Distinct roles of the equatorial and polar cortices in the cleavage of adherent cells

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Over the past 100 years, many models have been proposed and tested for cytokinesis [1]. There is strong evidence that the equator represents a unique region that receives cleavage signals from the mitotic spindle [2, 3]. The nature of such a signal and the mechanism of cleavage, however, remain poorly understood. To probe the contribution of different cortical regions in the cleavage of cultured epithelial cells, we applied cytochalasin D (CD), a known inhibitor of cytokinesis [4], in a highly localized manner to different regions of dividing NRK cells. Surprisingly, equatorial application of CD not only allowed cytokinesis to complete but also appeared to facilitate the process. Conversely, local application of CD near the polar region caused inhibition of cytokinesis. Our results suggest a mechanism that involves global coordination of cortical activities, including controlled cortical disassembly along the equator and possibly global cortical contraction.

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Results and discussion

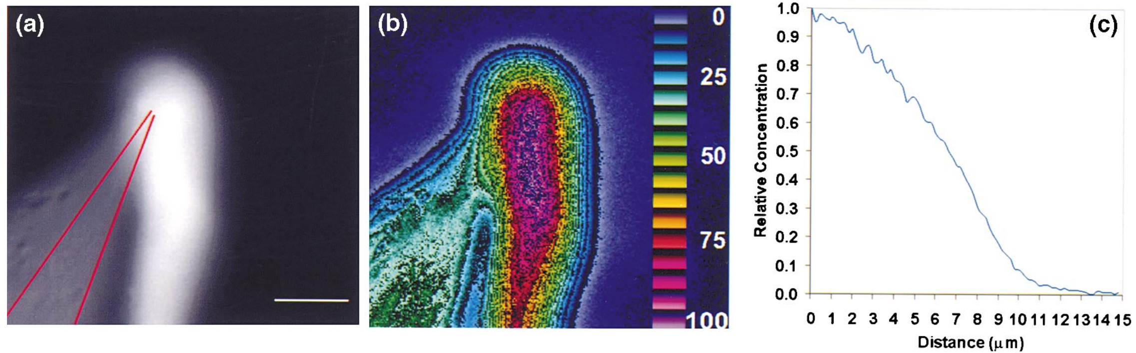
Based primarily on the presence of strong cortical forces in the equatorial region [5] and on the concentration of actin and myosin in the cleavage furrow [6, 7], it is generally believed that an equatorial 'contractile ring' pinches a cell in two. This model predicts that cytokinesis should be sensitive to local disruptions of the equatorial cortex. To probe the functional roles of different cortical regions, we created a highly localized field of cytochalasin D (CD), a known inhibitor of actin-based cellular functions, including cytokinesis [4, 8], by releasing the drug from a microneedle while withdrawing the medium from a nearby suction micropipette [9] (see Materials and methods). With proper positioning of the two needles, released drugs can be confined to a small region 15–20 μm in diameter in front of the tip of the needle, as shown by fluorescent dextrans included in the carrier solution (Figure 1).

We first titrated the concentration of CD required for the inhibition of cytokinesis of normal rat kidney (NRK) epithelial cells when applied globally to NRK cells. Concentrations above 2 μM CD were found to cause consistent inhibition, while lower concentrations induced progressively less-potent, less-consistent effects. CD at 5 μM was then applied to one side of the equator, starting at late metaphase to the beginning of anaphase before the initiation of cytokinesis. As shown in Figure 2 and in Supplementary Movie 1, this treatment had no detectable effect on the initiation of cytokinesis, with regard to both the positioning and timing of cortical ingression. Surprisingly, the CD-treated side of the equator developed a deep furrow, while cleavage on the opposite side appeared to be partially inhibited. Although local treatment with dimethyl sulfoxide (DMSO) alone also causes a slight increase in the amount of furrowing (Figure 2e–h and Supplementary Movie 2), the extent was much smaller than that induced by CD (Figure 2i). The lack of inhibition by equatorial treatment of CD suggests that either the equatorial cortex is resistant to CD or that the integrity of equatorial cortex is not essential for cytokinesis. Furthermore, the deep furrowing induced by CD suggests that cytokinesis may be facilitated by CD-induced weakening of the equatorial cortex.

In complementary experiments, we moved the release needle over the polar region of dividing cells at a similar stage of mitosis. In most experiments, the released CD solution was located between the spindle pole and the distal end of the cell but stayed away from the equator (Figure 3). This treatment caused an effective inhibition of cytokinesis (80%, $n = 10$; Figure 3 and Supplementary Movie 3; see Supplementary Movie 4 for the DMSO control). Cells appeared to initiate cytokinesis; however, the shallow furrow regressed later. Since equatorial treatment of CD caused no inhibition of cytokinesis, the inhibitory effect at the polar cortex cannot be explained by the diffusion of CD to the cleavage furrow.

CD caused disruptions to not only actin filaments but also cell-cell and cell-substrate adhesions, which in some systems are required for successful cytokinesis [10, 11]. To determine if the present observations were due to local dissociation of cell adhesions, we treated dividing NRK cells with trypsin, using the same needle release technique. The treatment opened a gap between the

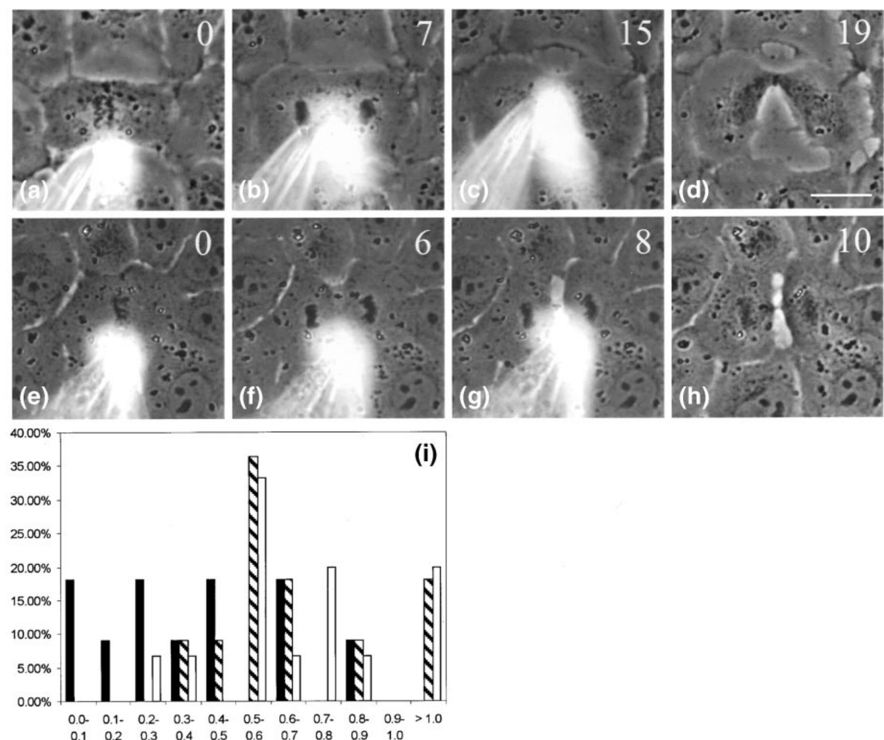
Figure 1



The local delivery of drugs. The distribution of molecules released from the microneedle is controlled by placing a suction pipette slightly behind the tip of the releasing needle. **(a)** Fluorescence image of released fluorescein dextran. The approximate position of the needle is indicated by red lines. **(b)** The same image rendered by color mapping. The color scale and numbers indicate the relative concentration of the released fluorescein. The bar represents 10 μm. **(c)** Figure showing the intensity of fluorescein dextran as a function of the distance from the release needle, as measured away from the tip of the needle along the 12 o'clock direction. A similar gradient is present toward left and right but not toward the 6 o'clock direction, where the drug extended for a longer distance while moving upward, due to the positioning of the suction needle at a higher elevation.

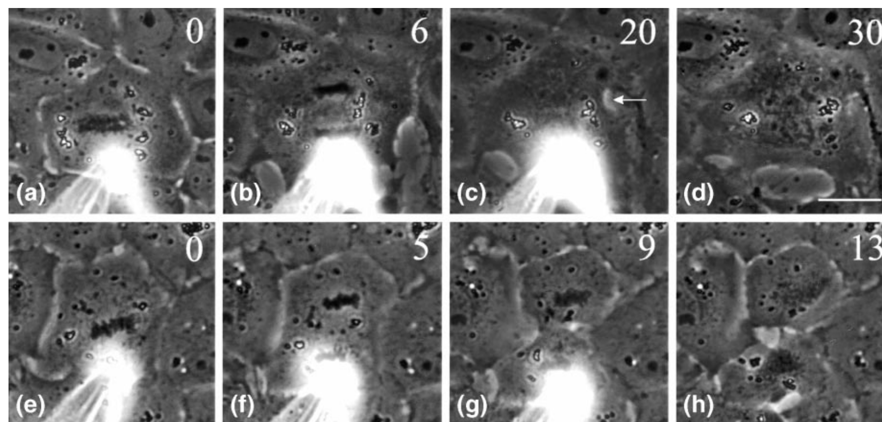
dividing cell and its neighbor and caused local release of cell-substrate adhesions at the cell periphery, as indicated by interference reflection microscopy (data not shown). However, neither equatorial nor polar treatment of trypsin caused the inhibition of cytokinesis (Figure 4). As with the DMSO control, the only detectable effect was a slight bias in cleavage, in favor of the side of the equator facing trypsin (Figure 2i). Therefore, although global removal of cell-substrate or cell-cell adhesions may inhibit cytokinesis [10, 11], limited dissociations as in the present study were not sufficient to account for the effects of CD. The negative results with trypsin are also consistent with the

Figure 2



(a–d) The effects of CD or **(e–h)** DMSO control released at the equator. Images in (a)–(c) and (e)–(g) were recorded with simultaneous phase and fluorescence illumination, showing both the distribution of released drug solution and the response of the cell. The treatment started at metaphase to early anaphase (t = 0), before the initiation of cleavage. Time in minutes is shown in the upper right corner. The side of the equator facing CD showed a deep ingression, while the opposite side barely ingressed. (d) A phase-dense midbody formed toward the end of cytokinesis. The bar represents 20 μm. The graph in **(i)** shows the histogram of the ratio of ingression between the untreated side and the treated side for cells treated with CD (11 cells; solid bars), DMSO alone (11 cells; striped bars), or trypsin (15 cells; open bars). Low value on the *x* axis indicates a bias toward the side facing the needle. This ratio showed an average of 0.8 for the DMSO control, indicating a slight bias toward the treated side (1.0 reflects no bias), as compared to 0.4 for CD-treated cells and 0.7 for trypsin-treated cells.

Figure 3

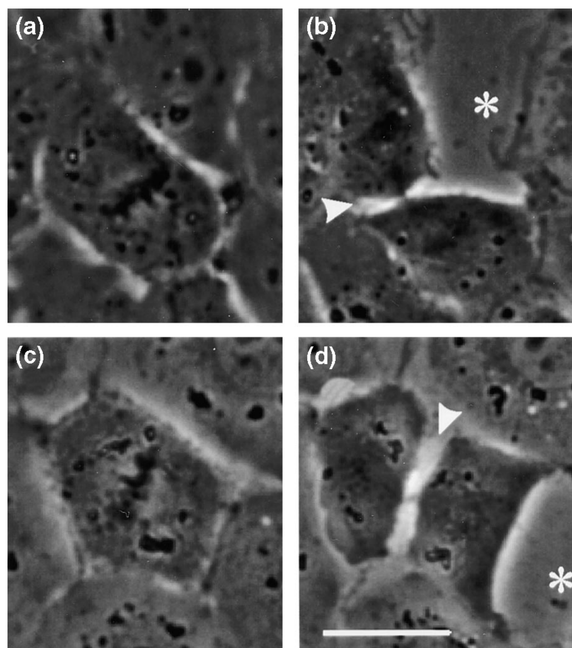


Inhibition of cytokinesis by CD released near the distal end of the cell. Images in **(a)–(c)** and **(e)–(g)** were recorded with simultaneous phase and fluorescence illumination, showing both the distribution of released drug solution and the response of the cell. The treatment started at metaphase to early anaphase (t = 0), before the initiation of cleavage. Time in minutes is shown in the upper right corner. Polar treatment with CD caused inhibition of cytokinesis (a–d), although a slight initial ingression was typically observed ([c], arrow). (e–h) Similar treatment with DMSO alone had no effect. The bar represents 20 μm.

observations with unmanipulated NRK cells — that cell-cell adhesions have no discernable positive or negative correlation with the ability of the cells to cleave [12].

Similar experiments were also performed with 5–6 μM latrunculin A (LatA), a sponge toxin that disrupts actin filaments through the sequestration of actin monomers [13]. LatA caused qualitatively similar effects as those seen with CD (see Supplementary Figure S1), although the effects were not as consistent. Inhibition of cytokinesis was observed in 50% of the cells treated near the pole. It is likely that the local effects of LatA were countered by the rapid diffusion of actin monomers in the cell, while CD was able to disrupt directly the integrity of actin filaments and/or actin-membrane interactions [4, 8]. Treatment with jasplakinolide (JASPA), a sponge toxin that permeablizes the cell membrane and nucleates/stabilizes actin filaments [14, 15], caused opposite effects. That is, polar release of JASPA had no effect on cytokinesis, while equatorial release caused inhibition of cytokinesis during late telophase (see Supplementary Figure S2). The interpretation of the effects of JASPA may be complicated, however, by its ability to disrupt preexisting filaments while nucleating new actin filaments [15].

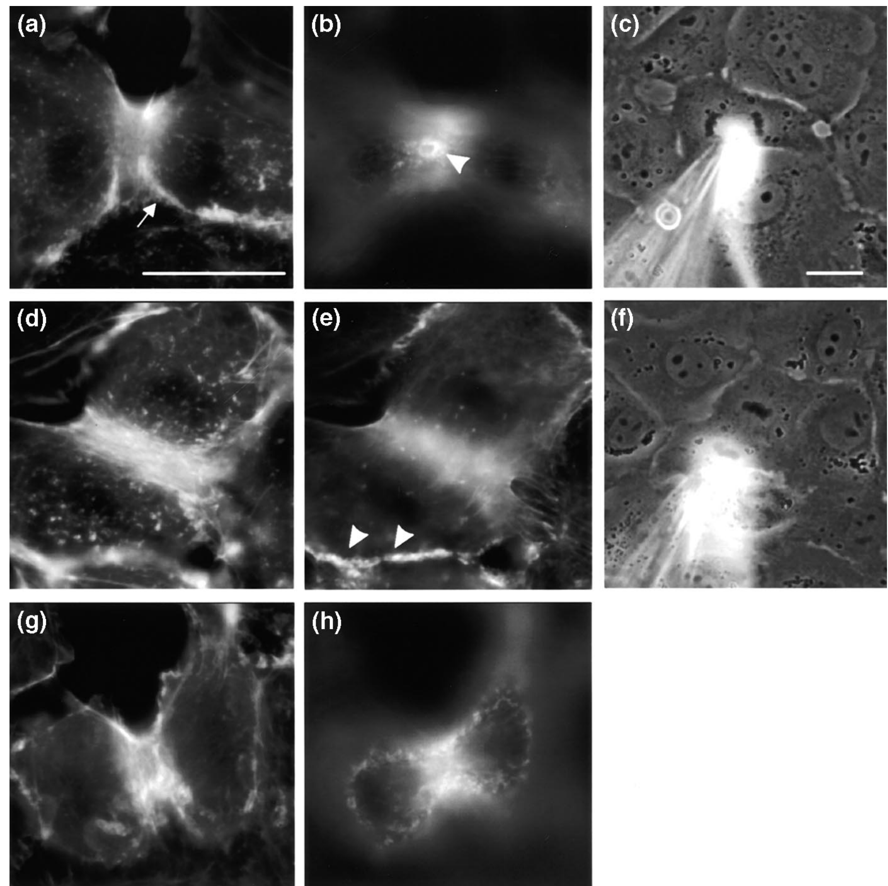
Figure 4



(a,b) Effect of trypsin released at the equator or **(c,d)** at the distal end of the cell. Images were recorded (a,c) before or (b,d) after the application of trypsin. Cells were treated locally with trypsin during metaphase to early anaphase, before the initiation of cleavage. The treatment created a gap (asterisks) between the mitotic cell and its neighboring cell (no longer in the field of view). The cleavage furrows are indicated by arrowheads. Cytokinesis is not affected by either treatment with trypsin. The bar represents 20 μm.

Cells treated locally with CD were fixed and stained with fluorescent phalloidin to determine if CD caused disruption of actin organization. As shown previously [12, 16], control epithelial cells formed a well organized band of actin and myosin II bundles on the ventral equatorial cortex. In addition, many small 'nuggets' of actin filaments were found throughout the cortex. Consistent with the effects of global CD application [17], prominent cortical actin aggregates or segments formed near the CD release site (Figure 5, arrowheads; Supplementary Movies 5 and 6), which were also seen in live cells microinjected with rhodamine phalloidin (see Supplementary Figure 3). In addition, equatorial treatment with CD caused the disappearance of most of the actin bundles that lie along the equator (n = 6; Figure 5a and Supplementary Movie 5). Although actin filaments were still concentrated in the furrow, there was an apparent decrease in equatorial actin concentration (Figure 5a). In contrast, cells treated with CD at the pole showed an apparent enhancement of equa-

Figure 5



(a,b) Organization of actin filaments in cells treated with CD at the equator or **(d,e)** at the pole. **(c)** and **(f)** show phase images recorded before fixation. Both cells were fixed 6–7 min after the initiation of drug treatment, which started near anaphase onset. **(g)** and **(h)** show a control cell. Images of fluorescent phalloidin staining were focused on either (a,d,g) the ventral cortex or (b,e) a higher plane, where CD-induced aggregation of actin filaments is visible. (h) shows the organization of actin filaments on the dorsal equatorial cortex of the control cell. (d) Large equatorial actin bundles are present on the ventral cortex of the cell treated at the pole. The cell treated at the equator shows a reduced concentration of actin filaments and no large bundle on the ventral cortex. The bright linear structures (arrow) reflect the contour of the cell at a slightly higher level of focus. Prominent aggregates of actin filaments are also present near the CD release site ([b,e], arrowhead). The bar represents 20 μm.

torial actin organization ($n = 4$; Figure 5d and Supplementary Movie 6).

Our results, suggesting that the integrity of the polar cortex is essential for cytokinesis, appear incompatible with the idea that the equatorial cortex is self-sufficient for executing the entire process of cytokinesis, as is implied in the contractile ring model. Neither can we easily explain the cleavage with the polar relaxation theory [18], which requires a stronger contraction along the equator than in other regions. The results, however, corroborate many previous observations that cytokinesis involves more than the assembly and contraction of an equatorial cortex. For example, the increase in membrane tension is known to occur over the entire cortex before the onset of cytokinesis [19]. In addition, cytokinesis of adherent *Dictyostelium* cells can take place at a proper time and place without myosin II and without a detectable equatorial organization of actin filaments [20]. Conversely, experiments with the yeast *S. pombe* [21] and with meiotic mammalian spermatocytes [22] indicated that the organization of actin and myosin along the equator does not guarantee a functional cleavage. It has also been shown that myosin II can concentrate to the equator of dividing *Dictyostelium*, without the involvement of its motor activities [23, 24]. Similarly, the concentration of actin filaments in the cleavage furrow may be related to such events as microtubule-dependent transport [20, 25, 26], polar cortical expansion [27], or membrane-vesicle fusion [28], instead of equatorial cortical contraction.

What might account for the cleavage of NRK cells? The inhibition of cytokinesis by polar application of CD emphasizes the importance of the coordination of the entire cortex during cytokinesis. For example, contractions of the polar cortex may contribute to the dissociation of actin filaments from the equator, and the disruption of this process may lead to the accumulation of actin along the equator and hamper the progress of cytokinesis (Figure 5d). Conversely, the biased cleavage induced by equatorial treatment suggests that a key step for cytokinesis involves the weakening of equatorial cortex relative to other regions, although some contractile activities may persist along the equator. The disassembly of equatorial cortex during cytokinesis was previously suggested, based on the thickness of the cortex during cleavage [29] and the organization of myosin II [30], and was viewed as a housekeeping function to avoid the accumulation of actin-

myosin II structures in the furrow. It was subsequently recognized that this process may be necessary and/or sufficient for successful cytokinesis, based on computer modeling [27] and on observations of the division of myosin II null *Dictyostelium* [20]. Moreover, mutations in cofilin were found to cause failure during late stages of cytokinesis in *Drosophila*, with large, stable aggregates of actin filaments located at the equator [31]. Therefore, one possibility is that cytokinesis may be driven by opposite traction forces generated outside the equator, which rip the cell apart along a weakened equatorial band. Although this process based on cell-substrate interactions may facilitate cleavage, our results suggest that adhesions alone cannot account for all the forces for cytokinesis, since, unlike polar CD treatment, release of polar adhesions with trypsin had no effect on cytokinesis. An alternative, global contraction-equatorial relaxation model was first proposed in the early 20th century (reviewed by Rappaport [1]), which suggests that inward contractile forces may develop throughout the cortex as the cell enters cytokinesis [19] and that a controlled, localized relaxation allows the cortex to collapse along the equator. The present results do not rule out the involvement of equatorial contraction in some aspects of cytokinesis, particularly the initial cortical ingression. They do, however, point to the importance of identifying processes that cause the disassembly of the equatorial cortex. It is possible that both equatorial contraction and relaxation/solation mechanisms are operational during cytokinesis, with their relative roles varying under different conditions to ensure a reliable cell division.

Conclusions

Although equatorial contraction may play a role in cytokinesis, the present results suggest that the integrity of the polar cortex is essential for the completion of cytokinesis in cultured adherent cells. Successful cell division requires the coordination of the entire cortex, including the disassembly of the equatorial cortex.

Materials and methods

Cell culture and microscopy

Normal rat kidney cells (NRK-52E; American Type Culture Collection) were grown on glass coverslips in Kaighan's modified F12 medium (Sigma) supplemented with 10% fetal calf serum (JRH Biosciences), 1 mM L-glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin. Cells were maintained at 37°C in a stage incubator built on top of an inverted microscope (Axiovert S100 TV; Carl Zeiss) and were viewed with a 40×/NA 0.75 plan-neofluar lens or a 100×/NA 1.30 fluar lens. All experiments were performed with well spread cells located within a colony. Images were acquired using a cooled charge-coupled-device camera (Roper Scientific) and custom software.

Local drug application

CD (Sigma) was kept as a frozen 100 μM stock in unsupplemented F12K media with 0.4% DMSO. Before use, it was diluted into 0.5 mg/ml fluorescein dextran (Molecular Probes) in unsupplemented F12K media to obtain a working concentration of 5 μM with 0.02% DMSO. LatA (Molecular Probes) and JASPA (Molecular Probes) were prepared similarly and were used at a final concentration of 5–6 and 7 μM, respectively, in 0.25% DMSO. Trypsin (GIBCO 840-7073) was dissolved at a concentration of 0.25% (w/v) in 0.5 mg/ml fluorescein dextran, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 30 mM Tris-Cl (pH 7.5) and was ultracentrifuged at 100,000 × g for 20 min in a Type 42.2Ti rotor (Beckman). The solutions were loaded into a release microneedle connected to a source of compressed air. Suction micropipettes were prepared by breaking and fire polishing the tip of a microneedle, using a microforge (Narishige). Positive and negative pressures were regulated with custom-made regulators. The release and suction micropipettes were mounted on a custom double micromanipulator that allowed both precise relative positioning of the two needles and their simultaneous movements. Generally, the suction pipette was positioned 20–40 μm behind the release needle at a slightly higher elevation. Highly localized distribution of the drug, as monitored with fluorescence optics, was obtained by adjusting the pressure, relative position, and tip size of the two needles. The release of CD, LatA, and JASPA was maintained throughout the period of observation, while trypsin was released for a period of no more than 5 min, sufficient to induce a gap between the target cell and its neighbor. To cause localized dissociation of cell-cell and cell-substrate adhesions, the release needle for trypsin was positioned just outside the boundary of the dividing cell.

Immunofluorescence

To visualize the organization of actin, treated cells were removed from the microscope and rushed to the hood, where they were rinsed with 37°C PBS and fixed for 1 min in 0.3% Tritron X-100, 1% formaldehyde, and 0.1% glutaraldehyde in cytoskeleton buffer (137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM PIPES, and 5.5 mM glucose [pH 6.1]). Cells were then postfixed in cytoskeleton buffer with 0.5% glutaraldehyde for 20 min, rinsed twice with buffer, and treated for 5 min with 0.5 mg/ml sodium borohydride. After washing for 10 min in PBS with 1% BSA, actin filaments were labeled with rhodamine phalloidin (Molecular Probes) diluted 1:50 in PBS.

Supplementary material

Supplementary data including time-lapse movies of the images shown in Figures 2 and 3, movies of the optical sections shown in Figure 5a–f, phase images of cells treated at the equator with LatA and JASPA, and fluorescence images of a living cell microinjected with rhodamine phalloidin and treated with LatA at the equator can be found at http://images.cellpress.com/supmat.supmatin.htm.

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