

Vesicle Accumulation and Exocytosis at Sites of Plasma Membrane Disruption

Katsuya Miyake and Paul L. McNeil

Department of Cellular Biology and Anatomy, The Medical College of Georgia, Augusta, Georgia 30912-2000

Abstract. Plasma membrane disruptions are resealed by an active molecular mechanism thought to be composed, in part, of kinesin, CaM kinase, snap-25, and synaptobrevin. We have used HRP to mark the cytoplasmic site of a mechanically induced plasma membrane disruption. Transmission electron microscopy revealed that vesicles of a variety of sizes rapidly (s) accumulate in large numbers within the cytoplasm surrounding the disruption site and that microvilli-like surface projections overlie this region. Scanning electron microscopy confirmed that tufts of microvilli rapidly appear on wounded cells. Three assays, employing the membrane specific dye FM1-43, provide quantitative evidence that disruption induces Ca²⁺-dependent exo-

cytosis involving one or more of the endosomal/lysosomal compartments. Confocal microscopy revealed the presence in wounded cells of cortical domains that were strikingly depleted of FM dye fluorescence, suggesting that a local bolus of exocytosis is induced by wounding rather than global exocytosis. Finally, flow cytometry recorded a disruption-induced increase in cell forward scatter, suggesting that cell size increases after injury. These results provide the first direct support for the hypothesis that one or more internal membrane compartments accumulate at the disruption site and fuse there with the plasma membrane, resulting in the local addition of membrane to the surface of the mechanically wounded cell.

THE cell's remarkable ability to survive a plasma membrane disruption is widely appreciated by the experimental cell biologist. For example, cultured mammalian cells recover rapidly from microinjection (Celis et al., 1984), syringe loading (Clarke and McNeil, 1992), and electroporation (Knight and Scruton, 1986), three techniques which produce disruptions in the plasma membrane in order to gain access to the cytosol of the living cell.

This capacity for surviving plasma membrane disruption is, more importantly, a biological necessity for many cells in vivo. Plasma membrane disruption, termed "cell wounding", is a widespread, common, and normal event that occurs in many mechanically challenged mammalian tissues. Cell types known to be subject to wounding in vivo under physiological circumstances include skeletal muscle cells, cardiac muscle cells, epidermal cells, endothelial cells, and gut epithelial cells (McNeil, 1993). A recent study identified as wounded ~20% of the myofibers of exercised rat muscle (McNeil and Khakee, 1992) and ~20% of the myocytes of unstimulated cardiac muscle (Clarke et al., 1995). This level rises to ~80% when heart rate and force of contraction is increased by administration of isoproterenol (Clarke et al., 1995). It therefore becomes of great biological interest to ask: what are the cellular and molecular adaptations required of cells residing in mechanically injurious environments? Specifically, do cells utilize mechanisms that facilitate survival of a plasma membrane disruption?

Survival of a plasma membrane disruption requires, in the first place, that the cell employ a rapid resealing mechanism. Plasma membrane disruptions that remain open for greater than ~15 s (Steinhardt et al., 1994; our unpublished observations) are not survived by smaller (fibroblast or endothelial) mammalian cells. Presumably this is due to loss during this interval of vital molecular constituents and, perhaps more importantly, to the influx of exogenous Ca²⁺. Sustained elevation of intracellular Ca²⁺ concentration above the normal level, ~10⁻⁷ M, is lethal to cells (Lemasters et al., 1987). Plasma membrane disruptions permit diffusion-mediated Ca²⁺ entry down an approximately 10⁴-fold concentration gradient, resulting in a rapid and striking elevation of intracellular Ca²⁺ concentration (McNeil and Taylor, 1985; Steinhardt et al., 1994).

Resealing of the erythrocyte plasma membrane and of manufactured lipid bilayers can be explained purely by reference to thermodynamic principles: resealing removes hydrophobic domains of phospholipid molecules from the aqueous environment and is therefore the energetically favored event. There is little reason to suspect that any additional, active mechanism is either available or used for facilitating resealing of either of these membrane systems.

Address all correspondence to P. L. McNeil, Department of Cellular Biology and Anatomy, The Medical College of Georgia, Augusta, GA, 30912-2000. Ph.: (706) 721-3065. Fax: (706) 721-6839.

K. Miyake's present address is Department of Anatomy, Fukushima Medical College, Fukushima, Japan.

By contrast, for cells more complex than the erythrocyte, e.g., sea urchin eggs and mammalian fibroblasts, successful resealing is an active process that employs a complex molecular mechanism (Steinhardt et al., 1994). Thus, injection of antibodies to kinesin and CaM kinase, as well as specific inhibitors of synaptobrevin and snap-25, all inhibited resealing in these two cell types. Moreover it has been known for some time that exogenous Ca^{2+} is required for resealing (Gingell, 1970; Yawo and Kuno, 1985).

These results suggested that resealing is accomplished by an exocytotic mechanism resembling that which mediates neurotransmitter release (Steinhardt et al., 1994). According to this hypothesis, resealing is initiated by calcium influx through the disruption, causing a local rise in cytosolic Ca^{2+} concentration and consequent local activation of CaM kinase, which is believed to promote the release of vesicles from actin filaments by phosphorylation of synapsin I (Robinson et al., 1994). Kinesin then powers delivery of vesicles on a microtubule track to the site of membrane disruption. Finally, vesicle docking and fusion, i.e., exocytosis, mediated by synaptobrevin, snap-25 (and of course possibly other proteins; see for review Sollner and Rothman, 1994) occurs locally at the site of plasma membrane disruption.

Undemonstrated, however, are several crucial elements of this hypothesis, including: (a) vesicle movement toward and accumulation around the disruption site; and (b) exocytosis and consequent membrane addition to the disrupted plasma membrane. In order to provide direct evidence that vesicles accumulate at the disruption site and that membrane is added to the cell surface following disruption, we developed a novel method for electron microscope visualization of the disruption site. We here show that within seconds after suffering a plasma membrane disruption, endothelial cells accumulate a dense array of vesicles in the cytoplasm bordering on a disruption site. Concomitantly at this site, dense tufts of microvilli or microvilli-like evaginations of plasma membrane emerge on the cell surface. Quantitatively demonstrable exocytosis is induced immediately by wounding, and appears to be localized to the disruption site.

Materials and Methods

Transient Disruption of Cell Plasma Membranes

Bovine retinal microvascular endothelial (BRME)¹ cells (passage 32–36), the kind gift of Dr. Ruth Caldwell (Medical College of Georgia, Augusta, GA), and NIH 3T3 cells were grown as previously described (Clarke et al., 1995). Scratch loading, used here for wounding adherent cells, was carried out as described (Swanson and McNeil, 1987). Briefly, cell monolayers (confluent) were scratched with a sterile 30-gauge needle so as to denude narrow strips of cells from the substratum. Syringe loading, used here for wounding cells in suspension, was carried out in an automated syringe loading device designed to inflict a reproducible level of mechanical stress on cells (Clarke et al., 1994). Briefly, cells ($\sim 5 \times 10^5$ per ml) in 1 ml culture medium were drawn up into and expelled from a sterile 1 ml syringe through a sterile 30-gauge needle by the action of a foot activated two-way valve under a constant pressure of 40 psi. When syringe loading was carried out on cell samples contained in a cuvette placed in a spectro-

fluorometer, the 30-gauge needle was mounted at the end of a 4-cm long, 20-gauge hypodermic needle, so that a connection could be made at a distance with the syringe mounted in the automated device (see above).

Electron Microscopy

Cells were fixed at various intervals after syringing by immersion in a formaldehyde–glutaraldehyde based fixative (Ito and Karnovsky, 1968) for 30 min at room temperature, postfixed in 1% OsO_4 at room temperature for 1 h, dehydrated in ethanol, transferred into isoamyl acetate, and, for scanning electron microscopy (SEM) critical point dried. For transmission electron microscopy (TEM), the samples were processed as above, except that the cells were syringed in the presence of culture medium containing horseradish peroxidase (10 mg/ml; Boehringer Mannheim, Indianapolis, IN) and were stained with 1% uranyl acetate for 1 h after osmication followed by embedding in epon-araldite. Ultra thin sections were cut on a Reichert Ultratuc E ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) and stained with a fresh mixture of equal parts acetone and saturated uranyl acetate. TEM was performed with a Zeiss EM902, SEM with a Hitachi S-800.

Spectrofluorometric Analysis of Exocytosis

Cells were incubated for 12 h with 4 ml of fresh culture medium containing FM 1-43 or FM 4-64 (Molecular Probes, Eugene OR) at a concentration of 2.5 $\mu\text{g/ml}$, trypsinized, washed by centrifugation, and dispensed in culture medium (2×10^5 per ml) into sterile tubes and kept on ice until use. Cells were syringed as described above at 37°C (unless otherwise indicated) while fluorescence was monitored in a spectrofluorometer (SLM Instruments, Inc., Urbana, IL). FM 1-43 fluorescence was excited at 488 nm (4-nm bandwidth) and emission measured at 565 nm (4-nm bandwidth). A background of slowly declining fluorescence (>100 times slower than response) was subtracted from FM 1-43 records before plotting the traces.

Flow Cytometry

Flow cytometric analyses were performed on a Coulter Epics Elite flow cytometer (Coulter Electronics Inc., Hialeah, FL). Excitation of FDx fluorescence was provided by a 488-nm argon ion laser line, and emission detected with a 525-nm bandpass filter in place. Excitation of FM 4-64 fluorescence was provided by a 596-nm argon ion laser line, and emission measured using a 674-nm bandpass filter. Cells labeled with FM 4-64 as described above were syringed in culture medium containing fluorescein dextran (70,000 mol wt, 2 mg/ml; Sigma Chem. Co., St. Louis, MO) or were incubated with the dextran but not syringed (as a control for pinocytosis of the probe). Approximately 15 s after syringing, or at later intervals, the cells were diluted into a large volume of ice cold PBS to halt further exocytotic/endocytotic events, and kept on ice until flow analysis. Forward angle scatter, right angle scatter and fluorescence intensities were recorded from 10,000–20,000 cells whose forward angle scatter fell above a threshold used to distinguish live from dead cells.

Image Analysis Quantitation of FM 4-64 and FDx Staining

Cells labeled with FM 4-64 as described above were syringed in the presence of fluorescein dextran (150,000, 10 mg/ml in PBS; Sigma Chem. Co.) and, approximately 15 s later, were chilled by dilution in ice cold PBS. After mounting the cells in microscope slide wells covered with a coverslip, the FM 4-64 (rhodamine-like) and FDx (fluorescein) fluorescence of cells present in numerous (~ 50) randomly selected fields was imaged (Dage-MTI 772 CCD camera) over a five minute period and stored on optical disc until analysis. Quantitative, paired measurements of FM 4-64 and FDx fluorescence were then made from each of 150 cells in the randomly collected fields using an Image One image analysis system (Universal Imaging Corp.) by methods previously described (Clarke et al., 1993), except that whole cell fluorescence was selected for measurement by drawing a measurement field defined by the outermost boundary of the FM 4-64 fluorescence. The correlation coefficient and statistical significance of the data thus obtained were determined using InStat software.

Confocal Microscopy of FM 4-64 Staining

Cells were prepared as above for flow cytometry, except that we waited for 30 min rather than 15 s before chilling on ice after wounding. After mounting in slide wells (as above) images of fluorescein (488 nm excita-

1. Abbreviations used in this paper: BRME, bovine retinal microvascular endothelial; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

tion) and FM 4-64 (525 nm excitation) fluorescence were acquired using a confocal laser microscope (Bio-Rad 600 using the COMOS software package and a K2 dual wavelength filter).

Results

Visualization of the Cytoplasmic Region Adjacent to a Plasma Membrane Disruption Site

Direct electron microscopic visualization of a plasma membrane disruption, which should be visible as a break in the

continuity of the characteristic 'unit' membrane, is theoretically achievable. In practice however we were unable to observe such breaks, probably because: (a) only a small number of disruptions may be induced per cell by syringing or scratching; and (b) the proportion of the total surface area occupied by each a break is very small. To overcome this problem, we wounded cells by syringing them in the presence of HRP and then rapidly fixed them at intervals of 5–30 s thereafter by squirting them directly out of the syringe needle into a large volume of fixative. HRP labeling was absent in cells not wounded by the syringing

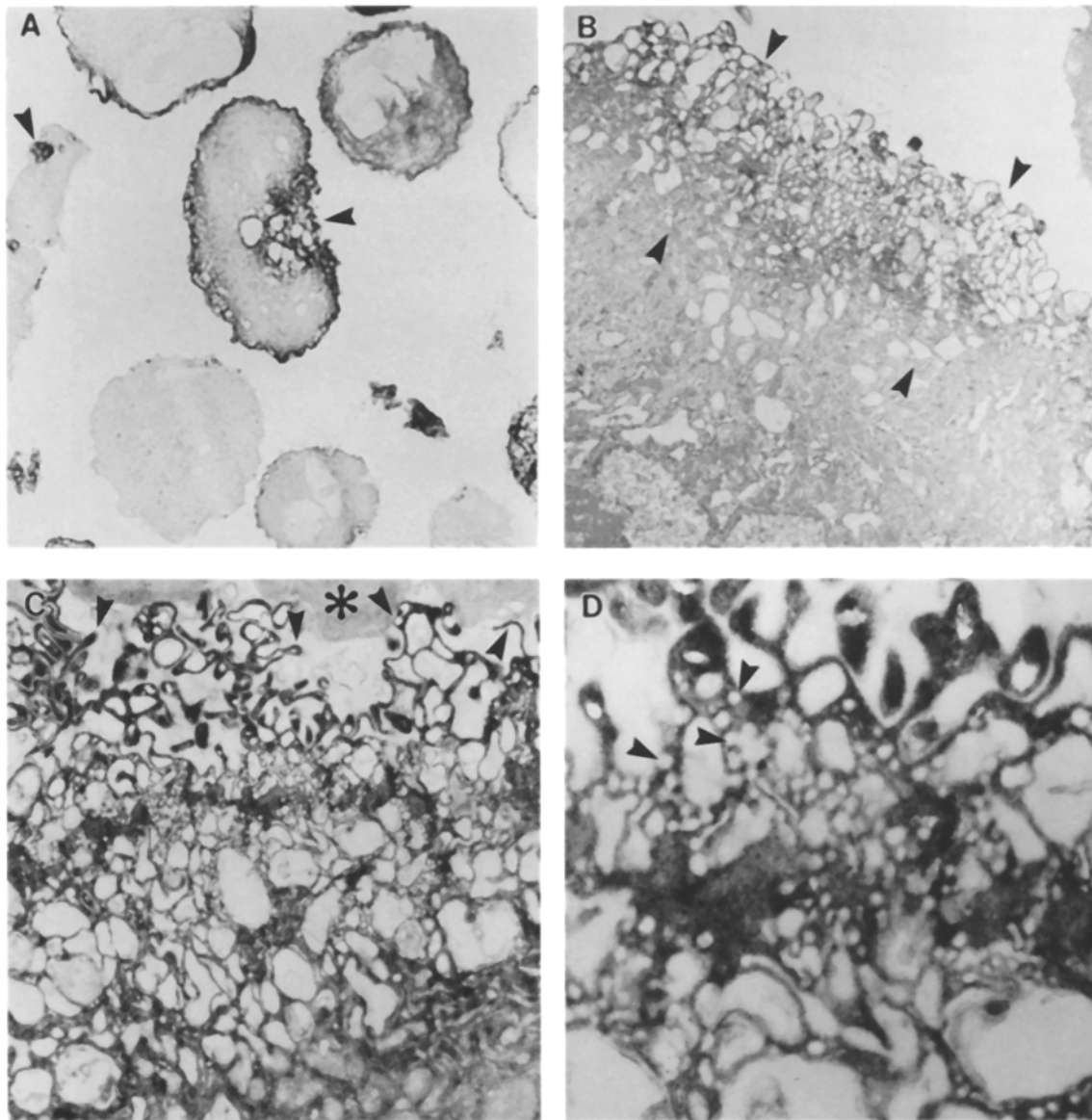


Figure 1. Dense accumulations of vesicles and microvilli are localized to sites of plasma membrane disruption. Endothelial cells were drawn up into and ejected from a 30-gauge syringe in the presence of horseradish peroxidase (HRP). On the fourth ejection the cells were squirted directly into fixative and prepared for transmission electron microscopy. (A) In some cells, localized cortical staining with HRP (arrowheads) identified sites at or near a plasma membrane disruption that was either still open at the time of fixation or had recently been resealed. (B) At such sites, transmission electron microscopy revealed striking accumulations of cytoplasmic vesicles (see area circumscribed by arrowheads). (C) Overlying these dense vesicular accumulations, we frequently observed numerous microvillar profiles (arrowheads). Note that a portion of another cell (asterisk), not labeled with HRP, possesses a low density of cortical vesicles by comparison to the HRP labeled zone just described. (D) Immediately adjacent to the plasma membrane surrounding the disruption site are numerous, small vesicles (arrowheads; diameter ~65 nm).

(Fig. 1 A) and was present in gradients in cells that did suffer membrane disruptions (Fig. 1 A, *arrowheads*). Regions of heavy labeling within a cell identify cytoplasmic sites that, at the time of fixation, are most accessible to the exogenous HRP. Since this tracer can enter the cytoplasm only via a plasma membrane disruption, sites of heavy HRP labeling were interpreted as being at or nearby a plasma membrane disruption site.

Electron microscopy revealed that regions of syringed endothelial cells labeled heavily with HRP invariably contained a striking accumulation of vesicles (Fig. 1 B). This accumulation was observed in cells fixed as soon as 5 s after syringing, indicating that vesicles had been mobilized to this region very rapidly. The vesicles were of a large range of sizes (65-nm–2- μ m diam) but alike in that most contained no inclusions or internal structure visible by electron microscopy. Most common were the smaller vesicles (~65 nm) which were often clustered immediately beneath the plasma membrane. Mitochondria, endoplasmic reticulum and other readily identifiable organelles were excluded from zones of vesicle accumulation. Vesicle accumulations were not observed in regions of endothelial cell cytoplasm lacking HRP labeling.

Visualization of Cell Surface Events Induced by Wounding

A typical result of exocytosis, which adds additional membrane to the plasma membrane, is the appearance of microvilli on the cell surface (Mazia et al., 1975; Eddy et al., 1978; Mia et al., 1994). To determine if microvilli appeared on endothelial cells after wounding, we syringed cells, fixed them at various intervals (5–30 s) thereafter, and then examined them using scanning electron microscopy. Control endothelial cells, that were not syringed, displayed few microvilli or similar cell surface protuberances (Fig. 2 A). Syringed endothelial cells by contrast were decorated with dense tufts of microvilli and/or microvillus-like protuberances (Fig. 2, B and C). Wound-induced microvillar appearance was not limited to the endothelial cell wounded in suspension by syringing; it also occurred in adherent endothelial cells wounded by scratching (data not shown). These patches of microvilli resided at or nearby sites of plasma membrane disruption. Thus in some cells examined by TEM, HRP labeled sites of vesicle accumulation (see above) were observed to be capped by characteristic microvillar profiles (Fig. 1 C).

Quantitative Demonstration of Wound-induced Exocytosis

The above morphological results suggested that exocytosis

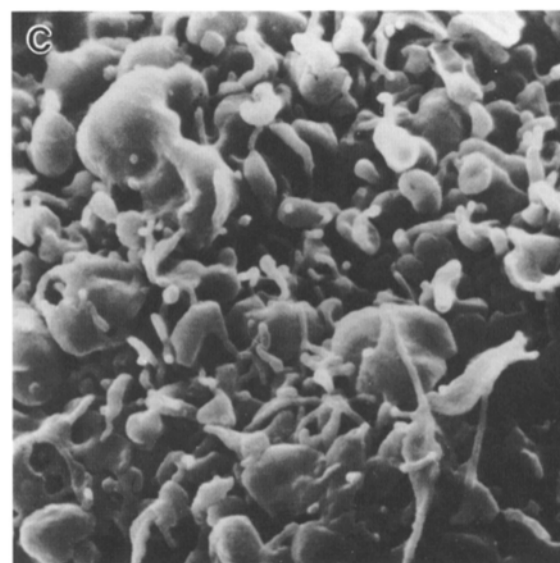
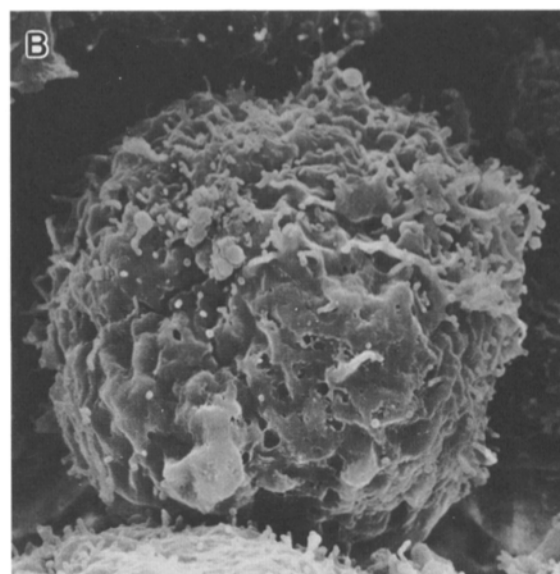
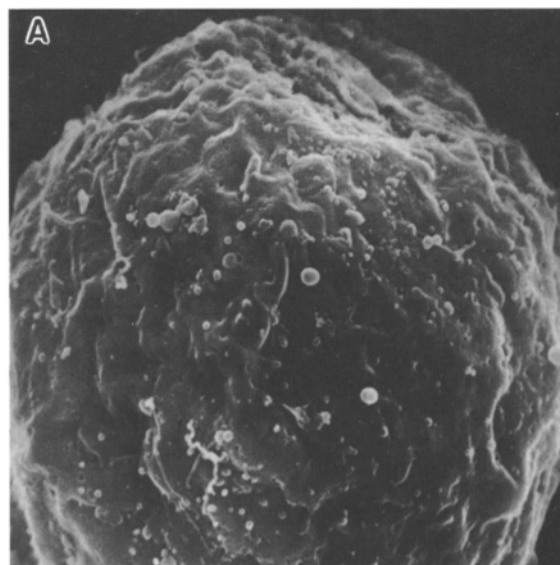


Figure 2. Tufts of microvilli (and other variously shaped surface projections) rapidly appear on the surfaces of endothelial cells suffering plasma membrane disruptions. Endothelial cells were syringed, fixed 5 s thereafter and prepared for scanning electron microscopy. (A) Control cells trypsinized but not subject to syringe loading typically possess few surface projections. (B) Wounded cells, by contrast, displayed tufts of microvilli and other surface projections. (C) A higher magnification view of a wounded cell, showing a region with a high density of microvilli-like, and other surface projections.

is provoked by wounding: vesicles accumulated in the cortex of the wounded cell near the plasma membrane and microvilli appeared on the wounded cell surface. To quantitatively demonstrate wound-induced exocytosis, we employed the lipophilic, membrane dye FM 1-43 in a recently developed fluorometric assay for exocytosis (Meffert et al., 1994). Cells were incubated in the dye, resulting in its

endocytosis and labeling of the luminal leaflet of endosomal vesicle membranes. Upon washing, following this endocytic pulse, the cells were wounded by syringing. Loss of cellular fluorescence in this assay is due to exocytotic dye delivery into the outer leaflet of the plasma membrane and thereafter into the aqueous medium where it loses (~50-fold) fluorescence (Meffert et al., 1994). Fluores-

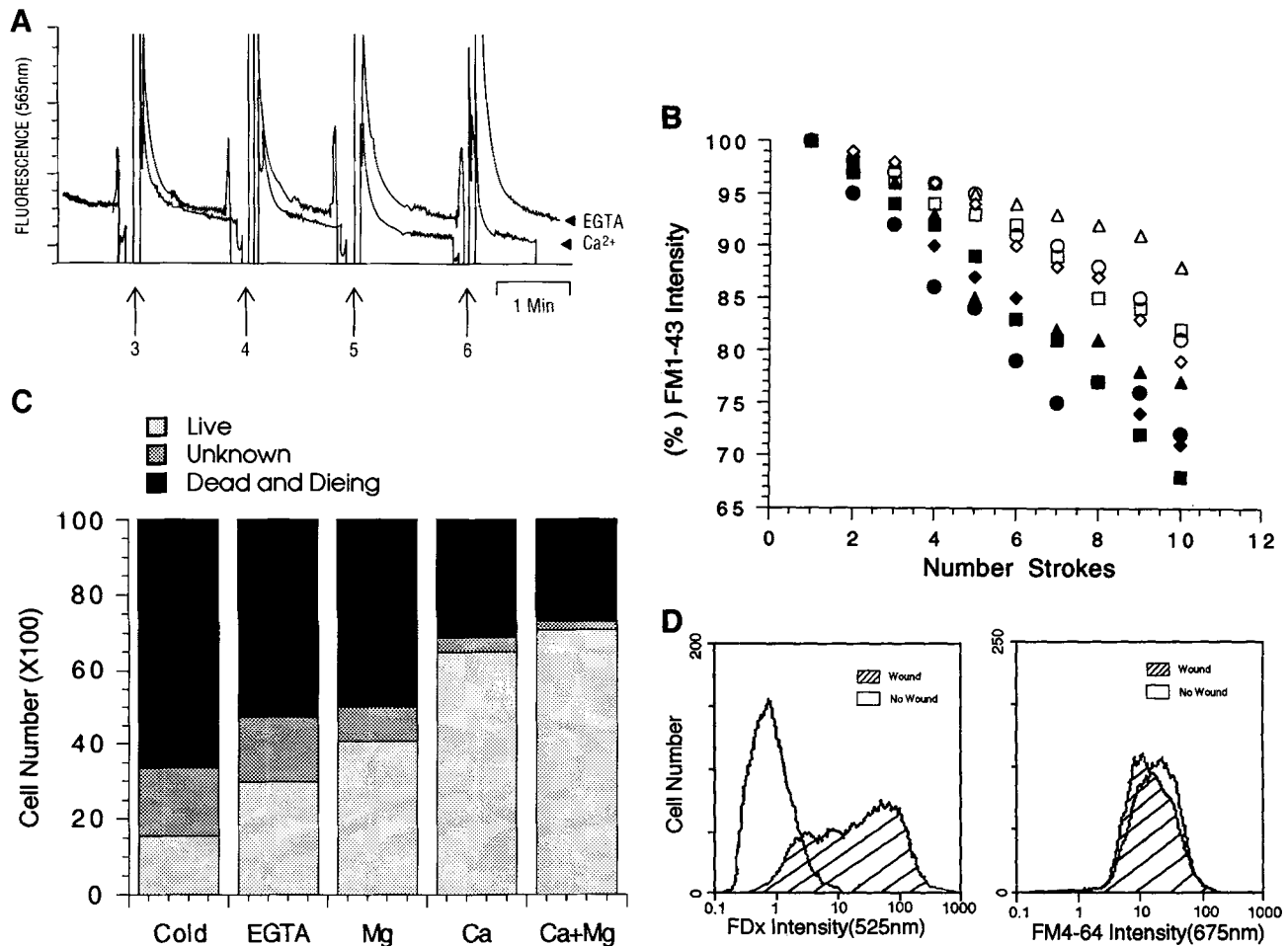


Figure 3. Plasma membrane disruption induces a Ca²⁺-dependent exocytotic event involving a component of the endosomal compartment. (A) Endothelial cells endocytosed the lipophilic plasma membrane dye, FM 1-43, and were then wounded by syringing while the fluorescence of the population was continuously monitored in a fluorometer. Uptake into the syringe of the FM labeled cells produced an artifactual downwards deflection on the record, as the fluorescent cells were removed from the cuvette, and expulsion an artifactual upwards deflection as the cells along with some bubbles were returned to the cuvette. Note that with each syringe stroke (arrows: numbered), there was an immediate decrease in the remaining, non-artifactual fluorescence on the chart record. This decrease was blunted when the syringing was carried out in Ca²⁺-free medium (containing EGTA). (B) From records such as that shown in A, the percentage change in fluorescence signal was calculated, relative to the signal measured at the experiment's beginning, after each of 10 syringe strokes (wounding events). The results of four separate experiments are shown, two using endothelial cells (circles, squares) and two NIH fibroblasts (triangles, diamonds). Wounding reproducibly reduced fluorescence intensity in all four populations, and the decrease measured was reproducibly larger when Ca²⁺ was present in the medium (filled symbols) than when it was absent (empty symbols). (C) Endothelial cell damage caused by syringing is greater in Ca²⁺-free medium than in Ca²⁺-containing medium. Cells were syringed in: (a) medium chilled on ice (Cold); (b) medium chelated with EGTA (5 mM) (EGTA); (c) medium containing added Mg²⁺ (5 mM) but not Ca²⁺ (Mg); (d) medium containing Ca²⁺ (5 mM) but not Mg²⁺ (Ca); or (e) medium containing both divalent cations (Ca+Mg), and the relative numbers of live and dead cells (detected by staining with ethidium homodimer) was determined by flow cytometry. (D) Wound-induced exocytosis can also be measured by flow cytometry. Endothelial cells endocytosed the plasma membrane dye, FM 4-64 (rhodamine like excitation, emission characteristics), and were then wounded by syringing in the presence of fluorescein dextran. Flow cytometry was used to measure the FM 4-64 and the dextran fluorescence of control, undisturbed cells and syringe-wounded cells. (Left) The fluorescence of 66% of the cells of the population syringed in the presence of the fluorescein dextran fell above a threshold set to exclude 95% of the population (pinocytosis control) that was not syringed, indicating that a majority of the syringed cells suffered plasma membrane disruptions. (Right) The FM fluorescence of the wounded cells was reduced by comparison to undisturbed controls. Identical results were observed in separate experiments.

cence of cells was monitored continuously during cell syringing in a spectrofluorometer. As can be seen in Fig. 3 A, FM 1-43 fluorescence decreased with each syringe stroke or wounding event. This result was highly reproducible (Fig. 3 B) and the decrease measured was always larger when Ca^{2+} was present in the medium than when it was absent (Fig. 3, A and B). This blunting of the fluorescence decrease in the absence of Ca^{2+} was not due to reduced cell damage in the Ca^{2+} -free medium. On the contrary, we found that the amount of fluorescence in a low speed supernatant, containing endosomes/lysosomes released from damaged cells, was twofold higher in cells syringed in Ca^{2+} -free medium (data not shown). Moreover, the amount of cell death induced was \sim twofold higher in the Ca^{2+} -free medium than in Ca^{2+} -containing medium (Fig. 3 C). Syringing in the cold similarly blunted the fluorescence decrease but caused increased cell damage (data not shown). Thus the decrease in fluorescence measured in the presence of Ca^{2+} is due to exocytosis and not cell damage.

We next used flow cytometry and another member of the FM family of membrane dyes, FM 4-64, to measure wound-induced exocytosis. As just described above, the cells were pulsed with dye and then chased in dye-free medium. In this experiment however they were syringed in the presence of FDx, a marker for a survival plasma membrane disruption event. Flow measurement revealed that \sim 65% of the cells, as judged by their increased FDx fluorescence, suffered survival plasma membrane disruptions as a result of syringing (Fig. 3 D, left). The FM 4-64 fluorescence of this population of wounded cells was reproducibly decreased relative to that of controls (Fig. 3 D, right), indicating increased exocytosis in the wounded population. Ca^{2+} -free medium and cold both blunted this decrease (data not shown).

Finally, we used image analysis to ask whether, as would be predicted for wound-induced exocytosis, the degree of fluorescence labeling with FM 4-64 (a negative marker of exocytosis) is inversely correlated with the degree of fluorescence labeling with FDx (a positive marker of cell wounding). We found a highly significant inverse correlation between these two fluorescence parameters (Fig. 4). That is, exocytosis was quantitatively correlated with the extent of wounding incurred.

Wound-induced Domains of Exocytosis

FM dyes have been used also as a microscopic marker for exocytosis (Betz et al., 1992; Terasaki, 1995). When we viewed wounded cells, identified by cytoplasmic labeling with fluorescein dextran, we observed a striking pattern of fluorescence staining. Highly localized domains of cytoplasm were devoid of punctate FM 4-64 staining (Fig. 5). This pattern of labeling suggests that a highly localized bolus of fluorescence-depleting exocytotic events occurs in the wounded cell. It was not observed in control cells that, as indicated by a lack of fluorescein dextran staining, had not suffered a plasma membrane disruption.

Cell Size Apparently Increases after Wounding

A predicted consequence of exocytosis is an increase in cell surface area and hence size. In fact, flow cytometry measurements revealed that cell forward scatter increases over

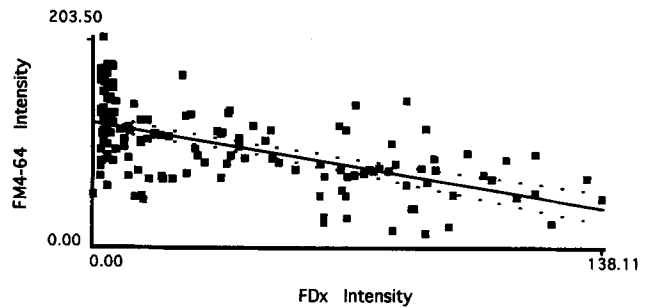


Figure 4. The amount of exocytosis induced by syringing correlates inversely with the degree of membrane disruption suffered by the cell. Endothelial cells were loaded endocytotically with FM 4-64 and then syringed in the presence of fluorescein dextran (FDx). Quantitative, paired measurement of the fluorescence of each dye on a cell by cell basis indicated a highly significant ($P < 0.001$, $n = 150$) negative correlation between FM 4-64 (rhodamine) and FDx (fluorescein) fluorescence.

time after syringing (Fig. 6). As forward scatter is proportional to cell size (Shapiro, 1988), this suggests that cell size increases after wounding. However, changes in forward scatter can also be due to other morphological alterations, such as the addition of microrvilli to the cell surface.

Discussion

Our results, and those of Bi et al. (1995; in this issue), provide crucial support for the hypothesis that an exocytosis-based mechanism is used to reseal plasma membrane disruptions. A first prediction of this hypothesis (Fig. 7), confirmed in this study using TEM to visualize and HRP to mark the disruption site, is that vesicles accumulate in the cytoplasm surrounding a disruption site. The vesicles observed by TEM were not derived from a rapid burst of endocytosis induced by the membrane disruption event. If this had been the case, then they would have been labeled (lumen) with HRP, which was present at the time of membrane disruption. Therefore they were derived from a pre-existing vesicular population, probably, in part, the endosomal-lysosomal compartment (see below).

A second important prediction of the exocytosis-based model for resealing—that exocytosis is evoked by plasma membrane disruption—has also been confirmed in the present study. Decreases in FM dye fluorescence have been shown previously to accurately monitor exocytosis of synaptic vesicles (Meffert et al., 1994). Here quantification of FM dye fluorescence by three separate methods (spectrofluorometry, flow cytometry and quantitative video microscopy) revealed a disruption-induced decrease in this exocytosis-dependent parameter. Importantly, the quantitative video data (Fig. 4) showed that exocytosis is quantitatively correlated with the size of the disruption suffered.

The FM dye assay used here (Meffert et al., 1994) reports exocytosis of endocytosed dye. Therefore one vesicular compartment involved in disruption-induced exocytosis is the endosomal/lysosomal compartment. Since the FM probes used in this study were endocytosed by cells over a prolonged period (hours), we cannot distinguish which subcompartments of the endosomal/lysosomal compartment are involved. The large variation in the size of

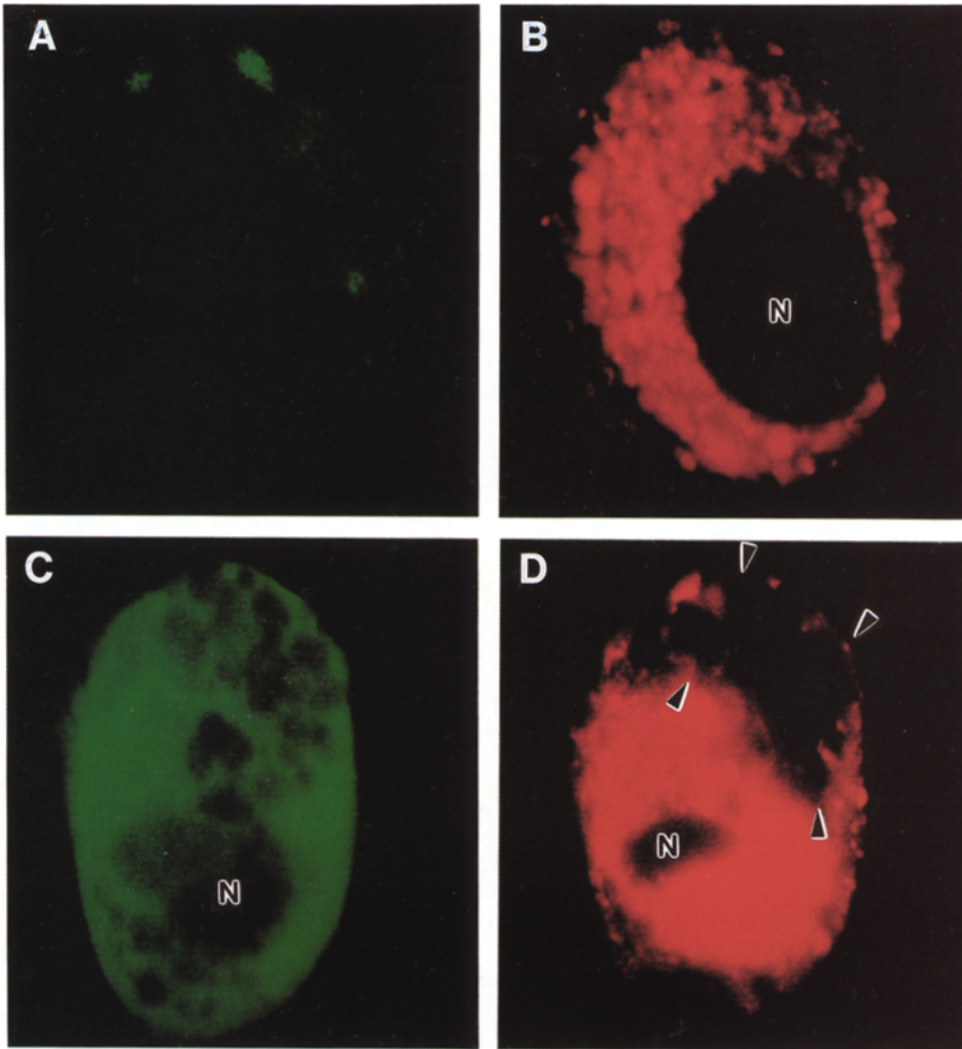


Figure 5. Localized domains of exocytosis of the endocytotic compartment appear in wounded cells. Shown are paired, confocal microscope images of the rhodamine and fluorescein fluorescence of each of two representative endothelial cells, non-wounded (*A* and *B*) and wounded (*C* and *D*), prepared as above in Fig. 4. (*A*) A cell that escaped membrane disruption during syringing is not labeled with fluorescein dextran. (*B*) It displays homogeneous FM labeling throughout its cytoplasm, except in the nucleus (*N*), which excludes the FM-labeled vesicular population. (*C*) A cell that suffered a membrane disruption during syringing is identified by its cytosolic (but not nuclear, *N*) labeling with fluorescein dextran. (*D*) It displays a zone (arrowheads) of cytoplasm free of the FM labeled vesicles.

the vesicles observed at the disruption site suggests that recruitment is not a highly selective process, and possibly involves most of the (variously sized) subcompartments of the endosomal/lysosomal compartment (Watts and Marsh, 1992). Moreover, it is possible that other membranous compartments are also involved in resealing. Kinesin may power radial movements of the endosomal/lysosomal compartment (Hollenbeck and Swanson, 1990), and so the involvement of this compartment in resealing is not unexpected. As synaptic vesicles are formed by endocytosis (Miller and Heuser, 1984), involvement of the endosomal/lysosomal compartment is further consistent with the synapse-like model for disruption-induced resealing.

A third prediction of the exocytosis model for resealing, documented here, is that membrane is rapidly added to the cell surface following a plasma membrane disruption. Thus, SEM and TEM showed that dense tufts of microvilli rapidly appear on the surface of cells suffering plasma membrane disruptions and that these microvilli were localized to disruption site. Moreover, flow cytometric measurements of cell forward scatter showed that this parameter, an accepted measure of cell size, increased over time after wounding. Exocytotic addition of membrane to the plasma membrane would be predicted to increase cell surface area and hence size.

Finally, we observed depletion of FM 1-43 labeling from remarkably discrete zones of the cytoplasm of wounded cells (Fig. 5). This suggests that a localized bolus of exocytosis is induced by wounding. This localized rather than global exocytosis is highly consistent with the localized accumulation of vesicles and microvilli that we observed using EM to visualize the disruption site. It is moreover strikingly consistent with the results of Bi et al. (accompanying paper), who, using an FM assay (Terasaki, 1995) that is essentially the converse of ours, observed the appearance of fluorescent "rings" on the plasma membrane, indicative of exocytosis, locally at sites of plasma membrane disruption in fertilized sea urchin eggs. It should be noted that localized, regulated exocytotic reactions are known to be evoked by the localized application of agonist in mast cells that, like endothelial cells, do not possess a polarized exocytotic apparatus (Lawson et al., 1978).

The mechanism used by giant cells to reseal extremely large disruptions may differ, in some respects, from that used by smaller cells resealing smaller disruptions, such as those studied here. Giant axons of squid and earthworm require hours rather than seconds to reseal after transection, which produces a membrane disruption hundreds of microns in diameter (Krause et al., 1994). Transection induces a Ca^{2+} -dependent, localized contraction (min) at the

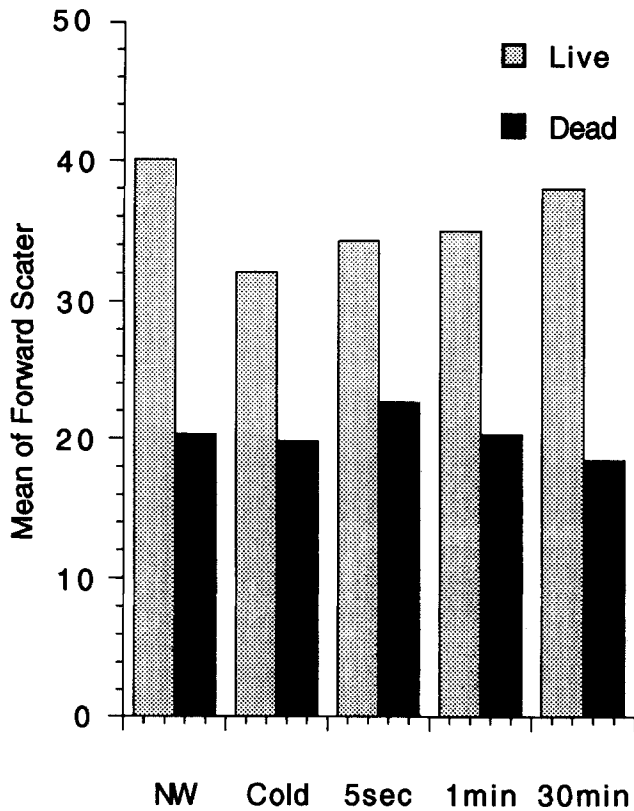


Figure 6. Cell diameter increases over time after syringe wounding. Endothelial cells were subject to syringing and at various intervals (5 s, 1 min, 30 min) thereafter placed on ice to prevent further membrane fusion events. As controls, an undisturbed population (NW) was measured, as well as a population wounded in the cold to prevent all post wound membrane fusion events. Flow cytometry was then used to measure the forward scatter, an accepted index of cell diameter, of 10,000 cells in each population. Live cells were distinguished from dead using ethidium homodimer as a stain for dead cells. The forward scatter (proportional to cell diameter) of the wounded cells increased over time after syringe wounding. Identical results were obtained in separate experiments.

cut end of the axon (Gallant, 1988), as well as the accumulation (min to h) of vesicles there (Fishman et al., 1990). It may therefore resemble morphologically, albeit on a much slower scale, the vesicle accumulation process described here for the wounded endothelial cell. Surprisingly, resealing of the squid giant axon is incomplete after 2.5 h, based on electrophysiological data (Krause et al., 1994). There is no evidence that transection evokes exocytosis in the giant axon. Rather it has been proposed that accumulated vesicles form a “plug” at the transected end, rather than contributing via exocytosis to reformation of plasma membrane continuity there (Krause et al., 1994).

It is possible that the actin-based cytoskeleton, in addition to the tubulin-based cytoskeleton, participates in resealing. The dense array of filamentous actin typically present in the cell cortex is hypothesized to act as a physical barrier that normally prevents vesicles from reaching the plasma membrane (Muallem et al., 1995). Considerable experimental evidence suggests that Ca^{2+} -activated dissolution of this barrier, mediated by gelsolin or proteins of

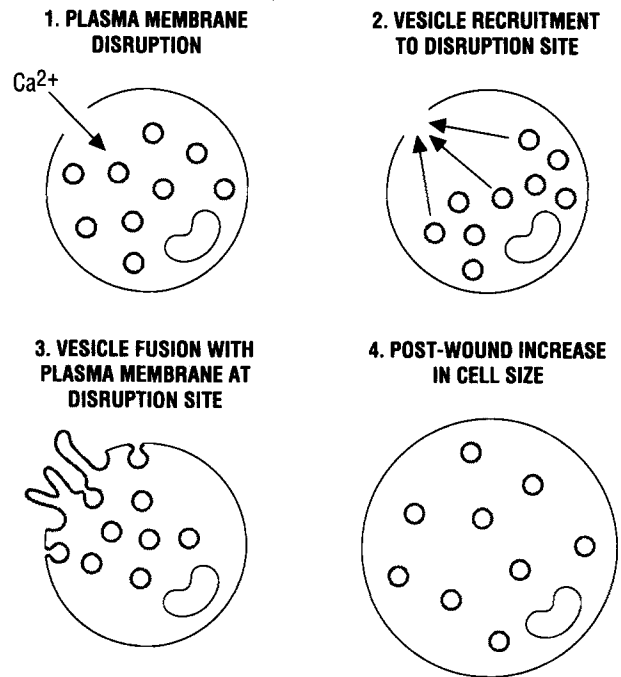


Figure 7. The model for resealing of the disrupted plasma membrane. See text for explanation.

similar function, is a necessary prelude to the completion of exocytosis, which requires that vesicles are positioned in close proximity to the plasma membrane. Therefore, Ca^{2+} influx through the plasma membrane disruption may cause a localized dissolution of cortical actin cytoskeleton, clearing the way for kinesin/tubulin-based vesicle transport there. Additionally, it should be pointed out that Ca^{2+} -mediated solation of the actin-based cytoskeleton is coupled, according to one hypothesis, to its contraction (Taylor and Fehcheimer, 1982). In the giant ameba (Jeon and Jeon, 1975), amphibian eggs (Gingell, 1970), and coenocytic algae (LaClaire, 1992), filamentous arrays of actin appear at sites of plasma membrane disruption, and contraction mediated by such filaments is proposed to aid in closing of the disruption. As mentioned above, transection of the giant axon elicits a contraction at the transection site. Therefore disruption-induced actin contractions, provoked again by Ca^{2+} influx, may be involved in resealing, especially of large disruptions occurring in giant cells.

We can at present only speculate how localized membrane addition to the disrupted plasma membrane facilitates resealing. One obvious possibility is that disruption-induced addition constitutes a kind of “patching” activity. Since membrane lipids are laterally mobile, they would be expected to flow over the disruption site, promoting resealing. However it is possible that this would not occur rapidly enough to insure cell survival. Erythrocytes take minutes to hours to reseal disruptions produced by osmotic lysis (Johnson et al., 1975; Lee et al., 1985), compared to the seconds taken by fibroblasts to reseal mechanically induced disruptions (Steinhardt et al., 1994). In cells more complex than the erythrocyte, leakage of cytoskeletal and other cytoplasmic contents through the disruption (Jeon and Jeon, 1975) might create an obstacle to lipid flow across the disruption site, delaying resealing. Patching therefore

could be a mechanism for accelerating the resealing process beyond the rate dictated by lipid flow alone.

Membrane addition might also conceivably have a post-resealing function. A disruption-induced increase in cell surface area might, for example, increase a crucial membrane-based survival activity, such as the pumping out of the cytosol of Ca^{2+} , and/or other ions, that entered through the disruption. In any case, the exocytosis induced by plasma membrane disruption may be an evolutionarily primitive form of this important cell activity. Resealing may have preceded communication or chemical modification of the external environment as the original function of exocytosis.

The frequent and widespread occurrence in vivo of plasma membrane disruption, or cell wounding, raises a host of biologically interesting questions in addition to those addressed in the present study. Disruptions, for example, provide a route for molecular traffic into and out of the cell that is independent of the classical routes, endocytosis and exocytosis. Growth factors that lack a signal sequence, such as basic and acidic fibroblast growth factor, are exported by this route, explaining how localized growth responses and consequent tissue adaptation are initiated by the imposition of increased mechanical load (McNeil, 1993; Clarke et al., 1995). Unexplored however is the nature of other forms of molecular traffic proceeding through plasma membrane disruptions, as well as the role of this molecular traffic in the biology of cells residing in mechanically challenging environments. We have moreover only begun to examine the molecular biological consequences of cell wounding. Unknown for example are its effects on gene expression and on consequent cell and tissue adaptation to mechanical load, a frequently changing variable in many tissues, such as skeletal muscle, cardiac muscle, skin and bone.

We wish to thank Mr. R. Khakee for providing technical assistance, Drs. Susumu Ito, Mark Terasaki, and Edward Basgall for advice and encouragement, and Dr. Richard Steinhardt for access to his unpublished results.

This work was supported by grants from the National Institutes of Health (GM 48091 to P. L. McNeil) and an American Heart Established Investigator Award (P. L. McNeil).

Received for publication 20 July 1995 and in revised form 16 October 1995.

References

- Betz, W. J., F. Mao, and G. S. Bewick. 1992. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J. Neurosci.* 12:362–375.
- Bi, G.-Q., J. M. Alderton, and R. A. Steinhardt. 1995. Calcium-regulated exocytosis is required for cell membrane resealing. *J. Cell Biol.* 131:1747–1758.
- Celis, J. E. 1984. Microinjection and organelle transplantation techniques. Academic Press, San Diego.
- Clarke, M. S. F., and P. L. McNeil. 1992. Syringe loading introduces macromolecules into living mammalian cell cytosol. *J. Cell Sci.* 102:535–541.
- Clarke, M. S. F., and P. L. McNeil. 1994. Syringe loading: a method for inserting macromolecules into cells in suspension. In *Cell Biology: A Laboratory Handbook*. Academic Press, San Diego, CA. 30–36.
- Clarke, M. F. C., R. N. Khakee, and P. L. McNeil. 1993. Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of

- normal and dystrophic muscle. *J. Cell Sci.* 106:121–133.
- Clarke, M. S. F., R. W. Caldwell, K. Miyake, and P. L. McNeil. 1995. Contraction-induced cell wounding and release of fibroblast growth factor in heart. *Circ. Res.* 76:927–934.
- Eddy, E. M., and B. M. Shapiro. 1978. Changes in the topography of the sea urchin egg after fertilization. *J. Cell Biol.* 71:35–48.
- Fishman, H. M., K. P. Tewari, and P. G. Stein. 1990. Injury-induced vesiculation and membrane redistribution in squid giant axon. *Biochim. Biophys. Acta.* 1023:421–435.
- Gallant, P. E. 1988. Effects of the external ions and metabolic poisoning on the constriction of the squid giant axon after axotomy. *J. Neurosci.* 8:1479–1484.
- Gingell, D. 1970. Contractile responses at the surface of an amphibian egg. *J. Embryol. Exp. Morph.* 23:583–609.
- Hollenbeck, P. J., and J. A. Swanson. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature (Lond.)* 346:864–866.
- Jeon, K. W., and M. S. Jeon. 1975. Cytoplasmic filaments and cellular wound healing in *Amoeba proteus*. *J. Cell Biol.* 67:243–249.
- Johnson, R. M. 1975. The kinetics of resealing of washed erythrocyte ghosts. *J. Membr. Biol.* 22:231–253.
- Knight, D. E., and M. C. Scruton. 1986. Gaining access to the cytosol: The technique and some applications of electroporation. *Biochem. J.* 234:497–506.
- Krause, T. L., H. M. Fishman, and G. D. Bitner. 1994. Axolemmal and septal conduction in the impedance of the earthworm medial giant nerve fiber. *Biophys. J.* 67:692–695.
- LaClaire, J. W. I. 1992. Contractile movements in the algae: the siphonocladales as model systems. In *The Cytoskeleton of Algae*. D. Menzel, editor. CRC Press, Boca Raton, FL. 239–251.
- Lawson, E., C. Fawcett, and M. Raff. 1978. Localized mast cell degranulation induced by concanavalin A-sepharose beads: implications for the Ca^{2+} hypothesis of stimulus-secretion coupling. *J. Cell Biol.* 79:394–400.
- Lee, B., K. McKenna, and J. Bramhall. 1985. Kinetic studies of human erythrocyte membrane resealing. *Biochim. Biophys. Acta.* 815:128–134.
- Lemasters, J. L., J. DiGiuseppi, A.-L. Nieminen, and B. Herman. 1987. Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. *Nature (Lond.)* 325:78–81.
- Mazia, D. G., G. Schatten, and R. Steinhardt. 1975. Turning on of activities in unfertilized sea urchin eggs: correlation with changes of the surface. *Proc. Natl. Acad. Sci. USA.* 72:4469–4473.
- McNeil, P. L. 1993. Cellular and molecular adaptations to injurious mechanical force. *Trends Cell Biol.* 3:302–307.
- McNeil, P. L., and R. Khakee. 1992. Disruptions of muscle fiber plasma membranes: role in exercise-induced damage. *Am. J. Path.* 140:1097–1109.
- McNeil, P. L., and D. L. Taylor. 1985. Aequorin entrapment in mammalian cells. *Cell Calcium.* 6:83–93.
- Meffert, M. F., B. A. Premack, and H. Schulman. 1994. Nitric oxide stimulates Ca^{2+} -independent synaptic vesicle release. *Neuron.* 12:1235–1244.
- Mia, A. J., L. X. Oakford, and T. Yorio. 1994. Surface membrane remodeling following removal of vasopressin in toad urinary bladder. *Tiss. Cell.* 26:189–201.
- Miller, T. M., and J. E. Heuser. 1984. Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. *J. Cell Biol.* 98:685–698.
- Robinson, P. J., J.-P. Liu, K. A. Powell, E. M. Fykse, and T. C. Sudhof. 1994. Phosphorylation of dynamin I and synaptic-vesicle recycling. *Trends Neurosci.* 17:348–353.
- Muallem, S., X. Xu, and H. L. Yin. 1995. Actin filament assembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J. Cell Biol.* 128:589–598.
- Shapiro, H. M. 1988. *Practical Flow Cytometry*. New York, Alan R. Liss, Inc.
- Sollner, T., and J. E. Rothman. 1994. Neurotransmission: harnessing fusion machinery at the synapse. *Trends Neurosci.* 17:344–348.
- Steinhardt, R. A., G. Bi, and J. M. Alderton. 1994. Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. *Science (Wash. DC)* 263:390–393.
- Swanson, J. A., and P. L. McNeil. 1987. Nuclear reassembly excludes large macromolecules. *Science (Wash. DC)* 238:548–550.
- Taylor, D. L., and M. Fechheimer. 1982. Cytoplasmic structure and contractility: the solution—contraction coupling hypothesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:185–197.
- Terasaki, M. 1995. Visualization of exocytosis during sea urchin egg fertilization using confocal microscopy. *J. Cell Sci.* 108:2293–2300.
- Watts, C., and M. Marsh. 1992. Endocytosis: what goes in and how? *J. Cell Sci.* 103:1–8.
- Yawo, H., and M. Kuno. 1985. Calcium dependence of membrane sealing at the cut end of the cockroach giant axon. *J. Neurosci.* 5:1626–1632.