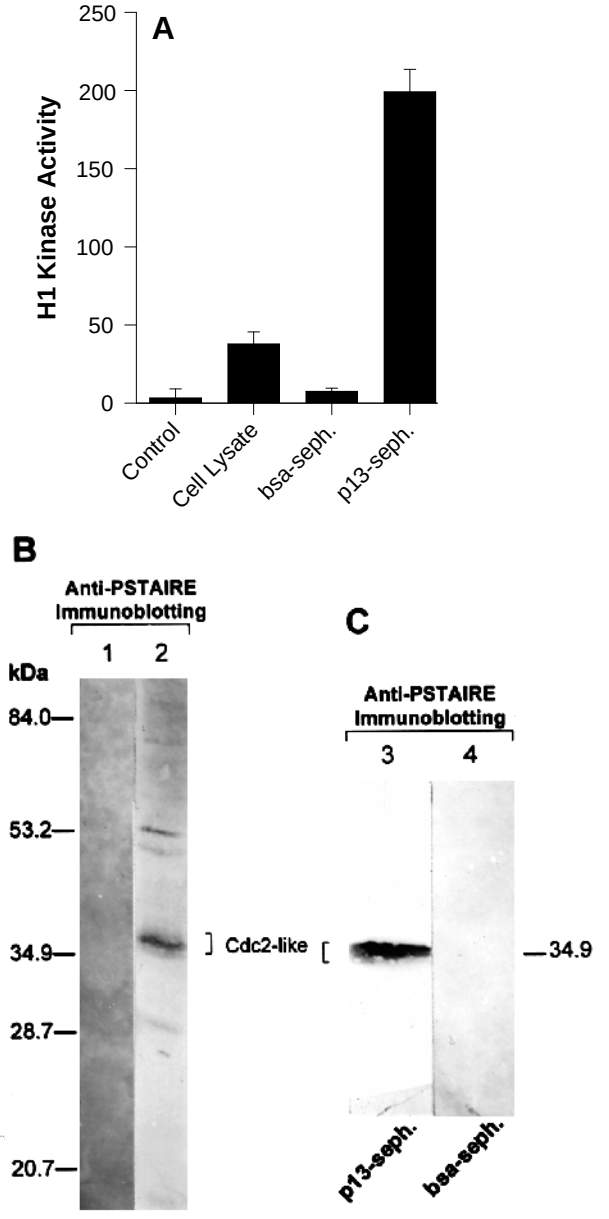

Fig. 1. Characterization of Cdc2-like kinase in unfertilized oocytes of *Ciona intestinalis*. (A) H1 kinase activity was measured on the total cell lysate (6 μg) as well as after p13^{suc1}-sepharose precipitation using the same cell lysate (50 μg). Bsa cross-linked to sepharose was used as a control for p13^{suc1}-sepharose precipitation. H1-peptide was used as the Cdc2 substrate. Immunoblotting of the total cell lysate (B), or p13^{suc1}- and bsa-sepharose precipitation (C) were probed with anti-PSTAIRE antibody (lane 2, 3, 4) or with anti-PSTAIRE antibody preincubated with the antigenic peptide (lane 1) as a control. (B,C) Cdc2-like bands were visualized using an alkaline phosphatase detection method. The molecular weight standards indicated were from Biorad.

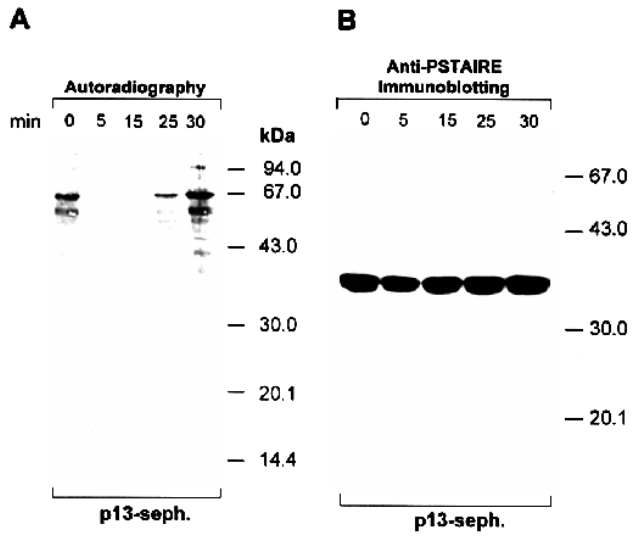

Fig. 2. Characterization of B-type cyclin in *Ciona intestinalis* oocytes at fertilization. (A) Oocytes were metabolically labeled with ^{35}S-amino acids before p13^{suc1}-sepharose precipitation. After electrophoresis, the samples were blotted to nitrocellulose and subjected to autoradiography. The film was exposed for 5 days at –70°C with intensifying screen. (B) The same nitrocellulose membrane was subsequently used to detect the presence of Cdc2-like protein(s). The bands on the immunoblotting were detected using a chemioluminiscence method. The molecular weight markers indicated were from Pharmacia.

1991). In fact, an anti-phospho tyrosine monoclonal antibody recognized ascidian Cdc2 on immunoblotting (Russo and Dale, unpublished data).

Cyclin B, the regulatory subunit of MPF, has been studied in many species; however, there is no data on the ascidian homolog of this protein. As for Cdc2, antibodies to cyclin B from different sources did not show any cross-reactivity towards ascidian cyclin B. To measure cyclin B levels in *Ciona intestinalis*, unfertilized oocytes were labeled with ^{35}S-amino acids for 70 minutes and aliquots were taken at different times after fertilization. The MPF complex was precipitated using p13^{suc1}-sepharose and the labeled proteins were analyzed by electrophoresis followed by blotting on nitrocellulose before autoradiography. Fig. 2A shows the presence of major protein of 67 kDa in unfertilized oocytes (metaphase I), that disappears 5 minutes after fertilization (anaphase I) and is synthesized again at metaphase II (25-30 minutes) implying it may be a B-type cyclin. In addition to the band at 67 kDa, other molecules at lower molecular weight are present with a similar pattern of synthesis and degradation and possibly related to different cyclinB-type proteins. The nitrocellulose membrane used for autoradiography in Fig. 2A, to detect cyclin synthesis, was also subjected to immunoblotting using anti-PSTAIRE antibody. It showed a double band in the p13^{suc1}-sepharose precipitates of 35 kDa corresponding to a Cdc2-like protein (Fig. 2B).

These experiments suggest that the MPF complex in ascidian contains Cdc2 and cyclin B-like molecules.

MAP kinase activity

Members of the MAP kinase family have been recently identified as key regulatory molecules in the control of meiosis (Haccard et al., 1993; Nebreda and Hunt, 1993; Minshull et al., 1994). There is no data on MAP kinase activity during fertilization in ascidian oocytes. The MAP kinase consensus sequence for phosphorylation overlaps that of the Cdc2/Cdk family of protein kinases and several putative substrates including myelin basic protein and histone H1 are targets for both kinases. To avoid cross-reactivity between Cdc2 and MAP kinase, we used a commercially available kit (Amersham) including as MAP kinase substrate a synthetic peptide not phosphorylated by Cdc2 kinase(s). Fig. 3 shows that MAP kinase activity is high in unfertilized oocytes of *Ciona intestinalis* and it increases to a maximal level at 5 minutes after fertilization followed by a decrease at the time of extrusion of the first polar body (telophase I). The enzyme activity did not show any further variation during meiotic division II. Under our assay conditions, p13^{suc1}-sepharose did not precipitate any significant MAP kinase activity (data not shown) as reported for other systems (Shibuya et al., 1992). Finally, using an antibody against mammalian p44 MAP kinase, we detected a band of 44 kDa in ascidian cell lysates whose intensity did not change during fertilization (Russo and Dale, unpublished data).

Intracellular calcium and H1 kinase activity

With our system, calcium measurements at 1 second intervals were possible for periods of up to 1 hour. As shown in Fig. 4 (middle panel), soon after the addition of spermatozoa to the bath, fluorescence intensity levels increased, peaked within 15-20 seconds and returned to resting values within 60-90 seconds (Fig. 3; n=20). A previous study showed that this fluorescence increase corresponds to peak calcium levels of approximately 10 μM (Brownlee and Dale, 1990). The initial wave, the activation wave, was followed by 5-7 (6±1.4; n=6) smaller transients that peaked in 10-15 seconds and returned to baseline in 10-15 seconds. The first group of oscillations lasted for a mean time of 4.9±0.9 minutes (n=6) and was nominated phase 1. The signal then remained at baseline values for a mean time of 5.0±1.0 minutes (n=6, phase 2), when a second series of small oscillations was initiated. These later transients (mean number

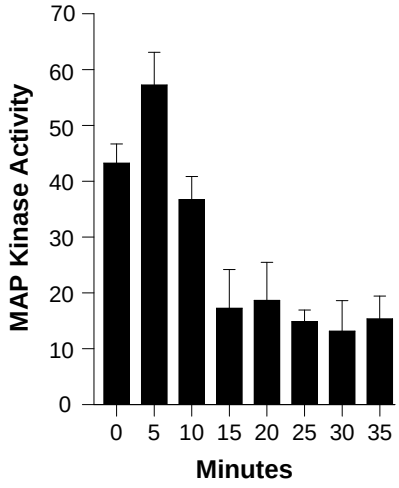

Fig. 3. MAP kinase activity in C*iona intestinalis* oocytes at fertilization. Enzyme activity was measured on the total cell lysate (10 μg) at the time points indicated after sperm addition.

7.8±1.3; *n*=6) had rise and fall times comparable to the phase 1 oscillations; however, the time interval between peaks was on average longer, measuring 105±5 seconds compared to 65±5 seconds for the phase 1 transients. The second group of calcium transients (phase 3), lasted for a mean time of 11.0±1.9 minutes (*n*=6).

The surface contraction occurred shortly after the first activation transient, as reported previously (Brownlee and Dale, 1990), while the first polar body was extruded at the end of the phase 1 transients, at a mean time of 7±0.9 minutes after the initiation of the first calcium transient. The second polar body appeared towards the end of the phase 3 transients, 15-17 minutes after extrusion of the first polar body (Fig. 4).

In several experiments, we whole-cell clamped the oocyte and measured plasma membrane currents simultaneously with the intracellular calcium signals (Fig. 5). Corresponding with the initial calcium transient oocytes generated an inward current of about 1-2 nA that peaked in 20 seconds and returned to zero current in 60 seconds. During the phase 1 calcium transients, the membrane conductance was stable; however, in correspondence with the second group of transients, phase 3, the membrane generated a series of current oscillations of 0.1-0.3 nA amplitude (Fig. 5).

To correlate the change in calcium levels with MPF, we measured Cdc2-like kinase activity at different times after spermatozoa addition. As shown in Fig. 4 (lower panel), H1 kinase activity is high in unfertilized metaphase I oocytes, decreases slowly to a minimum level at 20 minutes from insemination and stays low during the extrusion of the first polar body. Activity increases at 25-30 minutes after insemination corresponding to the end of the phase 3 oscillations.

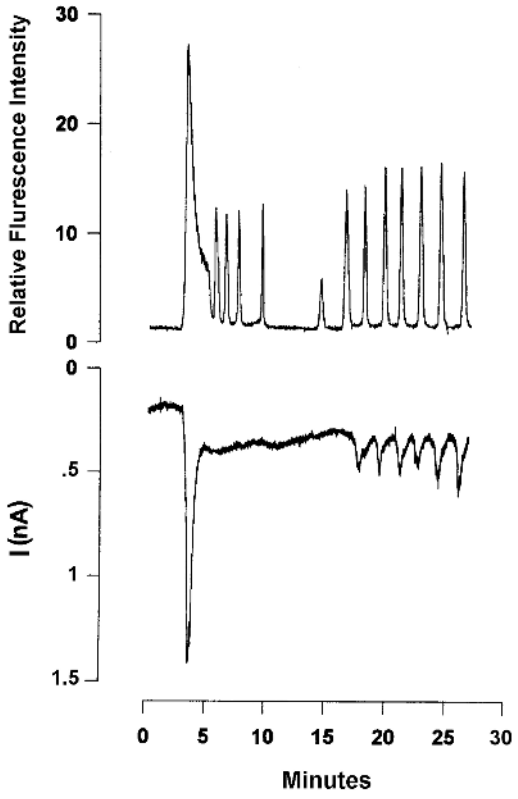

Fig. 5. Simultaneous recording of intracellular calcium levels and plasma membrane currents during fertilization and meiotic progression in an oocyte of *Ciona intestinalis*.

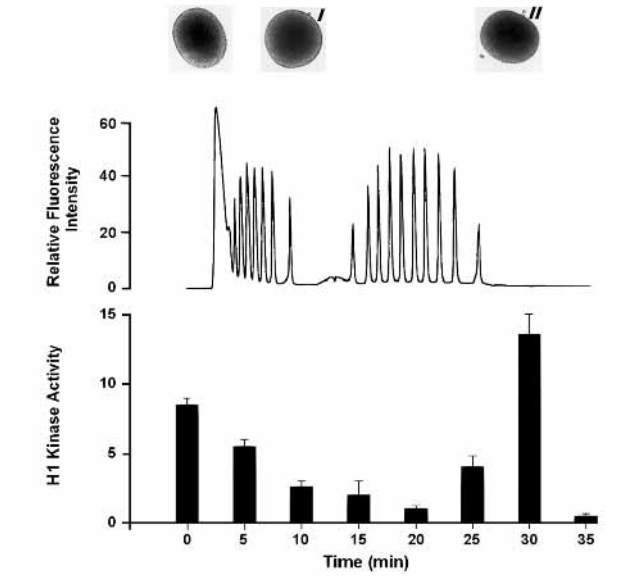

Fig. 4. Relationship between intracellular calcium, MPF activity and polar body extrusion in *Ciona intestinalis* oocytes at fertilization and progression through meiosis. The top panel shows the morphological changes during meiosis: contraction, extrusion of first (*I*) and second (*II*) polar body. The trace in the middle panel shows the relative fluorescence intensity in an oocyte loaded with Calcium Green-dextran, while the lower bar diagram shows H1 kinase activity measured towards H1-peptide and using the total cell lysate as enzymatic source.

Finally, the activity rapidly decreases again to baseline levels at 35 minutes post-insemination corresponding to telophase II, when 50% of total oocytes had extruded their second polar body. The two peaks of high Cdc2 activity correspond to metaphase I (time 0 minutes), and metaphase II (time 25-30 minutes; Satoh, 1994).

Since, we performed our kinase assays on whole-cell extracts prepared from a few oocytes, artifacts from different Cdc2-like activities or enzymatic activities not correlated to Cdc2 were possible. To exclude the latter, H1 kinase activity from cell extracts was precipitated using p13^{suc1}-sepharose (Fig. 6). The pattern of kinase activity in these experiments was similar to that observed for the cell extracts (Fig. 4).

To investigate the relationship between the calcium transients and MPF, metaphase I oocytes were loaded by microinjection with the calcium chelator BAPTA to an estimated concentration of 5 mM and inseminated. As expected, all calcium transients were inhibited, however Cdc2-like kinase activity decreased as in the controls injected with ICS (Fig. 7A) and in the untreated oocytes (Fig. 4, middle panel), remaining at low levels for at least 30 minutes. In addition, polar body extrusion was suppressed, while the fertilization current occurred as previously reported (Dale, 1987). Unfortunately, owing to the pigmentation of *Ciona intestinalis* oocytes, we do not know whether the oocytes leave metaphase I. In contrast, when BAPTA was injected into oocytes during phase 2, about 10-12 minutes after insemination, the second group of calcium waves, phase 3, was suppressed (Fig. 7B), the second polar

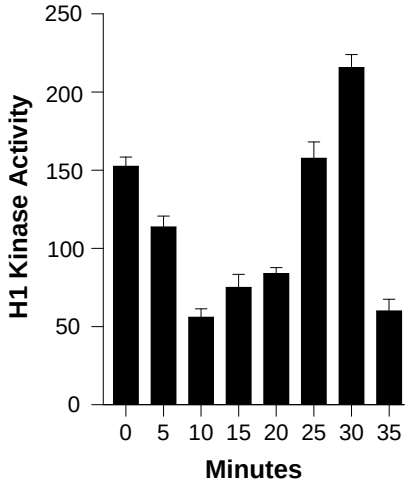

Fig. 6. H1 kinase activity associated with p13^{suc1}-sepharose at different times after fertilization in *Ciona intestinalis*. At the indicated time points from sperm addition, oocytes were collected and lysed as described in Materials and Methods. About 50 μg of total cell lysates were precipitated using p13^{suc1}-sepharose and H1 kinase activity was measured using H1-peptide.

body was not extruded and MPF kinase activity remained at low levels (Fig. 7C).

To determine the origin of the calcium transients, metaphase I oocytes were preloaded with heparin to a final concentration of 1-5 mM and then inseminated. Fig 8 shows that the first calcium transient, the activation peak, occurs as in control oocytes; however, the subsequent transients (phases 1 and 3) were suppressed, as were the surface contraction and polar body extrusion, while the fertilization current was generated as reported previously (Tosti and Dale, 1994).

DISCUSSION

Using biochemical and immunological assays, we have identified a Cdc2-like kinase in unfertilized oocytes of the ascidian *Ciona intestinalis* that phosphorylates histone H1 and several synthetic peptides containing a Cdc2 phosphorylation site. We measured Cdc2 activity in both total cell lysates and in p13^{suc1}-sepharose precipitates. Immunoblotting with the anti-PSTAIRE antibody produced a strong signal at 35 kDa corresponding to Cdc2-like molecules plus two other forms at 53 and 29 kDa possibly related to different members of the Cdk family. We also identified a B-type cyclin based on the following criteria: first, the molecule is present specifically at metaphase (I and II) and is degraded during telophase; second, it is precipitated by p13^{suc1}-sepharose together with the Cdc2 polypeptide and is the main labeled protein identified in the autoradiography (Fig. 2A,B); finally, the supposed molecular weight of 65-67 kDa is in the range of that expected for cyclin B (Pines and Hunter, 1991; Murray and Hunt, 1993). Although identification of ascidian cyclin B and Cdc2 requires cloning of the corresponding genes, the above indirect experiments suggest that the activity observed is indeed that of Cdc2 and cyclin B.

Ovulated oocytes resume meiosis in response to a signal from the fertilizing spermatozoon (Dale and DeFelice, 1990;

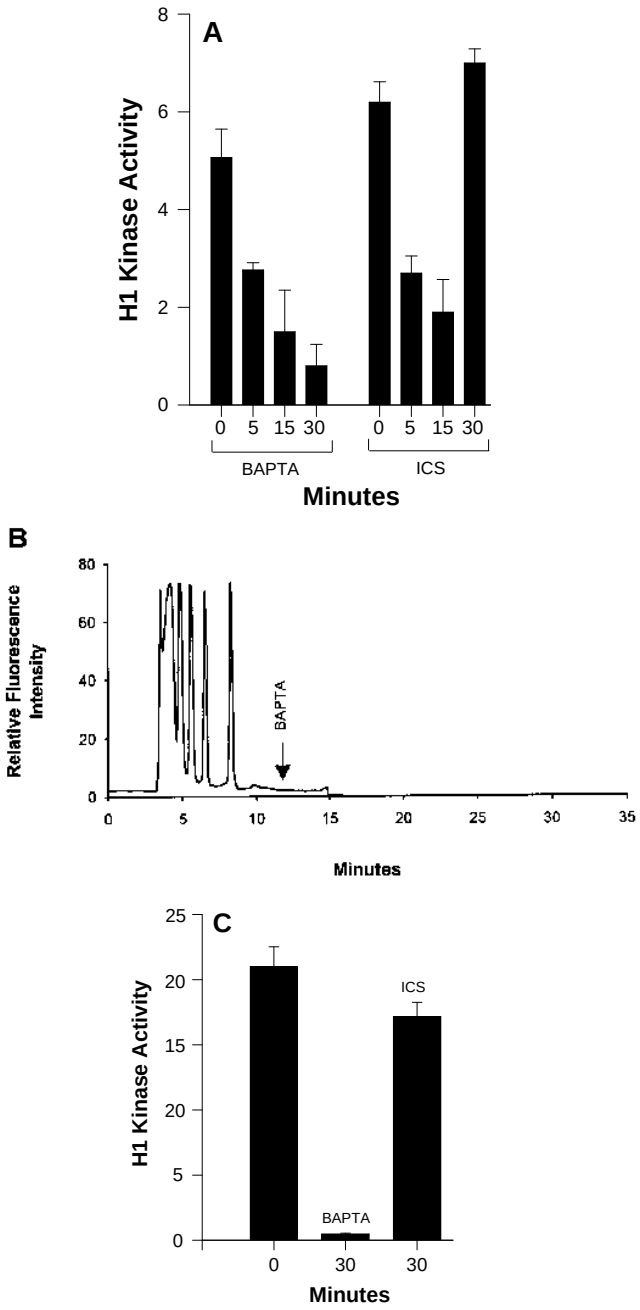

Fig. 7. The effect of BAPTA on calcium oscillations and H1 kinase activity in *Ciona intestinalis* oocytes at fertilization. H1 kinase activity decreased 5 minutes after insemination and remained low during meiosis II when oocytes were loaded with BAPTA compared to the controls injected with ICS that showed a normal pattern of activation of H1 kinase at metaphase II (A). BAPTA injected at phase 2, 10-12 minutes after insemination, eliminated both the phase 3 transients (B), and the increase in H1 kinase activity at 30 minutes postinsemination (C).

Whitaker and Patel, 1990). Here, we showed that metaphase I ascidian oocytes have high histone H1 kinase activity that decreases at fertilization, independent of intracellular calcium signals. In contrast, a later set of calcium oscillations trigger, and are essential for, an increase in H1 kinase activity during metaphase II. The role of calcium in meiosis resumption in

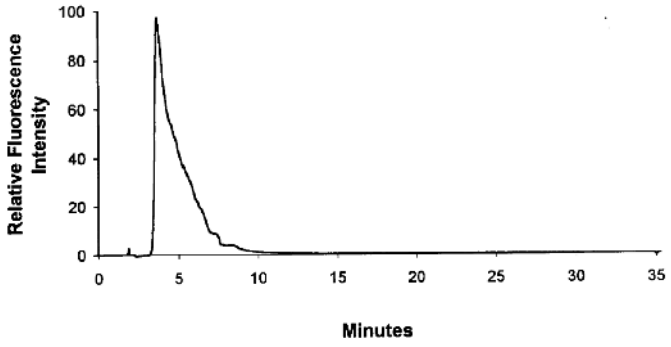

Fig. 8. Preloading a *Ciona intestinalis* oocyte with heparin eliminates all the calcium transients with the exception of the initial activation peak.

oocytes has been extensively studied both in vertebrates and invertebrates (Jaffe, 1985; Homa, 1995); however, many points need to be clarified. Differences encountered in vertebrate and invertebrate oocytes may be related to the stage of meiotic arrest (Monroy, 1965; Dale, 1983), or result from the different methods used to activate oocytes (sperm versus parthenogenesis), and whether the assays were performed in cell-free systems or on intact oocytes.

In ascidian oocytes, we have shown that progression through meiosis II is calcium dependent. Injection of BAPTA blocks calcium oscillations, increase of H1 kinase activity and extrusion of II polar body; however, we cannot exclude that the effect of BAPTA is due to chelation of other cations. In accordance with our observations, Kline and Kline (1992) showed that BAPTA-AM blocks calcium oscillations and meiosis resumption in metaphase II mouse oocytes. The increase in calcium seems to be correlated with cyclin destruction and CSF inactivation (Kubiak et al., 1993; Zernicka-Goetz et al., 1995), while exit from metaphase II requires the presence of a fully intact spindle during the release of intracellular calcium (Winston et al., 1995). There is also strong evidence that the calcium increase is responsible for the decrease in Cdc2 kinase activity in rabbit (Collas et al., 1995) and bovine oocytes (Collas et al., 1993). Finally, in a cell-free system of metaphase II *Xenopus* oocytes, an increase in intracellular calcium inactivates both CSF and MPF, and this event has been correlated with the activity of Ca^{2+}/calmodulin kinase II (Lorca et al., 1991; 1993). This kinase seems to be associated with sister chromatid segregation in CSF extracts (Morin et al., 1994). All these data are consistent with a key role for calcium in the resumption of meiosis from a metaphase II block, possibly mediated by Ca^{2+}/calmodulin kinase II.

Does intracellular calcium regulate progression throughout meiosis I? We have shown that inhibiting the phase I calcium oscillations in ascidian oocytes does not prevent the decrease in H1 kinase activity; however, the oocytes do not complete meiosis since they do not extrude the first polar body, nor is there an increase in H1 kinase activity at metaphase II. Although there are no comparable data on vertebrate oocytes, studies using parthenogenetic stimuli in mouse mutants show that metaphase I arrest is sustained by a mechanism different to that maintaining metaphase II arrest (Hampl and Eppig, 1995). In addition, in starfish (Witchel and Steinhardt, 1990) and in mouse oocytes (Tombes et al., 1992), although germinal vesicle breakdown is associated with MPF activation, it is calcium independent; the same authors also demonstrated that absence of calcium delays spindle formation at meiotic division I without arresting this process (Tombes et al., 1992). In starfish, the situation is more complicated since the ionophore A23187, fertilization or microinjection of calcium do not inactivate MPF (Capony et al., 1986; Dorée et al., 1990).

MPF activity is thought to be maintained high during metaphase II arrest by CSF. Several lines of evidence suggest that MAP kinase is a component of CSF together with the product of the c-*mos* proto-oncogene. It is possible that the regulation of CSF by calcium is different in ascidian oocytes at metaphase I and II. In *Xenopus*, MAP kinase and Mos are not sufficient to maintain metaphase I arrest, although their activities during this phase are very high (Haccard et al., 1993). On the contrary, Mos and MAP kinases do not require any other element to sustain MPF activity in metaphase II oocytes (Haccard et al., 1993). This suggests that MPF inactivation at metaphase I probably requires additional factors not regulated by calcium or able to overcome calcium regulation. In support of this view, the MAP kinase-dependent spindle assembly checkpoint identified recently in *Xenopus* oocyte extracts is also calcium independent since the addition of calcium to checkpoint-arrested mitotic extracts does not induce inactivation of MPF and MAP kinase or cause the formation of interphase nuclei (Minshull et al., 1994). We measured MAP kinase activity in ascidian oocytes at fertilization and we observed that activity is high in unfertilized oocytes, peaks at 5 minutes after fertilization and decreases during telophase I without any subsequent increase in meiosis II, similar to the pattern of MAP kinase activation reported in clams oocytes (Shibuya et al., 1992) and in the ascidian *Phallusia mammillata* (McDougall and Sardet, personal communication). This suggests that the role of MAP kinase in ascidians might be different at metaphase I compared to metaphase II, or that several members of the MAP-ERK kinase family are involved at different times in this transition; in addition, preliminary data obtained in our laboratory indicate that Mos is only present at meiotic division II and not at the metaphase-anaphase I transition (Russo and Dale, unpublished data). Studies are in progress to clarify the role of Mos and MAP kinase in the ascidian oocyte during the meiotic cycle and their possible regulation by calcium.

In ascidians, the activity of the proteosome complex 26S involved in the ubiquitin-dependent degradation of key molecules in cell cycle progression is regulated by calcium during the meiotic metaphase-anaphase transition (Kawahara and Yokosawa, 1994). At present, we do not know if the loss in H1 kinase activity observed after injection of BAPTA (Fig. 7A) is associated with protein degradation, including cyclin B. One hypothesis is that the inactivation of H1 kinase activity is calcium-independent, while proteolysis is calcium-dependent. According to this view, Holloway et al. (1993) demonstrated that, in *Xenopus* oocytes, anaphase is initiated by proteolysis rather than by MPF inactivation. Moreover, we may assume that calcium plays a role in the regulation of specific steps in the ubiquitin-mediated degradation pathway.

Calcium is a ubiquitous intracellular messenger that appears to regulate a host of cellular functions from exocytosis to nuclear membrane breakdown. How are these different functions orchestrated by a common signal? Spatial and temporal restrictions are obvious possibilities and, in fact, in the ascidian oocyte both mechanisms appear operative. In

Ciona intestinalis oocytes, we have shown that temporally distinct groups of calcium oscillations are associated with different phases of the meiotic cycle. Two major calcium release channels are implicated in the release of intracellular calcium: the inositol (1,4,5)-tris phosphate receptor ($InsP_3R$) and the ryanodine receptor (RYR) channels. Since heparin, which competes with $InsP_3$ (Berridge, 1993), eliminates only the phase 1 and 3 oscillations, the initial activation peak is generated at least in part by RYR channels, while the later oscillations are generated solely by $InsP_3$. Unfortunately, we were not able to measure H1 kinase activity in oocytes that had been injected with heparin owing to the fragility of the oocytes; however, these experiments show that the first activation peak of calcium is not sufficient to trigger progression through meiosis as indicated by the inhibition of polar body extrusion.

The phase 1 and 3 oscillations also differ. First, the phase 3 oscillations, which induce an increase in H1 kinase activity, occur simultaneously with a series of currents across the plasma membrane, while the phase 1 oscillations do not. Second, the time interval between the phase 3 transients is longer than in the phase 1 transients. Although we have no direct evidence, a possible explanation, in accordance with the results of McDougall and Sardet (1995), is that the two groups of transients are also spatially distinct. Although speculative, perhaps oocytes utilize spatially distinct calcium signals and different pools of Cdc2-related kinases to modulate meiosis I and meiosis II.

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