

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^{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^{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 pre-treatment 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^{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^{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 Ca^{2+} and PKC. We applied a fluorescent dye method (Angleton and Betz, 1997) to observe exocytotic events during this process, and propose that endocytotic compartments are mainly used at first wounding, and that new vesicles derived from the Golgi apparatus are used at sites of repeated woundings. 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 List Biological Labs (Campbell, CA). Commercially obtained neurotoxins are highly variable in activity. A 7 amino acid peptide, ASQFETS, which spans the tetanus toxin cleavage site for synaptobrevin, and which serves as control for specificity of action (Schiavo et al., 1992), was synthesized by the Microchemical Facility, Cancer Research Laboratory, UC Berkeley. Phorbol 12-myristate 13-acetate (PMA) and its inactive analog 4- α -phorbol 12-myristate 13-acetate (4 α -PMA) were from RBI (Natick, MA), and dissolved in DMSO at 1 mM as a stock. The specific inhibitors for PKC, bisindolylmaleimide I (BIS) and Gö-6976, were from Calbiochem (San Diego, CA), and dissolved in DMSO at 1 mM and 2 mM, respectively. Brefeldin A (BFA) was from LC Laboratories (Woburn, MA), and dissolved in DMSO to make a 30 mM stock solution. The nonionic surfactant, Pluronic F68 NF (BASF, Mount Olive, NJ), was dissolved in DMSO at 0.33 g/ml. Cytochalasin D was from Calbiochem, and dissolved in DMSO at 10 mM. Anti-mouse IgG (whole molecule) and bovine serum albumin were obtained from Sigma Chemical Co. Polystyrene beads (mean diameter = 1.02 μm) were from Bangs Laboratories Inc. (Fishers, IN).

Cell preparation and Fura-2 loading

3T3 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 8% (v/v) fetal bovine serum (Hyclone, Logan, UT, or Atlanta Biologicals, Norcross, GA) and 50 $\mu\text{g}/\text{ml}$ gentamicin sulfate (Gibco). Cells for experiments and microscopic observation were plated on glass coverslip-inserted plastic dishes (35 \times 10 mm), and were grown for 1 to 3 days before use. During experiments, 3T3 cells were maintained in Puck's saline solution or Rodent Ringer solution with the desired Ca^{2+} and Mg^{2+} concentrations. Ca^{2+} , Mg^{2+} -free Puck's saline solution consisted of 138 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO_3 , and 5.6 mM D-glucose. Ca^{2+} -free Rodent Ringer solution contained 138 mM NaCl, 2.7 mM KCl, 1.06 mM MgCl_2 , 5.6 mM D-glucose, and 12.4 mM Hepes (pH 7.25). Stock solutions of CaCl_2 and MgCl_2 (both 100 mM) were used to make solutions of varying Ca^{2+} and Mg^{2+} concentrations.

The calcium sensitive dye Fura-2 was introduced into the cells by AM-ester loading in most of experiments. When tetanus toxin was injected into 3T3 cells, the cell-impermeant Fura-2 salt was used as described below instead of Fura-2 AM. For Fura-2 AM loading into 3T3 cells, a 2 mM stock solution of Fura-2 AM was mixed with same volume of Pluronic F-127 (25% w/w in DMSO) before dilution to a final loading concentration of 1 μM in 1.8 mM Ca^{2+} Rodent Ringer solution containing 8% (v/v) fetal bovine serum. Fura-2 AM was loaded at room temperature (23–25°C) for 1 hour, and then washed with Puck's or Rodent Ringer solution containing the desired Ca^{2+} and Mg^{2+} concentrations for each experiment.

Microinjection and wounding procedure

25 μg tetanus toxin was mixed with 16 μl toxin injection buffer (100 mM potassium aspartate, 20 mM Hepes, and 5.4 mM Fura-2 salt) just before use at each experiment. For experiments with the 7-amino acid (ASQFETS) peptide substrate of tetanus toxin, 20 mM peptide was included in the toxin injection buffer described above. These solutions were spun through a 3 mm diameter syringe filter (pore size = 0.22 μm ; MSI, Westboro, MA) mounted in a 500 μl epi-tube. Final intracellular concentration of the toxin was about 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 heater. 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.

For tests of membrane resealing, 3T3 cells were wounded with a 1 mm diameter solid glass needle pulled with the Narishige PE-2. Wounding of cells was performed using the same system as for microinjection. The time setting for wounding was 0.3 seconds. Resealing was monitored by photometric measurement of Fura-2 emission fluorescence and subsequently confirmed visually as described previously (Steinhardt et al., 1994). A persistent decrease of 357 nm excited fluorescent intensity (as an indicator of dye loss) together with a persistent increase of the ratio of fluorescent intensity excited by 385/357 nm light (as an indicator of increasing intracellular Ca^{2+} concentration) indicated resealing failure. A transient decrease of 357 nm excited fluorescent intensity indicated successful resealing. All experiments were performed at 23–25°C unless otherwise mentioned.

Measurement of 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 endocytotic activity, 3T3 cells were incubated for 10 minutes with 4 μM FM1-43 and 100 nM phorbol ester (PMA or 4 α -PMA) in 1.8 mM Ca^{2+} 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 microinjection. 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 (Axioptan; 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 Ca^{2+} 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 \pm s.e.m. Statistics were calculated by Welch or Student *t*-test using InStat 2.00 (GraphPad Software, San Diego, CA).

RESULTS

Ca^{2+} -dependent facilitation of membrane resealing

Under favorable conditions, 3T3 cells can survive several woundings. To analyze membrane resealing during repeated woundings, Fura-2 loaded cells were wounded twice at two different external Ca^{2+} 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 reseat, 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 $P=0.0011$ for external $\text{Ca}^{2+} = 0.8$ mM, and $P=0.0146$ for external $\text{Ca}^{2+} = 0.4$ 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 ($k_{(\text{Ca}=0.8)} = 2.51 \pm 0.31$, $k_{(\text{Ca}=0.4)} = 1.49 \pm 0.16$). It should also be noticed that $k_{(\text{Ca}=0.8)}$ was significantly larger than $k_{(\text{Ca}=0.4)}$ (Welch *t*-test, $P=0.0067$). These results suggest that not only membrane resealing itself is Ca^{2+} -dependent (Steinhardt et al., 1994), this facilitated response is also Ca^{2+} -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

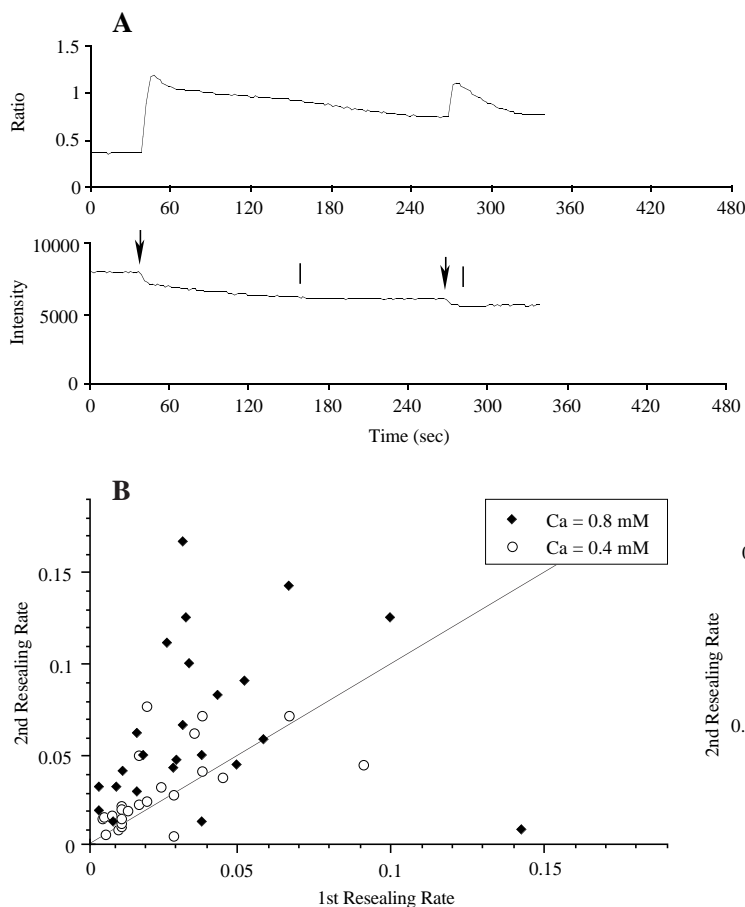


Fig. 1. Facilitation of membrane resealing. (A) A typical Fura-2 recording of double-wounding. Excitation wavelength 357 and 385 nm; emission 510 nm. Cells were wounded at arrows. Bars indicate the completion time of resealing. (B) Each point represents one experiment. The resealing rate was defined as the inverse of the resealing time in seconds. Points above the diagonal show facilitation after the first wounding (i.e. 2nd rate > 1st rate). (C) Data re-plotted in double-log scale. In this first figure, reduced calcium concentrations at two different values were used to show calcium dependence of facilitation. Two fitted lines are $y=0.75x-0.04$ for 0.8 mM Ca^{2+} Ringer (solid line) and $y=0.84x-0.12$ for 0.4 mM Ca^{2+} Ringer (dotted line). Temperature is 23–25°C in all experiments unless otherwise stated.

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 4 α -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 Ca^{2+} 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 (4 α -PMA, $n=22$). The peak values of PMA and 4 α -PMA treated cells were not significantly different (Welch t -test, $P=0.6368$ for PMA, $P=0.3457$ for 4 α -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 Ca^{2+} 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

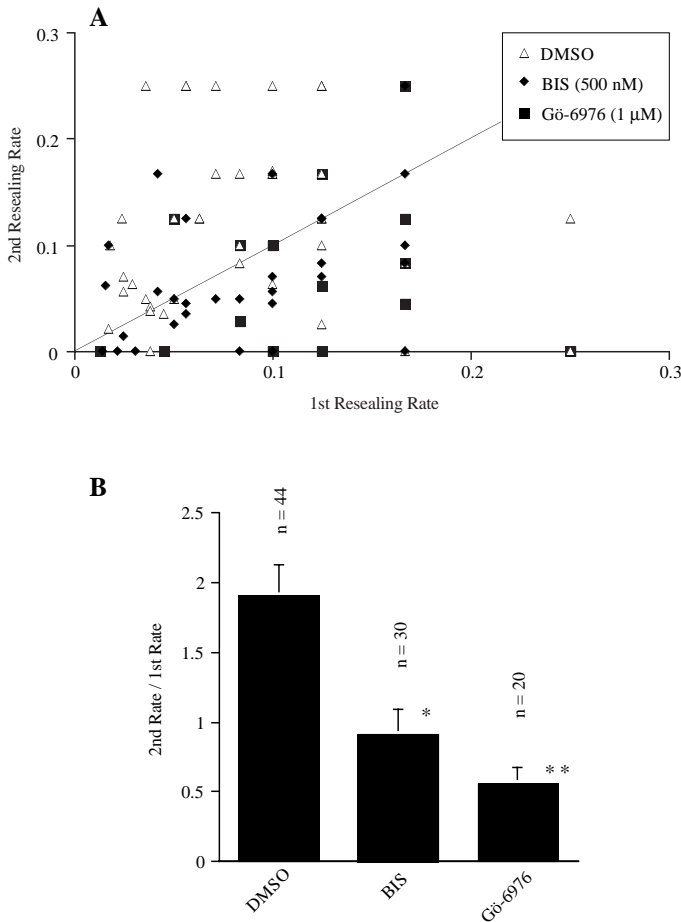


Fig. 2. Facilitation of membrane resealing is inhibited by PKC inhibitors. (A) Fura-2 loaded cells were wounded twice in the presence or absence of BIS (500 nM) or Gö-6976 (1 μ M) in normal 1.8 mM Ca^{2+} Ringer. In this and all subsequent figures, Ringer calcium concentrations were at 1.8 mM. Each point 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 \pm s.e.m. $P=0.0013$ (*) and $P<0.0001$ (**) using Welch *t*-test.

destaining was observed in 73.1% of resealed cells ($n=26$) as shown in the example in Fig. 4A, and average fluorescence change was $-2.00\pm 0.38\%$ ($n=26$) (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 $-0.3\pm 0.19\%$ ($n=12$) (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

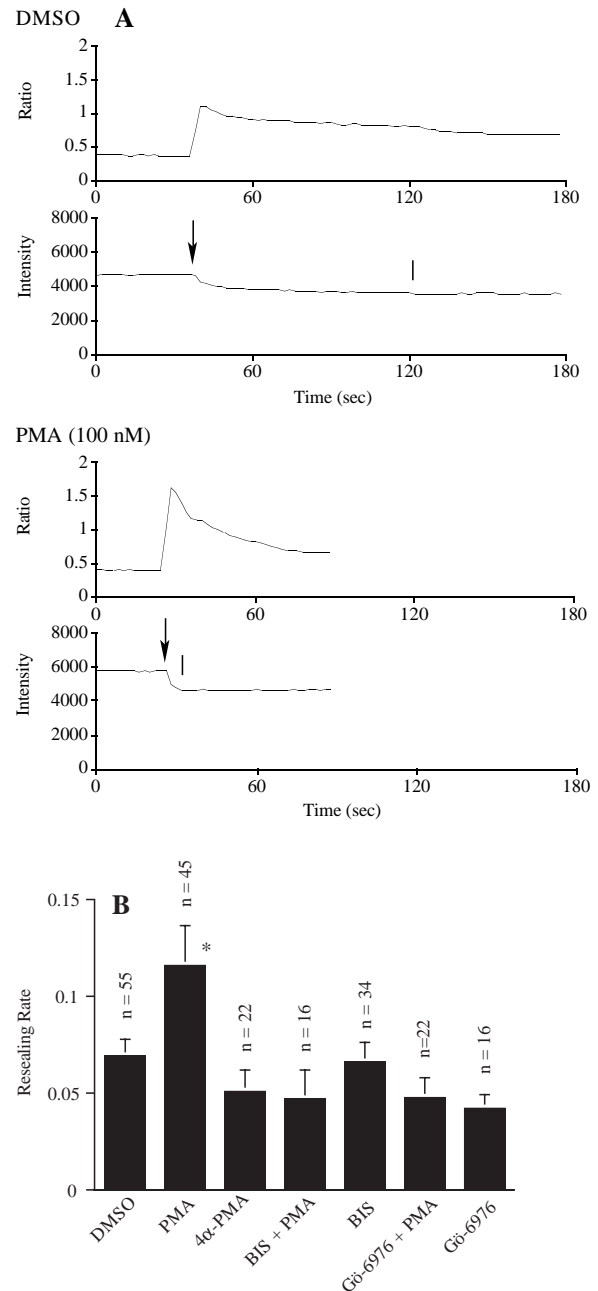


Fig. 3. PMA facilitates membrane resealing through activation of PKC. (A) Typical Fura-2 recordings of single-wounding in the presence of DMSO or 100 nM PMA. Cells were wounded at the arrows. Bars indicate the completion time of resealing. (B) Resealing rate was defined as the inverse of resealing time. 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 \pm s.e.m. $*P=0.0378$ using Welch *t*-test (compared with DMSO).

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

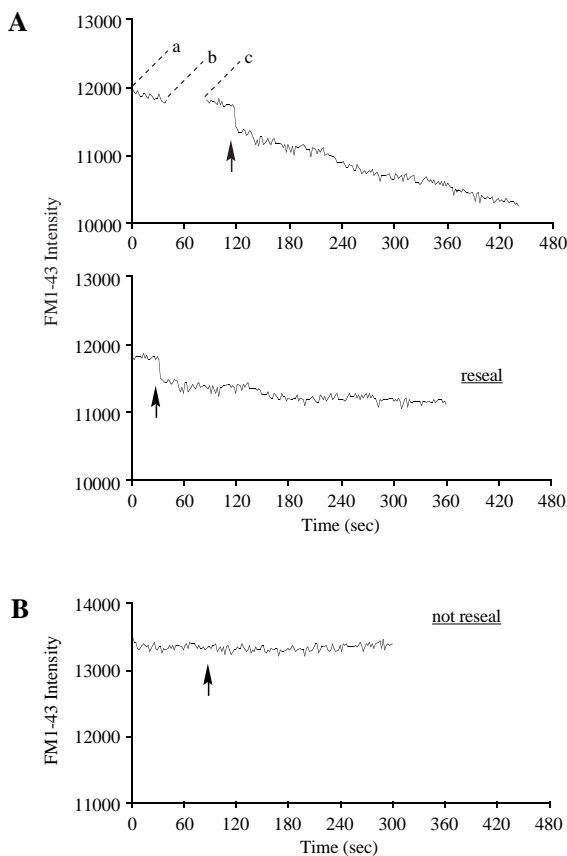


Fig. 4. Wound-induced decreases in FM1-43 fluorescence. FM1-43 loaded cells were wounded (arrows) in 1.8 mM Ca^{2+} Rodent Ringer solution. (A, upper trace) Raw recording of FM1-43 fluorescence (a-b). During b-c, excitation light is blocked to estimate rate of photobleaching. The photobleaching was subtracted from upper trace (A, lower trace). All subsequent data was processed in this manner to subtract photobleaching. Rapid destaining was observed in 73.1% of the cells that resealed. (B) Rapid destaining was not observed in those cells that failed to reseal.

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

	Destaining	Fluorescence change (%)*
Reseal	19/26	-2.00 ± 0.38
Not reseal	3/12	$-0.3 \pm 0.19 \ddagger$

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 \pm s.e.m.

$\ddagger P=0.0004$, Welch *t*-test.

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

Reagents injected	Resealing	Destaining
Toxin	1/6	2/6
Toxin + substrate	7/9	7/9

Tetanus toxin was injected with or without substrate peptide. Cells were wounded 91-120 minutes after toxin injection. Resealing was monitored by visual inspection.

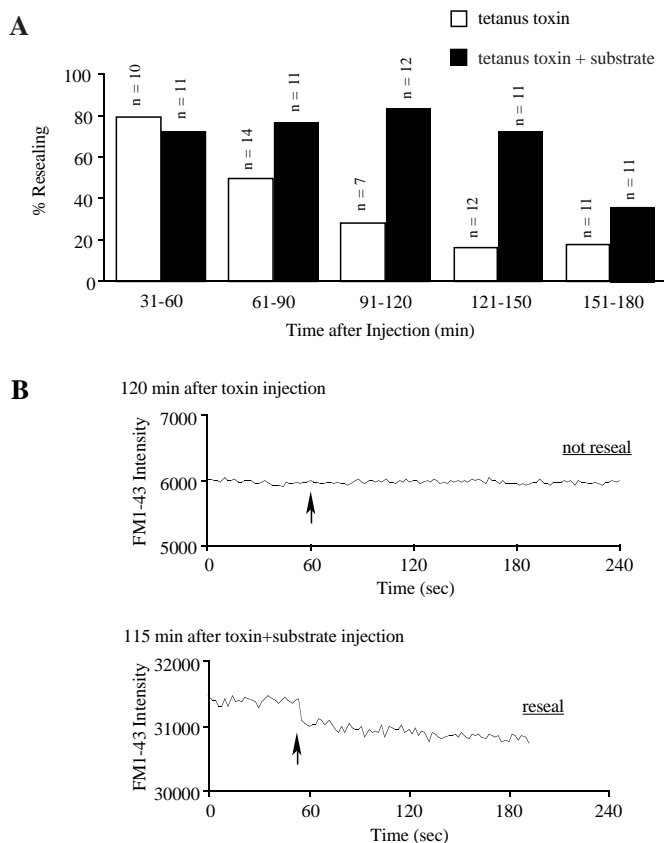


Fig. 5. Effect of tetanus toxin on membrane resealing and wounding-induced FM1-43 destaining. (A) Tetanus toxin was injected with or without substrate of the toxin. Membrane resealing was monitored by photometric measurement of Fura-2 fluorescence and visual inspection. Toxin had no effect on resealing until about 90 minutes after injection and the inhibition of resealing was not maximal until 180 minutes after injection. The effect of toxin was delayed by co-injection with substrate of the toxin. (B) Wounding-induced FM1-43 destaining is suppressed under conditions expected to inhibit vesicle exocytosis.

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.

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

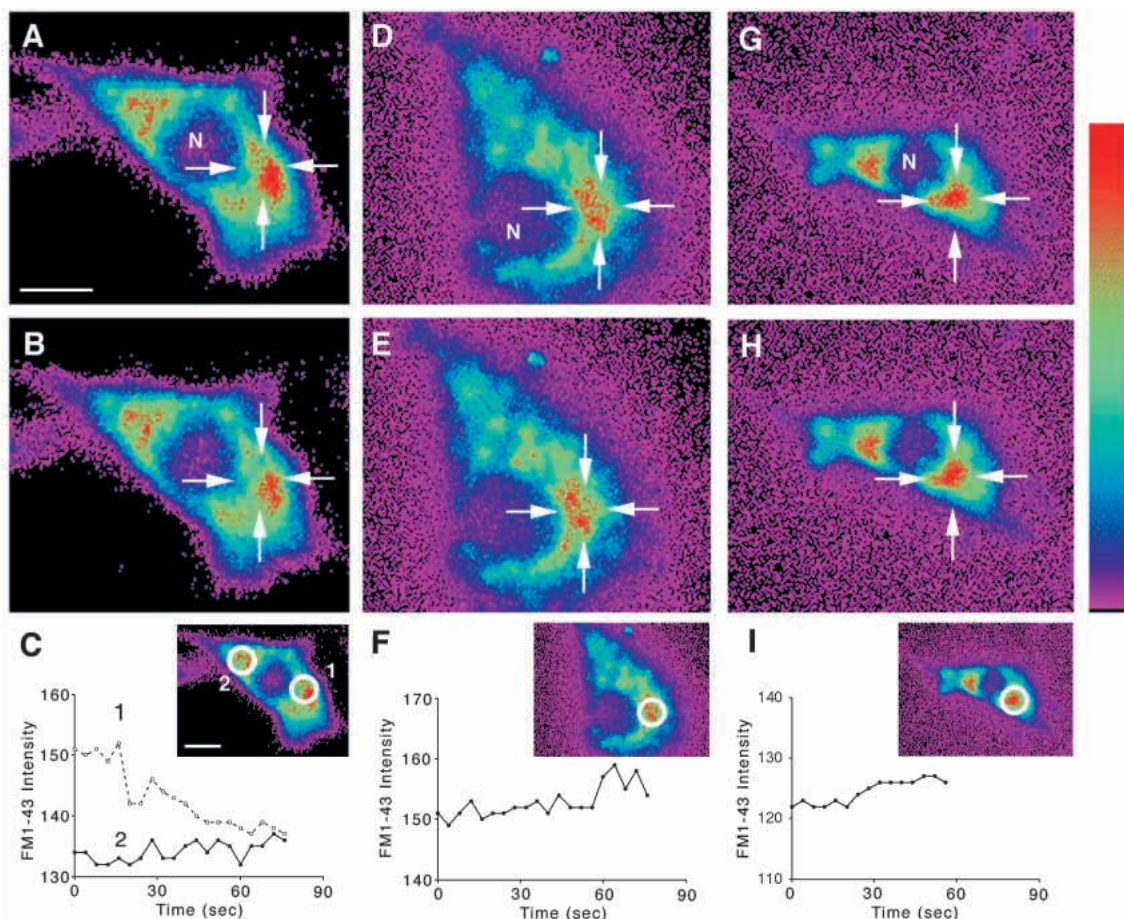


Fig. 6. Localized FM1-43 destaining is observed in a resealed cell. Pseudocolor images were acquired every 4-seconds. Cells were wounded in an area indicated by arrows between 5th (A,D,G) and 6th (B,E,H) image acquisition. (A,B) FM1-43 loaded cell was wounded in 1.8 mM Ca^{2+} Rodent Ringer solution. (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 μm .

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.

Table 3. Localized FM1-43 destaining at wounding

	Local destaining	Local fluorescence change (%)*
1.8 mM Ca^{2+} , reseal	24/29	-6.19 ± 0.99
Ca^{2+} -free, not reseal	0/6	0
Tetanus toxin, not reseal	0/11	0

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.

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.

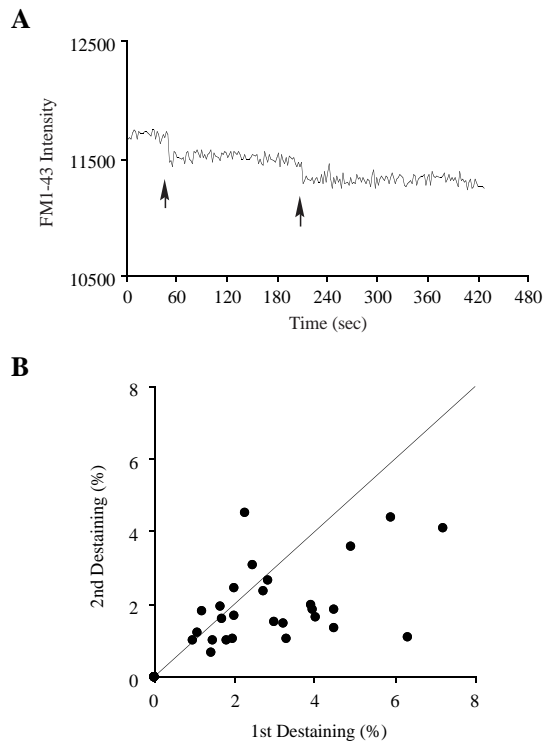


Fig. 7. FM1-43 destaining during double-wounding. (A) Typical recording of wounding-induced FM1-43 destaining. Arrows indicate the time of wounding. (B) Each point represents one experiment. Data were omitted when cells failed to reseal.

FM1-43 destaining in PKC activated cells

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 4 α -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 $-0.93 \pm 0.23\%$ ($n=27$) (Fig. 8B). In 4 α -PMA treated cells (Fig. 8B), on the other hand, the fluorescence change was $-1.94 \pm 0.44\%$ ($n=15$), 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

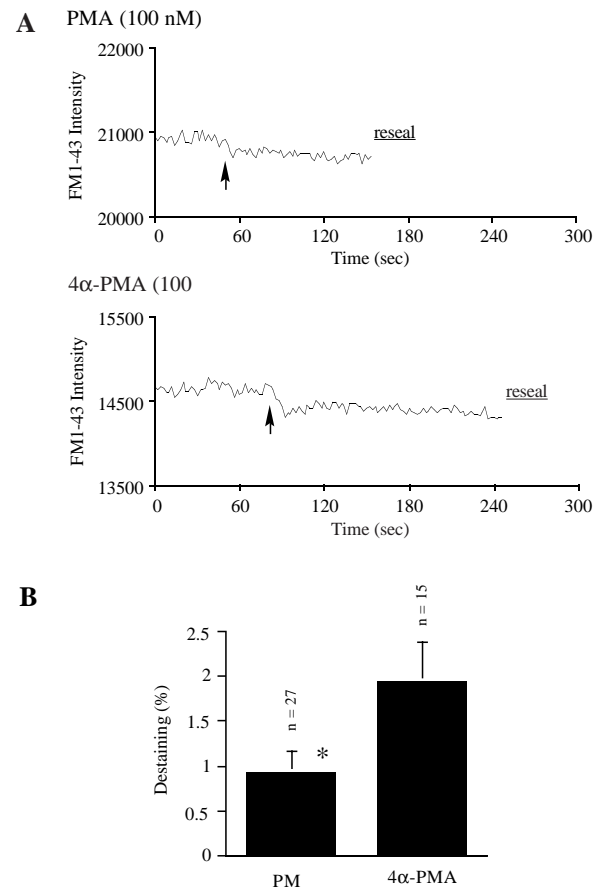


Fig. 8. Effect of phorbol esters on wounding-induced FM1-43 destaining. FM1-43 loaded cells were incubated with 100 nM PMA or 4 α -PMA for 10 minutes, and wounded. (A) Typical recordings of FM1-43 destaining. Arrows indicate the time of wounding. (B) The average fluorescence change in PMA and 4 α -PMA treated cells. * $P=0.0306$ using Student *t*-test.

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

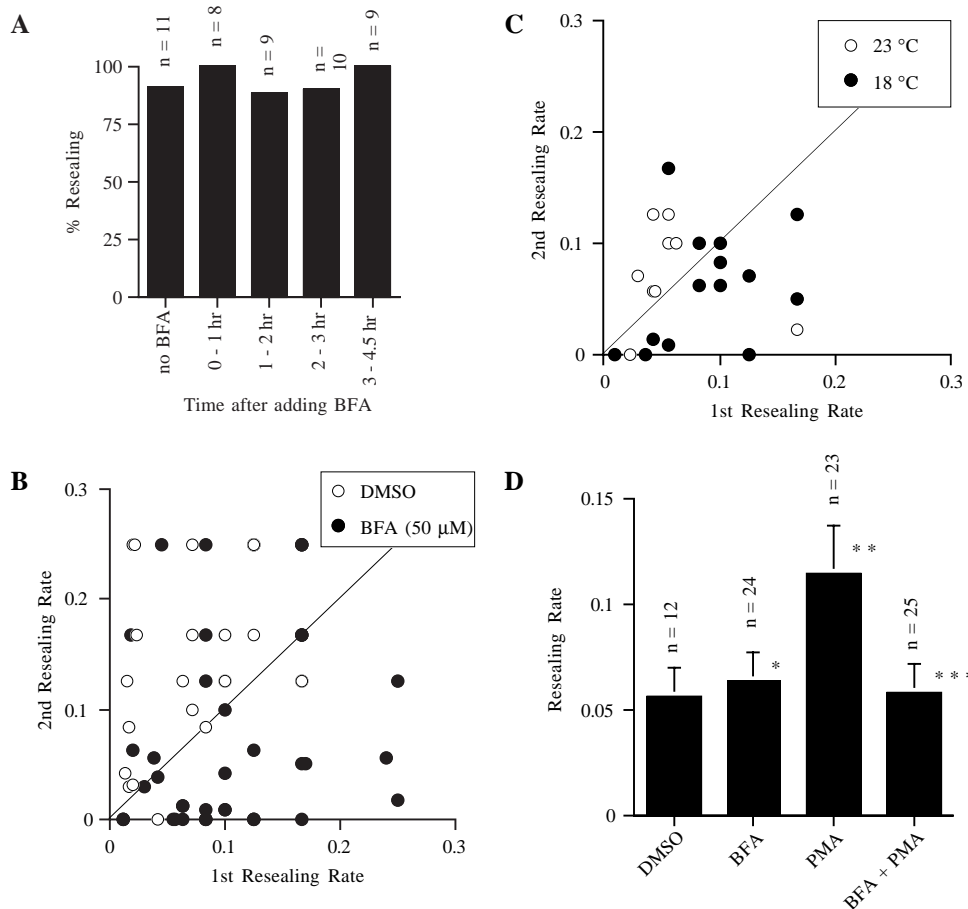


Fig. 9. (A) Effect of BFA on membrane resealing in 3T3 cells. No significant change in resealing ability was detected by 4.5 hours after adding BFA. (B,C) Effect of BFA (B) and low temperature (C) on facilitation of membrane resealing. Each point represents one experiment. Data were omitted when cells failed to reseal at first wounding. Points above the diagonal show facilitation after the first wounding (i.e. 2nd rate > 1st rate). (D) PMA does not facilitate membrane resealing in BFA treated cells. BFA treated and untreated cells were wounded after addition of PMA or DMSO. Pre-treatment with BFA blocked the facilitation of membrane resealing by PMA. Values are mean \pm s.e.m. $P=0.6974$ (*), $P=0.0326$ (**), $P=0.9256$ (***) using Welch *t*-test (compared with DMSO).

(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 4 α -PMA while fluorescence intensity was measured at 5 minutes intervals.

Fluorescence intensity of PMA treated cells was reduced to $96.6 \pm 1.0\%$ ($n=12$) at 20 minutes, whereas the intensity of 4 α -PMA treated cells was reduced to $99.2 \pm 0.9\%$ ($n=9$) 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 $140.8 \pm 4.2\%$ ($n=5$) by the treatment with PMA. Treatment with 4 α -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.,

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

	No. of resealed cells	Destaining	Fluorescence change (%)*
Control (DMSO)	8/11	8/8	-1.69±0.21
Cytochalasin D (20 µM)	17/18	4/17	-0.65±0.30‡

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.

1996). 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

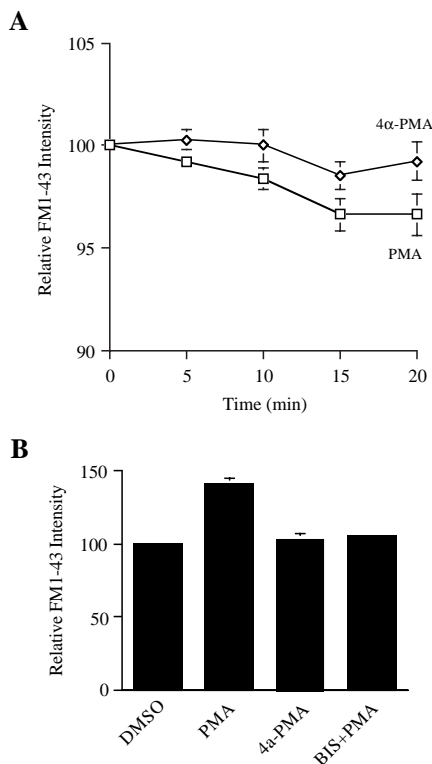


Fig. 10. (A) PKC activation by PMA accelerates FM1-43 destaining. Fluorescence intensity was measured at 5 minutes intervals. The shutter was closed between measurements to avoid photobleaching. The intensity at time zero was measured just before addition of phorbol esters. Bars are s.e.m. ($n=12$ for 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.

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

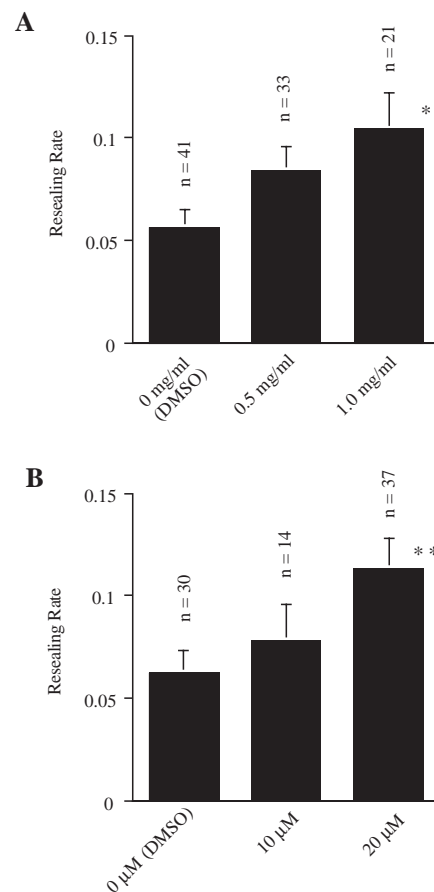


Fig. 11. Resealing is facilitated by surfactant and cytochalasin. (A) Cells were wounded in 1.8 mM Ca^{2+} Rodent Ringer solution with or without Pluronic F68 NF. * $P=0.0220$ using Welch *t*-test. (B) Cells were treated with cytochalasin D for 1 hour, and wounded in 1.8 mM Ca^{2+} Rodent Ringer solution.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

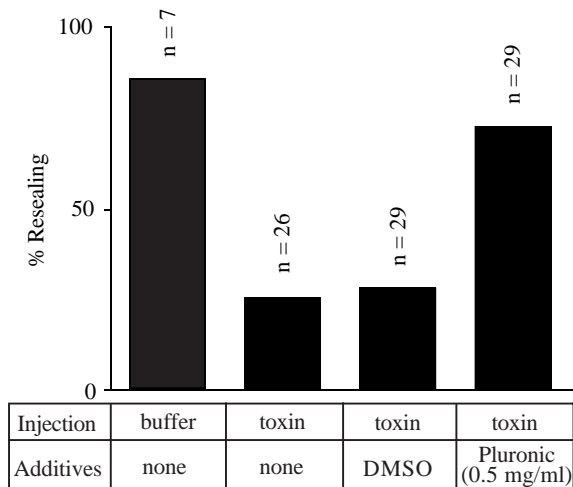


Fig. 12. Cells can reseal in the presence of surfactant under conditions which inhibit vesicle exocytosis. Tetanus toxin injected cells were wounded in 1.8 mM Ca^{2+} Rodent Ringer solution with or without Pluronic F68 NF at 121-180 minutes after the injection. Membrane resealing was monitored by photometric measurement of Fura-2 fluorescence and visual inspection.

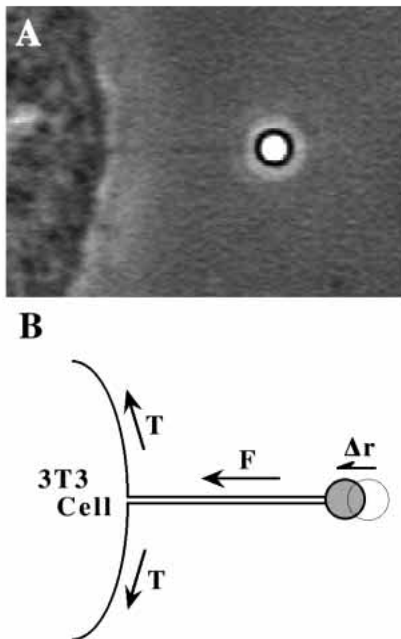


Fig. 13. (A) Membrane tether from a 3T3 cell. To form a tether from 3T3 cell, an IgG-coated bead was held on the cell surface for 2 seconds, and the bead was pulled away from the cell surface. (B) Distance of bead displacement in the laser trap (Δr) is dependent on the tether force (F) and surface tension (T).

equilibrium values. 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 (Angleton 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 Ca^{2+} and PKC in 3T3 cells (Figs 1, 2). These results suggest that exocytosis after repeated wounding 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). Phorbol ester 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 wounding 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 (Fig. 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 (Ktistakis 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, Gö-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 Ca^{2+} triggers exocytosis in various types of cells (Dan and Poo, 1992; Coorssen et al., 1996), and that endosomal compartments such as lysosomes are known to behave as Ca^{2+} -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 Ca^{2+} -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

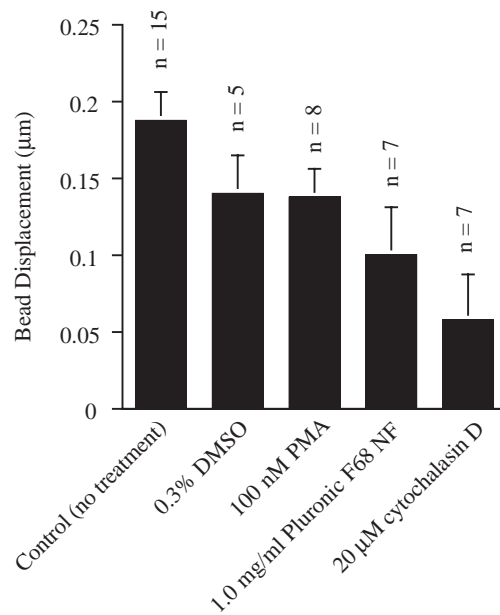


Fig. 14. Distance of bead displacement in the laser trap after the tether formation. Bead positions in the laser trap were compared before and after the tether formation. Tether length was 5–10 μm . Values are mean \pm s.e.m. $P=0.2158$ (DMSO), 0.1117 (PMA), 0.0225 (Pluronic F68 NF), and 0.0013 (cytochalasin D) using Student *t*-test (compared with untreated cells).

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 Ca^{2+} 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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