The mechanism of facilitated cell membrane resealing

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SUMMARY

Disruption of the plasma membrane evokes an exocytotic response that is required for rapid membrane resealing. We show here in Swiss 3T3 fibroblasts that a second disruption at the same site reseals more rapidly than the initial wound. This facilitated response of resealing was inhibited by both low external Ca\textsuperscript{2+} concentration and specific protein kinase C (PKC) inhibitors, bisindolylmaleimide I (BIS) and Gö-6976. In addition, activation of PKC by phorbol ester facilitated the resealing of a first wound. BIS and Gö-6976 suppressed the effect of phorbol ester on resealing rate. Fluorescent dye loss from a FM1-43 pre-labeled endocytotic compartment was used to investigate the relationship between exocytosis, resealing and the facilitation of resealing. Exocytosis of endocytotic compartments near the wounding site was correlated with successful resealing. The destaining did not occur when exocytosis and resealing were inhibited by low external Ca\textsuperscript{2+} concentration or by injected tetanus toxin. When the dye loaded cells were wounded twice, FM1-43 destaining at the second wound was less than at the first wound. Less destaining was also observed in cells pre-treated with phorbol ester, suggesting newly formed vesicles, which were FM1-43 unlabeled, were exocytosed in the resealing at repeated woundings. Facilitation was also blocked by brefeldin A (BFA), a fungal metabolite that inhibits vesicle formation at the Golgi apparatus. Lowering the temperature below 20°C also blocked facilitation as expected from a block of Golgi function. BFA had no effect on the resealing rate of an initial wound. The facilitation of the resealing by phorbol ester was blocked by pretreatment with BFA. These results suggest that at first wounding the cell used the endocytotic compartment to add membrane necessary for resealing. At a second wounding, PKC, activated by Ca\textsuperscript{2+} entry at the first wound, stimulated vesicle formation from the Golgi apparatus, resulting in more rapid resealing of the second membrane disruption. Since vesicle pools were implicated in both membrane resealing and facilitation of membrane resealing, we reasoned that artificial decreases in membrane surface tension would have the same result. Decreases in surface tension induced by the addition of a surfactant (Pluronic F68 NF) or cytochalasin D facilitated resealing at first wounding. Furthermore, Pluronic F68 NF restored resealing when exocytosis was blocked by tetanus toxin. These results suggest that membrane resealing requires a decrease in surface tension and under natural conditions this is provided by Ca\textsuperscript{2+}-dependent exocytosis of new membrane near the site of disruption.

Key words: Membrane resealing, Exocytosis, Protein kinase C, Golgi apparatus, Surface tension

INTRODUCTION

Disruptions of plasma membranes are usually resealed rapidly. The mechanism for membrane resealing is hypothesized to be dependent on an exocytotic reaction (Steinhardt et al., 1994; Miyake and McNeil, 1995; Bi et al., 1995, 1997; Terasaki et al., 1997). It is widely accepted that membrane traffic and exocytotic events are largely mediated by a number of proteins (Donaldson and Klausner, 1994; Südhof, 1995; Bennett, 1997; Goda, 1997; Goodson et al., 1997; Hanson et al., 1997). In fact, in sea urchin eggs and embryos, botulinum neurotoxins A, B, and C1, and tetanus toxin, which cleave the SNARE synaptic vesicle docking/fusion proteins (Schiavo et al., 1992, 1993; Blasi et al., 1993; Binz et al., 1994), inhibit the membrane resealing and block exocytosis at sites of membrane disruption (Steinhardt et al., 1994; Bi et al., 1995). Inhibition of kinesin or myosin, which are believed to be required for vesicle transport (Goodson et al., 1997), also inhibits membrane resealing and delivery of vesicles to sites of membrane disruption (Steinhardt et al., 1994; Bi et al., 1997). Direct confocal observations of exocytotic events in sea urchin eggs and embryos during membrane resealing confirmed that exocytotic vesicles were recruited by kinesin and myosin motors in a two-step process (Bi et al., 1995, 1997). In Swiss 3T3 fibroblasts, botulinum neurotoxins A and B inhibited membrane resealing (Steinhardt et al., 1994). The membrane resealing was also inhibited by blocking the function of kinesin (Steinhardt et al., 1994). However, the exocytotic processes during membrane resealing had not been observed in 3T3 cells and the inhibition of resealing in 3T3 cells had not yet been related to inhibition of exocytosis by direct observation. Miyake and McNeil (1995) have observed regional loss of
labeled endosomes after syringing fibroblasts, but their method could not correlate destaining directly with wounding sites, since the sites of wounding could only be estimated and there was evidence of massive endosomal fusion following these bigger ruptures.

Protein phosphorylation has been shown to regulate exocytotic pathways. Pools of available vesicles are regulated by calcium/calmodulin kinase II phosphorylation of synapsin I, which releases vesicles from actin-binding sites (Llinás et al., 1991; Benfenati et al., 1992; Ceccaldi et al., 1995). Membrane resealing of the fertilized sea urchin eggs and 3T3 cells was inhibited by suppressing the activity of calcium/calmodulin kinase II (Steinhardt et al., 1994; Bi et al., 1997). In addition to calcium/calmodulin kinase II, the role of protein kinase C (PKC) in modulating membrane traffic and exocytosis is well established (Vitale et al., 1995; Deeney et al., 1996; Gillis et al., 1996; Billiard et al., 1997; Haruta et al., 1997; Liu, 1997). However, its precise role in membrane resealing had not been investigated.

In the present study, we found that the resealing after repeated wounding was faster than that after the first wounding in 3T3 cells. This facilitated response of the membrane resealing was dependent upon both Ca2+ and PKC. We applied a fluorescent dye method (Angleson and Betz, 1997) to observe exocytotic events during this process, and propose that endocytic compartments are mainly used at first wounding, and that new vesicles derived from the Golgi apparatus are used at sites of repeated wounding. The need for new membrane area supplied by exocytosis appears to be to lower surface tension, since lowering surface tension by treatment with surfactant or cytochalasin D can facilitate membrane resealing to the same extent even when exocytosis is inhibited.

MATERIALS AND METHODS

Reagents

The fluorescent probes, Fura-2 pentapotassium salt (Fura-2 salt), Fura-2 AM, FM 1-43, and BODIPY FL C5-ceramide were purchased from Molecular Probes (Eugene, OR). Fura-2 salt was dissolved in aspartate buffer that consisted of 100 mM potassium aspartate and 20 mM Hepes (pH 7.2) to make a 27 mM stock solution. Fura-2 AM, FM1-43, and BODIPY FL C5-ceramide were dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co, St Louis, MO) at 2 mM, 4 mM, and 1 mM, respectively. Tetanus toxin was from LC Laboratories (Woburn, MA), and dissolved in DMSO to make a 138 mM stock solution containing 30 to 60 nM for injections of 5 to 10% of cell volume. The synthetic substrate to toxin ratio was about 13300:1.

Injection solutions were back-filled into borosilicate glass micropipettes made from 1-mm diameter tubing with filament (World Precision Instruments, Sarasota, FL). A PE-2 vertical pipette puller (Narishige Scientific Instrument Lab, Tokyo) was used with a setting of zero magnet and 12.75 μm. The pipette tip was broken to about 0.5 μm diameter on the coverslip bottom of the Petri dish containing the cells to be injected. Microinjection was performed using an Eppendorf 5242 microinjector and 5170 micromanipulator (Eppendorf, Madison, WI) mounted on a Zeiss IM-35 inverted microscope. Injection time was 0.2 to 0.3 seconds for injection of 5 to 10% of cell volume.

Electrode arrangement for FM1-43 destaining and uptake

To load FM1-43, 3T3 cells were incubated with 3 ml fresh culture medium containing 4 μM FM1-43 for 12-18 hours. Each dish was
washed with Rodent Ringer solution just before the experiment. FM1-43 fluorescence was excited at 490 nm and emission intensity was measured using a 510 nm cutoff filter at 2 seconds per data point either continuously or for about 10 seconds every 5 minutes. When the fluorescence intensity was continuously measured, a slowly declining fluorescence caused by photobleaching was subtracted from raw FM1-43 recordings before plotting the traces (see Fig. 1 for details). When brief measurements were performed at 5 minute intervals, photobleaching was not significant. Complete membrane resealing was confirmed by visual inspection 10-20 minutes after wounding. Cells that had not resealed were easily distinguishable visually at this time.

To investigate endocytic activity, 3T3 cells were incubated for 10 minutes with 4 μM FM1-43 and 100 nM phorbol ester (PMA or 4α-PMA) in 1.8 mM Ca2+ Rodent Ringer solution. Cells were incubated for 10 minutes at room temperature and washed three times with cold (4°C) Rodent Ringer solution. Fluorescence intensity of the cell was measured within 10 minutes after washing.

**FM1-43 imaging**

Image data acquisition was performed using a SIT68 video camera (MTI, Michigan City, IN) linked to a Zeiss IM-35 inverted microscope. Images were digitized by a Digidata 2000 AD board (Axon Instruments, Foster City, CA). FM1-43 fluorescence images were acquired at 4 second intervals by averaging four frames for each image. All processes were controlled by Axon Imaging Workbench 2.1 (Axon Instruments). Cells were wounded just after the 5th image acquisition using the same system as for the microscopy. Image data were edited by GraphicConverter 3.3 (Lemke Software, Germany) and Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA).

**Golgi apparatus staining and confocal microscopy**

To observe the Golgi apparatus, cells were incubated with or without 50 μM BFA at room temperature for 10 minutes before adding BODIPY FL C5-ceramide (final 1 μM). Images were taken within 1-2 hours after the dye addition. Confocal images were made with a MRC 600 (Bio-Rad Laboratories, Hercules, CA) equipped with an upright fluorescence microscope (Axioplan; Carl Zeiss) and an air-cooled Argon laser (5400; Ion Laser Technology, Inc., Fort Collins, CO). Images were acquired by averaging 16 to 24 frames, and edited by Adobe Photoshop.

**Laser optical trap estimates of relative surface tension**

To prepare IgG-coated beads, beads suspension (50 μl) was mixed with the same volume of PBS containing 1.0 mg/ml anti-mouse IgG (whole molecule), and incubated overnight at 4°C. The beads were pelleted by centrifugation. Then the beads were resuspended in PBS containing 1.0 mg/ml bovine serum albumin, rinsed by pelleting and resuspending with PBS four times, and kept in 200 μl PBS at 4°C.

For the experiments, the bead suspension was diluted 1:1000 in 1.8 mM Ca2+ Rodent Ringer solution. The trapping beam from an 899 Ring CW Titanium:Sapphire laser (Coherent Inc., Santa Clara, CA) at 820 nm was brought into a Zeiss Axiovert 135 M inverted microscope. To form a tether from the 3T3 cell, an IgG-coated bead was trapped with 60 mW of laser power, measured at the objective, held on the cell surface for 2 seconds, and pulled away from the cell surface by moving the cell 5-10 μm to one side forming a membrane tether. The cells and beads were imaged by a CCD camera (ZVS-47E; Optronics, Inc., Goleta, CA) equipped with the microscope. Images of bead before and after tether formation were acquired using the software of a Zeiss LSM 410 laser scanning microscope system. The position of the bead in the laser trap was compared between these images, and the distance of displacement of the bead in the trap was estimated using NIH-Image 1.61 (developed at the U.S. National Institutes of Health).

**Data analysis**

All data were indicated as mean ± s.e.m. Statistics were calculated by Welch or Student t-test using InStat 2.00 (GraphPad Software, San Diego, CA).

**RESULTS**

**Ca2+-dependent facilitation of membrane resealing**

Under favorable conditions, 3T3 cells can survive several wounding. To analyze membrane resealing during repeated wounding, Fura-2 loaded cells were wounded twice at two different external Ca2+ concentrations. As described previously (Steinhardt et al., 1994), woundings (arrows in Fig. 1A) are indicated by a sharp rise in the calcium-sensitive Fura-2 ratio, and the loss of dye results in a decrease in the fluorescence intensity of Fura-2 excited at a calcium-insensitive wavelength. When the cell resealed, the decrease in fluorescence intensity stopped (bars in Fig. 1A). We chose the first point when the signal reached a constant value (stopped declining), and defined that as the resealing time. We found that resealing after a second wounding was usually faster than that after the first wounding (Fig. 1A). Fig. 1B summarizes the results of the ‘double-wounding’ experiments. The resealing rate was defined as the inverse of the resealing time in seconds. For cells that failed to reseal, the rate was defined as zero. In the majority of cases of double wounding the second resealing rate was significantly faster than the first rate (paired nonparametric tests P=0.002 for external Ca2+ = 0.8 mM, and P=0.0146 for external Ca2+ = 0.4 mM). When plotted as second rate/first rate most of the data therefore fall well above the diagonal (Fig. 1B). When graphed in double-log scale, each data set can be fitted with a straight line with slope near 1 (Fig. 1C). Therefore, the two rates may satisfy a simple relationship:

\[ R_2=k R_1 \]

where k is a constant for a given condition \( k_{(Ca=0.8)=2.51±0.31, k_{(Ca=0.4)=1.49±0.16}. \) It should also be noticed that \( k_{(Ca=0.8)} \) was significantly larger than \( k_{(Ca=0.4)} \) (Welch t-test, \( P=0.0067). \) These results suggest that not only membrane resealing itself is Ca2+-dependent (Steinhardt et al., 1994), this facilitated response is also Ca2+-dependent.

**PKC inhibitors inhibit facilitation**

To test if PKC activity is involved in the facilitation of membrane resealing, double-wounding experiments were carried out in the absence or presence of specific PKC inhibitors BIS (Toullec et al., 1991) and Gö-6976 (Martiny-Baron et al., 1993). Fig. 2 summarizes the results of the double-wounding experiments. The majority of data points for BIS (500 nM) and Gö-6976 (1 μM) treated cells fell below the diagonal, indicating that the second resealing rate was lower than the first rate (Fig. 2). Six out of 30 BIS treated cells and 7 out of 20 Gö-6976 treated cells did not survive a second wound, but only 3 of 43 cells did not survive in control experiments (Fig. 2). The average ratio of first and second resealing rates (2nd/1st) of control cells was 1.87±0.22 (n=44). However, the ratio of BIS and Gö-6976 treated cells was 0.91±0.18 (n=30) and 0.56±0.12 (n=20), respectively. These results suggest that PKC activation after a first wounding is
required for subsequent facilitation of membrane resealing at a second wound.

PKC activation by phorbol ester facilitates membrane resealing at first wounding

We next tested the effect of PKC activation by phorbol ester on the membrane resealing at first wounding. Membrane resealing was facilitated by PMA treatment in a majority of the cells (Fig. 3A). Fig. 3B shows average resealing rate under various conditions. A 10 minute pre-treatment with 100 nM PMA facilitated membrane resealing to subsequent wounds in the hour following washout of PMA (resealing rate = 0.110±0.017, n=45), whereas an inactive analog 4α-PMA did not affect membrane resealing (resealing rate = 0.051±0.011, n=22). Furthermore, the specific PKC inhibitors BIS (500 nM) and Gö-6976 (1 μM) suppressed the effect of PMA on resealing rate. Resealing rates of BIS and Gö-6976 treated cells were 0.047±0.015 (n=16) and 0.048±0.01 (n=22), respectively. No changes of the ratio value of Fura-2 fluorescence were observed after the PMA application (n=4, data not shown), showing that intracellular free Ca²⁺ levels were unchanged by the addition of phorbol ester. There was also no significant change in calcium peak values after wounding. Peak values of Fura-2 ratios after wounding were 1.12±0.036 (DMSO, n=55), 1.15±0.053 (PMA, n=45), and 1.18±0.053 (4α-PMA, n=22). The peak values of PMA and 4α-PMA treated cells were not significantly different (Welch t-test, P=0.6368 for PMA, P=0.3457 for 4α-PMA) from controls. These results imply that PMA activated PKC, which then facilitated membrane resealing.

Wounding-induced decreases in FM1-43 fluorescence

A fluorescent dye method (Angleson and Betz, 1997) was applied to measure exocytosis during membrane resealing in 3T3 cells. The styryl dye FM1-43 intercalates into the outer leaflet of lipid bilayers but cannot cross the bilayer, and FM1-43 is much more fluorescent in hydrophobic than in hydrophilic environments. When cells are incubated with the dye and washed, dye remaining in the plasma membrane rapidly diffuses away, leaving only dye that is trapped in the luminal leaflet of endocytosed vesicle membranes. Subsequent delivery of the labeled endosomes into the plasma membrane by exocytosis, and diffusion of FM1-43 from the outer leaflet into the aqueous solution, results in a loss of cellular fluorescence.

The FM1-43 fluorescence intensity of single 3T3 cells was recorded at 2 second intervals during wounding in 1.8 mM Ca²⁺ Rodent Ringer solution. Before wounding, the slowly declining fluorescence of FM1-43 due to photobleaching was estimated by blocking the light from the excitation lamp for an interval leaving the rate of constitutive exocytosis in the absence of photobleaching (Fig. 4, points b-c). Fluorescence decreases by photobleaching estimated in this manner were subtracted in all subsequent figures. The resulting record is shown in Fig. 4A, lower trace. When cells were wounded, rapid
Facilitation of membrane resealing and protein kinase C

Facturation of membrane resealing and protein kinase C was observed in 73.1% of resealed cells (n=26) as shown in the example in Fig. 4A, and average fluorescence change was -2.00±0.38% (n=26) (Table 1). However, when cells failed to reseal, destaining was observed in only 25% of cells (n=12), and the average fluorescence change was only -0.3±0.19% (n=12) (Fig. 4B, Table 1).

Tetanus toxin and botulinum neurotoxin B cleave synaptobrevin at a specific single site and inhibit exocytosis at neurotransmitter release (Schiavo et al., 1992). Previous studies showed that injection of botulinum neurotoxins A and B inhibited the membrane resealing in 3T3 cells and in activated sea urchin eggs and embryos (Steinhardt et al., 1994; Bi et al., 1995). To investigate whether FM1-43 destaining is correlated with exocytosis and membrane resealing, tetanus toxin was injected into 3T3 cells, which were subsequently wounded. The percentage of membrane resealing in toxin injected cells was reduced to 17% (Fig. 5A). The onset of this block in membrane resealing was delayed about an hour after the injection, consistent with the time required for the

activation of the toxin protease. Co-injection of a 13,000-fold excess of a substrate peptide (ASQFETS) delayed the inhibitory effect of the toxin protease (Fig. 5A). Typical FM1-43 destainings of toxin injected cells are shown in Fig. 5B. When the toxin injected cells were wounded 91-120 minutes after the injection, one cell out of six resealed and rapidly

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**Fig. 2.** Facilitation of membrane resealing is inhibited by PKC inhibitors. (A) Fura-2 loaded cells were wounded twice in the presence or absence of BIS (500 nM) or Gö-6976 (1 μM) in normal 1.8 mM Ca2+ Ringer. In this and all subsequent figures, Ringer calcium concentrations were at 1.8 mM. Each point represents one experiment. Data were omitted when cells failed to reseal at first wounding. (B) Average ratios of first and second resealing rates (2nd/1st). Values are mean ± s.e.m. P=0.0013 (*) and P<0.0001 (**) using Welch t-test.

**Fig. 3.** PMA facilitates membrane resealing through activation of PKC. (A) Typical Fura-2 recordings of single-wounding in the presence of DMSO or 100 nM PMA. Cells were wounded at the arrows. Bars indicate the completion time of resealing. (B) Resealing rate was defined as the inverse of resealing time. For cells that failed to reseal, the rate was defined as zero. BIS and Gö-6976, selective inhibitors for PKC, blocked the facilitation of membrane resealing by PMA. Values are mean ± s.e.m. *P=0.0378 using Welch t-test (compared with DMSO).
When the substrate peptide was co-injected with the toxin and the cells were wounded 91-120 minutes after the injection, both resealing and rapid FM1-43 destaining were observed in seven of nine cells (Table 2). These results with FM1-43 destaining after wounding indicated that resealing was correlated with exocytosis.

**FM1-43 destaining is localized to the area nearest the wounding site**

We imaged FM1-43 destaining during membrane resealing to observe whether the exocytosis occurred locally at the wounding site in 3T3 cells. Typical imaging examples are shown in Fig. 6. Fluorescent images were acquired at 4 second intervals. Cells were wounded in an area indicated by arrows just after the 5th image was acquired. Localized destaining near the wound site was observed in the presence of normal extracellular Ca$^{2+}$ (compare Fig. 6A and B). FM1-43 destaining was localized only around the wounding site (Fig. 6C). On the other hand, the cells in Ca$^{2+}$-free, 2 mM Mg$^{2+}$ Rodent Ringer solution (Fig. 6D-F) and tetanus toxin injected destained (Table 2). When the substrate peptide was co-injected with the toxin and the cells were wounded 91-120 minutes after the injection, both resealing and rapid FM1-43 destaining were observed in seven of nine cells (Table 2). These results with FM1-43 destaining after wounding indicated that resealing was correlated with exocytosis.

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**Table 1. Wounding-induced FM1-43 destaining in 3T3 cells**

<table>
<thead>
<tr>
<th>Reseal</th>
<th>Destaining</th>
<th>Fluorescence change (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>19/26</td>
<td>-2.00±0.38</td>
<td></td>
</tr>
<tr>
<td>3/12</td>
<td>-0.3±0.19</td>
<td></td>
</tr>
</tbody>
</table>

FM1-43 loaded cells were wounded in 1.8 mM Ca$^{2+}$ Rodent Ringer solution. Membrane resealing was monitored by visual inspection.

\*Values are mean ± s.e.m.

\(p=0.0004\), Welch t-test.

**Table 2. Effect of tetanus toxin on cell membrane resealing and FM1-43 destaining in 3T3 cells**

<table>
<thead>
<tr>
<th>Reagents injected</th>
<th>Resealing</th>
<th>Destaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin</td>
<td>1/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Toxin + substrate</td>
<td>7/9</td>
<td>7/9</td>
</tr>
</tbody>
</table>

Tetanus toxin was injected with or without substrate peptide. Cells were wounded 91-120 minutes after toxin injection. Resealing was monitored by visual inspection.
Facilitation of membrane resealing and protein kinase C
cells (Fig. 6G-I) did not show localized destaining after
wounding. In this series of experiments, the percentages of
resealed cells were 78.4% \( (n=37) \) in 1.8 mM Ca\(^{2+}\) Rodent
Ringer solution, 0% \( (n=6) \) in Ca\(^{2+}\)-free Rodent Ringer solution,
and 15.4% \( (n=13) \) in toxin injected cells. When cells were
resealed in 1.8 mM Ca\(^{2+}\) Ringer solution, localized FM1-43
destaining was observed in 83% cells \( (n=29) \), and the average
fluorescence change of rapid destaining in the localized region
was \(-6.19 \pm 0.99\%\) (Table 3). However, no localized destaining
was observed when cells failed to reseal due to low external
Ca\(^{2+}\) concentration \( (n=6) \) or due to tetanus toxin injection
\( (n=11) \) (Table 3). These results indicate that the FM1-43
fluorescence changes that we observed were not due to shape
changes of the cell and/or loss of cytoplasm by cell disruption,
but due to localized exocytosis near the wounding site. We did
not observe recruitment of stained vesicles from other regions
of the cell, most probably because of the usual orientation of
the microtubule array outward from nucleus to periphery.

**FM1-43 destaining in double-wounded cells**
To establish the relationship between exocytosis and
facilitation of membrane resealing, FM1-43 destaining during
double-wounding was investigated. The amount of destaining
at the second wound was usually less than that at first wound.
Fig. 7B shows that over 70% of the cells had less destaining at
the second wound. These results suggested that the exocytosis
was reduced even though resealing was facilitated, or
alternately as we conclude below, that a FM1-43 unlabeled
vesicle pool was recruited for the second resealing.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Local destaining</th>
<th>Local fluorescence change (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8 mM Ca(^{2+}), reseal</td>
<td>24/29</td>
<td>(-6.19 \pm 0.99)</td>
</tr>
<tr>
<td>Ca(^{2+})-free, not reseal</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>Tetanus toxin, not reseal</td>
<td>0/11</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Localized FM1-43 destaining at wounding

The size of area for measurement is the same as shown in Fig. 3. Toxin injected cells were wounded 121-180 minutes after the injection. Membrane resealing was monitored by visual inspection 10-20 minutes after wounding.

*Fluorescent intensities were compared between the 5th and 6th images. Values are mean \( \pm \) s.e.m.

Fig. 6. Localized FM1-43 destaining is observed in a resealed cell. Pseudocolor images were acquired every 4-seconds. Cells were wounded in an area indicated by arrows between 5th (A,D,G) and 6th (B,E,H) image acquisition. (A,B) FM1-43 loaded cell was wounded in 1.8 mM Ca\(^{2+}\) Rodent Ringer solution. (C) Local fluorescence changes during wounding in 1.8 mM Ca\(^{2+}\) Rodent Ringer solution. FM1-43 destaining was observed only around the wounding site. Circle (1) indicates area monitored at wound site. Circle (2) indicates area monitored remote from wound site. (D,E) Cell was wounded in Ca\(^{2+}\)-free, 2 mM Mg\(^{2+}\) Rodent Ringer solution. (G,H) Tetanus toxin injected cell was wounded at 120 minutes after the injection. (F,I) Local fluorescence destaining did not occur under conditions expected to inhibit exocytosis. N, nucleus. Bar, 5 \( \mu \)m.
We further investigated FM1-43 destaining in PKC activated cells treated with phorbol ester. FM1-43 loaded cells were incubated for 10 minutes with either 100 nM PMA or 4α-PMA, and wounded after the drug was washed out. Typical recording examples are shown in Fig. 8A. When PMA treated cells were wounded, fluorescence change after wounding was $0.93±0.23\%$ ($n=27$) (Fig. 8B). In 4α-PMA treated cells (Fig. 8B), on the other hand, the fluorescence change was $1.94±0.44\%$ ($n=15$), a value almost identical to control cells (Table 1). These results also suggest that either PKC activated cells can reseal with less exocytosis, or, most probably, that they reseal by exocytosing a FM1-43 unlabeled compartment.

Treatment with BFA inhibits facilitation of membrane resealing

Treatment with BFA results in release of Golgi-associated coat proteins, redistribution of Golgi membrane into the endoplasmic reticulum and a block in secretion from Golgi apparatus (Klausner et al., 1992). In fact, the Golgi apparatus of 3T3 cells was completely disassembled within 1 hour after addition of BFA (data not shown). This allowed us to test whether newly Golgi derived vesicles are necessary for membrane resealing and the facilitation of resealing.

We first tested the percentage of 3T3 cells that successfully resealed after treatment with BFA. No effect was detected within the experimental time span of 4.5 hours (Fig. 9A). Resealing rates also did not change significantly (see Fig. 9D), indicating that the cells retained their resealing ability at least 3.5 hours after the disassembly of their Golgi apparatus. BFA apparently was not disrupting the resealing of a first wound which was dependent on exocytosis of the endocytotic compartment.

To determine whether BFA affects the facilitation of resealing to a second wounding, BFA treated and untreated cells were double-wounded (Fig. 9B). The majority of data points of BFA treated cells fall under the diagonal, indicating that the second resealing rate is significantly lower than the first rate. Twelve out of 43 BFA treated cells could not survive after the second wounding, however only one of 25 cells failed to reseal after the second wounding in control experiments (Fig. 9B). These results suggested that Golgi-derived vesicles are required for the facilitation of resealing of a second wound in the same region. In another test of Golgi function we dropped the temperature from 23°C down to 18°C. Lowering temperature to just below 20°C has been shown to block secretion of vesicles exiting from the Golgi to the plasma membrane, while near normal function is present at 23°C (Matlin and Simons, 1983; Saraste and Kuismanen, 1984). Facilitation was completely blocked at 18°C (Fig. 9C). In contrast, the resealing rate to the first wound at 18°C
Facilitation of membrane resealing and protein kinase C

(0.089±0.013, n=14) was not significantly different from the rate of resealing of the first wound at 23°C (0.058±0.014, n=9) (P=0.1214 using Student t-test). We concluded that the site of the BFA block of facilitation was at the Golgi apparatus.

To determine whether the action of PKC is dependent on a functional Golgi apparatus, BFA-treated and -untreated cells were wounded after 10 minutes exposure to PMA or DMSO (vehicle only), and resealing rates were compared (Fig. 9D). The average resealing rate of control (DMSO treated) cells was 0.056±0.014 (n=12). For an initial wound, resealing rate was not affected by BFA treatment, as described above (0.063±0.014, n=24). When cells were treated with PMA alone and wounded, the resealing rate was significantly increased to 0.115±0.022 (n=23) (see also Fig. 3). However, membrane resealing was not facilitated (resealing rate = 0.058±0.014, n=25) when cells were pre-treated with BFA for 1 hour before PKC activation by PMA. These results suggest that facilitation of membrane resealing by PKC activation requires a functional Golgi apparatus, namely, the target of PKC in facilitated resealing is recruitment of vesicles for exocytosis from the Golgi apparatus.

**Activation of PKC stimulates both exocytosis and endocytosis**

To measure the effect of PKC stimulation on exocytosis of FM1-43 labeled vesicles in unwounded cells, FM1-43 loaded cells were exposed to either 100 nM PMA or 4α-PMA while fluorescence intensity was measured at 5 minutes intervals. Fluorescence intensity of PMA treated cells was reduced to 96.6±1.0% (n=12) at 20 minutes, whereas the intensity of 4α-PMA treated cells was reduced to 99.2±0.9% (n=9) at 20 minutes (Fig. 10A) indicating a much slower rate of exocytosis in the control.

To investigate whether endocytosis is also stimulated by PKC activation, cells were co-incubated with phorbol esters and FM1-43. FM1-43 fluorescence was measured after washing the cells three times (Fig. 10B). FM1-43 uptake was accelerated to 140.8±4.2% (n=5) by the treatment with PMA. Treatment with 4α-PMA did not accelerate FM1-43 uptake. BIS inhibited the acceleration of FM1-43 uptake by PMA. These results suggest that endocytosis is also stimulated by PKC activation.

Since both exocytosis and endocytosis are stimulated it seemed unlikely that facilitation was simply persistence of previously exocytosed membrane, especially since, as noted above, the effect of a 10 minutes pretreatment with PMA lasted for the following hour (Fig. 3B).

Surfactant and cytochalasin D can facilitate membrane resealing and can substitute for exocytosis in exocytosis-inhibited cells

To confirm whether the function of exocytosis is to reduce cell surface tension, 3T3 cells were treated with the surfactant Pluronic F68 NF or cytochalasin D in order to decrease surface tension artificially. Cytochalasin is reported to decrease cell surface tension by 50% (Tsai et al., 1994; Hochmuth et al.,...
As shown in Fig. 11A, Pluronic F68 NF facilitated membrane resealing in a concentration-dependent manner. The resealing rates were 0.084±0.012 (*n=33) and 0.104±0.018 (*n=21) when cells were wounded in 0.5 and 1.0 mg/ml Pluronic F68 NF, respectively. Cytochalasin D also facilitated membrane resealing of 3T3 cells, and resealing rates were 0.078±0.018 (*n=14) and 0.113±0.015 (*n=37) when cells were treated with 10 and 20 μM cytochalasin D for 1 hour, respectively (Fig. 11B). Cytochalasin inhibited exocytosis as expected, but this effect was overridden and resealing was facilitated (Fig. 11B and Table 4). Tetanus toxin injected cells, in which exocytosis was inhibited, were wounded in the presence or absence of Pluronic F68 NF. The percentage of resealed cells when the toxin injected cells were wounded was 25.7% (*n=29), whereas the percentage was 72.4% (*n=29) when the toxin injected cells were wounded in the presence of 0.5 mg/ml Pluronic F68 NF (Fig. 12). These results suggest that decreases in cell surface tension by surfactant or cytochalasin D permitted membrane resealing to proceed even in the absence of exocytosis.

To compare cell surface tension under various conditions, membrane tethers (5-10 μm) were formed from 3T3 cells by applying a force with the laser tweezers to an IgG-coated bead attached to the plasma membrane (Fig. 13). Displacement of bead position in the laser trap was calculated by comparing bead positions before and after tether formation at constant laser power. Because distance of bead displacement is dependent on tether force it allows a relative measure of apparent cell surface tension (Dai and Sheez, 1998). When a tether was held for 20-30 seconds, bead displacement reached

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**Table 4. Effect of cytochalasin D on wounding induced FM1-43 destaining**

<table>
<thead>
<tr>
<th></th>
<th>No. of resealed cells</th>
<th>Destaining</th>
<th>Fluorescence change (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO)</td>
<td>8/11</td>
<td>8/8</td>
<td>-1.69±0.21</td>
</tr>
<tr>
<td>Cytochalasin D (20 μM)</td>
<td>17/18</td>
<td>4/17</td>
<td>-0.65±0.30‡</td>
</tr>
</tbody>
</table>

FM1-43 loaded cells were treated with 20 μM cytochalasin D or DMSO for 1 hour, and wounded. Membrane resealing was monitored by visual inspection.

*Values are mean ± s.e.m.

‡P=0.0093, Welch t-test.

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**Fig. 10.** (A) PKC activation by PMA accelerates FM1-43 destaining. Fluorescence intensity was measured at 5 minutes intervals. The shutter was closed between measurements to avoid photobleaching. The intensity at time zero was measured just before addition of phorbol esters. Bars are s.e.m. (*n=12 for PMA, 9 for 4α-PMA). (B) PMA enhances FM1-43 uptake through activation of PKC. Cells were co-incubated with FM1-43 and phorbol esters for 10 minutes, and the fluorescence intensity of the cell in cold Rodent Ringer solution was measured within 10 minutes after washing. In each experiment 15-20 cells were measured. Values are mean ± s.e.m. of five independent experiments.

**Fig. 11.** Resealing is facilitated by surfactant and cytochalasin. (A) Cells were wounded in 1.8 mM Ca2+ Rodent Ringer solution with or without Pluronic F68 NF. *P=0.0220 using Welch t-test. (B) Cells were treated with cytochalasin D for 1 hour, and wounded in 1.8 mM Ca2+ Rodent Ringer solution. **P=0.0101 using Welch t-test. Membrane resealing was monitored by photometric measurement of Fura-2 fluorescence and visual inspection. Values are mean ± s.e.m.
Facilitation of membrane resealing and protein kinase C

The average bead displacement in microns for untreated control cells was 0.19±0.019 (n=15) after tether formation. When 3T3 cells were treated with 1.0 mg/ml Pluronic F68 NF or 20 µM cytochalasin D, distance of bead displacement was significantly decreased to 0.1±0.03 (n=7) or 0.06±0.03 (n=7), respectively. However, no significant decreases were observed in DMSO and PMA treated cells (compared to untreated cells). The bead displacements in microns were 0.14±0.02 (n=5) or 0.14±0.02 (n=8) when the cells were treated with 0.3% DMSO or 100 nM PMA, respectively. These results suggest that Pluronic F68 NF and cytochalasin D decreased cell surface tension, and thereby permitted and facilitated membrane resealing.

DISCUSSION

Plasma membrane disruption has been previously found to evoke a rapid exocytotic response which is required for membrane resealing in invertebrate embryos (Steinhardt et al., 1994; Bi et al., 1995, 1997; Terasaki et al., 1997). To observe exocytosis during membrane resealing in mammalian cells, we applied the FM1-43 fluorescent dye method (Angleson and Betz, 1997). Our FM1-43 imaging experiments (Fig. 6) showed that rapid localized exocytosis near the wound site is correlated with membrane resealing in 3T3 cells. This observation is consistent with the previous observations of Miyake and McNeil (1995), who observed patches of FM4-64 destaining after mass wounding of fibroblasts and endothelial cells by syringing.

In the present study, we showed that membrane resealing of a second wound was faster than that after the first wound, and that this response of facilitated membrane resealing was dependent upon both Ca²⁺ and PKC in 3T3 cells (Figs 1, 2). These results suggest that exocytosis after repeated woundings is modulated by PKC activity. However, FM1-43 destaining at second wound was less than the first (Fig. 7), while the resealing at second wound was facilitated (Fig. 1). Phorbolester treated cells also showed less destaining and facilitated resealing at the first wounding (Figs 3, 8). The fluorescent method used in this study could only observe exocytosis of previously endocytosed dye and could therefore only monitor the fate of the pre-labeled endocytotic compartments. Therefore, we speculated that vesicles from a different source other than endocytotic compartments are used at repeated woundings for faster resealing, and that this process is stimulated by PKC.

The PKC target seems to be Golgi apparatus, since BFA, a fungal metabolite that inhibits vesicle formation at the Golgi apparatus (Klausner et al., 1992), inhibited the facilitation of membrane resealing (Fig. 9B). In addition, pre-treatment with BFA inhibited the facilitation of membrane resealing to a first wound induced by PMA (Fig. 9D). BFA does not affect membrane resealing to a first wound under control conditions in which cells apparently use just the endocytotic compartment for resealing (Figs 1, 9A,D), even though several studies have shown that BFA also induces membrane tubulation of endosomes and lysosomes (Klausner et al., 1992). Since BFA did not affect resealing rate or exocytosis to an initial wound, which depends on the endocytotic compartment, the effect of BFA on facilitation of membrane resealing seems to be inhibition of vesicle generation from Golgi apparatus. By lowering the temperature of our experiments from 23-25°C to 18°C, we again blocked facilitation as expected if facilitation was dependent on secretion from the Golgi apparatus (Fig. 9C) (Matlin and Simons, 1983; Saraste and Kuismanen, 1984).

It has been reported that PKC activity is implicated in the formation of post-Golgi vesicles. Ro 31-8220, a PKC inhibitor
interacting specifically with the catalytic domain, inhibits vesicle formation at the Golgi apparatus (Buccione et al., 1996; Westermann et al., 1996), suggesting that the phosphorylating activity of PKC is required for vesicle formation at the Golgi. Activation of PKC enhances binding of ADP ribosylation factor (ARF) and β-COP to Golgi membranes (De Matteis et al., 1993), and phospholipase D activity is stimulated by ARF and appears to be involved in vesicle formation at the Golgi (Kristakis et al., 1996). On the other hand, non-catalytic PKC activation of phospholipase D has been proposed to mediate vesicle formation in an in-vitro model system (Simon et al., 1996; Sabatini et al., 1996). Therefore, both the catalytic and non-catalytic PKC activities seem to be able to exert regulatory effects on exocytotic transport from the Golgi apparatus. In the present study, Gô-6976, believed to be a specific inhibitor of the calcium activated PKC isozymes (Martiny-Baron et al., 1993), along with the more general PKC inhibitor BIS (Toullec et al., 1991), inhibited facilitated resealing (Figs 2, 3). Since both inhibitors bind to the catalytic domain of PKC, phosphorylating activity of PKC may be required for the facilitation of membrane resealing in 3T3 cells.

It is known that elevated intracellular Ca2+ triggers exocytosis in various types of cells (Dan and Poo, 1992; Coorsen et al., 1996), and that endosomal compartments such as lysosomes are known to behave as Ca2+-regulated exocytotic vesicles in fibroblasts and epithelial cells (Rodríguez et al., 1997). Since cell membrane disruption is common in vivo (McNeil and Steinhardt, 1997), a ubiquitous Ca2+-regulated exocytosis may be involved in the membrane resealing in a large variety of eucaryotic cells. However, exactly how exocytotic processes facilitate membrane resealing had not previously been addressed experimentally. Resealing rates in liposomes have been correlated with surface tension: the lower the tension, the faster the rate (Zhelev and Needham, 1993). However, in real cell membranes, the existence of a closely linked rigid cytoskeleton greatly increases the surface tension, compared to simple lipid bilayers. This suggests that the function of exocytosis following membrane disruption may be to lower surface tension by the addition of new membrane in order to facilitate membrane repair. If so, we reasoned we should be able to get faster resealing by artificially lowering membrane surface tension and perhaps even get resealing under conditions which blocked exocytosis. We found that, indeed, the addition of the surfactant Pluronic F68 NF or cytochalasin D would facilitate membrane resealing and decrease the cell surface tension as predicted (Figs 11, 14). In addition, we found that adding the surfactant Pluronic F68 NF would restore successful membrane resealing even after it was otherwise blocked by tetanus toxin (Fig. 12). The same was true for cytochalasin D treatment which reduced exocytosis without inhibiting resealing (Fig. 11, Table 4). Indeed, cytochalasin greatly reduced surface tension in cultured cells (Fig. 14; see also Tsai et al., 1994; Hochmuth et al., 1996). In sea urchin embryos, exocytotic vesicles are recruited to the wound site by a two-step transport mechanism mediated by first kinesin and then myosin (Bi et al., 1997). Since kinesin is also implicated in membrane resealing of 3T3 cells (Steinhardt et al., 1994), vesicles may be recruited to the wounding site with a similar sequential use of kinesin and myosin. In this study, however, cytochalasin treatment did not inhibit membrane resealing in 3T3 cells, even though it greatly reduced exocytosis at the wound site. Apparently in these cultured cells the reduction of surface tension by cytochalasin is great enough that it can substitute for the additional membrane normally provided by exocytosis.

In summary, we propose that exocytosis is essential for plasma membrane resealing because it lowers surface tension by the addition of new membrane area. Membrane resealing following an initial wound appears to be dependent on the exocytosis of vesicles from an endocytotic compartment. A second wound within a few minutes reseals faster and appears to be dependent on a vesicle pool newly derived from the Golgi apparatus induced by PKC, which is activated by Ca2+ entry at the previous wound. What remains to be determined is the timing of the delivery of the second Golgi-derived vesicle pool in relation to the second wound. We have made a few preliminary measurements of surface tension after wounding, and these observations suggest that surface tension recovers to control values before the second wound in these experiments and therefore the exocytosis of new vesicles from the Golgi apparatus occurs after the second wound. This will be the focus of further studies.

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