Effect of wortmannin, an inhibitor of phosphatidylinositol 3-kinase, on the first mitotic divisions of the fertilized sea urchin egg

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SUMMARY

We have reported earlier that the polyphosphoinositide messenger system may control mitosis in sea urchin eggs. Besides phospholipase C activation and its second messengers, phosphatidylinositol (PI) 3-kinase has been proposed to affect a wide variety of cellular processes in other cellular systems. Therefore, we have investigated whether PI 3-kinase could play a role in regulating the sea urchin early embryonic development.

Our data presented here suggest that PI 3-kinase is present in sea urchin eggs. We found that wortmannin, an inhibitor of PI 3-kinase, led to arrest of the cell cycle. Chromosome condensation, nuclear envelope breakdown, microtubular aster polymerization, protein and DNA synthesis were not affected when fertilization was performed in the presence of the drug. However, maturation-promoting factor (MPF) activation was inhibited and centrosome duplication was perturbed preventing the formation of a bipolar mitotic spindle in wortmannin treated eggs. We discuss how PI 3-kinase might be involved in the cascade of events leading to the first mitotic divisions of the fertilized sea urchin egg.

Key words: Phosphatidylinositol 3-kinase, MPF, Mitosis, Egg, Sea urchin

INTRODUCTION

In all eukaryotes, cell cycle progression is under the control of an ubiquitous protein kinase, the maturation-promoting factor (MPF), composed of the cdc2 protein kinase (p34^{cdc2}) associated with a B-type cyclin (reviewed by Dorée and Galas, 1994; Draetta, 1993; Nigg, 1993; Pelech et al., 1990). Much of our understanding of the molecular mechanisms that regulate MPF activity comes from investigations of oocyte maturation and egg fertilization (reviewed by Kishimoto, 1996). After maturation, oocytes from many species including frog, starfish, and almost all vertebrates, are arrested at metaphase of meiosis I or II, a stage at which fertilization can occur (reviewed by Sagata, 1996). Unfertilized sea urchin eggs are arrested after completion of meiosis, at the START control point in G$_1$ that governs entry into S phase (Whitaker and Patel, 1990). After fertilization of these eggs, several cell cycle events can easily be followed: chromosome condensation during prophase, nuclear envelope breakdown (NEB) at entry in prometaphase, EXIT from mitosis at the metaphase-anaphase transition, and cytokinesis. The following embryonic cell cycles alternate rapidly between S-phase and M-phase.

Another protagonist that is involved in cell cycle regulation is calcium. Increases in intracellular free calcium (Ca$_i$) have been shown to be correlated with cell-cycle events in sea urchin eggs (Poenie et al., 1985; reviewed by Whitaker and Patel, 1990) and various other cell types (reviewed by Hepler, 1992): pronuclear migration, NEB, metaphase-anaphase transition, and cytokinesis. Recently, we reported that the PPI messenger system (reviewed by Downes and McPhee, 1990 and Bansal and Majerus, 1990) oscillates during the first mitotic divisions of the fertilized sea urchin egg. We observed that, at the time of mitosis, a phospholipase C (PLC) activity is stimulated (Ciapa et al., 1994), which hydrolyses phosphatidylinositol bisphosphate (PIP$_2$) to generate inositol trisphosphate (IP$_3$), responsible for the release of Ca$_i$, and diacylglycerol (DAG), activator of protein kinase C (reviewed by Divecha and Irvine, 1995). A PI kinase activity, which leads to the generation of phosphatidylinositol phosphate (PIP) (reviewed by Carpenter and Cantley, 1996), is also stimulated during mitosis (Ciapa et al., 1994; Pesando et al., 1995). These events lead to the cyclic increases in IP$_3$ that correspond to the mitotic fluctuations of Ca$_i$ (Ciapa et al., 1994). These cyclic variations of Ca$_i$ and PPI turnover were apparently independent of the activation of MPF (Ciapa et al., 1994), since they were not inhibited by protein synthesis inhibitors that block cyclin synthesis and prevent cell cycle progression in sea urchin embryos (reviewed by Whitaker and Patel, 1990). Clearly, two sets of events act in concert to control mitosis. One of these sets includes MPF and is suppressed by inhibitors of protein synthesis. The other

involves the centrosome duplication cycle (reviewed by Bailly and Bornens, 1992), fluctuations in Ca_i and PPI messenger system, and is independent of protein synthesis. How these two sets of events are interconnected is still unresolved.

PPI may then play an essential role at points of the cell cycle other than that stimulated by external factors, i.e. at times other than that of maturation or fertilization of oocytes where an increased turnover of all PPI was observed (Borg et al., 1992; Ciapa and Whitaker, 1986; Ciapa et al., 1992). Besides PLC activation and its second messengers as quoted above, a new class of lipids formed by a PI 3-kinase has emerged: the 3-phosphorylated inositol lipids (reviewed by Divecha and Irvine, 1995). PI 3-kinase is composed of two subunits (reviewed by Carpenter and Cantley, 1996), a regulatory 85 kDa subunit, which contains the SH2 domains, and a 110 kDa subunit displaying the catalytic site that confers sensitivity to wortmannin (Wt), a potent inhibitor of PI 3-kinase (Arcaro and Wymann, 1993; Okada et al., 1994; Shpetner et al., 1996). Although the role as second messengers of the 3-phosphorylated inositol lipids is still not fully understood, it is clear that PI 3-kinase is found in virtually all cell types. By treating cells with Wt, it has been shown that PI 3-kinase can affect a wide variety of cellular processes, including the oxidative burst in neutrophils, chemotaxis, formation of membrane ruffles, the endocytic pathway, and mitogenesis (reviewed by Carpenter and Cantley, 1996; Panayotou and Waterfield, 1992; Parker, 1995; Sheperd et al., 1996; Shpetner et al., 1996). At the molecular level, PI 3-kinase has been reported to affect the activities of p70S6 kinase (Karnitz et al., 1995), a number of PKC isoforms (Toker and Cantley, 1997) and MAP kinase (Cross et al., 1994).

For all these reasons, we have tested whether PI 3-kinase could be detected in sea urchin eggs and whether cellular events induced after fertilization could be altered by Wt. We found that treatment of eggs with this drug did not alter fertilization, but led to arrest of the cell cycle after chromosome condensation and NEB had occurred. MPF activation, centrosome duplication and formation of a bipolar mitotic spindle were inhibited by Wt, while protein and DNA synthesis were not affected by the drug. Our results suggest that PI 3-kinase may be part of the cascade of events that govern progression of the fertilized sea urchin egg through the mitotic cell cycle.

MATERIALS AND METHODS

Handling of gametes

Gametes were collected from the sea urchin *Paracentrotus lividus* and prepared as described previously (Payan et al., 1983; Ciapa and Epel, 1991). Eggs were kept in filtered sea water (FSW) at 13°C and dejellied by several passages through a 90 μm mesh nylon filter before use. A 4% (v/v) egg suspension in FSW was fertilized at room temperature. Sperm were stored dry at 4°C until use. Shortly before use, 10 μl of dry sperm were diluted in 1 ml FSW, and 2.5 μl of this sperm dilution were added per ml of egg suspension.

Determination of cleavage rates

A 10 mM stock solution of wortmannin (Wt, Biomol Research Lab., Inc) was made in DMSO and stored at −20°C. An intermediate dilution (100 μM) was first made in FSW. Various concentrations of Wt (1 to 20 μM final concentration) were added directly to the egg

suspension one minute before fertilization or at different times after sperm addition. In microinjection experiments, eggs were dejellied by successive filtrations, treated 1 minute with FSW, pH 5.0, and then rinsed with FSW, pH 8.0. Eggs were stuck to Petri dishes coated with 1% polylysine. Wt was diluted in a microinjection buffer (480 mM KCl, 0.1 mM EGTA, 20 mM Pipes, pH 7.0) containing 0.4 mM carboxyfluoresceine to allow vizualisation of injected eggs. The microinjection system we used resulted in the injection of approximately 1% of the egg volume (Rees et al., 1995). Elevated fertilization envelopes, formation of the streak stage and rates of cleavage were scored by observation under a light microscope. 0.2% DMSO (the highest concentration used) had no effect compared to untreated eggs.

Immunoprecipitations, western blots and PI kinase activity

All immunoprecipitation and western blot procedures have been previously described (Ciapa and Epel, 1991; De Nadai et al., 1996). Antiphosphotyrosine antibodies (PTyr-Abs) were from ICN (PY 20) and UBI Inc.

PI kinase activity was assayed following the protocol described by Varticovski et al. (1989) on anti-PTyr-immunoprecipitates (PTyr-Ips) which were prepared as described above. At the end of the rinse procedure, PTyr-Ips were transferred into 40 μl incubating medium (IM: 100 mM EGTA, 380 mM KCl, 20 mM Pipes, 72.3 mM $CaCl_2$, pH 7.4). Phosphotyrosine containing proteins were then dissociated from the immunoprecipitated complex by addition of 10 μl of a phenylphosphate solution (50 mM final concentration). 1 mg/ml PI (Sigma) was first dispersed by sonication in IM and 0.2 mg/ml of this solution was added to the immunoprecipitates. The reaction was started by addition of 20 μCi [γ-^{32}P]ATP and 50 μM final cold ATP, incubated 15 minutes at room temperature, and terminated by addition of 200 μl of $CHCl_3$/MeOH/HCl 11 N (1/2/0.05, v/v). The $CHCl_3$ phase containing the lipids was evaporated and analyzed by TLC chromatography as previously described (Ciapa et al., 1992). The spot on the TLC plate corresponding to PIP was scraped off and quantified by liquid scintillation counting.

A sample of egg PPI labelled with ^{3}H-inositol was used as standards and prepared as followed: 10 ml of a 5% suspension of sea urchin eggs were fertilized in FSW in the presence of 1 mCi ^{3}H-inositol (Amersham). We used this procedure since a rapid incorporation of ^{3}H-inositol into eggs and lipids occurs after fertilization (Ciapa et al., 1992). 4 hours after fertilization, eggs were rinsed with cold FSW and lipids extracted as described above. The $CHCl_3$ phase containing the lipids was evaporated, resuspended in 300 μl $CHCl_3$, and 50 μl was used for each TLC plate. All PPI have been previously identified using comigration with commercial standards (Ciapa et al., 1992).

Cytochemistry

The dispersion of nuclear envelopes was followed by Nomarski differential interference contrast (DIC) microscopy. So far, we have not been able to follow nuclear envelope breakdown by fluorescence because the W3-1 lamin antibody (a generous gift from Drs J. Holy and P. Collas) raised against sea urchin lamins (Holy et al., 1995) stained the *P. lividus* sperm nuclei but not the female pronuclei lamins during the segmentation stages (data not shown, and Holy et al., 1995). Observations were made on a Zeiss Axiophot using 20 or ×40 Plan Neofluar objectives and DIC optics, and pictures were taken on Kodak Tmax 100 film.

For cytolocalization, sea urchin eggs were taken at different times after fertilization, decanted and rinsed in artificial sea water deprived of Ca^{2+} (484 mM NaCl, 10 mM KCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 2.4 mM $NaHCO_3$, pH 8.2). Two different protocols were then used according to the structure to be localized.

For microtubules or chromatin, the eggs were fixed and permeabilized, for 30 minutes at room temperature, by dilution in a fixation medium composed of 2% formaldehyde, 20 mM Pipes, 5 mM

EGTA, 0.5 mM MgSO$_4$, 0.1% Triton X-100 (TX-100), pH 6.5 (Huitorel et al., 1996). The decanted eggs were then transferred to TBS (10 mM Tris-HCl, 140 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.1% TX-100, and were stored at 4°C or used immediately as described below.

For centrosomes or chromatin, the eggs were first extracted in a cytoskeleton stabilizing medium composed of 20 mM Pipes, 5 mM EGTA, 0.5 mM MgSO$_4$, 30% glycerol, 0.1% TX-100, pH 6.5, then fixed for 6 minutes at −20°C in methanol containing 1 mM EGTA, pH 7.4. The decanted eggs were transferred into TBS and stored at 4°C or used immediately as described below.

25 µl of egg suspension in TBS were layered on polylysine coated coverslip, rinsed in TBS 0.1% TX-100. Each specimen was then incubated with 10 µl of primary antibody for 30 minutes at room temperature, rinsed in TBS 0.1% TX-100, and incubated with 10 µl of the appropriate secondary antibody for 30 minutes at room temperature. After rinsing, the specimens were treated by 5 µg/ml Hoechst 33258 in the same buffer, and mounted in 90% glycerol containing 20 mM Tris-HCl, pH 8, and 0.1% *p*-phenylenediamine as an antibleach.

All antibodies were diluted in TBS, 0.1% TX-100, 1% normal donkey serum (Jackson). YL1/2, a rat monoclonal anti-tubulin antibody (Kilmartin et al., 1982) (Sera-Lab) was diluted 1/1,000. CTR 2611, a mouse monoclonal anti-centrosome antibody (a generous gift from Drs N. Bordes and M. Bornens; Bornens et al., 1987), was used as an undiluted supernatant. Donkey anti-rat or anti-mouse purified immunoglobulins labelled with fluorescein or Texas Red (Jackson) were diluted at 1/250.

The observations were performed on a Zeiss Axiophot, using a ×40, ×63 or ×100 Plan-Neofluar objective. Pictures were taken on Kodak T MAX 400 Film and processed with the Kodak T MAX developer for this sensitivity.

Determination of protein and DNA synthesis

Cumulative incorporation of [^{3}H]leucine into proteins or [^{3}H]thymidine into DNA was determined as follows. A 50% (v/v) suspension of jellied eggs was incubated for 2 hours in [^{3}H]leucine (5 µCi/ml) or [^{3}H]thymidine (5.7 µCi/ml) at 15°C. Eggs were then rinsed in cold FSW, dejellied, diluted as a 4% (v/v) suspension, and fertilized in the presence or absence of 20 µM Wt. 1 ml samples were taken before or after sperm addition and extracted with a 20% solution of trichloracetic acid (TCA) as already described (Pesando et al., 1995). Radioactivity was counted in a Packard liquid scintillation counter and protein concentration was determined by the Bradford assay (Bradford, 1976).

Measure of MPF activity

Histone H1 kinase activity, which reflects MPF activity, was measured following the same protocol described by Pelech et al. (1987) and using histone H1 (Sigma type III-S) as a substrate (1 mg/ml final concentration in the incubation medium). Results were expressed in cpm transferred from [γ-^{32}P]ATP to histone H1/mg egg protein per 15 minute incubation. Blank activity, i.e. activity of samples incubated in the absence of histone H1, was substracted from each value before report on the graphs.

When measured in total egg extracts, H1 kinase activity was determined in 5 µl samples prepared as follows. A 5% egg suspension was fertilized in the presence or absence of different concentrations of Wt. 500 µl samples were taken at different times before and after fertilization, rapidly centrifuged, and pellets resuspended in 200 µl homogenization buffer (HB: 60 mM β glycerophosphate, 15 mM nitrophenylphosphate, 25 mM MOPS, 15 mM EGTA, 15 mM MgCl$_2$, 1 mM dithiothreitol, 0.1 mM sodium fluoride, 0.1 mM sodium vanadate, pH 7.2). Samples were immediately frozen and stored in liquid nitrogen until the kinase assay was performed.

Histone H1 kinase activity was also measured on MPF purified with p9 Sepharose beads. *E. coli* (BL 31) carrying p9^{CKShs} was a generous gift from L. Meijer. Expression and purification of p9^{CKShs} and then association with Sepharose beads were performed according to the protocole described by Richardson et al. (1990) except that no polyethylene amine was added after lysis of bacteria. Around 1.5 µg p9 bound to beads were incubated for 30 minutes at 4°C with 200 µl egg samples prepared as described above. Beads were then rinsed with HB, incubated with 5 mg/ml histone H1 in the presence or not of Wt, and H1 kinase activity measured as explained above.

Synthesis of cyclins was measured as described below. A 4% egg suspension was incubated for 30 minutes with 0.03 mCi/ml translabelled [^{35}S]methionine (ICN Inc.). Eggs were then fertilized in the presence or not of 20 µM Wt. Eggs were rinsed 2 times with cold FSW when control eggs showed streak stage figures. 200 µl samples were taken at different times and eggs treated as described above for H1 kinase activity. The obtained egg extracts were diluted 2 times with bead buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 mM PMSF), incubated with around 1.5 µg p9 bound to beads for 30 minutes at 4°C, then rinsed 2 times with the bead buffer and used at the end with Laemmli buffer for SDS-PAGE analysis. Autoradiogram was performed and developed after one week exposure.

General phosphorylation of proteins

To determine the incorporation of ^{32}P into total proteins, we used the same protocol as that described above for H1 kinase activity measured on total egg extracts, except that no specific substrate was added during kinase assay. These samples were also separated on SDS-PAGE which was then exposed for autoradiography.

RESULTS

Characterization of a PI 3-kinase activity in sea urchin eggs

Since PI 3-kinase is known to be associated with proteins phosphorylated on tyrosine, we investigated whether proteins immunoprecipitated with anti-PTyr Abs (PTyr-Ips) contained a PI kinase activity. PTyr-Ips were made with unfertilized eggs or with fertilized eggs arrested 20 minutes or 50 minutes after fertilization, i.e. before and during mitosis. Fig. 1A shows a typical pattern of tyrosine phosphorylation obtained when immunoprecipitation and western blot were performed by using a mixture of two different anti-PTyr Abs as described in Materials and Methods. A large and sustained increase in the phosphorylation on tyrosine of several proteins occurred at these later times of development. These results confirm those reported earlier showing that fertilization triggers a rapid increase in the phosphorylation on tyrosine of several proteins (Ciapa and Epel, 1991; De Nadai et al., 1996). We observed that this increase with time in tyrosine phosphorylation of proteins was not significantly altered if eggs were treated at fertilization with 20 µM Wt, an inhibitor of PI 3-kinase (Fig. 1A).

These same PTyr-Ips were incubated in the presence of ^{32}P-labelled ATP and exogenous PI and incorporation of ^{32}PO$_4$ into PIP measured. Fig. 1B shows that PI kinase activity of PTyr-Ips in the control experiment increased with time after fertilization, and was dramatically reduced when the in vitro assay was performed in the presence of 20 µM Wt. We observed that PI kinase activity of PTyr-Ips made with eggs fertilized in the presence of 20 µM Wt was dramatically reduced when compared to that of PTyr-Ips of the control experiment, and became undetectable when the in vitro assay was further performed in the presence of 20 µM Wt (Fig. 1B).
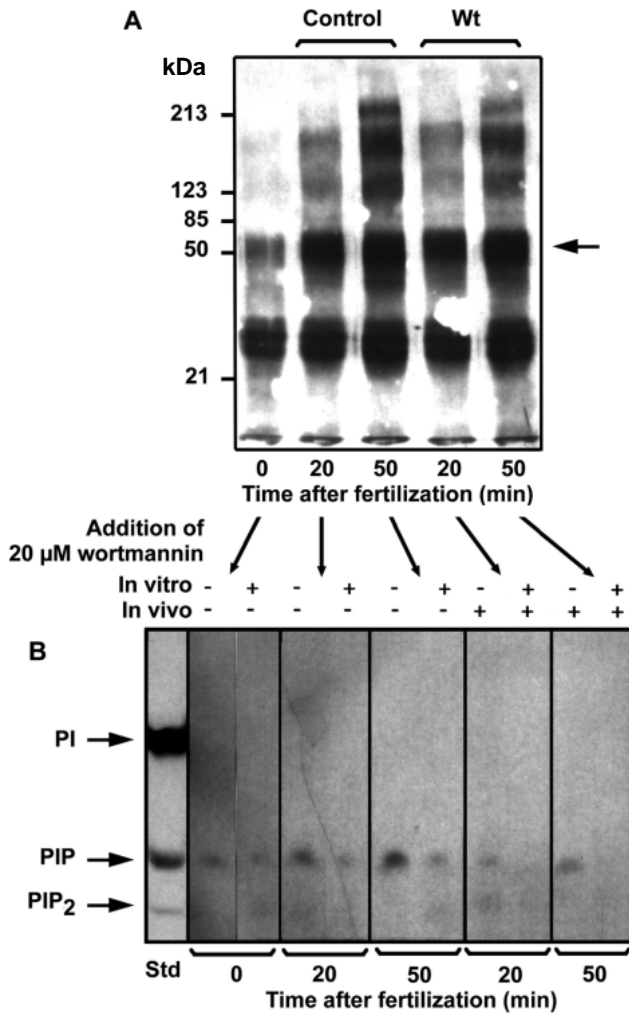
Fig. 1. Detection of PI kinase activity in PTyr Ips. Ips were obtained from unfertilized eggs (time zero) or eggs arrested 20 or 50 minutes after fertilization and treated with 20 μM Wt or not. (A) PTyr Ips were analysed by SDS-PAGE followed by western immunoblot as described in Materials and Methods using two anti-PTyr Abs. Film exposure was 1 minute. The arrow indicates the heavy chain of the IgG. Molecular mass standards (kDa) are indicated on the side of the panel. (B) The same PTyr Ips were assayed for PI kinase activity in the presence of PI and [^{32}P]ATP following the protocole described in Materials and Methods. The autoradiograph of the TLC separation of the reaction products is shown. Eggs were fertilized in the presence (addition in vivo) or absence of 20 μM Wt and PI kinase assay was performed in the presence (in vitro) or absence of the drug. On the left of the panel were run egg lipids labelled as explained in Materials and Methods with ^{3}H-inositol and used as standards (Std).

The same experiment was performed in the presence of decreasing concentrations of Wt. We found that full inhibition was obtained with 3-4 μM Wt and half inhibition with 1.5 μM Wt (data not shown). These results suggest that sea urchin eggs contain a Wt sensitive PI kinase activity that is associated with proteins phosphorylated on tyrosine.

Effect of wortmannin on the first divisions

Results presented above suggest that activation of a Wt sensitive PI 3-kinase activity occurred 20 and 50 minutes
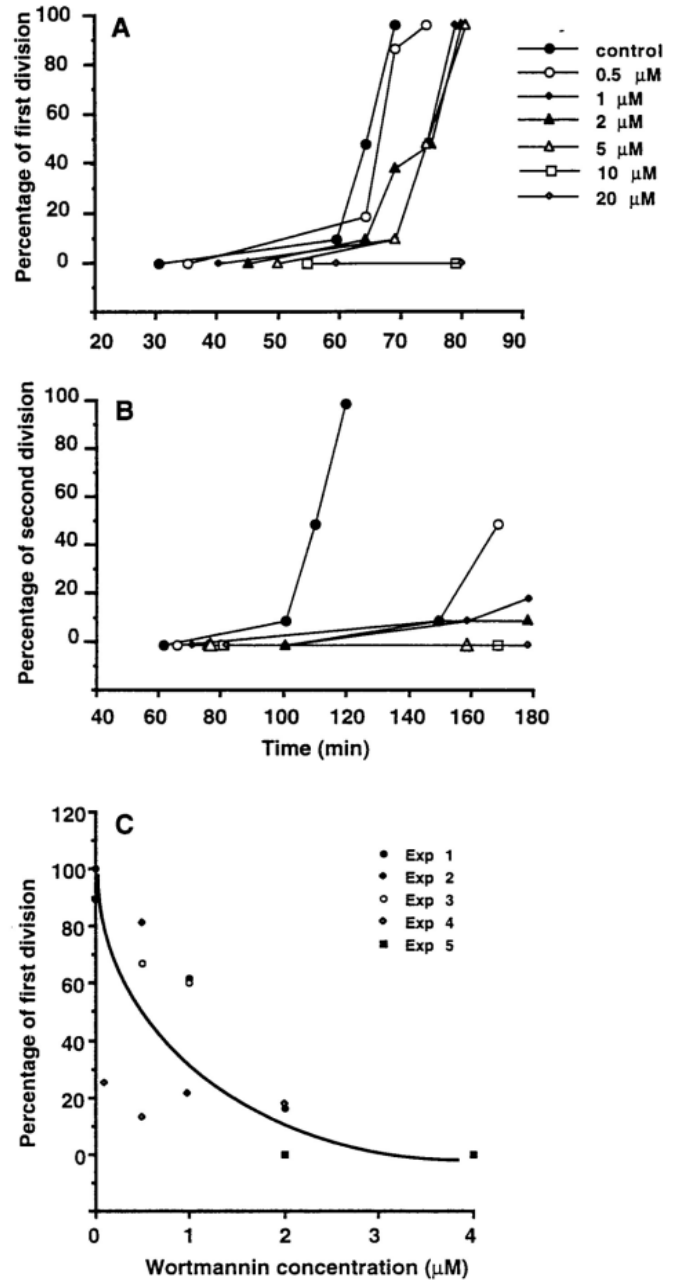

Fig. 2. Effect of increasing concentrations of Wt on the first (A,C) and the second (B) divisions. The drug was added prior to sperm addition and eggs were let to develop in this medium. Rates of cleavage were scored as described in Materials and Methods. (A and B) Addition of Wt to the egg suspension. (C) Microinjection of Wt into unfertilized eggs. The concentration of Wt indicated in the figure represents the intracellular concentration of the drug after taking into account the 1% dilution due to microinjection.

following fertilization and remained at a high level during mitosis. The question was, then, whether PI 3-kinase played a role during progression of the cell cycle. This led us to test whether treatment of eggs with Wt could affect fertilization and/or early mitotic cycles.

High concentrations of Wt of up to 20 μM or long pre-

incubations (up to 3 hours) in the presence of the drug did not affect fertilization, since all treated eggs showed normal elevated fertilization envelopes and were monospermic (data not shown). However, the presence of the drug affected the division cycle. A complete arrest of division was induced by 10 or 20 µM Wt, depending on the batch of eggs (Fig. 2A). Addition of such high concentrations of Wt within the first 30 minutes following fertilization led to arrest of eggs similar to that observed when fertilization was performed in the presence of the drug. When the drug was added between 30 and 50 minutes after fertilization, a significant proportion of eggs divided. When Wt was added 50 minutes after sperm addition, i.e. when the eggs showed anaphase figures under the light microscope, eggs divided a first time but were arrested during the second cell cycle (results not shown).

When eggs were fertilized and developed in FSW containing concentrations of Wt lower than 10 µM, the streak stage occurred with a 5-10 minute delay compared to the control and all eggs divided normally (Fig. 2A). However, when eggs were fertilized in the presence of these low concentrations of the drug, they did not divide a second time. This was probably due to the fact that Wt did not enter nor diffuse easily into the egg. In point of fact, 2 to 4 µM injected Wt were sufficient to completely arrest the first cell cycle after direct microinjection into the egg (Fig. 2C). This concentration also corresponds to that necessary to fully inhibit PI 3-kinase activity measured in PTyr-Ips and reported above. Taken together, our results suggest that Wt did not perturb fertilization nor cytokinesis but inhibited some step during mitosis itself.

Effect of wortmannin on the mitotic spindle assembly

In order to know more precisely at which particular stage embryos were arrested by Wt and which structures were perturbed, control and Wt treated embryos were followed over time by DIC microscopy. Fig. 3 shows that both control and Wt treated eggs went through sperm aster formation, pronuclear migration and fusion with the same timing, and nuclear envelope breakdown with a slight delay in treated eggs. However, treated eggs did not show mitotic spindles, which we confirmed by immunofluorescence.

Fig. 4 shows the staining for tubulin and chromatin of eggs arrested at different times from fertilization to cleavage. We observed that 20 µM Wt allowed normal pronuclear migration and fusion, in agreement with the normal formation and development of the sperm aster of microtubules. In Wt treated eggs, chromatin condensed normally, but at a slightly slower rate than in control eggs. These condensed chromosomes were progressively dispersed out of the former nuclear volume, since nuclear envelopes were broken (Fig. 3). At later times, these treated eggs showed a single array of microtubules radiating out of a volume containing the condensed chromosomes. These eggs remained at this stage for at least 3 hours and never formed any bipolar microtubular structure. As a consequence, chromosomes did not align along a metaphase plate and remained in a prometaphase-like stage for long times, while control eggs divided and cleaved (Fig. 4).

We then checked whether the absence of formation of a bipolar spindle in Wt treated eggs was due to abnormal duplication of the centrosome. Staining for centrosomal material is shown in Fig. 5. In control eggs, centrosomal material appeared organized in the form of two compact foci at the poles of the metaphase spindle (Fig. 5C). In Wt treated eggs, centrosomal staining was dispersed at time of fusion of pronuclei around a sphere corresponding to a volume larger than the nucleus (Fig. 3). Centrosome material of Wt treated eggs was progressively reorganized (Fig. 5E′) between the time
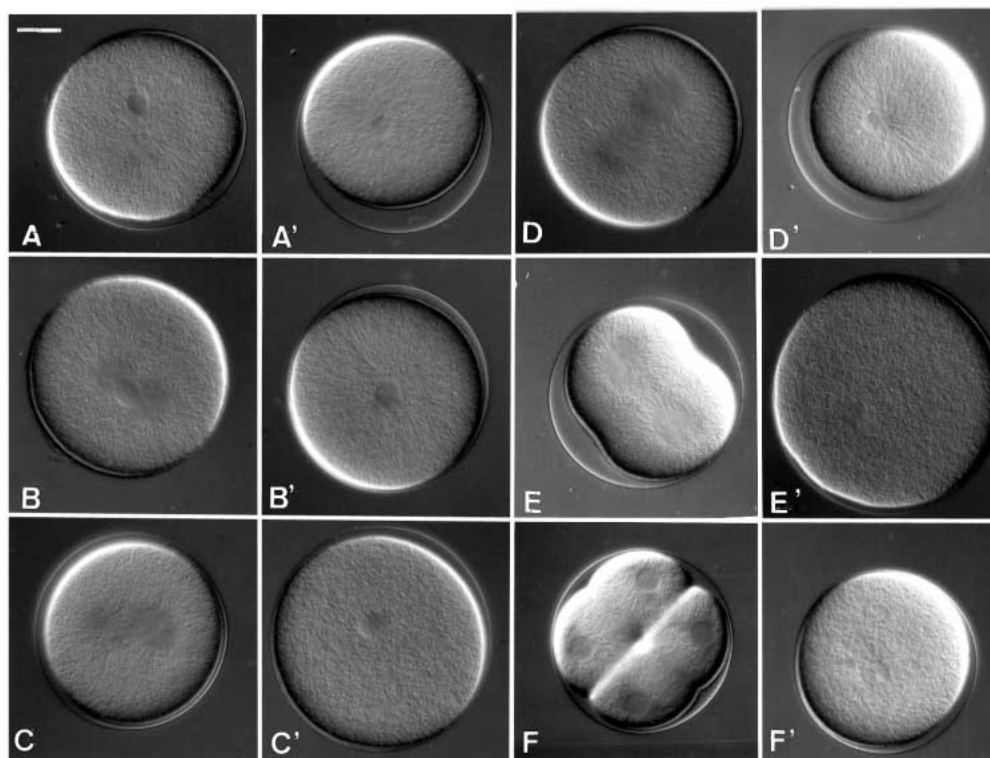

Fig. 3. Effect of Wt on the first mitotic cycle. Control (A-F) and 20 µM Wt (A′-F′) treated embryos were observed without fixation under DIC microscopy. At 15 minutes (A,A′), both specimen showed sperm aster formation and pronuclei migration; at 33 minutes (B,B′) pronuclei fusion was over and the microtubular aster was fully developed. At 51 minutes, nuclear envelope breakdown was evident in the control (C) and only started in Wt treated eggs (C′). Control eggs formed a bipolar mitotic spindle (C), but not Wt treated eggs (C′). At 55 minutes, mitosis progressed in the control (D) whereas nuclear envelope breakdown was evident in treated eggs (D′). At 57 minutes, control eggs (E) but not treated eggs (E′) started cytokinesis. At 95 minutes, control eggs divided a second time (F), whereas Wt treated eggs did not progress in mitosis (F′). Bar, 20 µm.
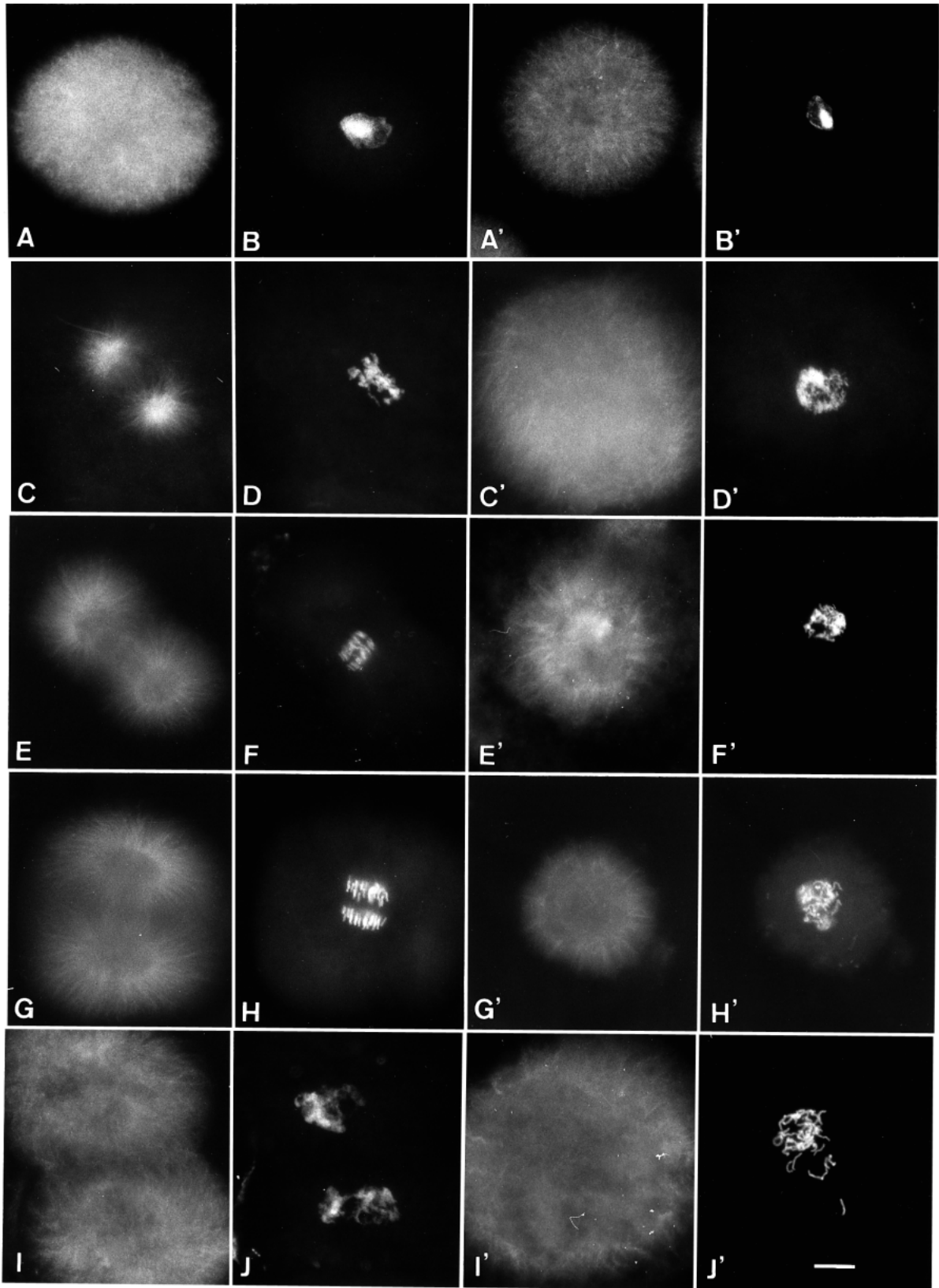
Fig. 4. Effect of Wt on mitotic spindle and chromosome organization. Control (A-J) and 20 μM Wt (A′-J′) treated embryos were fixed and double-labelled by anti-tubulin antibodies (A,C,E,G,I and A′,C′,E′,G′,I′) and Hoechst 33258 (B,D,F,H,J and B′,D′,F′,H′,J′) at 30 (A,B,A′,B′), 40 (C,D,C′,D′), 70 (E,F,E′,F′), 75 (G,H,G′,H′), or 85 (I,J,I′,J′) minutes after fertilization, respectively. Wt treated eggs show in D′ condensed chromosomes in a prometaphase-like state, where they stay (C′-J′), while control eggs go through metaphase (C,D), anaphase (E-H), then telophase-cytokinesis (I,I′). Bar, 20 μm.
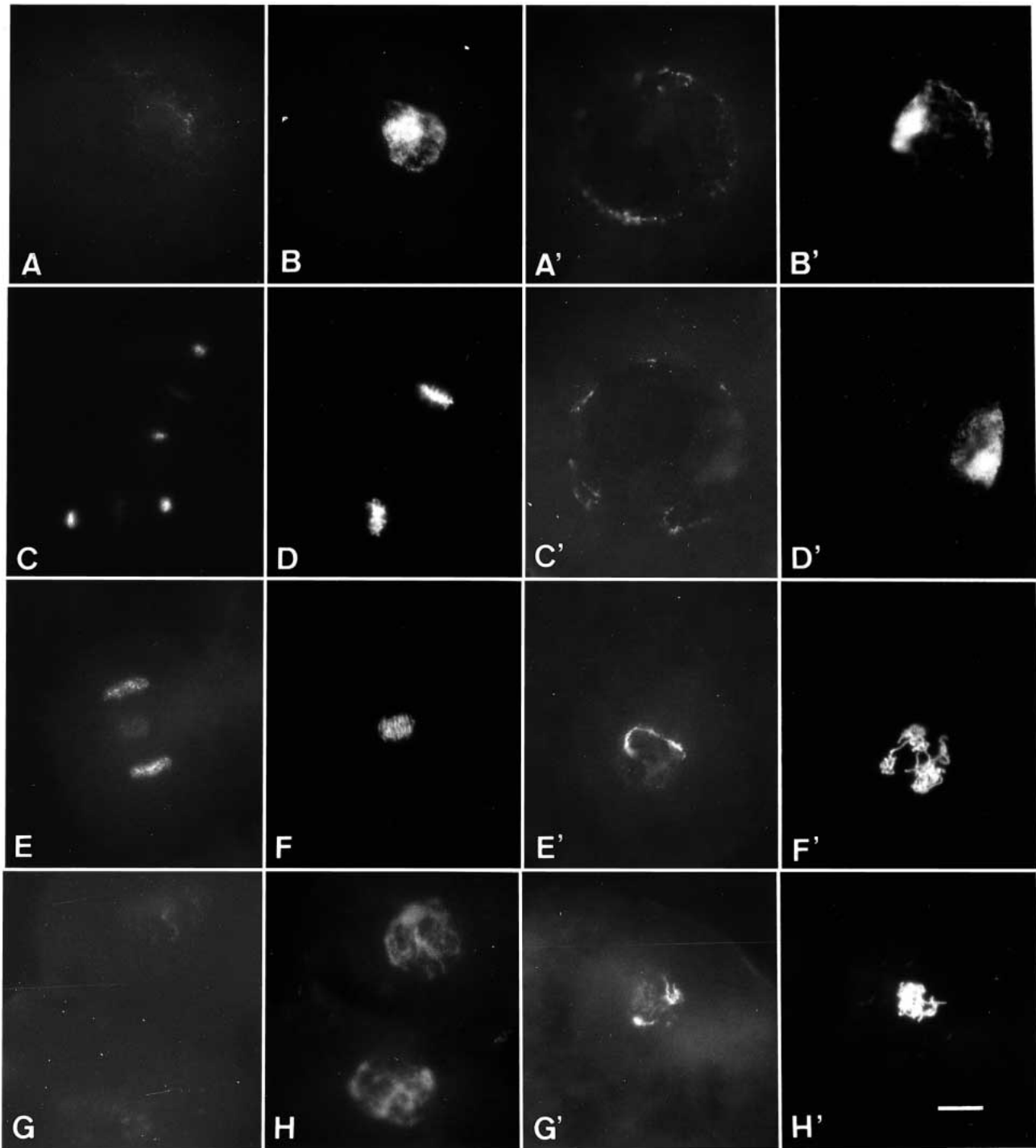
Fig. 5. Effect of Wt on centrosomal-associated material during the first mitotic cycle. Control (A-H) and 20 μM Wt (A′-H′) treated embryos were labelled as in Fig. 4 by anti-centrosome-associated material (A,C,E,G and A′,C′,E′,G′) and Hoechst 33258 (B,D,F,H and B′,D′,F′,H′) at 30 (A,B,A′,B′), 40 (C,D,C′,D′), 70 (E,F,E′,F′), or 85 (G,H,G′,H′) minutes after fertilization, respectively. Centrosome-associated material is localized at the poles of mitotic spindles during metaphase (C), anaphase (E), then telophase-cytokinesis (G) in control embryos, while it is dispersed in treated embryos (A′,C′,E′,G′). Bar, 20 μm.

of metaphase and cytokinesis of control embryos, but never showed two foci. This pattern of dispersed centrosomal material indicates abnormal duplication and separation of centrosomes, consistent with the formation of a radiating array of microtubules organized around chromosomes in the presence of the drug. Taken together, these results suggest that Wt inhibited some activities involving microtubule organization and necessary to progress in the mitotic cycle, but had no effect on events dependent on microfilaments, such as fertilization or cleavage. This led us to investigate which cell
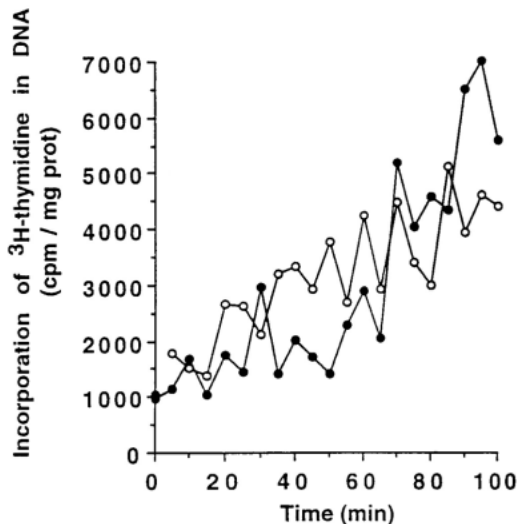
Fig. 6. Time course of DNA synthesis after fertilization of eggs treated (○) or not (●) with 20 μM Wt. Incorporation of [³H]thymidine was determined as explained in Materials and Methods.

cycle kinase activities, known to control progression through mitosis and stimulated at fertilization, were altered by Wt.

DNA synthesis in wortmannin treated eggs

Results presented above strongly suggest that Wt treated eggs entered mitosis. One of the control points of the cell cycle verifies whether DNA synthesis has been completed before progression through ENTRY in mitosis (reviewed by Nurse, 1994). We then measured whether DNA synthesis occurred in Wt treated eggs, in order to verify whether condensed chromosomes of these eggs observed in Figs 4 and 5 were also duplicated. Fig. 6 shows that incorporation of [³H]thymidine into DNA was not affected in Wt treated eggs.

Alteration of MPF stimulation in the presence of wortmannin

Alteration by Wt of MPF, the ubiquitous regulator of mitosis progression, might be one of the obvious causes responsible for egg arrest at the entry in first mitosis. The MPF activity due to cdc2/cyclin B is often referred to as histone H1 kinase, since MPF shows a pronounced activity towards this substrate and is the main source of histone H1 kinase activity in oocytes and eggs during meiosis (Gautier et al., 1988) and mitosis (Meijer et al., 1989).

We measured how H1 kinase activity evolved when fertilization was performed in the presence of Wt. Fig. 7A shows that 20 μM Wt drastically reduced the peak of H1 kinase activity observed in the control experiment and characteristic of each mitotic division in sea urchin (Meijer and Pondaven, 1988). However, H1 kinase activation was not totally cancelled, as a 2-fold increase in H1 kinase activity was observed at normal time of mitosis in Wt treated eggs instead of the 12-fold increase observed in control eggs (Fig. 7A,B,C). The drug concentration dependence reported in Fig. 7B indicates a half-maximal inhibition of 6-7 μM Wt, and a maximum effect obtained with 20 μM Wt.

We then analyzed whether inhibition of H1 kinase activity

by Wt was due to a direct effect of the drug on MPF. Eggs were treated or not with 20 μM Wt and arrested before fertilization or at time of mitosis. MPF from these extracts was purified with p9-Sepharose beads, and H1 kinase activity was measured in the presence or in the absence of 4 μM Wt in the in vitro assay. We observed that the increase in MPF activity of control eggs entering mitosis was not significantly altered when Wt was added to the in vitro assay (Fig. 7C). On the contrary, this increase in H1 kinase activity was markedly reduced when MPF was purified from eggs which were fertilized in the presence of the drug. These results suggest that the alteration of H1 kinase activity in Wt treated eggs was not due to a direct effect of the drug on MPF.

A possibility was that protein synthesis, and therefore synthesis of cyclins which are one component of MPF, might have been altered by Wt treatment. Fig. 7D shows that incorporation of [³H]leucine into proteins was similar in eggs treated or not by 20 μM Wt. Since we did not observe any alteration of the general protein synthesis by Wt, it was unlikely that the drug specifically acted on cyclin synthesis. We nevertheless verified this point by measuring the association of cyclins with cdc2 after protein labeling with [³⁵S]methionine. Results of an autoradiogram of labelled cyclins associated with cdc2 purified on p9 Sepharose beads are shown in Fig. 7E. We observed that the increase in cyclin synthesis that normally occurs after fertilization was not altered when eggs were treated with 20 μM Wt. An hypothesis is therefore that Wt altered the phosphorylation status of p34^{cdc2} which varies along the cell cycle.

Effect of Wortmannin on phosphorylation of proteins

All results presented above suggest that PI 3-kinase might control various events activated at time of mitosis and including formation of active MPF and of a bipolar spindle. It has recently been reported that PKB (also known as c-Akt) and particular isoforms of PKCs, such as PKCζ, PKCε or PKCη, could be some of the various possible targets of the 3-phosphorylated inositol lipids generated by PI 3-kinase (Stokoe et al., 1997; Toker and Cantley, 1997). This led us to investigate whether Wt modified the general phosphorylation state of proteins. Fig. 8 shows that incorporation of ^{32}P into proteins oscillates during successive cell divisions, as already described by Néant et al. (1989). We observed that the general level of protein labeling in Wt treated eggs was decreased compared to the control experiment, suggesting that part of the kinase activity was altered by the drug. However, a peak of incorporation of ^{32}P into proteins was still detected at a time corresponding to mitosis of the first cell cycle of control eggs (Fig. 8). Separation by SDS-PAGE of these labelled proteins confirmed this general decrease in phosphorylation by Wt treatment (data not shown).

DISCUSSION

By using Wt, a drug which has been reported in several other types of cells to block PI 3-kinase activity, we observed alterations of both morphological and biochemical events stimulated at fertilization. The arrest of the cell cycle induced by Wt treatment suggests that PI 3-kinase might be involved
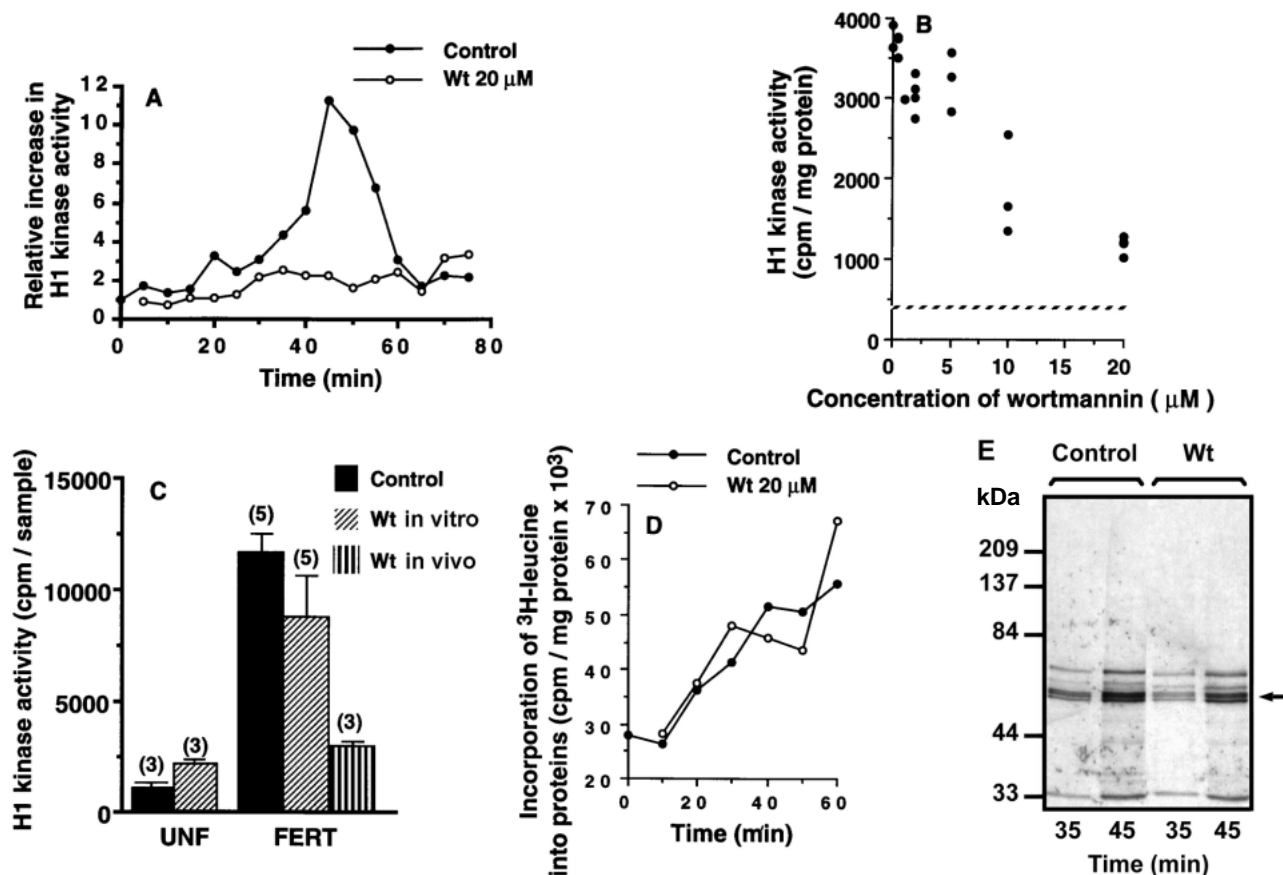
Fig. 7. Effect of 20 µM Wt on MPF. (A) Time course after fertilization of H1 kinase activity in eggs fertilized in the presence or not of the drug. Eggs were taken at different times after fertilization, and H1 kinase activity measured on extracts of eggs made as described in Materials and Methods. A clear increase in H1 kinase activity was detected at time of mitosis, i.e. between 40 and 60 minutes after sperm addition. (B) Effect of increasing concentrations of Wt on H1 kinase activity. Eggs were fertilized in the presence of the drug and H1 kinase activity measured on extracts of eggs arrested 50 minutes after sperm addition and as described in A. The activity determined in unfertilized eggs was 400 cpm/mg prot. (dashed line). (C) H1 kinase activity of MPF purified on p9 Sepharose beads. Eggs were arrested before fertilization (UNF) or at time of metaphase of control eggs (FERT). 20 µM Wt were added either in the egg suspension (in vivo) or during the in vitro assay. Results are means ± s.e.m. of n (number in brackets) determinations. (D) Time course of protein synthesis after fertilization of eggs treated or not with 20 µM Wt. Incorporation of [3H]leucine was determined as explained in Materials and Methods. (E) Cyclin synthesis of eggs treated or not with 20 µM Wt. Proteins were labelled with [35S]methionine and cyclins purified by binding MPF to p9 beads as described in Materials and Methods. Cyclins are visualized (arrow) by autoradiography after SDS-PAGE analysis.

in the cascade of events necessary to progress through the first mitotic divisions of the sea urchin egg. This leads to several questions concerning the nature of this cascade, how it is stimulated after fertilization and how it participates in the activation of MPF and the formation of the mitotic spindle.

The detection of a PI kinase activity in anti PTyr-immunoprecipitates which was sensitive to Wt argues for the presence of PI 3-kinase in sea urchin eggs. To our knowledge, no PI kinase other than PI 3-kinase can become phosphorylated on tyrosine after activation (von Willebrand et al., 1998) or contains an SH2 domain which can be associated with a phoshorylated tyrosine of another protein. All other PI kinases (PI 2, 4, or 5 kinase) do not contain these properties. Therefore, we find unlikely the existence of a PI kinase other than PI 3-kinase in the immunoprecipitates made with anti-phosphotyrosine antibodies. We found that this activity was stimulated very rapidly after fertilization, and remained activated at the time of mitosis. This corroborates our previous

results obtained after in vivo labeling of eggs, showing the stimulation of PI kinase activity at early times after fertilization (Ciapa et al., 1991, 1992) and during the first cell cycles (Ciapa et al., 1994; Pesando et al., 1995).

We observed that Wt was effective in experiments performed in vitro as well as in vivo. The concentration used in microinjections to obtain a maximal effect was around 2 µM, a relatively high concentration when compared to that used in other cell types, which can be explained by a poor diffusion of the drug inside the egg. A similar concentration of Wt was needed to inhibit PI 3-kinase activity in an in vitro assay. It is worthy to note that a number of other drugs, such as ouabain or amiloride, have to be used at concentrations a hundred times higher in sea urchin eggs than in somatic cells (Ciapa et al., 1984). The partial inhibition of PI 3-kinase activity by Wt used at a high concentration can be explained by the presence in sea urchin eggs of a PI 3-kinase related but not identical to the different isoforms already identified in other types of cells. The
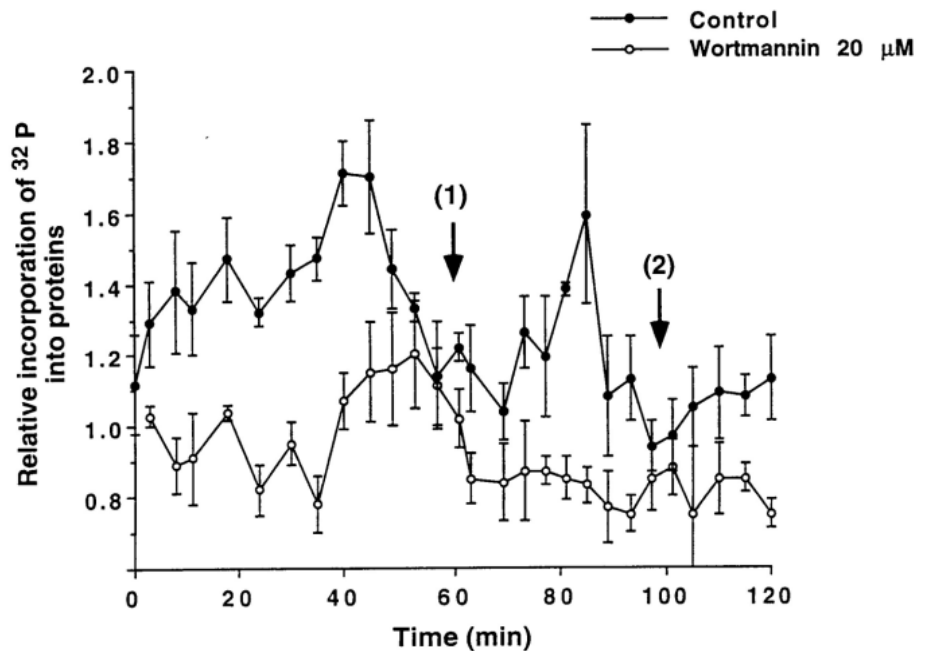
Fig. 8. Time course of general phosphorylation of proteins of eggs treated or not with Wt, measured by incorporation of ^{32}P into proteins during the two first cell cycles. Results are means ± s.e.m. of 3 determinations. Arrows indicate times at which all eggs were divided a first (1) then a second (2) time.

specificity of Wt may also be subject to discussion, although Wt is widely used in many different systems as a specific inhibitor of PI 3-kinase. In experiments described here, Wt seems to act on a limited number of cellular processes. For example, the drug does not act as a non specific inhibitor of all kinases and/or phosphatases. The increase of tyrosine phosphorylation observed in several proteins after fertilization and reported here was not significantly altered in eggs fertilized in the presence of the drug. Moreover, and despite a decreased level of incorporation of ^{32}P into proteins, Wt treated eggs still showed a peak of labeling at the normal time of the first mitosis in control eggs. Also, when added 50 minutes post-fertilization, Wt did not block cytokinesis but prevented entry in the second metaphase. The absence of an effect of Wt on cytokinesis in sea urchin embryos strongly suggests that Wt did not perturb myosin light chain kinase activity, a known target of Wt in other systems (Suzuki et al., 1996). Another example illustrating the lack of effect of Wt on other events is the occurence of nuclear envelope breakdown in Wt treated eggs. Since the calcium dependent kinase CaCamII kinase is involved in the control of this event in sea urchin eggs (Baitinger et al., 1990) and in other types of cells (reviewed by Lu and Means, 1993; Takuwa et al., 1995), it is improbable that Wt inhibits this type of kinases. Finally, we observed that Wt had no effect on the vanadate sensitive tyrosine phosphatase activity contained in sea urchin eggs (Chiri et al., 1998). Wt might also affect PI 4-kinase (Nakanishi et al., 1995) and phospholipase D (Carrasco-Marin et al., 1994). We do not know whether phospholipase D is present and stimulated at fertilization in sea urchin eggs, and whether it plays any role during development. In any case, our results establish a connection between PPI metabolism and cell cycle progression, which confirms and extends our previous observations (Ciapa et al., 1994; Pesando et al., 1995).

We observed that the fertilization process itself, including adhesion and fusion of the two gametes and elevation of fertilization envelopes, was not altered by Wt. This suggests that PI 3-kinase and/or another Wt-sensitive event did not play any role during these events. This also implies that a cascade involving PI 3-kinase was activated after fertilization through a route independent of that leading to the calcium signal. In other cell types, PI 3-kinase has been shown to bind to receptors with tyrosine kinase activity or to cytosolic tyrosine kinase linked to receptor complexes such as the T cell receptor (TCR) (reviewed by Parker and Waterfield, 1992). Recently, it has been proposed that PI 3-kinase could bind small GTP-binding proteins (reviewed by Parker, 1995). Therefore, the presence of PI 3-kinase in eggs cannot give any clue concerning the nature of the sperm receptor, a still debated question (Ciapa and De Nadai, 1996).

Treatment of eggs by Wt led to an 80% inhibition of H1 kinase activation. However, treated eggs built a normal aster of radiating microtubules, condensed their chromatin and disrupted their nuclear envelopes at a slower rate. This suggests that the residual MPF activity allowed eggs to enter mitosis. This fits with results reported by Geneviere-Garrigues et al. (1995) showing that a partial activation of MPF is sufficient for the G_2-M transition. Several hypotheses can explain the inhibition of MPF in Wt treated eggs. Since neither synthesis of cyclins nor their association with cdc2 were modified, it is possible that the decrease in MPF activity was due to alterations of the phosphorylation status of cdc2 (reviewed by Lew and Kornbluth, 1996). At the present time, we do not know whether Tyr and/or Thr phosphorylation of cdc2 is concerned. PI 3-kinase could modulate MPF activity either directly through its Ser/Thr kinase activity (Carpenter et al., 1993) or indirectly via other kinases such as some PKC isoforms (Nakanishi et al., 1993). As examples, PKCζ (Toker and Cantley, 1997) and PKB (Stokoe et al., 1997) can be controlled by the 3-phosphorylated inositol lipids generated by PI 3-kinase.

Even though Wt treated eggs entered mitosis (aster formation, chromosome condensation, nuclear envelope breakdown), they were unable to form any sort of bipolar microtubule spindle,

presumably due to the dispersion of centrosome-associated material normally attached to microtubule minus ends (Buendia et al., 1990). The reason for this dispersion is presently not known, but could be due to an altered phosphorylation level of some centrosomal components required for centrosome duplication and/or separation. For instance, it is known that MPF is partially associated with centrosomes during mitosis (Bailly et al., 1989) and that polo-like kinases, NuMA and the proper activity of several microtubular motors are required to build up normal spindle poles (Gaglio et al., 1996; Glover et al., 1996). This hypothesis is supported by the fact that the general phosphorylation status of proteins was altered by Wt. In the same manner, we could not detect a clear attachement of chromosomes to free microtubule plus ends, a fundamental property of mitotic kinetochores (Mitchison and Kirschner, 1985; Huitorel and Kirschner, 1988). As a consequence, chromosomes did not move outwards from the centrosome. The reason for that behavior is not known, but could also be due to an abnormal level of phosphorylation of kinetochore components, whether they have a motor activity or not. It has been shown for instance that kinetochores signal their proper position in the mitotic spindle by expressing a phosphoepitope at the kinetochores of moving chromosomes (Gorbsky and Ricketts, 1993), and that the presence of an unattached chromosome at metaphase prevents the metaphase-anaphase transition (Rieder et al., 1994) due to the presence of a checkpoint at this mitotic stage (Rudner and Murray, 1996). It is also possible that PI 3-kinase controls the formation of the mitotic spindle after fertilization of eggs by acting on PKCζ. The presence of this protein in sea urchin eggs (data not shown) and its association with the mitotic apparatus in primary cell cultures of the shark rectal gland that has been described (Lehrich and Forrest, 1994) argue in favor of such an hypothesis. Another possibility is that PI 3-kinase would not be involved in this process, and the lack of formation of the mitotic spindle would be due to the low MPF activity induced by Wt treatment.

By showing a radiating array of microtubules characteristic of prophase, a dispersed nuclear envelope and condensed chromosomes spread out in the cytoplasm, we propose that Wt treated eggs are arrested in a prometaphase-like (more precisely a monopolar prometaphase) stage, because there are several reasons (see above) why they are not allowed to enter metaphase. Some members of the MAP kinase family have been proposed to be components of the spindle assembly checkpoint (reviewed by Murray, 1994). Our results (see accompanying paper in this issue, Chiri et al., 1998) showing that a MAP kinase activity is present in sea urchin eggs and altered after Wt treatment may explain the arrest in mitosis induced by the drug.

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