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Calcium Oscillations in *Xenopus* Egg Cycling Extracts

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Abstract Cell cycle in various types of cells and in early embryos is often accompanied by transient changes in the concentration of free cytosolic calcium. In the present study, using fluorescent indicator fura-2, we demonstrate that Ca^{2+} oscillates cyclically with an amplitude of about 100 nM and a period of mitotic cycle in cell-free *Xenopus* egg cycling extracts. It peaks in early metaphase just preceding mitotic reactivation of Cdc2 kinase and MAPK and reaches a minimum in interphase. The source of Ca^{2+} in the extracts is a particulate fraction containing egg intracellular Ca^{2+} stores, since the addition of a calcium-mobilizing second messenger, inositol 1,4,5-trisphosphate (IP3), induced a transient increase in Ca^{2+}. The inclusion of heparin, an IP3 receptor antagonist, or ultrafiltration of the extracts prevented Ca^{2+}-releasing activity of IP3. The depletion of Ca^{2+} in the extracts by the calcium chelator BAPTA resulted in the blockade of cell cycle at different stages, depending on the time of drug administration. The addition of BAPTA late in interphase blocked cell cycle at mitotic entry in prophase, whereas its application in anaphase or telophase blocked the extracts in early interphase. BAPTA administration in metaphase before transition to anaphase brought about a metaphase-like arrest in the cycling extracts. Inhibition of IP3-induced calcium release by heparin also arrested cell cycle progression in the cycling extracts. J. Cell. Biochem. 82: 89–97, 2001.

Key words: calcium; cell cycle; *Xenopus*; protein kinase; BAPTA; heparin

Transient changes in the concentration of free cytosolic calcium have been established to promote cell cycle transitions in early embryonic division [Whitaker, 1997; Santella, 1998]. Unfertilized eggs of *Xenopus laevis* are arrested in metaphase of meiosis II with high activity of both maturation-promoting factor (MPF) and cytostatic factor (CSF) complexes. Fertilization or artificial activation of eggs induces a transient increase in free intracellular calcium that triggers the destruction of cyclin B, inactivation of MPF and CSF, and entry into the first embryonic interphase. Besides the first prominent increase, the periodic oscillations of free intracellular calcium have been detected, which occur with a frequency of cell cycle in the dividing *Xenopus* embryo [Grandin and Charbonneau, 1991; Kubota et al., 1993; Keating et al., 1994]. Calcium transients have been registered in the cleavage arrested embryos, suggesting that oscillations are associated with a periodic cytoplasmic activity independent of the furrowing process [Kubota et al., 1993; Keating et al., 1994]. Calcium transients in the cell cycle are dependent on intracellular stores and generated by an endogenous mechanism which does not involve plasma membrane receptors [Ciapa et al., 1994]. Further evidence for a regulatory role of calcium in mitotic events comes from the observation that the microinjection of calcium buffers into intact *Xenopus* blastomeres delays or blocks cell division in a dose-dependent manner [Snow and Nuccitelli, 1993].

Recently, cell-free cycling extracts, that provide large amounts of highly synchronized material, are being increasingly used in the cell cycle-related studies [Lohka and Masui, 1983; Murray, 1991; Desai et al., 1999]. These extracts demonstrate, in vitro, many of the hallmark events of the cell cycle, including chromatin reorganization, nuclear envelope breakdown, DNA replication, mitotic spindle assembly, oscillation of MPF activity, etc. Cycling extracts are prepared from activated eggs in the absence

Abbreviations used: MPF, maturation-promoting factor; CSF, cytostatic factor; MAPK, mitogen-activated protein kinase; IP3, inositol 1,4,5-trisphosphate; MBP, myelin basic protein.

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of EGTA and can undergo multiple cell cycles in vitro. Egg activation by electrical shock or calcium ionophore prior to extract preparation is accompanied by the release of intracellular calcium and is necessary to exit CSF-mediated metaphase arrest. In contrast, metaphase-arrested extracts from nonactivated eggs obtained in the presence of EGTA, referred to as CSF-arrested extracts, retain high activity of CSF and can be released into interphase by the addition of calcium.

In the present study, using fluorescent indicator fura-2 and a high frame digital CCD imaging system, we have registered low magnitude periodic changes in the concentration of free cytosolic calcium during cell cycle transition in *Xenopus* egg cycling extracts, but not in CSF-arrested extracts. These changes were coordinated with cell cycle events. Calcium concentration peaked in metaphase preceding the increase in Cdc2 kinase and MAPK activities and reached a minimum in interphase. Using the calcium-mobilizing second messenger, inositol 1,4,5-trisphosphate (IP3) and its antagonist, heparin, we demonstrated that calcium in the extracts is derived from a particulate material containing egg intracellular calcium stores. The addition of the calcium chelator BAPTA, as well as, the inhibition of IP3-induced calcium release by heparin blocked cell cycle in the extracts at different stages underlining the importance of calcium regulation for the normal mitotic transition.

MATERIALS AND METHODS

Cycling Extracts

The extracts were prepared essentially as described by Murray, [1991]. Briefly, *Xenopus* wild-type female frogs were primed by dorsal lymph sac injection of pregnant mare serum gonadotropin (Biogenesis, 50 U/animal) 5–10 days before ovulation. Ovulation was induced by injecting 500 U of human chorionic gonadotropin (Teikokuzoki, Japan) the previous night. Eggs were squeezed from the frogs into MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES, pH 7.8) immediately prior to extract preparation. Eggs were dejellied with 2% cysteine (Sigma), activated by an electric pulse (3.6 v/cm, 5 s, constant current), and washed extensively with extract buffer (XB), containing 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose, and 10 mM potassium HEPES, pH 7.7. Eggs were left at room temperature for 15 min to fulfill the activation, as judged by the pigment contraction of animal hemisphere, then transferred into centrifuge tubes containing XB buffer plus 100 μg/ml cytochalasin B (Sigma) and 10 μg/ml each leupeptin, pepstatin, and chymostatin. The tubes were put on ice for additional 15 min, centrifuged for 30 s at 1,000 rpm and then for 30 s at 1,500 rpm in TOMY microcentrifuge. All buffer was removed from the top of the tube and the eggs were crushed by a centrifugation for 15 min at 15,000 rpm at 2°C. The cytoplasmic layer was collected and subjected to the second clarifying centrifugation under the same conditions, then cytochalasin B, leupeptin, pepstatin, and chymostatin were added to a final concentration of 10 μg/ml each. Cycling was initiated by putting extracts to 23°C with simultaneous addition of 1/20 volume of energy mix (150 mM creatine phosphate, 20 mM ATP, 20 mM $MgCl_2$, 2 mM EGTA, pH, 7.7). Fifty μl of supplemented extract was taken for an individual reaction. Ultrafiltrated extracts in the experiments of Figure 3 were obtained by the additional high-speed centrifugation through a Millipore ultrafiltration membrane with a cut-off at 100 kD. EGTA and heparin (Nacalai Tesque, Japan) were added to the extracts just before IP3 administration.

In the experiments of Figure 4, BAPTA (Calbiochem) was added to the extracts at appropriate time points to a final concentration of 5 mM from a 0.5 M stock solution in water, pH 7.7.

Detection of Nuclear Morphology

Demembranated sperm nuclei were prepared as described earlier [Murray, 1991] and added to the extracts on ice prior to the initiation of cycling to a final concentration of 10^5/ml. Nuclear morphology was observed and scored by fluorescent microscopy after withdrawing 1 μl of extract at 15-min intervals and adding 4 μl of stain solution (1 μg/ml Hoechst 33342 (Sigma) in MMR buffer containing 10% formaldehyde and 50% glycerol).

Measurement of Ca^{2+}

UV-excitable ratiometric fluorescent calcium indicator fura-2 (Molecular Probes) was added to the extracts at the time of cycling initiation to a final concentration of 2 μM. The level of free calcium in the extracts was continuously

monitored by ratio-imaging microscopy using high frame digital CCD imaging ARGUS/HISCA system from Hamamatsu Photonics (Japan). Excitation wavelengths 340 and 380 nm were used while monitoring the emission at 510 nm.

Protein Kinase Assay

Cdc2 kinase and MAPK activities in the extracts were assessed by specific phosphate incorporation into histone H1 and myelin basic protein (MBP), respectively. One μl of the extract was five-fold diluted in a kinase dilution buffer (80 mM β-glycerophosphate, pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM NaF, 1 mM Na_3VO_4, 0.2 mM APMSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin) and stored at −80°C. The reaction mixture of protein kinase assay (20 μl) contained 20 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml histone H1 (Gibco), 0.5 mg/ml MBP (Sigma), 2 μM protein kinase A inhibitor peptide (Sigma), 100 μM [γ-32P]ATP (1 μCi), and 5 μl of diluted extract. Samples were incubated at 30°C for 10 min, then the reaction was stopped by the addition of concentrated Laemmli's buffer [Laemmli, 1970] and boiling. The reaction products were separated by SDS-PAGE, and radioactive bands of H1 and MBP were visualized by an image analyzer (BAS2000, FUJI Film).

Immunoblotting

Aliquots of the extracts in a kinase dilution buffer were mixed with SDS-PAGE sample buffer, boiled and subjected to immunoblot analysis using 100-fold diluted anti-phospho-MAPK antibody or 200-fold diluted anti-MAPK antibody (Biolabs), as described previously [Tokmakov et al., 1996]. Each sample for immunoblot analysis contained the material corresponding to 0.2 μl of the original extract.

RESULTS

Calcium Levels in *Xenopus* Egg Extracts

The changes of free Ca^{2+} level in the cycling extracts were recorded successively with 1 min intervals over 2 h (Fig. 1). As the activation induces a massive release of Ca^{2+} from intracellular stores which lasts for 15 −20 min, we usually started recording in 30 min after initiation of cycling. Typically, we could detect two low magnitude smooth oscillations of ratio signal over this period of time. The measurements taken simultaneously at different fields of the extract (parallel curves in Fig. 1) revealed essentially the same pattern of ratio signal changes throughout the whole extract. The lengthening of the period of oscillation in subsequent cycles was rather common. The periodic changes of the ratio signal could be detected in 11 out of 16 tested cycling extracts. By analyzing nuclear morphology of the extracts that failed to display oscillations, we confirmed that they came to arrest either in metaphase or in interphase of cell cycle presumably due to the low quality of eggs used for their preparation. Calcium oscillations could also be registered in the absence of the exogenous DNA (data not shown). We never detected the similar pattern of signal in CSF-arrested extracts obtained from nonactivated eggs in the presence of EGTA. These extracts showed somewhat lower level of signal in the absence of oscillations (Fig. 1, "non-cycling" curves). From the results presented in Figure 1, the amplitude of ratio signal changes was estimated to be at around 0.05 ratio unit. Using a calibration curve obtained by the addition of known Ca^{2+} concentrations to the extract and detecting the ratio signal (Fig. 1, insert) this value is estimated to correspond to around 100 nM change in the concentration of free calcium in the extract.

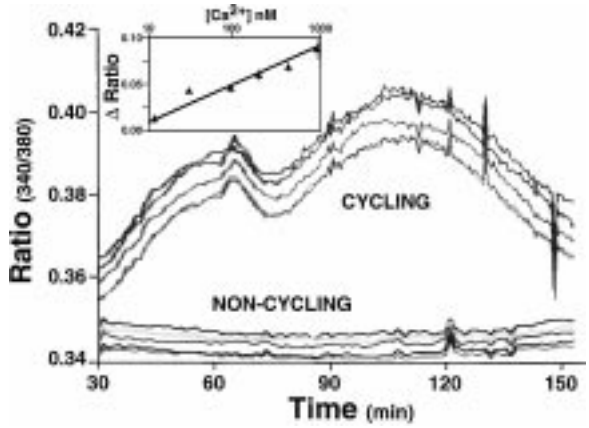

Fig. 1. Measurement of calcium levels in *Xenopus* egg extracts. Calcium level was registered in cycling and CSF-arrested (''non-cycling'') extracts using high frame digital CCD imaging ARGUS/HISCA system from Hamamatsu Photonics (Japan) and fluorescent calcium indicator fura-2 (2 μM). Five parallel measurements were taken in the detection field. Time after initiation of cycling is indicated. In the left upper corner, the calibration curve obtained by the addition of exogenous calcium to the extract is presented. Values are the mean±SE from three measurements.

Correlation of Calcium Transients With Cell Cycle Events

Remarkably, the detected calcium oscillations had the periodicity of mitotic cycle in the cycling extracts. Therefore, next we attempted to correlate them with the events of cell cycle. By simultaneous detection of nuclear morphology and calcium level in the extracts, we found that calcium concentration was lowest in interphase, started to increase early in prophase, and reached a maximum in early metaphase (Fig. 2). The level of calcium decreased gradually through telophase and early interphase reaching its minimum shortly before entering into prophase of the next mitotic cycle. Maximal rise in calcium concentration just preceded the peak of Cdc2 kinase activity, as detected by histone H1 phosphorylation. Also, it preceded a transient partial reactivation of MAPK at metaphase/anaphase transition, as revealed by the presence of activated Tyr-phosphorylated form of MAPK in immunoblotting (Fig. 2).

Calcium Stores in the Cycling Extracts

Next we investigated the source of calcium in the extracts. We found that the extracts obtained by the described method (see Materials and Methods) included a particular fraction containing intracellular calcium stores. The treatment of the extracts with IP3, a second messenger which releases calcium from intracellular stores [Berridge and Irvine, 1989], induced very fast, robust, and reversible increase in the concentration of free calcium up to the micromolar range. This pattern is typically detected upon IP3 receptor-mediated calcium release from intracellular stores [Twigg et al., 1988]. Consistently, the calcium-releasing effect of IP3 could be blocked by heparin, an IP3 receptor antagonist [Ghosh et al., 1988], and prevented by the inclusion of the calcium chelator EGTA in the extracts (Fig. 3). Importantly, ultrafiltrated extracts could not release calcium when treated with IP3 (Fig. 3), indicating the necessity of a particulate fraction. Although the procedure of the extract preparation by a low-speed centrifugation suggested strongly the presence of a vesicle-containing membrane fraction [Murray, 1991; Newmeyer and Wilson, 1991], our results demonstrate clearly that the cycling extracts contain egg intracellular IP3-regulated Ca^{2+} stores.

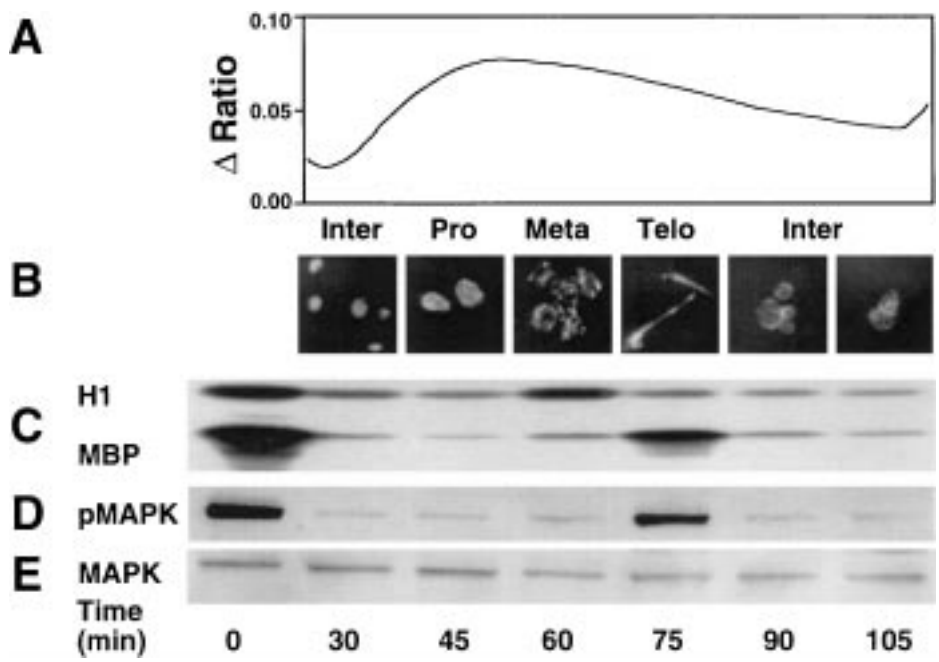

Fig. 2. Correlation of calcium oscillations with the events of cell cycle. Simultaneously with calcium measurements in cycling extracts (**A**), nuclear morphology of demembranated sperm nuclei (**B**), activity of Cdc2 kinase and MAPK (**C**), MAPK activation (**D**) and total content of MAPK (**E**) were determined. Activity of Cdc2 kinase and MAPK was assessed with histone H1 and MBP, respectively. MAPK activation was revealed by a phospho-specific antibody against catalytically active enzyme. Time ''0'' refers to the extract obtained from eggs before activation. The results are typical of three experiments with different extracts.

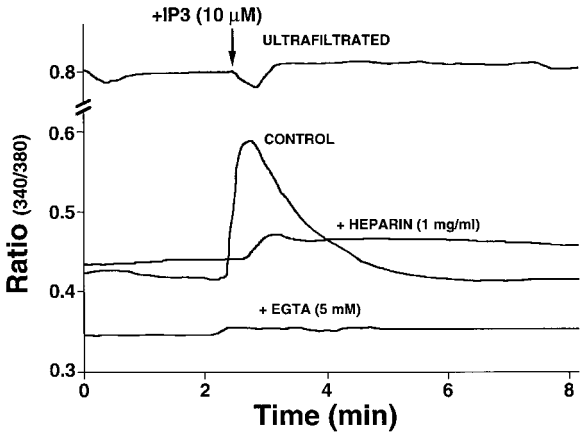

Fig. 3. Source of calcium in *Xenopus* egg extracts. At the time indicated by an arrow, 10 μM IP3 was added to control or treated extracts. Ratio level signal was recorded with 10 s intervals. Heparin and EGTA were added to the extracts at indicated final concentrations just prior to the IP3 administration.

Block of Cell Cycle in the Extracts Treated With BAPTA and Heparin

To investigate the importance of calcium for the normal transition of cell cycle in the chosen experimental model, we treated the *Xenopus* egg cycling extracts with the calcium chelator BAPTA at millimolar concentrations. The results of these experiments are presented in Figure 4. We found that calcium chelation could arrest cycling extracts in three different points of the mitotic cycle. Although the control extract proceeded through two mitotic cycles with periodic condensation of chromatin and reactivation of both Cdc2 and MAP kinases (Fig. 4A), the addition of BAPTA late in interphase or at the beginning of prophase brought about the block at the mitotic entry (Fig. 4B). In this case, the extracts could never progress to metaphase, however some partial and untimely delayed activation of Cdc2 kinase occurred in the absence of MAPK reactivation. The appropriate timing of BAPTA administration, exactly in metaphase before the transition to anaphase, arrested cycling extracts in metaphase with condensed chromatin and elevated activity of both Cdc2 kinase and MAPK (Fig. 4C). This state resembled the established metaphase arrest in CSF-arrested meiotic extracts, however the extent of Cdc2 kinase and MAPK activation was somewhat lower in metaphase-arrested mitotic extracts. When the extracts

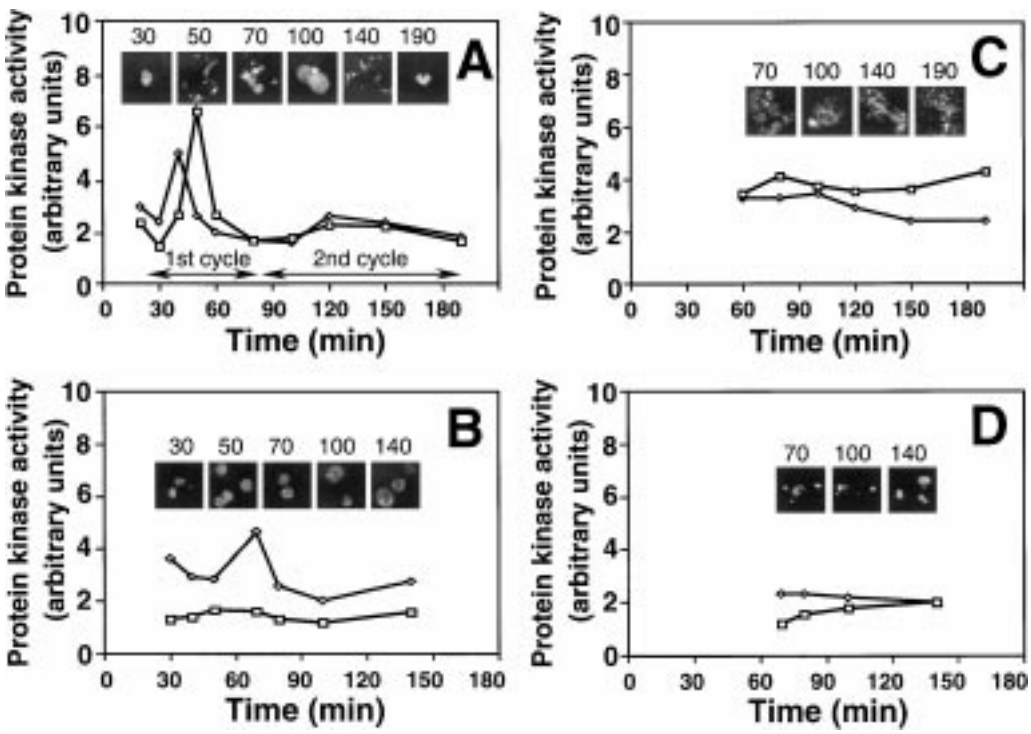

Fig. 4. Effect of BAPTA on cell cycle progression in cycling extracts. The morphology of demembranated sperm nuclei (pictures), Cdc2 kinase (◇), and MAPK (□) activities are shown in the control (**A**) and BAPTA treated extracts. Nuclear morphology was detected at the time indicated above pictures. BAPTA at a final concentration of 5 mM was added to the extracts at 20 min (**B**), 50 min (**C**), and 60 min (**D**) after initiation of cycling. Representative of two experiments with different extracts.

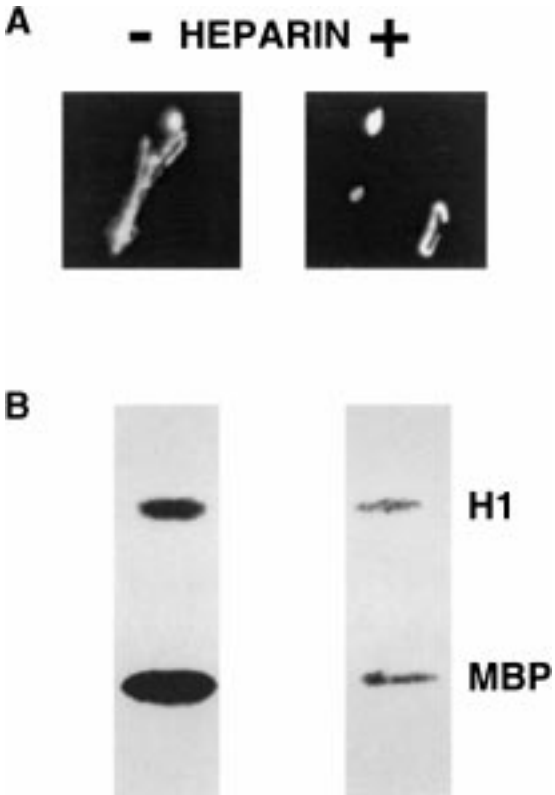

Fig. 5. Effect of heparin on cell cycle progression in cycling extracts. Heparin was added to the cycling extracts at the time of cycling initiation to a final concentration of 1 mg/ml. The morphology of demembranated sperm nuclei (**A**) and protein kinase activity (**B**) were detected as described in ''Materials and Methods'' after 60 min incubation of the control and treated extracts at 23°C. Both nuclear morphology and protein kinase activity were determined in triplicates.

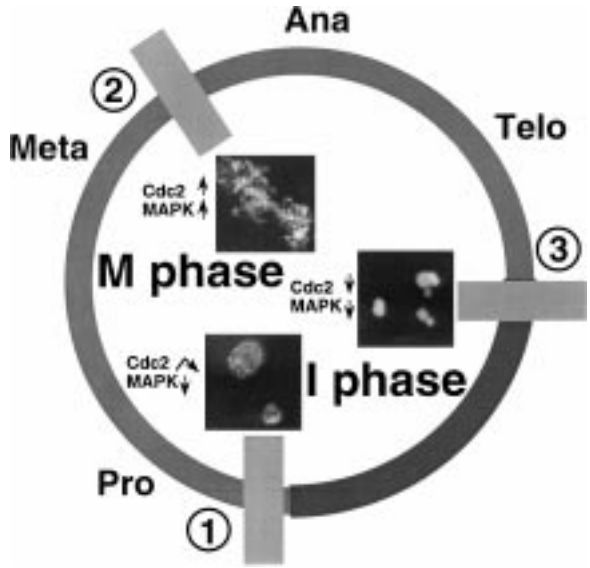

Fig. 6. Cell cycle blocks induced by calcium depletion in cycling extracts. Graphic representation of the data. Early mitotic (1), metaphase (2), and early interphase (3) blocks, that have different activities of Cdc2 kinase and MAPK, are indicated by nuclear morphology. See details in text.

were treated with BAPTA late in mitosis after metaphase-anaphase transition, they arrested at the interphase entry with very dense nuclei and the lowest activity of both Cdc2 kinase and MAPK (Fig. 4D). The results of the above experiments are summarized and further discussed in Figure 6.

The results presented in Figure 3 demonstrated that heparin suppresses IP3-induced calcium release in the cycling extracts. To establish the importance of IP3-induced calcium release in the cell cycle, we investigated the effect of heparin on the cell cycle transition in this experimental model. Heparin caused the complete arrest of cell cycle in the extracts when added in interphase at the time of cycling initiation. After 60 min incubation at 23°C, the treated extract remained in interphase whereas the control extract proceeded to metaphase, as judged by the nuclear morphology of demembranated sperm nuclei (Fig. 5A). Also, H1 kinase and MAPK activities remained low at the interphase level in the heparin-treated extract, although the mitotic reactivation of both protein kinases was evident in the control (Fig. 5B).

DISCUSSION

So far, there were no reports presented concerning calcium oscillations in cell-free *Xenopus* egg cycling extracts. However, periodic oscillations of intracellular free calcium in the dividing *Xenopus* embryos were registered that had a mean amplitude of 70 nM, around a basal level of 400 nM [Grandin and Charbonneau, 1991]. The amplitude of the relative change determined in the present study (around 100 nM) correlates well with the reported value (Fig. 1). Using fura-2, the level of free calcium in the *Xenopus* egg extracts was estimated to be similar to the value measured in intact *Xenopus* eggs in the range of 200–500 nM [Sullivan et al., 1993; Lindsay et al., 1995]. Our measurements also put the basal level of free calcium in the extracts into a submicromolar range, when the calibration of Figure 1 is applied (data not shown). Thus, it appears that free calcium in the

cycling extracts oscillates with a low magnitude around much higher basal level.

By correlating the time course of nuclear morphology and protein kinase activation with calcium oscillations, a maximum of free calcium in the cycling extracts might be mapped to early metaphase, just preceding the maximal activation of Cdc2 kinase and partial reactivation of MAPK (Fig. 2). The latter event occurs at mitotic spindle assembly checkpoint during metaphase/anaphase transition, as reported earlier [Guadagno and Ferrell Jr, 1998]. Noteworthy, in cleaving *Xenopus* embryos, calcium levels were also maximal during metaphase of mitosis and reached a minimum in interphase [Kubota et al., 1993]. MAPK activity has also been reported to increase at mitosis during the first embryonic cycles of the sea urchin embryo [Philipova and Whitaker, 1998]. As calcium increases just before mitosis in sea urchin embryos [Poenie et al., 1985; Wilding et al., 1996], it has been suggested that mitotic MAPK activity might be regulated by a calcium signal. The same chronology of cell cycle events in the *Xenopus* egg cycling extracts also suggests that calcium might play a role in mitotic regulation of MAPK.

Cycling extracts prepared by the described conventional method (see Materials and Methods) contain around 100 μM EGTA, that may buffer the level of the endogenous calcium in the extracts. Paradoxically enough, the addition of calcium chelators, such as EGTA and BAPTA, at millimolar concentrations does not dramatically affect the level of free calcium in *Xenopus* egg extracts which remained in the observed physiological range of 200–500 nM, as reported earlier [Sullivan et al., 1993; Lindsay et al., 1995]. It has been suggested, therefore, that chelators might suppress transient changes in calcium concentration rather than reduce the basal level of free calcium. Our experiments, however, failed to detect any short-time calcium transients in the cycling extracts (Figs. 1 and 2). Still, it is conceivable that the lack of ordered cellular compartmentalization in extracts could prevent the detection of short and strictly localized signals which might take place in the living cells.

Although we found that BAPTA at 5 mM concentration can block the mitotic cycle in the extracts (Fig. 4), EGTA too, at much higher concentration (up to 20 mM), was effective in blocking or delaying cell cycle progression in the extracts (data not shown). It has been noted, however, that EGTA loses its effectiveness at buffering calcium in the cytoplasm of sea urchin egg with time [Steinhardt and Alderton, 1988].

Microinjection of calcium buffers into two-cell *Xenopus* embryos has been reported to delay or prevent cleavage [Snow and Nuccitelli, 1993]. In the present paper, we found that *Xenopus* egg cycling extracts could be blocked by calcium chelation in three essentially different points of the mitotic cycle (Figs. 4 and 6), at the mitotic entry early in prophase, in metaphase, and at the interphase entry (marked as 1, 2, and 3, correspondingly, in Figure 6. Recently, measurement of MPF activity in embryos injected with calcium buffers at different times of cell cycle revealed two sensitive periods [Beckeling et al., 1999]: 1) injections before MPF activation blocked kinase activation and 2) injections just before the peak of histone kinase activity prevented kinase inactivation. When BAPTA was added to cycling extracts at the latter point, they arrested in mitosis with condensed chromatin and high activity of MPF [Beckeling et al., 1999]. This metaphase block has also been observed in our present study (denoted as 2 in Fig. 6). Also, we registered a block at mitotic entry which is characterized by lowered activity of mitotic histone kinase in the absence of chromatin condensation (denoted as 1 in Fig. 6). This block should occur in the time corresponding to the first sensitive period reported above, however, the interpretation of these experiments is complicated by possible effects of BAPTA on protein synthesis early in the first cell cycle. In addition, we observed a morphologically and enzymatically distinctive block at the interphase entry (denoted as 3 in Fig. 6) which has not been described in the *Xenopus* egg cycling extracts.

Previously, the inhibition of H1 kinase deactivation and cyclin degradation as well as slowdown of the kinase activation phase has been demonstrated in BAPTA-treated *Xenopus* prophase extracts [Lindsay et al., 1995]. It has also been found that the addition of calcium buffers at the end of H1 kinase activation phase did not prevent kinase inactivation. Thus, the exact timing of calcium chelator administration seems to be crucial for defining the point of cell cycle blockade in the extracts.

Calcium oscillations in the cell cycle are likely to be related to some events of mitosis. The evidence has been presented that cell cycle

calcium transients are linked to the inositide lipid cycle and driven by the changes in IP3 level. Reducing inositol lipid hydrolysis or IP3 receptor availability lengthens the duration of the cell cycle in *Xenopus* blastomeres [Han et al., 1992]. The antibody to IP3 receptor has been reported to block calcium wave and calcium oscillations in fertilized hamster eggs [Miyazaki et al., 1992]. The results presented in Figure 3 demonstrate that heparin at 1 mg/ml effectively inhibits IP3-induced calcium release in the *Xenopus* egg cycling extracts. Only partial inhibition of calcium release could be detected at a lower concentration of heparin (100 μg/ml), whereas the concentration of 10 μg/ml was not effective (data not shown). Similar dose response has been reported previously for IP3 receptor of *Xenopus* oocytes; heparin at 150 μg/ml displaced half of receptor-bound IP3 [Callamaras and Parker, 1994]. Subsequently we found that heparin could arrest cell cycle when added to the cycling extracts in interphase (Fig. 5). This result is consistent with the previous report that microinjection of heparin into one blastomere of the two-cell stage embryo caused partial or complete arrest of the cell cycle [Han et al., 1992]. Thus, both in the dividing embryos and in the cycling extracts inhibition of IP3-induced calcium release arrests cell cycle. Oscillations of inositol polyphosphate in the embryo cleavage cycle of *Xenopus* [Stith et al., 1993; Han, 1995] and sea urchin [Ciapa et al., 1994] have been reported. Most recently, phospholipase Cγ has been suggested to play a key role in this process [Shearer et al., 1999]. It is of interest, therefore, to investigate in more detail the involvement of phopsphoinositide messenger system in the regulation of calcium level in *Xenopus* eggs cycling extracts.

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