Continuous phosphatidylinositol metabolism is required for cleavage of crane fly spermatocytes

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Summary

Successful cleavage of animal cells requires co-ordinated regulation of the actomyosin contractile ring and cleavage furrow ingression. Data from a variety of systems implicate phosphoinositol lipids and calcium release as potential regulators of this fundamental process. Here we examine the requirement for various steps of the phosphatidylinositol (PtdIns) cycle in dividing crane fly (*Nephrotoma suturalis*) spermatocytes. PtdIns cycle inhibitors were added to living cells after cleavage furrows formed and began to ingress. Inhibitors known to block PtdIns recycling (lithium), PtdIns phosphorylation (wortmannin, LY294002) or phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] hydrolysis [U73122 (U7)] all stopped or slowed furrowing. The effect of these drugs on cytokinesis was quite rapid (within 0-4 minutes), so continuous metabolism of PtdIns appears to be required for continued cleavage furrow ingression. U7 caused cleavage furrow regression concomitant with depletion of F-actin from the contractile ring, whereas the other inhibitors caused neither regression nor depletion of F-actin. That U7 depletes furrow-associated actin seems counterintuitive, as inhibition of phospholipase C would be expected to increase cellular levels of PtdIns(4,5)P_2 and hence increase actin polymerization. Our confocal images suggest, however, that F-actin might accumulate at the poles of U7-treated cells, consistent with the idea that PtdIns(4,5)P_2 hydrolysis may be required for actin filaments formed at the poles to participate in contractile ring assembly at the furrow.

Movies available online

Key words: F-actin, PtdIns(4,5)P_2, Cytokinesis, U73122, Phospholipase C, *Nephrotoma suturalis*

Introduction

Successful cytokinesis in animal cells typically relies upon contraction of an underlying actomyosin ring that causes membrane furrowing. Bipolar filaments of myosin II draw actin filaments (F-actin) together in a purse string-like contractile ring to effect separation of the daughter cells (Satterwhite and Pollard, 1992). For the plasma membrane to remain linked to the constricting contractile ring, membrane must expand into the furrow region. In principle, such membrane could arrive either by cortical flow from the poles of the cell (Wang et al., 1994) or by membrane addition in the vicinity of the ingressing furrow. Indeed, furrow-associated vesicle fusion events at the plasma membrane promote cleavage of frog (*Xenopus laevis*) and worm (*Caenorhabditis elegans*) embryos and promote cellularization in fruit fly (*Drosophila melanogaster*) embryos (Burgess et al., 1997; Byers and Armstrong, 1986; Jantsch-Plunger and Glotzer, 1999; Skop et al., 2001) (reviewed by Edamatsu, 2001; Hales et al., 1999; Straight and Field, 2000). Many types of membrane transaction are important during cleavage, as mutations in proteins affecting endocytosis (e.g., dynamin, clathrin), secretion (syntaxin 1, syntaxin 5, COG5), lysosomal trafficking (lvsA) or recycling endosomes (Rab11, Nuf) cause cytokinesis or cellularization defects in various organisms (Farkas et al., 2003; Gerald et al., 2001; Kang et al., 2003; Kwak et al., 1999; Lauber et al., 1997; Riggs et al., 2003; Thompson et al., 2002; Xu et al., 2002). Furrow-associated vesicle-microtubule interactions have been described (reviewed in Straight and Field, 2000), but less is known about membrane interactions with components of the actin cytoskeleton.

Phosphatidylinositol (PtdIns) lipids are uniquely attractive as candidates to coordinate membrane-actin cytoskeleton interactions during cleavage. In particular, PtdIns and its phosphorylated derivatives, the phosphoinositides, serve as signals that direct many types of intracellular trafficking events (reviewed by Corvera et al., 1999; Czech, 2003; De Matteis et al., 2002; Heath et al., 2003; Simonsen et al., 2001). Phosphoinositides also regulate the localization and activity of proteins involved in a number of other cellular processes (Itoh and Takenawa, 2002). For example, PtdIns 4,5-bisphosphate [PtdIns(4,5)P_2] influences regulators of actin polymerization (e.g. profilin, cofilin, and capping protein): high levels of PtdIns(4,5)P_2 induce actin polymerization, whereas low levels block actin assembly or induce severing of actin filaments (for a review, see Yin and Janmey, 2003). PtdIns(4,5)P_2 also binds directly to cleavage furrow proteins such as septins and ezrin-radixin-moesin (ERM) family members in vitro and may regulate their polymerization state or function in the cell (Hirao et al., 1996; Matsui et al., 1999; Zhang et al., 1999).

The involvement of PtdIns lipids in cell regulation is via a complex metabolic cycle (depicted in Fig. 1). Synthesis of PtdIns requires any of three possible sources of inositol: de novo synthesis, acquisition from nutritional sources or a salvage pathway. PtdIns transferase proteins (PITP) insert PtdIns into cellular membranes, where it serves as a substrate for lipid kinases that phosphorylate the D-3, D-4 or D-5 position of the inositol ring to produce PtdIns 3-phosphate [PtdIns(3)*P*], PtdIns(4)*P* or PtdIns(5)*P*. These monophosphorylated lipids may in turn be phosphorylated by phosphoinositide kinases to yield PtdIns (3,4)-bisphosphate [PtdIns(3,4)P_2], PtdIns(4,5)P_2 or PtdIns(3,5)P_2. PtdIns(4,5)P_2 is one of several substrates for PtdIns 3-kinases, which produce PtdIns (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3], among other products (not shown). PtdIns(4,5)P_2 hydrolysis by phospholipase C (PLC) plays a key role in cell signaling by producing the second messengers inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG), which function in calcium release and activation of protein kinase C. PtdIns may then be resynthesized from Ins(1,4,5)P_3 and DAG through salvage pathways, completing the cycle.

Data from a variety of systems suggest that PtdIns lipids are important for cytokinesis. The first indication of such a role came from experiments showing that lithium (Li^+) blocks cytokinesis in sea urchin zygotes (Becchetti and Whitaker, 1997; Forer and Sillers, 1987). Li^+ blocks the conversion of Ins(1,4,5)P_3 to PtdIns by inhibiting the enzymes inositol monophosphatase (IMPase) and inositol polyphosphate-1-phosphatase (IPPase) (for reviews, see Naccarato et al., 1974; Parthasarathy and Eisenberg, 1986; Rana and Hokin, 1990) (steps 1 and 2 in Fig. 1). As the inhibitory effect of Li^+ on cytokinesis is reversed by addition of the precursor, *myo*-inositol, the PtdIns cycle is important for cleavage (Becchetti and Whitaker, 1997; Forer and Sillers, 1987). A second piece of evidence came from mammalian studies showing that Nir2, a PITP, is required for cytokinesis in tissue culture cells (Litvak et al., 2002). A third clue came from the study of mutations in the *Drosophila* gene *four wheel drive* (*fwd*), that cause a cytokinesis defect during male meiosis. As *fwd* encodes a predicted PtdIns 4-kinase type III β (Brill et al., 2000), these data suggest that synthesis of PtdIns(4)*P* is important for cytokinesis (step 3 in Fig. 1). The fission yeast (*Schizosaccharomyces pombe*) homolog of *fwd* also has been implicated in cytokinesis, indicating that the function of this PtdIns 4-kinase is evolutionarily conserved (Desautels et al., 2001). In *S. pombe*, both PtdIns(4)*P* 5-kinase and its product, PtdIns(4,5)P_2, localize to the medial ring of dividing cells. In addition, PtdIns(4)*P* 5-kinase mutants show defects in cytokinesis, suggesting that PtdIns(4,5)P_2 is important in this process as well (Zhang et al., 2000). Further evidence that PtdIns(4,5)P_2 is involved in cytokinesis came from experiments in which both depletion of PtdIns(4,5)P_2 and binding of PtdIns(4,5)P_2 with neomycin were shown to block cytokinesis in mammalian tissue culture cells (Zhang et al., 1999). Similarly, injection of cells with anti-PtdIns(4,5)P_2 antibodies results in multinucleate cells (Han et al., 1992).

We examined possible roles of the PtdIns cycle in cytokinesis of crane fly (*Nephrotoma suturalis*) spermatocytes. Hundreds of crane fly spermatocytes develop synchronously in a single testis (Forer, 1982), so it is relatively easy to obtain many cells undergoing cleavage at the same time. In addition, the cells are large and the timing of cytokinesis relative to meiotic events has been determined, as has its time course (Silverman-Gavrila and Forer, 2001). The ability to culture live cells held in a fibrin clot (Forer and Pickett-Heaps, 1998) made it possible to add inhibitors of different steps of the PtdIns cycle to living cells and observe how these drugs affected cytokinesis in real time. Our data suggest that multiple steps of the PtdIns cycle contribute to cytokinesis in these cells; that multiple rounds of the cycle occur during cleavage; and that ongoing PtdIns(4,5)P_2 hydrolysis is key to maintaining cleavage furrow stability.

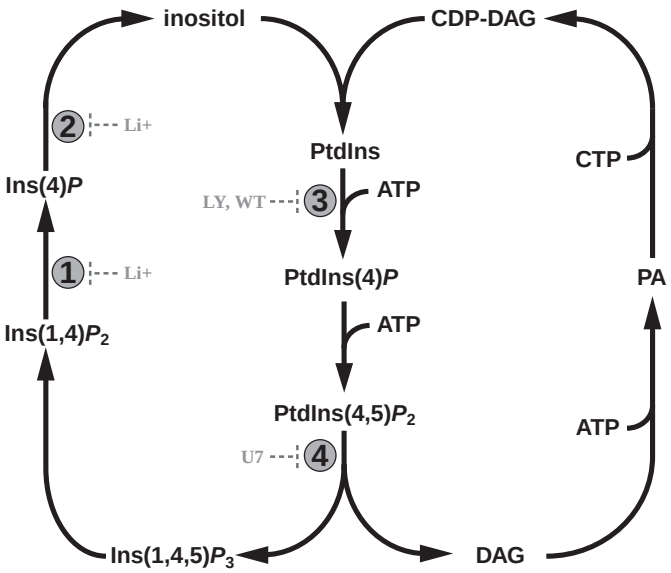

Fig. 1. Simplified version of the phosphatidylinositol cycle. Inositol is obtained from nutritional sources or de novo synthesis (not shown) or it is recovered from inositol trisphosphate [Ins(1,4,5)P_3] by a salvage pathway that involves sequential dephosphorylation of inositol phosphates by the enzymes inositol polyphosphate phosphatase (IPPase; indicated as step 1) and inositol monophosphate phosphatase (IMPase; step 2), both of which are inhibited by lithium. Phosphatidylinositol (PtdIns) is synthesized from inositol and CDP-diacyglycerol (CDP-DAG). Phosphatidylinositol is monophosphorylated to form PtdIns(4)*P* by type III PtdIns 4-kinase (step 3), which is inhibited by wortmannin (WT) and LY294002 (LY). PtdIns(4)*P* is phosphorylated by PtdIns(4)*P* 5-kinase to form phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. This in turn, is hydrolyzed by phospholipase C (PLC; step 4) to form Ins(1,4,5)P_3 and DAG in a reaction inhibited by U73122. DAG is then converted to phosphatidic acid (PA) and then CDP-DAG to complete the cycle. Note that WT and LY also inhibit PtdIns 3-kinase (not shown) and that a second type of PtdIns 4-kinase, the type II enzyme, is not inhibited by WT or LY.

Materials and Methods

Solutions and pharmacology

Insect Ringer's solution (0.13 M NaCl, 0.005 M KCl, 0.001 M $CaCl_2$, 0.003 M KH_2PO_4/Na_2HPO_4 buffer at pH 6.8) was prepared as individual concentrated stock solutions (10× salts, 10× buffer, 20× calcium), and stored in the freezer (–20°C) until dilution and use. Fibrinogen was freshly prepared and thrombin was in frozen aliquots, as described (Forer and Pickett-Heaps, 1998). LiCl was fully or partially substituted for NaCl in Ringer's solution, with the salt concentration (and tonicity) otherwise kept constant to avoid osmotic

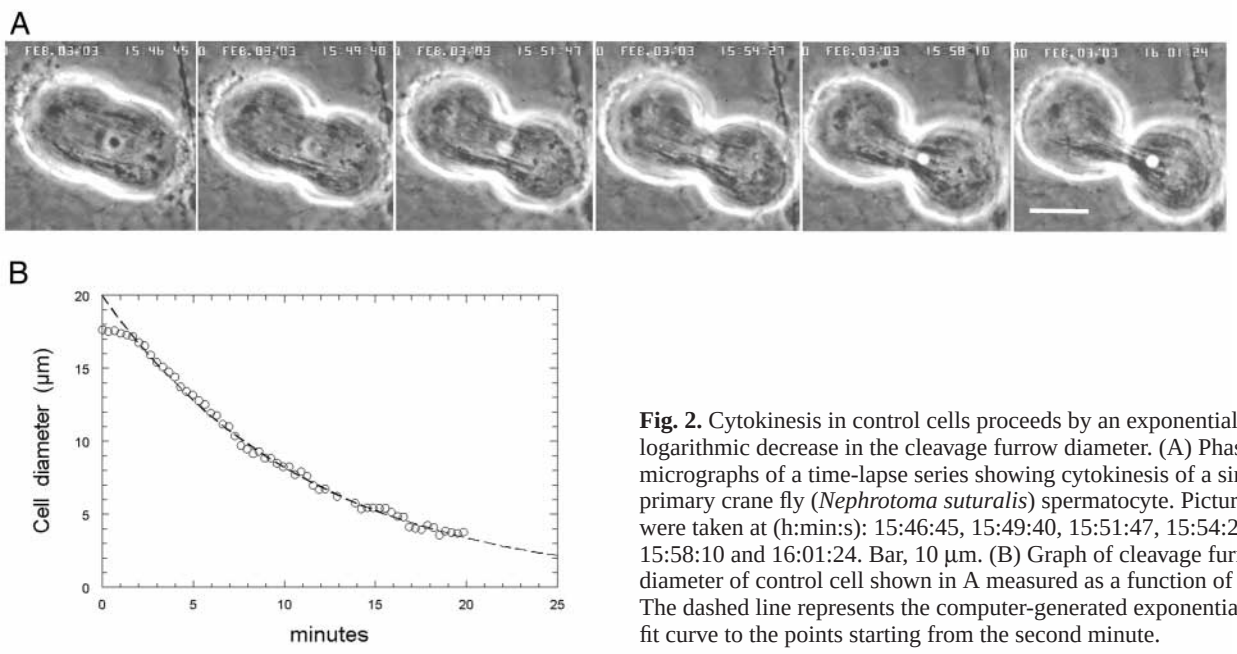

Fig. 2. Cytokinesis in control cells proceeds by an exponential or logarithmic decrease in the cleavage furrow diameter. (A) Phase micrographs of a time-lapse series showing cytokinesis of a single primary crane fly (*Nephrotoma suturalis*) spermatocyte. Pictures were taken at (h:min:s): 15:46:45, 15:49:40, 15:51:47, 15:54:27, 15:58:10 and 16:01:24. Bar, 10 μm. (B) Graph of cleavage furrow diameter of control cell shown in A measured as a function of time. The dashed line represents the computer-generated exponential best-fit curve to the points starting from the second minute.

effects. LiCl was used at a concentration of 0.13 M (100% Li^+), 0.065 M (50% Li^+, 50% Na^+) or 0.033 M (25% Li^+, 75% Na^+). *myo*-inositol (Sigma) was used at 10 mM by dissolving in Ringer's solution prior to use. *epi*-inositol (Sigma) was used at 20 mM. U73122 (U7) and its inactive isomer U73343 (both from Calbiochem) were dissolved at 5 mM in DMSO and stored in 10 μl aliquots at –20°C. Prior to use, they were thawed and dissolved in Ringer's solution to final concentrations of 1-5 μM. Wortmannin (WT; Sigma) was dissolved in DMSO at 10 mM, 100 μM or 10 μM and stored at –20°C in 5 μl aliquots. For experiments, stock WT was diluted with Ringer's solution to a final concentration of 10 μM, 100 nM or 10 nM and used within 2 minutes of dilution. As a precaution against possible photolability, fluorescent lights were kept off during the experiments. LY294002 (LY; Sigma) was dissolved in DMSO at 84 mM and stored at –20°C in 10 or 5 μl aliquots. It was dissolved in Ringer's solution to a final concentration of either 250 μM or 7.5 μM. We also studied brefeldin A (BFA; Sigma) dissolved in DMSO at 15 mg/ml. However, at concentrations of 15 μg/ml in Ringer's solution, a concentration reported to block cytokinesis in *C. elegans* embryos (Skop et al., 2001), BFA had no effect on cytokinesis in crane fly spermatocytes.

Crane fly spermatocytes and microscopy

Crane flies, *Nephrotoma suturalis* (Loew), were reared essentially as described (Forer, 1982). Living spermatocytes were placed in a fibrin clot in a perfusion chamber, as described (Forer and Pickett-Heaps, 1998). Cells were observed with phase-contrast microscopy, using an oil-immersion Nikon ×100 objective (N.A., 1.4). Individual primary spermatocytes in late anaphase/early telophase (pre-cleavage) were chosen for specific morphological characteristics (spherical, not flattened; healthy cytoplasmic streaming), to ensure both successful cleavage and consistent results. Inhibitors were added once the furrow began to form in a given dividing cell. For each experimental cell, we followed control cells from the same preparation (before addition of inhibitors) or from a preparation from the sister testis. Images were recorded in real time on videotape, and time-lapsed at 30 frames/minute using Adobe Premier 6.0 with an MPEG-4 codec. Time-lapsed movies were then analyzed using custom software and the data were analyzed using Slidewrite software. For presentation, still images were captured in Adobe Premier 6.0, exported to Adobe Photoshop, and modified only in brightness and contrast.

Histochemistry and fluorescence microscopy

Cells used for fluorescence microscopy were treated for 5 minutes with 100% Li^+; for 5 minutes with 250 μM LY; for 2.5 minutes with 5 μM U7; or for 3 minutes with 4 μM U7. After this they were lysed using a cytoskeleton stabilizing buffer, fixed with glutaraldehyde, treated with sodium borohydride and subsequently stained using procedures described elsewhere (Fabian and Forer, in preparation). In the experiments reported here, cells were stained for actin filaments using Alexa-488 phalloidin (Molecular Probes). Cells were studied using an Olympus Fluoview 300 confocal microscope, using a PlanApo ×60 oil immersion objective (N.A., 1.4), as described in Fabian and Forer (in preparation). Images for presentation were imported into Adobe Photoshop and processed as described above.

Results

Lithium stops or slows cytokinesis

Cytokinesis in non-treated control spermatocytes starts within a relatively short time after completion of autosomal anaphase (Silverman-Gavrila and Forer, 2001). The cell diameter decreases from around 15-20 μm to around 3-5 μm over the course of 10-20 minutes (Fig. 2; see Movies 1-3, http://jcs.biologists.org/supplemental/) (see also Silverman-Gavrila and Forer, 2001). The rate of change of diameter is described as exponential (Silverman-Gavrila and Forer, 2001), but the graphs of diameter as a function of time also fit logarithmic curves with similar ***r*** (goodness of fit) values. To determine if lithium (Li^+) affects cytokinesis, we added Li^+ to the cells during the first 1-4 minutes of furrow ingression, after which the furrow either stopped contracting or its rate of contraction slowed (Fig. 3). The effects were seen in all cells within 0-3 minutes (n=15), with Li^+ ranging from 100% to 25% substitution for sodium (Na^+). When the furrow was slowed or arrested in Li^+, the membranes in that region of the

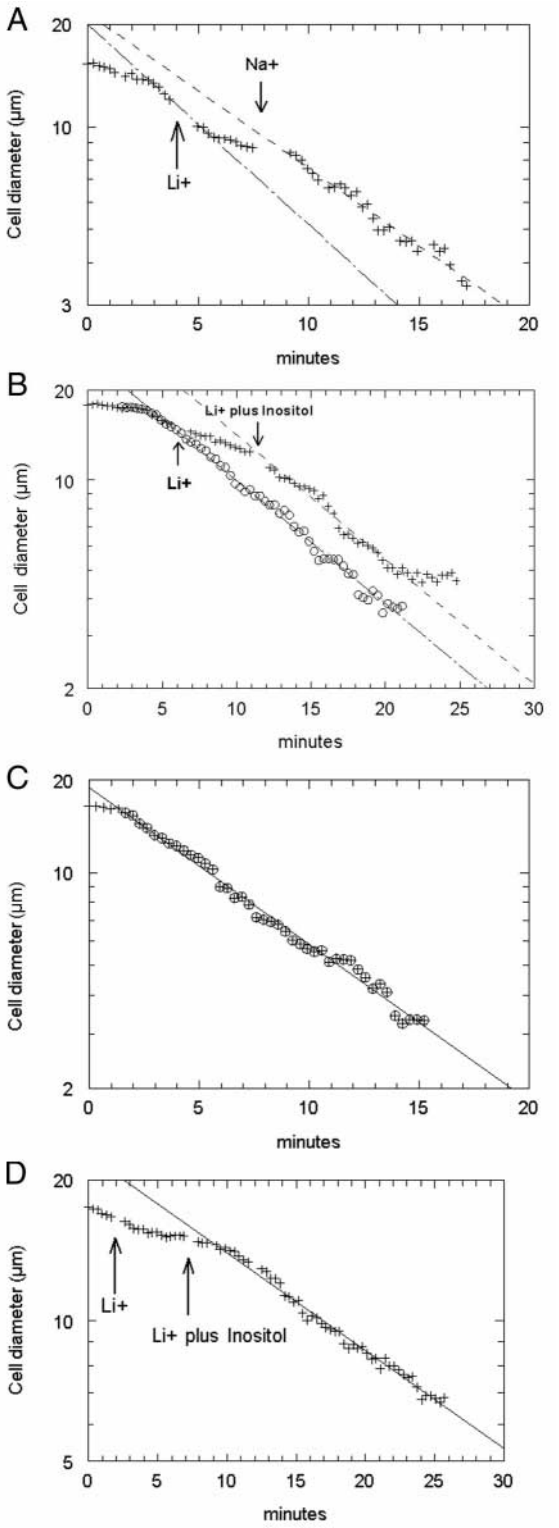

Fig. 3. Lithium dramatically slows the progression of cytokinesis and this effect is reversible by *myo*-inositol. Graphs of crane fly spermatocyte cell diameter measured at the cleavage furrow plotted on a logarithmic scale as a function of time for single cells treated with Li^+. (A) The effects of 100% Li^+ (added at the left arrow) and reversal with Ringer's solution (right arrow). The line on the left is the computer-generated best linear fit to the points between 2 and 4 minutes (i.e. expected curve in the absence of Li^+). The dashed line on the right is the computer-generated best linear fit to the included points. (B) The effects of 25% Li^+ Ringer's (added at the left arrow) and reversal with *myo*-inositol added to the Li^+ Ringer's (right arrow) for the series of points indicated by +; the dashed line is the best linear fit to the points after addition of *myo*-inositol. The circles and the other best-fit line are from a control cell. (C) A control cell maintained in Ringer's solution. The line is the best linear fit to the circled points. (D) A cell treated with Li^+, then with *myo*-inositol in Li^+, at the times indicated on the graph. Cleavage slowed a few minutes after adding Li^+ and sped up after *myo*-inositol was added. The line is the computer-generated best fit to the points after addition of *myo*-inositol.

cell changed configuration, often appearing to bleb along the length of the furrow and in nearby areas of the cell. When the Li^+ was washed out with normal Ringer's solution, furrow contraction resumed immediately (4/7 cells), or after 1-2 minutes (3/7 cells) and ended normally (Fig. 3A). To estimate the degree of slowing caused by Li^+, we plotted the logarithms (log_{10}) of cell diameters (in μm) versus time and measured the slopes of the resultant lines for control cells (Fig. 3B,C) and for Li^+-treated cells (Fig. 3A,B,D). The change in log of cell diameter per minute indicated that Li^+ reduced the rate of change to around 20% of the control value (Fig. 4).

To determine if the effect on cytokinesis was due to effects on the PtdIns cycle, we added *myo*-inositol to the Li^+ solutions. Cells undergoing cytokinesis were treated with Li^+ and furrow ingression slowed or stopped. *myo*-inositol (10 mM in Li^+ Ringer's) was added to the cells 4-6 minutes after the initial addition of Li^+. In all cells (n=7), *myo*-inositol reversed the effects of the Li^+ either immediately (6/7 cells; Fig. 3B) or within 2 minutes (1/7) after perfusion (Fig. 3D). We plotted log_{10} cell diameters (in μm) versus time to estimate the rate of cleavage after addition of *myo*-inositol (Fig. 3B,D). The slopes of the curves following addition of *myo*-inositol (Fig. 4) are similar to those prior to Li^+ treatment, but may be less than control values (P=0.02). To confirm the involvement of the PtdIns cycle, in similar experiments we added 20 mM *epi*-inositol (an inactive isomer). In no case did *epi*-inositol reverse the effect of Li^+ (n=3; not shown). When the *epi*-inositol/Li^+ Ringer's was washed out after 8-10 minutes with regular (Na^+) Ringer's solution, the ingression rate increased immediately to normal levels (not shown).

PtdIns 3/4-kinase inhibitors arrest cytokinesis

To determine if PtdIns phosphorylation (step 3 in Fig. 1) is important for cytokinesis, we employed two inhibitors that block the activity of PtdIns 3-kinases and PtdIns 4-kinases, wortmannin (WT) and LY294002 (LY) (Arcaro and Wymann, 1993; Nakanishi et al., 1995; Stack and Emr, 1994). We treated dividing crane fly spermatocytes with either WT or LY at concentrations predicted from the literature to block both PtdIns 3-kinase and PtdIns 4-kinase activity (Nakanishi et al., 1995; Sorensen et al., 1998) or with concentrations of LY (7.5 μM) or WT (100 nM or 10 nM) predicted to block PtdIns 3-kinase activity alone (Monfar et al., 1995). Cells were treated with either LY or WT within 2-6 minutes of onset of cleavage. LY at 7.5 μM (n=5 cells) or 250 μM (n=3 cells) blocked furrow ingression within 1-3 minutes of adding the drug. LY caused the furrow to remain at constant diameter or regress slightly

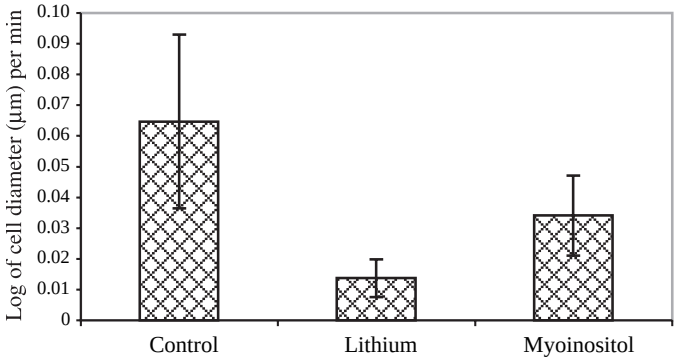

Fig. 4. The average slope of $\log_{10}$ diameter as a function of time for Li^+-treated crane fly spermatocytes is dramatically reduced compared to that of control cells and recovers substantially upon *myo*-inositol addition. The average slopes (±s.d.) are control 0.0647±0.0295 (n=12), Li^+ 0.0137±0.0065 (n=12) and Li^+ plus *myo*-inositol 0.0341±0.0140 (n=7). Error bars represent s.d. Values are significantly different for control versus Li^+ or Li^+ versus *myo*-inositol (P<0.0004, Student's t-test), whereas the difference between the control and *myo*-inositol was borderline significant (P=0.021, Student's t-test).

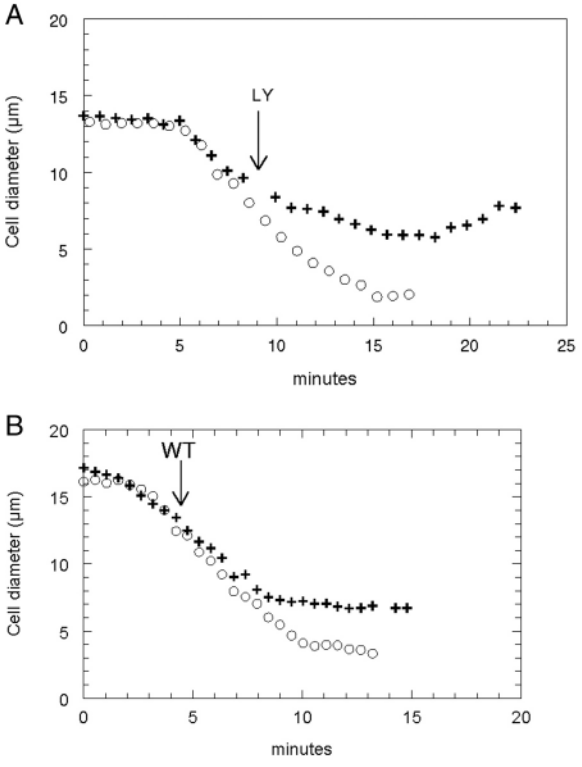

Fig. 5. LY294002 (LY) and wortmannin (WT) block further cytokinesis progression in crane fly spermatocytes. (A,B) Graphs of cleavage furrow diameter as a function of time for single cells treated with either (A) LY (added at the arrow) or (B) WT (added at the arrow). Cleavage furrow diameters in control cells (O) are shown for comparison.

(Fig. 5A, Fig. 6). WT at 10 μM blocked constriction consistently (6/6 cells); 100 nM WT blocked constriction about half the time (2/4 cells) and 10 nM WT did not block

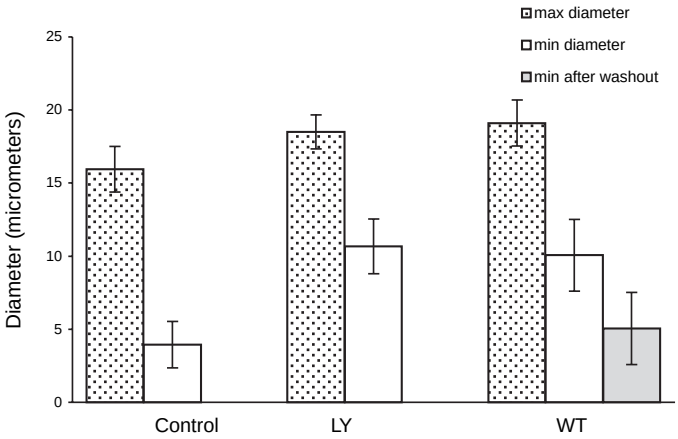

Fig. 6. LY294002 (LY) and wortmannin (WT) affect the extent of cleavage of dividing crane fly spermatocyte cells and the effect of WT is reversible. Bars with dots, average maximum diameters in μm (at the start of cleavage). Clear bars, average minimum diameter at the end of cleavage in control cells (n=8) and at the end of treatment in cells treated with LY (n=7) or WT (n=8), as indicated. Dark gray bars, average diameter after washing out WT (n=4). After washout of WT cytokinesis resumes and the diameters become smaller. Error bars represent s.d. Minimum diameters of control cells were not significantly different from those of washed out WT-treated cells (P=0.3, Student's t-test).

constriction (n=5). Ingression stopped about 3 minutes after adding the drug (Fig. 5B), with a range of 2-4 minutes (n=7) and did not proceed further in the presence of the drug, arresting at about 50% of the initial cell diameter (Fig. 6). In 4 of the 5 cells constriction resumed 10-15 minutes after washing out the drug and in the other cell resumed 6 minutes after washout. When LY was washed out, on the other hand, there was no indication that cleavage resumed, although only one of the 6 cells was followed for more than 7 minutes after washout. Cells treated with either WT or LY gave off large blebs of cytoplasm, erratically, over the surface of the cell and often in the furrows and pole regions (e.g. Fig. 7; see also Movies 4, 5, http://jcs.biologists.org/supplemental/). The blebbing continued for at least 15-20 minutes after the drugs were washed out and appeared even more vigorous after washout than in the presence of the drug.

PLC inhibition causes furrow instability

To determine if PtdIns(4,5)P_2 hydrolysis (step 4 in Fig. 1) is required for cleavage of crane fly spermatocytes, we treated cells with the aminosteroid U73122 (U7), an inhibitor of phospholipase C (PLC) (Wu et al., 1992; Yule and Williams, 1992). The effects of U7 occurred within a tight concentration range. Treatment with U7 at concentrations of 5 μM and 4 μM within the first 4-5 minutes of cleavage consistently blocked furrow ingression; concentrations below 3 μM had little or no effect (Fig. 8A). Blockage occurred within 1-2 minutes, and the furrows of cells treated with 4-5 μM U7 always regressed. Cells with blocked cleavage furrows had extensive blebbing, primarily at the poles. For cells treated with concentrations ⩽4 μM, blockage and regression were reversed after washing out the drug with normal Ringer's solution (Fig. 8B and Movie 6,

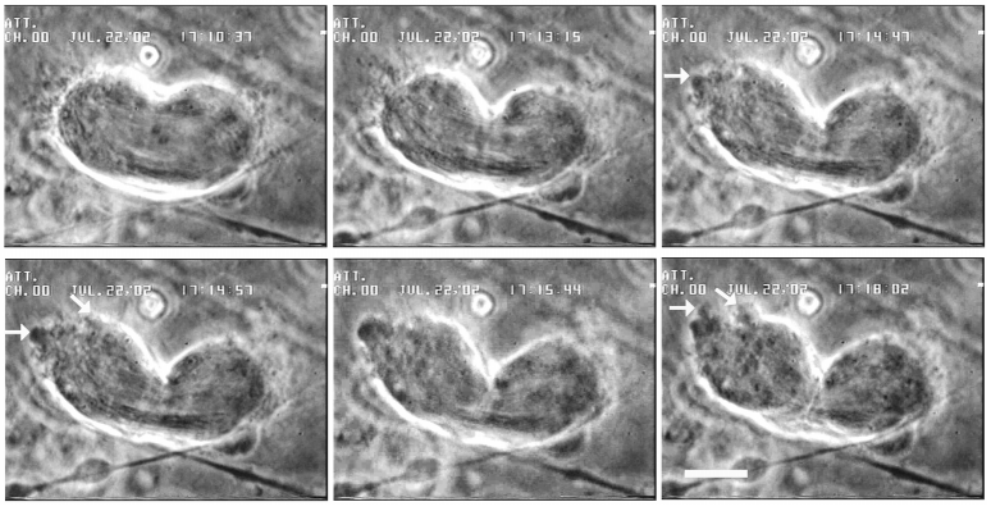

Fig. 7. Phase micrographs of a time-lapse series showing cytoplasmic blebbing in a single crane fly primary spermatocyte after treatment with wortmannin. Blebbing (arrows) is observed at the poles of the cell. The illustrations are at (h:min:s): 17:10:37, 17:13:15, 17:14:47, 17:14:57, 17:15:44 and 17:18:02. Bar, 10 μm.

http://jcs.biologists.org/supplemental/); however, there was no recovery for cells treated with 5 μM U7 (Movie 7, http://jcs.biologists.org/supplemental/). Treatment with an inactive isomer, U73343 (Agarwal et al., 1993; Chen et al., 1994; Jin et al., 1994), at a concentration of 5 μM had no effect on cytokinesis (not shown).

Effects of PtdIns cycle inhibitors on actin in the cleavage furrow

Since PtdIns(4,5)P_2 levels influence actin polymerization (reviewed by Yin and Janmey, 2003), we examined the effects of Li^+, LY and U7 on the organization of actin filaments in the cleavage furrow. In control cells, cortical actin filaments are arranged longitudinally around the cell until mid- to late-anaphase; after this, perpendicularly oriented filaments appear in the equatorial region. Subsequently, the filaments form increasingly compact equatorial bundles during telophase as the cells complete cytokinesis (Forer and Behnke, 1972; Silverman-Gavrila and Forer, 2003) (Fig. 9A-C). Li^+ and LY had no effect on the appearance of actin filaments in the cleavage furrow at any stage of cleavage (Fig. 9D,E). U7 on the other hand, had clear and unequivocal effects on cleavage furrow and polar actin, although spindle actin was unaffected (not shown). Late-cleavage (thick) contractile rings were unaffected, but in stages from late anaphase until mid-cleavage all equatorially arranged actin filaments were absent from the cleavage furrow (*n*=7; Fig. 9F,G) or in the last stages of disappearing, with only a few scattered filaments remaining (*n*=2; not shown). Other cortical filaments were present, but were reduced in number and less regularly arranged. In polar regions, U7 caused the usually linear-appearing actin to appear both linear and punctate or patchy (Fig. 9F,G) and staining in these regions appeared stronger than that of control cells.

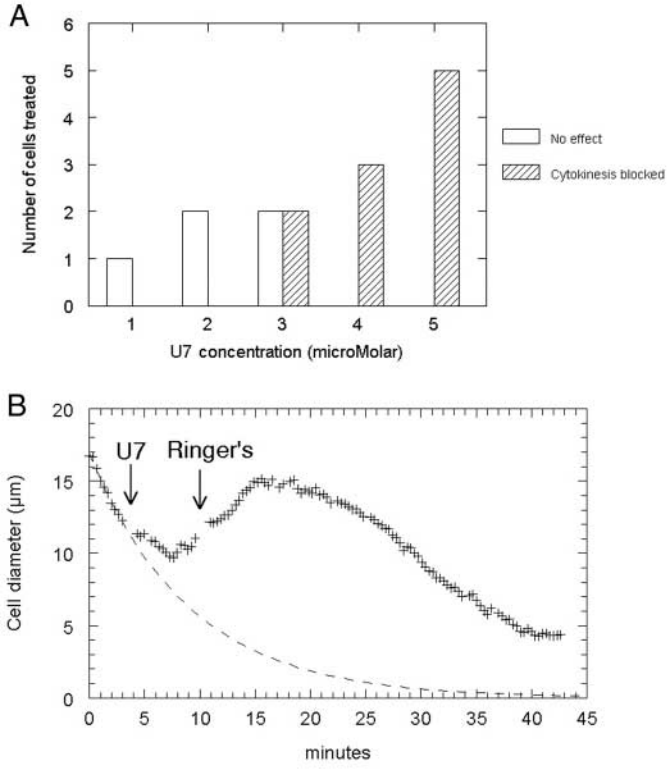

Fig. 8. U73122 (U7) causes reversible regression of cleavage furrows in crane fly primary spermatocytes. (A) Concentration dependence of U7 in blocking constriction. Clear bars, cells in which there was no effect on cleavage. Hatched bars, cells in which cytokinesis was blocked. (B) Graph of a single cell treated with 4 μM U7 (added at the left arrow), which was then washed out with Ringer's solution (right arrow). Note that after addition of U7, the furrow slows, regresses, and then resumes constriction approximately 8 minutes after washout. The dashed line is the computer-generated exponential best-fit curve through the points prior to addition of U7.

Discussion

Our data show that PtdIns recycling is necessary for efficient ingression, and that PtdIns phosphorylation and PtdIns(4,5)P_2 hydrolysis are important for furrow ingression and stability. Previous experiments on numerous organisms (described in the Introduction) suggested roles for different steps of the PtdIns cycle in cytokinesis. Our data are consistent with multiple steps

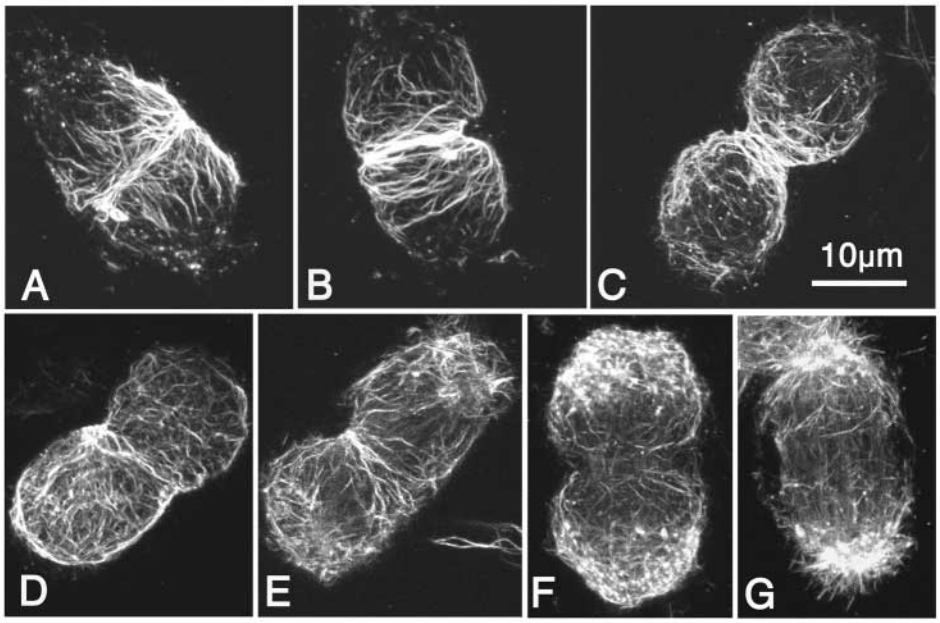

Fig. 9. Treatment with U73122 (U7) results in depletion of cleavage furrow F-actin. Confocal fluorescence micrographs of crane fly primary spermatocytes stained with Alexa-488 phalloidin to visualize F-actin. Each illustration is of a summed *Z*-series of confocal images. (A,B,C) Control cells in (A) late anaphase, (B) later anaphase and (C) telophase. (D) Cell treated with Li^+. (E) Cell treated with LY. (F,G) Cells treated with U7. Actin filaments appear normal in all cells except for those treated with U7, in which patchy cortical actin is prominent at the poles and actin filaments are absent or greatly reduced at the equator (F,G). Bar, 10 μm.

and, indeed, multiple cycles being required during cleavage. In addition, our observation that spermatocytes must hydrolyze PtdIns(4,5)P_2 to maintain cleavage furrow F-actin has broad-reaching implications for the mechanism of recruitment of furrow actin arrays.

Multiple PtdIns steps influence cytokinesis

Li^+ blocks cytokinesis in crane fly spermatocytes (Fig. 3), as it does in sea urchin embryos (Becchetti and Whitaker, 1997; Forer and Sillers, 1987). The ability of *myo*- but not *epi*-inositol to restore cytokinesis following Li^+ treatment suggests that Li^+ acts by blocking PtdIns recycling rather than exerting other effects, such as inhibition of glycogen synthase kinase-3 β (GSK3β), that are not rescued by *myo*-inositol (Hedgepeth et al., 1997). Thus, Li^+ acts on cytokinesis by preventing Ins(1,4,5)P_3 from forming new PtdIns (Fig. 1). Continued slow furrowing in the presence of Li^+ suggests that de novo PtdIns synthesis can support a small amount of cleavage, as animals are known to synthesize inositol from glucose by a mechanism that is only partially sensitive to Li^+ (Rana and Hokin, 1990). Although in some of our experiments we replaced the entire sodium in Ringer's solution with lithium (0.13 M), which should be sufficient to block PtdIns recycling, there can be discrepancies between external and internal Li^+ concentrations (e.g. Becchetti and Whitaker, 1997), so we cannot rule out the alternative interpretation that Li^+ does not completely block recycling. In any case, the drastic slowing (Fig. 4) indicates that the bulk of PtdIns used during cytokinesis derives from salvaged precursors.

PtdIns 3/4-kinase inhibitors, WT and LY, block cytokinesis in crane fly spermatocytes (Figs 5, 6). Previous experiments showed that the PtdIns 4-kinase type III β encoded by *fwd* is required for cytokinesis in *Drosophila*: spermatocytes mutant for *fwd* initiate cleavage, but the furrows are unstable and the daughter cells fuse together, producing multinucleate spermatids (Brill et al., 2000). In crane fly spermatocytes, both WT and LY stop further ingression. Although our experiments do not distinguish between inhibition of PtdIns 3/4-kinases, we note that low levels of LY and WT (predicted from experiments on mammalian cells to block PtdIns 3-kinase but not PtdIns 4-kinase activity) are sufficient to block cytokinesis. Further support for PtdIns 3-kinase involvement is that after washout of either LY or WT there is dramatic cellular blebbing, similar to previously reported effects of activation of PtdIns 3-kinase (Vemuri et al., 1996).

Our experiments using the PLC inhibitor, U7, demonstrate a role for PtdIns(4,5)P_2 hydrolysis in cytokinesis. PtdIns(4,5)P_2 has previously been implicated in cytokinesis in both *S. pombe* and mammalian cells (Han et al., 1992; Zhang et al., 1999; Zhang et al., 2000). In addition, calcium and Ins(1,4,5)P_3 are required for embryonic cleavage divisions in zebrafish (*Danio rerio*) (Chang and Lu, 2000) and calcium release has been observed during cleavage of *Xenopus* and fish (medaka and *Danio*) embryos (Chang and Meng, 1995; Fluck et al., 1991; Meng and Chang, 1994; Miller et al., 1993; Muto et al., 1996; Stricker, 1995; Webb et al., 1997), although its role remains controversial (cf. Noguchi and Mabuchi, 2002). The U7 aminosteroid we used can be highly poisonous to cells and indeed at 5 μM had pleiotropic toxic effects. Importantly, the effects were reversible at 4 μM (Movie 6, http://jcs.biologists.org/supplemental/), indicating that the cells were not poisoned. The effects of U7 were different from those of other inhibitors we used in that the cleavage furrows regressed, reminiscent of the phenotype of *D. melanogaster* spermatocytes mutant for *fwd* (Brill et al., 2000). However, regression of U7-treated furrows in crane fly spermatocytes appeared much more rapid than in *fwd* mutants (R. Wong and J.A.B., unpublished). Because U7 has been reported to interfere with the in vivo activity of multiple isoforms of PLC (Balla, 2001), the rapid effect of this drug on cleavage stability suggests that PtdIns(4,5)P_2 hydrolysis and perhaps the downstream products, DAG, Ins(1,4,5)P_3 and calcium, are continuously required for maintaining the cleavage furrow and for continuing ingression. Our results are compatible with a role for calcium in cleavage in this system, but direct

measurements of calcium in drug-treated and untreated crane fly spermatocytes would be needed to address this directly. We note that our preliminary results in *Drosophila* spermatocytes indicate that both PtdIns(4,5)P_2 hydrolysis and calcium are required for cleavage (R. Wong and J.A.B., unpublished).

Thus we have shown that multiple steps of the PtdIns cycle (or perhaps multiple PtdIns pathways) contribute to spermatocyte cell cleavage. Our data suggest that different steps of the PtdIns cycle play distinct roles in cleavage. Although we cannot formally rule out models in which a single step [e.g. PtdIns(4,5)P_2 hydrolysis] is critically required for cleavage and the rest of the cycle serves to produce the substrate for that step, it seems more likely that several of the metabolites in the PtdIns cycle co-ordinate distinct subsets of membrane trafficking and protein activities during cytokinesis because drugs that inhibit different stages in the PtdIns cycle induce morphologically distinct effects. Such a model is consistent with well-established data indicating that the various PtdIns lipids localize and function in different compartments within cells (c.f. Simonsen et al., 2001).

Multiple PtdIns cycles are required for cytokinesis

Cytokinesis was arrested (or slowed) rapidly when we blocked any of a number of steps in the PtdIns cycle during cytokinesis; i.e., adding the drug at any time within the first 5-6 minutes after constriction begins blocks cytokinesis. The first 5 minutes roughly corresponds to the first third of cytokinesis (both by time and by diameter), so our data indicate that the PtdIns cycle is required continuously for at least the initial third of cytokinesis and probably throughout. If, on the other hand, PtdIns lipids were required only once during cleavage (to initiate constriction, for example), one would have expected a sensitive period beyond which the PtdIns cycle would no longer be required. Similarly, a requirement for PtdIns lipids just once at a late stage of cleavage would be predicted to result in an identical endpoint regardless of when the various inhibitors were applied. However, this was not the case; instead, the time that ingression ceased was related to the time of drug addition and ingression terminated with variable cleavage furrow diameters. Thus the PtdIns cycle seems to be required continuously throughout cytokinesis. This conclusion is consistent with previous observations using the MLCK inhibitor ML7, which suggested that MLCK activity is required continually during cleavage (Silverman-Gavrila and Forer, 2001). It is also interesting in light of recent results that actin and two other cleavage furrow proteins, myosin essential light chain and tropomyosin, turn over quite rapidly (at least once every minute) in the medial ring of dividing *S. pombe* cells (Pelham and Chang, 2002). Perhaps turnover of membrane PtdIns lipids drives a constant reorganization of the actin cytoskeleton to enable successful constriction of the contractile ring.

PtdIns(4,5)P_2 hydrolysis regulates actin cytoskeletal organization during cleavage

The PLC inhibitor U7 profoundly affected furrow stability and F-actin organization. U7-treated cells had a dearth of actin filaments in the central portion of the cell after 2.5-3 minutes of treatment, concomitant with changes in actin organization at the poles, including fewer filamentous and more punctate elements. The lack of F-actin at the furrow suggests that PtdIns(4,5)P_2 turnover plays a role in either accumulation or stabilization of the long actin filaments that circumscribe the equator. This result directly implicates PtdIns(4,5)P_2 hydrolysis in maintaining the furrow and raises the possibility that the other inhibitors, especially Li^+ and LY (which have no obvious effect on furrow F-actin after 5 minutes of treatment), may have their most immediate effect on vesicle trafficking for example, rather than on actin cytoskeletal dynamics. This difference in the effect of the different PtdIns cycle inhibitors on F-actin in the furrow is thus consistent with the idea that each step of the cycle plays a distinct role in cytokinesis. However, we cannot rule out the possibility that the different phenotypes are due instead to a matter of timing; perhaps 5 minutes is not long enough for the actin filaments to disappear from Li^+- or LY-treated cells.

Our data do not address where in the cell PtdIns(4,5)P_2 must be hydrolyzed. Although it is attractive to imagine that PtdIns(4,5)P_2 hydrolysis occurs in the vicinity of the cleavage furrow, it is equally possible that PtdIns(4,5)P_2 is hydrolyzed at the poles of the cell. If the primary effect of U7 were to block PtdIns(4,5)P_2 hydrolysis at the poles of the cell, this might promote actin polymerization at the poles at the expense of contractile ring assembly at the equator. Indeed, preliminary experiments examining the effect of U7 on *Drosophila* spermatocytes show an apparent increase in PtdIns(4,5)P_2 at the cell poles (R. Wong and J.A.B., unpublished). This excess PtdIns(4,5)P_2 appears concentrated in membranous structures organized in a manner reminiscent of the actin foci observed at the poles of U7-treated crane fly spermatocytes (R. Wong and J.A.B., unpublished) (Fig. 9). To understand fully the role PtdIns(4,5)P_2 hydrolysis plays in cytokinesis, it will be of key importance to determine where PLC is active in dividing cells and to examine the localization and function of potential downstream effectors, Ins(1,4,5)P_3, DAG, calcium and protein kinase C, in this process.

Dynamic turnover of PtdIns(4,5)P_2: actin and the mechanism of cytokinesis

Several models have been proposed to explain cytokinesis: 1. The presence of an actomyosin contractile ring suggested a purse string-like mechanism of cleavage analogous to contraction in muscle (Satterwhite and Pollard, 1992); 2. Cleavage may be facilitated by relaxation of cortical tension at the poles relative to the equator (White and Borisy, 1983); 3. Cortical tension may be present everywhere in the cell except in the furrow, which undergoes equatorial collapse (Wang, 2001). Recent data examining cytokinesis in mammalian tissue culture cells are most consistent with aspects of model 3: for example, dividing cells maintain cortical tension at the poles (Matzke et al., 2001) and successful cleavage apparently requires actin polymerization at the poles (O'Connell et al., 2001). Myosin II (DeBiasio et al., 1996), cell surface receptors (Wang et al., 1994) and actin filaments (Cao and Wang, 1990a; Cao and Wang, 1990b) flow toward the equator during cytokinesis. In addition, furrow actin arrays are depolymerized during cytokinesis (O'Connell et al., 2001; Schroeder, 1972). Recent experiments in fission yeast indicate that this turnover may be even more dramatic than previously thought, occurring at least once per minute during constriction of the medial ring (Pelham and Chang, 2002). Despite this depolymerization of

furrow actin filaments, cell division is accompanied by increased stiffness in the furrow (Matzke et al., 2001) and this stiffness may be counteracted by compensatory contraction of the actomyosin ring (reviewed by Robinson, 2001).

Our U7 data indicate that PtdIns(4,5)P_2 needs to be turned over to maintain actin in the cleavage furrow. High levels of PtdIns(4,5)P_2 are associated with actin polymerization and we observe what appear to be increased numbers of actin filaments at the poles of U7-treated cells. Thus the requirement for PtdIns(4,5)P_2 turnover would be consistent either with a need to depolymerize actin at the poles (polar relaxation) or with PtdIns(4,5)P_2 hydrolysis somehow permitting recruitment of polymerized actin from the poles to the furrow. In mammalian cells, preformed actin filaments move toward the furrow and F-actin is initially perpendicular to the equator before becoming incorporated into an equatorial orientation in the contractile ring (Cao and Wang, 1990a; Cao and Wang, 1990b; Fishkind and Wang, 1993). Consistent with this, in the cortex of cleaving crane fly spermatocytes, early in cytokinesis F-actin is perpendicular to the furrow, whereas it later forms a contractile ring (Forer and Behnke, 1972). The absence of furrow filaments and apparent increase in F-actin at the poles in the presence of U7 might suggest that these filaments arise at the poles of the cell and are usually severed and transported to the equator for use in furrowing. In the absence of PtdIns(4,5)P_2 hydrolysis, the polar filaments are stabilized and accumulate.

In conclusion, our data indicate that at least three aspects of PtdIns metabolism influence the outcome of cytokinesis: Ins(1,4,5)P_3 recycling, phosphoinositide [PtdIns(3)*P* and/or PtdIns(4)*P*] synthesis and PtdIns(4,5)P_2 hydrolysis. As the block in cleavage occurs rapidly, continuous cycles of PtdIns synthesis and degradation are required for at least the first third of constriction. Changes in actin organization at poles and cleavage furrows in cells treated with the PLC inhibitor, U7, suggest that PtdIns(4,5)P_2 hydrolysis plays a key role in maintaining cleavage furrow F-actin arrays. It is notable that this requirement for PtdIns(4,5)P_2 hydrolysis is conserved: *Drosophila* spermatocytes and sea urchin embryos treated with PLC inhibitors have unstable cleavage furrows and fail cytokinesis (M. Ng and D. Burgess, personal communication; R. Wong, I. Hadjiyanni and J.A.B., unpublished). It is attractive to imagine that PtdIns(4,5)P_2 hydrolysis, through its effects on both intracellular calcium pools and F-actin assembly, may serve to coordinate such cellular responses as myosin II activation and vesicle fusion events with cytoskeletal organization at the furrow.

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