Current Biology, Vol. 15, 1401–1406, August 9, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.06.060

PIP2 Hydrolysis and Calcium Release Are Required for Cytokinesis in *Drosophila* Spermatocytes

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Summary

The role of calcium (Ca^{2+}) in cytokinesis is controversial [1, 2], and the precise pathways that lead to its release during cleavage are not well understood. Ca^{2+} is released from intracellular stores by binding of inositol trisphosphate (IP3) to the IP3 receptor (IP3R) [3], yet no clear role in cytokinesis has been established for the precursor of IP3, phosphatidylinositol 4,5-bisphosphate (PIP2). Here, using transgenic flies expressing PLCδ-PH-GFP, which specifically binds PIP2 [4–6], we identify PIP2 in the plasma membrane and cleavage furrows of dividing *Drosophila melanogaster* spermatocytes, and we establish that this phospholipid is required for continued ingression but not for initiation of cytokinesis. In addition, by inhibiting phospholipase C, we show that PIP2 must be hydrolyzed to maintain cleavage furrow stability. Using an IP3R antagonist and a Ca^{2+} chelator to examine the roles of IP3R and Ca^{2+} in cytokinesis, we demonstrate that both of these factors are required for cleavage furrow stability, although Ca^{2+} is dispensable for cleavage plane specification and initiation of furrowing. Strikingly, providing cells with Ca^{2+} obviates the need to hydrolyze PIP2. Thus, PIP2, PIP2 hydrolysis, and Ca^{2+} are required for the normal progression of cytokinesis in these cells.

Results and Discussion

Quantitative Features of Cytokinesis in Untreated Cells

To study cleavage in vitro and perturb the phosphatidylinositol (PI) pathway (Figure 1A) during cytokinesis, we cultured *Drosophila melanogaster* spermatocytes in fibrin clots inside a perfusion chamber, extending a method previously employed in crane-fly spermatocytes [7]. Dividing cells were identified by the morphology of their mitochondria and parafusorial membranes (multilayered nuclear membranes contiguous with the ER), which align along the spindle and appear dark by phase microscopy (Figure 2A, Movie S1 in the Supplemental Data available with this article online).

All morphologically normal control cells in meiosis I or meiosis II initiated cytokinesis, and most (91%) divided successfully (Figure 1B), forming stable intercellular bridges. Regression analysis (n = 34) suggested that constriction proceeded with linear kinetics over at least the first half of the process (e.g., Figure 2D). Constriction during meiosis I was significantly faster (1.2 ± 0.3 μm/min) than during meiosis II (0.8 ± 0.2 μm/min) ($p = 0.0002$, t test).

Localization of Phosphoinositides

We previously showed that PI4Kβ, encoded by the gene *four wheel drive* (*fwd*), is required for spermatocyte cytokinesis [8], implicating the PI pathway in this process. We used green fluorescent protein (GFP) fusions to PH domains that specifically bind PI(4)-phosphate (PI4P), PIP2, or PI(3,4,5)-trisphosphate (PIP3) to determine their localization during cleavage. Expression of these fusion proteins had no effect on the kinetics of cytokinesis, although PLCδ-PH-GFP, which binds PIP2, caused a weak cytokinesis defect (see below).

PIP2, but not PI4P or PIP3, was present in the plasma membrane of dividing spermatocytes. Distribution of PIP2 was uniform across the entire membrane, including the cleavage furrow. In contrast, PI4P localized to vesicular structures near the poles of the cell and PIP3 was not concentrated on cellular membranes during cleavage (Figure S1).

PIP2 Is Required for Cytokinesis

The presence of PIP2 in the plasma membrane suggested that it might participate in cytokinesis. To test this, we depleted PIP2 in the cell using three different methods (Figure 1A): dephosphorylation by the *Salmonella* phosphoinositide phosphatase SigD [9, 10], titration by PLCδ-PH-GFP [11], and sequestration by the cell-permeable PIP2 binding peptide, PBP10 [12]. All three treatments produced cytokinesis defects.

In two independent lines expressing SigD, PIP2 was depleted from the plasma membrane (not shown). SigD caused a dramatic cytokinesis defect, with 47.8%–72.0% of spermatids containing multiple nuclei (Table S1). That the majority of cells contained three or four nuclei indicated failure of cytokinesis during both meiosis I and II. Expression of PLCδ-PH-GFP caused a weaker cytokinesis defect, with 1.1%–1.9% of spermatids containing multiple nuclei and the majority of these containing two nuclei rather than four. For all lines tested, the multinucleate cells represented a statistically significant cytokinesis defect when compared to

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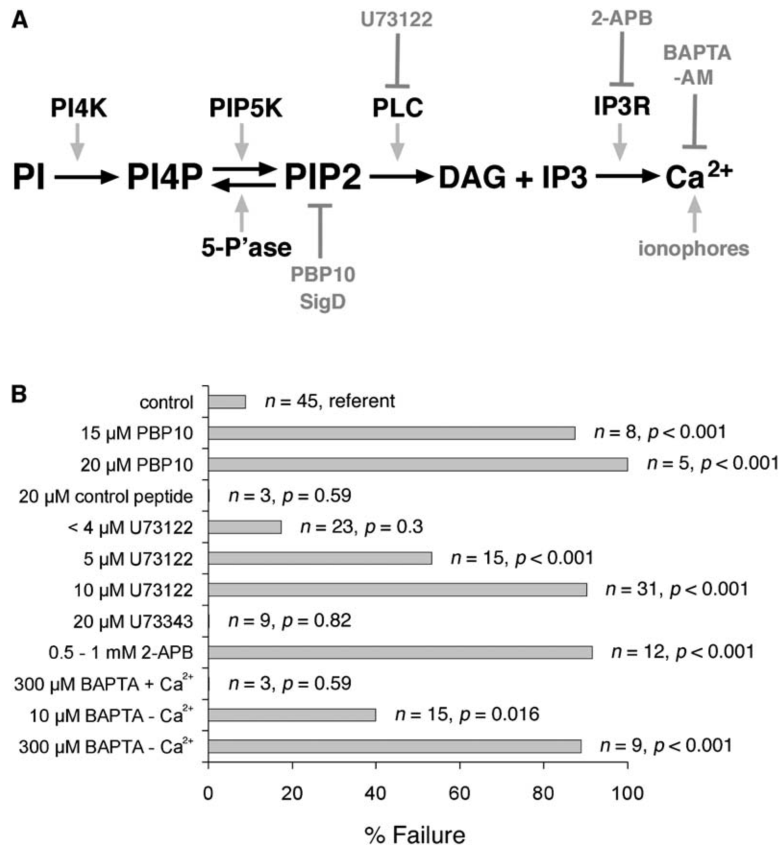


Figure 1. Inhibitors of Different Steps of the Phosphatidylinositol Pathway Block Cytokinesis

(A) Simplified pathway of phosphatidylinositol (PI) metabolism showing synthesis and hydrolysis of PI(4,5)P2 (PIP2). PI is phosphorylated by PI 4-kinase (PI4K) to generate PI 4-phosphate (PI4P). PI4P is phosphorylated by PIP 5-kinase (PIP5K) to make PIP2. PIP2 can be dephosphorylated by PI 5-phosphatase (5-P'ase) to form PIP4, phosphorylated by PI 3-kinases to produce PI(3,4,5)P3 (PIP3) (not shown), or hydrolyzed by phospholipase C (PLC) to form the second messengers I(1,4,5)P3 (IP3) and diacylglycerol (DAG). IP3 binds the IP3 receptor (IP3R), releasing calcium (Ca^{2+}). Gray: Pharmacological agents (PBP10, U73122, 2-APB, BAPTA-AM, ionophores) and phosphoinositide phosphatase (SigD) used in this study. Gray arrows: Enzymes or pharmacological agents that promote a step of the pathway or increase an end product, such as Ca^{2+}. Gray lines with bars: Enzymes or pharmacological agents that block a step of the pathway or that titrate or deplete a particular product.

(B) Treatments that block PIP2, IP3R, or Ca^{2+} cause cytokinesis failure. Control: Untreated control cells. PBP10: PIP2 binding peptide from gelsolin. Control peptide: Related peptide that does not bind PIP2. U73122: PLC inhibitor. U73343: Inactive isomer of U73122. 2-APB: IP3R antagonist. BAPTA-AM: Cell-permeable Ca^{2+} chelator. + Ca^{2+}: Cells incubated in buffer containing Ca^{2+}. – Ca^{2+}: Cells incubated in buffer lacking Ca^{2+}. % Failure: Percentage of cells failing cytokinesis. n, number of cells; p, p value.

wild-type (Yates' p value < 0.001). These results indicate that, as in mammalian cells [13], PIP2 is required for successful completion of cytokinesis.

To determine if PIP2 is required during furrow ingression, we treated cells after the onset of cleavage with 15–20 μM PBP10. Cytokinesis was compromised in the vast majority of cells (Figure 1B). PBP10 caused temporary arrest of furrowing, followed by regression (e.g., Figures 2B and 2D, Movie S2). Furrowing did not reinitiate over the course of the experiment, i.e., within 10 min. Other meiotic events, such as reformation of nuclei, occurred normally, indicating that other cellular processes were unaffected. Control cells, treated with 20 μM of a similar peptide that does not bind PIP2, divided normally (Figure 1B). PIP2 was not required for initiation of cytokinesis, as cells incubated with 30–40 μM PBP10 for up to 35 min before onset began cleavage normally but the furrows later regressed (n = 7), similar to the phenotype of spermatocytes lacking Fwd/PI4Kβ [8].

Thus, PIP2 is required dynamically following initiation of ingression. In addition, given its even distribution, PIP2 appears necessary, but not sufficient, for localization and/or activation of factors required during cytokinesis.

PIP2 Hydrolysis Is Necessary to Stabilize the Cleavage Furrow

To determine if PIP2 must be hydrolyzed during cytokinesis, we treated cells with the phospholipase C (PLC) inhibitor, U73122, at concentrations ranging from 0.25 to 20 μM. In a dose-dependent manner, U73122 caused rapid and complete cleavage furrow regression (Figures 1B, 2C, and 2D; Movie S3). The parafusorial membrane remained aligned along the spindle and continued to constrict, suggesting that the contractile ring was no longer associated with the plasma membrane (Figure 2C, arrows; not shown). In addition, blebbing at the equator was often observed (Figure 2C, arrowheads). In *Drosophila* spermatocytes treated with 5 μM U73122, similar to the IC_{50} in human cells [14, 15], cytokinesis was blocked 53% of the time (Figure 1B). At concentrations greater than 5 μM, cytokinesis was blocked in 90%–100% of cells. Cytokinesis failure was not reversed by washing out the inhibitor, although only a few cells were observed longer than 10 min. 20 μM U73343, an inactive isomer of U73122, had no effect (Figure 1B).

To confirm that U73122 treatment led to PIP2 accumulation, drug-treated spermatocytes expressing PLCδ-PH-GFP were examined by fluorescence microscopy (Figures 3A and 3A′, Movie S4). Upon membrane regression, PIP2 accumulated at the previous position of the cleavage furrow (Figure 3A′, right arrow). These PIP2 accumulations were adjacent to the mitochondria and parafusorial membranes in the plane of division, suggesting that the contractile ring remained present, perhaps binding the remaining PIP2 (compare Figures 3A and 3A′, last panel; not shown). Trails of PIP2 were seen leaving the aggregates at the equator and moving

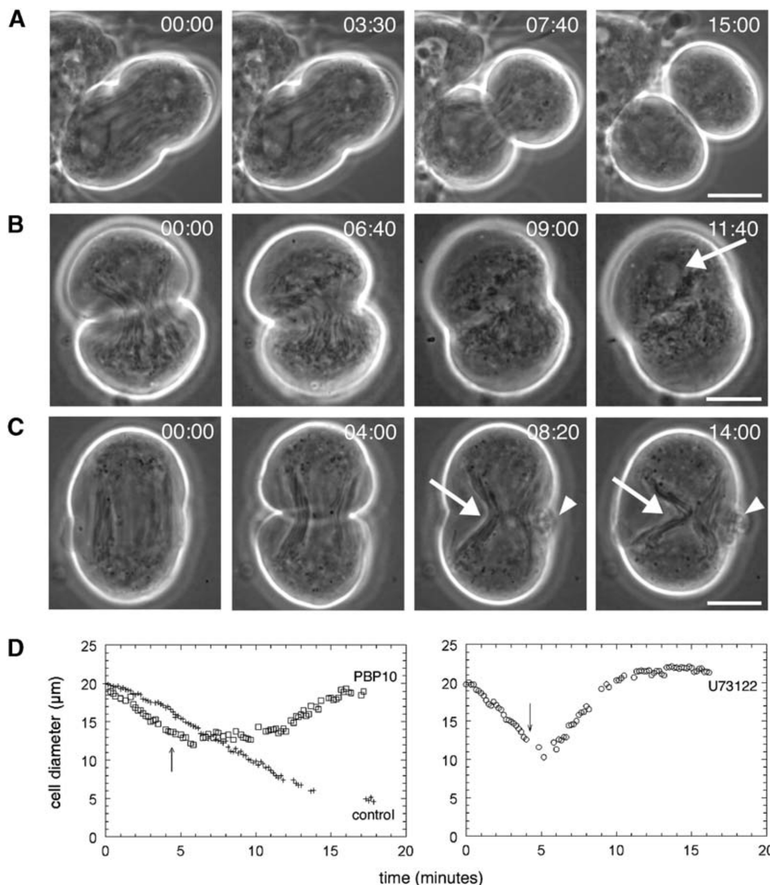


Figure 2. Spermatocyte Cytokinesis Requires Both PIP2 and PIP2 Hydrolysis

(A–C) Phase micrographs showing time course of meiosis I cytokinesis (times are min:s). Scale bars equal 10 μm.

(A) Control cell. 00:00: Onset of cytokinesis. Furrowing initiates at cell equator. Phase-dark mitochondria and parafusorial membranes align along the spindle and cell elongation occurs. 03:30, 07:40: Ingression. 15:00: Furrowing continues until daughter cells are connected by a small intercellular bridge. Parafusorial membranes appear constricted in the plane of division. See Movie S1.

(B) Cell treated with 15 μM PBP10 (PIP2 binding peptide). 00:00: Immediately after addition of PBP10. 06:40: Cytokinesis has stopped. 09:00: Cleavage furrow begins to regress. 11:40: Cleavage furrow has almost entirely regressed. Arrow: Reformed nucleus. See Movie S2.

(C) Cell treated with 10 μM U73122 (PLC inhibitor). 00:00: Onset of cytokinesis. 04:00: Cell in cytokinesis, just prior to addition of U73122. 08:20, 14:00: Cleavage furrow has regression after addition of U73122. Arrows: Parafusorial membranes continue to constrict. Arrowheads: Blebs of plasma membrane accumulate at the equator. See Movie S3.

(D) Plots of changes in cell diameter over time. Left: plus sign, control cell shown in (A); open square, PBP10-treated cell similar to cell shown in (B). Right: open circle, U73122-treated cell shown in (C). Arrows: times of PBP10 or U73122 addition.

to the sides of the cell or the poles (Figure 3A′, arrowhead). Large numbers of PIP2-containing tubular/vesicular structures also appeared at the poles of the cell (Figure 3A′, left arrow). As PIP2 was occasionally observed in similar structures in untreated cells (not shown), the apparent proliferation of these structures probably represents an accumulation of PIP2 on otherwise normal invaginations of the plasma membrane.

Thus, as previously demonstrated for crane-fly spermatocytes [16] and sea urchin embryos (M. Ng and D. Burgess, personal communication), PIP2 hydrolysis is crucial for cleavage furrow stability.

Calcium Is Needed for Completion of Cytokinesis

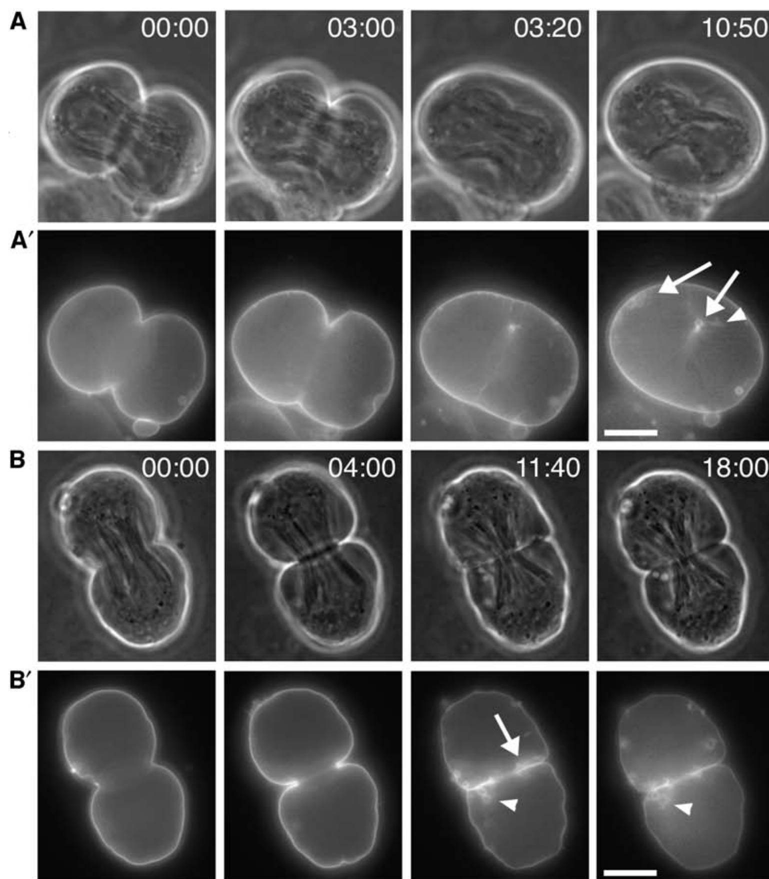
The effects of PIP2 hydrolysis are 2-fold: depletion of PIP2 from the plasma membrane and production of the second messengers, IP3 and DAG. As IP3 stimulates Ca^{2+} release from internal stores by binding IP3R on the ER, we investigated the roles of IP3R and Ca^{2+} in cytokinesis.

To determine if IP3R is required for cytokinesis, we treated dividing cells with the IP3R antagonist, 2-APB, shortly after the onset of ingression. Cells were treated with 0.5–1 mM 2-APB, approximately the same concentration used to inhibit cytokinesis in zebrafish embryos [3]. Cleavage furrows regressed in a large majority of cells (Figure 1B), indicating IP3R function is necessary for furrow stability (Figure 4A). Regression was similar to that observed with U73122, based on morphology of the mitochondria and parafusorial membranes. However, in some cells (4/11), only part of the furrow regressed and the remaining membrane remained constricted (Figure 4A, “after”).

To directly determine if Ca^{2+} is required for cleavage, we treated cells with the membrane-permeable Ca^{2+} chelator, BAPTA-AM. In the absence of BAPTA-AM, cells maintained in Ca^{2+}-free buffer divided and retained their normal morphology for at least 2 hr (Figure 1B). Treatment with BAPTA-AM in Ca^{2+}-free buffer, however, led to cytokinesis failure in 40% (10 μM) or 90% (300 μM) of cells observed over this period (Figures 1B and 4B). In the presence of Ca^{2+}, 300 μM BAPTA-AM had no effect (Figure 1B), indicating that these cells take up Ca^{2+} from the buffer during cleavage. In two of the cells treated at 10 μM, cleavage was reinitiated, but the furrows were again unstable and cytokinesis failed. These results indicate that, as in zebrafish embryos [3], Ca^{2+} is required in spermatocytes for furrow deepening rather than for initiation of cleavage.

Calcium Stabilizes the Cleavage Furrow

To probe the effects of increased cytoplasmic Ca^{2+} on cytokinesis, we used two Ca^{2+} ionophores, A23187 and ionomycin. Treatment with either ionophore during division caused the cell membrane to distort and undulate. Adjacent membrane from the nascent daughter cells



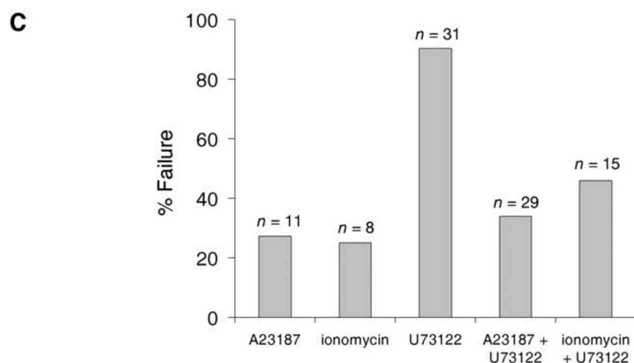


Figure 3. Effects of the PLC Inhibitor, U73122, and Ca^{2+} Ionophore Pretreatment on PIP2 Accumulation and Cytokinesis

(A and B) Phase and (A′ and B′) corresponding fluorescent images (PLCδ-PH-GFP) of dividing cells (times are min:s). Scale bars equal 10 μm.

(A and A′) PIP2 accumulates in cells treated with U73122.

(A) Cell treated with 10 μM U73122 (times are min:s). 00:00: Just prior to treatment. 03:00, 03:20: Regression.

(A′) 10:50: arrows, PIP2 accumulation at the poles and the equator. Arrowhead: PIP2 moving away from the equatorial accumulation. See Movie S4.

(B and B′) Pretreatment of cells with Ca^{2+} ionophores bypasses the requirement to hydrolyze PIP2.

(B) Cell pretreated with 10 μM ionomycin and then treated with 10 μM U73122. 00:00, pretreated cell. 04:00, just prior to addition of U73122. 11:40, 18:00, after treatment, furrow is stable.

(B′) 11:40, 18:00, arrow: PIP2-containing structures accumulate near the plasma membrane. Arrowheads: the largest of these (1.8 μm diameter) persisted for at least 20 min. See Movie S6.

(C) Cytokinesis outcomes for cells treated with different combinations of ionophores and U73122. Both A23187 and ionomycin allow U73122-treated cells to cleave. n, number of cells.

often came into contact (Figure 4C, "after," Movie S5) and sometimes fused. This ectopic fusion occurred outside the furrow and moved inward toward the furrow front until the two daughter cells had fused completely and was qualitatively different from regression caused by PBP10, U73122, 2-APB, or BAPTA-AM. As Ca^{2+} can activate myosin, these undulation and fusion events likely result from the triggering of increased contractility and the resulting chance apposition and fusion of adjacent cellular membranes.

The majority of cells treated with 10 μM A23187 maintained stable cleavage furrows (Figure 3C), although in four cells, furrowing stopped early. Drug treatment caused ectopic fusion in 27% of cells treated with 10 μM and the majority (2/3) of cells treated with higher doses of A23187 (not shown). Similarly, cells treated with 5–10 μM ionomycin exhibited perturbations in the membrane, with ectopic fusion occurring in 25% of cells (Figure 3C).

Although the ionophores modestly impaired cytokinesis, we hypothesized that ionophore-mediated Ca^{2+} influx could suppress aspects of the furrow ingression defect caused by loss of PIP2 hydrolysis by providing a downstream effector of the pathway. To test this, we treated cells with U73122 in the presence of A23187 or ionomycin (Figures 3B, 3B′, and 3C, Movie S6). Cells were treated with 10 μM A23187 at the onset of cytokinesis and then with 10 μM U73122 within 2–5 min. This concentration of U73122 is sufficient to cause furrow regression in 90% of single-drug treated spermato-

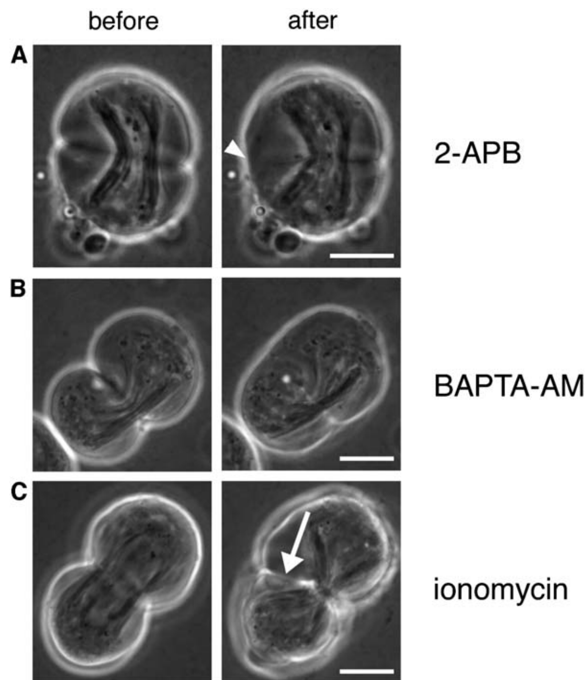


Figure 4. IP3R and Ca^{2+} Are Required for Cytokinesis

Phase micrographs of cells treated with various pharmacological agents. Before: immediately before treatment. After: after treatment.

(A) Effect of 0.5 mM 2-APB (IP3R antagonist). Before: note that tight apposition of membranes somewhat obscures the furrow front. After: regression is nonuniform. Arrowhead: side of furrow showing regression.

(B) Effect of 300 μM BAPTA-AM (Ca^{2+} chelator) in Ca^{2+}-free Ringer's.

(C) Effect of 10 μM ionomycin (Ca^{2+} ionophore). Arrow: ectopic fusion between adjacent membranes of daughter cells. See Movie S5. Scale bars equal 10 μm.

cytes (Figures 1B and 3C). However, the frequency of failed cytokinesis in cells treated with A23187 and U73122 was significantly lower (34%; $p < 0.01$, FET). Some of the cleavage furrows clearly regressed, whereas ectopic fusion occurred at a similar frequency as for cells treated with ionophore alone. In the remaining cells, the cleavage furrow was stable.

To confirm that treatment of cells with an ionophore could suppress the effects of U73122, we repeated these experiments using ionomycin (Figure 3C). Of cells pretreated with 5–10 μM ionomycin at the onset of cytokinesis and then treated with 10 μM U73122 within 1.5–5 min, 46.7% failed cytokinesis by either regression or ectopic fusion, significantly less than for cells treated with U73122 alone ($p < 0.01$, FET). The remaining cells continued to ingress to near completion and then stopped. As expected, Ca^{2+} was required for suppression of the U73122 effect, as cells treated with ionophore and U73122 in Ca^{2+}-free Ringer's solution failed cytokinesis ($n = 4$).

To visualize PIP2 localization in cells treated with an ionophore followed by U73122, we examined PLCδ-PH-GFP during treatment. After addition of U73122, PIP2-containing membrane accumulated near the cleavage furrow, which remained constricted (Figure 3B′, arrow, arrowhead). In addition, the cells exhibited microspike projections (Movie S6) and appeared highly contractile.

These results suggest that one function of PIP2 hydrolysis is to release Ca^{2+}, thereby promoting cleavage furrow ingression. Although the targets of Ca^{2+} regulation during cleavage are as yet unknown, elevated Ca^{2+} levels increase contractility by activating myosin and stimulate vesicle fusion events by activating SNAREs [17, 18]. Either or both of these processes could contribute to cytokinesis and may be the mechanism by which ionophore treatment bypasses the requirement to hydrolyze PIP2. However, we note that the concentration of Ca^{2+} used in our rescue experiments is significantly above physiological levels. In addition, we were unable to rescue U73122-treated cells by pretreatment with either thapsigargin (which releases Ca^{2+} from ER stores) or phorbol ester (which activates PKC) (not shown). Thus, we cannot exclude the possibility that ionophore-dependent Ca^{2+} influx bypasses the normal requirement for PIP2 hydrolysis in some other process, for example actin recruitment to the contractile ring [16].

Our experiments are the first to show a clear role for PIP2, PIP2 hydrolysis, IP3R activation, and Ca^{2+} during cytokinesis in a single cell type. As different steps of the PI pathway have been implicated in cytokinesis in a number of organisms, the function of this pathway in cell cleavage appears to be conserved. Because of the ease of manipulating *Drosophila* spermatocytes genetically and now in vitro, this system should prove ideal for dissecting molecular mechanisms by which the PI pathway and Ca^{2+} control this crucial process.

Supplemental Data

Supplemental Data include one figure, one table, Supplemental Experimental Procedures, and six movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/15/1401/DC1/.

Acknowledgments

The authors gratefully acknowledge H. Hoyle, E. Raff, T. Balla, S. Grinstein, B. Finlay, S. Marcus, T. Meyer, D. Alessi, M. Deak, M. Funaki, P. Janmey, and B. Edgar for sending plasmids, peptides, or flies. We are grateful to A. Forer for helpful advice on making clot preps, S. Grinstein for suggesting we use SigD, and T. Noguchi for his patience in teaching us about Ca^{2+}. We extend special thanks to M. Ng, D. Burgess, S. Field, and L. Cantley for communicating results prior to publication. We thank S. Field, L. Cantley, T. Noguchi, A. Forer, and W. Trimble for comments on the manuscript and J. Ashkenas for thoughtful experimental suggestions and help in editing the text. J.A.B. is indebted to M. Fuller, in whose lab this work was initiated. We apologize for leaving out many references due to space limitations. Funded by a Terry Fox Foundation grant of the NCIC (J.A.B.).

Received: February 2, 2005
Revised: June 15, 2005
Accepted: June 16, 2005
Published: August 9, 2005

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