

ERK1 activation is required for S-phase onset and cell cycle progression after fertilization in sea urchin embryos

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Summary

Fertilization of sea urchin eggs results in a large, transient increase in intracellular free Ca^{2+} concentration that is responsible for re-initiation of the cell division cycle. We show that activation of ERK1, a Ca^{2+} -dependent MAP kinase response, is required for both DNA synthesis and cell cycle progression after fertilization. We combine experiments on populations of cells with analysis at the single cell level, and develop a proxy assay for DNA synthesis in single embryos, using GFP-PCNA. We compare the effects of low molecular weight inhibitors with a recombinant approach targeting the same signalling pathway. We find that inhibition of the ERK pathway at

fertilization using either recombinant ERK phosphatase or U0126, a MEK inhibitor, prevents accumulation of GFP-PCNA in the zygote nucleus and that U0126 prevents incorporation of [³H]-thymidine into DNA. Abrogation of the ERK1 signalling pathway also prevents chromatin decondensation of the sperm chromatin after pronuclear fusion, nuclear envelope breakdown and formation of a bipolar spindle.

Key words: Cell cycle, GFP-PCNA, XCL100, ERK, Sea urchin, DNA replication

Introduction

MAP kinase signalling pathways play major roles in cell proliferation (reviewed by Hulleman and Boonstra, 2001; Roovers and Assoian, 2000; Ruderman, 1993). In mammalian (Meinecke and Krischek, 2003; Sun et al., 1999), frog (reviewed by Nebreda and Ferby, 2000) and starfish (Fisher et al., 1998) oocytes, MAP kinase activity is essential for proper progression through meiosis (Abrieu et al., 2001). Its activity then falls at fertilization as these oocytes leave meiotic metaphase. More recently, MAP kinase activation has been shown to occur during mitosis in embryonic cell cycles of frog (Chung and Cheng, 2003; Gotoh et al., 1991; Guadagno and Ferrell, 1998) and sea urchin (Chiri et al., 1998; Pelech et al., 1988; Philipova and Whitaker, 1998). In contrast to mammalian, frog and starfish oocytes, sea urchin eggs rest in interphase of the first mitotic cell cycle as they await fertilization. Three reports have documented changes in the activity of the MAP kinase pathway in sea urchin eggs immediately after fertilization. We have found that there is a substantial transient increase in ERK1 activity in the first few minutes after fertilization, triggered by the fertilization Ca^{2+} transient (Philipova and Whitaker, 1998). This increase in the activity of the ERK1 pathway is very distinct from the much later increase measured at mitosis of the first cell cycle. A second report (Chiri et al., 1998) documents a 50% increase in MAP kinase activation immediately after fertilization using myelin basic protein as a substrate, but this increase was not

seen using a Myc substrate that would be expected to detect an increase in ERK1/2 activity. A third report (Carroll et al., 2000) documents a fall in phosphorylated ERK at fertilization in sea urchin and finds that application of a MEK inhibitor, PD98059, initiates DNA synthesis in unfertilized eggs. The third report concludes that a fall in ERK pathway activity may trigger the onset of S phase. The aim of this report is to resolve the paradox in these data and to determine the role of the ERK pathway at fertilization in sea urchin eggs by comparing the effects of the recombinant ERK phosphatase XCL-100 with the low molecular weight inhibitors of the pathway that target MEK. In order to compare the actions of the soluble, cell-penetrating low molecular weight inhibitors with the non-penetrant recombinant ERK phosphatase, we have developed a single cell assay for S phase in sea urchin embryos using the proliferating cell nuclear antigen (PCNA) as a chimera with GFP (Leonhardt et al., 2000).

As its name implies, the appearance of PCNA immunofluorescence in the nucleus has for a long time been used as a marker of S phase (Takasaki et al., 1981). It has been shown by immunofluorescence to localise to the replication complex throughout DNA synthesis in *Xenopus* cell-free extracts and to dissociate as DNA synthesis is completed (Hutchison and Kill, 1989). This pattern has been confirmed in mammalian cells (Leonhardt et al., 2000; Somanathan et al., 2001) using the GFP chimera employed in the experiments reported here. PCNA forms a trimer that associates with DNA

polymerase δ and is the sliding clamp essential for the processivity of the polymerase. Aphidicolin, a DNA polymerase α , δ and ϵ inhibitor (Avkin et al., 2002) thus prevents association of PCNA with the replication complex provided it is present before PCNA is able to bind. By contrast, if aphidicolin is added once the replication fork has begun, it stalls the replication fork and prevents the dissociation of PCNA from chromatin (Dimitrova and Gilbert, 2000).

We demonstrate that nuclear localization and accumulation of GFP-PCNA is a proxy for S phase, in that it does not occur when DNA synthesis is prevented with the DNA polymerase inhibitor aphidicolin. Nor does it occur when eggs are treated with the recombinant ERK phosphatase (XCL-100) or with U0126. Nuclear localisation of GFP-PCNA is unaffected by U0124, an inactive analogue of U0126. The effects on S phase were confirmed by measuring incorporation of [3 H]-thymidine in embryos treated with the MEK inhibitor U0126. Abrogating the ERK pathway with both XCL-100 and U0126 also prevented decondensation of sperm chromatin within the zygote nucleus, as well as nuclear envelope breakdown, and the formation of a bipolar spindle. By contrast, at the concentrations previously used (20 μ M), PD98059 did not mimic the effects of either recombinant XCL100 or U0126. Instead, it provoked a very delayed accumulation of GFP-PCNA in the female pronucleus over a period of 2-3 hours and a correspondingly slow incorporation of [3 H]-thymidine.

We conclude that a rapid, Ca^{2+} induced stimulation of the ERK pathway immediately after fertilization is required for proper activation of the sperm head at fertilization and for triggering S phase and cell cycle progression, including nuclear envelope breakdown and mitotic spindle formation. We discuss these findings in the light of the previous data.

Materials and methods

Materials

U0126 and the polyclonal anti-active MAP kinase antibody were obtained from Promega; Hoechst 33342 dye from Sigma; PD98059, U0124, A23187 and aphidicolin from Calbiochem; MBP, histone H1 (hH1) and the monoclonal anti-active MAPK antibody from New England Biolabs; and rhodamine-labelled tubulin from Cytoskeleton. All other chemicals were from Sigma or BDH.

Gamete handling

All biochemical experiments were carried out in air conditioned room at 16-17°C during the *Lytechinus pictus* breeding season (May-October) to ensure reproducibility. Sea urchin eggs and sperm (*L. pictus*) were collected in artificial sea water (ASW) [410 mM NaCl, 39 mM MgCl₂, 15 mM MgSO₄, 2.5 mM NaHCO₃, 10 mM CaCl₂, 10 mM KCl, 1 mM EDTA (pH 8.0)].

Microinjection procedure and treatment with inhibitors

Proteins or mRNA were microinjected using borosilicate glass micropipettes (Harvard Apparatus GC150F-10) that were manipulated with an Eppendorf micromanipulator. Injections comprised 0.1-0.5% egg volume. Concentrations in the text are final concentrations in the egg. U0126 and U0124 treatments were applied 10-15 minutes before fertilization or ionophore activation.

Hoechst 33342 dye staining in vivo

The embryos were suspended as normal and allowed to undergo the cell cycle. Samples of 100 μ l cell suspension were regularly taken out and stained in vivo for 5 minutes before observation.

Expression constructs and purification of recombinant proteins

GFP-PCNA protein

GFP-fused human PCNA sequence was provided in pENeGFPPCNA2mut (Leonhardt et al., 2000). *Bam*HI-*Xba*I GFPPCNA fragment containing SV40 nuclear localisation signal at the N terminus was subcloned into pBCSK vector and the *Bam*HI-*Sac*I fragment inserted into pCal-n. The construct was confirmed by sequencing. The calmodulin tagged protein was purified on calmodulin affinity resin (Stratagene). For microinjection, the protein was dissolved in microinjection buffer (500 mM KCl; 20 mM PIPES; 100 μ M EGTA; pH 6.8).

GFPPCNA mRNA

GFP-PCNA *Bam*HI-*Not*I fragment from pBCSK+ vector was subcloned into pBluescript RN3 vector (Lemaire et al., 1995). To create pBluescript RN3GFP-PCNA the *Bg*III site was destroyed. Ambion mMessage mMachine kit with T3 RNA polymerase was used for in vitro transcription.

XCL100 protein phosphatase

The XCL100 gene was provided in pGEX vector. XCL100 was then subcloned into pBADB/gIII vector. The pBADXCL100 construct was used in the experiments of Fig. 4. Because of the low yield, in later experiments (Fig. 2) the *Nco*I-*Hind*III fragment from pBADXCL100 was subcloned into pCal-n vector and the protein was purified as described above. For microinjection, the XCL100 phosphatase was dissolved in PBS.

p13^{suc1} protein

The *S. pombe* p13^{suc1} gene was provided in pET21b. After gel filtration purification the protein was coupled to CNBr-Sepharose (Amersham Pharmacia, UK). The beads contained finally about 8 mg p13/ml gel and were used for isolation of cdk1/cyclin B followed by kinase assays. It has been shown (Sumerel et al., 2001; Moreau et al., 1998) that the two other kinases known to interact with p13, cdk2/cyclin E and cdk2/cyclin A do not cycle in sea urchins, which leaves only cdk1/cyclin B to be responsible for the measured activity.

Preparation of sea urchin cell lysates and treatment with U0126

All experiments were carried out in whole sea urchin embryos undergoing the mitotic cell cycle. Cell lysates from *Lytechinus pictus* were prepared as described earlier (Philipova and Whitaker, 1998). Lysates for MAP kinase assays were prepared in T buffer after two washes of eggs/embryos in Ca-free ASW and one wash in T buffer. These for hH1 kinase assays were prepared in H buffer (25 mM MOPS, 15 mM EGTA, 15 mM MgCl₂, 1 mM DTT, supplemented with protease and phosphatase inhibitors, pH 7.2). The final cell suspension contained not more than 50 μ l of eggs in 1 ml ASW. In each experiment, the batch was split in two: half of them as controls and the other half treated. The percentage of cells undergoing nuclear envelope breakdown (NEB) was always determined. Experiments were carried out only with batches of eggs showing at least 95% fertilization. The length of the cell cycle is slightly different for different batches of eggs, meaning that the timing of NEB varies from experiment to experiment. Stock solutions of 50 mM U0126 were always prepared immediately before use and were gradually diluted to 100 μ M and 20 μ M in ASW with vortexing at room temperature. The solution was then cooled down to 16°C.

In vitro protein kinase assays

MAP kinase and hH1 kinase assays were carried out as previously described (Philipova and Whitaker, 1998). The experiments involving inhibition with U0126 were carried out in the following way: the egg suspension was split in two aliquots for control and for U0126

treatment 10 minutes before fertilization (U_{-10}). All kinase assays were performed at the same time and the two gels were washed, dried, exposed and analysed together. Each result is representative of three or more independent experiments.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting experiments were carried out as previously described (Philipova and Whitaker, 1998), except that the immune complexes were not boiled. Instead, they were left for 15 minutes at room temperature in sample buffer and then frozen or loaded onto a gel for PAGE and blotting.

Measurement of [3 H]-thymidine incorporation

Incorporation of [3 H]-thymidine was measured as described previously (Kawahara et al., 2000). Pre-incubation in 1 μ Ci/ml [3 H]-thymidine was carried out for 30 minutes at 16°C.

Fluorescence measurements

In the experiments presented in Fig. 2A and Fig. 3G, GFP-PCNA fluorescence was observed using a Nikon Diaphot 300 inverted microscope with narrow band pass filters (Ex 480 \pm 15 nm, DM 505, Em 520 nm \pm 10 nm) controlled by the filter wheel (Sutter Instrument). Images were collected using a charge-coupled device camera (CCD; Photometrix Coolsnap fx, Roper Scientific) controlled by Metafluor software version 4 (Universal Imaging, West Chester, PA). Where DNA was stained with UV excited Hoechst 33342, labelled eggs were observed using narrow band-pass filters (Ex 355 \pm 50 nm, DM 400, Em 450 nm longpass). Pictures of the fluorescently labelled DNA and the differential interference contrast (DIC) images in Fig. 4 were taken using a Nikon Coolpix 950 digital camera. For CCD camera images the Nikon objective 40 \times /1.3NA oil was used. In the experiments shown in Fig. 1, Fig. 2C, Fig. 3A and Fig. 5, confocal inverted microscope (Leica Lasertechnik GmbH, Heilderberg, Germany) images were obtained. For single excitation scanning of GFP, the 488 nm line of an Argon-Krypton laser and 530 nm long-pass emission filter was used.

Confocal images of rhodamine-labelled tubulin were taken using 568 nm of Argon-Krypton laser line and 590 nm long-pass filter (Fig. 1A). For confocal images the Leica objective 40 \times /1.25 NA oil was used.

Image processing and analysis

Image processing was carried out using Metamorph software to obtain the average pixel intensity of the region of interest of the image. Fluorescence intensities were normalized to the point of earliest fluorescence detection. NEB was normalized to 70 minutes after fertilization.

Results

Aphidicolin-dependent accumulation of GFP-PCNA in the nucleus during S phase

Microinjection of mRNA encoding GFP-PCNA into unfertilized sea urchin eggs led to cell cycle-dependent nuclear accumulation of fluorescence after fertilization. Significant expression of fluorescent protein was seen only towards the end of the first cell cycle. Fig. 1A shows one blastomere of a two-celled embryo completing first mitosis, then undergoing the second cell cycle and entering the third. As the first mitosis ends, GFP-PCNA accumulates in the nuclear membrane-bounded micronuclei that form around the decondensing chromosomes (102'), then in the reformed nucleus once fusion of the micronuclei has taken place. GFP-PCNA disappears from the nucleus as nuclear envelope breakdown occurs and spindle microtubules invade the nucleus (118'). The pattern is repeated in the third cell cycle. These

observations demonstrate a pattern of nuclear accumulation in S phase, which in sea urchin embryos begins before anaphase is complete and a full nucleus reformed (Hinegardner et al., 1964; Ito et al., 1981). The intensity of the accumulated nuclear signal is comparable from one cell cycle to the next. It is also clear that these concentrations of exogenous GFP-PCNA do not themselves interfere with cell cycle progression over three cell division cycles.

In order to study the pattern of GFP-PCNA accumulation during the first cell cycle, we microinjected recombinant GFP-PCNA protein to a concentration of 60 nM. Fig. 1B illustrates the accumulation of chimera during S phase of the first cell cycle. GFP-PCNA is detectable in the male pronucleus as early as 5 minutes after fertilization (not shown) and by 20 minutes it is accumulating in both male and female pronuclei. After pronuclear fusion, GFP-PCNA associated with sperm chromatin is gradually distributed evenly throughout the nucleus. By the time of NEB, there has been a 20-fold accumulation of GFP-PCNA compared with its first detectable levels; at NEB the chimera is released into the cytoplasm. The DNA polymerase α and δ inhibitor aphidicolin, which prevents DNA synthesis in sea urchin embryos (Ikegami et al., 1979; Nishioka et al., 1984), completely abolishes accumulation of GFP-PCNA into both male and female nuclei (Fig. 1B). These data demonstrate that nuclear accumulation of GFP-PCNA is a proxy for DNA synthesis.

The ERK phosphatase XCL100 prevents nuclear accumulation of GFP-PCNA and decondensation of sperm chromatin

The recombinant ERK phosphatase CL100 inactivates ERK1 *in vitro* (Alessi et al., 1993); CL100 was shown to block ERK activation *in vitro* at a concentration of around 2 μ M (Lewis et al., 1995). mRNA encoding the *Xenopus* CL100 homologue, XCL100, injected into G2 arrested *Xenopus* oocytes has been shown to delay or prevent progesterone-induced meiotic maturation (Sohaskey and Ferrell, 2002). It has been reported that much lower concentrations (75 nM) of XCL100 have no apparent effect on mitotic progression (Wang et al., 1997).

Microinjection of the recombinant phosphatase to a final concentration of 1.5 μ M had a small but not significant effect on nuclear localization of GFP-PCNA, while a concentration of 2.5 μ M significantly ($P < 0.0001$, Fig. 2B) reduced nuclear accumulation to around 20% of controls at 30 minutes, 12% at 50 minutes and 20% at the time of NEB, 65 minutes (Fig. 2A,B). GFP-PCNA fluorescence in zygotes injected with 2.5 μ M XCL100 was not uniformly distributed (see Fig. 2A, arrow). As in untreated zygotes (Fig. 1B), residual GFP-PCNA fluorescence was brightest in the region of sperm chromatin, presumably reflecting its lesser degree of decondensation relative to the female-derived chromatin in the zygote nucleus. NEB did not take place in embryos injected with 2.5 μ M XCL 100.

These data indicate by proxy that the ERK phosphatase prevents DNA synthesis and suggest that inactivation of ERK1 prevents decondensation of sperm chromatin within the zygote nucleus.

XCL 100 prevents nuclear GFP-PCNA accumulation in eggs activated with the Ca $^{2+}$ ionophore A23187

Treating unfertilized sea urchin eggs with the Ca $^{2+}$ ionophore A23187 generates a Ca $^{2+}$ transient comparable in magnitude to

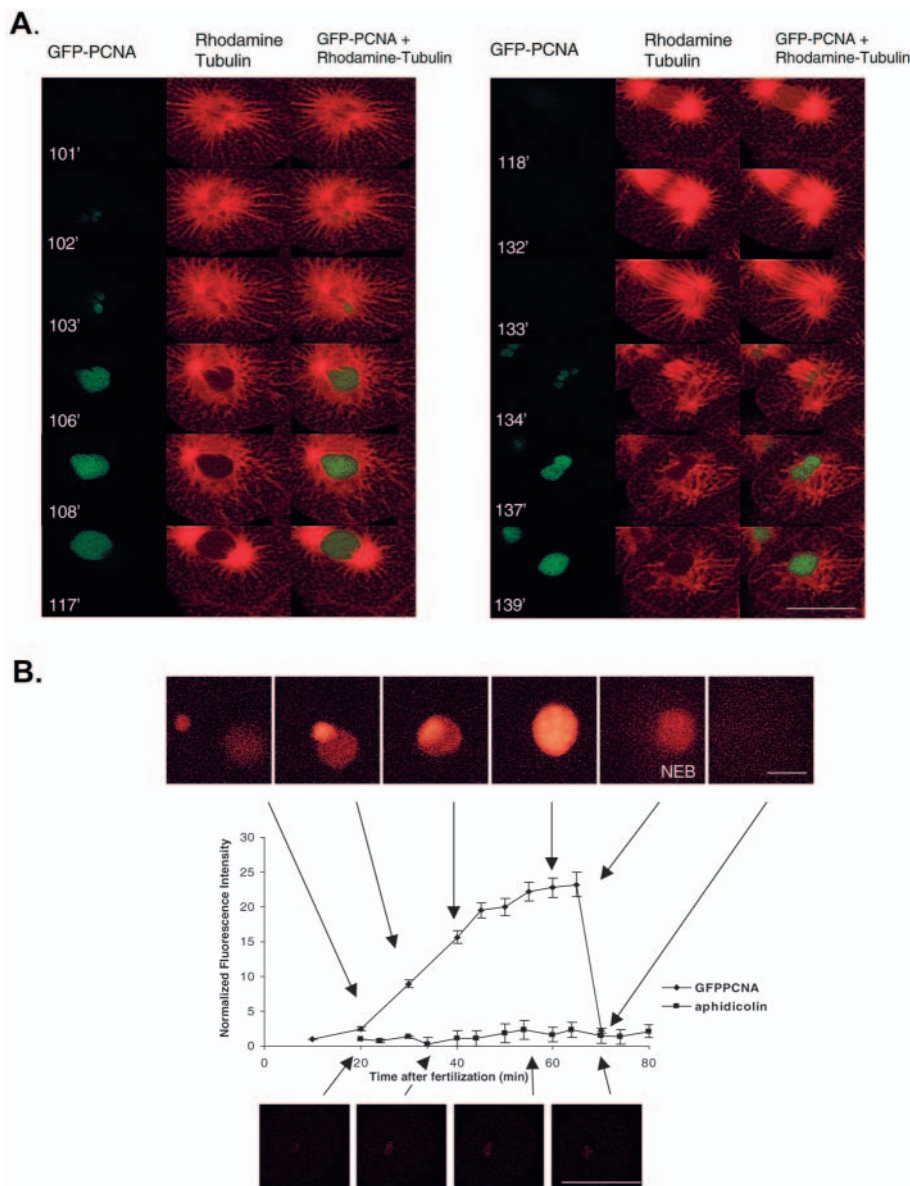


Fig. 1. GFP-PCNA accumulates in the embryonic nucleus during S phase through an aphidicolin-dependent process. (A) mRNA encoding GFP-PCNA (green, final concentration 0.0013 $\mu\text{g}/\mu\text{l}$), together with rhodamine-labelled tubulin (red, final conc. 0.01 $\mu\text{g}/\mu\text{l}$) were microinjected 100 minutes before two-channel confocal image recording. Time after fertilization is indicated. Second cell cycle, 101–133 minutes; S phase of the third cell cycle, 134–139 minutes. Scale bar: 50 μm . (B) GFP-PCNA protein was microinjected before fertilization. Confocal images of the nuclei at various times during the first cell cycle of control embryos (upper panel; scale bar, 15 μm) and embryos treated with aphidicolin (20 $\mu\text{g}/\text{ml}$ at 15 minutes pre-fertilization: lower panel; scale bar, 100 μm) are shown. The measured average intensity of fluorescence of the female nucleus throughout the first cell cycle in both conditions is also shown. The time of each image corresponds to the time indicated on the graph. Aphidicolin caused a cell cycle block and NEB was not observed. The graph shows mean \pm s.e.m. from six control experiments and from three experiments with aphidicolin treatment. For each experiment a different female was used.

that at fertilization and leads to the activation of ERK1, onset of S phase and then nuclear envelope breakdown, though no spindle forms in the absence of the centrosomes that are donated by the sperm at fertilization (Philipova and Whitaker, 1998; Whitaker and Steinhardt, 1982). When unfertilized eggs

injected with GFP-PCNA are activated using 20 μM A23187, GFP-PCNA accumulates in the nucleus as it does in fertilized eggs, though its onset was advanced slightly relative to fertilized eggs (Fig. 2C). The extent of accumulation by the time of NEB in A23187-treated eggs is comparable with that in fertilized eggs.

Microinjection of XCL-100 to a concentration of 2.5 μM prevented A23187-induced nuclear accumulation of GFP-PCNA (Fig. 2C).

These results indicate that Ca^{2+} -triggered ERK1 activation is required for accumulation of GFP-PCNA in the nucleus.

Nuclear accumulation of GFP-PCNA, [^3H]-thymidine incorporation and MAP kinase activation are all prevented by U0126

The soluble MEK inhibitor U0126 has been shown to act specifically to block ERK activation at concentrations of 50–100 μM (Gross et al., 2000; Walter et al., 2000). Inhibition of ERK activation during mitosis has been reported to require 100–400 μM U0126 (Chung and Cheng, 2003; Walter et al., 2000). We added U0126 to unfertilized eggs suspended in ASW and fertilized the eggs 10 minutes later. Fig. 3A,B shows that a concentration of 20 μM U0126 did not significantly alter the progressive localization of GFP-PCNA in the male and female pronuclei and the zygote nucleus. By contrast, 100 μM U0126 completely abolished the localization, while the inactive analogue at the same concentration was without effect. Neither 20 μM nor 100 μM U0126 when alone added to unfertilized eggs led to nuclear accumulation of GFP-PCNA (Fig. 3B). U0126 (100 μM) also completely blocked the incorporation of [^3H]-thymidine into DNA in fertilized eggs (Fig. 3C). This concentration of U0126 did not stimulate [^3H]-thymidine incorporation when added to unfertilized eggs in the absence of sperm (Fig. 3D). U0126 (100 μM) prevented the post-fertilization increase in MAP-kinase activity (Fig. 3E). We have previously shown that the increase in MAP kinase activity immediately after fertilization is due to activation of a sea urchin ERK1 (Philipova and Whitaker, 1998). Fig. 3F confirms that the phosphorylation of ERK1 dimers (Cobb and Goldsmith, 2000; Khoklatchev et al., 1998) correlates with measured MAP kinase activity, being absent in eggs treated with 100 μM U0126.

These data demonstrate that MEK inhibition by U0126 prevents activation of ERK1, the onset of DNA synthesis and nuclear localization of GFP-PCNA after fertilization, suggesting that activation of the ERK signalling pathway is necessary for DNA synthesis. They also independently confirm that the nuclear localization and accumulation of GFP-PCNA correlates with the presence or absence of DNA synthesis in sea urchin embryos. We found no evidence that inhibition of the MEK pathway in unfertilized eggs led to DNA synthesis measured either as nuclear accumulation of GFP-PCNA or [³H]-thymidine incorporation.

U0126 prevents nuclear GFP-PCNA accumulation and [³H]-thymidine incorporation in eggs activated with Ca²⁺ ionophore

Treatment with the Ca²⁺ ionophore A23187 leads to GFP-PCNA accumulation in the nucleus. Accumulation in the nucleus induced by A23187 is prevented by incubation with 100 μM U0126 (Fig. 3G). A23187 stimulates incorporation of [³H]-thymidine into DNA (Nishioka and Magagna, 1981) (Fig. 3H); as with GFP-PCNA accumulation, the onset of incorporation appears to be slightly earlier than in fertilized eggs, though the reasons for this are unknown. U0126 (100 μM) also prevents A23187-induced incorporation of [³H]-thymidine into DNA (Fig. 3H).

These data are consistent with our finding that ERK1 activation at fertilization can be triggered by A23187 (Philipova and Whitaker, 1998) and confirm that the ERK pathway is essential for the initiation of DNA synthesis.

XCL100 and U0126 have identical effects on sperm chromatin decondensation within the zygote nucleus

We investigated the effects of ERK pathway inhibition on sperm chromatin decondensation using both U0126 and XCL100. Fig. 4 shows that sperm chromatin is readily visible as a region of accented staining with the DNA dye Hoechst 33342 at 25 minutes after fertilization and that by 55 minutes sperm chromatin has dispersed within the nucleus, giving uniform staining with H33342. By contrast, both 2.5 μM XCL100 and 100 μM U0126 remarkably delay sperm chromatin dispersal with the zygote nucleus. At 70 minutes, when, in control

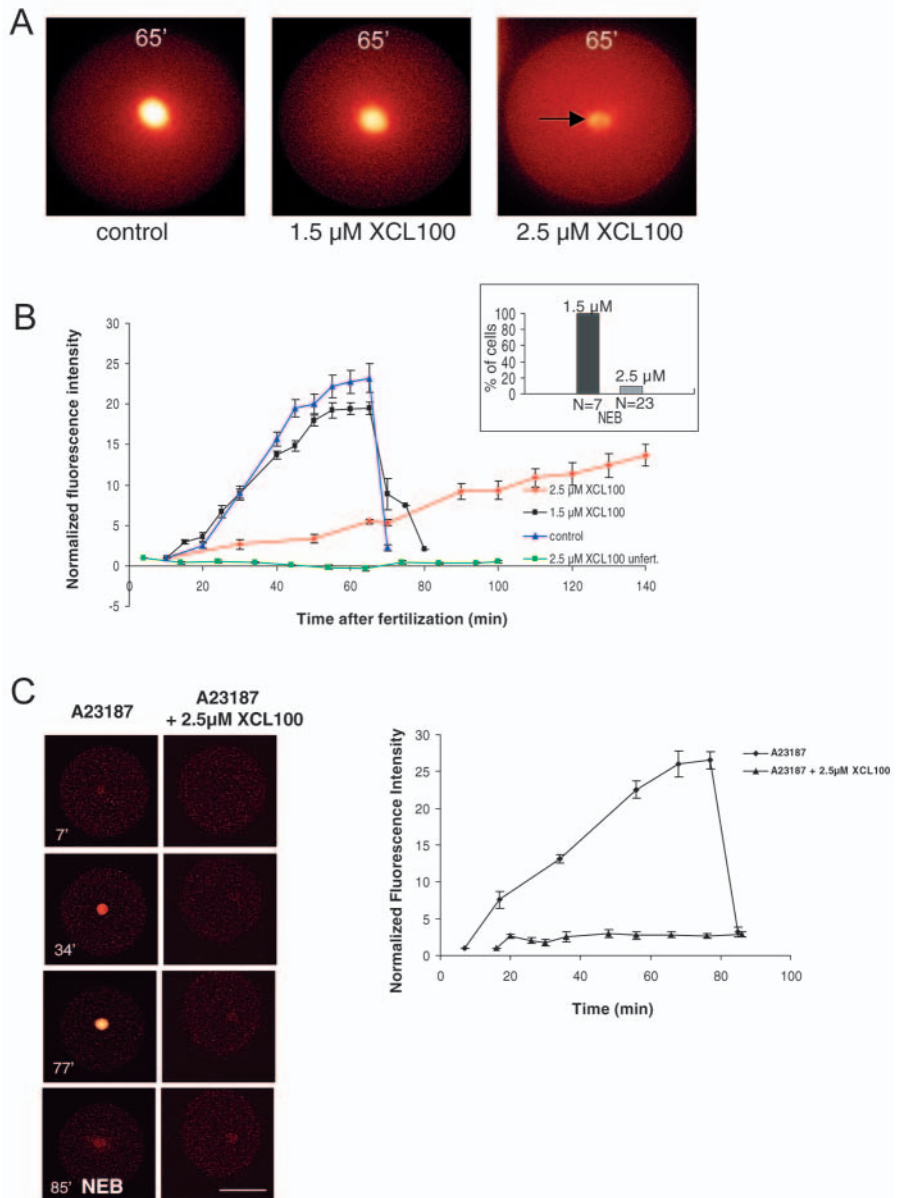


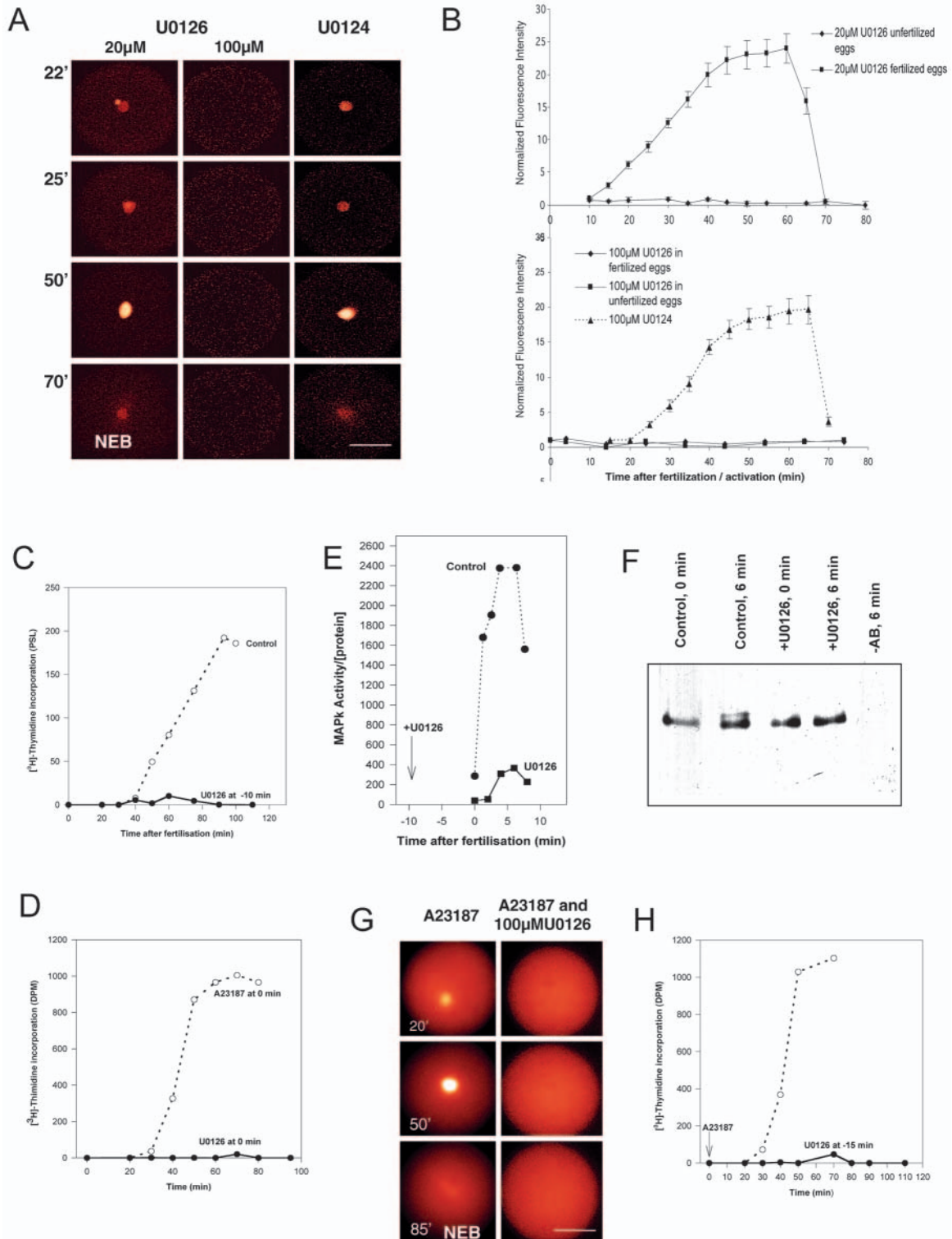
Fig. 2. XCL100 phosphatase prevents both sperm chromatin decondensation in early embryos and nuclear accumulation of GFP-PCNA in embryos and Ca²⁺ ionophore-activated eggs. (A) Wide-field fluorescence microscopy of embryos microinjected with GFP-PCNA (control) and, in addition, with XCL100 (1.5 μM and 2.5 μM final concentration) before fertilization and imaged at 65 minutes after fertilization, just before NEB. XCL100 (2.5 μM) blocked the cell cycle and NEB did not subsequently occur. Arrow indicates uncondensed sperm chromatin. (B) Nuclear GFP-PCNA fluorescence intensity (mean ± s.e.m., control, *n*=6; 1.5 μM XCL100, *n*=3; 2.5 μM XCL100, *n*=4; 2.5 μM XCL100 unfertilized eggs, *n*=3) measured during the course of the cell cycle in the experiments shown in A. Five different females were used in experiments with embryos (graphs and bars) and three females in the experiments with unfertilized control eggs. Inset shows that 2.5 μM XCL100 prevented NEB. (C) Confocal images of nuclear GFP-PCNA in control (*n*=3) and XCL100-microinjected eggs (*n*=4) activated with A23187 (20 μM). The time-course of GFP-PCNA fluorescence intensity in the nucleus is also shown. NEB was not observed in the presence of XCL100. Scale bar: 50 μm.

embryos, chromatin is beginning to condense throughout the nucleus just before NEB, treated embryos still maintain sperm chromatin in an undispersed state. Treated embryos fail to undergo NEB and show evidence of continued failure to

disperse sperm chromatin even at 120 minutes, when control embryos are undergoing first cleavage and are in S phase of the second embryonic cell cycle. Evidence of a degree of chromatin condensation comparable with control embryos at 70 minutes is seen only at 170 minutes inside a still intact nucleus, when control embryos are in S phase of the third cell cycle. No mitotic spindle formed in the embryos treated with U0126 or microinjected with XCL100, an observation that we

confirmed using embryos injected with rhodamine-labelled tubulin (data not shown).

These data confirm that inhibition of the ERK pathway prevents dispersal of the sperm chromatin within the zygote nucleus and demonstrate that the low molecular weight U0126 and the recombinant protein XCL100 give rise to an identical phenotype with identical timing. They also indicate that inhibition of the ERK pathway arrests the cell cycle at some



point before nuclear envelope breakdown and spindle formation.

Activation of Cdk1 at mitosis is prevented by treatment with U0126

It has been established that preventing DNA synthesis using aphidicolin in sea urchin embryos prevents nuclear envelope breakdown and formation of bipolar mitotic spindles (Nishioka et al., 1984). Full activation of Cdk1 at the time of mitosis does not occur in the presence of aphidicolin (Patel et al., 1997), though active Cdk1 gradually accumulates to levels seen during mitosis over a period of 2 hours [(Genevriere-Garrigues et al., 1995; Hinchcliffe et al., 1999); our unpublished data]. We therefore measured both ERK1 and Cdk1 activities in embryos treated with U0126 at the time that control embryos were undergoing mitosis. We found, as expected, that ERK1 activation at the time of mitosis (Philipova and Whitaker, 1998) was prevented by incubation with 100 μM U0126, as was Cdk1 activity (see Fig. S1 in the supplementary material).

These observations indicate that inhibition of the ERK pathway immediately after fertilization prevents DNA synthesis and that, as expected, blocking DNA synthesis prevents the timely activation of Cdk1 (Genevriere-Garrigues et al., 1995).

The effects of the ERK pathway inhibitor PD98059 do not mimic those of the recombinant ERK phosphatase, in marked contrast to U0126

PD98059 is an inhibitor of MEK at concentrations of 20 μM . It has been shown to induce DNA synthesis in unfertilized sea urchin eggs using a fluorescence method involving the use of bromo-deoxyuridine (Carroll et al., 2000). We added 20 μM PD98059 to unfertilized eggs microinjected with GFP-PCNA. We found a very slow, aphidicolin-sensitive accumulation of GFP-PCNA in the egg pronucleus. Accumulation at 55 minutes, the time at which accumulation had reached 20-fold in fertilized controls, was comparable with that detected at 15 minutes in these controls (Fig. 5A,B). Nuclear levels then rose to around 15-fold by 130 minutes after addition, before levelling off. These results were confirmed by measurements of [^3H]-thymidine incorporation in unfertilized eggs treated with 20 μM PD98059 (Fig. 5C).

Aphidicolin prevented PD98059-induced nuclear

accumulation of GFP-PCNA in unfertilized eggs (Fig. 5B), demonstrating once more that this accumulation is a reliable proxy for DNA synthesis.

Microinjection of 2.5 μM XCL100 into unfertilized eggs did not lead to nuclear accumulation of GFP-PCNA (Fig. 2B), unlike PD98059. Neither did U0126 cause nuclear accumulation of GFP-PCNA (Fig. 3B).

PD98059 at the same concentration (20 μM) was not able to prevent DNA synthesis in fertilized eggs, as judged by nuclear accumulation of GFP-PCNA (Fig. 5D).

These results demonstrate that the effects of PD98059 do not in any way resemble those of the MAP kinase phosphatase XCL-100, unlike the effects of U0126, which are identical to those of XCL-100.

Discussion

Nuclear localization of GFP-PCNA is a proxy for DNA synthesis

Our observations using GFP-PCNA are entirely consistent with the reported close correlation between PCNA binding to chromatin and the onset of DNA synthesis. In each experiment in which we measured both [^3H]-thymidine incorporation and nuclear accumulation of GFP-PCNA, we found accumulation when incorporation could be registered and no accumulation when it could not.

We did not observe discrete foci of GFP-PCNA within the nucleus that might correspond to spatially clustered replicons with coordinated replication timing, such as were seen with GFP-PCNA in mammalian cell nuclei (Leonhardt et al., 2000; Somanathan et al., 2001). In non-mammalian early embryos (*Xenopus*, *Drosophila*), replication origins are closely spaced at 10 kb (DePamphilis, 1999) and replicate simultaneously. This might explain the lack of focal sites in sea urchin embryos.

The accepted view in the field is that the onset of DNA synthesis occurs after syngamy, that is, the fusion of male and female pronuclei (Longo and Plunkett, 1973). However, we observe substantial accumulation of GFP-PCNA in the male pronucleus some time before pronuclear fusion. Accumulation can be prevented by aphidicolin. This implies that it is the formation of replication complexes rather than an excess of GFP-PCNA that lead to accumulation of GFP-PCNA in the male pronucleus. GFP-PCNA fluorescence intensity is fivefold higher in the male pronucleus compared with the female

Fig. 3. U0126 inhibits nuclear accumulation of GFP-PCNA, activation of ERK1, and DNA synthesis in early embryos (A-F) and also in ionophore-activated eggs (G-H). (A) Nuclear accumulation of GFP-PCNA and subsequently NEB are prevented by 100 μM U0126, but not by 20 μM U0126 or 100 μM U0124 in early embryos. Times after fertilization are indicated. Scale bar: 50 μm . (B) Intensity of GFP-PCNA nuclear fluorescence in early embryos treated as in A and, in addition, in unfertilized eggs incubated in 20 μM and 100 μM U0126. Each graph represents the mean fluorescence value from three to five different experiments; in each experiment different females were used. (C) DNA synthesis is prevented by 100 μM U0126 in embryos. Incorporation of [^3H]-thymidine in embryos (control, white circle; treated with U0126, black circle). (D) U0126 alone does not trigger DNA replication in unfertilized eggs (black circle), but treatment with the Ca^{2+} ionophore A23187 (20 μM) as a control does induce DNA synthesis (white circle). (E) U0126 (100 μM) applied 10 minutes before fertilization abolishes the post-fertilization peak in ERK1 activity. We measured MAP kinase activity in cell extracts during the first 8 minutes after fertilization: control, black circle; after U0126 treatment, black square. (F) Western blotting of immunoprecipitated active MAP kinase dimers, detected with anti-dual phosphorylated MAP kinase antibody (New England Biolabs). Control, 0 and 6 minutes; 100 μM U0126 treated (+U0126), 0 and 6 minutes; control immunoprecipitation in the absence of antibody (-AB, at 6 minutes). (G) Wide-field fluorescence images and intensity of nuclear fluorescence of unfertilized eggs microinjected with GFP-PCNA, incubated in 100 μM U0126 and activated with Ca^{2+} ionophore (A23187, 20 μM), together with controls. Nuclear fluorescence was not observed in eggs treated with U0126. Scale bar: 50 μm . (H) Incorporation of [^3H]-thymidine in unfertilized eggs treated with 20 μM Ca^{2+} ionophore A23187. Ionophore activated eggs (white circle); eggs treated with U0126 15 minutes before ionophore activation (black circle).

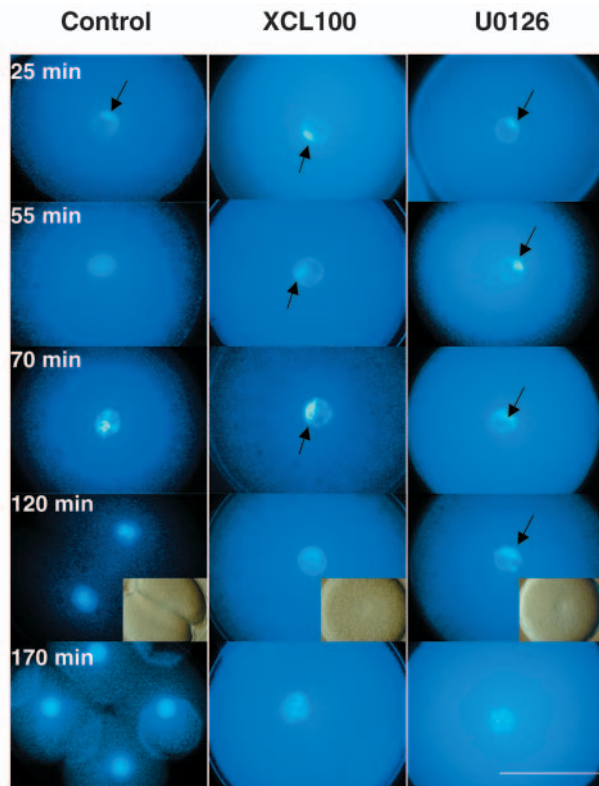


Fig. 4. XCL100 and U0126 inhibit decondensation of sperm chromatin after pronuclear fusion. Hoechst 33342 dye DNA staining of control embryos, embryos microinjected with 2.5 μM recombinant XCL100 and embryos incubated with 100 μM U0126. The times indicated in the Control panels apply to all three conditions. Arrows indicate sperm chromatin within zygote nucleus. Inset at 120 minutes shows the control embryo undergoing cytokinesis, while the XCL100- and U0126-treated embryos have not undergone NEB. Scale bar: 50 μm .

pronucleus prior to syngamy. This difference is almost certainly due to the difference in the density of chromatin in the two pronuclei, as the DNA-staining Hoechst dye 33342 shows a similar four- to sevenfold higher intensity in the male pronucleus (not shown).

We were keen to confirm that the distribution of GFP-PCNA after fertilization mirrored that of the endogenous sea urchin protein. However, none of three different antibodies raised against mammalian PCNA crossreacts with the sea urchin protein. The concordance of our data with those obtained using the same construct in HeLa cells (Leonhardt et al., 2000) and the effects of aphidicolin on nuclear accumulation nonetheless gives us confidence that the PCNA chimera reflects DNA synthetic activity.

It has been previously shown that cyclin E is associated predominantly with the male pronucleus of the sea urchin zygote; it appears in the female pronucleus only after syngamy (Schnackenberg and Marzluff, 2002). However, cyclin A/Cdk2 is found to be predominantly localized to the female pronucleus (Moreau et al., 1998; Schnackenberg and Marzluff, 2002). The female pronucleus is able to initiate DNA synthesis in the absence of sperm, if activated by Ca^{2+} ionophore. In other words, it possesses the machinery necessary for

activation of DNA synthesis. Further work is needed to test the possible roles of Cdk2, cyclin E and cyclin A in the formation of replication complexes and initiation of S phase after syngamy.

ERK1 activation at fertilization is required for nuclear accumulation of GFP-PCNA, the initiation of DNA synthesis and progress through the cell cycle

Microinjection of recombinant XCL-100, an ERK phosphatase, prevented accumulation of GFP-PCNA in both male and female pronuclei. This implies that formation of replication complexes requires the activation of ERK1 after fertilization and suggests a mechanism in which activation of ERK1 promotes the accumulation of PCNA and possibly cyclin E in the male pronucleus.

The effects of the MEK inhibitor U0126 are identical to those of the ERK phosphatase XCL-100. Both prevent nuclear accumulation of GFP-PCNA, decondensation of male chromatin within the zygote nucleus and the formation of a bipolar spindle. Experiments with U0126 demonstrate also that blocking activation of the ERK pathway prevents incorporation of [^3H]-thymidine into DNA. The increases in ERK1 and Cdk1 activity that would accompany mitosis are absent in embryos treated with the MEK inhibitor U0126. Overall, the results presented here suggest that the first embryonic cell cycle arrests before S phase in the absence of ERK1 activation at fertilization.

The effects of another MEK inhibitor, PD 98059, do not conform to the effects of the ERK phosphatase XCL-100. The differences observed between the effects of PD98059 and XCL-100 can be summarised as follows: PD98059 did not prevent nuclear accumulation of GFP-PCNA after fertilization or ionophore treatment, unlike XCL-100; PD98059 did not prevent male pronuclear decondensation and bipolar spindle formation, unlike XCL-100; PD98059 induced a slow accumulation of GFP-PCNA in the nucleus of unfertilized eggs, unlike XCL-100. Thus, PD98059 acts on a target distinct from that targeted by XCL-100 and U0126. It has been shown that PD98059 is less effective than U0126 as an inhibitor of the MEK-ERK pathway (Favata et al., 1998; Gross et al., 2000; Pitts et al., 1998).

We confirm the finding (Carroll et al., 2000) that treatment of unfertilized eggs with PD98059 leads to the events of DNA synthesis. However, we show that PD98059 does not induce a physiological S phase: the onset of DNA synthesis and its rate of increase lags very substantially behind that observed in fertilized (diploid) or ionophore-activated (haploid) eggs. These observations demonstrate that PD98059 is acting on a different aspect of the ERK signalling pathway than the ERK phosphatase XCL-100 and the MEK inhibitor U0126. In fact, it has previously been shown that 2.5 μM PD98059 was sufficient to induce a fall in phosphorylated ERK in unfertilized eggs comparable with that measured at fertilization (Carroll et al., 2000). We have determined that this concentration is not sufficient to induce any detectable replication complex formation over a period of 2.5 hours. An effect of DNA synthesis has been reported previously only at 20 μM (Carroll et al., 2000), as confirmed in this study. There is thus no correlation between a fall in measured ERK activity and DNA synthesis induced by PD98059.

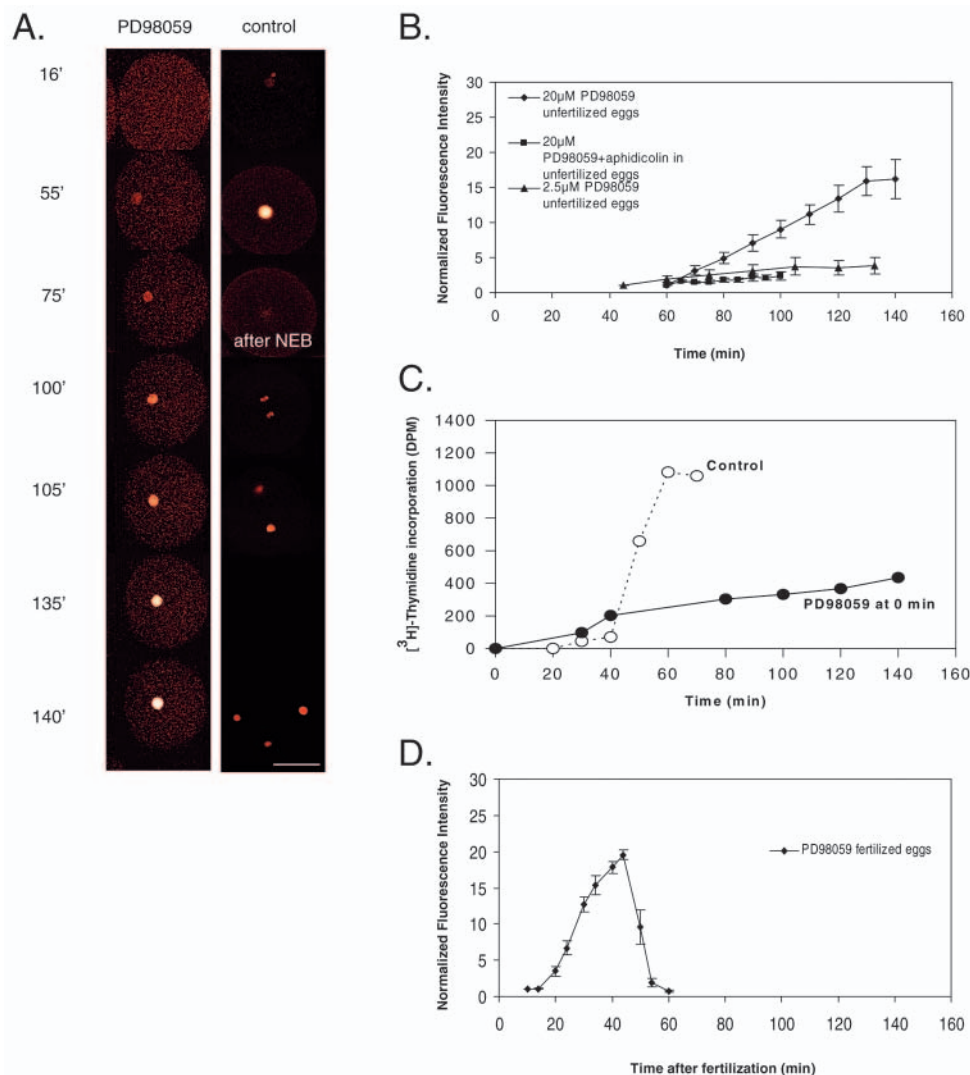


Fig. 5. Effects of PD98059 on nuclear accumulation of GFP-PCNA in both unfertilized and fertilized eggs. GFP-PCNA fusion protein was microinjected into sea urchin eggs and the fluorescence intensity was observed in the presence of MEK inhibitor PD98059. (A) Confocal images of GFP-PCNA nuclear fluorescence in 20 μM PD98059-treated and control eggs. Scale bar: 50 μm . (B) Intensity of nuclear GFP-PCNA fluorescence in unfertilized eggs treated with 2.5 μM and 20 μM PD98059, and with 20 μM PD98059 in the presence of aphidicolin (20 $\mu\text{g}/\text{ml}$ 15 minutes before addition of PD98059). (C) $[^3\text{H}]$ -Thymidine incorporation in unfertilized eggs treated with ionophore (Control) or 20 μM PD98059. Representative of three independent experiments. (D) PD98059 (20 μM) does not inhibit the nuclear accumulation of GFP-PCNA in embryos after fertilization. Each graph in B and D represents mean \pm s.e.m. from three or five experiments; in each experiment different females were used.

The role of MAP kinase at fertilization in sea urchin eggs: reconciliation and a synthesis

The inhibition of nuclear GFP-PCNA uptake in embryos by an ERK phosphatase implies that ERK activation is necessary for the onset of DNA synthesis and so supports the original observation that ERK1 activity increases rapidly and transiently immediately after fertilization (Philipova and Whitaker, 1998). This study found that the major in-gel kinase activity was due to a 44 kDa protein that was identified by peptide sequencing as ERK1. The post-fertilization increase was accounted for by threefold increase in MBP phosphorylation that was immunoprecipitable using an anti-ERK1 antibody, comparable with that seen during mitosis. A contemporary study reported a 50% post-fertilization increase in MBP activity followed by another peak at mitosis, though the increase was not reflected in phosphorylation of GST-Myc (Chiri et al., 1998). A later study reported a small though not significant increase at 5 minutes after fertilization using an anti-phosphoERK antibody (Carroll et al., 2000). Statistical analysis of the data from this study indicates that it would be predicted to detect a threefold increase with statistical significance with 80% power, that is, four out of five times. In

parallel experiments, no increase in activity was detected using immunoprecipitation with a pan anti-ERK monoclonal and Elk1 as substrate. A further study (Kumano et al., 2001) confirmed the report of a 43 kDa MAP kinase active in unfertilized eggs and falling after fertilization or Ca^{2+} ionophore treatment. The activity was sensitive to low (0.5 μM) doses of U0126 and did not reappear during mitosis. The authors conclude that this activity is distinct from the activity we reported previously (Philipova and Whitaker, 1998) and measure in the present study. We concur and also conclude that this distinct activity does not control DNA synthesis, as we show here that treatment of unfertilized eggs with U0126 induces neither GFP-PCNA accumulation nor incorporation of $[^3\text{H}]$ -thymidine.

Our current cell physiology data point to an increase in ERK1 activity as essential at fertilization. Can the differences in the biochemical data be explained? All three studies used different antibodies to identify and/or immunoprecipitate a 43-44 kDa protein with ERK substrate specificity. Only one study determined that the major component of MBP activity was ERK1 by peptide sequencing (Philipova and Whitaker, 1998). This study measured soluble ERK1 activity, while the other

two used whole cell samples. It appears therefore that it is soluble ERK1 activity that correlates with activation of DNA synthesis after fertilization.

In mammalian somatic cells, it has been shown that activation of Cdk2 is accompanied by the formation of MAP kinase-Cdk2 complexes and that inactivation of MAP kinase prevents activation and nuclear localization of Cdk2 and entry into S phase (Blanchard et al., 2000). Cdk2 association with cyclins E and A are essential for entry and progression through S phase (Coverley et al., 2002) and ERK activation controls the activating phosphorylation of Cdk2 Thr-160 (Chiariello et al., 2000). Sea urchin eggs are arrested in a G1-like stage before fertilization, unlike most other oocytes (Whitaker, 1996). So although a fall in ERK activity is detected in frog, ascidian and mouse oocytes as they exit a meiotic division, it would be predicted that sea urchin eggs, by analogy with somatic cells, would exhibit an increase in ERK activation as they enter S phase after fertilization (Whitaker, 1996; Whitaker and Patel, 1990; Whitaker and Steinhardt, 1982).

The Ca²⁺ signal is alone sufficient to induce the nuclear accumulation of GFP-PCNA and the onset of DNA synthesis in unfertilized eggs

We have previously demonstrated that treatment of eggs with the Ca²⁺ ionophore A23187 activates ERK1 (Philipova and Whitaker, 1998). A23187 generates a Ca²⁺ transient in eggs comparable with that recorded at fertilization (Whitaker and Steinhardt, 1982). Here, we show that the ionophore causes nuclear accumulation of GFP-PCNA and incorporation of [³H]-thymidine into DNA (Steinhardt and Epel, 1974; Whitaker and Steinhardt, 1982). Nuclear accumulation of GFP-PCNA in response to A23187 is prevented by microinjection of the recombinant ERK phosphatase XCL-100 and also by treatment with the MEK inhibitor U0126, as is [³H]-thymidine incorporation into DNA. Thus, the activating Ca²⁺ signal causes the onset of DNA synthesis because of activation of the ERK1 pathway.

It has been known for some time that syngamy is not an essential pre-requisite for DNA synthesis in the female pronucleus (Longo and Plunkett, 1973). The role of cyclin E in parthenotes is unclear. It has been suggested that cyclin E/Cdk2 activity may relicense replication in the male pronucleus and that the DNA in female pronucleus may have been relicensed during meiosis (Schnackenberg and Marzluff, 2002). It may be that in parthenotes, the assembly of replication complexes and the onset of processive DNA synthesis is under the sole control of cyclin A/Cdk2 (Schnackenberg and Marzluff, 2002).

Conclusion

This study demonstrates that GFP-PCNA can be used as a surrogate marker for S phase and that the ERK1 pathway activated by the fertilization Ca²⁺ transient controls the onset of DNA synthesis and cell cycle progression in sea urchin embryos.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/3/579/DC1>

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