Okadaic acid suppresses calcium regulation of mitosis onset in sea urchin embryos

Rajnikant Patel and Michael Whitaker

Department of Physiology
University College London
London WC1E 6BT, United Kingdom

We show that a phosphatase inhibitor, okadaic acid, induces premature and persistent mitosis during the first cell cycle in sea urchin embryos. Okadaic acid-induced mitosis requires protein synthesis, suggesting that it activates the protein synthesis-requiring mitotic H1 kinase. By microinjecting the calcium chelators BAPTA and EGTA and by measuring Ca_i using fura-2, an indicator dye, we show that okadaic acid-induced mitosis is independent of the calcium signal that usually triggers mitosis onset in sea urchin embryos. Disabling the calmodulin kinase II that is thought to respond to the mitotic Ca_i signal using a peptide inhibitor fails to prevent mitosis in response to okadaic acid. These data suggest that okadaic acid bypasses calcium regulation of mitosis by inducing constitutive phosphorylation of a site on the H1 kinase that is normally under the control of the calmodulin-regulated kinase.

Introduction

The unfertilized sea urchin egg is arrested in interphase of the cell cycle, before S-phase. After fertilization, the early embryonic cell division cycles alternate rapidly between S-phase and M-phase. The transition into M-phase is dependent on the actions of protein kinases and protein phosphatases in a wide variety of cell types (reviewed by Cyert and Thorner, 1989). There are cycles of protein phosphorylation and dephosphorylation during the early embryonic cell cycles of the sea urchin that correlate with entry into and exit from mitosis (Neant *et al.*, 1989); and two protein kinases have been identified whose activities are necessary for entry into mitosis: a histone H1 kinase (H1 kinase) (Meijer and Pondaven, 1988; Meijer *et al.*, 1989) and a calcium/calmodulin (CaM)-dependent protein kinase (Baitinger *et al.*, 1990).

H1 kinase is a calcium- and cyclic nucleotide-independent enzyme that is activated only at each metaphase (Bradbury *et al.*, 1974; reviewed by Hohmann, 1983). The sea urchin H1 kinase has been purified and shown to be a dimer comprising cyclin and p34, the latter a homologue of the yeast *cdc2*/CDC28 gene product (Meijer *et al.*, 1989). p34^{cdc2} is the central kinase that regulates mitosis in eucaryotic cells (reviewed by Nurse, 1990). The level of p34^{cdc2} does not vary during the sea urchin cell cycle, but its kinase activity increases abruptly at M-phase (Meijer *et al.*, 1989). Two conditions must be fulfilled to activate the kinase activity of p34^{cdc2}. First, cyclin needs to be synthesized and accumulated to a threshold level (Evans *et al.*, 1983; Draetta *et al.*, 1989; Murray and Kirschner, 1989; Murray *et al.*, 1989; Solomon *et al.*, 1990); in sea urchin embryos cyclin is also phosphorylated and there is good correlation between the appearance of H1 kinase activity and the phosphorylation of cyclin (Meijer *et al.*, 1989). Second, dephosphorylation of a tyrosine residue on p34^{cdc2} is necessary (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Solomon *et al.*, 1990). At the end of mitosis, the kinase activity of the p34^{cdc2}-cyclin complex is rapidly switched off via degradation of the cyclin subunit (Draetta *et al.*, 1989; Meijer *et al.*, 1989; Murray *et al.*, 1989). Recent evidence indicates that the active p34^{cdc2}-cyclin complex itself initiates cyclin destruction (Felix *et al.*, 1990b).

The partially purified sea urchin calcium/calmodulin-dependent kinase (CaM kinase) is a multimeric enzyme (405 kDa; Baitinger *et al.*, 1990) that may be homologous to the multifunctional CaM kinase required during early development of the sea urchin embryo (Chou and Rebhun, 1986). Microinjection into sea urchin eggs of either a synthetic peptide corresponding to the autoinhibitory domain of the neuronal CaM kinase or an antibody to the neuronal enzyme inhibits or delays nuclear envelope breakdown (NEB)¹ (Baitinger *et al.*, 1990). The CaM

¹ Abbreviations: ASW, artificial sea water; BAPTA, bis-(o-

kinase is the probable target for the mitosis ENTRY calcium transient that is the immediate trigger for M-phase onset in sea urchin embryos (Poenie *et al.*, 1985; Steinhardt and Alderton, 1988; Twigg *et al.*, 1988; reviewed in Whitaker and Patel, 1990) and mammalian cells (Kao *et al.*, 1990). Preventing the mitosis ENTRY calcium transient in sea urchin embryos by the use of calcium chelators blocks mitosis onset (NEB; Steinhardt and Alderton, 1988; Twigg *et al.*, 1988), whereas precociously inducing an intracellular calcium transient by microinjecting calcium buffers or the calcium-releasing phosphoinositide messenger inositol trisphosphate (InsP_3) causes premature entry into mitosis. These data point to activation of the CaM kinase as the step controlling mitosis onset in sea urchin embryos.

On the other hand, very little is known about the role of protein phosphatases in the sea urchin cell cycle. In yeast, *Aspergillus* and *Drosophila* mutations in genes coding for either protein phosphatase 1 or 2A arrest the cell cycle in mitosis (Booher and Beach, 1989; Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990; Kinoshita *et al.*, 1990). In *Xenopus* oocytes and embryos, a mitotic inhibitor INH is reported to show homology to protein phosphatase 2A (Solomon *et al.*, 1990). That protein phosphatase activity negatively regulates mitosis onset has also been demonstrated by the use of okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A (Bialojan and Takai, 1988; Cohen *et al.*, 1989; Haystead *et al.*, 1989; Felix *et al.*, 1990a; Solomon *et al.*, 1990; Yamashita *et al.*, 1990). Microinjection of okadaic acid into *Xenopus*, mouse, and starfish oocytes causes maturation (entry into mitosis) and activation of H1 kinase (Goris *et al.*, 1989; Picard *et al.*, 1989; Pondaven *et al.*, 1989; Gavin *et al.*, 1991).

The purpose of this study was to investigate the consequences of inhibiting phosphatase activity in the cell cycle of early sea urchin embryos by the use of the phosphatase inhibitor okadaic acid in both fertilized and parthenogenetically activated sea urchin eggs. We were especially interested in the links between phosphatase activity and the mitosis entry Ca_i signal. We report that okadaic acid induces premature mitosis onset (NEB) and subsequently arrests

the cell cycle in mitosis. Okadaic acid-induced mitosis is blocked by a protein-synthesis inhibitor, emetine. We also find that okadaic acid-induced NEB is calcium-independent and insensitive to a CaM kinase inhibitory peptide, which indicates that phosphatase inhibition bypasses the calcium signaling system normally responsible for triggering mitosis in sea urchin embryos.

Results

Okadaic acid does not restart the cell cycle in unfertilized eggs

Microinjection of okadaic acid ($1 \mu\text{M}$) into unfertilized eggs had no visible effects over a 3-h period. This is in agreement with earlier results (Picard *et al.*, 1989) and indicates that okadaic acid cannot substitute for the START Ca_i transient at fertilization and induce reinitiation of the cell cycle in early interphase. When okadaic acid-injected eggs are fertilized, cortical granule exocytosis and elevation of the fertilization envelope are inhibited in a dose-dependent manner, although the fertilization Ca^{2+} -transient is not affected (Whalley *et al.*, 1991). The lack of a fertilization envelope results in polyspermic eggs at normal sperm densities. In the following experiments a low sperm density was used to fertilize okadaic acid-injected eggs to prevent polyspermy.

Okadaic acid advances mitosis entry in fertilized and ammonia-activated eggs and arrests the cell cycle in mitosis

Okadaic acid ($10 \mu\text{M}$) had no effect on fertilization or the cell cycle when added to the bathing medium before fertilization. In contrast, when microinjected just before fertilization, it caused premature NEB, advancing entry into M-phase by up to 20 min at a final concentration of $1 \mu\text{M}$ (Figure 1). Doubling the concentration of okadaic acid did not reduce further the time of onset of mitosis (NEB). Figure 1A also shows that okadaic acid at concentrations $> 0.25 \mu\text{M}$ blocked the cell cycle in mitosis. The arrested eggs did not reform their nuclear envelopes and the chromatin remained condensed for up to 3 h after fertilization (Figure 1B). These effects contrast with the lack of effect of okadaic acid in unfertilized eggs and are similar to the consequences of microinjecting okadaic acid into starfish oocytes, where it induces germinal vesicle breakdown (GVBD) and arrests the cell cycle with condensed chromosomes and high

aminophenoxy)-*N,N,N',N'*-tetraacetic acid; EGTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEB, nuclear envelope breakdown; PMA, phorbol 12-myristate 13-acetate; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

B

Figure 1. Okadaic acid-induced mitosis in sea urchin embryos. (A) Cytoplasmic concentration effect curve for the advance of mitosis onset and the block in mitosis. Each point represents the mean of three to five separate experiments; 10–18 eggs were microinjected in each experiment. Okadaic acid was diluted into injection buffer from a 2 mM stock in dimethylsulfoxide (DMSO). The injection volume (0.5 μl ; 0.1% egg volume) was constant. Microinjection of 0.2% DMSO alone did not affect the cell cycle. (B) Photomicrographs of eggs treated with the vital DNA stain H33342. Left: An egg fertilized after injection with 1 μM okadaic acid underwent NEB at 55 min and was photographed at 160 min. The chromatin remained condensed over a period of ≥ 3 h; neither did the nuclear envelope reform. Right: a DMSO-injected control embryo at the four-cell stage 160 min after fertilization, with nuclear envelopes and decondensed chromatin. The scale bar indicates 20 μm . *L. pictus*. 16°C.

H1 kinase and mitosis promoting factor (MPF) activities (Picard *et al.*, 1989). The cell cycle is arrested after S-phase in immature star-

fish oocytes, just before the onset of meiosis, in contrast to sea urchin eggs, which are arrested in interphase before S-phase (Whitaker

and Patel, 1990). The comparison suggests that okadaic acid has its effects at the mitosis ENTRY cell cycle control point and that the cell cycle control mechanism is primed to respond to okadaic acid by some event that follows fertilization, perhaps protein or DNA synthesis.

Parthenogenetic activation of eggs with artificial seawater (ASW) containing ammonium ions stimulates protein and DNA synthesis (Epel *et al.*, 1974; Mazia and Ruby, 1974). Ammonia-activated eggs do not divide but undergo cycles of DNA replication, chromatin condensation, and NEB (Mazia, 1974; Patel *et al.*, 1989). Microinjection of okadaic acid (0.5 μ M) followed by activation of the eggs with ammonium chloride resulted in premature NEB. As in fertilized eggs, the okadaic acid-injected eggs arrested in the cell cycle with condensed chromatin and did not reform their nuclear envelopes (Figure 2A). Okadaic acid-induced NEB was advanced to the same degree in NH_4Cl -activated and fertilized eggs (Figures 2 and 5, legend).

Okadaic acid-induced mitosis entry requires protein synthesis

Previous work in starfish oocytes suggests that the factor required for okadaic acid-induced mitosis entry is a cell cycle protein (Picard *et al.*, 1989). In *Xenopus* oocytes and egg extracts, it has been shown that cyclin is the only protein that is required to induce entry into M-phase (Swenson *et al.*, 1986; Minshull *et al.*, 1989; Murray and Kirschner, 1989). In sea urchins, in-

hibiting protein synthesis with emetine has been shown to prevent cyclin synthesis (Evans *et al.*, 1983), MPF and H1 kinase activation (Meijer and Pondaven, 1988; Arion and Meijer, 1989), and NEB (Twigg *et al.*, 1988; Whitaker and Patel, 1990). Emetine (100 μ M) is known to completely inhibit protein synthesis (>99%; Wagenaar and Mazia, 1978) without affecting DNA synthesis. We looked at the effect of emetine on fertilized eggs that had been microinjected with okadaic acid. When protein synthesis was completely blocked, okadaic acid was unable to induce NEB (Figure 2B), suggesting that okadaic acid relies for its effects on a protein synthesized after fertilization, most probably cyclin.

Increased protein synthesis becomes a sufficient stimulus for mitosis entry in okadaic acid-injected eggs

We also looked at the effects of okadaic acid in eggs treated with either low concentrations of ammonia (2 mM NH_4Cl) or the phorbol ester phorbol 12-myristate 13-acetate (PMA). Both PMA and 2 mM ammonia stimulate protein synthesis by increasing pH_i (Epel *et al.*, 1974; Whitaker and Steinhardt, 1982; Swann and Whitaker, 1985). Although cyclin and phosphorylated cyclin (Pcyclin) are present in almost equimolar proportions in PMA- and 2 mM NH_4Cl -treated eggs, the rapid conversion of cyclin to Pcyclin that occurs in fertilized eggs is not seen, nor is there a mitosis ENTRY Ca^{2+}_i transient or NEB in these eggs when they are observed for periods of several hours (Whitaker

Figure 2. Okadaic acid advances mitosis onset in parthenogenetically activated eggs and requires protein synthesis. (A) NEB in ammonia-activated eggs (15 mM NH_4Cl in ASW, pH 9 for 15 min) microinjected with okadaic acid (0.5 μ M) before activation. In two experiments, NEB occurred 71 and 74 min after activation, an advance of 10 and 16 min, respectively, compared with controls. Fourteen to 16 eggs were microinjected with okadaic acid in each experiment. (B) The protein synthesis inhibitor emetine (100 μ M) was added to okadaic acid-injected eggs (1 μ M) and uninjected controls. In two separate experiments, one of which is shown, it prevented NEB in both okadaic acid-injected eggs and controls. Ten to 14 eggs were microinjected with okadaic acid in each experiment. *L. pictus*. 16°C.

Figure 3. Increasing protein synthesis by raising pH_i leads to NEB in okadaic acid-injected eggs. (A) Increasing pH_i using ammonium chloride (2 mM NH₄Cl in ASW, pH 9 for 15 min) in eggs previously injected with okadaic acid (1 μM). Twelve to 18 eggs were microinjected with okadaic acid for each experiment. (B) Increasing pH_i using the phorbol ester PMA (250 nM in ASW) in eggs previously injected with okadaic acid (1 μM). Ten to 14 eggs were microinjected with okadaic acid in each experiment. *L. pictus*. 16°C.

and Patel, 1990). Under normal circumstances, then, stimulating protein (cyclin) synthesis alone does not lead to resumption of the cell cycle or mitosis in unfertilized sea urchin eggs: these treatments are not parthenogenetic activators. However, in both PMA- and 2 mM NH₄Cl-treated eggs, microinjection of okadaic acid led to NEB (Figure 3).

Okadaic acid-induced mitosis entry is calcium independent

The above results suggest that okadaic acid-induced NEB is independent of a mitosis ENTRY Ca_i transient. To determine whether a mitotic Ca_i transient was required for NEB in fertilized eggs injected with okadaic acid, we microinjected the calcium chelator ethylene glycol-bis(aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) into fertilized eggs preinjected with okadaic acid. It failed to inhibit NEB (Table 1). We also injected another calcium chelator, bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), into 15 mM NH₄Cl-activated eggs that had been preinjected with okadaic acid. Eggs microinjected with 2 mM BAPTA underwent NEB at the same time as eggs microinjected with okadaic acid alone (Figure 4). In the same experiments, these chelators delayed or blocked NEB in control embryos. Microinjection of 2 mM BAPTA is known to inhibit the Ca²⁺_i transient at NEB, but it does not alter the resting level of Ca²⁺_i (Whitaker and Patel, 1990). These data indicate that okadaic acid uncouples NEB from the mitosis ENTRY Ca_i transient.

Okadaic acid-induced mitosis bypasses the CaM kinase

Antibody and peptide inhibitors directed against the multifunctional CaM kinase delay or block NEB in sea urchin embryos, suggesting that this kinase is the target of the mitosis ENTRY calcium transient (Baitinger *et al.*, 1990). We microinjected the inhibitory CaMK(273–302) peptide (Payne *et al.*, 1988) and confirmed that it delayed NEB at a concentration of 30 μM (Figure 6A). The noninhibitory CaMK(284–302) peptide (a shorter fragment of the CaM kinase auto-inhibitory domain) was ineffective. In the same batch of eggs, the timing of okadaic acid-induced NEB was not affected by microinjecting the inhibitory peptide (Figure 5B). These data indicate that the multifunctional CaM kinase is

Table 1. Effects of EGTA and okadaic acid on mitosis onset in early sea urchin embryos

	Nuclear envelope breakdown (80 min postfertilization)	
	Expt 1	Expt 2
EGTA (4 mM)	1/8	2/12
Okadaic acid (1 μM)	10/10	13/13
EGTA (4 mM) + okadaic acid (1 μM)	4/5	5/6
Uninjected controls	89/90	119/124

The experimental protocol was similar to the protocol in the legend to Figure 4. EGTA was microinjected 25–40 min after fertilization.

Figure 4. The calcium chelator BAPTA does not affect okadaic acid-induced NEB in parthenogenetically activated eggs. Two separate experiments are shown. In each, 22–24 eggs were microinjected with okadaic acid (1 μ M) and activated with ammonium chloride (15 mM NH_4Cl in ASW, pH 9 for 15 min) along with controls. Fifty-five to 65 (experiment 1) and 60–70 (experiment 2) min after activation, half (10–11) the okadaic acid-injected eggs were microinjected with BAPTA (1 M, pH 7.3) to a final concentration of 2 mM, along with 6–8 control eggs. At 100 min, when all the control eggs had undergone NEB, 1/8 (experiment 1) and 0/6 (experiment 2) control/BAPTA-injected eggs had undergone NEB, confirming that the calcium chelator delays or blocks NEB in ammonia-activated eggs (Patel *et al.*, 1989). *L. pictus*. 16°C.

not part of the mechanism of okadaic acid-induced NEB.

Okadaic acid suppresses the mitosis ENTRY Ca_i transient

We have also looked at the effects of okadaic acid on the mitosis ENTRY Ca^{2+}_i transient in eggs parthenogenetically activated with 15 mM NH_4Cl . As in fertilized eggs, this transient increase in Ca^{2+}_i is necessary for NEB and chromatin condensation (Patel *et al.*, 1989). Measurements of Ca^{2+}_i by the use of the fluorescent calcium-sensitive dye fura-2 show that Ca^{2+}_i increases transiently at NEB (Figure 5A). In okadaic acid-injected eggs activated with 15 mM NH_4Cl , no transient increase in Ca^{2+}_i was observed when the eggs underwent premature NEB. Moreover, there was no change in Ca_i at the time at which NEB would normally have occurred (Figure 6B). This result confirms that no increase in Ca^{2+}_i above basal levels is required for NEB in the presence of okadaic acid and indicates that okadaic acid suppresses or preempts the mechanism responsible for generating the mitosis ENTRY Ca_i transient.

Timing of mitosis onset in okadaic acid-injected eggs

The timing of mitosis onset in the first embryonic cell cycle in sea urchins is determined un-

der normal circumstances by the mitosis ENTRY Ca_i transient (Poenie *et al.*, 1985; Steinhardt and Alderton, 1988; Twigg *et al.*, 1988; Whitaker and Patel, 1990). The onset of mitosis can be delayed by slowing the rate of protein (cyclin) synthesis, both in control (Whitaker and Patel, 1990) and okadaic acid-injected eggs (Figure 7A). At concentrations of emetine that delay but do not block NEB in fertilized eggs (Whitaker and Patel, 1990), okadaic acid injection advanced NEB and arrested the cell cycle in mitosis.

Sufficient protein synthesis to support mitosis onset has occurred by 40 min after fertilization (Wagenaar, 1983; Twigg *et al.*, 1988). To determine how long it took for okadaic acid to activate the mitotic kinase once the cyclin requirement of the kinase was satisfied, we injected fertilized eggs with okadaic acid at 50 min after fertilization. Mitosis onset occurred some 10 min later (Figure 7B). In eggs injected with maximal concentrations of okadaic acid just before fertilization, NEB occurs 53 min later, that is, ~ 10 min after the cyclin requirement is satisfied. These data suggest that the timing of mitosis onset in eggs injected with okadaic acid before fertilization is set by the availability of cyclin, with a short lag period of 10 min that intervenes once sufficient cyclin is present in the egg cytoplasm. A similar lag period for H1 kinase activation after

A

B

Figure 5. The CaMK(273-302) inhibitory peptide does not affect okadaic acid-induced mitosis onset. (A) A control experiment. The CaMK(273-302) peptide (30 μ M) delays mitosis onset by 40 min when present in early embryos. Microinjecting the CaMK(284-302) peptide (43 μ M) as a control showed it to be without effect at this concentration. (B) The inhibitory CaMK(273-302) peptide does not affect the timing of okadaic acid (1 μ M)-induced NEB in early embryos. Both experiments were made on the same batch of eggs. A second identical experiment gave the same result. Ten or 11 eggs were microinjected in each experiment. *L. pictus*. 16°C.

cyclin accumulation has been observed in a cell-free system isolated from *Xenopus* embryos (Solomon *et al.*, 1990).

Okadaic acid-induced NEB was slower in eggs

in which cyclin synthesis was induced by treatment with phorbol ester (74 ± 5.8 min; mean $\pm$ SE, $n = 5$) than in fertilized and ammonia-activated eggs. Moreover, if okadaic acid was microinjected into eggs 70–80 min after treatment with phorbol ester (a time at which the level of cyclin synthesis is comparable with that in control eggs at NEB: Whitaker and Patel, 1990), then NEB occurred only 60–70 min later (Figure 7C). This observation contrasts markedly with the short delay we observed in fertilized eggs. It implies that the inactive but cyclin-bound kinase is less susceptible to activation by okadaic acid in eggs activated by phorbol ester, despite the presence of equivalent amounts of cyclin, and that, in PMA-treated eggs, it is not cyclin accumulation that is the major rate-limiting step for kinase activation.

Discussion

Newly synthesized protein is necessary and sufficient for okadaic acid-induced mitosis onset

The unfertilized sea urchin egg is metabolically quiescent and arrested in the cell cycle before S-phase. The egg is activated at fertilization by a global Ca_i transient and an increase in pH_i that derepress the egg's metabolism and restart the cell cycle (Whitaker and Steinhardt, 1982). The fertilization Ca_i transient triggers the increase in pH_i . The pH_i increase is in turn responsible for stimulating protein synthesis. It has been known for some time that mitosis onset in sea urchin embryos requires newly synthesized proteins (Wagenaar and Mazia, 1978). Experiments with weak bases indicate that protein synthesis, although necessary, is not a sufficient condition for mitosis onset in sea urchin embryos. The parthenogenetic activator ammonium chloride induces DNA synthesis and chromatin condensation cycles only at concentrations > 10 mM; below this concentration it quite adequately stimulates protein synthesis by increasing pH_i but fails to trigger mitosis (Epel *et al.*, 1974; Whitaker and Patel, 1990). A similar result obtains with the phorbol ester PMA, which increases pH_i , and thus protein synthesis, by activating the egg's plasma membrane sodium-hydrogen antiporter but does not restart the cell cycle (Whitaker and Patel, 1990).

We show here that the phosphatase inhibitor okadaic acid promotes mitosis onset in sea urchin eggs, provided protein synthesis is stimulated by increasing pH_i . We use emetine, a protein synthesis inhibitor, to demonstrate that okadaic acid requires protein synthesis to bring

Figure 6. Okadaic acid suppresses the mitosis ENTRY Ca_i transient in ammonia-activated eggs. (A) Intracellular free calcium ion concentration in an ammonia-activated (15 mM NH_4Cl in ASW, pH 9 for 15 min) egg, measured with the fluorescent calcium indicator dye fura-2 (10 μM). The Ca_i transient occurred 48 min after activation; NEB occurred 2.6 min later. In five similar experiments, the mitosis ENTRY Ca_i transient occurred 65 ± 4.2 min (mean $\pm$ SE) after activation and NEB 5.5 ± 1.8 min later. (B) Intracellular free calcium ion concentration ([fura-2]: 15 μM) in an egg activated with ammonia after microinjection of okadaic acid (1 μM). NEB occurred 40 min after activation, but no Ca_i transient was observed. In five similar experiments, eggs underwent NEB 46 ± 2.8 min (mean $\pm$ SE) later, without any detectable alteration in Ca_i . *L. pictus*. 16°C.

about mitosis, just as in the normal embryo. The striking difference between okadaic acid-induced mitosis onset and its natural counterpart

is that, for the former, protein synthesis is a sufficient condition. The consensual view is that the mitotic protein synthesis requirement re-

Figure 7. Factors governing the timing of mitosis onset in okadaic acid-injected eggs. (A) Partial inhibition of protein synthesis using emetine (25 μM) delays mitosis onset in early embryos. Okadaic acid (1 μM) reduces the delay. (B) Comparison of the effects of okadaic acid (1 μM) on the timing of mitosis onset when microinjected before fertilization (before cyclin accumulation) or at 50 min (after cyclin accumulation). (C) Comparison of the effects of okadaic acid (1 μM) on the timing of mitosis onset (NEB) in PMA-activated eggs. When injected just before PMA treatment (before PMA), NEB occurs at 60 min. When injected at 80 min, after cyclin accumulation (after PMA, bar), NEB occurs 60–70 min later. One of two sets of experiments. *L. pictus*. 16°C.

flects the need for cyclins, cell cycle proteins that are synthesized during interphase and form one component of the H1 kinase activity that drives the nucleus into mitosis, the other component being p34^{cdc2} (Murray, 1987; Murray and Kirschner, 1989; Murray *et al.*, 1989). We have shown previously that increasing pH_i in unfertilized eggs using PMA or low concentrations of ammonia leads to the synthesis of cyclin B at rates comparable to those in early embryos (Whitaker and Patel, 1990). The dependence of okadaic acid-induced mitosis onset on protein synthesis in sea urchin embryos implies that critical levels of cyclin must be generated before mitosis onset can occur. A similar conclusion has been reached in comparable protein synthesis-inhibition experiments in starfish oocytes (Picard *et al.*, 1989). The study also reports that the cell cycle in okadaic acid-injected starfish oocytes is arrested in mitosis, a result we confirm in sea urchin embryos.

Okadaic acid-induced mitosis onset is independent of calcium and the CaM kinase II

Mitosis onset in early sea urchin embryos follows very closely on an intracellular Ca_i transient (the mitosis ENTRY Ca_i transient) and is blocked by calcium chelators that suppress the Ca_i transient without affecting resting levels of cytoplasmic calcium (Poenie *et al.*, 1985; Steinhardt and Alderton, 1988; Twigg *et al.*, 1988; reviewed in Whitaker and Patel, 1990). The mitosis ENTRY Ca_i transient targets a multifunctional CaM kinase: it has been shown in sea urchin embryos that antibody and peptide inhibition of the CaM kinase prevents mitosis (Baitinger *et al.*, 1990). We show here that okadaic acid bypasses the calcium-requiring step at mitosis. Okadaic acid-induced mitosis is unaffected by calcium chelators and is independent of the CaM kinase, in that it is immune to suppression by the CamK(273–302) inhibitory peptide that delays or blocks mitosis onset in control embryos (Payne *et al.*, 1988; Baitinger *et al.*, 1990). These data suggest that phosphatase inhibition by okadaic acid substitutes for the CaM kinase II activity in bringing about mitosis, that is, that a critical phosphorylation normally dependent on the transient activation of the CaM kinase at the time of mitosis is brought about in okadaic acid-injected embryos by the constitutive inhibition of phosphatases.

Site of action of the okadaic acid-sensitive phosphatase

Okadaic acid binds to protein phosphatases 1 and 2A at nanomolar concentrations, but we see effects on the sea urchin embryo cell cycle only at concentrations > 250 nM. The most likely explanation for this apparent quantitative discrepancy is that inhibition by okadaic acid is due to stoichiometric binding to phosphatases that are present at concentrations of ~0.1–1 μM within the cytoplasm (Cohen *et al.*, 1989). This should be compared with the cytoplasmic concentrations of p34^{cdc2} and cyclin (2–5 and 0.2 μM, respectively: Meijer *et al.*, 1989). At these concentrations, okadaic acid does not distinguish between the phosphatase 1 and 2A subtypes (Bialojan and Takai, 1988; Cohen *et al.*, 1989); either or both might act as mitotic suppressors. In starfish oocytes, selective inhibition of phosphatase 1 did not lead to meiosis onset (Picard *et al.*, 1989), but genetic deletion experiments in yeast and *Aspergillus* suggest that deletion of either phosphatase 1 or phosphatase 2A loci is sufficient to induce a block in mitosis similar to that seen with okadaic acid. On this evidence, it is uncertain whether either phosphatase subtype alone governs a rate limiting step in mitosis onset, although it is very likely that in our experiments okadaic acid is inhibiting both.

Others have shown that okadaic acid promotes cyclin-dependent H1 kinase activity in cell extracts, suggesting that okadaic acid-sensitive phosphatases control the activity of the p34/cyclin heteromer (Felix *et al.*, 1990a; Solomon *et al.*, 1990). A threonine phosphate on p34 and an unidentified phosphate on cyclin are candidate regulators of H1 kinase activity, which has been shown to be negatively regulated by INH, a protein with phosphatase 2A homology (Solomon *et al.*, 1990). We show here that okadaic acid substitutes for the CaM kinase activation that is the immediate trigger of mitosis in untreated sea urchin embryos (Baitinger *et al.*, 1990). The obvious corollary is that the CaM kinase positively regulates the phosphate that is the substrate of INH, converting the assembled H1 kinase to its active form. Cyclin phosphorylation coincident with mitosis onset is blocked when sea urchin embryos are microinjected with a calcium chelator that abolishes the mitosis ENTRY Ca_i transient (Whitaker and Patel, 1990): it may be the cyclin phosphate that is regulated by the CaM kinase and INH, although this is no more than a reasonable conjecture.

Okadaic acid can in part substitute for the START cell cycle Ca_i transient at fertilization

The cell cycle resumes at fertilization in sea urchin eggs in response to two ionic signals: a Ca_i transient (the START Ca_i transient) and a sustained increase in pH_i (Whitaker and Steinhardt, 1982; Whitaker and Patel, 1990). Both are necessary; increasing pH_i increases cyclin synthesis but does not lead to mitosis. We show here that microinjecting okadaic acid into unfertilized eggs does not in itself restart the cell cycle. However, it can partially substitute for the START Ca_i transient in that the pH_i signal is a sufficient signal for the cell cycle to resume in okadaic acid-injected eggs. This effect of okadaic acid at the START cell cycle control point seems to be distinct from its effects at the mitosis ENTRY control point. Its kinetics are much slower (Figure 7) and are rate limiting for mitosis onset. These observations are consistent with previous observations that suggest that three conditions must be satisfied for successful mitosis onset in sea urchin embryos: a proximate mitosis ENTRY Ca_i transient; cyclin synthesis due to the START pH_i signal; and prior phosphorylation of p34 by the START Ca_i signal (Whitaker and Patel, 1990). It appears that okadaic acid can substitute for the first and the last of these, the last rather more slowly than the first.

Cell cycle timing and the cell cycle pacemaker

The timing of mitosis is governed by a cytoplasmic mechanism that generates periodic H1 kinase activity that in turn leads to dissolution of the nuclear envelope and chromatin condensation. One widely canvassed hypothesis, arrived at from the properties of in vitro models of mitosis in cell-free systems, suggests that the bursts of H1 kinase activity are generated by the cyclin-abundance cycle (Murray, 1987, 1989; Murray and Kirschner, 1989; Murray *et al.*, 1989). Our results support this idea inasmuch as they suggest that the timing of mitosis onset in okadaic acid-injected embryos is determined by the accumulation of cyclin and that cyclin accumulation is the sole requirement and rate-limiting step. However, okadaic acid-injected embryos differ from normal sea urchin embryos in that they have lost their mitosis ENTRY Ca_i transient and their mitosis has escaped from the control of the CaM kinase. It is this latter control that appears to be responsible for the timing of mitosis under normal circum-

Table 2. Kinetics of okadaic acid-induced mitosis onset and the START Ca_i transient

	Time to 50% NEB after injection of okadaic acid (min)	
	Fertilized (START Ca_i + pH_i)	PMA-treated (pH_i alone)
OA injection before cyclin accumulation	53 ± 3.1 (5) ^a	74 ± 5.8 (5) ^b
OA injection after cyclin accumulation	16 ± 2.7 (4) ^a	60 (2)

Values are means $\pm$ SE; numbers in parentheses are numbers of experiments, each on ≥ 10 eggs.

^a See Figure 7B.

^b See Figure 7C.

stances, in which there is a close temporal correlation between the mitosis ENTRY Ca_i transient, inhibition of mitosis by calcium chelators, and inhibition of the CaM kinase and in which cyclin accumulation is a necessary but not sufficient condition for mitosis onset (Baitinger *et al.*, 1990; Whitaker and Patel, 1990). Simply stated, okadaic acid appears to create in ovo a simulacrum of cell cycle control that pertains in vitro.

Materials and methods

Obtaining gametes—egg activation

Eggs of the sea urchin *Lytechinus pictus* (Marinus, Long Beach, CA) were obtained by intracoelomic injection of 0.5 M KCl and collected in ASW (435 mM NaCl, 40 mM MgCl_2 , 15 mM MgSO_4 , 11 mM CaCl_2 , 10 mM KCl, 2.5 mM NaHCO_3 , 1 mM EDTA, pH 8.0). The eggs were dejellied by passage through Nitex mesh (pore size 100 μm) four or five times, washed twice with ASW, and resuspended in ASW. For microinjection the eggs were attached to coverslips precoated with 0.05 mg/ml poly (L-lysine) (Sigma, Poole, UK). Sperm was collected "dry" and stored at 4°C until required. For fertilization 5 μl of the dry sperm was diluted in 1 ml of ASW and 100 μl of this dilute suspension was added to the bath containing the eggs. To facilitate microinjection of fertilized eggs the eggs were fertilized in ASW containing 1 mM para-aminobenzoic acid (PABA). After 15 min the PABA ASW was replaced with normal ASW. Before microinjection the ASW was replaced with ASW containing 1.5% wt/vol dextran. After microinjection the dextran ASW was replaced with normal ASW. Eggs were parthenogenetically activated by incubating them for 15 min with 15 mM NH_4Cl in ASW (pH 9.0). After 15 min the eggs were washed twice with ASW before being placed in ASW. For activation with PMA (Sigma), the eggs were continuously incubated with 250 nM PMA in ASW. In all experiments reported here, >95% of the eggs raised fertilization envelopes and developed to the four-cell stage. All experiments were conducted at 16°C. Control and experimental cell cycle times were normalized to 50% NEB at 70 min.

Microinjection of eggs

Eggs were microinjected as described before (Swann and Whitaker, 1986) except that the micropipette was held in a hydraulic manipulator (Narashige, Tokyo, Japan) mounted on the stage of an inverted Diaphot fluorescence microscope (Nikon, Telford, UK). Microinjections were performed by the use of bright-field microscopy with a 40 $\times$, 0.65-NA achromat objective. Stages of the cell cycle were scored by the use of DIC optics. The injection volume was estimated by measuring the cytoplasmic displacement of the injected fluid with an eyepiece graticule. The injection buffer was 0.5 M KCl, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid (PIPES), 1 mM EGTA, pH 6.7, except for injection of the CaMK(273–302) and CaMK(284–302) peptides, where 130 mM KCl, 10 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0 was used.

Fluorescence measurement of Ca_i

Ca_i was measured in single eggs using the fluorescent calcium-sensitive dye fura-2 (Pentapotassium salt, Molecular Probes, Eugene, OR). Fura-2 (10 mM in 0.5 M KCl/20 mM PIPES, pH 6.7) was microinjected into eggs to give a cytoplasmic concentration of 10–20 μ M. The fluorescence from the egg was measured as described before (Swann and Whitaker, 1986).

Visualization of DNA

Eggs were incubated with 20 μ M Hoechst 33342 (Calbiochem, Nottingham, UK) in ASW for 30 min. After 30 min the dye was removed and the eggs were washed twice with ASW. DNA staining was observed with a Nikon Diaphot fluorescence microscope.

Sources of reagents

All chemicals were of analytical grade and from BDH (Poole, UK) unless otherwise indicated.

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