An actin barrier to resealing

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SUMMARY

Plasma membrane disruption is a common form of cell injury in many normal biological environments, including many mammalian tissues. Survival depends on the initiation of a rapid resealing response that is mounted only in the presence of physiological levels of extracellular Ca^{2+}. Vesicle-vesicle and vesicle-plasma membrane fusion events occurring in cortical cytoplasm surrounding the defect are thought to be a crucial element of the resealing mechanism. However, in mammalian cells, the vesicles used in this fusion reaction (endosomes/lysosomes) are not present in a 'pre-docked' configuration and so must be brought into physical contact with one another and with the plasma membrane. We propose that a requisite prelude to fusion is the disassembly in local cell cortex of the physical barrier constituted by filamentous actin. Consistent with this hypothesis, we found that rat gastric epithelial (RGM1) cell cortical staining with phalloidin was apparently reduced at presumptive disruption sites. Moreover, flow cytofluorometric analysis of wounded RGM1 populations revealed a small, but significant, Ca^{2+}-dependent reduction in whole cell phalloidin staining. The functional significance of this disruption-induced depolymerization response was confirmed in several independent tests. Introduction into RGM1 cells of the filamentous actin-depolymerizing agent, DNase1, enhanced resealing, although cytochalasin treatment, by itself, had no effect. By contrast, when the filamentous actin cytoskeleton was stabilized experimentally, using phalloidin or jasplakinolide, resealing was strongly inhibited. Cells in wounded cultures displayed an enhanced cortical array of filamentous actin, and resealing by such cells was enhanced strongly by both cytochalasin and DNase 1, demonstrating the specific reversibility of a biologically mediated, polymerization-induced inhibition of resealing. We conclude that localized filamentous actin disassembly removes a cortical barrier standing in the way of membrane-membrane contacts leading to resealing-requisite homotypic and exocytotic fusion events.

Key words: Plasma membrane, Disruption, Resealing, Exocytosis, Actin

INTRODUCTION

Plasma membrane disruption is a common event for cells residing in unpreturbed mammalian gut (McNeil and Ito, 1989), skin (McNeil and Ito, 1990), skeletal muscle (McNeil and Khakee, 1992), cardiac muscle (Clarke et al., 1995), aorta (Yu and McNeil, 1992) and inner ear (Mulroy et al., 1998). Such disruptions are large enough that macromolecules normally present outside of cells, such as serum albumin, can diffuse into cell cytosol where they can be trapped by resealing. The resultant cytosolic 'labeling' with these normally extracellular macromolecules serves to identify the 'wounded' cell in vivo. Physiologically generated mechanical force is thought to be the cause of these membrane disruptions since their frequency increases as a function of this variable (McNeil and Steinhardt, 1997).

Rapid resealing of a disruption limits influx of potential toxins such as Ca^{2+} and/or prevents excessive loss of essential cytosolic constituents, permitting survival. Survival is possible even when disruptions occur on a massive scale: the sea urchin egg, for example, can restore plasma membrane continuity within approximately 5 seconds after thousands of square microns of its plasma membrane are ripped off or dissolved away; such eggs can then be fertilized and will go on to divide (McNeil et al., 2000; Terasaki et al., 1997). This remarkable capacity for resealing represents an essential animal cell adaptation, and its early evolutionary development in the ancestral eukaryote may have been the crucial first step, after loss of the cell wall, towards exploitation by wall-less eukaryotes of nature's many mechanically challenging environments (McNeil and Terasaki, 2001).

Rapid resealing has an absolute requirement for physiologic levels of external Ca^{2+} (Heilbrunn, 1930b; Steinhardt et al., 1994). Exocytosis is evoked locally at disruption sites in both sea urchin eggs and mammalian cells, and quantitatively correlates in extent with disruption size (Bi et al., 1995; Miyake and McNeil, 1995). Moreover, botulinum toxins, which cleave/inactivate SNARE family protein components of the fusion machinery, inhibit resealing in both eggs and fibroblasts (Steinhardt et al., 1994). Finally, depletion of cytoplasmic vesicles from egg peri-disruption cortex blocks resealing (Terasaki et al., 1997). Thus one hypothesized role of Ca^{2+} in resealing is to evoke vesicle-plasma membrane (exocytotic) fusion events.

The function in resealing of these fusion events, leading to deposition of internal membrane into the plasma membrane, is

proposed, in the case of smaller disruptions (1 μm or less in diameter), to be the lessening of surface tension and hence the promotion of lipid flow over the disruption (Togo et al., 1999; Togo et al., 2000). For larger disruptions, we propose that resealing occurs by a 'patch' mechanism (McNeil et al., 2000; Terasaki et al., 1997). Ca^{2+} entering through a plasma membrane disruption initiates vesicle-vesicle and vesicle-plasma membrane (exocytotic) fusion events locally in peri-disruption cell cortex. In this way, large 'patch' vesicles are created and joined, by exocytotic fusion events, to plasma membrane surrounding the defect site. The disruption is, we propose, 'patched' over with internally derived membrane. The strongest evidence supporting this hypothesis is that cytoplasm (of the sea urchin egg) can, by itself, rapidly (seconds) erect extensive membrane boundaries in response to Ca^{2+} (Terasaki et al., 1997), as can an isolated vesicle population (yolk granules) (McNeil et al., 2000).

Importantly, recent work has established that it is an 'undocked' vesicle population (lysosomes in mammalian cells (Andrews, 2000; Reddy et al., 2001), yolk granules in eggs (McNeil and Terasaki, 2001)), that is available for these homotypic and exocytotic fusion events. Therefore, a rapid mechanism for bringing the relevant membranes into intimate physical contact must be activated by a disruption. Vesicle transport powered by myosin and kinesin is hypothesized to be required for resealing, since resealing is inhibited by function blocking antibodies against these motor proteins (Bi et al., 1997; Steinhardt et al., 1994). Disruption-induced vesicle transport may promote resealing not only by bringing vesicles into intimate contact with one another and/or the plasma membrane, but also by recruiting additional vesicles, for use in fusion events, into peri-disruption cytoplasm (Miyake and McNeil, 1995).

We hypothesize that the cytoskeleton itself, especially the actin-filament-rich, gel-like cortical cytoskeleton of mammalian epithelial cells, can present a physical obstacle to the vesicle transport and contact events required for resealing. Previous observations have failed to resolve this question. Thus, treatment of fertilized sea urchin eggs and embryonic cells with cytochalasin is reported to inhibit resealing (Bi et al., 1997), whereas cytochalasin treatment of fibroblasts promotes resealing (Togo et al., 2000). Here we test several predictions of this hypothesis by variously manipulating the actin polymerization state in living, gastric epithelial cells and measuring the effect on resealing.

MATERIALS AND METHODS

Reagents

Fluorescein isothiocyanate (FITC)-labeled phalloidin, cytochalasin B, tetramethylrodamin isothiocyanate (TRITC)-phalloidin and phalloidin were purchased from Sigma (St Louis, MI). Fixable Texas Red-labeled dextran (M_r 70,000; TRDx), jasplakinolide and Alexa 633-labeled phalloidin were from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP) and DNase1 were from Boehringer Mannheim (Mannheim, Germany); and an 'ATP detection kit' was from Toyo Ink. (Tokyo, Japan). Fixable FITC-dextran conjugated to lysine (M_r 10,000; FDx) was made as previously described (Gimlich and Braun, 1985).

Cell culture

The rat gastric mucosal epithelial cell line, RGM1, established by H. Matsui from normal Wistar rat gastric epithelium (Kobayashi et al., 1996), was kindly supplied by the Riken Cell Bank (Tsukuba, Japan). RGM1 cells were cultured to confluence in a 1:1 mixture of Dulbecco's modified eagle medium and Ham's F12 medium supplemented with 20% fetal calf serum, and used after their 25th passage.

Plasma membrane disruption

For microscope experiments with adherent cultures, cells (~20,000) were plated onto sterile coverslips or into wells of Teflon coated slides (Eric Scientific, Portsmouth, NH) and allowed to attach overnight. Cell monolayers (confluent) were scratched with sterile 18-gauge needle (Swanson and McNeil, 1987), or alternatively, treated with glass beads (McNeil and Warder, 1987), to induce plasma membrane disruptions, and then processed for microscopy at various intervals afterwards as described below.

For flow cytofluorometric analysis of phalloidin staining, sub-confluent RGM1 cultures were scraped from 100 mm diameter culture plates using a soft rubber policeman as previously described (McNeil et al., 1984).

For luciferin-based assays of disruption survival, plasma membrane disruptions were induced in an automated syringe loading device designed to inflict a reproducible level of mechanical stress on cells (Clarke and McNeil, 1992). Briefly, cells (~5×10^5 per ml) in 1 ml of culture medium were drawn up into and expelled from a sterile 1 ml syringe through a sterile 30 gauge needle (Becton-Dickinson, Ruterford, NJ) by the action of a foot activated two-way valve under a constant pressure of 1 kg/cm^2.

F-actin staining

For rapid fixation for microscopy, cells were immersed in PBS containing formaldehyde-glutaraldehyde based fixative (Ito and Karnovsky, 1968) for 10 seconds, washed in ice cold PBS and then fixed for a further 10 minutes in 4% paraformaldehyde (freshly generated). For flow cytometery, cells were fixed with paraformaldehyde (2.5%) only. Fixed cells were washed three times in PBS, permeabilized in 0.2% (vol/vol) Triton X-100 in PBS for 10 minutes, incubated in 10 mM FITC, TRITC or Alexa 633 phalloidin dissolved in PBS at room temperature for 20 minutes and then washed thoroughly in PBS before microscopic analysis by confocal laser microscopy (Olympus, Fluorview System, Tokyo, Japan) or by flow cytofluorometry.

Semi-quantitative evaluation of cell F-actin content by flow cytofluorometry

Flow cytometric analysis was performed on a FACS flow cytometer (Becton Dickinson) using a 488 or 632 nm argon ion laser excitation line and appropriate band-pass filters. Forward angle scatter and fluorescence intensities were recorded from 10,000 cells, and dead cells eliminated from data sets based on their forward angle scatter.

Electron microscopy

Cells were syringed (taken up into and expelled twice from a 30 gauge needle) in the presence of 1 ml of culture medium containing HRP (10 mg/ml) and fixed ~15 seconds later by immersion in the formaldehyde-glutaraldehyde fixative for 10 minutes at room temperature. HRP reaction product was developed using diaminobenzedine as a substrate and the cells were then postfixed in 1% OsO_4 at room temperature for 1 hour. Ultra thin sections (cut on an MT1 ultramicrotome, Sorval) were examined in a JOEL (1210, Tokyo, Japan) transmission electron microscope.

Luminometer quantification of cell injury

This assay is based on one previously described (Miyake and McNeil, 1998) in which release of ATP from cells is used as an index of damage. After cells (20,000) were syringed or otherwise manipulated, 50 μl of a luciferin/luciferase solution (from the Toyo kit) were added to the cell suspension, and the cuvette containing this mixture then

immediately inserted into a luminometer (Yamato Scientific, Compactlumi VS500, Tokyo, Japan) for measurement of light intensity.

RESULTS AND DISCUSSION

Evidence for disruption-induced filamentous actin dissolution and 'patch' vesicle formation in RGM1 cells

If actin depolymerization is induced locally by plasma membrane disruption, this might be detectable microscopically as a local decrease in cortical staining with a fluorescently labeled phalloidin, which binds with high affinity and specificity to filamentous actin. Therefore we examined RGM1 cells bordering on scratch wound sites, and fixed within ~15 seconds after this plasma membrane disruption-inducing injury. The scratch injury was carried out in the presence of a fixable fluorescein-labelled dextran (FDx), so that those cells bordering on the scratch site, which incurred a plasma membrane disruption, could subsequently be identified, and so that the boundaries of cortical cytoplasm could be visualized in these wounded cells. We observed in some of these wounded cells localized cortical domains stained with FDx (Fig. 1a) but exhibiting apparently reduced TRITC-labeled phalloidin staining (Fig. 1b). By contrast, in cultures fixed for 1 minute (Fig. 1c,d) or, more dramatically, 30 minutes (Fig. 1e,f) after the disruption-inducing scratch injury to the monolayer, there was an apparent increase in cortical TRITC-phalloidin staining in the FDx-positive cells.

To confirm these results quantitatively, we assessed phalloidin staining by flow cytofluorometry (Cano et al., 1992). RGM1 cells were scraped from their culturing substratum to induce plasma membrane disruptions (Fig. 2a) and to free them for subsequent flow analysis, and then fixed ~15 seconds later. Scraping (and fixation) was carried out either in the presence of normal levels of extraceullular Ca^{2+} (1.5 mM), or in Ca^{2+}-free media (no added Ca^{2+}, 1.0 mM EGTA). The flow data revealed a small but highly significant and reproducible decrease in the level of Alexa 633-labeled phalloidin staining under the resealing permissive (plus Ca^{2+}) condition compared with the resealing non-permissive (minus Ca^{2+}) condition (Fig. 2b,c). RGM1 cells analyzed by flow at later time points (5 or 60 minutes), showed, by comparison, significantly enhanced levels of phalloidin staining, consistent with the confocal imaging results (data not shown).

To confirm that RGM1 cells, like other cells studied previously (Miyake and McNeil, 1995), form large, cortically disposed 'patch' vesicles, we used electron microscopy to image regions of RGM1 cell cytoplasm bordering on disruption sites. RGM1 monolayers were wounded by scratching them in the presence of horseradish peroxidase (HRP) and then fixed immediately (<15 seconds after scratching). Cells that incur plasma membrane disruptions are strongly labeled with HRP under this condition, and within such cells a gradient of HRP labeling is observed to mark the general cytoplasmic domain beneath the disruption site. Compared with unlabeled RGM1 cell cytoplasm (Fig. 3a), strongly HRP-labeled cytoplasm contained an abundance of cortical and abnormally enlarged vesicles, some of which were apparently fused with the plasma membrane (Fig. 3b). Thus, electron microscopy suggests that wounded RGM1 cells, like endothelial cells and sea urchin eggs, possess within the cell cortex a greatly enlarged and abundant vesicle population, as would be predicted by the patch hypothesis.

Functional tests of a resealing requirement for F-actin depolymerization

In functional tests of whether actin polymerization state influences resealing capacity, RGM1 cultures were subject to two or more independent actin filament depolymerizing or stabilizing treatments, and the effect on disruption survival evaluated by several independent methods.

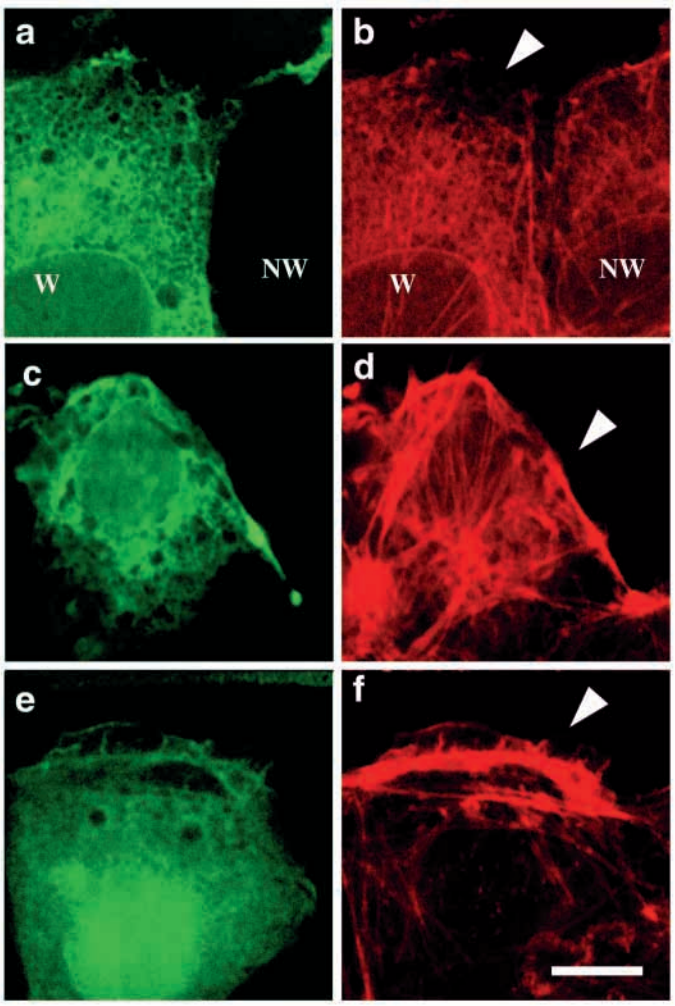


Fig. 1. Confocal imaging of phalloidin staining of wounded RGM1 cells. At 15 seconds (a,b), 1 minute (c,d) or 30 minutes (e,f) after scratching a monolayer in medium containing FDx, cultures were fixed and stained with TRITC-phalloidin. Shown are paired, confocal microscope images of fluorescein fluorescence indicating cell permeation with FDx as a result of a plasma membrane disruption event (a,c,e) and rhodamine fluorescence indicating F-actin distribution (b,d,f). At 15 seconds post-disruption-injury, FDx-labeled zones of cytoplasm bordering on the scratch site were (a) often apparently depleted of F-actin (b, arrowhead). By contrast, at 1 or 30 minutes post disruption-injury, FDx labeled cells that had survived a disruption (c,e) characteristically displayed very strong phalloidin staining in cortex bordering on the scratch site (d,f, arrowheads). Bar, 10 μm. W, wounded; NW, not wounded.

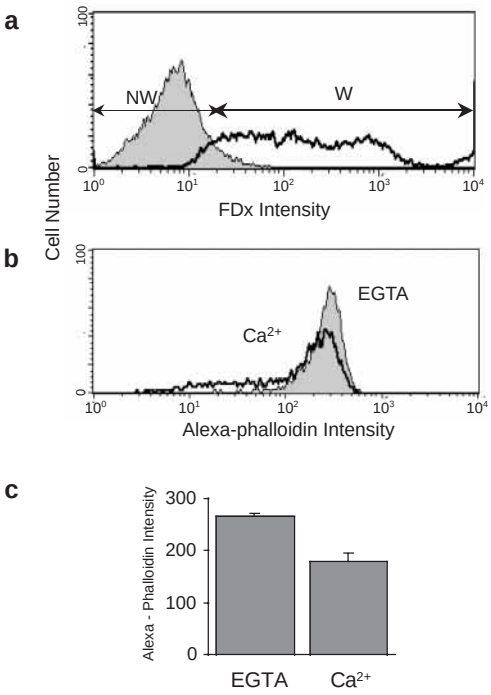


Fig. 2. Flow cytofluorometric analysis of phalloidin staining of RGM1 cells as a function of the presence or absence of Ca^{2+} during wounding. (a) Cells were wounded by scraping them from the substratum or incubated undisturbed in FDx for an equivalent interval. Flow cytometry was then used to measure the fluorescence of 10,000 cells in the undisturbed (gray field) and scraped (bold trace) populations. Greater than 92% of the scraped (W, demarcated by right arrow) population fell above a fluorescence threshold set to contain 95% of the undisturbed population (NW, demarcated by left arrow), indicating that this percentage incurred plasma membrane disruptions as a result of scraping. (b) Cells were scraped from the substratum in PBS containing 1.5 mM Ca^{2+} (Ca^{2+}; bold trace) or containing no added Ca^{2+} and 1.0 mM EGTA (EGTA; gray filled). Flow cytometry was used to measure the fluorescence of 10,000 cells in each population. (c) The mean (and s.d.) of the FITC-phalloidin fluorescence measured by flow cytofluorometry from 4 populations scraped separately from four dishes, in EGTA (EGTA)- or Ca^{2+} (Ca^{2+})-containing medium. Applying the Kruskal-Wallis test to these two data sets gave $P<0.05$.

We first examined the effect of cytochalasin on resealing, as previously it had been reported to both enhance and inhibit resealing (Bi et al., 1997; Togo et al., 2000). Monolayers of RGM1 cells were scratched with a sharp implement and, as above, this was done in the presence of FDx, so that cells incurring and resealing a plasma membrane disruption could subsequently be identified microscopically by virtue of their cytosolic staining with this marker. In monolayers pre-treated with the actin depolymerizing agent, cytochalasin B, actin depolymerization was evident by TRITC-phalloidin staining as a loss of cortical actin and stress fiber staining (Fig. 4a,b) and, by phase-contrast imaging, as a failure of wound closure (Fig. 4c,d). However, the densities of FDx-positive cells along scratch sites made with or without cytochalasin B were qualitatively (Fig. 4e,f) and quantitatively (Fig. 4g) indistinguishable. In a positive control, monolayers were pre-treated with EGTA to reduce extracellular Ca^{2+} levels below the ~0.1 mM threshold required for resealing (Steinhardt et al., 1994). FDx-positive cell density was reduced by approximately twofold in the absence of Ca^{2+} relative to control (normal Ca^{2+}) values (Fig. 4g).

In a second, independent test of the effect of filamentous actin depolymerization on resealing, we induced plasma membrane disruptions by syringing RGM1 cells (Clarke and McNeil, 1992). Resealing effectiveness was assessed quantitatively by monitoring release of a small but normally membrane-impermeant cytosolic molecule, ATP (Miyake and McNeil, 1998). When cells were loaded with the actin-depolymerizing enzyme, DNase1 (Hitchcock et al., 1976), through the induction of plasma membrane disruptions in a first syringing event, resealing was significantly enhanced during a second syringing event (Fig. 5a). However, cytochalasin treatment was found, as in the above assay, not to significantly effect resealing (Fig. 5b).

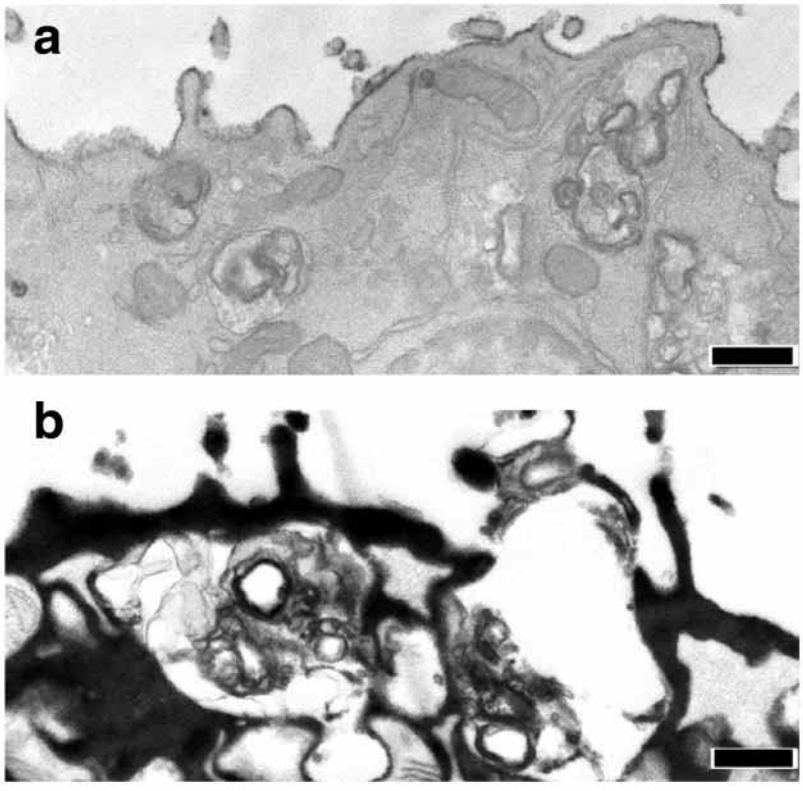
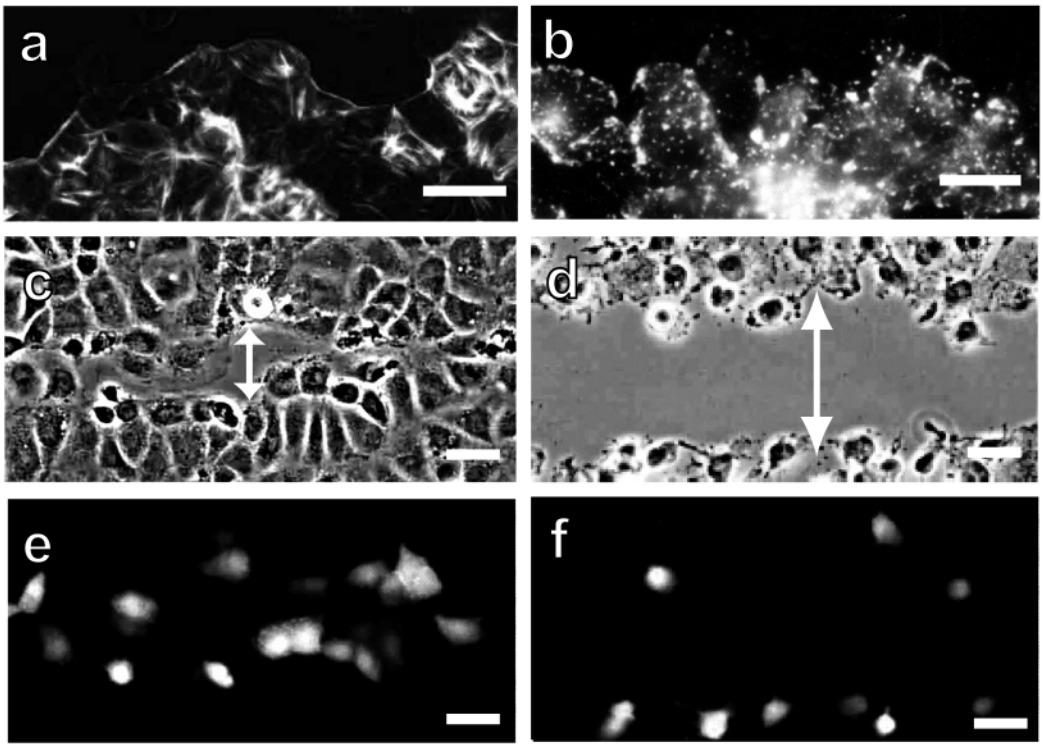


Fig. 3. Electron microscope comparison of native RGM1 cytoplasm with cytoplasm surrounding a disruption site. (a) A section through the cortex of a RGM1 cell not subject to shear stress but incubated in HRP. Note the lack of HRP staining of cytoplasm typical of such an incubation carried out in the absence of concurrent plasma membrane disruption. (b) A section through the cortex of a cell syringed in HRP. Cytosolic labeling with HRP indicates that this cell experienced a plasma membrane disruption. Typical of such cells is the striking accumulation of abnormally large vesicles in cortical cytoplasm. Bars, 0.5 μm.



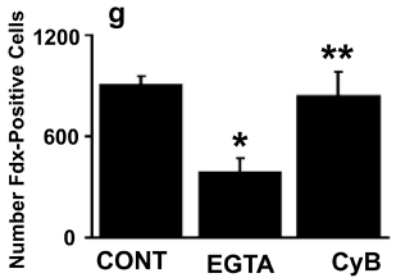


Fig. 4. Cytochalasin B treatment of undisturbed RGM1 cells does not effect resealing. (a) Control RGM1 cells stained with TRITC-phalloidin. (b) Cytochalasin B (5 minutes, 3 μg/ml)-treated cells stained with TRITC-phalloidin. Note that, by comparison with the untreated cells in (a), there is a marked disruption of cortical and stress fiber F-actin staining in these cells. (c) Nearly complete closure of monolayer injury sites was observed 2 hours after scratching a cover slip in PBS. (d) Closure failure was observed when cytochalasin B (3 μg/ml, added 5 minutes before scratching) was added to the PBS. (e) Fixable FDx (M_r 10,000)-positive cells, which suffered and survived a plasma membrane disruption, line a control wound site created in the presence of this marker (10 mg/ml) added to PBS. (f) FDx-positive cells, in apparently equivalent density, line a wound site in a culture treated with cytochalasin. (g) Quantitation of the density cell survivors of PMD along scratch sites. Scratch-loading was performed in PBS containing FDx and no additive (Cont), 5 mM-EGTA (EGTA) or 5 μg/ml cytochalasin B added 5 minutes before monolayer injury (CyB), and the number of FDx-positive cells lining a 100 mm distance along scratch edges counted for each condition. The mean and s.d. of three separate experiments are shown. Comparison of the EGTA treated and control samples yielded $P<0.05$ (*) (Kruskal-Wallis test), and for cytochalasin and control samples $P=0.51269$ (**). Bars, 30 μm.

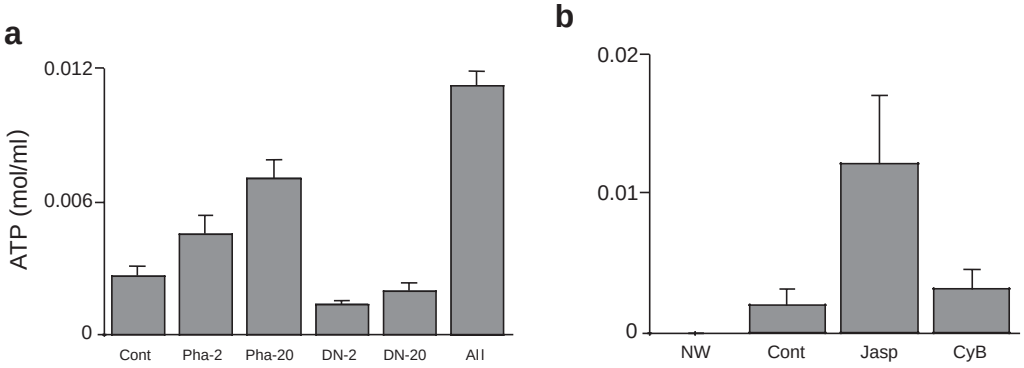


Fig. 5. DNase1-mediated actin depolymerization can enhance, and phalloidin and jasplakinolide stabilization can inhibit, resealing of RGM1 cells. (a) The total amount of ATP released by cells upon syringe injury was measured in a chemiluminesence assay as a function of phalloidin (Pha-2; 2 μg/ml; or Pha-20; 20 μg/ml) or DNase1 (DN-2; 2 μg/ml; or DN-20; 20 μg/ml) loading by a first syringing event. Control (Cont) cells were subject to the loading procedure (syringing) carried out in PBS with no additives. Total cell population content of ATP was measured after lysis initiated by the addition of a kit-supplied cell lysis reagent (All). Values plotted represent mean and s.d. of three experiments, and differed significantly from the control (Cont) condition ($P<0.05$, Kruskal-Wallis test). (b) The total amount of ATP released upon no injury (NW), and upon syringe injury of cells pre-incubated for 20 minutes in plain PBS (Cont), jasplakinolide (Jasp; 3 μg/ml) or cytochalasin B (CyB; 3 μg/ml). Values represent the mean and s.d. of three experiments. Comparison of the control and jasplakinolide samples yields $P<0.05$ (Kruskal-Wallis test). Plasma membrane disruptions were induced in an automated syringe-loading device designed to inflict a reproducible level of mechanical stress on cells (Clarke and McNeil, 1992).

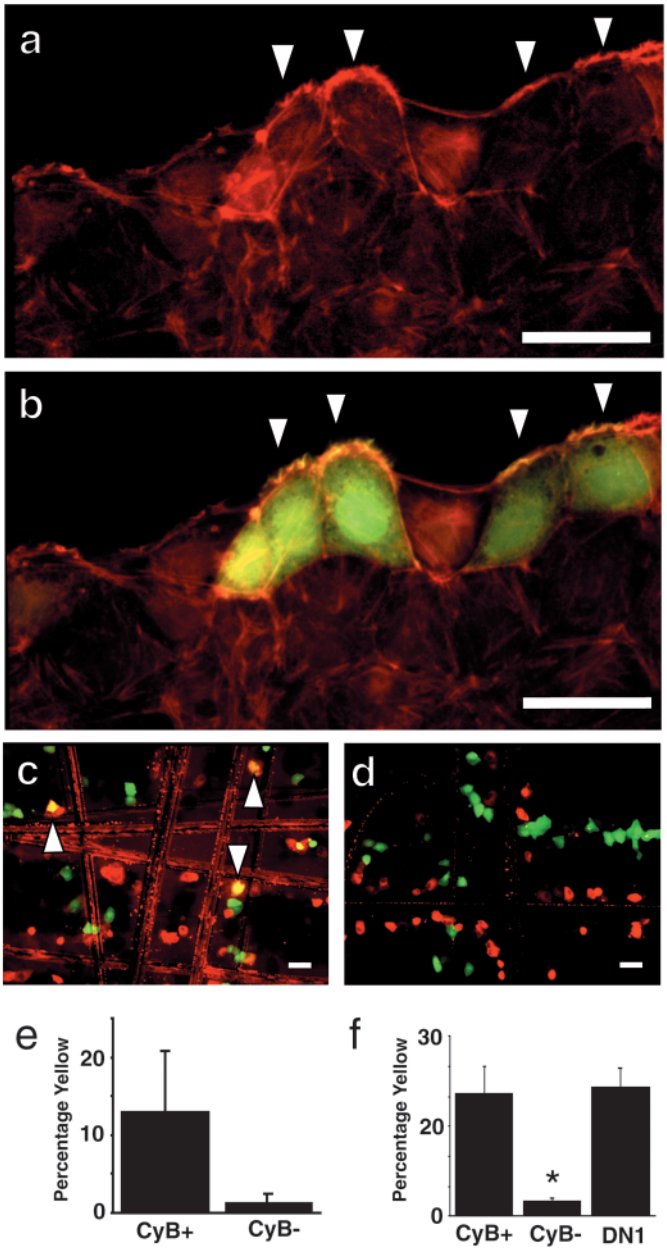


Fig. 6. Inhibition of resealing by biologically mediated actin polymerization is reversible with cytochalasin or DNase1. (a) RGM1 cells lining a scratch site 30 minutes after its creation were heavily stained in their cortices with TRITC-phalloidin. (b) When this scratch maneuver was carried out in FDx, so that those cells along the scratch site incurring plasma membrane disruptions could be identified, those cells most heavily labeled with TRITC-phalloidin were co-labeled with the FDx marker of a plasma membrane disruption event (arrowheads). (c) A monolayer was scratched first in PBS containing cytochalasin B (3 μg/ml) and FDx (10 mg/ml), and then rinsed with fresh medium minus cytochalasin. Two hours later it was scratched a second time (scratch lines approximately at right angle to first set) in the presence of PBS containing fixable Texas Red-labeled dextran (TRDx; M_r 70,000; 10 mg/ml). Double-labeled (yellow) cells (arrows) are clearly present in the population and represent those that survived two PMD events. (d) A monolayer was treated identically to that above, except that it was scratched first in PBS minus cytochalasin. No double-labeled (yellow) cells are observed. (e) The percentage of yellow (double-labeled) relative to green cells (single label from first scratch only) observed as a function of scratching the first time plus (CyB+) or minus (CyB–) cytochalasin B (3 μg/ml). The number of yellow cells relative to green were scored along a total of 200 mm of scratch site length in five separate experiments. The mean and s.d. of these measurements are plotted. Comparison of the two data sets yielded P=0.00882 (Kruskal-Wallis test). (f) Analysis of cells surviving two disruptions made by glass beads instead of scratching the monolayer. During the first plasma membrane disruption event induced by beads, the PBS medium contained no additives (CyB–), or cytochalasin B at 3 μg/ml (CyB+) or DNase1 at 100 μg/ml (DN1). The data represents the mean and s.d. of five experiments. The CyB– (*) sample differs significantly from the other two samples (P< 0.01, Kruskal-Wallis test). Bars, 20 μm.

To test the effect of filamentous actin stabilization on resealing, RGM1 cells were incubated in the membrane-permeant, cyclic peptide derived from a marine sponge, jasplakinolide, or loaded with membrane-impermeant phalloidin. Both of these drugs are known to stabilize filamentous actin (Bubb et al., 1994; Lengsfeld et al., 1974). Phalloidin loading into cells by a first syringing event decreased resealing upon a second syringing event by approximately threefold compared with controls syringed a first time in PBS only (Fig. 5a). Jasplakinolide pre-incubation decreased resealing by approximately eightfold (Fig. 5b). Thus, stabilization of filamentous actin by drug treatment strongly inhibits resealing.

The inhibitory effects of phalloidin and jasplakinolide could be ascribed to nonspecific, drug-induced alterations unrelated to actin polymerization state. Owing to the high affinity constants of phalloidin and jasplakinolide for filamentous actin, reversing these treatments was not a feasible option (Ayscough, 2000). Therefore, for this purpose we used a biologically induced increase in filamentous actin, namely, that initiated in certain mammalian cells by monolayer scratch wounding (Bement et al., 1999; Martin and Lewis, 1992). Fig. 6a shows that RGM1 cells, too, lining a scratch wound are heavily stained in their cortices with TRITC-phalloidin. Many of the most heavily labeled (with TRITC-phalloidin) of these had suffered plasma membrane disruptions during the scratch, as indicated by their staining with FDx (Fig. 6b). Moreover, flow cytofluorometric analysis of TRITC –phalloidin staining intensity of cells from RGM1 cultures wounded with glass beads clearly revealed a quantitative increase, relative to unwounded controls, in staining with this specific probe for filamentous actin (data not shown). Therefore, we next made a first, actin polymerization-inducing round of disruptions by scratching RGM1 monolayers in presence of FDx, so that those cells in which disruptions were produced by this first scratch event could subsequently be identified by virtue of their labeling with this ‘green’ fluorescence dye. Alternatively, we induced a first round of disruptions using glass beads. This first round of disruptions served to load membrane impermeant DNase1 into cells. Two hours later a second round of scratches was made on the slides, or a second bead treatment was performed, this time in the presence of Texas Red-labeled dextran (TRDx). In combined fluorescein and Texas Red fluorescence images, yellow cells are those that incurred and survived two plasma membrane disruptions, and hence retained both the ‘green’ (first disruption) and ‘red’ (second disruption) fluorescent tracers (Fig. 6c,d). Therefore the proportion of

yellow/green will quantitatively reflect whether successful resealing of the second disruption was modulated by conditions present during the first disruption. In cytochalasin-treated (Fig. 6c) or DNase1-loaded (not shown) cultures, the proportion of yellow cells was apparently greatly enhanced, compared with controls (Fig. 6d). Fig. 6e,f presents a quantitative analysis of this difference: the presence of cytochalasin B, or DNase1, during the first disruption increased the yellow/green proportion by seven- to eightfold. Thus, the resealing inhibitory effect of a biologically induced increase in filamentous cortical actin was readily reversible with two different depolymerizing agents.

The mechanism of resealing: role of F-actin depolymerization

Hypothetically, the Ca^{2+} requirement for rapid resealing can be explained as a direct role for this ion in activating a protein 'sensor' component of the fusion machinery itself. Such a role, independent of any effect on the cytoskeletal apparatus, is indeed supported by work in resealing and other, more intensely studied model systems. Thus, in cell- and cytosol-free systems that reconstitute vesicle fusion in vitro, and obviously lack cortical arrays of filamentous actin, fusion can be initiated simply by adding Ca^{2+} ions to the isolated vesicles (Mayorga et al., 1994; McNeil et al., 2000). Although proof of the molecular identity of the Ca^{2+} 'sensor' has remained elusive even in the most intensely studied systems, several candidates have been put forward (Burgoyne and Morgan, 1998). Most interesting among these, from the standpoint of this work, is the protein synaptotagmin VII, whose activity is required for Ca^{2+}-dependent exocytosis of lysosomes (Reddy et al., 2001; Martinez et al., 2000): as mentioned above, lysosomes are one of the vesicle populations used in the homotypic and eoxcytotic fusion events leading to resealing.

Our results suggest that a second key function of Ca^{2+} during resealing is to regulate actin assembly state. First, we confirmed the prediction that resealing can be facilitated by experimental manipulations favoring filamentous actin dissolution (e.g. by loading the actin-depolymerizing enzyme, DNase1, into RGM1 cells). Our failure using RGM1 cells to confirm the enhancing effect of cytochalasin treatment on resealing, previously reported in fibroblasts (Togo et al., 2000), may reflect differences between these two cell types and/or the methods used for wounding and the evaluation of resealing. It is, in any case, worth noting that cytochalasin, which binds to barbed ends of actin filaments, is generally considered to be a less effective depolymerizing agent than other drugs, such as latruculin or DNase1, which display a high affinity for monomeric actin (Wakatsuki et al., 2001). Second, we confirmed the previously untested and crucial prediction that resealing can be inhibited by manipulations favoring filamentous actin stabilization (e.g. using two different actin stabilizing agents, phalloidin and jasplakinolide). Third, we confirmed the prediction that a resealing deficit, mediated by a biologically induced increase in filamentous actin, can be reversed by cell treatment with filamentous actin destabilizing agents (e.g. cytochalasin and DNase 1). Fourth, we showed that filamentous actin content is quantitatively reduced in a Ca^{2+}-dependent fashion shortly (~15 seconds) after wounding. Our results parallel those of many studies that have similarly provided structural and functional evidence that actin filament disassembly is required for various other exocytotic responses (Muallem et al., 1995; Sontag et al., 1988; Trifaro et al., 2000). They can moreover be understood in terms of the well-documented capacity, at both the cell and molecular level (Taylor and Fechheimer, 1982; Yin et al., 1981), of Ca^{2+} to stimulate actin depolymerization.

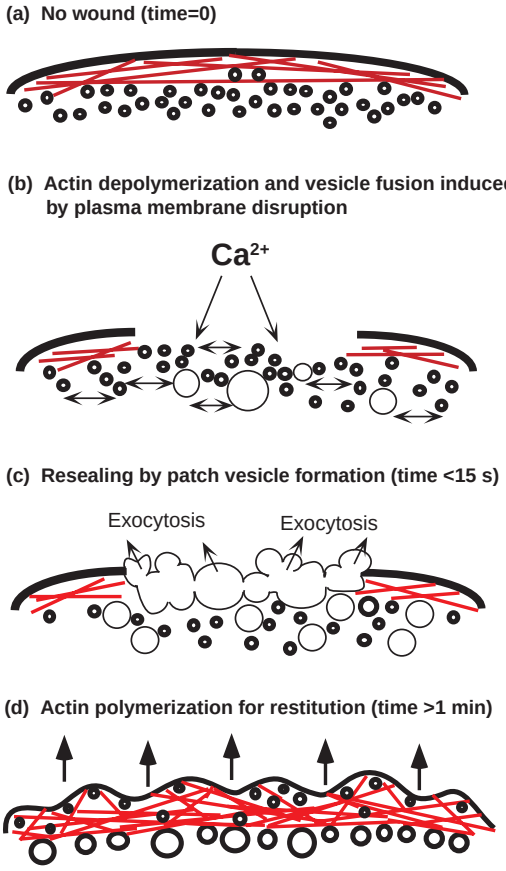


Fig. 7. Proposed role of actin depolymerization in resealing. (a) A cortical barrier to vesicle movement consisting, in part, of filamentous actin is present in undisturbed cells. (b) A plasma membrane disruption allows entry of Ca^{2+} that promotes consequent Ca^{2+}-induced actin depolymerization locally in peri-disruption cortex. (c) This facilitates Ca^{2+}-induced membrane fusion events required for resealing by removing an obstacle to vesicle-vesicle and vesicle-membrane interaction. (d) Actin polymerization occurring in wounded and other cells lining injury sites many minutes after PMD events may be required for successful restitution or repair of monolayer injury.

The requirement for actin depolymerization may be a particularly strong in the case of resealing-based homotypic fusion and exocytosis. Resealing is a crisis response to an event that does not occur with predictable timing or location. It uses vesicles, lysosomes (Andrews, 2000; Reddy et al., 2001) and possibly endosomes (Bi et al., 1995; Miyake and McNeil, 1995), which are not 'pre-docked' with their appropriate fusion partners; yet fusion of these partners must occur rapidly (seconds). Active transport (Bi et al., 1997) of endosome/lysosomes, most of which are >100 nm in diameter, into cell cortex bordering on a disruption, may function to rapidly concentrate internal membrane locally when and where needed (Miyake and McNeil, 1995). However, direct physical contact of these vesicles with one another is then required for the ensuing

fusion events that form an enlarged (many >1 μm diameter) 'patch' vesicle population (Miyake and McNeil, 1995; Terasaki et al., 1997). Similarly, exocytotic fusion of these large patch vesicles cannot occur until they make direct physical contact with the plasma membrane. The mean pore diameter of cell cytosol is estimated to be 30-40 nm, and the filamentous actin component of the cytoskeleton contributing to this size limit on particle diffusion is strikingly enriched in the cortex of most cells (Luby-Phelps, 2000). Based on these theoretical considerations and the experimental data presented here, we propose a new element of the 'patch' hypothesis for explaining how mammalian cells possessing an actin-rich cortex reseal: Ca^{2+}-regulated disassembly of the actin-based cortical cytoskeletal barrier is an essential prelude to vesicle docking and fusion beneath the disruption site (Fig. 7).

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