Activation of protein kinase C alters p34^{cdc2} phosphorylation state and kinase activity in early sea urchin embryos by abolishing intracellular Ca²⁺ transients

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The p34^{cdc2} protein kinase, a universal regulator of mitosis, is controlled positively and negatively by phosphorylation, and by association with B-type mitotic cyclins. In addition, activation and inactivation of p34^{cdc2} are induced by Ca²⁺ and prevented by Ca²⁺ chelators in permeabilized cells and cell-free systems. This suggests that intracellular Ca²⁺ transients may play an important physiological role in the control of p34^{cdc2} kinase activity. We have found that activators of protein kinase C can be used to block cell cycle-related alterations in intracellular Ca²⁺ concentration ([Ca²⁺]_i) in early sea urchin embryos without altering the normal resting level of Ca²⁺. We have used this finding to investigate whether [Ca²⁺]_i transients control p34^{cdc2} kinase activity in living cells via a mechanism that involves cyclin B or the phosphorylation state of p34^{cdc2}. In the present study we show that the elimination of [Ca²⁺]_i transients during interphase blocks p34^{cdc2} activation and entry into mitosis, while the

elimination of mitotic [Ca²⁺]_i transients prevents p34^{cdc2} inactivation and exit from mitosis. Moreover, we find that [Ca²⁺]_i transients are not required for the synthesis of cyclin B, its binding to p34^{cdc2} or its destruction during anaphase. However, in the absence of interphase [Ca²⁺]_i transients p34^{cdc2} does not undergo the tyrosine dephosphorylation that is required for activation, and in the absence of mitotic [Ca²⁺]_i transients p34^{cdc2} does not undergo threonine dephosphorylation that is normally associated with inactivation. These results provide evidence that intracellular [Ca²⁺]_i transients trigger the dephosphorylation of p34^{cdc2} at key regulatory sites, thereby controlling the timing of mitosis entry and exit.

Key words: cyclin B, embryonic cell cycle, Ins(1,4,5)P₃, mitosis, phorbol esters.

INTRODUCTION

In all eukaryotic cells that have been studied, protein phosphorylation serves as a key regulator of cell cycle events. Sequential phases of the cell cycle are induced by the sequential activation and inactivation of cyclin-dependent kinases, which in turn phosphorylate a wide range of cellular substrates [1]. Entry and exit from the M phase of the cell cycle are triggered universally by the activation and inactivation of p34^{cdc2}, a cyclin-dependent kinase that binds B-type mitotic cyclins. During interphase the association of p34^{cdc2} with cyclin B causes p34^{cdc2} to be phosphorylated at activating (Thr¹⁶¹) and inhibitory (Thr¹⁴ and Tyr¹⁵) sites, leading to the accumulation of inactive p34^{cdc2}–cyclin B complexes. Dephosphorylation of the inhibitory sites at the G2/M transition by the cdc25 phosphatase activates p34^{cdc2}, while the dephosphorylation of Thr¹⁶¹ and proteolytic destruction of cyclin B at the metaphase/anaphase transition inactivates p34^{cdc2} [2].

The idea that Ca²⁺ is an important regulator of the M phase of the cell cycle is supported by studies showing that the intracellular Ca²⁺ concentration ([Ca²⁺]_i) increases transiently at nuclear envelope breakdown (NEBD), anaphase onset and cytokinesis

[3,4]. Further evidence that Ca²⁺ regulates cell division comes from studies in which Ca²⁺ chelators, or inhibitors of Ins(1,4,5)P₃-mediated Ca²⁺ mobilization, delay or prevent cell cycle progression [5–8]. The effects of the chelators are reversed by Ca²⁺ and, in some cases, cell cycle events are triggered prematurely by an experimentally induced [Ca²⁺]_i transient [9–12]. However, [Ca²⁺]_i transients are not observed in all cells under all conditions, suggesting that they may often be too localized to detect, or that they do not serve a regulatory function [11,13].

In oocytes arrested at meiotic metaphase, a key function of the fertilization [Ca²⁺]_i transient is to induce exit from meiosis by triggering cyclin B proteolysis and p34^{cdc2} inactivation [14]. Ca²⁺ may control the activity of p34^{cdc2} during mitotic cell cycles as well, based on our previous finding that the precocious elevation of [Ca²⁺]_i induces premature p34^{cdc2} inactivation in permeabilized sea urchin embryos [15]. In addition, Ca²⁺ chelators block activation and inactivation of p34^{cdc2} in interphase and mitotic extracts of *Xenopus* embryos [16]. However, because Ca²⁺-induced p34^{cdc2} inactivation is not correlated with cyclin B proteolysis in these studies [15,16], Ca²⁺ and p34^{cdc2} may be linked by different mechanisms in mitosis and meiosis.

To determine the molecular basis for the regulation of p34^{cdc2}

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; [Ca²⁺]_i, intracellular Ca²⁺ concentration; diC₈, 1,2-dioctanoyl-*sn*-glycerol; CFASW, calcium-free artificial sea water; DTT, dithiothreitol; NEBD, nuclear envelope breakdown; PKC, protein kinase C; PLC, phospholipase C; TCA, trichloroacetic acid.

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activity by Ca^{2+} during mitotic cell cycles, we have experimentally inhibited cell cycle $[\text{Ca}^{2+}]_i$ transients in synchronous populations of early sea urchin embryos. Previously, it has been shown in a variety of cells that manipulations that activate protein kinase C (PKC) inhibit receptor-coupled mobilization of Ca^{2+} from intracellular stores [17–19]. We found that in early sea urchin embryos, activation of PKC eliminates cell cycle $[\text{Ca}^{2+}]_i$ transients without altering the normal resting level of $[\text{Ca}^{2+}]_i$. Using this approach, potential Ca^{2+} requirements for $\text{p34}^{\text{cdc}2}$ activation, inactivation, complex formation with cyclin B, and the phosphorylation state of important regulatory amino acids may be directly identified by biochemical analysis. In the present study our results provide evidence that $[\text{Ca}^{2+}]_i$ transients trigger the dephosphorylation of $\text{p34}^{\text{cdc}2}$ at key regulatory sites, thereby controlling $\text{p34}^{\text{cdc}2}$ activity and the timing of mitosis entry and exit.

MATERIALS AND METHODS

Reagents

PMA and 4zPMA were purchased from LC Laboratories (Woburn, MA, U.S.A.). Stock solutions were prepared in DMSO at a concentration of 2 mM and were stored frozen under N_2 to inhibit oxidation. For experiments, aliquots were diluted to 20 μM with DMSO, so that 1% (v/v) DMSO would be present to increase the solubility of the compounds in sea water at the final working concentration of 200 nM. 1,2-Dioctanoyl-*sn*-glycerol (diC_8), from Avanti Polar Lipids (Alabaster, AL, U.S.A.), dissolved in chloroform was dried under a stream of N_2 and dissolved in DMSO as a 100 $\times$ stock solution (5–10 mM) to be used the same day. Staurosporine and the Ca^{2+} ionophore A23187 (Calbiochem, La Jolla, CA, U.S.A.) were prepared as 100 $\times$ stock solutions in DMSO. Emetine (Sigma, St. Louis, MO, U.S.A.) was dissolved in sea water at a concentration of 10 mM. PKC(19–31) peptide (LC Laboratories) was dissolved in either intracellular buffer (see below) at a concentration of 1 mM, or in microinjection buffer (300 mM potassium gluconate, 300 mM glycine, 10 mM NaCl, 2 mM MgSO_4 and 2 mM EGTA, pH 7.2) at a concentration of 9 mM. Except for diC_8 , the stock solutions were stored as aliquots at -70°C . Protease inhibitors were obtained from Boehringer Mannheim (Lewes, East Sussex, U.K.). Antimycin, oligomycin, phosphocreatine and creatine phosphokinase were purchased from Sigma (Poole, Dorset, U.K.).

Embryo culture and micro-injection

Eggs were collected from the sea urchin *Lytechinus pictus* (Marinus, Long Beach, CA, U.S.A.) in artificial sea water (435 mM NaCl, 40 mM MgCl_2 , 15 mM MgSO_4 , 11 mM CaCl_2 , 10 mM KCl and 5 mM Hepes/NaOH, pH 8.0) following intracoelomic injection of 0.5 M KCl. The eggs were harvested by centrifuging for 30 s at 250 g and were treated with sea water adjusted to pH 5.0 (with HCl) for 3 min to remove the jelly layer. These were then suspended to 1% (v/v) in sea water and stirred continuously at 16°C using a motorized paddle or shaking water bath. Fertilization was accomplished by introducing a few drops of sperm diluted with sea water. Batches of eggs exhibiting less than 95% fertilization were discarded. For determinations of NEBD, embryos were fixed in ethanol/acetic acid (3:1, v/v) and were examined using differential interference contrast microscopy. A number of embryos (100) were evaluated per time point. Chromosomes were stained in fixed embryos using 70% (v/v) acetic acid saturated with orcein (Sigma).

$[\text{Ca}^{2+}]_i$ was measured using a fluorescence ratio method [20] in

embryos injected with fura 2 conjugated to 10 kDa dextran (Molecular Probes, Eugene, OR, U.S.A.), according to the method of Ciapa et al. [21]. The embryos were treated with PMA (or DMSO) 10 min after fertilization, while in suspension. After 20 min these were affixed to polylysine-coated cover slips for injection and measurements.

Injectations of PKC(19–31) were performed as described by Sluder et al. [22]. Injection volumes were determined from the diameter of equivalent oil droplets used to cap the micropipettes. Stock solution (9 mM) was introduced (4–12 pl), yielding an intracellular concentration of 50–150 μM for a hollow sphere equal to the embryo in diameter (110 μm).

Measurements of protein and DNA synthesis

To measure rates of protein synthesis, suspensions of eggs were pre-loaded with 15 $\mu\text{Ci/ml}$ [^{35}S]methionine (1200 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) for 20 min. The eggs were then washed three times with sea water, suspended to 1% (v/v) and fertilized. At intervals, samples were precipitated with 10% (w/v) trichloroacetic acid (TCA; 60 min at 4°C) and collected by centrifugation (5000 g for 2 min). Aliquots of the TCA-soluble supernatants were mixed with Aquasol (Packard Instruments, Meriden, CT, U.S.A.) for liquid-scintillation counting in order to determine the specific radioactivity of the intracellular methionine pool. TCA precipitates were washed three times with 10% TCA containing 1 mg/ml methionine, solubilized in 1 M NaOH, and counted to determine ^{35}S incorporation. The incorporation is expressed as a percentage of the intracellular methionine pool. For determinations of cyclin B abundance, eggs were pre-loaded for 20 min with 25 $\mu\text{Ci/ml}$ [^{35}S]methionine and washed as above. Following fertilization, samples were analysed by SDS/PAGE and autoradiography [15].

To monitor DNA synthesis, 20 $\mu\text{Ci/ml}$ [^3H]thymidine (80 Ci/mmol; Amersham) was added to eggs 10 min before fertilization. At intervals after fertilization, samples were washed three times with sea water at 4°C and precipitated with 10% TCA containing 0.5 mg/ml thymidine. The precipitates were washed three times with 10% TCA containing thymidine and were subsequently solubilized in 1 M NaOH. Aliquots of the TCA-soluble and TCA-insoluble fractions were analysed by liquid-scintillation counting to determine the percentage of incorporation, as above. The fraction of TCA-soluble radioactivity corresponding to [^3H]thymidine triphosphate is essentially constant at this stage of development.

Kinase assays

Embryos were washed and re-suspended to 1% (v/v) in intracellular buffer [250 mM potassium gluconate, 250 mM *N*-methyl-D-glucamine, 50 mM Hepes/acetate (pH 7.2), 10 mM EGTA, 6.7 mM MgCl_2 and 1 mM dithiothreitol (DTT)] at 4°C , and were lysed using three 10-s pulses of a Branson Sonifier fitted with a stepped micro-tip (Branson Ultrasonics, Danbury, CT, U.S.A.). $\text{p34}^{\text{cdc}2}$ kinase activity and protein concentrations were measured as described previously [15] except that 50 μM PKC(19–31) was included in the kinase assays.

Anti-phosphotyrosine immunoblots

Embryos were lysed in adsorption buffer (intracellular buffer without MgCl_2 , supplemented with 5 mM EDTA, 1 mM Na_3VO_4 , 1 μM microcystin-LR, 0.5 mM PMSF, 2 $\mu\text{g/ml}$ leupeptin and 2 $\mu\text{g/ml}$ pepstatin) using ultrasonic disruption. To enrich for $\text{p34}^{\text{cdc}2}$, volumes of lysate containing 1 mg of protein

were adsorbed to 35 μ l of p13^{suc1}-Sephacel beads for 60 min at 4 °C (5 mg of p13^{suc1}/ml of beads; Upstate Biotechnology, Lake Placid, NY, U.S.A.). After adsorption, the beads were washed three times with adsorption buffer and twice with buffer containing 50 mM Tris/HCl (pH 6.8), 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM Na₃VO₄, 1 μ M microcystin-LR, 0.5 mM PMSF, 2 μ g/ml leupeptin and 2 μ g/ml pepstatin. Bound proteins were eluted in 125 μ l of SDS/PAGE sample buffer, resolved by SDS/PAGE, and transferred on to nitrocellulose membranes [15]. Transfers were blocked for 2 h at room temperature with TBS [10 mM Tris/HCl (pH 8.0)/150 mM NaCl] containing 5% (w/v) BSA (Sigma; 'essentially fatty acid-free') and 1% (w/v) ovalbumin, and were labelled overnight at 4 °C with an anti-phosphotyrosine monoclonal antibody diluted to 2 μ g/ml in blocking solution (clone PY20; ICN, Costa Mesa, CA, U.S.A.). The transfers were subsequently labelled for 90 min at room temperature with affinity-purified rabbit anti-mouse IgG (Pierce, Rockford, IL, U.S.A.) at 1 μ g/ml, followed by ¹²⁵I-labelled Protein A (Amersham) at 10 ng/ml, in blocking solution. After each labelling step, the transfers were washed four times with TBS (10 min per wash). Autoradiography was performed at -70 °C with an intensifying screen.

Measuring Ca²⁺ pumping and release in microsomes

Calcium homeostasis in an endoplasmic reticulum preparation from sea urchin embryos was investigated essentially as described previously [23]. Microsomes were prepared from control or PMA-treated embryos 60 min after fertilization (200 nM 4 α PMA or 200 nM PMA added 24 min after fertilization). PMA or 4 α PMA was included at concentrations of 200 nM in all buffers used for microsome preparation and subsequent analysis. Embryos produced from the fertilization of 5 ml of eggs were collected by centrifugation and suspended in calcium-free artificial sea water (CFASW; 445 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 10 mM KCl, 1 mM EDTA, 2 mM EGTA and 10 mM Hepes/NaOH, pH 8.0). Eggs were washed twice with CFASW and then suspended 1:1 (v/v) in ice-cold isolation buffer (250 mM *N*-methyl glucamine, 250 mM potassium gluconate, 20 mM Hepes and 1 mM MgCl₂, adjusted to pH 7.2 with acetic acid). The embryos were homogenized with 10 strokes of a tight-fitting teflon pestle in a Potter homogenizer. The homogenate was centrifuged at 10000 *g* for 10 s and the pellet was discarded. The resulting microsome fractions were used immediately for analysis of calcium homeostasis.

Embryo homogenate (1 ml) was added to 1 ml of fluorimeter buffer (a solution comprising isolation buffer supplemented with 1 mM NaN₃, 1 μ g/ml oligomycin and antimycin, 0.1 mg/ml soybean trypsin inhibitor, 200 μ g/ml leupeptin, 200 μ g/ml aprotinin, 1 mM ATP, 10 mM phosphocreatine and 10 units/ml creatine phosphokinase). This mixture was incubated for 1 h at 16 °C. This was then added to 8 ml of fluorimeter buffer and the mixture was incubated at 16 °C for 4 h. Fluorimetry was performed using a Perkin Elmer Luminescence Spectrometer (LS50-B). A 3 ml aliquot of microsomes was added to a quartz cuvette and 1 μ M fluo 3 (Molecular Probes) was added. The mixture was stirred at full speed using a stirring cuvette holder and the temperature was maintained at 16 °C. Fluorescence was measured using an excitation wavelength of 490 nm and an emission wavelength of 590 nm.

Phosphopeptide mapping and phospho amino acid analysis

Embryo suspensions were supplemented with 0.13 mCi/ml carrier-free ³²PO₄ (Amersham), 20 min after fertilization.

Samples were washed extensively, lysed in adsorption buffer and incubated with p13^{suc1} beads as outlined above. Eluted proteins were resolved by SDS/PAGE, transferred on to Immobilon-P membranes (Millipore, Bedford, MA) in the presence of 20% (v/v) methanol and 0.1% SDS, and visualized by autoradiography. The band corresponding to p34^{cdc2} was excised and digested with 2 $\times$ 4 μ g of tosylphenylalanylchloromethane-trypsin (Pierce) according to the protocol of Luo et al. [24]. Peptide mapping was performed on ³²P-labelled tryptic digests as described by Boyle et al. [25]. Samples were applied to 100 μ m thin-layer cellulose plates and were electrophoresed at pH 1.9 for 25 minutes at 1 kV. Separation in the second-dimension was by chromatography in phospho chromatography buffer. For phospho amino acid analysis, tryptic digests were hydrolysed in constant-boiling 6 M HCl for 6 h at 110 °C to enrich for phosphothreonine (see the Results section). Liberated phospho amino acids were separated by TLC [26] and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). For each time point p34^{cdc2}-associated ³²P was normalized to account for the specific radioactivity of the intracellular TCA-soluble pool. The size of the intracellular ATP pool does not change during these experiments [27], and the turnover of the protein phosphate *in vivo* is rapid since the amount of ³²P incorporated into the TCA-insoluble fraction closely parallels the specific radioactivity of the intracellular ³²P pool (results not shown).

RESULTS

Activators of PKC block mitosis entry and exit

Embryos of the sea urchin *L. pictus* were treated at different stages of the cell cycle with 200 nM PMA, a potent activator of PKC [28]. As a control embryos were also treated with 200 nM 4 α PMA, an isomer of PMA that does not activate PKC. When 4 α PMA was applied during G1 phase (24 min after fertilization), embryos underwent multiple rounds of division (Figures 1A and 1E) with timing that was typical of untreated populations. In contrast, the application of PMA during G1 phase arrested the cell cycle at a stage preceding mitosis; NEBD (Figures 1B and 1F) and chromosome condensation (results not shown) were blocked for at least 2 h. The fact that DNA is replicated in the PMA-treated embryos (Figure 2B) indicates that the cell cycle is blocked in G2 phase.

When PMA was administered during prophase or pro-metaphase (72 min after fertilization), embryos entered mitosis but were unable to exit; nuclear envelope re-formation did not occur (Figures 1C and 1G), and chromosomes remained condensed (Figure 1H). The application of PMA during telophase (96 min after fertilization) did not interfere with the completion of mitosis, but arrested the cell cycle before the next mitosis (Figure 1D). Identical results were obtained with the PKC activator, diC₈, at concentrations of 50–100 μ M (see below). The cell cycle arrest imposed by activators of PKC was not due to a general repression of cell metabolism, as neither protein nor DNA synthesis was inhibited (Figure 2). Thus activators of PKC block entry into or exit from mitosis, depending on the cell cycle stage at which they are applied.

Because PKC activation arrested the cell cycle, we determined the consequences of inhibiting PKC by injecting the selective peptide inhibitor PKC(19–31) [29] during interphase of the first cell cycle (25–50 min after fertilization). An intracellular concentration of 70–150 μ M peptide was used, since $\geq$ 60 μ M has been shown to block 98% of the cytoplasmic alkalization induced by PMA in unfertilized *L. pictus* eggs [30]. Embryos injected with PKC(19–31) divided at least three times, with the

Figure 1 PMA blocks cell cycle progression in a stage-dependent manner

Embryos were treated with 200 nM 4 α PMA (A) or 200 nM PMA (B–D) at the times indicated (arrows). At intervals, samples were fixed and examined microscopically to determine the kinetics of NEBD. (E) Photomicrograph of (A) at 120 min. (F) and (G) Embryos from (B) and (C) at 120 min. (H) Embryo from (C) at 120 min stained to visualize chromatin. Scale bars represent 50 μ m in (E–G) and 10 μ m in (H). Results typical of 5 experiments are shown.

Figure 2 Effect of PMA on protein and DNA synthesis

PMA (■) or 4 α PMA (○) (200 nM) was added to embryos during G1 phase (arrow). Protein synthesis (A) and DNA synthesis (B) were measured as described in the Materials and methods section. Typical results are shown.

same timing as control embryos injected with buffer ($n = 12$). Therefore apart from its role in cytoplasmic alkalization at fertilization [31,32], PKC is not essential for cell cycle progression in sea urchin embryos. As diacylglycerol would be generated when Ins(1,4,5) P_3 is produced prior to each $[Ca^{2+}]_i$ transient, one might also expect PKC to be activated at each $[Ca^{2+}]_i$ transient. Our data suggest that this activation, if it takes place, is not essential for cell cycle progression. Perhaps PtdIns(4,5) P_2 hydrolysis is localized to regions of the cell where PKC is absent or, alternatively, it is possible that diacylglycerol is rapidly metabolized before PKC activation can take place.

PKC activation inhibits cell cycle $[Ca^{2+}]_i$ transients

The fact that activators of PKC block the cell cycle in a manner similar to Ca^{2+} chelators [3], and the reported inhibition of receptor-coupled Ca^{2+} mobilization by PKC [17–19], suggested that PKC might inhibit cell cycle-related $[Ca^{2+}]_i$ transients. To directly test this possibility, embryos were continuously treated with PMA (or with DMSO, as a control) beginning in G1 phase, and were injected with fura 2-dextran, so that $[Ca^{2+}]_i$ could be measured in individual embryos using dual wavelength confocal microscopy. In the controls, $[Ca^{2+}]_i$ fluctuated over the course of the cell cycle, exhibiting sustained increases (> 2 min) of 50–125 nM that were associated with NEBD, anaphase onset, and cytokinesis (Figure 3A), as reported previously [20,21]. PMA

Figure 3 PMA eliminates cell cycle $[Ca^{2+}]_i$ transients

$[Ca^{2+}]_i$ was measured continuously in individual embryos that were treated with 1% DMSO (A) or 200 nM PMA (B) during G1 phase (10 min after fertilization) and subsequently injected with fura 20-dextran. Each recording in (A) is from an embryo of the same batch as the corresponding recording in (B). The results shown are representative of 9 trials in which the embryos remained viable throughout the course of the experiment. In four additional trials, the combination of PMA treatment and micro-injection resulted in distortion of the embryos, gross movements of the cytoplasm, and relocation of the nucleus to the cell periphery. Symbols indicate $[Ca^{2+}]_i$ transients associated with NEBD (▼) anaphase onset (▼) and cytokinesis (●).

treatment did not significantly alter the resting level of $[Ca^{2+}]_i$ in the embryos; however, it eliminated all sustained increases in $[Ca^{2+}]_i$ for at least 40 min (Figure 3B). In four out of four control experiments, an NEBD-associated calcium transient occurred 68.6 ± 0.92 min after fertilization. Its peak height was 66 ± 10 nM and its duration was 5.9 ± 1.06 min. In seven experiments in which $[Ca^{2+}]_i$ was monitored from 30 to 90 min after fertilization, in embryos treated with PMA at 24 min, the largest and longest deviation in $[Ca^{2+}]_i$ from baseline was a 30 nM excursion of 1.1 min duration that occurred 40 min after fertilization. The mean of the excursions from baseline in PMA-treated embryos between 64.92 and 72.28 min after fertilization (two standard deviations either side of the mean time of occurrence of the NEBD transient in controls) was 22.5 ± 4.66 nM. Analysis of the control and PMA-treatment NEBD $[Ca^{2+}]_i$ data using an unpaired *t*-test showed the differences to be significant at the $P \leq 0.001$ level. Thus it can be seen that activation of PKC leads to a suppression of cell cycle associated $[Ca^{2+}]_i$ transients. The absence of $[Ca^{2+}]_i$ transients might explain the arrest of the cell cycle in PMA-treated embryos.

We used an *in vitro* egg endoplasmic reticulum microsome preparation [23] to determine whether PMA treatment had any effect on either Ca^{2+} -pumping or $Ins(1,4,5)P_3$ sensitivity. Microsomes were prepared from control or PMA-treated embryos

Table 1 PKC activation does not affect Ca^{2+} pumping or sensitivity to $InsP_3$ in endoplasmic reticulum microsome homogenates

Embryos were treated with 4 α PMA (control) or PMA 24 min after fertilization. Microsomes were prepared 36 min after these additions. $[Ca^{2+}]_i$ was monitored using fluo 3 fluorescence and when a steady baseline reading was achieved Ca^{2+} pumping rate and $InsP_3$ sensitivity were determined. Protein concentrations in control and PMA-treated microsomes were adjusted so that they were identical. The rate of Ca^{2+} pumping was determined by adding 10 nmol of Ca^{2+} and measuring the decrease in $[Ca^{2+}]_i$ over the following 10 min. $InsP_3$ sensitivity was determined by measuring the increase in $[Ca^{2+}]_i$ following the addition of 100 nM $InsP_3$. For both Ca^{2+} pumping and $InsP_3$ sensitivity determinations the data were obtained from duplicate experiments from three separate batches of embryos.

Condition	Rate of Ca^{2+} pumping by sea urchin embryo microsomes (nmol/min) means $\pm$ S.E.M., <i>n</i> = 6	Extent of Ca^{2+} release in response to 100 nM $InsP_3$ (nmol), means $\pm$ S.E.M., <i>n</i> = 6
Control	0.89 ± 0.15	4.13 ± 0.45
PMA-treated	0.92 ± 0.13	3.99 ± 0.34

60 min after fertilization (200 nM 4 α PMA or 200 nM PMA added 24 min after fertilization) and both the Ca^{2+} -pumping rate and $Ins(1,4,5)P_3$ sensitivity were determined. As shown in Table 1 there was no difference in either parameter between the PMA-treated and control embryos.

PKC stimulation prevents p34^{cdc2} activation and inactivation

Since entry and exit from mitosis are controlled by the p34^{cdc2} protein kinase, we investigated whether the activation of PKC blocks cell cycle progression by interfering with the activation and inactivation of p34^{cdc2}. In this regard, it was important to establish that we could accurately measure the protein kinase activity of p34^{cdc2} in embryos treated with activators of PKC, since PKC phosphorylates histone H1, the substrate used to assay p34^{cdc2} activity. Therefore we determined the $[Ca^{2+}]_i$ dependence of sea urchin PKC in crude lysates using the selective peptide substrate, [Ser²⁵]PKC(19–31) [29]. We found that PKC activity was only $9.1 \pm 0.6\%$ maximal (mean $\pm$ S.D.) at 40 nM Ca^{2+} , the concentration present in p34^{cdc2} kinase assays. This result is consistent with the previous identification of *L. pictus* PKC as a Ca^{2+} -requiring isoform based on biochemical properties [33] and sequence analysis [34]. As shown in Figure 4(A), the inclusion of 50 μ M PKC(19–31) in p34^{cdc2} kinase assays of PMA-treated embryos eliminated the low level of Ca^{2+} -independent PKC activity without inhibiting p34^{cdc2}.

When 4 α PMA was applied to embryos during G1 phase, p34^{cdc2} kinase activity underwent cyclical oscillations, increasing 8–10-fold prior to mitosis and then rapidly decreasing to the interphase level during mitosis (Figure 4B). In contrast, p34^{cdc2} kinase activity increased only slightly over a period of 3 h when PMA was applied during G1 phase and entry into mitosis was blocked (Figure 4B). Thus the failure of these embryos to enter mitosis correlated with their inability to activate p34^{cdc2}.

When PKC was activated during prophase to block exit from mitosis, p34^{cdc2} kinase activity remained at the elevated mitotic level for at least 2 h (Figure 4C). Thus the failure of these embryos to exit mitosis correlated with their inability to inactivate p34^{cdc2}. Elevated protein kinase activity was responsible for the

Figure 4 PMA blocks activation and inactivation of p34^{cdc2}

(A) Selectivity of the p34^{cdc2} kinase assay. Lysates were prepared from embryos treated with 200 nM PMA for 5 min, and from untreated embryos, during G1 phase (24 min after fertilization) and prophase (75 min after fertilization). In both cases, the phosphorylation of histone H1 was assayed in the presence and absence of 50 μ M PKC(19–31). (B) PKC activation during G1 phase blocks p34^{cdc2} activation. p34^{cdc2} kinase activity was measured in lysates prepared every 12 min from embryos which received additions of 200 nM PMA (■) or 4 α PMA (○), 24 min after fertilization (arrow). Control (4 α PMA-treated) embryos completed two cycles of NEBD and assembly, while those treated with PMA did not undergo NEBD. (C) PKC activation during prophase blocks p34^{cdc2} inactivation. p34^{cdc2} kinase activity was monitored in embryos which received additions of 200 nM PMA (■), or 4 α PMA (○), 75 min after fertilization (arrow). Control embryos completed a second mitosis, whereas embryos treated with PMA did not exit from the first mitosis. Typical results are shown.

block to mitosis exit, since it was reversed by staurosporine, an inhibitor of serine/threonine protein kinases. As shown in Figure 5 the addition of 5 μ M staurosporine induced nuclear envelope re-formation in > 95 % of embryos arrested in mitosis as a result of PMA or diC₈ treatment.

Elevated [Ca²⁺]_i restores p34^{cdc2} activation and inactivation in PMA-treated embryos

To establish that the effects of PMA on p34^{cdc2} activation and inactivation were due to the absence of [Ca²⁺]_i transients, rather than to a direct effect of PKC (or PMA) on p34^{cdc2}, we used the Ca²⁺ ionophore A23187 to elevate [Ca²⁺]_i in PMA-treated embryos. As shown in Figures 6(A) and 6(B), the addition of Ca²⁺ ionophore to embryos blocked in interphase with low p34^{cdc2} kinase activity led to an approximately 2-fold activation

Figure 5 Reversibility of the mitotic arrest induced by activators of PKC

Embryos (■) were treated with 200 nM PMA (A) or 100 μ M diC₈ (B) during prophase (first arrow). After 24 min (second arrow), 5 μ M staurosporine was administered to half of each population (△). The percentage of embryos exhibiting NEBD was determined for all four populations. Typical results are shown. DAG, diC₈.

of p34^{cdc2}. When PMA was applied later in the cell cycle, to arrest embryos with elevated p34^{cdc2} kinase activity, ionophore treatment triggered a 65 % decrease in activity (Figure 6C). Moreover, it is significant that the same ionophore treatment promoted activation of p34^{cdc2} when activation was blocked by PMA, and induced inactivation of p34^{cdc2} when inactivation was blocked by PMA. In both of these cases, while there was an alteration in the level of p34^{cdc2} kinase activity upon addition of ionophore there was neither entry into nor exit from mitosis. This might be due to the fact that ionophore-induced [Ca²⁺]_i transients differ from normal cell cycle [Ca²⁺]_i transients, in terms of both magnitude and spatial localization. In an attempt to more faithfully replicate normal cell cycle [Ca²⁺]_i transients, Ins(1,4,5)P₃ (1 μ M) was injected into embryos that had been arrested in interphase by the addition of PMA 24 min after fertilization. In 20 experiments, in 3 batches of embryos, microinjection of Ins(1,4,5)P₃ did not permit entry into mitosis as determined by scoring for NEBD (results not shown).

PKC activation uncouples p34^{cdc2} activity and cyclin B abundance

Since the activation of p34^{cdc2} requires its binding to cyclin B, while inactivation normally requires cyclin B proteolysis [35,36], we determined whether PKC activation had effects on the synthesis and destruction of cyclin B. Embryos were pulse-labelled with [³⁵S]methionine prior to fertilization and levels of cyclin B were monitored by SDS/PAGE and autoradiography. In control embryos, treated with 4 α PMA during G1 phase, cyclin B migrated on SDS gels with an apparent molecular mass of 50 kDa, and was converted into a 53 kDa form during late G2 and early M phases (Figure 7A; control, 48–84 min) due to phosphorylation by p34^{cdc2} [3,37]. During anaphase, cyclin B was completely destroyed (Figure 7A; control, 96–108 min), and

Figure 6 Ca²⁺ promotes p34^{cdc2} kinase activation and inactivation in PMA-blocked embryos

Batches of embryos were treated with 200 nM PMA at three successive stages of the cell cycle. In each instance, p34^{cdc2} kinase activity was determined 12 min after the addition of PMA (first open bar) and 60 min after PMA (second open bar). Ca²⁺ ionophore, A23187 (5 µg/ml), was added to half of each batch 12 min after PMA addition, and p34^{cdc2} kinase activity was measured 48 min later (i.e. 60 min after PMA addition; solid bar). Times of PMA addition correspond to: (A) anaphase and (B) telophase of the first mitosis; (C) interphase of the second cell cycle. A typical result is shown.

then began to re-accumulate in advance of the next mitosis (Figure 7A; control, 120 min). Cyclin B was synthesized at the same rate in embryos in which PKC was activated during G1 phase; however, it did not become phosphorylated (consistent with the block to p34^{cdc2} activation) and was not destroyed (Figure 7A; PMA added at 24 min). Furthermore, cyclin B co-adsorbed with p34^{cdc2} on to immobilized p13^{suc1} (Figure 7B), indicating that it was bound to p34^{cdc2} in the PMA-treated embryos. Therefore the activation of PKC during interphase inhibits the pathway controlling p34^{cdc2} activation at a step which follows formation of the p34^{cdc2}-cyclin B complex. Since we have shown that the application of Ca²⁺ ionophore activates p34^{cdc2} in embryos treated with PMA in interphase, the simplest interpretation of these data is that interphase [Ca²⁺]_i transients are required for activation of p34^{cdc2} following the formation of the p34^{cdc2}-cyclin B complex.

When PKC was activated during prophase in order to arrest the cell cycle in mitosis (with high p34^{cdc2} activity), cyclin B nevertheless was destroyed at the same time as in control embryos

Figure 7 [Ca²⁺]_i transients are not required for the synthesis and destruction of cyclin B

(A) Levels of dephosphorylated and phosphorylated cyclin B (cycB and P-cycB) in control embryos treated with 200 nM 4αPMA during G1 phase (24 min after fertilization), and in embryos treated with 200 nM PMA during G1 phase or prophase (24 or 79 min after fertilization). The control embryos completed mitosis, while those treated with PMA at 24 min did not enter mitosis and those treated at 79 min did not exit mitosis. (B) Levels of cyclin B bound to p34^{cdc2} were determined by adsorption to p13^{suc1}-Sepharose. Typical results are shown.

(Figure 7A; PMA added at 79 min). This result demonstrated that the destruction of cyclin B and inactivation of p34^{cdc2} could be temporally uncoupled in sea urchin embryos, such that p34^{cdc2} remained active in the absence of cyclin B. A similar result has been observed in frog oocytes treated with okadaic acid [36]. Since we have shown that artificially elevating [Ca²⁺]_i in embryos arrested in mitosis led to a decrease in p34^{cdc2} activity we conclude that Ca²⁺ is required to regulate the pathway controlling p34^{cdc2} inactivation at a step which is distinct from cyclin B proteolysis.

Ca²⁺ requirements for p34^{cdc2} dephosphorylation

The data presented so far suggest that PKC activation abolishes the cell cycle [Ca²⁺]_i transients that normally regulate p34^{cdc2} activation and inactivation. They also indicate that the cell cycle dynamics of cyclin B are not affected by the abolition of cell cycle [Ca²⁺]_i transients. A general paradigm for mitotic regulation is that the association of p34^{cdc2} with cyclin B during interphase promotes p34^{cdc2} phosphorylation at both inhibitory (Thr¹⁴ and Tyr¹⁵) and activating (Thr¹⁶¹) sites. Dephosphorylation of the inhibitory sites during G2 phase activates p34^{cdc2}, while dephosphorylation of the activating site during anaphase is essential for inactivation [36,38]. Since the cell cycle dynamics of cyclin B did not explain the Ca²⁺ requirement for p34^{cdc2} activation and inactivation in our study, we analysed how the elimination of [Ca²⁺]_i transients through PKC activation might influence the phosphorylation state of p34^{cdc2} at putative regulatory sites.

In early sea urchin embryos p34^{cdc2} undergoes cell cycle-dependent tyrosine phosphorylation, which is at least partially dependent on newly synthesized cyclin B [39]. As shown in

Figure 8 $[Ca^{2+}]_i$ transients are required for cell cycle-dependent $p34^{cdc2}$ dephosphorylation

(A) Anti-phosphotyrosine Western-blot of $p34^{cdc2}$ in embryos treated with 200 nM PMA or 200 nM 4 α PMA during G1 phase (24 min after fertilization). The PMA-treated embryos did not undergo NEBD. (B) Phosphothreonine content of $p34^{cdc2}$ in embryos treated with 200 nM PMA and 100 μ M emetine (■) during prophase (arrow), and in control embryos which were similarly treated with 4 α PMA and emetine (○). The PMA-treated embryos did not exit from mitosis.

Figure 8(A) (control), $p34^{cdc2}$ exhibited a normal pattern of tyrosine phosphorylation and dephosphorylation over the course of the cell cycle in 4 α PMA-treated control embryos. The phosphotyrosine content of $p34^{cdc2}$ gradually increased after fertilization, and was maximal during interphase prior to $p34^{cdc2}$ activation (0–60 min). Phosphotyrosine was absent during mitosis (80–100 min), but reappeared during the next interphase (120–140 min). In contrast, the level of $p34^{cdc2}$ tyrosine phosphorylation continued to increase throughout the experiment when PMA was applied during G1 phase (Figure 8A; PMA). Therefore $p34^{cdc2}$ did not undergo the tyrosine dephosphorylation necessary for activation in PMA-treated embryos that lacked interphase $[Ca^{2+}]_i$ transients.

To study whether or not Ca^{2+} was also involved in regulating $p34^{cdc2}$ phosphorylation at an activating site, equivalent to Thr¹⁶¹, two-dimensional phosphopeptide mapping and phospho amino acid analysis were performed on tryptic digests of $p34^{cdc2}$ that were isolated from ^{32}P -labelled embryos. Interestingly, a Thr¹⁶¹-containing phosphopeptide was not evident in two-dimensional maps; perhaps because Thr¹⁶¹ phosphorylation is difficult to detect by ^{32}P labelling *in vivo* [40], and because only 20% of the $p34^{cdc2}$ -associated ^{32}P was phosphothreonine (results not shown). Therefore we subjected ^{32}P -labelled sea urchin $p34^{cdc2}$ to acid hydrolysis (6 M HCl at 110 °C) for 6 h, since the half-life of phosphothreonine under these conditions is > 25 h, while the half-lives of phosphoserine and phosphotyrosine are 8 h and 2 h respectively [41,42]. This procedure improved the yield of free

phosphothreonine relative to phosphoserine and phosphotyrosine. Levels of ^{32}P -labelled phosphothreonine were measured using TLC. As shown in Figure 8(B), the phosphothreonine content of $p34^{cdc2}$ increased more than 2-fold prior to mitosis in control embryos, and decreased to a similar extent as the population returned to interphase. In contrast, phosphothreonine remained at the elevated mitotic level for at least 90 min when PMA was added during prophase to block $p34^{cdc2}$ inactivation and exit from mitosis (Figure 8B). The addition of PMA did not induce extensive phosphorylation of $p34^{cdc2}$ by PKC, since additional tryptic phosphopeptides were not present in two-dimensional maps of $p34^{cdc2}$ isolated from PMA-treated embryos (results not shown). In addition, our results were not influenced by the re-phosphorylation of sites equivalent to Thr¹⁴ and Thr¹⁶¹ in preparation for the next mitosis. These events require cyclin B [36,38], and emetine was added to both the control and the PMA-treated embryos during prophase to block the *de novo* synthesis of cyclin B after it was destroyed. Therefore in PMA-treated embryos where PKC activation leads to an abolition of mitotic $[Ca^{2+}]_i$ transients, $p34^{cdc2}$ does not undergo the threonine dephosphorylation that is normally associated with its inactivation following cyclin B degradation.

DISCUSSION

PKC activation causes cell cycle arrest in sea urchin early embryos

PKC has been implicated as a regulator of the cell cycle in many cell types. In oocytes, PKC activation has been shown to both inhibit [43–45] and stimulate [46,47] meiotic maturation. We examined the effects of activators of PKC on the early embryonic cell cycles of sea urchins. We found that treatment of sea urchin early embryos with activators of PKC (PMA or diC₈) resulted in cell cycle arrest that was dependent on the time of application of the inhibitor. The administration of PKC activators prior to the beginning of S phase blocked entry into mitosis, while administration during prophase blocked exit from mitosis. The concentration of PMA used in the present study (200 nM) is sufficient to activate PKC *in vivo*, since similar concentrations induce cytoplasmic alkalinization in unfertilized sea urchin eggs; a process that involves activation of a Na⁺/H⁺ antiporter by PKC [30–32]. To learn more about the inhibitory effects of PKC on the cell cycle we analysed the effects of PKC activation on $[Ca^{2+}]_i$, cyclin B levels, $p34^{cdc2}$ kinase activity and its phosphorylation state.

PKC activation blocks cell cycle $[Ca^{2+}]_i$ transients

We have shown that the activation of PKC eliminates cell cycle $[Ca^{2+}]_i$ transients in populations of early sea urchin embryos without altering the normal resting level of $[Ca^{2+}]_i$. This method arrests the cell cycle in a manner similar to Ca^{2+} chelators injected into single cells [3,4]. In our hands, PMA was more effective than the membrane-permeant Ca^{2+} chelator, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) tetrakis(acetoxymethyl ester), at blocking cell cycle progression in sea urchins. A number of our results show that it is unlikely that PMA arrests the cell cycle due to a non-specific toxic effect. First, the embryos arrest at different defined stages of the cell cycle depending on the time of PMA application. Second, the PMA isomer, 4 α PMA, has no effect on cell cycle progression. Third, rates of protein and DNA synthesis are not altered by PMA treatment. Fourth, PMA does not alter the resting level of $[Ca^{2+}]_i$.

How does the activation of PKC block cell cycle [Ca²⁺]_i transients? In early frog and sea urchin embryos, there is compelling evidence that these transients are generated by periodic increases in the intracellular concentration of Ins(1,4,5)P₃, which causes Ca²⁺ to be released from the endoplasmic reticulum [8,21]. In cultured cells, PKC activation blocks the production of Ins(1,4,5)P₃ by receptor-coupled PtdIns(4,5)P₂ hydrolysis in response to growth factors and other extracellular signals [17–19]. This inhibition is due to PKC phosphorylating phospholipase C (PLC), the enzyme that catalyses PtdIns(4,5)P₂ hydrolysis, or to PKC phosphorylating the cell surface receptor. These phosphorylations prevent the subsequent activation of PLC by tyrosine phosphorylation [17,19] or G proteins [18]. It is intriguing in light of this finding that PMA-induced cell cycle arrest at G2/M in cultured mammalian cells involves PKC phosphorylation of the epidermal growth factor receptor [48]. We have shown that endoplasmic reticulum microsomes prepared from PMA-treated embryos sequester Ca²⁺ at the same rate as microsomes prepared from control embryos. Furthermore, endoplasmic reticulum microsomes prepared from PMA-treated embryos have the same sensitivity to Ins(1,4,5)P₃ as controls. These observations are consistent with the idea that PKC activation blocks [Ca²⁺]_i transients by reducing levels of Ins(1,4,5)P₃ during the cell cycle. In sea urchin embryos, the increases in Ins(1,4,5)P₃ during the cell cycle are probably due to the periodic activation of a PLCγ isoform [49] and this is clearly a potential target for phosphorylation by PMA-activated PKC.

[Ca²⁺]_i transients required for p34^{cdc2} activation and inactivation

We took advantage of the finding that PKC activation appeared to inhibit embryonic cell cycles by preventing normal [Ca²⁺]_i transients, to determine how [Ca²⁺]_i transients might affect levels of p34^{cdc2} kinase activity. In the first cell cycle of sea urchin embryos, [Ca²⁺]_i is transiently elevated at pro-nuclear migration (G1/S), NEBD, anaphase onset, and cleavage [3,8,20,21]. The capacity to eliminate these [Ca²⁺]_i transients using PMA has enabled us to directly show that Ca²⁺ is required *in vivo* for activation and inactivation of the key cell cycle regulatory protein, p34^{cdc2}. The effect of PMA on p34^{cdc2} activity is due to its effect on [Ca²⁺]_i transients, since both the activation and inactivation of p34^{cdc2} are partially restored in PMA-treated embryos when Ca²⁺ ionophore A23187 is used to elevate [Ca²⁺]_i. Ca²⁺ may be required for the p34^{cdc2} activation pathway as early as the G1/S transition, since the administration of PKC activators at later stages of the cell cycle does not block mitosis entry. Therefore it is possible that one or both of the interphase [Ca²⁺]_i transients (G1/S and NEBD) participate in the activation of p34^{cdc2}.

Ca²⁺ is required for the inactivation of p34^{cdc2} at the metaphase/anaphase transition, since the administration of PKC activators during telophase does not block exit from mitosis. This suggests that the metaphase/anaphase [Ca²⁺]_i transient participates in the p34^{cdc2} inactivation pathway. Although it is well established that elevated [Ca²⁺]_i triggers p34^{cdc2} inactivation in oocytes arrested at meiotic metaphase [14], there have been few reports of Ca²⁺ regulating p34^{cdc2} activity during mitosis. Activation and inactivation of p34^{cdc2} are inhibited by Ca²⁺ chelators in cell-free preparations of frog embryos [16], and elevated physiological levels of Ca²⁺ induce precocious inactivation of p34^{cdc2} in permeabilized sea urchin embryos [15]. The latter finding implies that the metaphase/anaphase [Ca²⁺]_i transient is not only required for p34^{cdc2} inactivation, but that it controls the timing of inactivation.

Although artificially elevating [Ca²⁺]_i by the application of ionophore led to a partial restoration of both activation and inactivation of p34^{cdc2}, this did not allow cell cycle progression to take place. Microinjection of Ins(1,4,5)P₃ into arrested embryos was similarly without effect. This might be because neither of these artificial methods of elevating [Ca²⁺]_i faithfully recreates the temporal and spatial aspects of cell cycle [Ca²⁺]_i transients that are required for entry into or exit from mitosis. Alternatively, it is possible that PKC activation might also inhibit cell cycle progression through alternative mechanisms in addition to its effect on cell cycle [Ca²⁺]_i transients. For example, it is possible that PKC activation might result in the abnormal sub-cellular distribution of key Ca²⁺-binding proteins, such as calmodulin [50].

Mechanism of p34^{cdc2} activation by Ca²⁺

In systems where it has been analysed, the activation of p34^{cdc2} involves several sequential events. In the first step, p34^{cdc2} binds to cyclin B and is phosphorylated at a site required for activity (Thr¹⁶¹), and at sites that inhibit its activity (Thr¹⁴ and Tyr¹⁵). In the second step, the cdc25 phosphatase activates p34^{cdc2} by dephosphorylating Tyr¹⁵ and Thr¹⁴ [2]. The amino acid sequence of sea urchin p34^{cdc2} has not been determined; however, it undergoes cycles of tyrosine phosphorylation during interphase and dephosphorylation at G2/M, consistent with the presence of an inhibitory site equivalent to Tyr¹⁵ ([39] and Figure 8A). We have shown in the present study that the elimination of interphase [Ca²⁺]_i transients does not inhibit cyclin B synthesis, formation of the cyclin B–p34^{cdc2} complex, or the phosphorylation of p34^{cdc2} on tyrosine. However, p34^{cdc2} is not activated and does not undergo the tyrosine dephosphorylation that normally accompanies activation. These results imply that elevated Ca²⁺ is required for the dephosphorylation of p34^{cdc2} at an inhibitory site equivalent to Tyr¹⁵. If so, the role of Ca²⁺ may be to increase cdc25 phosphatase activity, or to decrease the activity of Wee1, the protein kinase that phosphorylates p34^{cdc2} at Tyr¹⁵. In HeLa cells, the human tyrosine phosphatase p54(cdc25-c) is phosphorylated at the G2/M phase transition through a Ca²⁺/calmodulin-dependent pathway [51]. It is possible that in sea urchin embryos, cdc25 activity is affected by the [Ca²⁺]_i transient at the G2/M phase transition through a similar Ca²⁺-dependent mechanism.

Mechanism of p34^{cdc2} inactivation by Ca²⁺

The inactivation of p34^{cdc2} during M phase involves proteolytic destruction of its cyclin B subunit and dephosphorylation at Thr¹⁶¹ [36]. In frog oocytes arrested at meiotic metaphase, the fertilization [Ca²⁺]_i transient induces proteolysis of cyclin B and inactivation of p34^{cdc2} [14]. In permeabilized sea urchin embryos, elevated [Ca²⁺]_i induces p34^{cdc2} inactivation without cyclin B proteolysis, thereby demonstrating that these two processes can be uncoupled in a mitotic system, and that p34^{cdc2} inactivation, but not cyclin destruction, is controlled by Ca²⁺ [15]. This conclusion is supported by the work of Lindsay et al. [16], who found that the addition of Ca²⁺ to mitotic extracts prepared in the presence of the Ca²⁺ chelator, BAPTA, could induce rapid inactivation of p34^{cdc2} without cyclin B proteolysis. An important prediction of our hypothesis is that the elimination of mitotic [Ca²⁺]_i transients should block inactivation of p34^{cdc2} without blocking the destruction of cyclin B. In the present study, we show that this prediction is correct; the use of PMA to eliminate [Ca²⁺]_i transients during mitosis blocks p34^{cdc2} inactivation, but cyclin B is destroyed at the same time as in control embryos. This is further evidence that p34^{cdc2} inactivation and cyclin B pro-

Figure 9 The effects of Ca^{2+} on p34^{cdc2} kinase activation and inactivation in embryonic cell cycles

The various p34^{cdc2} –cyclin B complexes formed during the early embryonic cell cycles are shown. Inactive p34^{cdc2} is shown as an oval and active p34^{cdc2} is shown as a star shape. In interphase, p34^{cdc2} and cyclin B associate and p34^{cdc2} is phosphorylated on Tyr¹⁵ (Y on the left side of cdc2) by Wee1 kinase. Thr¹⁶¹ (T on the right side of cdc2) is phosphorylated by cdc2 -activating kinase (CAK). At the onset of mitosis, the conversion from inactive into active p34^{cdc2} kinase is accomplished through the dephosphorylation of Tyr¹⁵ by the tyrosine phosphatase cdc25 . Activation of p34^{cdc2} kinase leads to the stimulation of the anaphase-promoting complex (APC) leading to cyclin destruction by proteasomes. As mitosis ends, p34^{cdc2} is rendered inactive by the activity of Thr¹⁶¹ phosphatase (T161P). The data presented in the present study suggest that Ca^{2+} is important in the activation of p34^{cdc2} kinase either through the activation of cdc25 or inactivation of Wee1. Ca^{2+} is also required for Thr¹⁶¹ phosphatase activation and thus p34^{cdc2} inactivation.

teolysis are distinct, separable processes during mitosis. In contrast, we show that in the absence of mitotic $[\text{Ca}^{2+}]_i$ transients, p34^{cdc2} does not undergo threonine dephosphorylation that is normally associated with its inactivation and exit from mitosis. Lorca et al. [36] previously reported that okadaic acid (an inhibitor of type 1 and type 2A serine/threonine protein phosphatases) blocked p34^{cdc2} inactivation, but not cyclin B proteolysis, in cell-free extracts of frog embryos. In their study, p34^{cdc2} remained active in the absence of cyclin B, because it did not undergo dephosphorylation at Thr¹⁶¹. Our results provide evidence that the metaphase/anaphase $[\text{Ca}^{2+}]_i$ transient controls the timing of p34^{cdc2} inactivation in early sea urchin embryos by inducing the dephosphorylation of p34^{cdc2} at a site required for activity (the equivalent of Thr¹⁶¹). A model of the possible interactions of Ca^{2+} with cell cycle control proteins in early embryonic cell cycles is shown in Figure 9.

If Ca^{2+} inactivates p34^{cdc2} via dephosphorylation, what is the role of cyclin B proteolysis? Since the phosphorylation of p34^{cdc2} at Thr¹⁶¹ requires p34^{cdc2} binding to cyclin B [36,38], and because the Thr¹⁶¹ kinase is constitutively active during early development [52], cyclin B proteolysis ensures that p34^{cdc2} remains inactive until the appropriate stage of the next cell cycle, when cyclin B has re-accumulated. This is especially important in view of the transient nature of the metaphase/anaphase increase in $[\text{Ca}^{2+}]_i$. A return of $[\text{Ca}^{2+}]_i$ to resting levels in the continued presence of cyclin B might be expected to re-activate p34^{cdc2} and thereby prevent the completion of mitosis, as occurs with non-degradable

cyclin B mutants [35,53–55]. In permeabilized sea urchin embryos, elevated $[\text{Ca}^{2+}]_i$ inactivates p34^{cdc2} without cyclin B proteolysis; however, $[\text{Ca}^{2+}]_i$ is maintained at an elevated level throughout the course of these experiments by means of Ca^{2+} /EGTA buffers [15]. Although the phosphothreonine content of p34^{cdc2} was not determined in the present study, Ca^{2+} was found to induce dissociation of cyclin B– p34^{cdc2} complexes [15]. Since this complex is stabilized by the phosphorylation of p34^{cdc2} at Thr¹⁶¹ [56], its dissociation may well be a consequence of Ca^{2+} -induced Thr¹⁶¹ dephosphorylation. How Ca^{2+} plays a role in p34^{cdc2} activation at one stage of the cell cycle, and triggers inactivation at another stage, is an important question. The present study suggests that component(s) of the pathway that govern p34^{cdc2} dephosphorylation at Thr¹⁶¹ are not functional, or do not have access to p34^{cdc2} , during the NEBD $[\text{Ca}^{2+}]_i$ transient. In contrast, these component(s) are enabled, or are accessible to p34^{cdc2} at the time of the metaphase/anaphase $[\text{Ca}^{2+}]_i$ transient.

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