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The Relationship between Calcium, MAP Kinase, and DNA Synthesis in the Sea Urchin Egg at Fertilization

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Fertilization releases the brake on the cell cycle and the egg completes meiosis and enters into S phase of the mitotic cell cycle. The MAP kinase pathway has been implicated in this process, but the precise role of MAP kinase in meiosis and the first mitotic cell cycle remains unknown and may differ according to species. Unlike the eggs of most animals, sea urchin eggs have completed meiosis prior to fertilization and are arrested at the pronuclear stage. Using both phosphorylation-state-specific antibodies and a MAP kinase activity assay, we observe that MAP kinase is phosphorylated and active in unfertilized sea urchin eggs and then dephosphorylated and inactivated by 15 min postinsemination. Further, Ca^{2+} was both sufficient and necessary for this MAP kinase inactivation. Treatment of eggs with the Ca^{2+} ionophore A23187 caused MAP kinase inactivation and triggered DNA synthesis. When the rise in intracellular Ca^{2+} was inhibited by injection of a chelator, BAPTA or EGTA, the activity of MAP kinase remained high. Finally, inhibition of the MAP kinase signaling pathway by the specific MEK inhibitor PD98059 triggered DNA synthesis in unfertilized eggs. Thus, whenever MAP kinase activity is retained, DNA synthesis is inhibited while inactivation of MAP kinase correlates with initiation of DNA synthesis. © 2000 Academic Press

Key Words: sea urchin; fertilization; MAP kinase; calcium; DNA synthesis.

INTRODUCTION

Mitogen-activated protein kinases (MAP kinases) or extracellular signal-related kinases (ERKs) are regulated through cascades of conserved kinases and have been implicated in a variety of cellular responses (Cobb and Goldsmith, 1995; Robinson and Cobb, 1997; Garrington and Johnson, 1999). In the eggs of most metazoans, MAP kinase becomes activated during oocyte meiotic maturation and inactivated following fertilization (Ruderman, 1993; Sagata, 1996, 1998; Guadagno and Ferrell, 1998; Kishimoto, 1998; Murray, 1998). It has been suggested that one of the major functions of MAP kinase in unfertilized oocytes and eggs is to act as a brake on the cell cycle for the prevention of parthenogenetic development, perhaps functioning at dif-

ferent cell cycle stages, depending on the species (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Picard *et al.,* 1996; Sagata, 1996, 1998; Shibuya *et al.,* 1992; Tachibana *et al.,* 1997; Kishimoto, 1998; Sadler and Ruderman, 1998).

Determining the mechanism by which MAP kinase activity might regulate entry into the first mitotic cell cycle is complicated by the fact that meiosis must be completed first (Sagata, 1996, 1998). Fertilization triggers the first S phase, which often occurs in parallel with maturation (Nomura *et al.,* 1991). Recent work using starfish eggs has attempted to overcome this complication by causing the oocytes to complete meiosis II and then fertilizing the matured oocytes (Abrieu *et al.,* 1997b; Fisher *et al.,* 1998; Picard *et al.,* 1996; Sadler and Ruderman, 1998; Tachibana *et al.,* 1997). Starfish oocytes normally are fertilized prior to completion of meiosis (Meijer and Guerrier, 1984; Kanatani, 1985). Interestingly, when starfish oocytes are fertilized normally (that is, before the first polar body is extruded prior to meiosis II), inactivation of MAP kinase is delayed until after completion of meiosis I, suggesting that the

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fertilization "signal" to inactivate MAP kinase is retained over time (Tachibana *et al.,* 1997). However, if starfish oocytes are exposed to the maturation hormone 1-methyladenine but not fertilized, they complete meiosis and exhibit high levels of MAP kinase activity. But, the stage at which these matured, unfertilized eggs arrest differs depending on the species of starfish (reviewed in Kishimoto, 1998). In *Asterina* species, unfertilized eggs arrest at G1 with elevated levels of MAP kinase activity, which depends on continuous protein synthesis (Sadler and Ruderman, 1998; Tachibana, *et al.,* 1997). Fertilization triggers a decrease in MAP kinase activity and S phase follows, with normal embryogenesis occurring. MAP kinase activity is both sufficient and necessary to prevent DNA synthesis (Sadler and Ruderman, 1998; Tachibana *et al.,* 1997). In contrast, *Marthasterias glacialis* and *Astropecten aranciacus* complete meiosis, duplicate the maternal genome, and then arrest in the G2 phase, even though levels of MAP kinase activity remain high (Picard *et al.,* 1996; Fisher *et al.,* 1998). After fertilization, MAP kinase activity decreases and is followed by M phase. If MAP kinase activity is maintained experimentally (e.g., by expression of constitutively active MAP kinase kinase), there is no transition to M phase. If MAP kinase is inactivated, for example, by expression of the MAP kinase phosphatase Pyst-1, the G2 arrest is released, even in the absence of fertilization (Fisher *et al.,* 1998). So, although it is likely that MAP kinase activity is involved in the prevention of parthenogenesis in both types of starfish, the result of this block has different consequences (see Kishimoto, 1998). The relationship between MAP kinase activity and DNA synthesis may be quite distinct in the two types of starfish eggs. How signal transduction at fertilization actually modulates MAP kinase activity, DNA synthesis, and progression through the cell cycle is not clear.

It is possible that Ca^{2+} could play a role in the regulation of MAP kinase activity since both Ca^{2+} (Whitaker and Patel, 1990; Whitaker and Swann, 1993) and MAP kinase activity (Kishimoto, 1998) have been implicated in cell cycle progression, particularly DNA synthesis. Ca^{2+} has been shown to be sufficient for DNA synthesis in sea urchin eggs (Steinhardt and Epel, 1974), although the mechanism by which this occurs is not known. Inhibition of Ca^{2+} release by microinjection of a dominant-negative PLCγ SH2 domain fusion protein blocks DNA synthesis and also maintains MAP kinase activity in fertilized sea urchin eggs (Carroll *et al.,* 1999). Inhibition of PLCγ, however, also prevents the rise in cytoplasmic pH (Carroll *et al.,* 1999), making it difficult to establish if it is the rise in Ca^{2+}, pH, or both which is prerequisite to MAP kinase inactivation and DNA synthesis.

The sea urchin egg is an excellent model system in which to study the role of MAP kinase in the first cell cycle without the complication of meiosis, since fertilization normally occurs in the G1 (pronuclear) stage. Recently, MAP kinase activity was reported to increase at the first mitosis in sea urchin eggs (Chiri *et al.,* 1998; Philipova and

Whitaker, 1998), similar to what is observed in *Xenopus* eggs (Guadagno and Ferrell, 1998) and suggesting a role for MAP kinase in M phase of the embryonic cell cycle. Chiri *et al.* (1998) found that unfertilized sea urchin (*Paracentrotus lividus*) eggs contain a minimal level of MAP kinase activity that decreases rapidly after fertilization and then is activated again just prior to first mitosis. Their assay was the phosphorylation of a specific MAP kinase substrate, Myc. Using an in-gel myelin basic protein (MBP) kinase assay, Philipova and Whitaker (1998) observed that unfertilized sea urchin eggs (*Lytechinus pictus*) do not contain active MAP kinase but that MAP kinase activity is activated prior to first mitosis. Further, treatment of unfertilized eggs with the Ca^{2+} ionophore A23187 was sufficient to activate MAP kinase (Philipova and Whitaker, 1998). This is an interesting and important observation given that in all other systems studied to date, MAP kinase is active in unfertilized eggs and that this activity appears to be necessary to prevent parthenogenetic development (see above, and review by Kishimoto, 1998).

We therefore have assessed MAP kinase activity in unfertilized and fertilized sea urchin eggs (*Strongylocentrotus purpuratus, L. pictus,* and *L. variegatus*), focusing on the immediate first 30 min after insemination, in an effort to further understand the relationship that MAP kinase activity may have with the prevention of parthenogenetic development. Two different, specific assays for MAP kinase activity were used: (1) the phosphorylation state of MAP kinase on both Thr 202 and Tyr 204, which is a direct indicator of its activity, and (2) the phosphorylation of a specific MAP kinase substrate, Elk-1. We also have investigated the role of Ca^{2+} in regulating MAP kinase phosphorylation, activity, and DNA synthesis. Using the two assays, we find that MAP kinase is active in unfertilized sea urchin eggs and that Ca^{2+} is both necessary and sufficient for its rapid inactivation. Further, inactivation of MAPK is sufficient to trigger DNA synthesis in unfertilized eggs.

MATERIALS AND METHODS

Animals, Gamete Collection, and Fertilization

Adult sea urchins were collected from the Santa Barbara Channel and maintained in open system aquaria at 10°C for *S. purpuratus* and at ambient temperature (~18°C) for *L. pictus*. Adult *L. variegatus* were a gift from Mark Terasaki (Marine Biological Laboratory, Woods Hole, MA) and were maintained in running seawater tables at ambient temperature. Spawning was induced by intracoelomic injection of 0.55 M KCl. For all of the experiments, gametes were used within a few hours after collection. Eggs were collected by inverting the female over a beaker of filtered sea water (FSW; natural seawater was filtered through glass fiber filters and then through 0.22-μm nitrocellulose filters and stored at 10°C). Sperm were pipetted from the gonadopores into Eppendorf tubes and stored on ice. For biochemistry experiments, eggs were washed by gravity settling in large volumes of FSW three times at 10°C followed by dejellying by multiple passage through a Nitex mesh (120 μm for *S. purpuratus* and 210 μm for *L. pictus*). For microin-

jection experiments, eggs were washed and then were passed through 210-μm mesh Nitex to remove debris prior to use. *Lytechinus* spp. were used for microinjection experiments because they are more easily injected than the eggs of *S. purpuratus.*

For fertilization, eggs were resuspended at ~10% (v/v) suspension in 50 ml of FSW. Dry sperm (15 μl) was diluted into 50 ml of FSW/jelly water and added to the eggs with gentle mixing. Fertilization envelopes were observed within 1 min of insemination and a small portion of the fertilized eggs was monitored for proper development.

Ionophore and BAPTA or EGTA Treatment of Eggs

Ionophore treatment of eggs was carried out using eggs in FSW as a 5% (v/v) suspension and final concentration of A23187 was 1 μM. Fertilization envelopes were observed immediately upon addition of A23187. In some experiments, eggs were injected (see below) with 3 mM BAPTA (Molecular Probes, Inc., Eugene, OR) + 0.9 mM CaCl$_2$ to inhibit the increase of intracellular free Ca^{2+} after fertilization. Because of concerns regarding the long-term effect of BAPTA on the sea urchin egg (see below), we also used microinjection of 2 mM EGTA and 1 mM Pipes, pH 6.8, as an alternate way to inhibit the increase of intracellular free Ca^{2+} following fertilization.

Preparation of Samples, SDS–PAGE, and Immunoblots

Samples of egg suspensions were briefly centrifuged (2000g) to collect the eggs. The seawater was removed and the eggs were lysed in a buffer composed of 60 mM β-glycerophosphate, 20 mM Hepes, pH 7.0, 1% NP-40, 150 mM NaCl, 15 mM EGTA, 10 mM Na$_3$VO$_4$ and protease inhibitors (PIC; 10 μM each leupeptin, aprotinin, and benzamidine). After solubilization on ice for 20 min, samples were centrifuged at 13,000g and the soluble material was transferred to a new tube. Protein concentration was determined by the BCA method (Pierce Biochemicals, Inc.) using BSA as a standard.

Proteins were dissolved in Laemmli SDS sample buffer (Laemmli, 1970), heated at 95°C for 5 min, and separated by electrophoresis in 10 or 12% polyacrylamide Tris–glycine SDS gels (see figure legends) followed by transfer to nitrocellulose (Towbin *et al.,* 1979). A monoclonal panERK antibody (No. E17120; Transduction Laboratories) and a polyclonal anti-ERK1 (No. sc-93; Santa Cruz Biotechnology) were used to detect MAP kinase. Both antibodies consistently recognized a protein of ca. M_r 43K and in some instances, a protein of ca. M_r 85K. The p85, ERK-related protein is recognized by many ERK antibodies, but its function is not known; conceivably, the p85 protein may be ERK5 (Kültz, 1998). A rabbit polyclonal anti-phosphoMAP kinase (Thr 202/Tyr 204) antibody (No. 9101; New England BioLabs) and a mouse monoclonal anti-phosphoMAP kinase (Thr 202/Tyr 204) antibody (E10; No. 9106; New England BioLabs) were used to detect phosphorylated MAP kinase. HRP-conjugated anti-mouse IgG was from Cappel, Inc., and HRP-conjugated anti-rabbit IgG was from Transduction Laboratories. All antibodies were used at the concentration recommended by the supplier. Antibody binding was detected using the Super Signal enhanced chemiluminescence kit (Pierce Biochemicals, Inc.). Blots were stripped with 62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS; washed extensively; blocked; and reprobed as indicated in the figure legends.

MAP Kinase Activity Assays

MAP kinase activity was assessed by phosphorylation of the specific substrate, Elk1 (MAP Kinase Assay Kit; New England BioLabs, Inc.; see also Marais *et al.,* 1993; Zinck *et al.,* 1995). Active MAP kinase was immunoprecipitated from 200 μg of NP-40 lysate (see above), and the precipitates were washed three times in NP-40 buffer and once in kinase buffer (25 mM Hepes, pH 7.0, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na$_3$VO$_4$, 10 mM MgCl$_2$, 1 μM PIC). Final resuspension was in 25 μl of kinase buffer with 100 μM ATP. GST–Elk1 fusion protein (1 μg) was added and the sample was incubated at 30°C for 30 min. The reaction was terminated by the addition of Laemmli sample buffer followed by electrophoresis and transfer to nitrocellulose. Blots were probed with anti-phosphoElk1 (Ser 383; Cat. No. 9181; New England BioLabs, Inc.). As a positive control, egg lysates were spiked with 20 ng of active MAP kinase (Erk2; New England BioLabs, Inc.) prior to precipitation. Negative controls included precipitation with protein G–Sepharose alone and a mock immunoprecipitation (no lysate).

Treatment of Eggs with PD98059

Dejellied eggs were washed twice with artificial seawater (ASW; 484 mM NaCl, 10 mM KCl, 11 mM CaCl$_2$–2H$_2$O, 29 mM MgSO$_4$–7H$_2$O, 27 mM MgCl$_2$–6H$_2$O, 2.4 mM NaHCO$_3$, pH 8.0) and used as a 1% suspension in small glass beakers. Varying amounts of the MEK inhibitor PD98059 (Cal Biochem, Inc.) dissolved in DMSO and diluted in ASW were added to the eggs. The eggs were resuspended gently using a paddle every few minutes and samples were removed at various times for analysis. We noticed that when PD98059 was added at greater than 25 μM, a precipitate formed. To quantify the amount of MAP kinase phosphorylation in eggs treated with inhibitor, immunoblots from at least three separate experiments (see figure legends) were scanned (using a PDI 420e scanner) and normalized to the total amount of MAP kinase present on the same blot, which was stripped and reprobed with the pan-ERK antibody.

DNA Synthesis

DNA synthesis was measured using the technique of fluorescent nucleotide incorporation into the newly synthesized DNA (see Carroll *et al.,* 1999 for details). *L. variegatus* eggs were injected at 18–20°C with 1 μM Oregon Green 488-5–dUTP (C-7630; Molecular Probes Inc.) or co-injected with 3 mM BAPTA + 0.9 mM CaCl$_2$ + 1 μM Oregon Green dUTP. Sperm were added 10–60 min after microinjection in the cases in which fertilization was desired. We noted that, over time, the morphology of eggs injected with BAPTA was different from that seen in control eggs (see below). Eggs were visualized using a confocal microscope (MRC600; Bio-Rad Laboratories, Hercules, CA). Fluorescence of Oregon Green dUTP that was not incorporated into DNA was reduced by photobleaching the live eggs. The eggs were positioned so that the region of egg cytoplasm exposed to the photobleaching was at least 20 μm from the nucleus. The confocal laser was set to 100% power. The eggs were scanned on zoom 10, with three sets of 30 passes on the slow scan rate. Images of the eggs before and after photobleaching were taken with laser power set to 10%, zoom 1, and saved to the hard disk. Adobe PhotoShop 4.0 (Adobe Systems, Inc., Seattle, WA) was used to make Fig. 5.

Ca²⁺ Measurements

Eggs were injected with 10 μM Ca^{2+}-green 10-kDa dextran (Molecular Probes) alone, or in combination with BAPTA/Ca, while being held between two coverslips as described by Kiehart (1982). For fertilization experiments, the eggs were positioned in the chambers so that they were within one egg diameter ($\sim$110 μm) of the coverslip edge. This was performed so that sperm access to the eggs was kept relatively constant. For experiments in which PD98059 or A23187 was added, the eggs were positioned at the coverslip edge to minimize the distance the pharmacological compounds had to diffuse to contact the eggs. The eggs were kept at 16–18°C before, during, and for 10 min after microinjection. To measure the Ca^{2+}-green fluorescence of *L. pictus* eggs, the chambers containing the injected eggs were moved to an Olympus BX60 microscope equipped with a photomultiplier. The eggs were fertilized (or treated) by replacing the entire volume of seawater in the chamber with the indicated sperm (or PD98059 or A23187) dilution; the fertilization and fluorescence recording were done at 20–23°C. Total light intensity gathered by the photomultiplier was collected every 200 ms using the MCS Plus system, Microsoft Windows software, Version 1 (EG&G ORTEC, Oak Ridge, TN). MCS Plus data files were converted to Macintosh text files using MCS file conversion software written by Dr. Steven Haddock (Marine Science Institute, UCSB, Santa Barbara, CA). This software is available for download at http://lifesci.ucsb.edu/~haddock. Data were plotted and analyzed using the graphing software Kaleida-Graph Version 3.0.2 (Abelbeck Software).

Microinjection

Quantitative microinjection was performed using mercury-filled micropipets (Hiramoto, 1962; Kiehart, 1982), allowing injection of precisely calculated picoliter volumes into the eggs. Further details of this method are available at http://www2.uchc.edu/~terasaki/injection/index.html. Injection volumes were 3% of the total egg volume. The volume of the *L. pictus* and the *L. variegatus* egg was calculated to be 700 pl based upon a diameter of 110 μm. Unless stated otherwise, eggs were injected with 21 pl (3% of the total egg volume). Injections were made 10–40 min before insemination. The time between injection and stimulation by sperm did not influence the results observed.

RESULTS

MAP Kinase Is Phosphorylated and Active in Unfertilized Eggs and Is Dephosphorylated within 15 min after Sperm Addition

Antibodies (see Materials and Methods) raised against mammalian ERK1 and ERK2 were used to assess sea urchin eggs for the presence of MAP kinase. Using an anti-panERK monoclonal antibody, a protein of ca. M_r 43K was detected in sea urchin eggs (Fig. 1A). A different anti-ERK1 antibody, (which has been used to detect MAP kinase in starfish oocytes; Sadler and Ruderman, 1998), also recognized a protein of ca. M_r 43K (data not shown). This is in good agreement with the predicted M_r (42K and 44K) for ERKs in most species (Keyse, 1998; Kültz, 1998).

In order to assess the phosphorylation state of MAP kinase in eggs, it is common to observe a slight shift in M_r and to use phospho-specific antibodies. We observed that the M_r shift was unreliable in our gel system. Therefore, a monoclonal and a polyclonal antibody specific for the dual phosphorylated (active) form of p42/44 MAP kinase were used. These antibodies recognize MAP kinase only when it is phosphorylated on both of the highly conserved residues Thr 202 and Tyr 204. Both of these phospho-specific MAP kinase antibodies recognized a sea urchin egg protein of ca. M_r 43K (Fig. 1B, lane 1). In order to assess the phosphorylation state of MAP kinase after fertilization, samples were prepared at various times after sperm addition. By 10 min post-sperm addition, the level of phosphorylation clearly had decreased and by 15 min, phosphorylation was barely detectable (Fig. 1B). The total amount of MAP kinase protein in the eggs, however, remained constant (Fig. 1A). Scanning densitometry was used to determine the relative amount of phosphorylation versus total amount of MAP kinase for each time point (Fig. 1C). Although some variation in the precise timing of MAP kinase dephosphorylation was observed (Fig. 1C), a statistically significant reduction was observed consistently by 15 min postinsemination. We also sampled eggs at shorter intervals immediately after sperm addition since rapid and transient changes in MAP kinase could be missed in the longer intervals (Fig. 1D). No significant change in MAP kinase phosphorylation was detected during this early period.

Dephosphorylation of MAP Kinase Correlates with Loss of MAP Kinase Activity

Phosphorylation on Thr 202 and Tyr 204 is required to activate MAP kinase (Keyse, 1998). Although this phosphorylation event is a commonly used indicator of MAP kinase activity, we wanted to ensure that the phosphorylation state of sea urchin egg MAP kinase was correlated with enzymatic activity. We used a MAP kinase activity assay in which MAP kinase was immunoprecipitated and incubated with a specific substrate, Elk-1. Phosphorylation of the substrate was then detected using an antibody specific for the phosphorylated form of Elk-1 (Marais *et al.,* 1993; Zinck *et al.,* 1995). Although Elk-1 also can be phosphorylated by JNK, the MAP kinase antibody is not known to precipitate JNK (New England BioLabs, Inc.) nor were we able to detect active (phosphorylated) JNK in unfertilized sea urchin eggs (data not shown). Therefore, Elk-1 phosphorylation appears to be a good indicator of MAP kinase activity in these assays. Unfertilized eggs contain phosphorylated MAP kinase that is able to phosphorylate the Elk-1 substrate in this assay (Fig. 2A). For each experiment, the phosphorylation state of MAP kinase was determined by immunoblot analysis (data not shown). In unfertilized eggs, the Elk-1 substrate is phosphorylated, coincident with the phosphorylation of MAP kinase (Fig. 2). At 5 min post-sperm addition, there is a marked decrease in phosphorylation of Elk-1, coincident with a decrease in MAP kinase phosphorylation (Fig. 2B). By 20 min postfertilization, when phosphorylated MAP
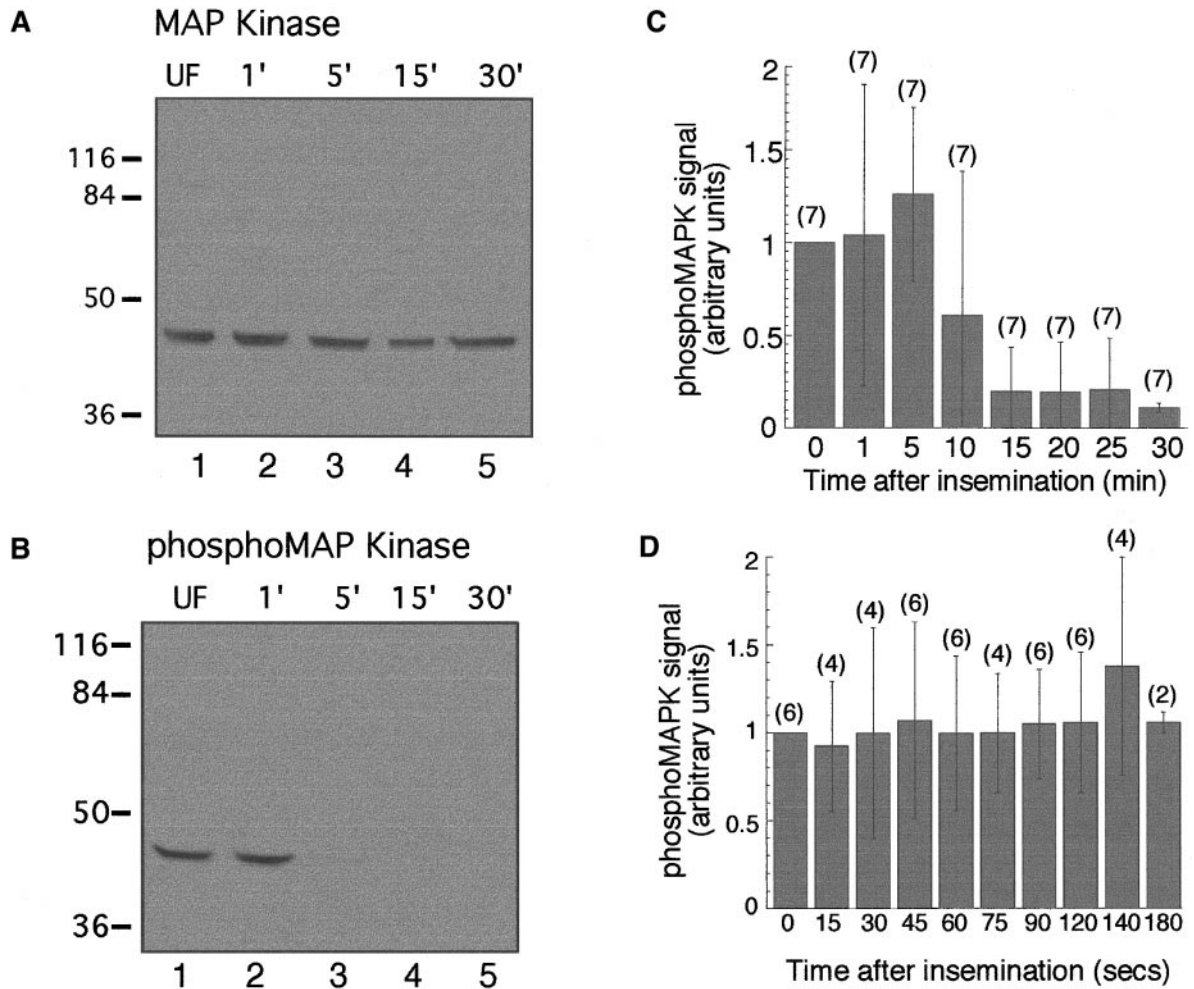
FIG. 1. MAP kinase dephosphorylation following fertilization. Eggs of *S. purpuratus* were lysed before fertilization and at various times after sperm addition as described under Materials and Methods. Fifty micrograms of protein was loaded per lane, electrophoresed through a 10% polyacrylamide–SDS reducing gel, and transferred to nitrocellulose. The blot was probed with a polyclonal antibody specific for phosphorylated MAP kinase (B) and then stripped and reprobed with a monoclonal anti-ERK IgG (A). Antibody binding was detected with goat anti-rabbit Ig (B) or sheep anti-mouse Ig (A) conjugated to HRP followed by enhanced chemiluminescence using Pierce Supersignal. M_r markers are indicated on the left ($\times 1000$ Da). (C) Quantification of MAP kinase dephosphorylation/inactivation at fertilization was determined by scanning immunoblots using a PDI 420e densitometric scanner, normalizing each scan to the total amount of MAP kinase present (as measured by the panERK antibody). Error bars represent the standard deviation. The numbers in parentheses indicate the number of times each time point was sampled (independent experiments). A statistically significant decrease was observed by 15 min post-sperm addition ($P < 0.001$). The same quantification was carried out for experiments using a tighter time course immediately after sperm addition (D). No statistically significant change in MAP kinase phosphorylation was observed ($P > 0.5$). The same results were observed using the monoclonal anti-phosphoMAP kinase IgG. The same results were observed in eggs *of L. pictus* (8 experiments).

kinase is no longer detectable, there is a concomitant loss of activity (Fig. 2). We observed some variation from experiment to experiment, particularly with regard to the precise timing of inactivation (Fig. 2B), but in each case, activity always correlated with the phosphorylation state of MAP kinase. Thus, as expected, the MAP kinase phosphorylation state is related directly to enzymatic activity and serves as a marker for activity in sea urchin eggs.

Calcium Release Is Sufficient and Necessary for MAP Kinase Inactivation

In order to determine if Ca^{2+} is sufficient to inactivate MAP kinase, eggs were treated with the Ca^{2+} ionophore A23187 and at various times, samples were prepared and assessed by SDS–PAGE and immunoblot analysis with the polyclonal and monoclonal phosphoMAP kinase antibodies. By 10 min post-addition of the ionophore, MAP kinase
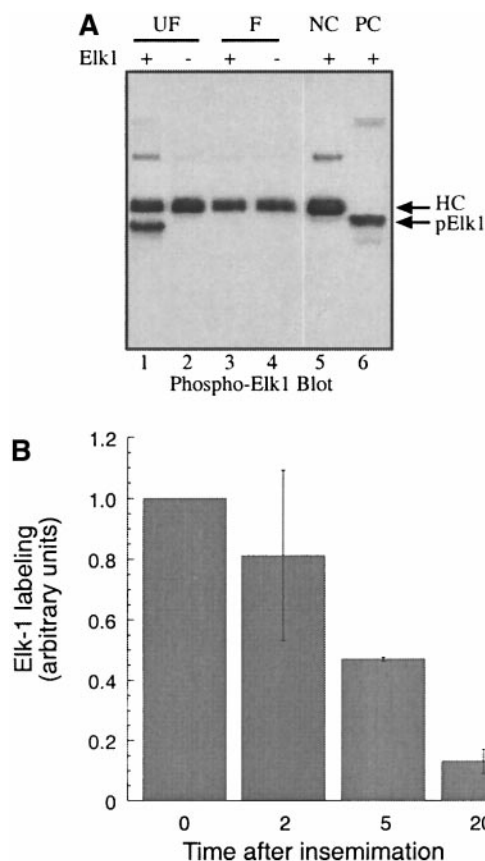
FIG. 2. MAP kinase enzymatic activity decreases after fertilization. Unfertilized (UF) or fertilized (F) *S. purpuratus* eggs (20 min post-sperm addition) were lysed in buffer containing NP-40. Anti-MAP kinase immunoprecipitates were subjected to an *in vitro* kinase assay in the presence (+) and absence (−) of a recombinant GST–Elk-1 fusion protein as substrate (see Materials and Methods). Other controls included a mock immunoprecipitation and kinase assay in the absence of egg lysate (lane 5) and incubation of purified, recombinant active MAP kinase with the substrate (lane 6). The samples were electrophoresed on a 12% polyacrylamide–SDS reducing gel and transferred to nitrocellulose. Phosphorylation of Elk-1 was detected by probing the blot with an antibody specific for phosphoElk-1 and antibody binding was detected with goat anti-rabbit Ig conjugated to HRP followed by enhanced chemiluminescence using Pierce Supersignal. Total soluble protein was assessed by immunoblot analysis for relative amounts of phosphorylated MAP kinase (data not shown) and total MAP kinase protein. M_r markers are indicated on the left ($\times 1000$ Da). Quantification of MAP kinase dephosphorylation/inactivation at fertilization was determined by scanning immunoblots using a PDI 420e densitometric scanner. The labeling of Elk-1 was arbitrarily set to a value of 1 for each experiment and the Elk-1 labeling at the other time points was normalized to this. Error bars represent the standard deviation. The experiment was repeated eight times for *S. purpuratus* and twice for *L. pictus,* with the same results.

was dephosphorylated (Fig. 3A), although the level of MAP kinase protein did not change (data not shown). Parallel experiments assessing MAP kinase activity directly using the Elk-1 phosphorylation assay yielded the same results (data not shown). Eggs treated with the ionophore elevated fertilization envelopes, indicating that Ca^{2+} release had occurred. Thus, Ca^{2+} is sufficient to inactivate MAP kinase.

In order to determine the necessity of Ca^{2+} for MAP kinase inactivation, eggs were injected with a mixture of buffered Ca^{2+} and the Ca^{2+} chelator, BAPTA and then fertilized (see Materials and Methods). The eggs were then removed from the injection chambers and prepared for SDS–PAGE 30–45 min after sperm addition to the chambers. BAPTA-injected eggs did not elevate fertilization envelopes, indicating that an increase in internal Ca^{2+} levels had been prevented (data not shown). Immunoblot analysis revealed that these eggs retained phosphorylated, active MAP kinase (Fig. 3B, lane 4), indicating that internal Ca^{2+} is necessary to inactivate/dephosphorylate MAP kinase. The MAP kinase in control-injected, fertilized eggs was not phosphorylated (Fig. 3B, lane 3). To confirm these results, eggs injected with 2 mM EGTA in 1 mM Pipes, pH 6.8, were also examined for the presence of phosphorylated MAPK before and after fertilization. As with the BAPTA injections, microinjection of EGTA prevented the dephosphorylation of MAPK following fertilization (Fig. 3C, lane 4). These results indicate that a rise in intracellular Ca^{2+} is required for the inactivation of MAP kinase.

Inactivation of MAP Kinase by the Specific Inhibitor PD98509

The above data indicate that Ca^{2+} is both necessary and sufficient to inactivate MAP kinase in sea urchin eggs. However, the mechanism of how Ca^{2+} exerts its effects is unknown. There are at least two basic models. One is that Ca^{2+} activates a phosphatase which inactivates MAP kinase either directly or indirectly. The other is that Ca^{2+} inactivates (directly or indirectly) an activator of MAP kinase, such as MEK (MAP kinase kinase) which is "on" in unfertilized eggs. It is also possible that both models could be operating (Keyse, 1998).

To begin to investigate the control of MAP kinase activity, eggs were incubated with the MEK inhibitor PD98059 as a means to inactivate MAP kinase (Cohen, 1997; Dudley *et al.,* 1995; Pang *et al.,* 1995). In eggs that were injected with EGTA and then subjected to PD98059 treatment, MAP kinase was inactivated (Fig. 3C, lane 6). This indicates that EGTA does not block the inhibitory function of PD98059 and suggests that in unfertilized eggs, there is maintenance of active MAP kinase via a kinase cascade.

To fully characterize the effect of PD98059 on sea urchin egg MAP kinase, concentration-dependence and time-course experiments were conducted. Eggs were soaked in various concentrations of PD98059 in artificial seawater. After 30 min, eggs were collected by centrifugation, lysed, and subjected to immunoblot analysis (Fig. 4A). Even at the
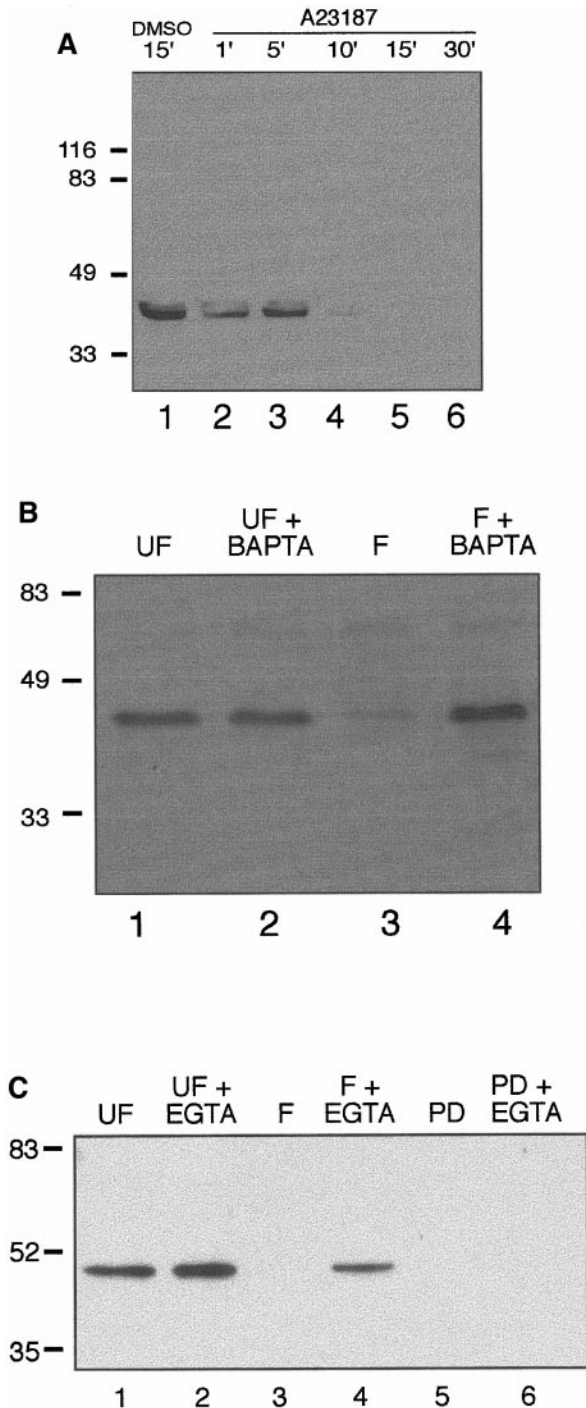
lowest PD98059 concentrations used (0.075 μM), a slight decrease in MAP kinase phosphorylation was observed. However, eggs treated with the vehicle (DMSO) alone maintained the same level of MAP kinase phosphorylation as untreated eggs (Fig. 4A). The inhibitor had no effect on the levels of MAP kinase protein, which was determined by stripping and reprobing the blot with the panERK antibody (data not shown). Using PD98509 at 2.5 μM, a time course experiment was conducted to investigate the kinetics of MAP kinase inactivation (Fig. 4B). Eggs were incubated in the inhibitor and samples were removed at various times and analyzed by immunoblotting. Within 10 min of application of the inhibitor to unfertilized eggs, a decrease in MAP kinase activity was detected (Fig. 4B). Thus, inactivation of MEK leads to rapid inactivation of MAP kinase in unfertilized eggs, again indicating maintenance of MAP kinase activity in eggs.

MAP Kinase Activity, Calcium, and DNA Synthesis

Previous work using eggs of the sea star (*Asterina* sp.) has suggested that MAP kinase activity acts to prevent DNA synthesis in unfertilized eggs (Tachibana *et al.,* 1997; Sadler and Ruderman, 1998). Also, inhibition of PLCγ activity (and thus the rise in Ca^{2+} and pH) at fertilization blocks MAP kinase dephosphorylation and DNA synthesis in sea urchin eggs (Carroll *et al.,* 1999). These data are in contrast to those of Philipova and Whitaker (1998), who observed that MAP kinase was not active (determined using a MBP kinase

2 and 4) or buffer (lanes 1 and 3). 10–30 min later, sperm were added to the eggs (F, fertilized) (lanes 3 and 4). At 30 min after sperm addition, the eggs were collected from the chambers, dissolved in sample buffer, electrophoresed through a 10% polyacrylamide–SDS reducing gel, and transferred to nitrocellulose. (C) Unfertilized eggs (5/lane) were injected with the calcium chelator EGTA (lanes 2, 4, and 6) or left uninjected (lanes 1, 3, and 5). 10–30 min later, sperm (lanes 3 and 4; F), or 10 μM PD98059 (lanes 5 and 6; PD), was added to the eggs. At 15 min after sperm or PD98059 addition, the eggs were collected from the chambers, dissolved in sample buffer, electrophoresed through a 10% polyacrylamide–SDS reducing gel, and transferred to nitrocellulose. (A, B, and C) The blots were probed with a polyclonal antibody specific for phosphorylated MAP kinase. The same results were obtained using the monoclonal anti-phosphoMAP kinase antibody (data not shown). Antibody binding was detected with goat anti-rabbit Ig conjugated to HRP followed by enhanced chemiluminescence using Pierce Supersignal. M_r markers are indicated on the left ($\times$1000 Da). The blots in A and B were stripped and reprobed with the panERK antibody to ensure that equivalent amounts of MAP kinase were present (data not shown). The ionophore experiment (A) was repeated six times for *S. purpuratus* and four times for *L. pictus,* with the same results. The BAPTA experiments (B) were repeated three times for *L. pictus,* with the same results. The EGTA experiments (C) were repeated twice for *L. pictus* with the same results.
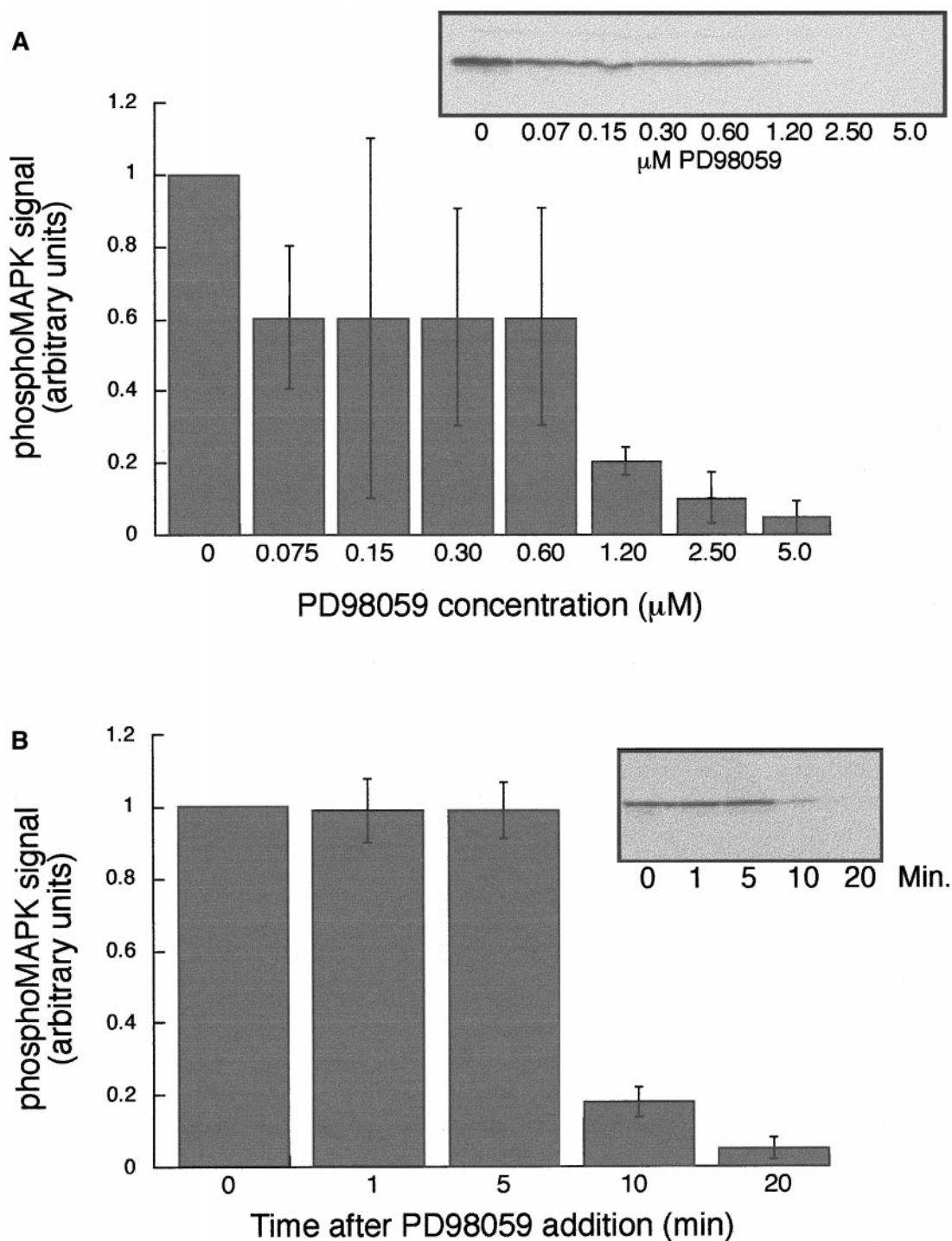
FIG. 3. Calcium is necessary and sufficient for inactivation of MAP kinase at fertilization. (A) Unfertilized eggs were treated with DMSO alone or with the calcium ionophore A23187 (1 μM) for various times. Eggs were washed and lysed and the protein concentration was determined. Fifty micrograms of protein was loaded per lane, electrophoresed through a 10% polyacrylamide–SDS reducing gel, and transferred to nitrocellulose. (B) Unfertilized (UF) eggs (25–28/lane) were injected with the calcium chelator BAPTA (lanes

FIG. 4. Inactivation of MAP kinase in unfertilized eggs by treatment with a specific MEK inhibitor. (A) Unfertilized eggs were incubated with DMSO alone or with various amounts of the MEK inhibitor PD98059. After 30 min, eggs were washed and lysed and the protein concentration was determined. (B) Unfertilized eggs were incubated with 2.5 μM PD98059 for the times indicated. The eggs were then washed and lysed and the protein concentration was determined. Fifty micrograms of protein was loaded per lane and electrophoresed through a 10% polyacrylamide–SDS reducing gel and transferred to nitrocellulose. The blots (insets) were probed with a polyclonal antibody specific for phosphorylated MAP kinase. Antibody binding was detected with goat anti-rabbit Ig conjugated to HRP followed by enhanced chemiluminescence using Pierce Supersignal. Both blots were stripped and reprobed with the panERK antibody to ensure that equivalent

assay) in unfertilized sea urchin eggs but was stimulated by Ca^{2+} release. Therefore, we sought to investigate further the relationship between Ca^{2+}, MAP kinase activity, and DNA synthesis in sea urchin eggs. To measure DNA synthesis, we monitored the incorporation of a fluorescent nucleotide, Oregon Green dUTP, into the newly synthesized DNA (see Materials and Methods). First, we assessed whether inactivation of MAP kinase by PD98059 led to DNA synthesis. Control eggs underwent DNA synthesis by 47 min postinsemination (Figs. 5A and 5B, Table 1). Unfertilized eggs treated with 10 μM PD98059 also underwent DNA synthesis by 50 min (Fig. 5C, Table 1). Thus, inhibition of MAP kinase activity via the specific MEK inhibitor PD98059 leads to entry into S phase, even in the absence of fertilization.

To determine if the MEK inhibitor had any effect on Ca^{2+} release in eggs, we monitored Ca^{2+} in unfertilized eggs that had been injected with Ca^{2+}-green dextran and incubated in PD98059. No detectable rise in Ca^{2+} was observed (Fig. 6A). Eggs that had been soaked in the inhibitor were still able to be fertilized and showed a normal Ca^{2+} release in response to sperm (Fig. 6A).

We also confirmed that treatment of unfertilized sea urchin eggs with the calcium ionophore A23187 leads to DNA synthesis, under the conditions we have used, as has been shown by Steinhardt and Epel (1974). DNA synthesis occurred within 46 min of ionophore addition to unfertilized eggs (Fig. 5D, Table 1). Neither untreated eggs nor eggs treated with the vehicle alone (DMSO) underwent DNA synthesis (Table 1). Thus, Ca^{2+} is sufficient to both inactivate MAP kinase (Fig. 3A) and trigger S phase.

Because Ca^{2+} also is necessary for the inactivation of MAP kinase (Figs. 3B and 3C), we next investigated the effects on DNA synthesis of blocking the rise of internal Ca^{2+} at fertilization with BAPTA. Eggs were microinjected with a mixture of buffered Ca^{2+}/BAPTA and then fertilized (under these conditions, MAP kinase remains active; Figs. 3B and 3C). These eggs did not exhibit DNA synthesis (Fig. 5E). Multiple fertilization cones indicated that sperm had entered these eggs, ruling out the possibility that they were not fertilized (data not shown). Control-injected eggs that were fertilized underwent normal Ca^{2+} release, as judged by the Ca^{2+} release profile (Fig. 6B) and fertilization envelope elevation, and synthesized DNA. Using Ca^{2+}-green dextran to monitor Ca^{2+} release revealed that BAPTA did indeed inhibit the rise in Ca^{2+} at fertilization (Fig. 6B). However, we were concerned about the effect that BAPTA may have on the overall health of sea urchin eggs. As mentioned above, we noted that the eggs microinjected with BAPTA exhibited an altered morphology. As shown in Fig. 5E, the
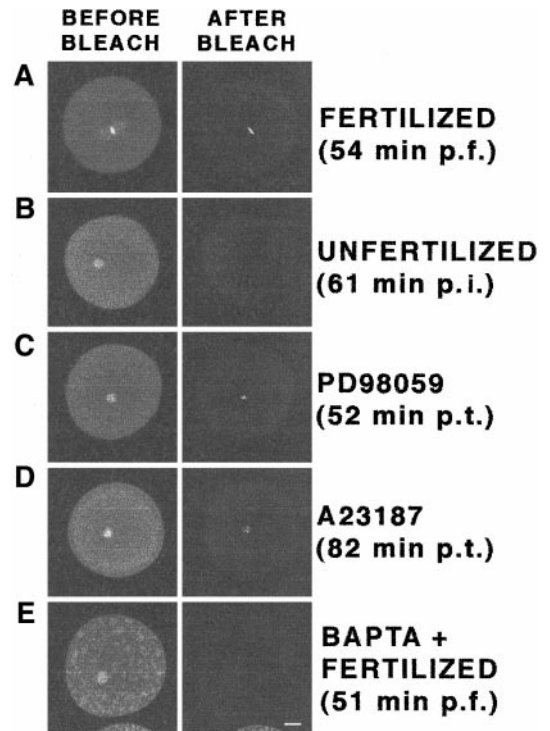

FIG. 5. Both calcium and MAP kinase are upstream regulators of DNA synthesis. *L. variegatus* eggs that were injected with the fluorescent nucleotide OG-dUTP were subjected to various treatments and DNA synthesis was monitored by the incorporation of the OG-dUTP into DNA (see Materials and Methods). The micrographs shown depict representative eggs at various times postincubation (p.i.), post-sperm addition (p.f.), or other treatment (p.t.). See Table 1 for a summary of the DNA synthesis data. Fertilized eggs (A) underwent DNA synthesis while control, unfertilized eggs (B) did not. Eggs in which MAP kinase was inactivated via incubation in 20 μM MEK inhibitor PD98059 (C) or by Ca ionophore (1 μM) treatment (D) underwent DNA synthesis in the absence of fertilization. Eggs injected with BAPTA and then fertilized (E) did not undergo DNA synthesis. Scale bar, 20 μm.

OG-dUTP was not evenly distributed throughout the cytoplasm of the BAPTA-injected eggs; rather the dye gathered in large (5–10 μm) patches that began to appear between 20 and 30 min following microinjection. BAPTA commonly is used for the study of free Ca^{2+} regulation in many cell types, but its suitability for long-term use in eggs has not been examined carefully. Therefore, although these results suggest that the intracellular Ca^{2+} increase is necessary for DNA synthesis to occur, we cannot state this with cer-

amounts of MAP kinase were present (data not shown). Quantification of MAP kinase dephosphorylation/inactivation in response to PD98059 was determined by scanning immunoblots ($n = 4$) using a PDI 420e densitometric scanner, normalizing each scan to the total amount of MAP kinase present (as measured by the panERK antibody). Error bars represent the standard deviation.

Treatment[a]	Ratio of eggs with DNA synthesis	Time observed after treatment (min)[b]	No. eggs, No. animals
Sperm	7/7	54–70	7, 3
1 µM A23187	11/12	46–92	12, 2
20 µM PD98059	8/9	50–132	9, 2
Unfertilized	0/11	52–76	11, 2

[a] See Materials and Methods for conditions.
[b] DNA synthesis was monitored by incorporation of OG-dUTP. The time indicated refers to the time elapsed between sperm addition or initiation of the treatment and the time of observation of OG-dUTP incorporation.

tainty until new methods are devised to buffer intracellular free Ca^{2+} in eggs.

DISCUSSION

The results presented here support the hypothesis that MAP kinase activity is acting as a brake on the cell cycle in unfertilized eggs, presumably as a block to parthenogenetic activation (Sagata, 1996; Kishimoto, 1998). As in the starfish *Asterina pectinifera* (Tachibana *et al.,* 1997) and *A. miniata* (Sadler and Ruderman, 1998), MAP kinase activity is necessary to prevent DNA synthesis in haploid sea urchin eggs (*S. purpuratus, L. variegatus,* and *L. pictus*). Further, the data suggest that Ca^{2+} release at fertilization plays a role in regulating MAP kinase activity and thus DNA synthesis in sea urchin eggs. These eggs contain phosphorylated, active MAP kinase and Ca^{2+} is both necessary and sufficient to inactivate MAP kinase. Further, Ca^{2+} is sufficient and, as the BAPTA experiments suggest, necessary for DNA synthesis. Finally, inactivation of MAP kinase by the MEK inhibitor PD98059 stimulated DNA synthesis in unfertilized eggs. Together, these data provide evidence in support of the model (Sagata, 1996; Kishimoto, 1998) that calcium release at fertilization impinges upon MAP kinase activity and that MAP kinase activity regulates DNA synthesis, either directly or indirectly.

Using specific antibodies, we have detected a MAP kinase isoform in sea urchin eggs which may correspond to the "myelin basic protein kinase" activity previously described in unfertilized eggs of *S. purpuratus* (Pelech *et al.,* 1988). Using the MBP activity assay, Pelech *et al.* (1988) established that the kinase was inactivated by 5 min postfertilization and that activity resumed just prior to the first cell division. Recently, others have found that MAP kinase activity increases at the first mitosis in sea urchin eggs (Chiri *et al.,* 1998; Philipova and Whitaker, 1998). We have not looked carefully at MAP kinase activity during the first mitosis (roughly 80–90 min postinsemination), but our

results do indicate that MAP kinase activity is present in the unfertilized egg and that calcium is both necessary and sufficient to inactivate MAP kinase after fertilization. This is in contrast to the results of Philipova and Whitaker (1998); using an in-gel MBP kinase assay, they observed that sea urchin eggs contained very little MBP kinase activity and that fertilization and ionophore treatment rapidly
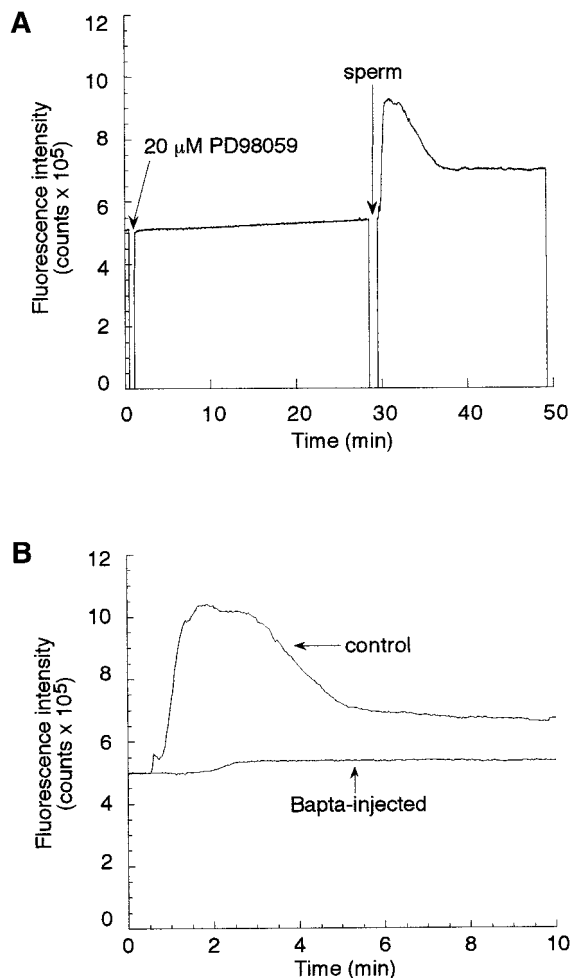

FIG. 6. Calcium release in eggs treated with PD98059 and in eggs injected with BAPTA. (A) Unfertilized *L. pictus* eggs were soaked in 20 µM PD98059 after being injected with Ca^{2+}-green dextran. After 30 min of recording, sperm were added to the eggs. A representative recording is shown. Normal calcium release was observed. The eggs elevated fertilization envelopes and went on to divide (not shown). (B) Unfertilized *L. pictus* eggs were either injected with Ca^{2+}-green dextran only (control) or co-injected with BAPTA/Ca^{2+} and Ca^{2+}-green dextran (BAPTA-injected). After 10 min, sperm were added to the chamber. A representative recording is shown for each. The peak amplitude of the intracellular Ca^{2+} increase (which represents the change in CaG fluorescence after fertilization) was 1.19 ± 0.1 for control eggs ($n = 3$) and 0.05 ± 0.04 for BAPTA-injected eggs ($n = 4$).

stimulated this activity. This inconsistency may be due to differences in the antibodies used to precipitate MAP kinase and in the difference in the assay used to measure MAP kinase activity. Although the MBP phosphorylation assay is widely accepted as an indicator of MAP kinase activity, other kinases can utilize MBP as a substrate (Philipova and Whitaker, 1998; Cohen, 1997), whereas the Elk-1 and Myc phosphorylation assays are thought to be more specific for MAP kinase activity (Cohen, 1997; Zinck *et al.,* 1995). It is possible that ionophore treatment stimulates some other kinase which is capable of phosphorylating MBP. Using phosphorylation of the specific MAP kinase substrate Myc to assess MAP kinase activity in *P. lividus* sea urchin eggs, Chiri *et al.* (1998) found that unfertilized eggs do contain some level of MAP kinase activity, which decreases immediately after fertilization, in agreement with our findings.

As in the eggs of the starfish *A. pectinifera* (Tachibana *et al.,* 1997) and *A. miniata* (Sadler and Ruderman, 1998), our results show that MAP kinase activity appears to correlate with suppression of DNA synthesis in sea urchin eggs. Inactivation of MAP kinase, even in the absence of fertilization, leads to DNA synthesis in sea urchin eggs (Fig. 5, Table 1). This finding is in contrast to eggs of other starfish species (*A. aranciacus* and *M. glacialis*), which maintain MAP kinase activity after meiosis II yet undergo DNA synthesis in the absence of fertilization and then arrest in the G2 stage of the first cell cycle (Picard *et al.,* 1996; Fisher *et al.,* 1998). This difference may reflect the diversity of function that has evolved for MAP kinase signaling (Fisher *et al.,* 1998; Kishimoto, 1998; Murray, 1998; Sadler and Ruderman, 1998; Sagata, 1998). Despite the differences in terms of the stage of cell cycle arrest, the common aspect is that unfertilized eggs do arrest, probably as a way to prevent parthenogenetic activation (Kishimoto, 1998; Sagata, 1996, 1998; Murray, 1998).

It has been known for some time that Ca^{2+} is sufficient to trigger DNA synthesis (Steinhardt and Epel, 1974). Here, we show that Ca^{2+} also is sufficient to inactivate MAP kinase (as well as stimulate DNA synthesis) and, further, that Ca^{2+} is necessary for MAP kinase inactivation. It therefore seems likely that Ca^{2+} somehow causes the inactivation of MAP kinase in the fertilized egg, thus "releasing the brake." Presumably, the MEK inhibitor PD98059 initiates DNA synthesis in unfertilized eggs by bypassing the need for Ca^{2+}. DNA synthesis in the absence of fertilization also was observed in starfish eggs that had been allowed to complete meiosis and then were exposed to PD98059 (Sadler and Ruderman, 1998). The sea urchin egg appears to be more sensitive to this inhibitor; 1–5 μM was sufficient to inhibit MAP kinase completely in sea urchin eggs (Fig. 4A) while a minimum of 50 μM was required to inhibit MAP kinase activity in starfish eggs (Sadler and Ruderman, 1998). Because it is difficult to determine how much inhibitor is taken up into the quiescent sea urchin egg, a precise calculation of the IC50 is difficult. In mammalian cells, the IC50 value of this inhibitor is roughly 4–10 μM, depending

on the cell type and conditions (Dudley *et al.,* 1995; Pang *et al.,* 1995). The inhibitor, which is a flavone compound, is thought to work by binding to inactive forms of MAP kinase kinase at a site which blocks access to activating enzymes (Dudley *et al.,* 1995; Pang *et al.,* 1995; Cohen, 1997). Its effect thus is exerted upstream of MAP kinase in a kinase signaling cascade. This suggests that in unfertilized sea urchin eggs, there is a MAP kinase kinase that is actively maintaining the phosphorylated, active state of MAP kinase.

Although inhibiting a MAP kinase kinase (presumably a MEK1 family member) appears to down-regulate MAP kinase rapidly in unfertilized eggs, it is likely that the activation of a phosphatase at fertilization plays a role as well; it is thought that Type 2 serine/threonine phosphatases are major regulators of MAP kinase pathways (Ruderman, 1993; Keyse, 1998). In addition, a new class of threonine/tyrosine dual-specificity phosphatases, the MAP kinase phosphatases or MKPs, have been described in mammalian cells, *Drosophila,* and yeast (reviewed in Keyse, 1998). The activity of one of these MKPs, Pyst-1, is sufficient to drive unfertilized, mature *Xenopus* eggs (Abrieu *et al.,* 1997a,b) and starfish (*M. glacialis* and *A. aranciacus*) eggs (Fisher *et al.,* 1998) into first mitosis. The regulation of MKPs is not well understood (Keyse, 1998).

As in other systems (reviewed in Waskiewicz and Cooper, 1995), perhaps there is a balance between a kinase and phosphatase activity(s) that maintains MAP kinase phosphorylation in unfertilized eggs. Although Ca^{2+} is necessary and sufficient for MAP kinase inactivation at fertilization (Fig. 3), the observation that PD98059 treatment can overcome the chelator block (Fig. 3C) suggests that MAP kinase activity is being actively maintained in eggs. A working model is that active maintenance of MAP kinase activity is occurring through a kinase signaling cascade in unfertilized eggs, and then at fertilization, a Ca^{2+}-dependent inactivation occurs, perhaps mediated by a Ca^{2+}-dependent phosphatase.

Phosphatase activity has been implicated in a number of fertilization events. Okadaic acid, which inhibits type 1 and 2A phosphatases (Bialojan and Takai, 1988; Hardie *et al.,* 1998) prevents pronuclear fusion, microtubule dynamics, and cell division in sea urchin eggs at fertilization (Wright and Schatten, 1995). Interestingly, an okadaic acid-sensitive phosphatase(s) activity appears to be responsible for the dephosphorylation of two proteins of ca. M_r 40K and 42K by 15 min postfertilization (Wright and Schatten, 1995); one of these may be MAP kinase. Treatment of *Xenopus* eggs with the tyrosine phosphatase inhibitor vanadate inhibits egg activation and prevents the dephosphorylation of p42 MAP kinase (Sato *et al.,* 1998). It will be interesting to test whether phosphatase inhibitors specifically block DNA synthesis as well.

Because sea urchin eggs naturally are arrested in the pronuclear stage prior to fertilization and because they can be isolated in large quantities and synchronously fertilized, they offer a good model system for studying the relationship

between MAP kinase and DNA synthesis. Our data indicate that calcium-mediated inactivation of MAP kinase in sea urchin eggs at fertilization leads to DNA synthesis. Zhang and Ruderman (1993) demonstrated that unfertilized sea urchin egg extracts are deficient in DNA synthesis initiation, but not elongation, activity. Taken together with the data presented in this paper, it is plausible that potential MAP kinase targets in unfertilized eggs are proteins involved in the initiation of DNA replication.

ACKNOWLEDGMENTS

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