Inactivation of MAPK in mature oocytes triggers progression into mitosis via a Ca^{2+}-dependent pathway but without completion of S phase

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Summary

Unfertilized sea urchin eggs that are arrested at G1 phase after completion of meiosis contain a highly phosphorylated mitogen-activated protein (MAP) kinase (MAPK), the ERK-like protein (ERK-LP). Several data including our previous results show that ERK-LP is inactivated after fertilization, which agrees with results obtained in other species including *Xenopus*, starfish and mammals. The question is to elucidate the function of a high MAPK activity in sea urchin eggs. We report here that dephosphorylation of ERK-LP with very low concentrations of two MEK inhibitors, PD98059 or U0126, triggers entry into mitosis. Under these conditions, recurrent oscillations of the phosphorylation of ERK-LP and of a tyrosine residue in Cdc2 occur, and the intracellular Ca^{2+} level (Ca^{2+}_i) progressively and slowly increases. Nuclear envelope breakdown and all mitotic events initiated after dephosphorylation of ERK-LP are inhibited when changes in Ca^{2+}_i are prevented; however, they are independent of the intracellular pH. These results suggest that inactivation of a MEK-ERK pathway, normally induced after fertilization of sea urchin eggs, triggers entry into mitosis by altering Ca^{2+}_i but cannot trigger full DNA replication. We discuss the hypothesis that neither inactivation nor activation of a MEK-ERK pathway is required for S phase completion in sea urchin egg.

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Key words: MAP kinase, Egg, Oocyte, Sea urchin, ERK, Mitosis, Calcium, MPF, Fertilization

Introduction

The mitogen-activated protein (MAP) kinase (MAPK) family member ERK2 (extracellular regulated kinase 2) is well known to control meiotic maturation in oocytes (Abrieu et al., 2001). In eggs of starfish (Tachibana et al., 2000; Kishimoto, 2004) and *Xenopus* (Tunquist and Maller, 2003), fertilization results in the inactivation of this MAPK. High levels of activated MAPK prevent eggs from entering a program of mitotic divisions, as well demonstrated in starfish (Tachibana et al., 2000). ERK activity in unfertilized oocytes has also been suggested to prevent DNA synthesis but results obtained in starfish seem to contradict this hypothesis. In oocytes of the starfish *A. pectinifera*, where fertilization occurs at G1 after meiosis, ERK inactivation has been correlated with stimulation of DNA synthesis (Tachibana et al., 1997; Tachibana et al., 2000). On the contrary, in oocytes of the starfish *M. glacialis* and *A. aranciacus*, MAPK inactivation was neither required for premature DNA replication after the first meiotic cell cycle nor for DNA replication after completion of meiotic maturation (Picard et al., 1996). In those species, the calcium ionophore A23187 could inactivate ERK in oocytes arrested at G2 without inducing DNA synthesis (Fisher et al., 1998).

What is the situation in sea urchin eggs fertilized after completion of meiosis, in G1 phase? Our previous data (Chiri et al., 1998; Zhang et al., 2005) and those reported by others (Carroll et al., 2000; Kumano et al., 2001) strongly suggest that, at this stage, unfertilized eggs contain a highly active ERK-like protein (ERK-LP) that is inactivated after fertilization. Inactivation of a Ca^{2+}-sensitive MAP kinase kinase (MEK) pathway has been proposed to inactivate ERK-LP at fertilization (Kumano et al., 2001). This situation looks similar to that described for the starfish *A. pectinifera* because Carroll et al. also showed that treatment of eggs with the specific MEK inhibitor PD98059 triggers initiation of DNA synthesis (Carroll et al., 2000). However, one group found opposite results; Philipova and Whitaker reported that unfertilized sea urchin eggs do not contain active ERK (Philipova and Whitaker, 1998). Rather, fertilization after the activation of ERK-LP is required for S-phase onset and cell-cycle progression, whereas inactivation of this MAPK by XCL100 (a MAPK phosphatase) or the MEK inhibitor U0126 does not induce DNA synthesis in unfertilized eggs (Philipova et al., 2005a). Whether DNA synthesis depends on ERK inactivation at G1 and how the MEK-ERK cascade is involved in cell-cycle progression after fertilization of sea urchin eggs are then two important issues that need to be clarified.

We report here the behavior of unfertilized eggs in which ERK-LP dephosphorylation is induced with U0126 or PD98059 (Davies et al., 2000). These eggs showed microtubule polymerization, chromatin condensation and nuclear envelope breakdown (NEB), which corresponds to entry into mitosis. However, no division and no real development were observed. Since a transient increase in intracellular free Ca^{2+} (Ca^{2+}_i), triggers entry in mitosis in sea urchin embryos (Wilding et al., 1996), we tested whether inactivation of ERK-LP leads to changes in Ca^{2+}_i. We found that M-phase entry is indeed inhibited in eggs deprived of phosphorylated ERK-LP after injection of EGTA. Altogether, our results suggest that inhibition of the MEK-ERK pathway in unfertilized sea urchin eggs generates M-phase entry and recurrent oscillations of the M-phase-promoting factor (MPF) by altering the Ca^{2+}_i level, but does not induce complete DNA replication.

Results

Inactivation of ERK-LP triggers mitotic events but not embryogenesis

We first investigated whether inactivation of the highly phosphorylated ERK-LP present in unfertilized sea urchin eggs can drive eggs into early parthenogenesis, as it is the case in starfish oocytes arrested in meiosis I (MI) expressing an antisense-Mos (Tachibana et al., 2000). We hypothesized that the situation is different in these two species, because unfertilized sea urchin eggs arrested at G1 do not contain any centrosome, whereas MI-arrested starfish oocytes contain two centrosomes (Schatten, 1994; Kato et al., 1990).

Treatment with the MEK inhibitors U0126 or PD98059 induced changes in the general morphology of unfertilized eggs. Observation under a light microscope showed that eggs of the sea urchin *P. lividus* that had been treated with 1-2 μM U0126 or with 2.5-5 μM PD98059 underwent NEB (93±3%; n=13 experiments or 50±5%; n=15 experiments, respectively) 3 hours after administration of the drugs (Fig. 1A, top panels), indicating entry into mitosis. At this time, 5-20% and 7-25% of eggs, depending on the batch of eggs treated with UO126 and PD98059, respectively, showed figures of constriction and blisters more or less accentuated (Fig. 1A, top panels). Very similar results were obtained using another sea urchin species *L. pictus* (Fig. 1A, bottom panels). Changes in morphology took longer to occur when concentrations of MEK inhibitor higher than 50 μM were used (not shown). Although a few eggs showed attempts of division, complete cytokinesis was never observed and parthenogenesis did never occur. Finally, some *P. lividus* eggs treated with 2.5 μM PD98059 (four out of 15 batches) or with 1 μM U0126 in (9 out of 13 batches) showed a very thin fertilization membrane (5-10% or 4-28%, respectively) (Fig. 1A, top panels). Similar results were obtained in *L. pictus* eggs (Fig. 1A, bottom panels).

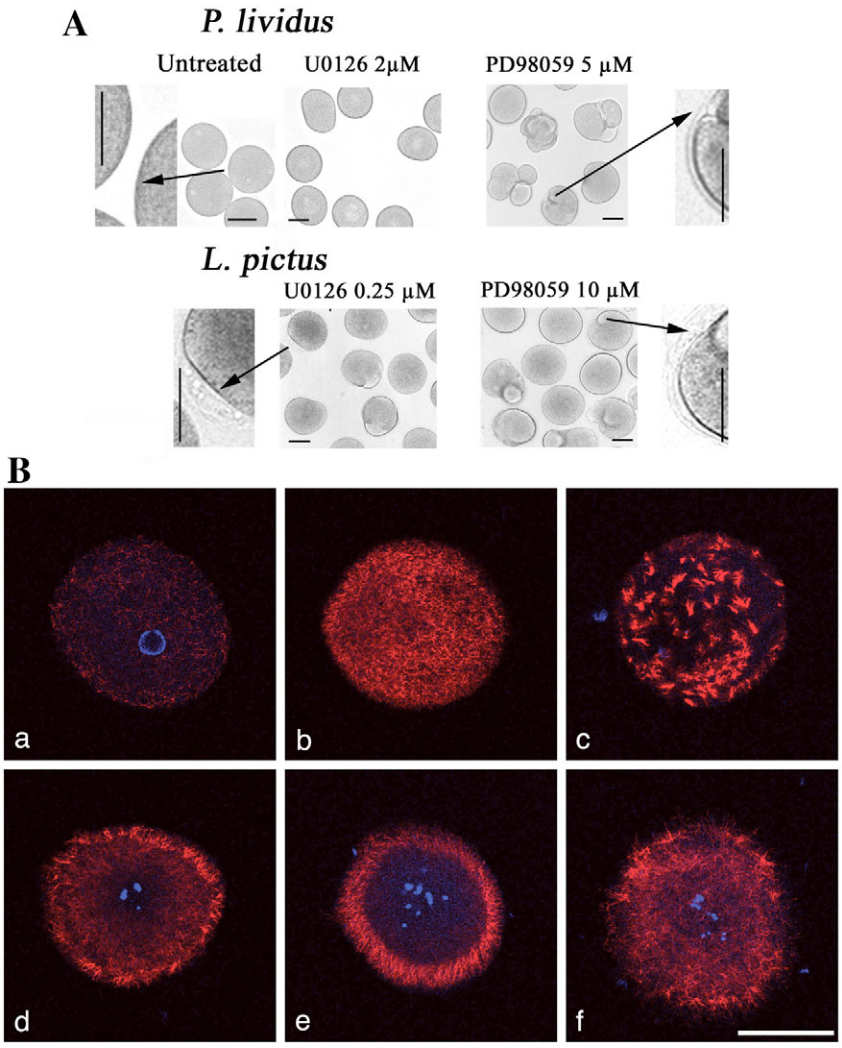

Fig. 1. Effect of two MEK inhibitors on egg morphology. (A) Light-microscopy images of changes in the general morphology of eggs from two sea urchin species, *P. lividus* (top) and *L. pictus* (bottom), induced after a 3-hour treatment with U0126 or PD98059. Untreated eggs showed a clear nucleus and no apparent fertilization membrane (upper left inset), whereas figures of constrictions and blisters, NEB and a thin fertilization membrane for a few eggs (lower left and right insets) were induced in treated eggs of both species. Bars, 50 μm. (B) Confocal microscopy images of microtubule polymerization and chromatin condensation induced in unfertilized eggs of *P. lividus* after treatment with 1 μM U0126. Chromatin was labeled with Hoechst 123 (blue) and microtubules were labeled with an anti-tubulin antibody (red). Eggs were observed (a) before treatment with U0126 or (b) 30 minutes, (c) 1 hour, (d) 2 hours, (e) 3 hours or (f) 4 hours afterwards. Bar, 50 μm. Similar results were obtained in the presence of PD98059 (supplementary material Fig. S1, Table S1).

P. lividus eggs treated or not with 1 μM U0126 (Fig. 1B) and fixed at different times for microtubule and chromatin staining showed the following kinetics of events: A fast microtubule polymerization step took place in the cortical area within 30 minutes of treatment (Fig. 1Bb) that later led to formation of arrow-shaped bundles generated from several points of 'nucleation' at the periphery of the egg (Fig. 1Bc). Microtubule polymerization then continued, showing fewer

bundles but a deeper and denser network that either remained cortical (Fig. 1Bd,e) or was both cortical and localized at the periphery of condensed chromatin (Fig. 1Bd,f). Chromatin progressively condensed, until individualization of chromosomes within 2 hours of treatment (Fig. 1Bd). Chromosomes were then dispersed and microtubules entered the nuclear space, indicating that NEB had occurred between 2 and 3 hours of treatment (Fig. 1Be,f). Even though some kind of chromosome attachment to microtubules – as well as a number of bipolar spindles – was occasionally observed, we never observed a real metaphase spindle that led to anaphase entry. Very similar results were obtained with eggs treated with 2.5 μM PD98059 (supplementary material Fig. S1, Table S1).

Several conclusions can be drawn from these results. (1) Unfertilized eggs deprived of MEK activity enter mitosis. (2) In contrast to starfish oocytes arrested in meiosis I, early development cannot be induced in G1-arrested sea urchin eggs by only inactivating MEK. (3) Elevation of a thin fertilization envelope in some eggs that had been treated with MEK inhibitor suggests changes in Ca^{2+}_i levels, because cortical exocytosis is Ca^{2+}-dependent (Zimmerberg et al., 1999).

Inactivation of MAPK does not induce full DNA replication

Since unfertilized sea urchin eggs whose MEK activity was blocked entered mitosis, we thought they first went through S phase. Therefore, DNA replication by BrdU incorporation (see Materials and Methods) was estimated in unfertilized eggs treated with 1 μM U0126. Under these conditions, we observed some BrdU incorporation; however, it was similar in the presence or in the absence of aphidicolin, an inhibitor of DNA polymerase α (Sheaff et al., 1991) (Fig. 2). No detectable incorporation of BrdU was measured in untreated unfertilized eggs at this point in time (data not shown). Therefore, BrdU incorporation induced by U0126 was not due to complete replication of DNA. Block of the MEK-ERK pathway induced entry into mitosis without completion of S phase.

Dephosphorylation of the ERK-LP induces MAPK and oscillations of MPF

We then questioned whether a lack of MPF fluctuations, as reported in starfish oocytes (Tachibana et al., 2000), explains the absence of early development in sea urchin eggs after inactivation of the MEK-ERK pathway.

To compare our results with those obtained by Philipova et al. who used *L. pictus* (Philipova et al., 2005a; Philipova et al. 2005b), we first verified that all antibodies we used to detect ERK recognized the same protein in *P. lividus* and *L. pictus*. Results obtained in unfertilized eggs are reported in Fig. 3A. In both species, the anti-ERK and the anti-panERK antibodies labeled a doublet of proteins at approximately 44 kDa, widely accepted to represent the non-phosphorylated (lower band) and the phosphorylated form (upper band) of ERK (Fig. 3Aa), and which we therefore named ERK-LP. As a matter of fact, the upper band of the doublet and the protein labeled by the antibody against phosphorylated MAPK (anti-phosphoMAPK antibody) migrated at the same molecular mass. Furthermore, the proteins revealed in both species had the same molecular mass (Fig. 3Aa). We observed that the anti-panERK antibody also revealed a protein at a molecular mass of approximately 81 kDa, in *L. pictus* (Fig. 3Aa) as well as in *P. lividus*,

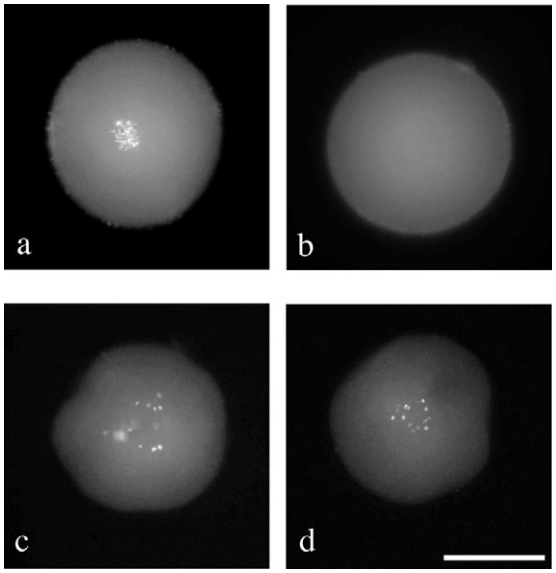

Fig. 2. Effect of U0126 on DNA synthesis. DNA replication was monitored by incubating *P. lividus* sea urchin eggs with BrdU as described in Materials and Methods. Untreated eggs, analyzed 1 hour after fertilization, show major BrdU incorporation (a). This was not observed when eggs were treated with 20 μM aphidicolin (b). Unfertilized eggs treated with 1 μM U0126 for 3 hours, and then incubated (d) with or (c) without 20 μM aphidicolin showed similar BrdU incorporation. Bar, 50 μm.

depending on the exposure of the gel during ECL procedure (see Materials and Methods) (Fig. 3Aa vs Ab right panel). As shown in our previous report (Zhang et al., 2005), a decrease in the phosphorylation of ERK-LP occurred in *P. lividus* eggs after treatment with PD98059 (Fig. 3Ab left panel). This decrease in phosphorylation was not due to a change in the total amount of ERK-LP because labeling intensity did not vary when using the anti-panERK antibody (Fig. 3Ab right panel) and because the decrease in phosphorylation was also observed after treatment with alkaline phosphatase (Zhang et al., 2005). Finally, a very faint signal was detected with the anti-phosphoMAPK antibody at approximately 81 kDa, which did not vary after addition of PD98059 (Fig. 3Ab left panel), similarly to the signal detected at this molecular mass with the anti-panERK antibody under the same conditions (Fig. 3Ab right panel). Altogether, we are confident that the ERK-LP detected in our assays in eggs of *P. lividus* and *L. pictus* is the same and that the anti-phosphoMAPK antibody is specific against the phosphorylated form of ERK-LP.

We then analyzed the variations of ERK-LP phosphorylation after treatment of eggs with the MEK inhibitors. Unfertilized eggs from the sea urchin *P. lividus* were first treated with 2.5 μM PD98059. As expected, this induced a rapid dephosphorylation of the ERK-LP. Surprisingly, a transient peak of ERK-LP phosphorylation was observed at approximately 180 minutes, and a second peak was observed approximately 240 minutes after addition of PD98059 (Fig. 3Ba, upper panel). Unfertilized eggs contained a substantial amount of Cdc2 phosphorylated at tyrosine residue 15 (Cdc2-Tyr*P*) and a low H1 kinase activity, corresponding to a low MPF activity. Addition of PD98059 induced a rapid decrease in the

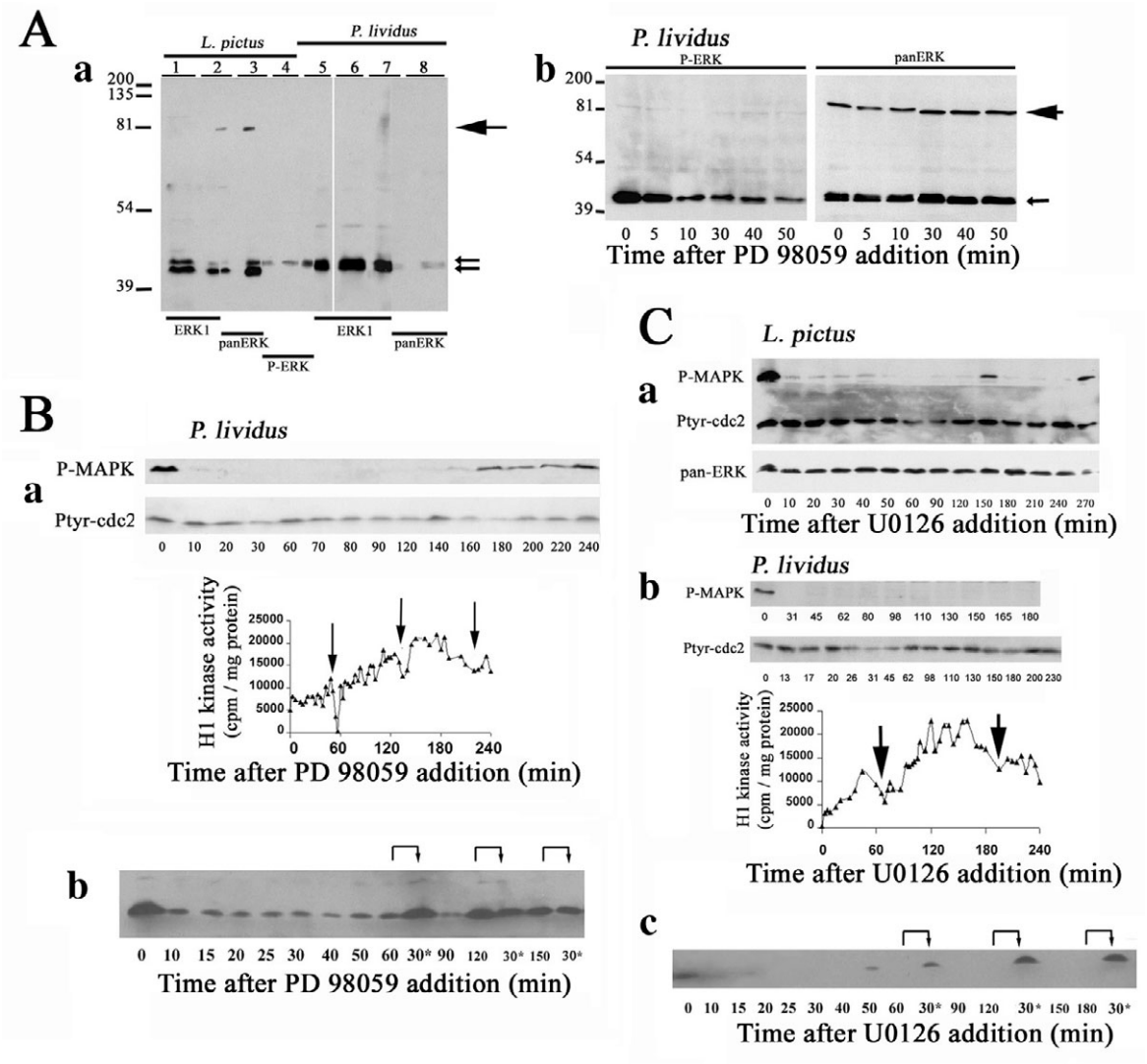

Fig. 3. Effect of PD98059 or U0126 on phosphorylated MAPK (P-MAPK) and MPF activity in unfertilized eggs of *P. lividus* and *L. pictus*. (A) Comparison of ERK-LP in eggs of two sea urchin species using three different antibodies. (Aa) Samples 2, 3, 5 and 7 were cut in two halves after transfer and before blotting with various antibodies, and then re-aligned before ECL. The anti-ERK1 (ERK1) and the anti-panERK (panERK) antibodies detected in both species a doublet at approximately 44 kDa (small arrows) with an upper band corresponding to the protein detected with the anti-phosphoMAPK42/44 antibody (P-ERK). The anti-panERK antibody also revealed a single protein of approximately 81 kDa (large arrow). (Ab) Detection in *P. lividus* eggs of changes in the phosphorylation of the 44 kDa ERK-LP using the anti-phosphoMAPK42/44 antibody (left panel). A decrease in this signal was observed during the 1-hour treatment with 5 μM PD98059 but no change (small arrow) was observed after stripping and re-blotting with the anti-panERK antibody (right panel). A protein was detected by both antibodies (especially by the anti-pan ERK antibody) at approximately 81 kDa (large arrow). (B) Effect of 2.5 μM PD98059 on P-MAPK and MPF activity. (Ba) Fluctuations in the phosphorylation of ERK-LP were detected in *P. lividus* eggs with the anti-phosphoMAPK42/44 antibody (P-MAPK) and that of Cdc2-Tyr*P* using the anti- Cdc2-Tyr*P* antibody (upper panel). H1 kinase activity (lower panel) slowly increased after addition of the inhibitor, with transient decreases at times when Cdc2 was phosphorylated at tyrosine residue 15 (arrows). (Bb) Progressive loss of PD98059 activity after dilution in ASW. Changes after 1 μM PD98059 addition in P-ERK-LP after western blot using the anti-phosphoMAPK42/44 antibody. The inhibitor was added in the egg suspension at *t*=0. The supernatant, containing the inhibitor, was taken and added to fresh eggs at different times (arrow) that were then analyzed 30 minutes later (star). PD98059 was unable to act on the MAPK pathway after a 60-minute dilution in ASW. (C) Effect of 1 μM U0126. (Ca) *L. pictus*. U0126 addition induced oscillations in ERK-LP and in Cdc2-Tyr*P* phosphorylation similar to those observed with PD98059 (Fig. 3B). No change was detected with the anti pan-ERK antibody after stripping and reprobing of the western blot performed with the anti-phosphoMAPK42/44 antibody. The same gel was cut around 38 kDa after transfer in order to blot the upper part for P-MAPK and the lower part for Cdc2-Tyr*P* detection. (Cb) *P. lividus*. The upper panel shows that ERK-LP was dephosphorylated after addition of U0126, but was not phosphorylated again. Oscillations in Cdc2-Tyr*P* similar to those observed in (Ca, upper panel) or after PD98059 (Fig. 3B) corresponded to a gradual increase in H1 kinase activity (lower panel) that showed decreases at times when Cdc2-Tyr*P* level was high (arrows). (Cc) Progressive loss of U0126 activity after dilution in ASW. Changes after 0.5 μM U0126 addition in P-ERK-LP after western blot using the anti-phosphoMAPK42/44 antibody. The inhibitor was added in the egg suspension at *t*=0. The supernatant containing the inhibitor was taken and added to fresh eggs at different times (arrow) that were analyzed 30 minutes later (*). A complete loss of U0126 activity was observed after a 3-hour dilution in ASW.

Tyr phosphorylation of Cdc2 that was followed by two peaks of phosphorylation, occurring at times earlier than those observed for P-MAPK, namely 60 minutes and 140 minutes after drug addition (Fig. 3Ba, upper panel). Concomitantly, H1 kinase activity increased gradually, but recurrent partial inactivation was observed when phosphorylation of Cdc2-Tyr*P* was increased (Fig. 3Ba lower panel). The level of H1 kinase activity reached after a 3-hour treatment with the MEK inhibitor, corresponded to a third of activity measured at mitosis in fertilized eggs (De Nadai et al., 1998) (data not shown). Similar results were obtained in *L. pictus* (data not shown).

These results could be explained by a gradual loss of activity of PD98059 present in the external medium. A complete loss of the drug activity was measured at approximately 1-2 hours after dilution (Fig. 3Bb), which explains the fact that ERK-LP is phosphorylated again 1-2 hours after drug addition (Fig. 3Bb). However, this cannot explain the later peak of ERK-LP phosphorylation.

Experiments with 1 μM U0126 gave results similar to those obtained with PD98059. At 1 μM, U0126 induced inactivation of the ERK-LP, which was followed by two peaks of reactivation 150 minutes and 270 minutes after addition of the drug in *L. pictus* (Fig. 3Ca) and in *P. lividus* (data not shown). The initial decrease in phospho-ERK-LP was not due to a loss or degradation of the protein itself during that period, because no change of total ERK was observed when using the pan-ERK antibody. As observed for PD98059, the level of Cdc2-Tyr*P* progressively decreased to reach a very low level 1 hour after addition of U0126; peaks of Cdc2-Tyr*P* were then observed 150 minutes and 240 minutes after addition of the drug. These data indicate that inhibition of the MEK-ERK cascade is sufficient to trigger MAPK and MPF oscillations in unfertilized eggs. Therefore, the question arises whether both types of oscillations are linked to each other?

Oscillations in the phosphorylation of ERK were not always seen in experiments using U0126. In some experiments, ERK was dephosphorylated and no reactivation was seen thereafter, whereas oscillations of Cdc2-Tyr*P* still occurred (Fig. 3Cb). A progressive decrease in Cdc2-Tyr*P* was first observed under these conditions and reached a low 30 minutes after addition of U0126. Two later peaks of Cdc2-Tyr*P* were then detected, after approximately 100 minutes and 200 minutes (Fig. 3Cb upper panel). As observed with PD98059, a gradual increase in H1 kinase activity, showing transient waves, was measured after addition of U0126, and lower activity was observed after approximately 60 minutes and 200 minutes when Cdc2-Tyr*P* was high (Fig. 3Cb lower panel).

As shown above for PD98059, a gradual loss of U0126 activity was achieved at 1 hour and a complete loss 2-3 hours after dilution (Fig. 3Cc). This loss of activity of the MEK inhibitor thus explains that ERK-LP could be phosphorylated again within 3 hours in U0126-treated eggs.

In conclusion, oscillations in MPF activity can be observed in eggs treated with U0126 that did not show any re-phosphorylation of ERK-LP. Therefore, MPF oscillations triggered by an initial inactivation of MAPK appear to be independent of MAPK.

Inactivation of MAPK induces entry into mitosis by Ca-dependent mechanism(s)

We tested the hypothesis that, in oocytes, active MAPK prevents entry into mitotic cycles by maintaining a low level of Ca^{2+}_i, which can be overcome by the fertilization Ca^{2+}_i signal (Tunquist and Maller, 2004). Experiments were performed using *P. lividus eggs*. Although no modification in the level of Ca^{2+}_i was recorded in control eggs during 150 minutes, the usual fertilization Ca^{2+}_i transient was observed and addition of U0126 induced a slow and progressive rise in Ca^{2+}_i levels (Fig. 4A). Small oscillations seemed to occur during the slow rise because the average values of approximately 62 minutes (0.46±0.02, n=9) and 106 minutes (0.50±0.03, n=5), were significantly higher (Student's test, P<0.05) than the average value measured at 82 minutes (0.42±0.04, n=10) (Fig. 4A). The increase in Ca^{2+}_i levels after 3 hours of U0126 treatment could reach a level corresponding to the smallest (or half of the largest) fertilization Ca^{2+}_i signal that we measured (Fig. 4A). This corroborates the appearance of a fertilization membrane in a small number of eggs (Fig. 1A).

Is this increase in Ca^{2+}_i necessary to trigger entry in mitosis in eggs treated with MEK inhibitors? We first determined the accurate amount and concentration of EGTA buffer that needs to be injected to inhibit activation after fertilization (data not shown). Injection of EGTA buffer did not induce any activation by itself (Fig. 4B). It blocked NEB in 17 of 22 egg batches that were injected and then treated with PD98059 but it reduced the number of eggs entering mitosis in only five of 22 egg batches (Table 1). Similar results were obtained with U0126 (Table 1). An experiment that led to inhibition of NEB (Fig. 4B) showed that all EGTA-injected eggs that were further treated with U0126 showed neither NEB nor mitotic figures, whereas non-injected eggs did.

These changes in Ca^{2+}_i levels could either be due to modifications in Ca^{2+} permeability of the endoplasmic reticulum or an influx of external Ca^{2+} into the egg. Therefore, we tested whether the sensitivity to inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] was modified in eggs treated with 2 μM U0126 or with 10 μM PD98059. Ins(1,4,5)P_3 injection activated unfertilized eggs in a concentration-dependent manner. Treatment with each inhibitor reduced the percentage of activated eggs, when less than 10 μM Ins(1,4,5)P_3 was injected (Fig. 4C). This suggests that a block in MEK activity reduced the sensitivity of intracellular compartments to Ins(1,4,5)P_3.

Finally, the absence of external Ca^{2+} did not inhibit entry in mitosis in eggs treated with U0126, because eggs treated at a low external Ca^{2+} concentration showed NEB and morphological alterations that seemed even more severe (Fig. 4D). Similar results were obtained with PD98059 (data not shown).

All these results suggest that entry in mitosis, triggered by a block of the MEK-ERK cascade in unfertilized eggs, depends on an increase of Ca^{2+}_i. Moreover, they also suggest that variations in Ca^{2+}_i triggered by MEK inhibition were due to modifications of Ca^{2+} transport inside the egg and not to a stimulation of Ca^{2+} influx into the egg.

Entry into mitosis after inactivation of MEK is independent of intracellular pH changes

The mitotic events that are induced after ERK-LP dephosphorylation could be due to an increase in intracellular pH (pH_i), as it is the case after fertilization or after ammonia treatment. We used the DMO technique, which has been

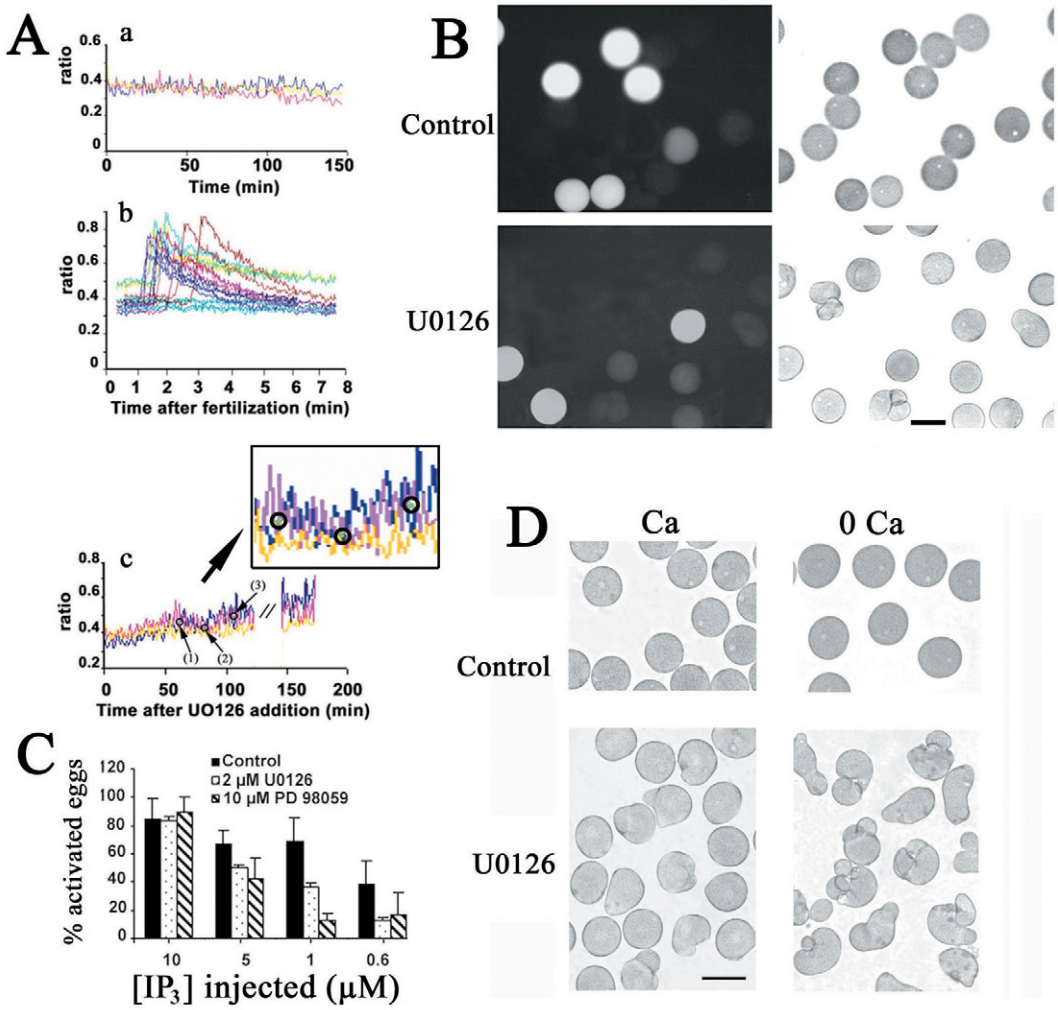

Fig. 4. Role of Ca^{2+} in mitosis entry after MAPK inactivation in *P. lividus* eggs. (A) The time course of Ca^{2+}_i changes in (a) unfertilized control eggs, (b) fertilized eggs or (c) unfertilized eggs treated with 1 μM U0126. The fluorescence ratio (340 nm to 380 nm) measured using fura-2 dextran indicate relative Ca^{2+}_i changes. Each color corresponds to one egg: a, no clear change was recorded in unfertilized control eggs during 3 hours; b, all Ca^{2+}_i signals were recorded in eggs showing elevation of the fertilization membrane, whereas absence of Ca^{2+}_i signal corresponded to eggs that were not fertilized; c, eggs treated with U0126 showed a slow increase in Ca^{2+}_i ; calculations of average ratios indicated with an arrow at (1) 62 minutes, (2) 82 minutes and (3) 106 minutes (enlarged in inset) are given in the text and suggest small oscillations of Ca^{2+}_i level. (B) Ca-EGTA inhibits entry in mitosis triggered by 1 μM U0126. The injection buffer containing Ca-EGTA and carboxyfluorescein allowed to visualize the injected eggs (left panels). All eggs, including the injected ones, were treated with the MEK inhibitor (UO126) or not treated (Control) and observed 3 hours after treatment. Injected eggs did not show NEB, whereas non-injected eggs entered mitosis and sometimes showed constrictions. (C) Effect of U0126 or PD98059 on egg activation induced by injection of Ins(1,4,5)P_3. MEK inhibitors reduced egg activation, as seen by elevation of the fertilization membrane, in eggs injected with less than 10 μM Ins(1,4,5)P_3. (D) Effect of the absence of external Ca^{2+} on U0126-treated eggs. Eggs were treated with 5 μM U0126 (UO126) or not (Control, upper panels) in ASW containing 10 mM Ca^{2+} (Ca, left panels) or not (0 Ca, right panels) for 3 hours. Entry into mitosis was induced by U0126 even at zero external Ca^{2+}, with deformations of the eggs that were even more severe.

widely used to determine variations of pH_i in sea urchin eggs (Johnson and Epel, 1981) and gives results similar to those obtained using fluorescent probes (Dube and Eckberg, 1997). Treatment of eggs with concentrations of PD98059 as high as 50 μM did not trigger any increase in pH_i and eggs behaved like untreated eggs. An increase of 0.35 pH units was, as expected, detected after fertilization (Payan et al., 1983) but was not altered by PD98059 (Fig. 5A).

Does an increase in pH_i that is normally induced by ammonia (Dube and Epel, 1986) trigger ERK-LP dephosphorylation? Although complete ERK-LP dephosphorylation was observed after fertilization or, as expected, after treatment of eggs with the Ca^{2+} ionophore A23187 (Carroll et al., 2000), no modification was seen (even after 1 hour) when eggs where treated with 10 mM ammonia (Fig. 5B).

Altogether, these results strongly suggest that, in unfertilized sea urchin eggs, mitotic events triggered by ERK dephosphorylation are independent of the pH_i status.

Discussion

Our present data, which were equally reproducible by using U0126 or PD98059 in the two different sea urchin species *P.*

Table 1. Effect of MEK inhibitors and Ca^{2+}_i on entry in mitosis

	PD98059			U0126		
	n	%NEB	% Division	*n*	% NEB	% Division
Number of batches (with NEB)	5			5		
EGTA-injected eggs	24	42	0	11	64	0
Non-injected eggs	38	60	16	67	92	1
Number of batches (without NEB)	17			9		
EGTA-injected eggs	34	0	0	23	0	0
Non-injected eggs	198	70	19	110	75	9
Injected eggs (total)	58	17	0	34	21	0
Non-injected eggs (total)	236	68	19	177	81	6

Percentage of eggs that showed nuclear envelope breakdown (NEB) or constrictions after treatment with 5 μM PD98059 or 1 μM U0126 and that before had been injected or not with Ca^{2+}-EGTA buffer. *n* indicates the number of batches with and without NEB (not all batches necessarily contain the same number of eggs), or the total number of eggs injected or not. Five batches treated with PD98059 and five batches treated with U0126 gave a few eggs that underwent NEB even after injection with Ca^{2+}-EGTA buffer (Exp NEB). In 17 and nine batches treated with PD98059 or U0126, respectively, none of the eggs injected with Ca^{2+}-EGTA buffer underwent NEB.

lividus and *L. pictus*, show that inactivation of a MEK-ERK cascade in unfertilized eggs triggers oscillations of phosphorylated ERK (ERK-*P*) and MPF, and leads to entry into mitosis without full DNA replication. All these events were mediated by changes in Ca^{2+}_i and not by changes in pH_i. This artificial situation may help to understand the role of MAPK inactivation in cell-cycle progression after fertilization. It is clear that location and activity of MAPK in eggs differ radically before and after fertilization, suggesting not only different targets but also different regulating pathways upstream MEK-MAPK before and after fertilization. (1) The unfertilized egg contains very high levels of active ERK-like protein, which is highly expressed all over the egg, in the cytosol and the nucleus (Zhang et al., 2005), and which, as shown here, prevents eggs to enter a mitotic program. (2) Fertilization induces a rapid decrease in MAPK activity, that is followed at time of mitosis by a very small reactivation, corresponding to a tenth of the unfertilized level (Zhang et al., 2005). ERK-like protein is located at that time in very small areas, in the vicinity of centrosomes and insures formation of a normal mitotic spindle, but controls neither entry into nor exit from first mitosis (Zhang et al., 2005).

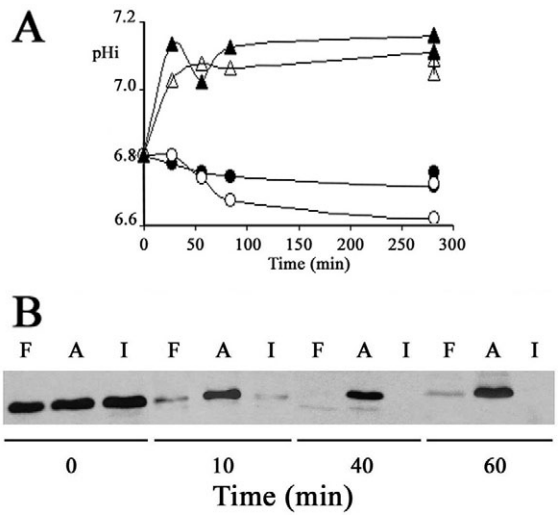

Fig. 5. No effect of PD98059 was seen on pH_i and P-MAPK. (A) Change in pH_i. Eggs were fertilized (▲, △) or not (●, ○) in the presence (△, ●) or not (▲, ●) of 50 μM PD98059. PD98059 did not modify the increase in pH_i observed after fertilization, nor did it change the small acidification observed in unfertilized eggs. (B) Changes in the phosphorylation of the ERK-LP detected by western blot using the anti-phosphoMAPK42/44 antibody after fertilization (F) or treatment with 10 mM ammonium (A) or with 10 μM A23187 (I). MAPK was rapidly dephosphorylated after fertilization or activation with the Ca^{2+} ionophore, but no change was observed after treatment with ammonium.

The MEK-ERK pathway and the control of DNA replication

In unfertilized eggs, inactivation of the MEK-ERK pathway triggers chromatin condensation. Some DNA replication (measured through BrdU incorporation) occured, which was similar in the presence or in the absence of aphidicolin – an inhibitor of DNA polymerase α, which inhibits the processive elongation of nascent DNA strands but does not prevent the initiation of replication and the formation of short (100-500 bp) primers (Marheineke and Hyrien, 2001; Maiorano et al., 2006). Inactivating MAPK might have led to initiation but not to progression of DNA replication. These results corroborate data by Philipova et al. reported for sea urchin (Philipova et al., 2005a) but contradicts those obtained by Carroll et al., who report to have detected DNA replication (Carroll et al., 2000). We, however, believe they might have detected DNA repair. Our results also corroborate those obtained in *Xenopus* egg extracts (Abrieu et al., 1997) and oocytes of the starfish *M. glacialis* and *A. aranciacus* (Picard et al., 1996; Fisher et al., 1998), but contradict those reported for the starfish *A. pectinifera* (Tachibana et al., 1997). However, the phosphatase XCL100, used by the latter group to inactivate MAP kinase, might act upstream of DNA synthesis on a target other than ERK and, although injection of constitutively active MEK repressed the initiation of DNA synthesis following fertilization, 30% of these eggs did escape G1 arrest and developed until G2 (Tachibana et al., 1997; Abrieu et al., 1997). Altogether, these results suggest that ERK inactivation is not required for full DNA replication during first mitosis of early sea urchin embryos.

The very opposite hypothesis has been proposed recently by Philipova et al., who suggest that a rise in ERK activity occurring after fertilization is required for S phase (Philipova et al., 2005a). First, the authors detected an ERK protein that is not active in unfertilized eggs, which contradicts the well-established view that "downregulation of MAP kinase is a universal consequence of fertilization in the animal kingdom" (Fisher et al., 1998). Philipova's view could be explained if two types of ERK were expressed in the sea urchin egg. However, this hypothesis seems unlikely because all anti-ERK antibodies used in our study detected a single protein in two sea urchin species, including *L. pictus*, which was used by these authors (Philipova et al., 2005a; Philipova et al., 2005b; Philipova and Whitaker, 1998; Philipova and Whitaker, 2005). Their so-called ERK1 formed dimers that were detected at a molecular mass of approximately 90 kDa (Philipova and Whitaker, 2005). We also detected a band of approximately 80 kDa with the anti-pan ERK and the anti-phosphoERK antibodies in *P. lividus* as well as *L. pictus*, under reducing electrophoresis conditions, but we believe that this high-molecular mass protein is either a non-specific signal or ERK5. Second, 100 μM U0126 were necessary to block DNA replication and MAPK activity (Philipova et al., 2005a), but this huge amount of U0126 might non-specifically alter other components of MEK-MAPK pathways including ERK5 or SAPK2α (stress-activated protein kinase 2α/p38) (Davies et al., 2000), which act on DNA synthesis (Dehez et al., 2001) and on cell-cycle checkpoints (Pearce and Humphrey, 2001). Moreover, it is surprising that PD98059 could not inhibit several biological processes inhibited by UO126 (Philipova et al., 2005a). Third, the MAPK phosphatase XCl 100 used by Philipova et al. to cancel DNA synthesis after fertilization might also, as discussed above, act on targets other than ERK. Our hypothesis is that neither inactivation nor activation of the MEK-ERK cascade act on DNA replication during first mitosis of early sea urchin embryos. Both Ca^{2+}_i and pH_i signals triggered by fertilization have been widely described to be upstream regulators of DNA synthesis (Mazia and Ruby, 1974; Nishioka and Magagna, 1981; Schomer-Miller and Epel, 1999), and might upregulate DNA synthesis through pathways others than the MEK-ERK cascade.

Inactivation of the MEK-ERK cascade, stimulation of MPF and entry into mitosis

The unfertilized *P. lividus* sea urchin egg contains a non-negligible MPF activity, as well as a pool of Cdc2-Tyr*P* that is dephosphorylated after inactivation of the MEK-ERK cascade. This leads to a general increase in MPF activity without any stimulation of the low rate of protein synthesis that can be measured in unfertilized eggs (data not shown) and also to entry into mitosis. This is in agreement with the idea that a Mos-MEK-MAPK cascade downregulates a mechanism that inactivates cyclin B-cdc2 kinase in matured oocytes (Abrieu et al., 1997). The small amount of MPF may be sufficient (Geneviève-Garrigues et al., 1995) and is obviously necessary to trigger entry into M phase in unfertilized eggs deprived of MEK-ERK activity (this report) and in fertilized eggs (Abrieu et al., 2001; Philipova et al., 2005b). Oscillations in MPF activity and Cdc2-Tyr*P* that were then generated, seem to be independent of ERK-LP variations, which corroborates data obtained in *Xenopus* egg extracts (Takenaka et al., 1997; Guadagno and Ferrell, Jr, 1998); alternatively, these oscillations might control activity of ERK-LP, as suggested by Abrieu et al. (Abrieu et al., 2001).

Full inactivation did not occur between peaks of H1 kinase activity, which could explain why eggs did not undergo complete mitotic cycles, never divided or developed, as reported for starfish oocytes (Tachibana et al., 2000). The presence of a centrosome in immature MI-arrested starfish oocytes can also possibly explain this difference between starfish (MI-arrested) and sea urchin (G1-arrested) oocytes, because the remaining centrosome in immature oocytes is incompetent for reproduction and meant to disappear at the end of meiosis, as it is the case in sea urchin unfertilized eggs (Uetake et al., 2002).

Morphological events, such as egg constrictions, occurred either more rapidly after induction of a faster ERK re-phosphorylation, for example after rinsing off the inhibitor or with a delay when concentrations higher than 50 μM were used (data not shown), which probably delays ERK-LP re-phosphorylation. MEK inhibitor used at such a high level might act non-specifically on pathways that lead to NEB and mitotic events. In another hypothesis, MAPK re-activation acts as a 'facilitator' of mitosis, as reported during the first mitosis of the sea urchin embryo (Zhang et al., 2005) or in somatic cells (Roberts et al., 2002). The longer time that is necessary to get rid of a higher amount of inhibitor can then explain the delay in these two hypotheses. It then seems that reactivation of the MEK-ERK cascade regulates, but does not trigger, mitotic events, which, again, contradicts recent data obtained by Philipova et al. (Philipova et al., 2005b), but questions similar to those pointed above concerning DNA replication can be raised.

Unfertilized sea urchin eggs deprived of MEK-ERK activity can enter M phase without complete replication of DNA. This is in agreement with the fact that aphidicolin does not inhibit MPF activation, and suggests that factors required for both DNA replication and checkpoint activation (Takeda and Dutta, 2005) are present in fertilized sea urchin eggs and absent in unfertilized eggs (Sluder et al., 1995; Geneviève-Garrigues et al., 1995; Philipova et al., 2005b) (our unpublished results). Finally, 4N chromosomes and the probable absence of a DNA checkpoint machinery in MI-arrested starfish oocytes might explain the situation in these oocytes where aphidicholin does not block the first cleavages when fertilization is induced in MI (Nagano et al., 1981), and where development can go as far as bona fidae larvae when MAPK is inactivated (Tachibana et al., 2000; Sadler et al., 2004).

The MEK-ERK cascade is independent of events related to pH_i

Ammonia did not trigger dephosphorylation of ERK-LP, and ERK-LP dephosphorylation did not trigger an increase in pH_i. Dephosphorylation of Cdc2-Tyr*P* induced after inactivation of the MEK-ERK pathway is pH_i-independent, as reported after fertilization (Edgecombe et al., 1991). Microtubule polymerization, chromosome condensation and NEB might not be strictly pH_i-dependent events. Ammonia, which has been reported to stimulate these events (Bestor and Schatten, 1982), might act via a pH_i-independent pathway or might be directly affected by amines, as suggested by others (Dube and Epel, 1986; Rees et al., 1995).

The MEK-MAPK pathway and the control of Ca^{2+}_i

Inactivation of the MEK-MAPK pathway led to a progressive and slow increase in Ca^{2+}_i that was necessary for progression of eggs into mitosis. This is consistent with results showing that chromatin condensation (Twigg et al., 1988) and NEB (Philipova et al., 2005b; Wilding et al., 1996) that occur after fertilization are Ca-dependent events. It is unlikely that this increase in Ca^{2+}_i was due to entry of external Ca^{2+}, because entry into mitosis was not inhibited in zero-Ca^{2+} external medium. Therefore, the rise in Ca^{2+}_i could only be due to alterations in Ca^{2+} transport into and out of intracellular compartments. We cannot rule out the possibility of non-specific effects of the MEK inhibitors on Ca^{2+} channels or transporters. However, to our knowledge, all non-specific effects that have been reported were obtained at concentrations higher than those used in our study (Pereira et al., 2002). Moreover, U0126 does not seem to act non-specifically on store-mediated Ca^{2+} entry, as reported for human platelets (Rosado and Sage, 2001).

Inactivation of the MEK-ERK pathway at fertilization

We show here the induction of mitosis entry in unfertilized eggs, i.e. under conditions where artificial ERK-LP inactivation and MPF stimulation occurred at the same time. Normally, fertilization in sea urchin eggs triggers inactivation of both activities in the first minutes after fertilization (Chiri et al., 1998), and mitosis occurs after completion of S phase, when MPF is stimulated (Meijer and Pondaven, 1988). At fertilization, the decrease in MAPK activity must then occur together with another event that delays stimulation of MPF and entry in mitosis, to allow S-phase completion before entry into mitosis. How to compare the situation triggered by chemical MEK inhibition in unfertilized eggs with that where ERK-LP is dephosphorylated by fertilization (Fig. 6)? The large fertilization Ca^{2+}_i peak that induces MEK-ERK initial inactivation is by-passed in eggs treated with MEK inhibitors. Two essential elements must then be considered. The first one concerns the level of Ca^{2+}_i, reached in unfertilized eggs after a 30-minute treatment with MEK inhibitor, which is much smaller than the Ca^{2+}_i measured at entry in mitosis in fertilized eggs (Ciapa et al., 1994; Philipova et al., 2005b; Wilding et al., 1996). Under similar conditions, this small increase was – although detected – not acknowledged by others (Carroll et al., 2000). The MEK-ERK cascade might act on nuclear (Gerasimenko and Gerasimenko, 2004) or mitochondrial (Bianchi et al., 2004) Ca^{2+} signaling mechanisms to generate the small Ca^{2+} signals that are localized in the peri-nuclear area, have been detected before NEB and might be at the origin of the larger NEB Ca^{2+}_i transient (Wilding et al., 1996). The second element refers to the role of a Src-PLCγ pathway that is activated at fertilization and leads to Ins(1,4,5)P_3 synthesis necessary for mitotic Ca^{2+} transients (Ciapa et al., 1994; Shearer et al., 1999). Fertilization strongly stimulates the egg metabolism and various signaling pathways, including a constant increase in tyrosine phosphorylation during the first cell cycle (Ribot, Jr et al., 1984). A large amount of Ins(1,4,5)P_3, due to a high level of upstream tyrosine activity attained at mitosis, must then be compensated by a decrease in Ins(1,4,5)P_3 sensitivity to generate small Ca^{2+} peaks. The decrease in Ins(1,4,5)P_3 sensitivity of intracellular stores in unfertilized eggs after MEK inactivation might be due to an altered phosphorylation status of Ins(1,4,5)P_3 receptors that is known to play a role in the specificity of the Ca^{2+}_i signals (Yule et al., 2003). This could also explain why the amount of Ins(1,4,5)P_3 that generates the small and slow mitotic Ca^{2+}_i signal at entry of mitosis (i.e. when ERK-LP phosphorylation is low) is much higher than that produced during the large and rapid fertilization Ca^{2+}_i signal (Ciapa et al., 1994) (Fig. 6).

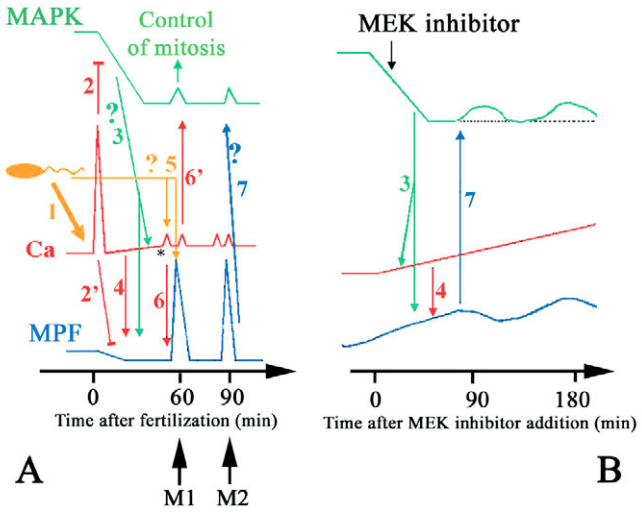

Fig. 6. Models of the relation between Ca^{2+}_i, MAPK and MPF activity changes during egg activation. (A) Schematic representation of egg activation at fertilization. Activated MAPK and MPF activities, and Ca^{2+}_i levels are indicated in green, blue and red, respectively. Changes induced by each of them are given by arrows of corresponding color. A high MAPK activity (green line) is present in unfertilized egg. A t=0, the sperm triggers (large yellow arrow 1) a sharp Ca^{2+}_i signal (red line) that desactivates MAPK by a Ca-dependent pathway (red arrow 2) (Carroll et al., 2000). The decreasing MAPK activity brings the Ca^{2+}_i level (green arrow 3) higher (*), to an almost undetectable point (Wilding et al., 1996), that precludes the mitotic Ca^{2+}_i transients at 60 and 90 minutes, times of first (M1) and second (M2) mitosis, respectively. This change in Ca^{2+}_i level (red arrow 4), together with inactivation of MAPK (green arrow 3), induces oscillations of MPF activity (blue line). These MPF oscillations would be shaped in the fertilized eggs by the sperm: (1) The huge Ca^{2+}_i fertilization signal would first desactivate the low amount of active MPF already present in the unfertilized egg by a Ca-dependent pathway (red arrow 2') (Tunquist and Maller, 2003); (2) factor(s) of spermatic origin would act at mitosis by undetermined pathway(s), perhaps involving the polyphosphoinotide messenger system (thin yellow arrow 5) (Ciapa et al., 1994) to generate the Ca^{2+}_i mitotic transients that would themselves induce the huge MPF mitotic activation (red arrow 6 and 6'); this pathway of spermatic origin could also act directly on MPF oscillations (thin yellow arrow 5). MPF oscillations would induce MAPK oscillations (blue arrow 7). (B) Egg activation after treatment with a MEK inhibitor. Only pathways 3, 4 and 7 are activated, pathways 1, 2, 5 and 6, under sperm control, being absent. The MEK inhibitor induces MAPK inactivation, which triggers a slow rise in Ca^{2+}_i (arrow 3), itself responsible for MPF oscillations (arrow 4). Those oscillations would themselves induce MAPK oscillations (arrow 7). This would explain why MPF and MAPK oscillations are wider and of smaller amplitude in this condition. The level of Ca^{2+}_i reached in such treated eggs (red line) reaches a high level that cannot be attained in fertilized eggs.

In conclusion, we believe that ERK inactivation induced

after fertilization sets up an endogenous $Ca^{2+}{}_i$ oscillator, independent of pH_i, which controls MPF oscillations and early embryonic division, as suggested by Swanson et al. (Swanson et al., 1997). The frequency and intensity of the $Ca^{2+}{}_i$ spikes, and full activation of MPF during mitosis, may be regulated by another oscillator system. This second mechanism might be of spermatic origin, stimulated by the fertilization $Ca^{2+}{}_i$ signal and might involve the phosphoinoisitide messenger system (Ciapa et al., 1994) including PLCγ (Shearer et al., 1999) (Fig. 6).

Materials and Methods

Handling of gametes

Paracentrotus lividus sea urchins were collected in the bay of Marseille or Villefranche-sur-mer (France) and *Lytechinus pictus* were collected in California (Marinus Sci. Inc.). Gametes were collected, prepared and fertilized either in natural sea water (NSW) or in artificial sea water (ASW; Reef Crystals Instant Ocean) as described (Chiri et al., 1998; De Nadai et al., 1998).

Inhibitors

Eggs were treated or not with PD98059, U0126 or U0124 (inactive analog of U0126) (Calbiochem), which were made as a 15 mM stock in DMSO. The appropriate addition of DMSO to the egg culture was used as a control of all experiments.

Western blot analysis

Preparation of egg samples, separation of proteins by SDS-PAGE and western blotting were performed as described previously (Chiri et al., 1998; De Nadai et al., 1998; Zhang et al., 2005). All the antibodies were diluted in blocking buffer as follows: mouse anti-phospho-MAPK isoforms 42 and 44 (Thr202 and Tyr204, respectively; Cell Signalling) at 1:2000, mouse anti-pan ERK antibody (BD Transduction laboratories) at 1:1000, rabbit anti-phospho-Cdc2 (Tyr15, Cell Signalling) at 1:500, rabbit anti-Cdk1/Cdc2 (PSTAIR, UBI) at 1:1000, the anti-mouse horseradish-peroxidase (HRP)-conjugated goat IgG (ICN) and the anti-rabbit HRP-conjugated goat IgG (ICN) secondary antibodies at 1:1000 and 1:10 000, respectively. Proteins were revealed by enhanced chemoluminescence (ECL, Amersham).

H1 kinase activity

H1 kinase activity was measured in egg extracts obtained before and at different times after addition of MEK inhibitor using the protocolt described by Meijer and Pondaven (Meijer and Pondaven, 1988). Since it has been established that H1 kinase activity essentially reflects MPF activity in eggs and early embryos (Levasseur and McDougall, 2000; Levasseur and McDougall, 2003), the word MPF is used in that sense.

Monitoring of DNA replication by BrdU incorporation

To monitor DNA replication, 5-bromo-2-deoxyuridine (BrdU) was added to unfertilized egg suspension at a concentration of 0.1 mg/ml in presence of 1 mM ATA. Unfertilized eggs were treated with 1 μM U0126 with or without 20 μg/ml aphidicholin and harvested after 3 hours. Control-fertilized eggs were harvested 60 minutes after fertilization. Eggs were transferred to 0.2 mg/ml pronase in seawater to remove the fertilization membrane of fertilized eggs. The pronase reaction was stopped by addition of 1% BSA and eggs were rinsed in Millipore-filtered sea water (MFSW) and fixed in 4 N HCl for 2 hours. After post-fixation in methanol for 30 minutes, eggs were washed in PBS-Tween for 1 hour and incubated in anti-BrdU mouse monoclonal antibodies (Amersham) for 2 hours at room temperature. They were washed in PBS-Tween for 1 hour and then incubated with a 1:250 dilution of FITC-conjugated anti-mouse IgG antibody (Sigma) for 1 hour at room temperature. After washing in PBS-Tween for 1 hour at room temperature, eggs were mounted on pre-washed glass plates in Mowiol and observed by fluorescence microscopy on a Zeiss microscope.

Cytochemistry

Eggs were cultured in ASW or NSW, treated or not with PD 98059 or U0126, and taken for fixation and labeling.

For microtubule and chromatin labeling, eggs were taken at different times after U0126 or PD 98059 addition, then fixed, labeled and observed by confocal microscopy as previously explained (Zhang et al., 2005). Microtubules were labeled with the monoclonal rat anti-tubulin antibody YL1/2 (Chemicon) and chromatin with Hoechst-33258 dye as described (Zhang et al., 2005).

Injection

De-jellied eggs were stuck on Petri dishes coated with polylysine (0.5 mg/ml), and were maintained at 18°C. Injection volumes were 1-2% of the egg volume and were performed under N_2 atmosphere using a Narishige IM 300 microinjector. We used zero-Ca^{2+} injection buffer (10 mM EGTA, 480 mM KCl, 20 mM PIPES, pH 7.0) to avoid $Ca^{2+}{}_i$ changes.

Ca^{2+} imaging

$Ca^{2+}{}_i$ measurements were made in eggs injected as described above with injection buffer (480 mM KCl, 0.1 mM EGTA, 20 mM PIPES, pH 7.0) containing 5 mM 10 kDa-Fura-2 dextran (Molecular Probes). Eggs were then treated with U0126 or PD98059 and $Ca^{2+}{}_i$ fluorescence was detected under a Nikon Eclipse TE300, equipped with a ×20 plan Fluor Nikon objective, a Lambda-10 (Sutter Inst.) shutter and a Hamamastu CCD camera C2400-80. Signals were analyzed using a Dynamic Fluorescence and Ion Imaging software (Axon Instruments, Inc.).

Determination of pH_i

Variations of pH_i were measured following the protocol as previously described in detail (Payan et al., 1983).

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