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Calcium requirements during mitotic cdc2 kinase activation and cyclin degradation in *Xenopus* egg extracts

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SUMMARY

Activation of p34^{cdc2} kinase is essential for entry into mitosis while subsequent deactivation and cyclin degradation are associated with exit. In *Xenopus* embryos, both of these phases are regulated by post-translational modifications and occur spontaneously on incubation of extracts prepared late in the first cell cycle. Even though high levels of calcium buffer were initially used to prepare these extracts, we found that free calcium levels in them remained in the observed physiological range (200-500 nM). Further addition of calcium buffers only slightly reduced free calcium levels, but inhibited histone H1 (cdc2) kinase deactivation and cyclin degradation. Higher buffer concentrations slowed the kinase activation phase. Reducing the free buffer concentration by premixing with calcium reversed the effects of the buffer, indicating that the inhibitory effects arose from the calcium-chelating properties of the buffer rather than non-specific side effects. Furthermore, additions of calcium buffer at the end of the H1 kinase activation phase did not prevent deactivation. From these results, and the order of effectiveness of different calcium buffers in disrupting the H1 kinase cycle, we suggest that local transient increases in free calcium influence the rate of cdc2 kinase activation and are required to initiate the pathway leading to cyclin degradation and kinase inactivation in mitotic cell cycles.

Key words: BAPTA, calcium, cell division cycle, cyclin, CaM kinase

INTRODUCTION

Entry into mitosis is induced by the activation of maturation promoting factor (MPF; Masui and Markert, 1971). Purification of MPF showed it to be a complex containing a serine/threonine kinase p34^{cdc2} and a B type mitotic cyclin (Gautier et al., 1988; Labbe et al., 1989). Activation of mitotic *cdc2* kinase requires association of p34^{cdc2} and cyclin B, but this is not sufficient to activate the kinase (reviewed by Coleman and Dunphy, 1994). Regulation of p34^{cdc2} in higher eukaryotes involves phosphorylation and dephosphorylation of analogous tyrosine and threonine residues to those in fission yeast. However, phosphorylation occurs not only on Tyr15 (as in yeast) but also on the adjacent threonine residue Thr14 (Krek and Nigg, 1991; Norbury et al., 1991). Phosphorylation of either of these residues reduces the activity of p34^{cdc2} kinase as both must be dephosphorylated for full activation (Norbury et al., 1991). Phosphorylation of threonine residue 161 is essential for kinase activity. In *Xenopus* eggs, tyrosine phosphorylated forms of p34^{cdc2} accumulate during interphase of the first cell cycle (Ferrell et al., 1991) such that, just before mitosis, high speed supernatant extracts can be prepared that spontaneously undergo one round of kinase activation, deactivation and specific cyclin degradation on incubation at room temperature (prophase extracts; Felix et al., 1989). Extracts prepared earlier in interphase do not spontaneously activate (Felix et al., 1989). What causes the transition from accumulation of tyrosine-phosphorylated p34^{cdc2} to loss of these forms and activation is not understood (Coleman and Dunphy, 1994).

Fertilization of *Xenopus* eggs stimulates a rise in the intracellular free calcium concentration (Busa and Nuccitelli, 1985), leading to cyclin degradation by the ubiquitin proteolysis system (Glotzer et al., 1991; King et al., 1995) and the inactivation of MPF and cytostatic factor (CSF; Meyerhof and Masui, 1977; Watanabe et al., 1991). Extracts, prepared from metaphase arrested eggs using EGTA-containing buffer, retain H1 (cdc2) kinase activity which can be deactivated, together with specific cyclin degradation, by addition of calcium (Murray et al., 1989; Lorca et al., 1991). Deactivation can be inhibited by adding a peptide corresponding to the autoinhibitory domain of calcium/calmodulin dependent kinase II (CaM kinase II) while the requirement for calcium can be overidden by adding a constitutively active form of CaM kinase II (Lorca et al., 1993).

In mitotic cell cycles transient increases in intracellular free calcium are temporally linked to nuclear envelope breakdown and the metaphase/anaphase transition in sea urchins (Poenie et al., 1985). These events can be blocked by calcium chelators or CaM kinase II inhibitory peptides, suggesting a requirement for CaM kinase II in the sea urchin mitotic cell cycle (Baitinger

et al., 1990; Whitaker and Patel, 1990; Patel and Whitaker, 1991). In *Aspergillus nidulans* limiting levels of extracellular calcium or intracellular calmodulin in a calmodulin conditional strain, prevent entry into mitosis and block activation of both p34^{cdc2} and NIMA kinases (Lu and Means, 1993). Expression of constitutively active forms of CaM kinase II produced G_2 arrest in mouse cells (Planas-Silva and Means, 1992). These apparently contradictory results might be resolved if a short-lived increase in CaM kinase II activity was required.

Calcium and pHi fluctuations that have a periodicity equal to that of the early embryonic cell cycle have been detected in *Xenopus* eggs (Grandin and Charboneau, 1991a,b). These Ca^{2+} fluctuations are small and slow in comparison with the short lived Ca^{2+} increase observed at fertilization (5-10 minutes; Busa and Nuccitelli, 1985). However, injection of calcium buffers can prevent, delay or alter the normal pattern of cleavage furrow formation depending on the time of injection and the concentration of buffer (Baker and Warner, 1972; Han et al., 1992; Snow and Nuccitelli, 1993; Miller et al., 1993). Of several BAPTA (1,2-bis(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid) buffers tested, dibromo-BAPTA was the most potent (Miller et al., 1993). This BAPTA derivative was also the most effective in blocking cleavage and rhizoid outgrowth in fucoid eggs (Speksnijder et al., 1989) and in preventing membrane fusion during nuclear assembly in *Xenopus* egg extracts (Sullivan et al., 1993). The calcium dissociation constant (K_D) of dibromo-BAPTA is 1.2 μM under the ionic conditions of the *Xenopus* extracts used (Sullivan et al., 1993). Around this concentration of free calcium dibromo-BAPTA should be maximally effective in suppressing calcium concentration changes (Speksnijder et al., 1989). These injection experiments have been interpreted to indicate that local or transient changes in free calcium are needed for cell cycle progression. Though these calcium buffers prevented cleavage when injected during interphase, the nuclear division cycle and MPF activation were not analysed.

A role for any of the calcium dependent phosphatases or kinases in the regulation of H1 kinase activity in egg extracts has been considered unlikely since the extraction buffers used often contain relatively high concentrations of EGTA (60 mM; Felix et al., 1989). We have prepared late interphase extracts of activated eggs using a buffer in which EGTA was replaced with BAPTA. These extracts underwent one cycle of H1 kinase activation, deactivation and cyclin degradation. However, further addition of BAPTA prevented the deactivation of histone H1 kinase and at higher concentrations slowed kinase activation. These effects were achieved by the calcium chelating activity of BAPTA though the measured free calcium in such extracts was in the expected physiological range. Dibromo-BAPTA was more effective than BAPTA in disrupting the H1 activation cycle. The data indicate that calcium mediated events influence the rate of cdc2 kinase activation and are required to initiate cyclin degradation. We suggest that local transient increases in free calcium stimulate both steps.

MATERIALS AND METHODS

Materials

BAPTA (Calbiochem) was dissolved at 500 mM in water after first neutralising to pH 7.0 with HCl. Dibromo-BAPTA (Calbiochem) was dissolved at 100 mM in water. EGTA (Sigma) was dissolved as a 500 mM solution, pH 7.2. CaM kinase II inhibitory peptide (Calbiochem) was dissolved in water at 5 mM. PKC inhibitory peptide (19-36) was the generous gift of Dr John Kay. Other chemicals used were molecular grade and purchased from either Sigma, Pharmacia, Calbiochem, BDH or Boehringer.

Preparation of high-speed extracts from activated eggs

Extracts were prepared using the protocol of Felix et al. (1989) with several modifications. Female *Xenopus* were injected and eggs collected, dejellied and washed as described previously (Hutchison et al., 1987). They were transferred to a shallow glass beaker in 25% saline tapwater containing 0.25 μg/ml ionophore A23187 to induce synchronous activation, which was identified by the contraction and subsequent relaxation of the animal pigment and the swelling of the fertilization membrane. Following activation, the eggs were washed three times in 25% saline tapwater to remove the ionophore and allowed to incubate at 22°C for 10-45 minutes for interphase extracts, or 50-60 minutes for prophase extracts (all timings taken from the point of ionophore addition). After incubation, the eggs were washed twice with distilled water and once in ice-cold extraction buffer (100 mM K-acetate, 2.5 mM Mg-acetate, 5 mM BAPTA, 1 mM DTT, 20 mM Hepes, pH 7.2, 250 mM sucrose, and 5 μg/ml cytochalasin B). Some extracts were prepared with 60 mM EGTA (as Felix et al., 1989) instead of BAPTA. The eggs were transferred to 5 ml centrifuge tubes containing extraction buffer, excess buffer removed, and centrifuged at 10,000 rpm for 10 minutes at 2°C using a SW50.1 rotor in a Beckman L2 ultracentrifuge. The cytoplasmic layer was centrifuged again at 48,000 rpm for 2 hours at 2°C in the same rotor. The clear supernatant fraction was removed by side puncture using a 25 gauge needle, snap frozen and stored in liquid nitrogen. On thawing, the extract was supplemented with an energy regeneration system: 1 μl of each of the following stock solutions per 50 μl of extract, 500 mM creatine phosphate, 4 mg/ml creatinephosphokinase (50% glycerol in H_2O), and 50 mM ATP (pH 7.0 in 20 mM Hepes).

Extract incubations and histone kinase assays

Aliquots of extract were pooled, supplemented with the energy regeneration system (see above) and divided into 30 μl aliquots. Additions were made at 4°C (never more than 20% of the extract volume). Aliquots were incubated at 22°C. Timepoint samples, taken as indicated, were diluted either 1/25 for H1 kinase assays and snap frozen, or in SDS sample buffer for SDS-PAGE and immunoblotting.

Histone H1 kinase assays were performed as described by Felix et al. (1989) with minor modifications. Duplicate 10 μl aliquots of diluted timepoint samples were incubated with 10 μl of reaction cocktail containing 0.6 mM ATP, 2 mg/ml histone H1 (calf thymus: Sigma), 75 μCi/ml [γ-^{32}P]ATP (Amersham) for 15 minutes at 22°C. Assays were stopped by spotting 15 μl of each sample onto P81 paper (Whatman) and processed as described (Felix et al., 1989). Aliquots of MPF extract were included with each experiment and H1 kinase activity expressed relative to this, after subtracting the counts registered in samples incubated without added extract. Duplicate assays were routinely within 2% of each other. Estimates of H1 kinase activity were in the range observed by Felix et al. (1989), with peak activities of 8-10 pmoles phosphate transferred per minute per μl extract.

p13^{suc1} bead depletion of extracts

p13^{suc1} protein purified from *E. coli* was coupled to CNBr activated Sepharose as previously described (Chevalier et al., 1995). Prior to use beads were washed 3 times with 200 μl of bead wash buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P40, 10 μg/ml of aprotinin, leupeptin, soyabean trypsin inhibitor and 100 μM benzamidine) and once in the histone kinase buffer. The volume of beads used was 75% of the undiluted volume of extract to be depleted. Incubations were for 90 minutes at 4°C with

mixing every 5 minutes. The beads were pelleted by spinning for 5 seconds in a microfuge. The pellet was washed three times in 200 μl bead wash buffer and either assayed for histone H1 kinase activity or processed for immunoblotting. Protein was stripped from the beads by boiling with SDS sample buffer and run on 12% polyacrylamide gels.

Preparation of [^{35}S]methionine labelled cyclin

Capped mRNAs were prepared from linearised plasmids as described by Mathews and Colman (1991). *Xenopus* cyclin clones (B_1 and B_2) in the expression vector pGEM (Minshull et al., 1990) were a generous gift from Dr Tim Hunt. [^{35}S]methionine labelled *Xenopus* cyclins were prepared in message dependent reticulocyte lysate (gift of Dr T. Patrick) programmed with the respective cyclin mRNA. Maximally 2 μl of this was added to 30 μl prophase extract to follow cyclin degradation.

SDS-PAGE and immunoblotting

SDS-PAGE was carried out using the method of Laemmli (1970) with the modifications of Knowland (1981). Gels were fixed and Coomassie stained in 10% glacial acetic acid, 40% methanol, and 0.1% Brilliant Blue R. After de-staining to visualize the molecular mass markers, gels were dried down and exposed to Hyperfilm β-max X-ray film (Amersham). Alternatively gels were processed for fluorography using the enhance system (Dupont). Treated gels were exposed at −70°C to Hyperfilm-MP (Amersham) preflashed to 0.15 Au.

For immunoblotting, proteins were transferred in transfer buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol) overnight at 10 V onto nitrocellulose. The filter was blocked by incubating in blot rinse buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20) containing 4% fat-free milk protein (blotto) for 6 hours at 4°C. Filters to be probed with anti-phosphotyrosine antibodies were blocked in a solution of blot-rinse buffer containing 4% bovine serum albumin. Filters were incubated overnight in blot rinse buffer containing 0.1% FCS and the respective primary antibody. The membrane was incubated in blotto containing an alkaline phosphatase-conjugated secondary antibody (Dako 1:400) for 2 hours at room temperature. Bands were visualised using 5 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 2 μg/ml nitro blue tetrazolium (NBT) in a buffer containing 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$. PSTAIRE monoclonal antibody (diluted 1:4,000) was a gift from Dr Y. Yamashita (Yamashita et al., 1991). Anti-phosphotyrosine antibody (diluted 1:1,000) was a gift from Dr G. Peaucellier. p34^{cdc2} C-terminal antibody was kindly provided by Dr Tim Hunt.

Calcium measurements

Measurement of free calcium was made essentially to the method of Swann and Whitaker (1986) with modifications to accommodate measurements in extracts as opposed to intact sea urchin eggs. Extracts were inoculated with fura 2 to a final concentration of 50 μM; 10 μl of this extract was then transferred to a microscope slide and pulsed with fluorescent light wavelengths of 350 and 380 nm. Addition of calcium chloride or calcium chelators directly to extract on the microscope slide was found to be unreliable due to the formation of air bubbles in the extract. For this reason additions were made to the extract in an 1.5 ml Eppendorf tube and then immediately transferred to the slide. The ratio of the fura 2 emissions at these two wavelenths was used to calculate the free Ca^{2+} concentration. [Ca^{2+}] was measured for 5 minutes or until a steady reading was achieved. Free [Ca^{2+}] was calculated by the equation $K(R–R_0)/(R_s–R)$ where R_0 is the ratio of fluoresence at 380 nm and 350 nm at zero Ca^{2+} and R_s is the fluorescence at saturating Ca^{2+}. K represents $K_D(F_0/F_s)$ where K_D is the dissociation constant for fura-2 in the appropriate ionic conditions. F_0 is the fluorescence at 380 nm in zero Ca^{2+} while F_s is the fluorescence at 380 nm at saturating Ca^{2+} (Poenie et al., 1985). R_0 and R_s were obtained by pulsing fura-2 reference solutions with light at both wavelengths (zero calcium solution: 10 mM EGTA, 100 mM KCl, 1 mM $MgCl_2$, pH 7.0, 20 mg/ml BSA; saturating calcium: same solution but containing 40 mM $CaCl_2$). Data was recorded and analysed using the CRS-400 software (V.2.4B; C. Regan PhD, Bio-Rad Microscience Ltd). Estimates of cytoplasmic Ca^{2+} and Mg^{2+} concentrations in the presence of various chelators were calculated using the Max Chelate software (V4.63; Chris Patton, Hopkins Marine Station, Stanford University, Pacific Grove, California, USA).

RESULTS

Changes in the phosphorylation state of p34^{cdc2} during activation of histone H1 kinase

Previous studies have shown that high speed supernatant extracts of *Xenopus* eggs prepared 55-60 minutes after egg activation (prophase extracts) display a single oscillation of histone H1 kinase activity and efficiently degrade exogenous cyclin protein during the kinase deactivation phase. This kinase activity is attributable to the p34^{cdc2}-cyclin B kinase complex (Felix et al., 1989). In order to modulate the free calcium concentration of such extracts, the extraction buffer was modified by substituting 5mM BAPTA for the 60 mM EGTA in the original buffer. Since the eggs are packed under gravity, and excess buffer removed before preparing the extract the final concentration of BAPTA in the extract is less than 2 mM. A typical time course of H1 kinase activation and deactivation during incubation of an extract prepared in this way is shown in Fig. 1A. At different times during the incubation aliquots were taken, incubated with suc1-beads, and the washed pellets analysed by western blotting (Fig. 1B). As is observed with unfractionated extracts (Fig. 2A), five bands are detected at the start of incubation by a PSTAIRE-peptide antibody (Fig. 1B, 0 minutes). The four slower-migrating bands are also detected at similar relative intensities by p34^{cdc2} specific antibodies (Fig. 2B and data not shown). The fastest migrating band detected by PSTAIRE antibody has previously been identified as p33^{cdk2} (Solomon et al., 1991). The presence of four distinguishable p34^{cdc2} bands suggests that the prophase extracts, as expected, contain p34^{cdc2} in several modified forms.

As H1 kinase activated (Fig. 1A) the two slowest migrating cdc2 bands disappeared while the intensity of the other two bands increased (Fig. 1B). Scanning densitometry of such blots showed that the total cdc2 protein remains essentially constant during the incubation (not shown). Thus it is unlikely that we were observing degradation events but rather the interconversion of forms of p34^{cdc2}. As the activation of p34^{cdc2}/H1 kinase is known to involve dephosphorylation on key tyrosine and threonine residues duplicate aliquots of the p13^{suc1} bead pellets were run on the same gel and the blot probed with an anti-phosphotyrosine antibody (Fig. 1B, right panel). Prior to kinase activation (zero time when 5 PSTAIRE bands are clearly detected) there were two distinct bands recognised by the anti-phosphotyrosine antibody that co-migrated with the two slowest cdc2 bands. As activation proceeded, both these bands and the phosphotyrosine bands disappeared simultaneously (Fig. 1B). In extracts (prepared earlier in interphase) where incubation did not result in spontaneous H1 kinase activation, the two slowest migrating p34^{cdc2} bands did not disappear (data not shown). Thus by the criteria of co-precipitation with p13^{suc1} Sepharose and detection by PSTAIRE, p34^{cdc2} specific

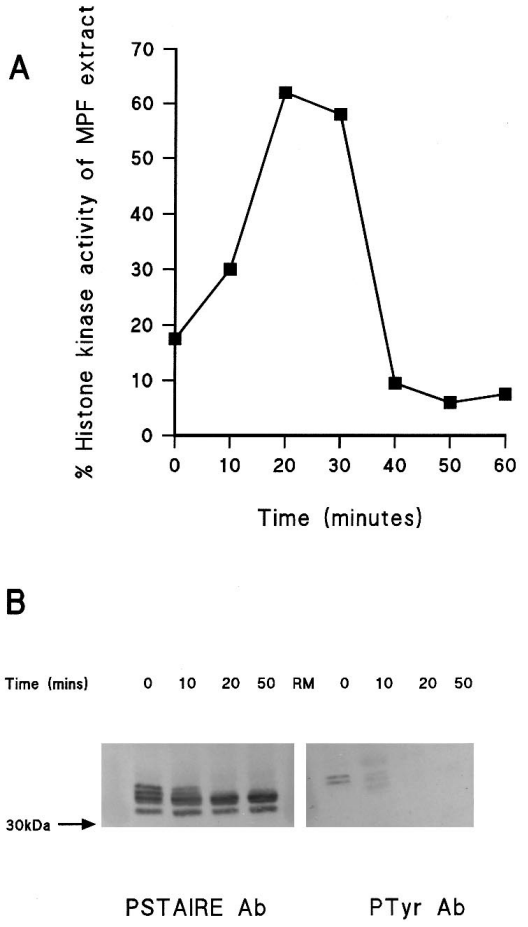

Fig. 1. Time course of H1 kinase activity and western blotting of $p13^{suc1}$ bead pellets with anti-phosphotyrosine/PSTAIRE antibodies. (A) Pattern of H1 kinase activity of prophase extract incubated at 22°C. Samples were taken and assayed for kinase activity on histone H1. (B) Western blots of $p13^{suc1}$ bead pelleted material probed with PSTAIRE and anti-phosphotyrosine antibody. Samples taken at the times indicated from the incubation shown in A were subjected to $p13^{suc1}$ Sepharose depletion. Duplicate samples (2 μl cytoplasm equivalents) were processed for immunoblotting. The nitrocellulose membrane was divided and probed with the respective antibodies. RM, molecular mass marker.

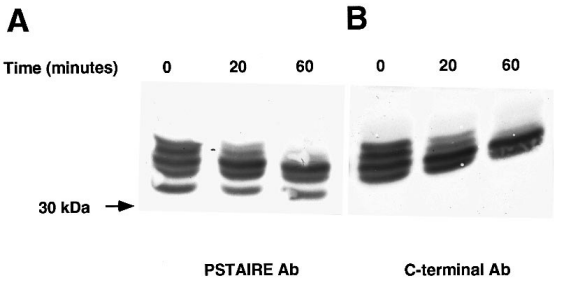

Fig. 2. Western blot of a prophase extract with PSTAIRE (A) or $p34^{cdc2}$ C-terminal antibody (B). Duplicate timepoint samples were taken during incubation and processed using SDS-PAGE and immunoblotting. The filter was then divided and probed with the respective antibodies.

and phosphotyrosine antibodies, these bands are indicative of the tyrosine phosphorylated forms of $p34^{cdc2}$ that are subsequently lost as $p34^{cdc2}$ kinase activates. The rate of loss of these forms can be conveniently followed by the loss of the slowest migrating bands detected with PSTAIRE antibody under these gel conditions.

The calcium chelator BAPTA prevents H1 kinase deactivation and cyclin degradation

Aliquots of a prophase extract were incubated in the presence of various additional concentrations of BAPTA (Fig. 3A). As the concentration of BAPTA is increased, kinase deactivation is delayed, until kinase activity remained high for the remainder of the incubation. Though the standard incubation time was 100 minutes, high levels of H1 kinase activity can be maintained in the presence of BAPTA for at least two hours. These levels were at least as high as the maximum achieved by the control extract, implying that the failure to inactivate was not a consequence of an inability to reach the required threshold level of kinase activity (Felix et al., 1990).

Xenopus cyclin B_2 added to the reactions remained stable during kinase activation until H1 kinase deactivation (Fig. 3B). In this example, addition of 1 mM BAPTA delayed both kinase deactivation and cyclin degradation by 20 minutes. At a concentration of 3 mM, once H1 kinase activity had risen, it remained high and cyclin B_2 remained stable for the rest of the incubation. Different extract preparations showed some variability in the timing of the kinase activation-inactivation cycle (data not shown; Felix et al., 1989) and in their sensitivity to BAPTA. In eight extracts between 1 and 5 mM BAPTA was required to inhibit kinase deactivation.

PSTAIRE immunoblots of samples taken during these incubations showed that the loss of the phosphotyrosine forms of $p34^{cdc2}$ was slightly retarded at BAPTA concentrations which prevented kinase inactivation (Fig. 3C). This retardation also correlated with the time required to reach maximum kinase levels. By 50 minutes, the loss of these forms has been completed and H1 kinase activity has reached levels similar to the control. Despite this level of kinase activity, 3 mM BAPTA prevented the deactivation of H1 kinase and the degradation of cyclin. These results suggest that increased calcium chelator levels inhibit the normal deactivation phase of mitotic $p34^{cdc2}$ cyclin B.

If BAPTA were having a non-specific effect on kinase deactivation then the extent of inhibition at a given concentration should be independent of the level of free BAPTA. To address this, BAPTA was mixed with $CaCl_2$ such that when added to a prophase extract the final concentration of BAPTA would be expected to prevent kinase deactivation. Using the Max Chelate software (V4.63; Chris Patton, Hopkins Marine Station) ratios of BAPTA to Ca^{2+} were chosen such that the calculated free Ca^{2+} of the mix was 140 nM, which is in the range of free Ca^{2+} measured in these extracts (see below). This mixture (3 mM BAPTA/1.5 mM Ca^{2+} after dilution in the extract) should not affect the resting level of Ca^{2+} in the extract, but will reduce the free BAPTA by 1.5 mM. Addition of 3 mM BAPTA alone prevented deactivation of H1 kinase (Fig. 4A). When the same total BAPTA concentration was added after premixing with calcium, the deactivation phase was restored. Though slightly delayed in relation to the control, the effect of this mixture was similar to that of adding a lower

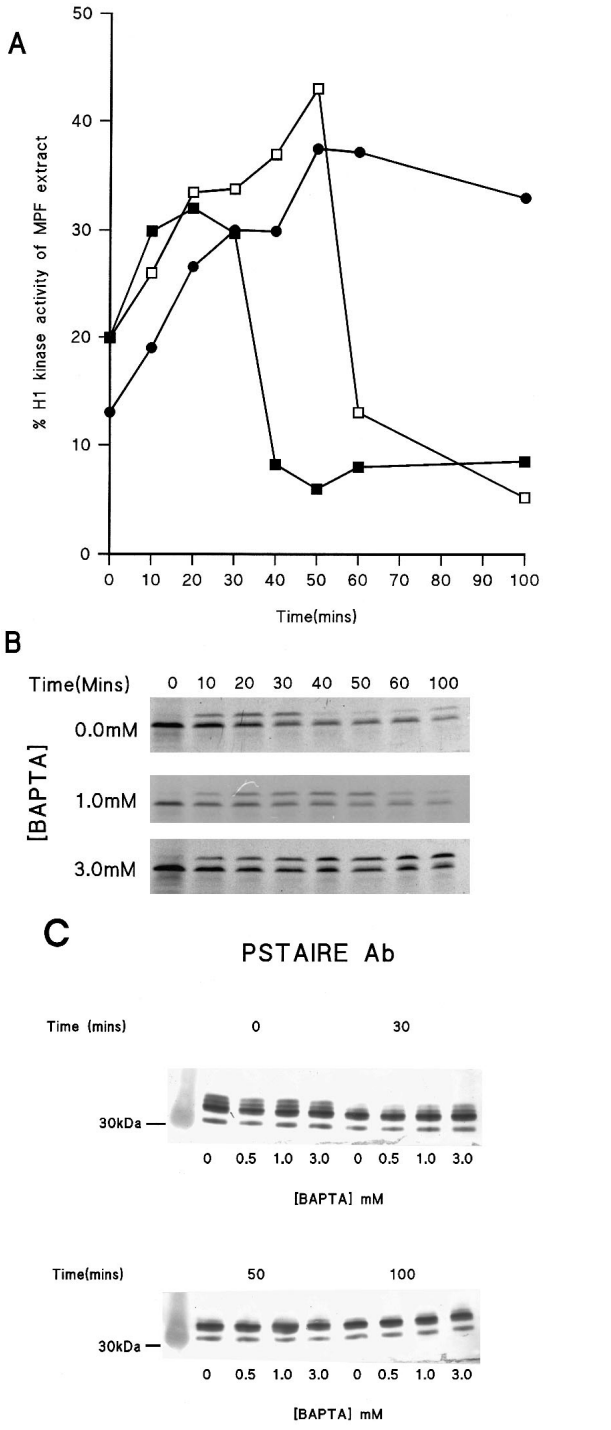

Fig. 3. Effect of the calcium chelator BAPTA on the H1 kinase activity in prophase extracts. (A) Aliquots of prophase extract were incubated at 22°C in the presence of exogenous [^{35}S]methionine labelled *Xenopus* cyclin B_2 and various concentrations of added BAPTA; 0 mM (■); 1 mM (□); 3 mM (●). Samples taken at different times were assayed for H1 kinase activity. At 50 minutes the kinase activity in the sample containing 3 mM BAPTA was 8.8 pmols/min/μl extract. The results with different concentrations of BAPTA are typical of ten experiments with three different extracts. (B) Fluorographs showing changes in the stability of exogenous cyclin B_2 during incubation in prophase extract treated with different BAPTA concentrations. (C) Western blotting using the PSTAIRE antibody on samples taken from the incubation described in A.

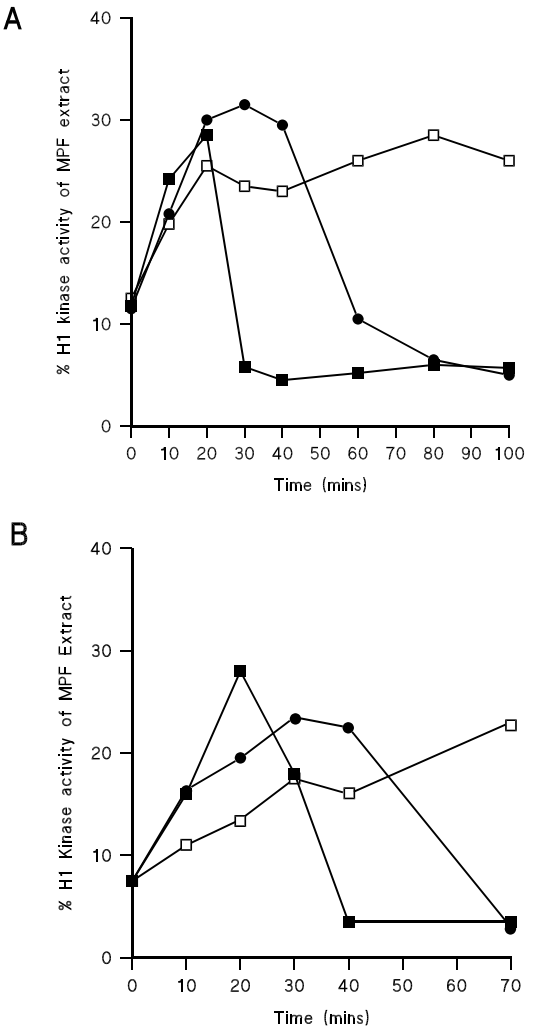

Fig. 4. The inhibition of deactivation and activation of H1 kinase by BAPTA can be reversed by reducing the concentration of free BAPTA. (A) BAPTA/$CaCl_2$ mixes were added to a prophase extract, incubated at 22°C and samples removed for H1 kinase assay. The final concentrations of BAPTA and $CaCl_2$ were: 0.0 mM BAPTA/0.0 mM $CaCl_2$ (■); 3 mM BAPTA/0.0 mM $CaCl_2$ (□); 3.0 mM BAPTA/1.5 mM $CaCl_2$ (●). (B) A different extract, in which the kinase activation phase was inhibited by BAPTA, was used. The experiment was performed as in A. The final concentrations of BAPTA and $CaCl_2$ were: 0.0 mM BAPTA/0.0 mM $CaCl_2$ (■); 2.0 mM BAPTA/0.0 mM $CaCl_2$ (□); 2.0 mM BAPTA/1.0 mM $CaCl_2$ (●).

concentration of BAPTA alone (e.g. 1 mM, Fig. 3A). Decreasing the Ca^{2+} concentration while maintaining the BAPTA concentration increased the delay in kinase deactivation (data not shown). Therefore by changing the ratio of BAPTA/Ca^{2+} it was possible to alter the timing of kinase deactivation in a way that reflected the calculated free BAPTA concentration.

In Fig. 4A, addition of BAPTA slowed the activation phase slightly, but completely inhibited deactivation. In other experiments, using extracts with somewhat slower kinase activation profiles (Fig. 4B) or using higher concentrations of BAPTA (data not shown), the activation phase was markedly inhibited by additional BAPTA (Fig. 4B). H1 kinase activity peaked between 20 and 30 minutes in the control, but took 70 minutes

to reach similar levels after addition of BAPTA. When the free BAPTA concentration was reduced by premixing with Ca^{2+}, the rate of activation was restored such that kinase activity peaked between 30 and 40 minutes (Fig. 4B). These results indicate that both the activation phase and the deactivation phase of the H1 kinase cycle can be inhibited by BAPTA. The rescue of both phases obtained by reducing the free BAPTA concentration with Ca^{2+} indicates that BAPTA is achieving both effects through its calcium chelating properties and not by some non-specific effect.

The effect of different calcium chelators on H1 kinase and free calcium levels in prophase extracts

BAPTA was chosen for these experiments because its binding of calcium is largely pH independent and its selectivity for calcium over other divalent cations is high (Tsien, 1980). As important is the hypothesis that BAPTA, and derivatives of it, buffers against local transient changes in free Ca^{2+} in the micromolar range, and does so more effectively than EGTA (Miller et al., 1993; Snow and Nuccitelli, 1993). Of the BAPTA derivatives, 5,5′-dibromo-BAPTA is the most potent form for inhibiting fucoid egg development, cleavage in *Xenopus* eggs and nuclear membrane fusion in egg extracts (Speksnijder et al., 1989; Miller et al., 1993; Sullivan et al., 1993). We therefore analysed the effects of EGTA and dibromo-BAPTA on H1 kinase activity in prophase extracts.

When extracts were incubated with EGTA, deactivation of H1 kinase was increasingly delayed as the concentration of EGTA was increased. However, some 15-20-fold higher concentrations of EGTA than BAPTA were required to elicit equivalent delays. The extract shown in Fig. 5A required 20 mM EGTA to inhibit kinase deactivation for the remainder of the incubation, while 10 mM produced only a 20 minute delay compared to the control. In this particular extract, only 1 mM BAPTA was required to inhibit kinase deactivation completely. These results indicate that BAPTA is substantially more effective in inhibiting H1 kinase deactivation than EGTA, even though estimates of their calcium dissociation constants (K_D) under ionic conditions similar to those in these extracts are similar (0.12 μM for both; Sullivan et al., 1993) or lower for EGTA (0.01 μM, EGTA; 0.17 μM, BAPTA; Pethig et al., 1989). The greater effectiveness of BAPTA may reflect the time scale of Ca^{2+} concentration changes needed to initiate kinase deactivation since BAPTA chelates calcium more rapidly than EGTA (Tsien, 1980).

Dibromo-BAPTA has a K_D which is some ten-fold higher than BAPTA (1.5 μM and 0.17 μM, respectively; Pethig et al., 1989). When added to a prophase egg extract (Fig. 5B) it inhibited deactivation and activation of H1 kinase at lower concentrations than either BAPTA or EGTA. For this extract, inhibition of kinase deactivation required 5 mM BAPTA but only 0.5 mM dibromo-BAPTA. At these respective concentrations, both BAPTA (not shown) and dibromo-BAPTA also noticeably slowed kinase activation. Dibromo-BAPTA at 1 mM decreased the rate of activation further while 5 mM dibromo-BAPTA completely inhibited the activation phase (Fig. 5B and data not shown). Though the highest concentration of EGTA tested (40 mM) did not significantly slow activation, the observation that dibromo-BAPTA is 10-fold more effective than BAPTA, which in turn is 15-20-fold more effective than EGTA suggests that even more EGTA would be required to

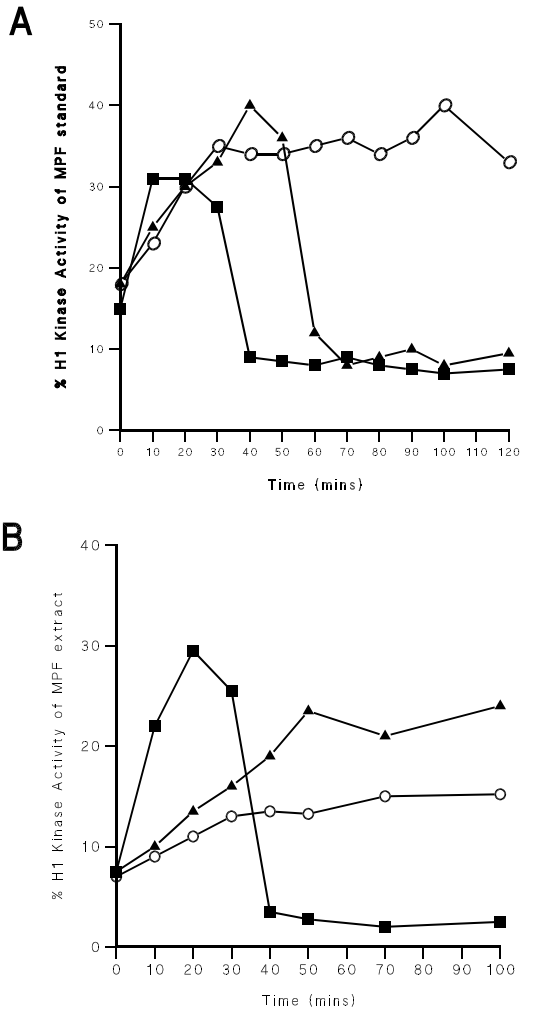

Fig. 5. The effect of EGTA and 5 5′ dibromo-BAPTA on H1 kinase activity in prophase extract. Aliquots of a prophase extract were incubated in the presence of EGTA or dibromo-BAPTA at the concentrations indicated. (A) EGTA was added to final concentrations of 0.0 mM control (■), 10 mM (▲) and 20 mM (○). (B) Dibromo-BAPTA was added to a final concentration of 0.0 mM control (■), 0.5 mM (▲), 1.0 mM (○).

detect an effect on activation. The results suggest that the absolute level of intracellular free Ca^{2+} is not critical for the successful activation/deactivation of $p34^{cdc2}$ kinase. The increasing efficacy of these calcium chelators to inhibit the deactivation and activation of $p34^{cdc2}$ kinase is consistent with the hypothesis that transient local changes in Ca^{2+} concentration are involved in both stages. The maximal inhibitory effect observed with dibromo-BAPTA appears to reflect its particular affinity for Ca^{2+}.

Free calcium levels in extracts and the effect of chelator addition on these levels was measured using the fluorometric calcium indicator fura2. Twelve extracts, made using either the EGTA or BAPTA extraction buffers were analysed. Free calcium was found in the range 180-510 nM across extracts made with both buffer types (Table 1). These levels are consistent with levels of free Ca^{2+} recorded in vivo (Grandin and Charbonneau, 1991a; Kubota et al., 1993), and in vitro (Lorca et al., 1991). Table 2 shows the free calcium concentrations

Table 1. Free calcium concentrations in *Xenopus* extracts

Extract type	Initial free [Ca^{2+}] (nM)
BAPTA	250
	260
	230
	180
	210
	200
	200
	240
	250
EGTA	230
	510
	340

Free calcium was measured using fura2 on nine extracts made with BAPTA extraction buffer and three extracts made using EGTA (see Materials and Methods).

Table 2. Effect of BAPTA and EGTA on free calcium concentration in *Xenopus* extracts

Extract	Initial free [Ca^{2+}] (nM)	Addition	Resulting free [Ca^{2+}] (nM)	Delay (minutes)
(1)	170	0.5 mM BAPTA	150	10
		1.0 mM BAPTA	140	40
		5.0 mM BAPTA	130	>80
		5.0 mM EGTA	160	20
		15 mM EGTA	120	40
(2)	200	1.0 mM BAPTA	160	>60
(3)	250	5.0 mM BAPTA	260	20
		15 mM BAPTA	70	>50

Free calcium was measured before and after addition of the various concentrations of BAPTA or EGTA. Delay is the time (in minutes) between kinase deactivation of control incubations and deactivation in the presence of added chelator. (The > sign indicates that deactivation had still not occurred by the end of the experiment.)

observed before and after addition of various concentrations of BAPTA or EGTA. Though some reduction in free Ca^{2+} levels were observed, they did not reflect the delays in kinase deactivation obtained (Table 2). For example, addition of 5 mM BAPTA produced a similar reduction in free Ca^{2+} as 15 mM EGTA. However, this concentration of EGTA delayed kinase deactivation by 40 minutes compared to over 80 minutes achieved by the lower concentration of BAPTA. These measurements indicate that H1 kinase deactivation is not dependent on the global free calcium concentration, but may require transient changes that are more effectively buffered by BAPTA than EGTA.

Addition of BAPTA to prophase extract during the activation of H1 kinase

It has been shown that a threshold level of H1 kinase activity is required to trigger cyclin degradation and kinase inactivation (Felix et al., 1990). Although phosphotyrosine forms of $p34^{cdc2}$ disappeared to control levels during activation in the presence of sufficient BAPTA to prevent deactivation (Fig. 3C), it seemed possible that BAPTA might be preventing the full activation of H1 kinase and consequently causing a delay

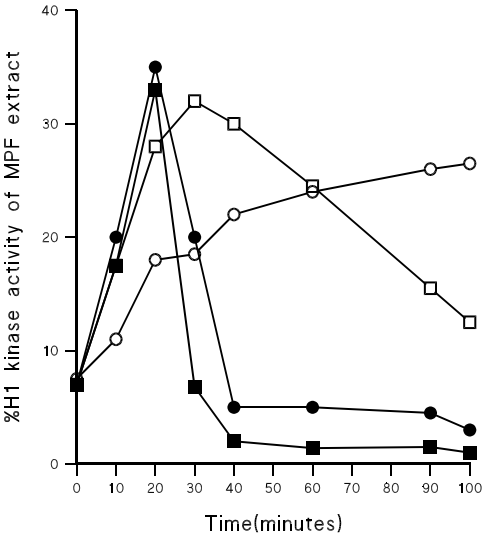

Fig. 6. Addition of BAPTA to prophase extract during the activation of H1 kinase. BAPTA (2 mM) was added to aliquots of a prophase extract at different times during the activation phase. Additions were made at 0 minutes (○), 10 minutes (□) and 20 minutes (●). BAPTA addition at 25 minutes is not shown as it was the same as the water addition control (■).

in deactivation. To address this, BAPTA was added to a prophase extract at different times during kinase activation (Fig. 6). Addition of BAPTA at the start of the incubation slowed the activation of H1 kinase and prevented deactivation. When BAPTA was added at 10 minutes, H1 kinase activation was similar to the control. However, the subsequent deactivation phase was slowed considerably. Thus, despite essentially normal activation of H1 kinase in the presence of BAPTA, the rate of kinase deactivation was reduced. Addition of BAPTA at 20 minutes or later did not affect the rate of kinase deactivation. This indicates that, once initiated, kinase deactivation is insensitive to BAPTA. These results suggest that, during activation, a calcium step that is independent of the overall kinase level determines the initiation and rate of kinase deactivation.

Addition of a peptide corresponding to the calmodulin binding domain of CaM kinase II delays deactivation of H1 kinase and the degradation of cyclin

Calmodulin is an ubiquitous intracellular Ca^{2+}-binding protein responsible for translating Ca^{2+} concentration into enzyme activity. The binding of Ca^{2+} to calmodulin produces a complex capable of activating a wide range of kinases and phosphatases (reviewed by Lu and Means, 1993). In view of the evidence that CaM kinase II is involved in initiating cyclin degradation in CSF extracts it seemed plausable that the effects of calcium chelators on H1 kinase activity were caused by the inhibition of Ca^{2+}/calmodulin dependent enzymes (Lorca et al., 1993). The amino acid sequences responsible for binding Ca^{2+}/calmodulin have been identified for a number of Ca^{2+}-dependent enzymes. The addition of peptides corresponding to these calmodulin binding domains would be expected to saturate cellular calmodulin and prevent it activating target enzymes. Therefore, a synthetic peptide corresponding to the

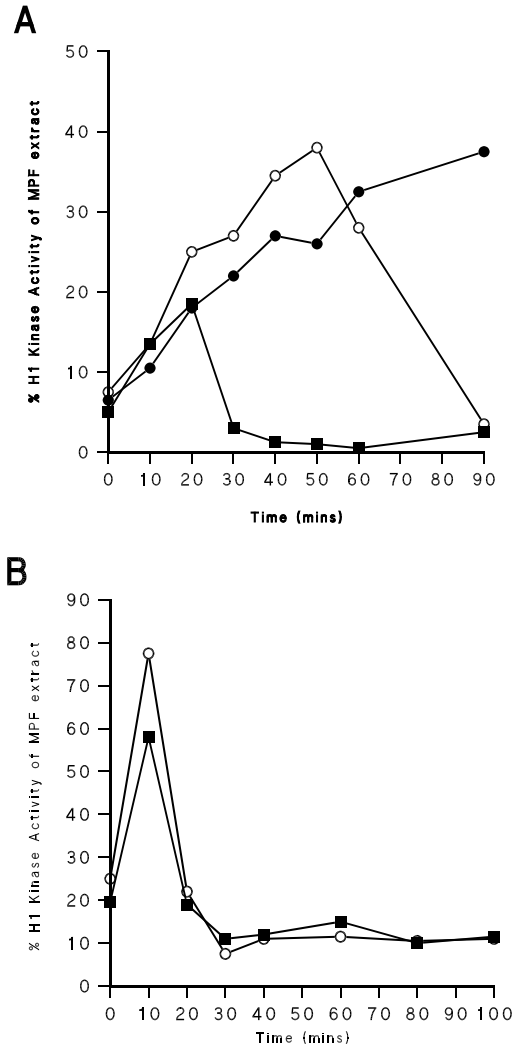

Fig. 7. Effect of a calmodulin binding peptide on the H1 kinase activity of a prophase extract. (A) Prophase extract was incubated with the CaM kinase II synthetic peptide at a concentration of 0.0 μM (■); 330 μM (○): 500 μM (●). (B) Peptide corresponding to the auto-inhibitory domain of PKC (19-36) was added to prophase extract at a final concentration of 500 μM (○). Control (■).

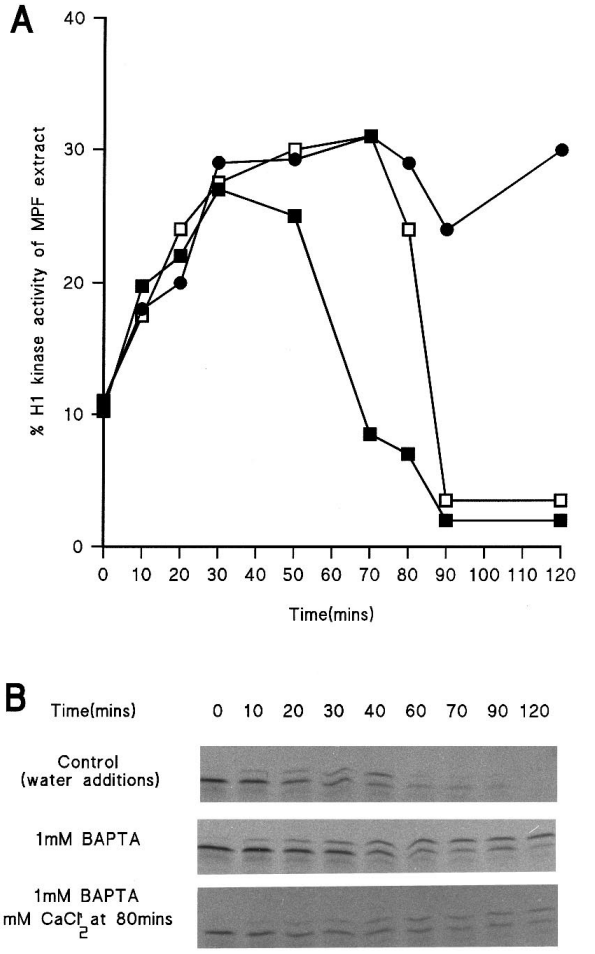

Fig. 8. Calcium addition to a BAPTA arrested prophase extract leads to inactivation of the H1 kinase activity while exogenous cyclin remains stable. (A) Prophase extract was incubated in the presence of sufficient BAPTA (1 mM) to prevent H1 kinase deactivation. After 80 minutes $CaCl_2$ was added at 0.0 mM (●); 1.0 mM (□). Control without added BAPTA (■). (B) Fluorographs showing the stability of exogenous cyclin B_2 under the above conditions.

calmodulin binding domain of Ca^{2+}/calmodulin dependent kinase II (residues 290-309; Payne et al., 1988) was added to a prophase extract. Increasing peptide concentration delayed the deactivation of H1 kinase in a dose dependent manner (Fig. 7A). The delay in H1 kinase deactivation was associated with the accumulation of higher kinase activity than the control. The results indicate that the peptide was interfering with the induction of the deactivation pathway.

As a control for the high concentration of CaM kinase peptide required to inhibit deactivation, a synthetic peptide corresponding to the auto-inhibitory domain of protein kinase C (residues 19-36) was added to a prophase extract at a concentration of 500 μM. While the CaM kinase inhibitory peptide increased levels of H1 kinase activity and delayed the timing of H1 kinase deactivation, the PKC peptide had no effect on the timing of H1 kinase deactivation (Fig. 7B) with respect to the condition run in the absence of either peptide. This also suggested that PKC is not involved in either the activation or deactivation of H1 kinase.

H1 kinase deactivation in the absence of cyclin degradation

BAPTA appears to prevent the deactivation of H1 kinase by buffering transient increases in free Ca^{2+} concentration. Once arrested with BAPTA, subsequent addition of Ca^{2+} might rescue deactivation, particularly if the added calcium was rapidly chelated by the excess BAPTA already present. Various concentrations of calcium were added to a prophase extract that had been incubated with sufficient BAPTA (1 mM, Fig. 8) to prevent kinase deactivation. Ca^{2+} was added at 80 minutes, after the control without BAPTA had deactivated and degraded cyclin (Fig. 8A,B). Addition of 0.4 mM calcium (final concentration) did not affect either H1 kinase levels or the stability of exogenous cyclin B_2 (not shown). Addition of 1 mM calcium induced a rapid loss of kinase activity, but surprisingly cyclin remained stable (Fig. 8B). Addition of 2 mM calcium produced loss of kinase activity and also cyclin degra-

Table 3. Effect of BAPTA and calcium addition on free calcium concentration in *Xenopus* extract

Basal free Ca^{2+} (μM)	+1 mM BAPTA (μM)	+1 mM Ca^{2+} (μM)	+0.4 mM Ca^{2+} (μM)
0.201	0.167	8.77	–
0.203	0.181	4.87	–
0.201	0.181	–	0.441
	0.211	–	0.360

Prophase extract was treated with BAPTA concentrations (previously determined) that prevent kinase deactivation and free calcium was then measured. The BAPTA treated extract was then treated with $CaCl_2$ to the final concentrations stated and the resulting free calcium measured.

dation. However, this cyclin degradation was non-specific as a radiolabelled control protein ($p34^{cdc2}$) included in the incubation was also rapidly degraded (not shown). Therefore the addition of 0.4 mM Ca^{2+}, which is sufficient to induce kinase inactivation and specific cyclin degradation in meiotic metaphase CSF extracts (Lorca et al., 1991), is unable to rescue these events in BAPTA arrested prophase extracts. Further increases in added Ca^{2+} induced the inactivation of H1 kinase either in the absence of cyclin degradation or with non-specific proteolysis. Rapid kinase inactivation has been observed in CSF extracts following addition of calcium to levels above the micromolar range (Lorca et al., 1993). We tried to limit the duration of elevated free calcium by adding excess (10 mM) BAPTA one minute after adding 1 mM Ca^{2+}. Ten minutes later H1 kinase was reduced but recovered over the next twenty minutes, consistent with a failure to degrade cyclin (data not shown).

Measurement of free calcium in the extract again shows that BAPTA slightly reduces the free calcium levels in the extract (Table 3). Measurements were made over several minutes starting as soon as possible (about 15 seconds) after the appropriate addition, and were stable through this period. The addition of 0.4 mM calcium produced a threefold increase in the level of free Ca^{2+}. While such an increase is quite large the resulting level (440 nM) is within the range of free Ca^{2+} measured in untreated extracts (Table 1). This increase in free Ca^{2+} did not change the level of kinase activity and cyclin remained stable. Addition of 1 mM calcium raised the free calcium to 5-9 μM, which was above levels usually seen in vivo (1.0-1.5 μM at fertilization; Busa and Nucitelli, 1985; Kubota et al., 1987) but not to millimolar levels. This was sufficient to deactivate H1 kinase without cyclin degradation and did not switch on the non-specific proteolysis observed with higher calcium concentrations. These results suggest that either a narrow window of calcium concentration between 0.5 and 4 μM is required to initiate the cyclin degradation pathway or perhaps that global addition of calcium is not an appropriate way to reverse inhibition by BAPTA.

DISCUSSION

The results presented here indicate, firstly, that calcium chelators can disrupt the normal activation/deactivation cycle of H1 (cdc2) kinase and the degradation of cyclin. Preincubation of BAPTA with Ca^{2+} rescues both activation and deactivation of H1 kinase indicating that BAPTA is achieving these effects by chelating Ca^{2+}. The precise effect depends on the concentration of free chelator and the particular chelator used. Secondly, measurement of free Ca^{2+} shows that Ca^{2+} chelators do not dramatically reduce free calcium levels, suggesting that chelators may act to suppress transient changes in calcium concentration rather than the resting levels of free Ca^{2+}. Similar observations in which Ca^{2+} buffers do not change free Ca^{2+} concentration significantly have been made in mammalian cells (Kao et al., 1990) and *Xenopus* extracts (Lorca et al., 1991; Sullivan et al., 1993). Thirdly, BAPTA addition late during H1 kinase activation did not prevent subsequent deactivation, indicating that in these extracts an event critical for the completion of a kinase cycle occurs during activation. Fourthly, the addition of a peptide that interferes with calmodulin function delayed the deactivation of H1 kinase. This is consistant with the presence of a Ca^{2+}/calmodulin coordinated event required to switch on the deactivation/cyclin degradation pathways.

Dibromo-BAPTA was more effective at inhibiting the H1 kinase cycle than BAPTA, which in turn was more effective than EGTA. In studies where cleavage in *Xenopus* was slowed or prevented by injection of BAPTA derivatives, dibromo-BAPTA was also the most effective (Miller et al., 1993; Snow and Nuccitelli, 1993). These authors proposed that the BAPTA buffers were acting to eliminate localised Ca^{2+} gradients. The effectiveness of a particular buffer would be dependent on its ability, not only to bind Ca^{2+} but to release it rapidly in areas of low Ca^{2+} (Speksnijder et al., 1989). Comparison of several BAPTA derivatives showed that either side of an optimal K_D value the effective buffer concentration increased, as was found for halting fucoid egg development and nuclear vesicle fusion in *Xenopus* egg extracts (Snow and Nuccitelli, 1993; Speksnijder et al., 1989; Sullivan et al., 1993). Where EGTA has been tested, it is either ineffective or required at much higher concentrations than BAPTA (Baker and Warner, 1972; Vincent et al., 1987; Sullivan, et al., 1993). The order of effectiveness of these buffers in preventing kinase deactivation and slowing kinase activation is consistent with these observations and suggests that both kinase activation and the pathway to cyclin degradation are stimulated by local changes in free Ca^{2+}. A requirement for local calcium transients may explain why changes in free Ca^{2+} are difficult to detect and relate reproducibly to specific cell cycle events (reviewed by Hepler, 1994).

The free Ca^{2+} concentration in prophase extracts is similar to those measured in vivo and in vitro (Grandin and Charbonneau, 1991a; Lorca et al., 1991). Addition of Ca^{2+} buffers to extracts does not produce reductions in free Ca^{2+} that reflect the resulting effect on activating H1 kinase (i.e. similar reductions in free Ca^{2+} produced by BAPTA and EGTA do not produce similar delays in H1 kinase deactivation). This further suggests that localised changes in free Ca^{2+} rather than total free Ca^{2+}concentrations are important for kinase activity and that dibromo-BAPTA and BAPTA are more efficient than EGTA in disrupting these changes.

As the concentration of free BAPTA in an extract is increased, the time at which cyclin degradation occurs is first delayed and then inhibited (Fig. 3). A threshold level of cdc2 kinase activity is required to initiate degradation and a minimum lag of some fifteen minutes is observed before cyclin

degradation occurs (Felix et al., 1990). A failure to reach the required threshold of kinase activity is not responsible for the inhibition of deactivation since the stable level of kinase activity reached in the presence of BAPTA can be as high or higher than the maximum reached by the control extract. However, H1 kinase activation is also slowed in the presence of BAPTA buffers, though deactivation is more sensitive. This slowing reflects reduced tyrosine dephosphorylation of p34^{cdc2} by p80^{cdc25} or increased tyrosine phosphorylation by the wee1 inhibitory pathway since we observed a reduced rate of loss of tyrosine phosphorylated forms during kinase activation. Even though kinase activation is slowed, tyrosine phosphorylated forms reduce to control levels when kinase activity reaches its maximum level. It was also noticeable that the addition of low concentrations of BAPTA (producing short delays in deactivation) resulted in the generation of more kinase activity before deactivation was finally initiated (Fig. 3A). Under this condition the pathway to cyclin degradation is slowed and kinase activates above control levels before degradation cuts in. At a BAPTA concentration that blocks the pathway to cyclin degradation, kinase activation is also slowed, generating a stable activated level which depends on the new balance affecting Thr14 and Tyr15 phosphorylation and Thr161 phosphorylation. The observed effects of calcium chelators suggest that two calcium-mediated events are involved in cdc2 kinase activation and inactivation (Fig. 9). We suggest that the first (A in Fig. 9) stimulates cdc2 kinase activation locally. The second event (B, Fig. 9) occurs later, in the presence of the required threshold level of active kinase and initiates the pathway to cyclin destruction. The model depicts the effects of adding BAPTA (or dibromo-BAPTA) at different times relative to the two proposed events. We suggest that events A and B are local transient increases in free calcium. In the volume of the incubated extract the periods during which these calcium transients occur might overlap in time but not space.The second event appears to be sensitive to lower concentrations of chelator.

The addition of the peptide corresponding to the calmodulin binding domain of CaM kinase II inhibited the deactivation of H1 kinase. This suggests that a Ca^{2+}/calmodulin dependent activity is required during kinase activation to allow subsequent deactivation and cyclin degradation. While inhibitors that bind calmodulin will affect the activity of a range of Ca^{2+} dependent enzymes (reviewed by Soderling, 1990) CaM kinase II activates the pathway to specific cyclin degradation following Ca^{2+} addition to CSF extracts (Lorca et al., 1993). Subsequent anaphase sister chromatid separation in both CSF and mitotic extracts can be prevented by competitive inhibition of cyclin degradation (Holloway et al., 1993; Morin et al., 1994). These observations are consistent with the view that, during cdc2 kinase activation in mitotic cycles, transient increases in free calcium stimulate CaM kinase II activity which initiates the pathway to cyclin degradation and anaphase separation. We anticipate that in fertilised *Xenopus* eggs, low concentrations of dibromo-BAPTA injected at 0.5 of the first cell cycle will cause a delay or arrest at metaphase, while higher concentrations will delay nuclear envelope breakdown (NEBD) by slowing cdc2 kinase activation. In sea urchins, inhibitory peptides and antibodies to CaM kinase II, as well as calcium buffers delayed NEBD (Steinhardt and Alderton, 1988; Baitinger et al., 1990; Twigg et al., 1988; Whitaker and Patel, 1990; Patel and Whitaker, 1991). However, the peptide we have used did not slow kinase activation in the *Xenopus* cell-free system. This may reflect the greater resistance of this step to inhibition, as was observed with calcium buffers, or indicate that activation is not affected by Ca^{2+}/calmodulin dependent enzymes.

When BAPTA was added at different times during the kinase activation/deactivation cycle, no effect was observed at or after the time of maximal kinase activation (Fig. 6, modelled in Fig. 9). This indicates that the pathway to cyclin degradation is initiated during kinase activation. Indeed, addition of BAPTA during activation slows, but does not substantially delay, deactivation and cyclin degradation. As a threshold level of cdc2 kinase activity is required to induce cyclin degradation (Felix et al., 1991), this suggests that the local level of kinase

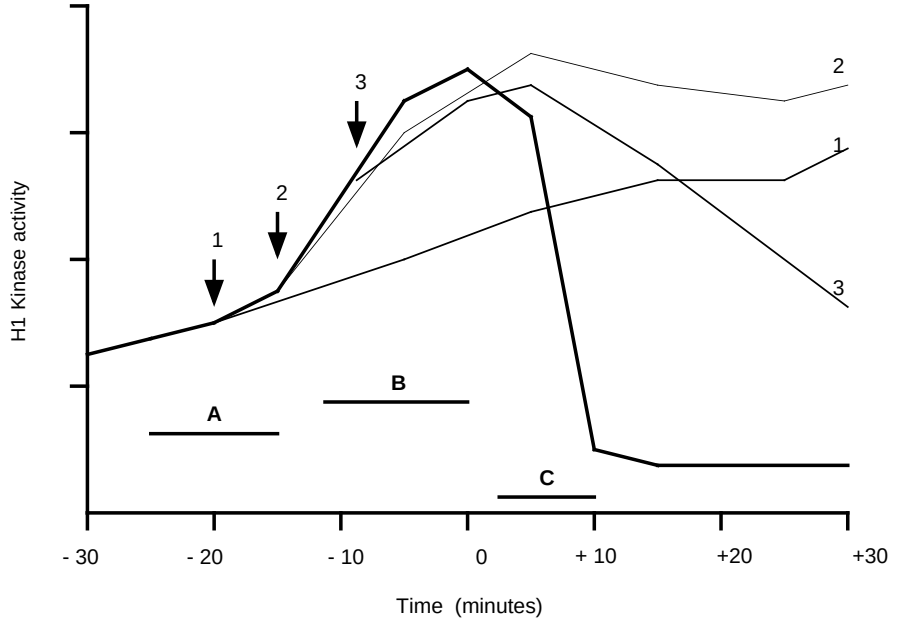

Fig. 9. Model of the timing of two calcium mediated events (A and B) relative to changes in overall H1 kinase activity and the period of cyclin degradation (C). (A) We envisage that calcium transients during this period stimulate H1 kinase activation. Extracts are prepared about 20 minutes before mitosis, which is during this period. This may explain why activation is more resistant than deactivation to a given concentration of added BAPTA. (B) This period terminates by the time global kinase activity has peaked (Fig. 6). We suggest that calcium transients occur locally during periods A and B rather than globally throughout these periods. (C) The window during which cyclin B is degraded in an untreated extract. Arrows 1,2,3: predicted behaviour of H1 kinase if BAPTA were added at the times indicated by the arrows (see Figs 3A, 4A,B and 6).

activation meets this requirement, and, with a transient increase in free calcium, initiates the pathway to inactivation. When BAPTA is added during activation (at 10 minutes, Fig. 6) the pathway has already been initiated in some regions and is subsequently inhibited in others. The outcome is that deactivation occurs more slowly, but without delay. Since the lag time between addition of a threshold level of cdc2 kinase and onset of cyclin degradation is minimally fifteen minutes (Felix et al., 1990), this further implies that the event induced by calcium (CaM kinase II activation) is only required early in the pathway.

In injection experiments, EGTA is particulary ineffective in blocking cellular events because it becomes mostly saturated with calcium drawn from intracellular stores or the extracellular medium (Speksnijder et al., 1989; Miller et al., 1993). This may provide an explanation for the failure of an estimated 20 mM EGTA present during preparation of prophase extracts to prevent subsequent cdc2 kinase activation and inactivation on a similar time-scale to these events in vivo (Felix et al., 1989). Much of the membrane fraction present in eggs is left in the pellet fraction when the high-speed supernatant is taken. Subsequent addition of 20 mM EGTA to this supernatant inhibits kinase deactivation (Fig. 5A). Even though most of the membrane fraction has been removed, a membrane-containing particulate fraction essential for kinase activation can be separated from the supernatant by further centrifugation (Felix et al., 1989; Leiss et al., 1992). In the extracts we have prepared, if free calcium is increased by calcium addition, the free calcium level drops again during subsequent incubation, indicating that calcium sequestration occurs (data not shown). Perhaps the particulate fraction contains a calcium store needed for kinase activation and inactivation. If local activation and inactivation of cdc2 kinase is stimulated by local calcium release it might generate regional differences in MPF activity particularly in the large cells of early embryos. Regional differences in MPF activity have been observed in frog and newt eggs (Masui, 1972; Iwao et al., 1993). Local targeted destruction has also been suggested to explain, in early syncitial *Drosophila* embryos, the occurrence of nuclear division cycles when global H1 kinase levels are stably elevated (Edgar et al., 1994). In *Beroe*, regional differences in microtubule behaviour leading to axis establishment prior to first cleavage may reflect local activation and spreading of mitotic activity (Houliston et al., 1993). High speed extracts, though far removed from the structure of intact eggs, offer the possibility of detection and manipulation of local calcium changes.

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