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Lithium blocks cell cycle transitions in the first cell cycles of sea urchin embryos, an effect rescued by *myo*-inositol

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SUMMARY

Lithium is a classical inhibitor of the phosphoinositide pathway and is teratogenic. We report the effects of lithium on the first cell cycles of sea urchin (*Lytechinus pictus*) embryos. Embryos cultured in 400 mM lithium chloride sea water showed marked delay to the cell cycle and a tendency to arrest prior to nuclear envelope breakdown, at metaphase and at cytokinesis. After removal of lithium, the block was reversed and embryos developed to form normal late blastulae. The lithium-induced block was also reversed by *myo*- but not *epi*-inositol, indicating that lithium was acting *via* the phosphoinositide pathway. Lithium microinjection before fertilization caused arrest prior to nuclear envelope breakdown at much lower concentrations (3-5 mM). Co-injection of *myo*-inositol prevented the block. Microinjection of 1-2 mM lithium led to block at the cleavage stage. This was also reversed by coinjection of *myo*-inositol. Embryos blocked by lithium microinjection proceeded rapidly into mitosis after photolysis of caged inositol 1,4,5-trisphosphate. These data demonstrate that a patent phosphoinositide signalling pathway is essential for the proper timing of cell cycle transitions and offer a possible explanation for lithium's teratogenic effects.

Key words: lithium, sea urchin, *myo*-inositol, cell cycle, phosphoinositides, mitosis

INTRODUCTION

Increasing evidence points to the phosphoinositide signalling pathway as an important regulator of the embryonic cell division cycle (Hepler, 1992, 1994; Whitaker and Patel, 1990; Whitaker, 1995). In sea urchin eggs, injection of inositol 1,4,5-trisphosphate ($InsP_3$) stimulates premature nuclear envelope breakdown (NEB) and chromatin condensation (Twigg et al., 1988). Oscillations in $InsP_3$ mass accompany the calcium transients that occur just prior to the mitotic transitions of the cell cycle and the $InsP_3$-antagonist heparin blocks both calcium transients and entry into mitosis (Ciapa et al., 1994). In *Xenopus* oocytes, $InsP_3$ mass increases at first cleavage (Stith et al., 1993) and injection of monoclonal antibodies against phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) greatly slows the blastomere division cycle, while heparin blocks it (Han et al., 1992). The same anti-$PtdInsP_2$ antibodies suppress mitosis in several mammalian cell lines (Fukano et al., 1988; Huang et al., 1991; Matuoka et al., 1988; Uno et al., 1988).

Lithium is a classic tool for the study of phosphoinositide-dependent cellular processes (Berridge et al., 1989); by inhibiting the *myo*-inositol-1-phosphatase, it blocks the dephosphorylation of myo-inositol-1-phosphate to *myo*-inositol. This prevents the synthesis of phosphoinositides and leads to depletion of $PtdInsP_2$ and loss of the $InsP_3$ signal (Berridge and Irvine, 1989). Lithium is known to respecify the dorsoventral axis in amphibia (Kao et al., 1986) and zebrafish (Stachel et al., 1993) and to produce vegetalization of sea urchin embryos (Herbst, 1892; Livingstone and Wilt, 1989, 1990). These effects can be reversed by the addition of *myo*-inositol (Berridge et al., 1989; Busa, 1988; Busa and Gimlich, 1989). However, the effects of lithium on early embryonic cell cycles have not been much attended to. Cell cycle effects may contribute to the mechanism of this interference in morphogenetic pattern (Izquierdo and Becker, 1982). Early observations made by Mazia et al. (1972) suggested that substitution of NaCl with LiCl could block early cleavages in the sea urchin *Strongylocentrotus purpuratus*. Subsequently, Forer and Sillers (1987) extended these observations in the sea urchin *Lytechinus pictus* and showed that incubation of eggs with 400 mM LiCl (at 22°C) blocked the first cell cycle. This effect was sometimes reversed by 10-100 μM *myo*-inositol, implying an involvement of the phosphoinositide pathway in the regulation of the sea urchin cell cycle, but the effect was hard to reproduce. The results with inositol were extremely variable, indeed inconclusive. The levels of *myo*-inositol were two orders of magnitude lower than others have used (Berridge and Fain, 1979; Balla et al., 1984; Downes and Stone, 1986; Godfrey, 1989; Markram and Segal, 1992), moreover, *myo*-inositol is poorly taken up by sea urchin embryos (Ciapa et al., 1992). Nor was it clear which phases of the cell cycle were affected by lithium.

Because a robust rescue by *myo*-inositol is essential if it is to be inferred that lithium is affecting the phosphoinositide cycle, we have determined conditions under which it occurs.

We have confirmed a lithium-induced block of the sea urchin early cell cycles at a more physiological temperature (16°C) and obtained a reproducible rescue by *myo*-inositol at concentrations 1-2 orders of magnitude higher (1-10 mM) than that used by Forer and Sillers (1987). By following the first cell cycle in the presence of the fluorescent DNA dye Hoechst 33342, we have demonstrated that the lithium-delayed embryos paused predominantly before NEB, at metaphase and during cytokinesis. Rescue of the block by $InsP_3$ indicates that lithium treatment arrests embryos just prior to NEB. These observations constitute further independent evidence of the involvement of the phosphoinositide signalling pathway in the regulation of the cell cycle transitions and are consistent with and predicted by the existence of intracellular calcium transients and $InsP_3$ peaks that precede mitotic events in sea urchins and other organisms (Ciapa et al., 1994; Hepler, 1992, 1994; Kao et al., 1990; Poenie et al., 1985; Whitaker, 1995; Wilding et al., 1996).

MATERIALS AND METHODS

All experiments were performed at 16°C. Unless otherwise indicated, data represent averages of several experiments and are usually plotted as mean±standard error of the mean (s.e.m.). One- tailed Student's *t*-test was applied to test for significance. Legend for *t*-test: *=0.1≤*P*≤0.05; **=0.05≤*P*≤0.01; *** = 0.01≤*P*.

Solutions

Artificial Sea Water (ASW)

450 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 50 mM $MgCl_2$, 2.5 mM $NaHCO_3$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 (adjusted with NaOH). When needed, LiCl (up to 400 mM) and *myo*-inositol or *epi*-inositol (up to 10 mM) were substituted for an equiosmolar amount of NaCl.

Control injection solutions

0.5-0.7 M KCl, 20 mM piperazine-NN′-bis-2-ethane sulphonic acid (Pipes), 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), pH 6.7.

Lithium injection solution

0.2, 0.3 or 0.5 M LiCl (osmolarity corrected with KCl), 20 mM Pipes, 1 mM EGTA, pH 6.7. When necessary, 0.1-0.8 M *myo*-inositol (or *epi*-inositol) was substituted for KCl.

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical (St Louis, MO, USA) or BDH Limited (Poole England).

Gamete handling and fertilization

Eggs from the sea urchin *Lytechinus pictus* (Marinus, CA, USA) were obtained by intracoelomic injection of 0.5 M KCl and collected in ASW. The eggs were dejellied by several passages through Nitex mesh. Sperm was collected 'dry' and stored at 4°C for no more than 3 days. For fertilization, 5 μl of the dry sperm was diluted in about 100 μl of ASW and this dilute suspension was added to the bath containing the eggs. The extent of fertilization was always checked 2-4 minutes after sperm addition; in all experiments reported here, >95% of the eggs raised fertilization envelopes. When removal of fertilization membrane was necessary, eggs were fertilized in ASW supplemented with 1 mM *p*-aminobenzoic acid (to prevent toughening of the fertilization envelope); after elevation of the fertilization envelope (1-2 minutes), the envelope was removed by running the egg suspension through Nitex mesh. Eggs were then plated onto coverslips coated with 50 μg/ml poly-D-lysine.

Lithium incubation

All experiments were started within 15 minutes of eggs being shed. Immediately after fertilization (which was usually complete within 1-2 minutes), the eggs were washed several times to remove the sperm. Then, equal portions of the eggs were placed in beakers containing either ASW (controls) or a lithium-containing solution (with or without *myo*- or *epi*-inositol). The whole procedure usually took about 5-10 minutes. When required, eggs were preincubated with the LiCl containing solution for 1 hour before fertilization. Samples of eggs were placed on a glass slide and scored for cell cycle stage at the desired intervals (usually at least 40-50 embryos for every time point) on a Zeiss bright-field microscope.

H33342 treatment

Eggs were incubated with 5 μM Hoechst 33342 (Arndt-Jovin and Jovin, 1989) in ASW for 30 minutes; for lithium preincubation experiments, the dye was added to the LiCl-containing solution 30 minutes before fertilization. The dye-containing medium was removed by washing twice before fertilization. Fertilized eggs were placed onto glass coverslips (after sperm removal) and viewed on a Nikon Diaphot 300 fluorescence microscope, equipped with a Nikon camera.

Microinjection experiments

Unfertilized eggs were seeded inside a fertilization chamber whose glass floor had been previously coated with 0.01 mg/ml of poly-L-lysine. Control or LiCl-containing solutions were then injected in 4-8 eggs for each kind of treatment. The amount injected was adjusted to obtain a final intracellular [LiCl] between 1 and 5 mM. The eggs were fertilized immediately after injection. Elevation of the fertilization envelope always took place within a few minutes and at the same time in controls and Li^+-treated eggs, indicating that neither the injection procedure nor the presence of LiCl affected the fertilization process.

The injection procedure was performed in two steps: micropipette penetration using a piezo translator PMZ (Märzhäuser Wetzlar, Germany) was followed by injection using gas pressure; the micropipette was then withdrawn by the same piezoelectric system. Borosilicate glass capillaries with inner filament (Clark Electromedical Instruments, England) were pulled by a standard vertical puller (BioScience, Sheerness, Kent, UK) and backfilled using a Hamilton syringe. The normal cleavage of control-injected zygotes gave us confidence that the injection process had not been significantly harmful to the embryos.

Release of caged $InsP_3$

A solution containing LiCl (300 mM) with or without D-myo-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)-ethyl ester, trisodium salt (caged $InsP_3$; Calbiochem) was injected into sea urchin eggs immediately before fertilization to a final concentration of 3 mM. The concentration of caged $InsP_3$ in the pipette was 10-50 μM. Before each experiment, we verified that this solution, when injected, could only induce the elevation of fertilization envelopes after flashing.The flash was applied by a Cairn Flash Photolysis system (Cairn, UK) when the control eggs had completed NEB.

RESULTS

Cell cycle lithium block is reversed by myo-inositol

A concentration of 400 mM lithium chloride in sea water prevents embryos reaching the 4-cell stage (Fig. 1). Lower concentrations are less effective. The sea water also contained 50 mM sodium chloride to maintain an appreciable inward sodium gradient (E_{Na} = 40 mV, assuming an intracellular sodium concentration of 10 mM) and permit the normal functioning of the

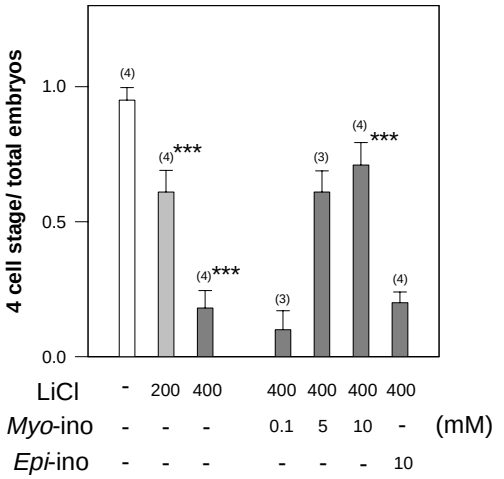

Fig. 1. LiCl block of the early cell cycles is reversed by millimolar concentration of *myo*-inositol. Bars represent the fraction of embryos that reached the 4-cell stage, under different experimental conditions, 270 minutes after fertilization. Legend: *Myo*-ino, *myo*-inositol; *Epi*-ino, *epi*-inositol. Bars represent means (±s.e.m.) of the number of experiments indicated. Student's one-tailed *t*-test for both LiCl versus Ino 10 mM and Controls versus LiCl gives $P \leq 0.01$ (***); LiCl and Epi-ino were not significantly different.

sodium/proton antiporter (Whitaker and Patel, 1990) and the sodium/inositol cotransporter. In fact, sea urchin embryos develop at least to blastula stage when sodium is completely substituted by choline, provided that millimolar sodium or lithium are present during the first 10 minutes after fertilization (Chambers, 1976). The lithium block is reversed by addition of 1-10 mM *myo*-inositol to the sea water at the start of the incubation. The inactive *epi*-inositol isomer is ineffective at a concentration of 10 mM (Fig. 1). These data indicate that lithium treatment blocks the cell cycle and that the block can be reversed by inositol, demonstrating that the effect of lithium is largely due to blockage of the phosphoinositide pathway.

The fraction of embryos traversing the first and second cell cycles is shown in Fig. 2A. A little over half the embryos are blocked in the first cell cycle and 82% by the second cell cycle, only 18% reaching the 4-cell stage. *Myo*-inositol rescues both the first and second cell cycle block: 71% now reach the 4-cell stage at 270 minutes, compared to 95% of the control, untreated embryos. After 4 hours in 400 mM lithium chloride, the intracellular lithium concentration will have reached levels much greater than those required to block *myo*-inositol 1-phosphatase (Ciapa and Maggio, 1993), whose K_i is 1-5 mM (Atack et al., 1993; Berridge et al., 1989). This raises the possibility that lithium might be causing toxic effects unrelated to its actions on the phosphoinositide cycle (Erlich and Diamond, 1980; Atack et al., 1995).

Lithium is not toxic in the early stages of development

The 65-70% rescue of the lithium block by *myo*-inositol indicates that the lithium block is specific to the phosphoinositide pathway but, though this degree of rescue compares well with other published data (Balla et al., 1984; Busa and Gimlich, 1989; Godfrey, 1989), it seemed possible that an element of the response to lithium might involve irreversible

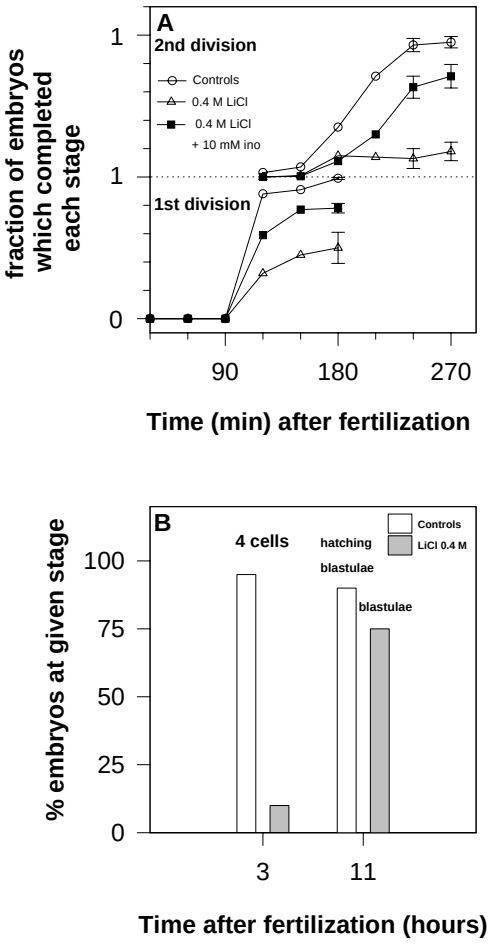

Fig. 2. (A) Time course of LiCl (0.4 M) block and *myo*-inositol (10 mM) rescue on the early cleavages, during the first 4.5 hours after fertilization. Data points represent the fraction of embryos that completed each stage (1st or 2nd division) at the indicated time. Each curve was obtained by averaging data from 4 experiments. Error bars (s.e.m.) are shown for the 180, 240 and 270 minute data points; *t*-test for values at 180 minutes gives $P \leq 0.01$ for controls versus 400 mM LiCl, and $0.01 \leq P \leq 0.05$ for 400 mM LiCl versus 400 mM LiCl plus 10 mM inositol. See Fig. 1 for significance values at 270 minutes. Legend: open circles, controls (ASW); open triangles, LiCl (400 mM); filled squares, *myo*-inositol (10 mM). (B) Recovery from cell cycle block after LiCl removal 3.5 hours after fertilization. One of two experiments is shown in which 0.4 M LiCl strongly inhibited the first two cell cycles. At 3 hours, 10% of the lithium-treated embryos had cleaved, compared to 95% in ASW. After LiCl removal, embryos started to cycle again. Around 75% of the treated embryos reached the blastula stage ~11 hours after fertilization, with a delay of ~2 hours compared to the controls (~85% reach blastula).

toxicity. We tested this by incubating embryos with 400 mM lithium chloride for 3.5 hours after fertilization before returning them to normal sea water (Fig. 2B). One of two comparable experiments is illustrated. The treatment induced a robust cell cycle block (fewer than 10% of the embryos had cleaved to 4 cells at 180 minutes, compared to 95% of the controls). After return to normal sea water, the embryos began to divide again. They were followed to late blastula stage. A comparable proportion of embryos reached this stage as in controls, though development to this stage was delayed by

Table 1. LiCl incubation delays the completion of the stages of cell division

	Cell cycle phases in 0.4 M LiCl						
	First cell cycle				Second cell cycle		
	pre-NEB	Metaph.	Anaph.	Cytok.	pre-NEB	Metaph.	Anaph.
120-150 min	8.5%	35%	48%	8.5%	0.00	0.00	0.00
180-210 min	7%	22%	6%	14%	10%	21%	20%
% remaining at 180-210 min	82%	63%	12.5%	165%			

Unfertilized eggs were loaded with the DNA stain Hoechst 33342 as described in Materials and Methods; they were then observed after fertilization during the first two cell cycles. The percentage of embryos blocked in the different stages at two fixed observation periods (when control embryos had completed the first or second cell cycle) is shown for the 1st and 2nd cell cycles. Embryos were found in every stage, but with a prevalence of metaphase embryos. The bottom line shows the % of embryos that remained in the indicated stage at 180-210 minutes compared to those originally in that stage. Data are mean values from 5 experiments (3 for the first and 2 for the second cell cycle; a total of 152 embryos were scored). Legend: pre-NEB, before NEB; Metaph., metaphase; Anaph., anaphase; Cytok., cytokinesis.

around 2 hours. These experiments indicate that the effects of lithium on the first cell cycle are reversible.

Lithium causes cell cycle arrest prior to NEB, at metaphase and at cleavage

Progress through the cell cycle in sea urchin embryos is regulated by checkpoints just prior to NEB, at metaphase and at cleavage (Whitaker and Patel, 1993; Whitaker, 1995). $InsP_3$-driven calcium signals determine progress through the checkpoints (Ciapa et al., 1994; Wilding et al., 1996). It might be expected that a lithium-induced block of the phosphoinositide signalling pathway would result in delay or block at these checkpoints. We therefore looked to see whether lithium treatment led to delay or arrest at specific cell cycle stages or merely slowed the cell cycle overall.

We followed the chromatin condensation/decondensation cycle using Hoechst 33342, a vital fluorescent DNA stain. When all controls had completed the first cell cycle, around 60% of the lithium-treated embryos were still at a precleavage stage. We scored the proportion of embryos at each cell cycle stage at this time, 2-2.5 hours after fertilization. 83% of the embryos were in metaphase and anaphase and 8.5% had not undergone NEB (Table 1). When the control embryos had completed the second cell cycle, we scored the lithium-treated embryos again (Table 1). The treated embryos were found in every mitotic stage of the two cell cycles. In the hour that had elapsed beween observations, 80% of the embryos blocked pre-NEB in the first cell cycle had not progressed. The corresponding figures for metaphase, anaphase and cytokinesis of the first cycle cycle are 63%, 13% and 164%. These data are consistent with the idea that, when embryos cease progression through the cell cycle, they arrest predominantly pre-NEB, at metaphase or at cleavage and rarely in anaphase. However, it is clear that some embryos escape the lithium block completely in the first cell cycle.

The rate of uptake of lithium by early sea urchin embryos is slow (Ciapa and Maggio, 1993). We therefore preincubated unfertilized eggs in 400 mM lithium chloride for 1 hour before fertilizing them in the continuing presence of lithium. In this experiment, we scored the cell cycle stages continuously throughout the experiment, using H33342 (Fig. 3). NEB was substantially delayed in lithium-treated embryos compared to controls (121±15 minutes compared to 69±2.5 minutes, n=3, Fig. 3A) and the embryo population lingered longer in metaphase and in cytokinesis (Fig. 3B-D). The time spent by the population in anaphase was not significantly affected. The time spent in interphase before NEB might reflect delays in any or all of G_1, S and G_2 (see below). The increased time spent in metaphase and cytokinesis confirms the idea that lithium is hindering progress at specific points in the cell cycle, since the alternative, an overall increase in cell cycle time or in dispersion in the population, would have led to an increase in the time spent in anaphase.

We confirmed a metaphase block by observing individual embryos after lithium treatment. Of three embryos in the same field, two were in metaphase (Fig. 4A,C) and one in anaphase (Fig. 4B) at 90 minutes after fertilization. 25 minutes later, when 93% of controls had cleaved, the two embryos were still in metaphase, while the anaphase embryo was in telophase. A further observation was made 15 minutes later: the two embryos were still in metaphase, showing that in the presence of lithium, metaphase can persist for 40 minutes, while anaphase proceeds normally. Metaphase in *individual* control embryos lasts 5 minutes or less. Fig. 4 also shows examples of embryos blocked pre-NEB (G) and at cleavage (H,I). The same results were found in three different egg batches (4 metaphase, 1 anaphase; 3 metaphase, 1 anaphase; 3 metaphase, 1 anaphase) with metaphase always persisting at least 30 minutes, the time limit of some of our observations (not shown). In all, 10 of 10 embryos remained in metaphase and 3 of 3 in early anaphase proceeded through anaphase (the probability of this observation occurring by chance is 0.0125).

Lithium microinjection blocks mitosis at the G_2 stage

Bath application of lithium chloride is widely used to alter developmental pattern in embryos. Our experiments indicate that bath application of lithium leads to a block of the cell cycle at metaphase and cytokinesis. However, high concentrations of lithium chloride are required externally (the effect on AV axis in the sea urchin involves treatment with 20 mM lithium chloride for 6-8 hours, at the 16-cell stage; Livingston and Wilt, 1989) and we are unable to tell whether the pre-NEB block occurs in G_1, S or G_2. We therefore turned to microinjection to address these points.

We microinjected lithium chloride to a final concentration of 3-5 mM (recall that the K_i of inositol 1-phosphatase is 1-5 mM: Berridge et al., 1982; Atack et al., 1993) just before fertilization. This blocked embryos pre-NEB (Fig. 5A). The block was reversed by coinjection of 1-8 mM *myo*-inositol, but not by *epi*-inositol (Fig. 5A). When lithium chloride was injected 50 minutes after fertilization, no pre-NEB arrest was seen (Fig. 5A). These experiments confirm our supposition that high con-

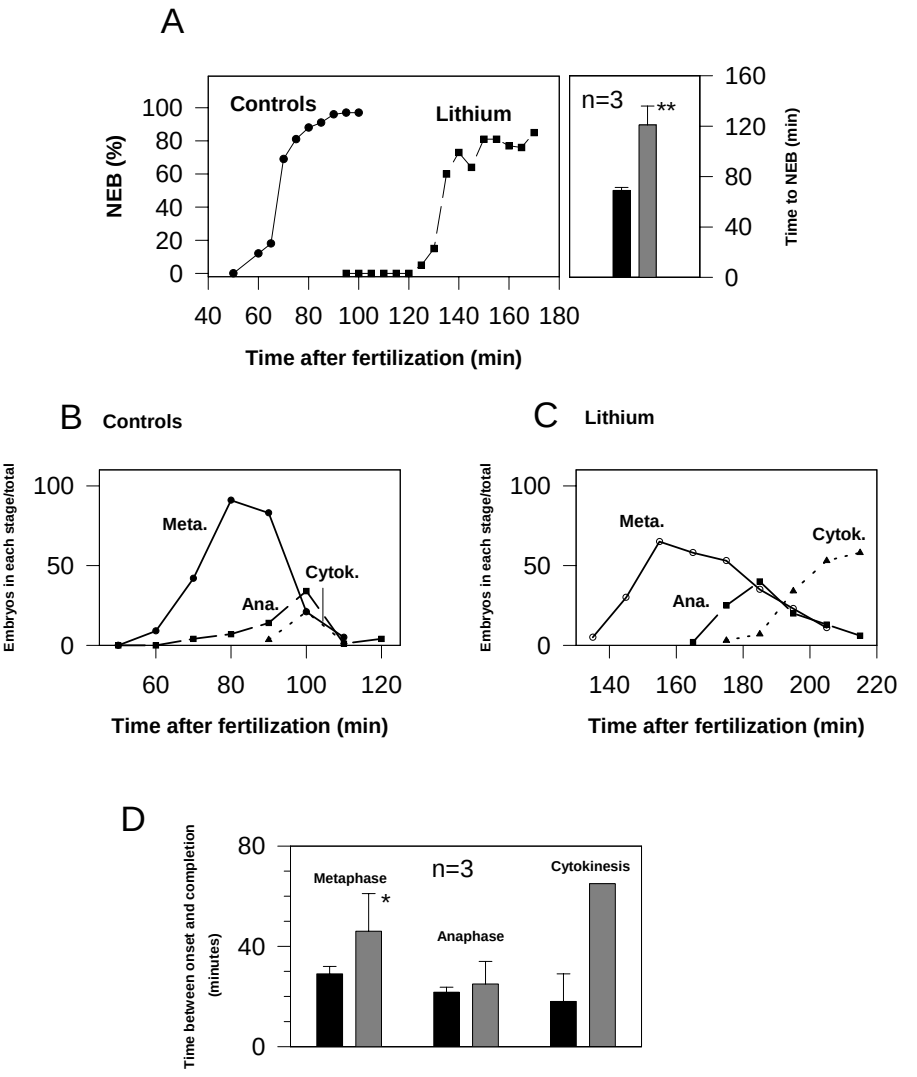

Fig. 3. (A) Left panel: representative example of the time course of first NEB in sea urchin embryos treated or not with 0.4 M LiCl (treatment started 1 hour before fertilization). Lithium considerably delays NEB. Circles, controls; squares, LiCl. Right panel: mean time to 50% NEB in controls or LiCl-treated embryos. Black, controls; gray, LiCl. $P \leq 0.05$ (**; Student's *t*-test, $n=3$). (B) Representative example of the time course of first cell cycle stages. The percentage of the embryos at the different stages was plotted as a function of time after fertilization (minutes). The cell cycle stage was scored by using the DNA stain Hoechst 33342. Legend: circles, metaphase; squares, anaphase; triangles, cytokinesis. (C) As in B, but with embryos treated with LiCl (0.4 M). LiCl treatment started 1 hour before fertilization. Legend: open circles, metaphase; squares, anaphase; triangles, cytokinesis. (D) Duration of the cell cycle phases in control and Li^+-treated embryos. The mean durations of metaphase, anaphase and cytokinesis were plotted for control and Li^+-treated embryos. Lithium appears to delay metaphase and cytokinesis. Black bars, controls ($n=3$); gray bars, Li^+-treated (1 hr pre-incubation, 400 mM, $n=3$). Student's *t*-test for metaphase, $P \leq 0.1$. Values for anaphase were not significantly different. With these s.e.m.s, a 30% increase would have been significant at the $P \leq 0.1$ level. In two of the three experiments, embryos were completely blocked in cytokinesis and are not included. Note that 30 minutes after the end of these experiments, embryos became misshapen.

centrations of lithium chloride are needed extracellularly simply because of the poor uptake of lithium into sea urchin embryos (Ciapa and Maggio, 1993) and also suggest either that the lithium-sensitive stage is early in the cell cycle or that lithium takes some time to exert its effects.

We used caged $InsP_3$ to determine at which stage lithium blocked the cell cycle before NEB. If lithium is blocking the cell cycle by interfering with phosphoinositide metabolism, then the block should be bypassed by the phosphoinositide messenger $InsP_3$. Microinjection of $InsP_3$ will cause calcium release at all stages of the cell cycle, but triggers NEB only in G_2 (Twigg et al., 1988). We microinjected one set of embryos before fertilization with lithium and a second set with lithium and caged $InsP_3$. 85 minutes after fertilization, when more than 90% of control embryos had undergone NEB, we applied a brief and intense flash of ultraviolet light to both experimental sets and scored for NEB over the next 30 minutes. Photorelease of $InsP_3$ led to a peak of NEB 15 minutes after the flash (Fig. 5C). These experiments demonstrate that the lithium block pre-NEB can be overcome by $InsP_3$, consistent with the idea that lithium is interfering with $InsP_3$ production. They also demonstrate that the pre-NEB block is in G_2.

Lithium microinjection blocks cleavage

Microinjecting lithium to a final concentration of 5 mM at 50 minutes after fertilization does not block NEB (Fig. 5A). Our results with caged $InsP_3$ imply that this is because the blocking effects of lithium are slow in onset, presumably because phosphoinositide lipid depletion must occur before $InsP_3$ production is prevented (Berridge et al., 1989). Consistent with this idea, we find that lithium injection at 1-3 mM at fertilization merely delays NEB but blocks cytokinesis in 60% of the embryos injected in the experiments shown (Figs 5B, 6). The block is overriden by coinjection of 8 mM *myo*-inositol, but not *epi*-inositol (Fig. 6). These experiments indicate that a critical level of phosphoinositide messenger precursor is required for progress through cytokinesis.

DISCUSSION

Treatment with lithium chloride blocks cell cycle progression

The cell cycle block obtained with lithium chloride treatment is progressive. Overall, in all our experiments, 86±3.4% of

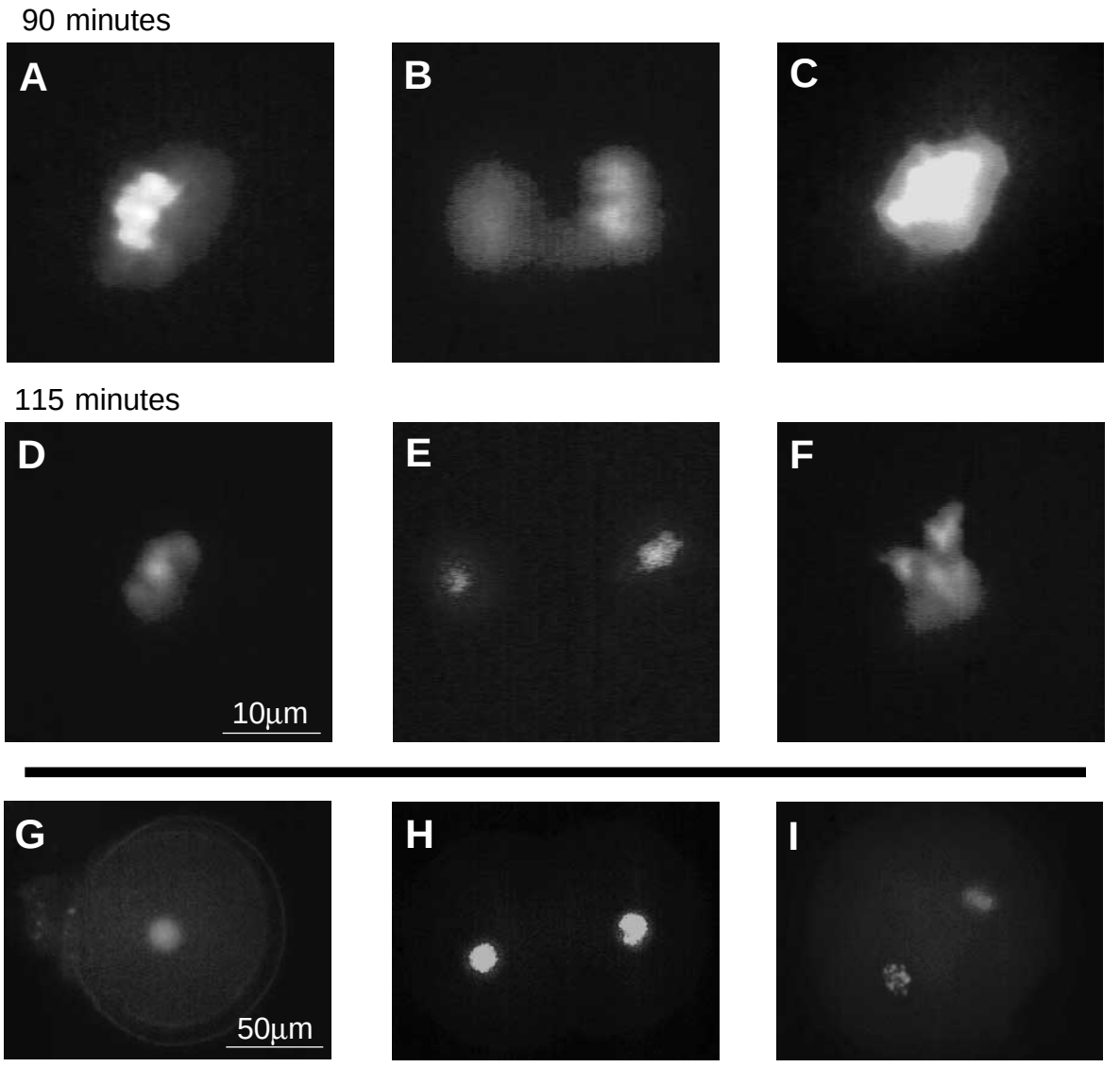

Fig. 4. LiCl (400 mM, added 5 minutes after fertilization) strongly prolongs metaphase in the first cell cycle. Eggs were loaded 30 minutes before fertilization with the DNA stain Hoechst 33342 as described in Materials and Methods and chromatin condensation during the first cell cycle was followed. (A-C) Pictures taken 90 minutes after fertilization showing the nuclei of adjacent zygotes; embryos in A and C were in metaphase, while B is a cell in early anaphase. After 25 minutes, the former embryos were still in metaphase (D,F), while the latter was in telophase (E). The remaining pictures show examples of embryos blocked before NEB of the first or second cell cycle (G,H, respectively) or in cytokinesis (I) about 3 hours after fertilization.

embryos were blocked by the 4-cell stage but, even when not arrested completely, embryos spent longer, on average, in reaching NEB, in mitosis and in cytokinesis. Anaphase was not affected significantly. When embryos did arrest, they stopped in metaphase or cytokinesis, or in an interphase stage that we could not define using H33342 staining alone.

The progressive block was largely relieved by cotreatment with inositol. Rescue of lithium block by inositol at millimolar concentration is the diagnostic test for an effect of lithium on phosphoinositide lipids (Balla et al., 1984; Downes and Stone, 1986; Berridge et al., 1989; Busa and Gimlich, 1989; Godfrey, 1989). The extent of rescue by inositol in our experiments is comparable to previously published data (Balla et al., 1984; Busa and Gimlich, 1989; Godfrey, 1989) and argues that lithium is not causing non-specific toxic effects. We did observe a marked deterioration of embryos that were maintained in lithium continuously after 5-6 hours. On the contrary, embryos kept for 3.5 hours in lithium and then returned to normal sea water showed a robust cell cycle block, but then developed quite normally to late blastulae, showing that the effect of lithium is reversible, at least up to 3.5 hours, the time relevant to blocks observed in the first cell cycles.

Our observations are consistent with the known properties and kinetics of the lithium uptake mechanism in early sea urchin embryos (Ciapa and Maggio, 1993). When we preincubated embryos with lithium before fertilization to preload the egg with lithium, we found that cell cycle transitions in the first cell cycle were more effectively inhibited than when lithium

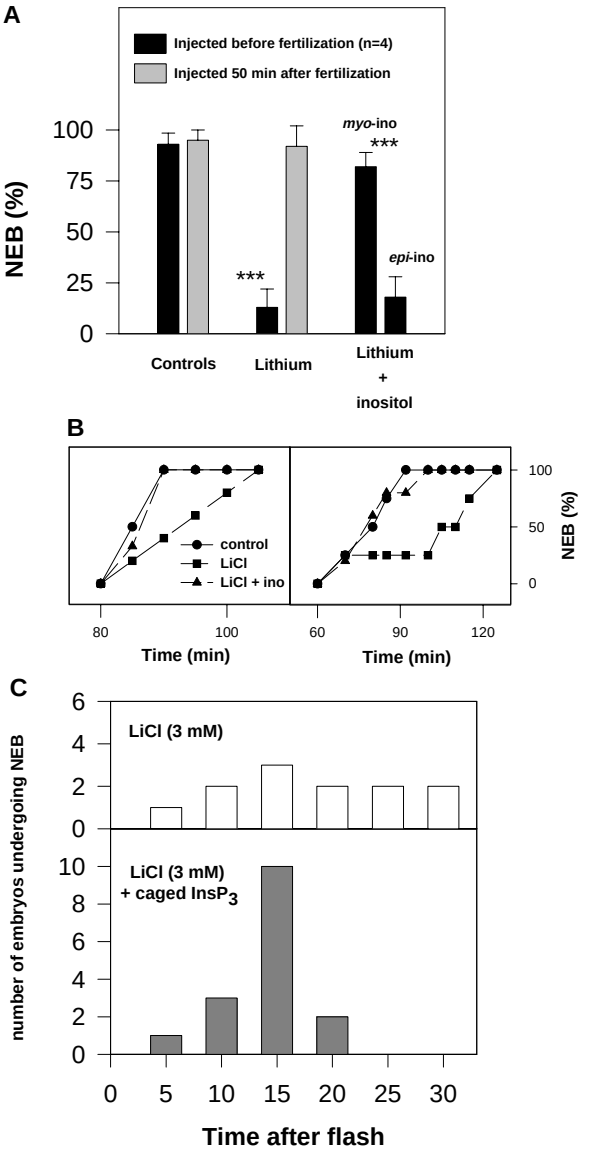

Fig. 5. LiCl microinjection delays NEB. (A) LiCl injection (~5 mM) before fertilization strongly delays the completion of NEB (black bars represent mean % of NEB at the time when the controls had completed NEB). Only 40% of the lithium-treated eggs ever completed cleavage, indicating an additional block in mitosis, besides the delay before NEB. Data points summarize the results obtained from 16 control and 18 lithium-injected embryos in four experiments. Controls and Lithium + *myo*-inositol (but not *epi*-inositol) are significantly different from lithium data ($P \leq 0.01$, ***). LiCl was not effective on NEB when injected 50 minutes after fertilization (gray bars). However, cytokinesis was blocked (not shown), showing that the lithium effect downstream to NEB is independent of the effects at NEB. (B) Representative examples of the time course of NEB in embryos injected with 2-3 mM LiCl with or without 8 mM *myo*-inositol (the controls were injected with KCl). This LiCl concentration produced a delay in NEB timing, which was completely rescued by *myo*-inositol treatment. (C) Photorelease of caged IP_3 in LiCl-treated embryos produced a peak of NEB within 15 minutes of flashing. Bars represent the number of embryos undergoing NEB in a 5 minutes time window (end time indicated on abscissa). The flash was applied when ~90% of the controls had undergone NEB.

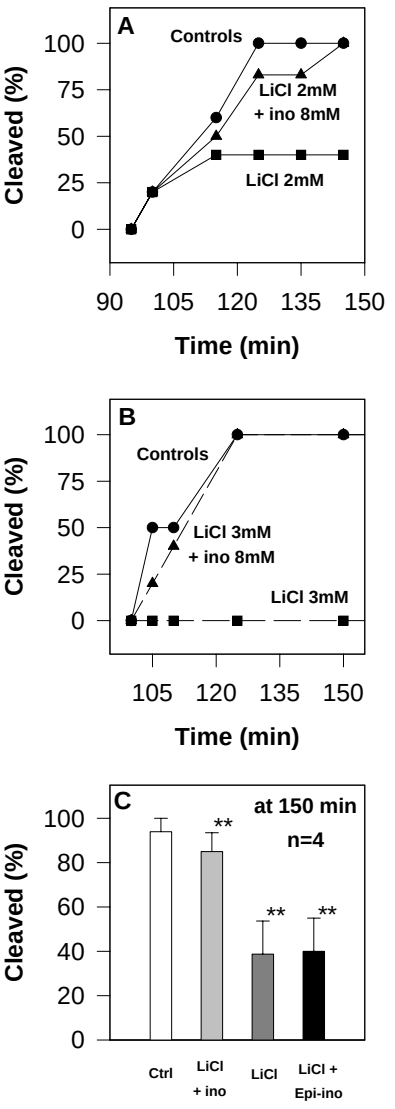

Fig. 6. *Myo*-inositol rescues the cleavage block produced by LiCl. Embryos were injected before fertilization with KCl (Controls) or LiCl (final concentration 2-3 mM) supplemented or not with *myo*-inositol or *epi*-inositol (~8 mM). (A,B) Time course of cleavage in the indicated experimental conditions in two representative experiments; (A) [LiCl]=~2 mM; (B) [LiCl]=~3 mM. Legend: circles, controls; triangles, LiCl + *myo*-inositol; squares, LiCl. (C) Summary of the results of 4 experiments (~40 embryos for each treatment were scored, [LiCl] was always ~2 mM). Legend: Ctrl, controls; ino, *myo*-inositol; *epi*-ino, *epi*-inositol. LiCl and LiCl + ino were significantly different ($0.01 \leq P \leq 0.05$, *t*-test, **).

was added after fertilization. The 3.5 hour incubation with lithium led to a 2 hour delay in development to blastula. This is consistent with the kinetics of lithium extrusion from embryos after return to normal sea water (Ciapa and Maggio, 1993). Uptake of inositol into sea urchin eggs is also relatively slow (Ciapa et al., 1992).

Forer and Sillers (1987) have previously reported rather variable effects of bath treatment with both lithium and inositol on the first sea urchin embryo cell cycle. We did not observe the variability that they found in the effect of lithium, perhaps because we continued our observations into the second cell

cycle. However, we could observe no effects of inositol at the concentrations that they used. The inositol transport parameters of sea urchin embryos support the idea that these lower concentrations could be only marginally effective.

Lithium microinjection

Our microinjection experiments confirm that low intracellular concentrations of lithium in the range required to inhibit inositol 1-phosphatase are effective and confirm that *myo*-inositol effectively reverses the lithium block in the first cell cycle, whereas *epi*-inositol is ineffective. Rescued embryos underwent NEB and cleavage at the same time as controls, in contrast to embryos rescued by bath treatment, which were delayed relative to controls. Inositol rescue by microinjection was more effective than bath application, with rescue approaching 100%. These differences perhaps indicate that microinjection is a more effective and efficient method for delivering lithium and inositol to the cell than bath application.

The microinjection experiments also demonstrate a second time-dependent process in the block that presumably reflects the relatively slow depletion of phosphoinositide lipid pools (Berridge et al., 1982; Downes and Stone, 1986; Godfrey, 1989; Markram and Segal, 1992). This is reflected in our experiments with lithium injection late in the first cell cycle, which blocks not NEB but cytokinesis and in our injection of a lower lithium concentration which again spares NEB but blocks cleavage. Lithium is a use-dependent inhibitor and requires turnover of the phosphoinositide cycle before its effects are manifest. Rapid turnover of phosphoinositides begins only at fertilization (Ciapa et al., 1992) and is stimulated periodically in phase with cell cycle transitions, so that one cell cycle transition may be responsible for the depletion that blocks the next (Ciapa et al., 1994).

Caged $InsP_3$

NEB triggered by releasing $InsP_3$ by photolysis fulfills the prediction that an $InsP_3$-induced calcium transient should release the lithium block. It also shows that the embryos had arrested in G_2, since earlier in the cell cycle, NEB cannot be evoked by injecting $InsP_3$ (Twigg et al., 1988), the embryos not being competent to enter mitosis.

Lithium and cell cycle transitions

Our data point to block at specific cell cycle stages. Embryos observed in metaphase and cytokinesis in the first cell cycle after lithium block largely remained in metaphase or cytokinesis, while embryos in anaphase progressed to cytokinesis and beyond. Lithium-treated embryos on average spent longer in metaphase and cytokinesis than controls, while the length of time spent in anaphase was comparable to controls. Individual embryos were observed to remain in metaphase for at least 30 minutes, compared to 5 minutes for controls, while anaphase embryos in the same experiments progressed normally. These observations indicate that lithium treatment blocks cells in metaphase and cytokinesis.

The pre-NEB block is more ambiguous in these bath experiments, in the absence of a morphological marker for G_2 embryos. However, the experiments with caged $InsP_3$ demonstrate unambiguously that these interphase embryos are blocked in G_2. It is evident that lithium blocks at the cell cycle stages that would be predicted from our knowledge of $InsP_3$ and calcium transients, which occur just prior to NEB, metaphase and cytokinesis (Ciapa et al., 1994; Wilding et al., 1996).

Phosphoinositide messengers and cell cycle

The only other investigation on the effects of lithium on mitotic cell cycles that we are aware of was performed by Wolniak on stamen hair cells of *Tradescantia* (Wolniak, 1987, 1988). He found that cells treated with lithium before NEB (the experimental condition closest to ours, among those that he used) were either arrested in prophase for >60 minutes or proceeded to prometaphase and arrested in metaphase. This is in reasonable agreement with our results, even though the differences in species (in kingdom, actually) and in experimental conditions prevent too detailed a comparison.

While it is generally accepted that $InsP_3$ triggers the intracellular calcium signal necessary for cell cycle resumption at fertilization (Galione et al., 1993; Whitaker and Swann, 1993; Whitaker and Irvine, 1984; Whitaker and Patel, 1990), the importance of $InsP_3$-induced calcium transients during the cell cycle is less widely accepted (Hepler, 1992, 1994; Whitaker, 1995). Nonetheless, strong evidence on this point has been accumulating for the early sea urchin embryo cell cycles. As we indicated in the Introduction, $InsP_3$ injection stimulates premature NEB and chromatin condensation (Twigg et al., 1988); furthermore, cell cycle calcium transients have been detected that accompany NEB, the metaphase-anaphase transition and cytokinesis (Browne et al., 1992; Ciapa et al., 1994; Poenie et al., 1985; Wilding et al., 1996); these transients are driven by oscillations in $InsP_3$ mass, since the $InsP_3$-antagonist heparin blocks both calcium transients and entry into mitosis (Ciapa et al., 1994; Whitaker, 1995). The present paper presents further evidence to support the hypothesis. The lithium block, which can be reversed by myo-inositol, appears to affect precisely those cell cycle transitions that have been demonstrated to be strictly correlated with $InsP_3$-induced calcium transients. Thus the classical lithium experiment is good independent evidence of the involvement of the phosphoinositide cycle in the regulation of the timing of early embryonic cell cycle transitions.

Our findings also raise the important question of whether the widespread teratogenetic effect of LiCl can be at least partly ascribed to an effect on the timing of embryonic cell cycles, an issue that merits further investigation.

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