

# Calcium-regulated Exocytosis Is Required for Cell Membrane Resealing

Guo-Qiang Bi,\* Janet M. Alderton,<sup>†</sup> and Richard A. Steinhardt\*<sup>‡</sup>

\*Group in Biophysics; and <sup>†</sup>Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3200

**Abstract.** Using confocal microscopy, we visualized exocytosis during membrane resealing in sea urchin eggs and embryos. Upon wounding by a laser beam, both eggs and embryos showed a rapid burst of localized  $\text{Ca}^{2+}$ -regulated exocytosis. The rate of exocytosis was correlated quantitatively with successfully resealing. In embryos, whose activated surfaces must first dock vesicles before fusion, exocytosis and membrane resealing were inhibited by neurotoxins that selectively cleave the SNARE complex proteins, synaptobrevin, SNAP-25, and syntaxin. In eggs, whose cortical vesicles are already docked, vesicles could be reversibly undocked with externally applied stachyose. If cortical vesicles were undocked both exocytosis and plasma

membrane resealing were completely inhibited. When cortical vesicles were transiently undocked, exposure to tetanus toxin and botulinum neurotoxin type C1 rendered them no longer competent for resealing, although botulinum neurotoxin type A was still ineffective. Cortical vesicles transiently undocked in the presence of tetanus toxin were subsequently fusion incompetent although to a large extent they retained their ability to redock when stachyose was diluted. We conclude that addition of internal membranes by exocytosis is required and that a SNARE-like complex plays differential roles in vesicle docking and fusion for the repair of disrupted plasma membrane.

CELL membranes are able to reseal after mechanical injury or disruption by microinjection, chemical permeabilization, or electroporation, but the mechanism of resealing has not been well-characterized (Cajal, 1928; Celis, 1984; Yawo and Kuno, 1985; McNeil and Ito, 1989; Xie and Barrett, 1991; McNeil, 1991; Tsong, 1991; McNeil and Khakee, 1992; Yu and McNeil, 1992; Clarke et al., 1993; Spira et al., 1993; Weaver, 1993; Krause et al., 1994). Our previous study of plasma membrane repair led to the hypothesis that a calcium-regulated fusion of vesicles is necessary for resealing (Steinhardt et al., 1994). We found that membrane resealing in sea urchin eggs, early embryos, and Swiss 3T3 fibroblasts required threshold levels of extracellular  $\text{Ca}^{2+}$  that could be antagonized by  $\text{Mg}^{2+}$ . In addition, we found that both botulinum neurotoxin types B (BNT-B)<sup>1</sup> and A (BNT-A) inhibited membrane resealing in Swiss 3T3 fibroblasts and in sea urchin embryos. BNT-B and BNT-A are known to be proteases that block neurotransmission by specific cleavages of the SNARE synaptic vesicle docking/fusion proteins, synaptobrevin, and SNAP-

25, respectively (Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994; Jahn and Niemann, 1994; Schiavo et al., 1994). Furthermore, kinesin and calcium/calmodulin kinase II appear to be involved in this resealing process because function-blocking antibodies or autoinhibitory peptides against these proteins also blocked resealing. Based on these results, we proposed that a  $\text{Ca}^{2+}$ -regulated exocytosis might be required for membrane resealing that was similar in many respects to the vesicle recruitment, docking, and fusion seen in neurotransmission (Steinhardt et al., 1994). Although  $\text{Ca}^{2+}$ -regulated exocytosis is a well-known fast membrane process that is also antagonized by magnesium (Hubbard et al., 1968), it has been regarded as a specialized process that exists only in certain cell types. Our findings, on the contrary, seemed to require that calcium-regulated exocytosis be ubiquitous and that vesicles that have other specialized uses could also be used as needed in rapid membrane repair (Steinhardt et al., 1994).

To establish the causal relationship between exocytosis and resealing, it is necessary to visualize directly the exocytotic events during membrane resealing and show that delivery of internal membranes to the surface is required for resealing. Using cell-impermeant lipophilic fluorescent dyes such as FM 1-43, it has been possible to study synaptic vesicle recycling by optically monitoring fluorescent spots representing clusters of hundreds of synaptic vesicles (Betz et al., 1992; Betz and Bewick, 1992; Ryan et al., 1993; Ribchester et al., 1994). Terasaki (1995) has further developed this method to study the exocytosis of individual cortical

Address all correspondence to Dr. Richard Steinhardt, 391 LSA, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200. Tel.: (510) 642-3517. Fax: (510) 643-6791. E-mail: rick@mendel.berkeley.edu

1. *Abbreviations used in this paper:* AB, aspartate buffer; ASW and NSW, artificial and natural sea water, respectively; BNT-A, BNT-B, and BNT-A LC, botulinum neurotoxin types A and B, and BNT-A light chain, respectively; HB, HEPES buffer; Tetx, tetanus toxin.

vesicles during sea urchin egg fertilization. Based on the excellent depth discrimination of confocal microscopy and the fact that exocytosis of large cortical vesicles results in semi-stable open pockets (Eddy and Shapiro, 1976; Chandler and Heuser, 1979), individual exocytotic events triggered by elevated intracellular calcium during fertilization could be easily distinguished. The unique ability to observe directly individual exocytotic events, combined with the fact that the unfertilized egg has thousands of predocked cortical vesicles that the activated embryo lacks, make the sea urchin egg and embryo ideal preparations for studies of exocytosis and membrane resealing. In the literature, the secretory vesicles predocked at the plasma membrane of the unfertilized egg are usually referred to as cortical granules. For clarity, we refer to them here as cortical vesicles, to emphasize their inclusion in the pool of vesicles capable of participating in plasma membrane resealing.

An unresolved issue in our previous studies was whether unfertilized eggs used the same exocytotic mechanism to reseal as did embryos, since reagents that blocked resealing in embryos and other cells did not affect unfertilized eggs, whose cortical vesicles are already docked at the plasma membrane. A previous study has shown that high osmolarity caused by extracellular 0.8 M stachyose in sea water can undock the cortical vesicles in unfertilized sea urchin eggs and that the undocked vesicles are unable to exocytose upon calcium ionophore stimulation (Chandler et al., 1989). Since this undocking method was fully reversible when stachyose was diluted, we were able to compare docking status with resealing ability in unfertilized eggs and also to transiently expose the otherwise inaccessible docking/fusion machinery of cortical vesicles to neurotoxins.

## Materials and Methods

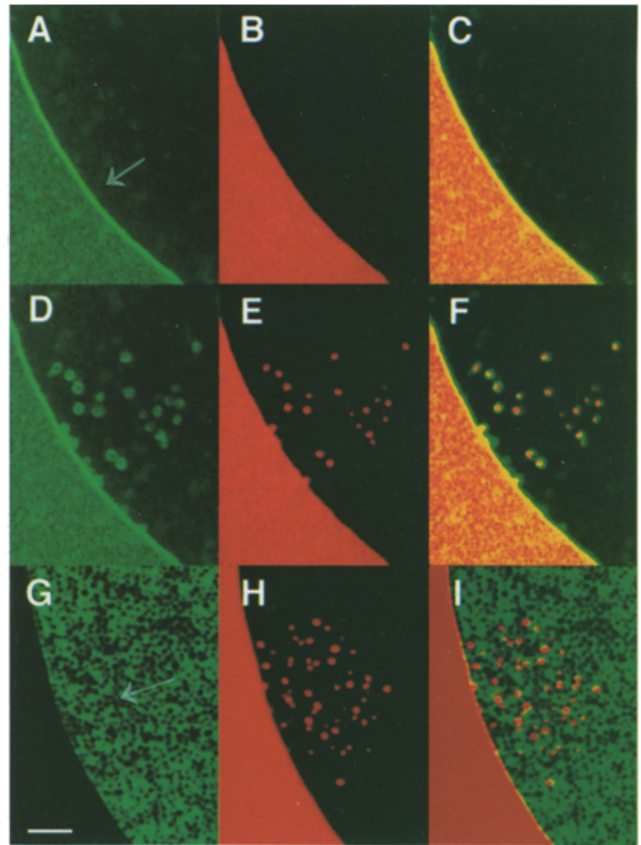
### Cell Preparation and Mechanical Wounding

*Lytechinus pictus* sea urchins were from Marinus Inc. (Long Beach, CA) and were handled as previously described (Baitinger et al., 1990). Microinjection and resealing tests were performed at 21°C on the stage of an inverted microscope (IM35; Carl Zeiss, Inc., Thornwood, NY) with a 40× lens (UV-F; Nikon Inc., Instrument Group, Melville, NY) as described (Steinhardt et al., 1994). Briefly, washed eggs were allowed to settle onto glass coverslips treated with poly-DL-lysine (Sigma Chemical Co., St. Louis, MO, 10 mg/ml in glass distilled water). Reagents mixed with the fluorescent dyes calcium green dextran (10,000 mol wt) and/or fura-2, pentapotassium salt (both from Molecular Probes, Eugene, OR) were pressure injected into unfertilized or fertilized sea urchin eggs (injection was done within 10 min after fertilization). Injection solutions for resealing tests contained ~5 mM fura-2 salt unless otherwise stated. Injection vol was ~3–5% of cell vol. Mechanical wounding for tests of resealing were done by advancing a glass micropipette towards the egg to create a dimple ~20% of the egg diameter deep. A tap was applied to the micromanipulator to wound the egg. The micropipette was withdrawn after wounding and subsequent resealing was monitored by photometric measurement of fura-2 fluorescence at 510 nm excited with 357- and 385-nm UV light. The wound size could not be measured directly and was variable even if a standard force was applied. Based on the size of the microneedle, we estimate the wound size should have been in the range of 1–3 μm. Persistent decrease of 357 nm excited fluorescent intensity (as an indicator of dye leakage out of the cell) together with persistent increase of the ratio of fluorescent intensity excited by 385/357-nm light (as an indicator of increasing intracellular Ca<sup>2+</sup>) indicated resealing failure.

### Confocal Microscopy and Laser Wounding

Confocal microscopy was performed using a confocal microscope (MRC 600; Bio-Rad Laboratories, Hercules, CA) equipped with an upright fluorescent

microscope (Axioplan; Carl Zeiss, Inc.) and an air-cooled Argon laser (5400; Ion Laser Technology, Inc., Fort Collins, CO) with total output of ~25 and ~10 mW for either 488-nm or 514-nm line. A 40× oil immersion lens (Neofluor; Carl Zeiss, Inc.) (NA 1.3) was used in the experiments. Unfertilized eggs or early embryos (4–8-cell stage) were settled (before fertilization for embryos) onto poly-lysine-coated glass coverslips as described above. Injections (when applicable) were also performed separately as described in the last section, except that the injection solution contained 1 mM calcium green dextran 10,000 in addition to 5 mM fura-2 salt. The



**Figure 1.** Wounding-induced exocytosis of cortical vesicles in unfertilized sea urchin eggs. Eggs were imaged with a confocal microscope with the focal plane just below the egg surface attached to the coverslip. An egg wounded by 10–20 s of laser irradiation showed a burst of exocytosis of cortical vesicles near the wound site. As the cell-impermeant fluorescent dyes in the sea water diffused into the open pocketlike structures formed by the exocytosed vesicles, these structures became visible in the scanning images. The lipophilic dye FM 1-43 appears as bright circles, while the hydrophilic dye rhodamine dextran appears as bright disks. Comparison with the image of cortical vesicles before wounding demonstrates that the disks correspond to exocytosed vesicles. (A–F) Dual-channel confocal microscopy using FM 1-43 (A and D) and rhodamine dextran (B and E). A and B are an image pair taken before wounding. C was produced by merging A with B. D and E are a pair taken ~6 s after wounding. Green circles in D and red disks in E represent exocytosed cortical vesicles (see text). D and E were merged to form F, showing colocalization of green circles with red disks. Arrowheads in this and the following figures indicate the position of laser wounding. (G–I) Dual-channel imaging using injected calcium green dextran (G) and extracellular rhodamine dextran (H). G was taken before laser wounding. H was taken ~10 s after irradiation. Dark disks in G indicate predocked cortical vesicles. I is a merged image from G and H. The colocalization of bright disks in H with dark disks in G verifies that these disks do represent exocytosed vesicles. Bar, 5 μm.

coverslip was then mounted into a handmade chamber that held ~300  $\mu$ l natural sea water (NSW). The chamber was later placed on the stage of the confocal microscope and was cooled to ~19–21°C by ice in glass beakers on the metal stage during experiments.

To image exocytosis, the confocal scanning plane was focused just inside the cell surface attached to the glass coverslip. 1  $\mu$ M FM 1-43 (Molecular Probes) and/or 100  $\mu$ M rhodamine dextran (3,000 mol wt, Molecular Probes) were dissolved in the sea water (NSW or artificial [ASW]) in the chamber. Both these dyes are cell impermeable, but they can readily diffuse into the exocytic vesicle pockets that retain their shape for a length of time sufficient for dye transfer and for recording. For all time series experiments, zoom 8 and 1/8 window size (192  $\times$  256 square pixels), with F1 slow scan mode was used. Scanning and storage of each image frame took ~3 s. Scanning only (without storage) ran at ~1.5 s per frame. The dye transfer appeared to be instantaneous at our imaging frame rate. Our system has a spatial resolution limit of ~0.3  $\mu$ m, and a temporal resolution limit of ~1 s. Too transient events or too small vesicles may escape detection. The exocytic nature of these recordings was directly verified in the case of cortical vesicles (Fig. 1, G–I).

Laser wounding was done by “parking” the beam at a selected position for 10–30 s, depending on dye composition and laser power, to produce a wound that could trigger subsequent exocytosis in normal conditions. Similar to needle puncture, the exact size of a laser wound was also variable and could not be measured directly. We can estimate based on the laser beam that the laser wound size should be ~0.5–1  $\mu$ m. The actual injury from laser wounding also depends on the irradiation time, the laser power, and the dye composition. Cells were continuously scanned after wounding, and frames were stored when new exocytotic events occurred or after a certain period of time (which varied from 15 s to several min depending on conditions) without new events. A semiautomated macro program was written to perform these tasks. Dual channel experiments were done in a similar way except that window size was doubled (384  $\times$  256 square pixels) and in some experiments, a Kalman filter was used to average over three (as in Fig. 1, G–I) or six (as in Fig. 6) sequential frames to obtain sharper images.

### Stachyose Treatment

The stachyose solution was made by dissolving stachyose hydrate (Sigma Chemical Co.) into NSW. 0.8 M stachyose (which causes undocking of cortical granules in unfertilized sea urchin eggs) was 0.53 g stachyose plus 1 ml sea water. 0.53 g stachyose was dissolved in 0.6 ml sea water by vortexing. This solution was then gently mixed into 0.4 ml sea water on the coverslip containing the sea urchin eggs (held in place by poly-lysine). Gentle mixing was continued until the solution appeared uniform. 0.4 M stachyose (which does not cause undocking) was 0.26 g stachyose plus 1 ml sea water. For pulsed stachyose treatments, unfertilized eggs in NSW were injected with toxin or buffer, incubated at 21°C for ~1 h (the time required for neurotoxins to inhibit resealing in fertilized eggs (Steinhardt et al., 1994), then exposed to 0.8 M stachyose. After 30 min of exposure, the stachyose was gently washed out, and the eggs recovered their normal appearance.

### Buffers and Solutions

ASW solutions with varied  $\text{Ca}^{2+}$  concentrations were made as previously described (Steinhardt et al., 1994). Fura-2 salt solution was one part 55 mM fura-2, pentapotassium salt in 10 mM KOH, and one part aspartate buffer (AB). AB was 100 mM potassium aspartate and 20 mM Hepes at pH 7.2. The calcium green dextran stock solution was 2 mM in AB.

BNT-C1 stock solution (Wako Bioproducts, Richmond, VA) was 1 mg/ml (~1.5  $\mu$ M) in 200 mM NaCl, 50 mM sodium acetate, pH 6.0. This BNT-C1 solution was mixed 4:1 with fura-2 salt solution to make the BNT-C1 injection solution. A 9-amino acid peptide, SDTKKAVKY, spanning the BNT-C1 cleavage site for syntaxin (Schiavo et al., 1995) was 20 mM in Hepes buffer (HB). HB was 100 mM KCl, 10 mM Hepes (pH 6.0). The peptide solution was mixed 4:4:1 with BNT-C1 stock solution and fura-2 salt solution to make the BNT-C1 + syntaxin peptide injection solution. Control experiments substituted the syntaxin peptide with an equal concentration of VAMP/synaptobrevin peptide, ASQFETS, a 7-amino acid peptide that spans the tetanus toxin (Tetx) cleavage site for synaptobrevin (Schiavo et al., 1992). Peptides were synthesized by the Microchemical Facility, Cancer Research Laboratory, University of California, Berkeley, purified by high pressure liquid chromatography, and confirmed by sequencing or mass spectroscopy.

Pure BNT-A stock solution without hemagglutinin (generously provided by Dr. B. R. DasGupta, University of Wisconsin, Madison) was 5

mg/ml in HB. The toxin stock was mixed 1:10:1 with HB and fura-2 salt solution to make the BNT-A injection solution. A 17-amino acid peptide, TRIDEANQRATKMLGSG, spanning the BNT-A cleavage site for SNAP-25 (Schiavo et al., 1993) was 5.4 mM in HB (generously provided by Dr. Raymond Stevens, University of California, Berkeley). For control experiments, the SNAP-25 peptide was substituted with a 19-amino acid peptide of CaM kinase, QETVDCLKKFNARRKLGKA, at 20 mM in HB (Baitinger et al., 1990).

BNT-A light chain-lyophilized powder (List Biological Laboratories, Campbell, CA) was reconstituted just before injection. The solution contained about 10  $\mu$ M toxin in 100 mM KCl, 10 mM Hepes, 40 mM sodium phosphate (pH 6.0), 20 mM NaCl with or without 5 mM DTT. Tetanus toxin lyophilized powder (List Biological Laboratories) was also freshly reconstituted for each experiment. This solution contained about 8  $\mu$ M toxin in 60 mM sodium phosphate (pH 7.5), 100 mM KCl, 10 mM Hepes. Both toxin solutions were mixed 2:1 with fura-2 stock for resealing tests or 4:1:1 with fura-2 salt (27 mM in AB) and calcium green dextran stock (2 mM in AB) for confocal imaging.

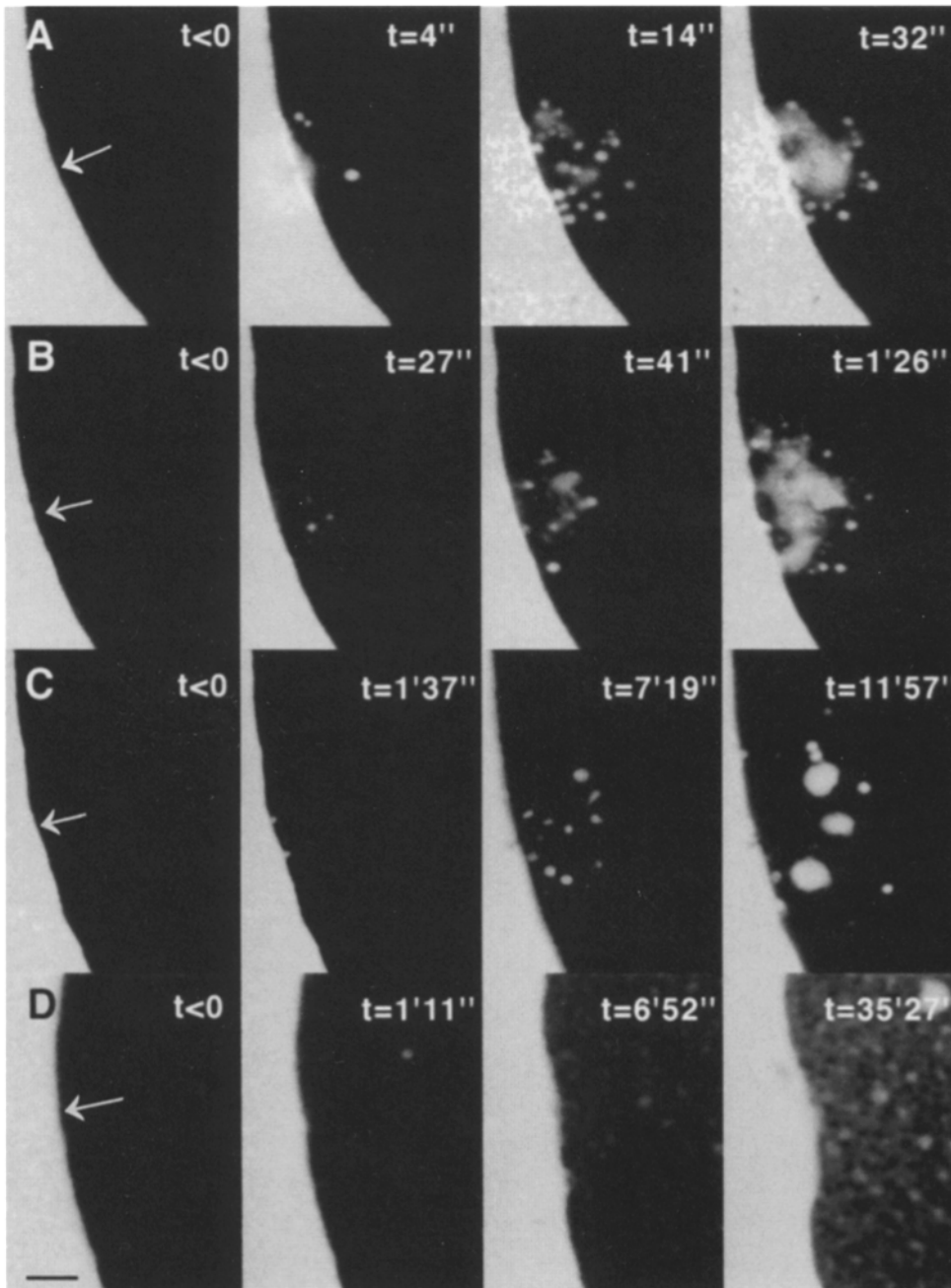
## Results

### Membrane Resealing in Sea Urchin Eggs Is Accompanied by a Burst of Cortical Vesicle Exocytosis Localized Near the Wound

We wounded cells with the laser beam of the confocal microscope by focusing the laser at a selected point on the cell surface for 10–20 s. The effective size of the wound could be controlled by adjustment of the power of the laser. The location of the lesion could also be confirmed by local bleaching of dye at the cell surface. At normal calcium concentrations, plasma membrane wounds triggered a burst of local exocytosis and resealed (Fig. 1). With lipophilic dye FM 1-43 in the sea water, exocytosed vesicles appeared as bright rings (Fig. 1 D). With water-soluble rhodamine dextran (3 K mol wt), they appeared as bright disks (Fig. 1 E). The colocalization of rings and disks became apparent when the dual-channel images were merged (Fig. 1 F). To make sure what we saw was indeed exocytosis of cortical vesicles, we injected the fluorescent dye calcium green dextran into unfertilized eggs and added rhodamine dextran to the sea water. The injected dye revealed cortical vesicles as dark disks in the bright background in the green fluorescence channel (Fig. 1 G). After wounding, bright disks emerged around the wound site viewed in the rhodamine fluorescence channel (Fig. 1 H). The exact colocalization of these bright disks with the dark disks in the green fluorescence channel was verified by merging these two images (Fig. 1 I). This demonstrated that the bright disks we saw using extracellular rhodamine dextran represented exocytosed vesicles. Many red disks appear larger than the dark disks because the vesicles have expanded after exocytosis (Zimmerberg and Whitaker, 1985). This expansion can also be seen in (Fig. 2).

### Distinct Spatial and Temporal Patterns of Exocytosis When Membranes Reseal

We investigated the spatial and temporal dynamics of cortical vesicle exocytosis during membrane wounding and resealing under different conditions. We selected the rhodamine dextran assay because of its better image quality. Fig. 2 shows a series of confocal images during the time course of cortical vesicle exocytosis in unfertilized sea urchin eggs after wounding at different extracellular calcium concentrations. Consistent with our previous experiments



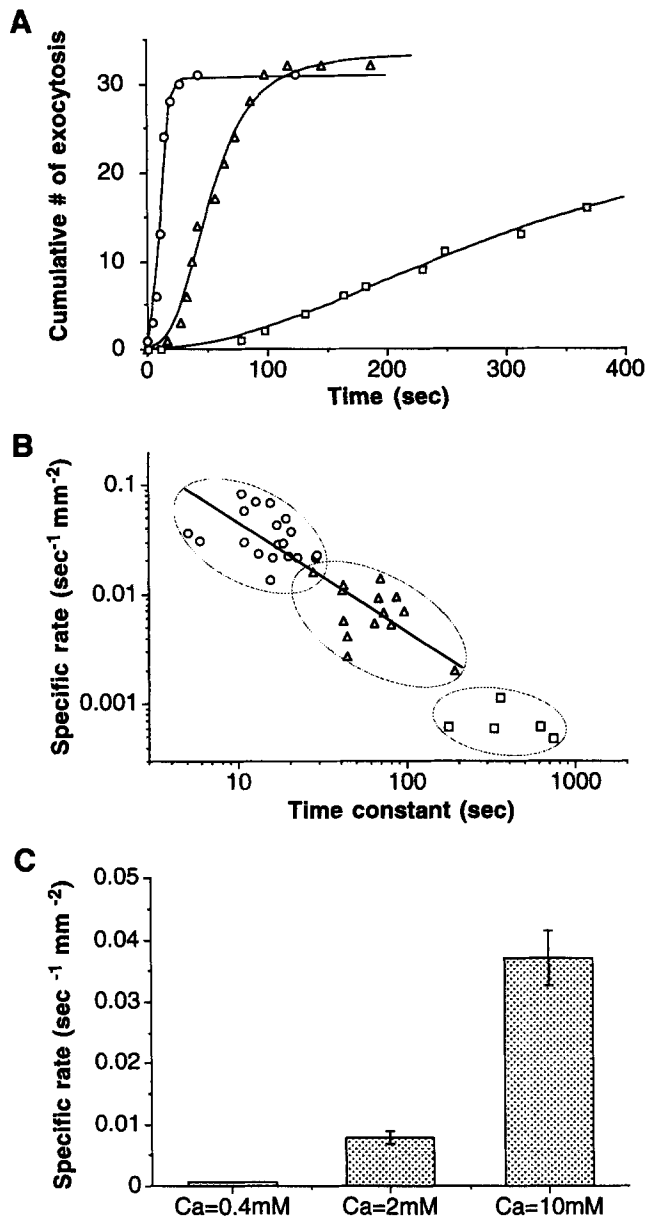
**Figure 2.** Time series images of wounding-induced exocytosis in unfertilized eggs in varying external calcium concentrations. For all time series images, extracellular rhodamine dextran (100  $\mu$ M) was used. Confocal scanning sections were set  $\sim 0.5$   $\mu$ m inside the cell surface that was adhering to the glass coverslip (and was therefore relatively flat).  $t = 0$  is defined as the time point immediately after laser wounding. The first image of each series ( $t < 0$ ) was obtained before wounding. Different time series were used in this selection of images to show the full range of events under different conditions. (A) In NSW in which  $[Ca^{2+}]_o$  was  $\sim 10$  mM. (B) In ASW with  $[Ca^{2+}]_o = 2.0$  mM. Eggs in both A and B resealed (in  $\sim 15$  and 70 s, respectively). After exocytosis of many vesicles, the secreted material trapped between the cell surface and the coverslip caused depressions of the cell membrane. In confocal images, these depressions appear as large bright areas because dye molecules can freely diffuse into these regions. (C) In ASW with  $[Ca^{2+}]_o = 0.4$  mM. This cell visually lysed  $\sim 30$  min after wounding. (D) In ASW with  $[Ca^{2+}]_o = 0$  mM. Dye leakage into the cell is apparent in the last image ( $t = 35'27''$ ). This cell did not reseat. Bar, 5  $\mu$ m.

with micropipette punctures, the eggs resealed successfully in NSW containing  $\sim 10$  mM  $Ca^{2+}$  and in ASW containing 2 mM  $Ca^{2+}$ , but failed to reseat when external  $Ca^{2+}$  was too low. This failure was confirmed by a persistent dye leakage into the cell that was usually detectable several minutes after the wounding (Fig. 2 D). Sometimes the leaked-in dye formed a blob first at the wound site and eventually diffused throughout, followed by complete lysis of the cell.

One clear feature from this set of experiments is the distinct spatial and temporal patterns of exocytosis at different  $Ca^{2+}$  concentrations. In NSW, exocytosis started rapidly, localized tightly around the wounding site, and terminated in  $\sim 10$ –20 s (Fig. 2 A). We also noticed that extensive endocytosis of vesicles, usually smaller than the cortical vesicles, often followed the rapid exocytosis (data not shown). These endocytotic vesicles could be easily distinguished by

their intracellular position and their Brownian-like movement. At a lower calcium concentration ( $\sim 2$  mM in Fig. 2 B), exocytosis occurred at a slower rate, still localized near the wounding site, and lasted for a couple of minutes. When the calcium level was reduced below the level required for cell resealing (Steinhardt et al., 1994), exocytosis occurred very slowly and persisted for many minutes (Fig. 2, C and D). At reduced concentrations of calcium, these slow or infrequent exocytotic events distributed diffusely in a larger area, indicating partial failure of calcium control due to prolonged leakage through the wound.

Because  $Ca^{2+}$  influx is the direct trigger of exocytosis, and because, in most cases, the urchin cells can buffer and pump  $Ca^{2+}$  very efficiently, a distinct cessation of exocytosis indicates either the end of  $Ca^{2+}$  influx or the depletion of the vesicle pool. The latter is unlikely in most of our ex-



**Figure 3.** Quantification of wounding-induced exocytosis in unfertilized sea urchin eggs. (A) Sample traces of exocytosis in eggs in different external calcium concentrations. For each series, the number of newly emerged exocytosis disks in each image frame was counted. This number plus the numbers from all previous frames gave the cumulative number for this time point. (B) Summary of all traces of exocytosis in sea urchin eggs in different external calcium concentrations. Each data point stands for a series of  $\sim 10$ – $20$  frames of images from an individual cell which were counted and the curves fitted using the Hill equation  $y = a/[1 + \{b/(x + 10)\}^c]$ .  $x + 10$  was used instead of  $x$  to better fit the curve and to be consistent with the fact that membrane injury was actually made before the defined  $t = 0$  point. The same method of quantification is used in Fig. 5. Time constant is the fitted  $b$  value, which is the expected time needed for half-maximum exocytosis. Specific rate of exocytosis was calculated in three steps: (1) Calculating the total rate of exocytosis at the half-maximum point using formula  $R = ac/(4b)$ . (2) Choosing the image at, or near, this time point to measure the area occupied by all current and previous exocytosis events by drawing a polygon just encircling all exocytosis disks. (3) Dividing  $R$  by this area to give the specific rate of exocytosis. The three ellipses in this graph indicate the

experimental conditions since we could repeatedly wound at the same site with successfully resealing. Therefore the cessation of exocytosis marks completion of the resealing process. For this reason, the time constant from the kinetics of exocytosis (as derived below) is a suitable measure of the kinetics of resealing.

To understand the exact relationship between the kinetics of resealing and the rate and spatial pattern of exocytosis, Fig. 3 quantifies and summarizes 40 experiments. Fig. 3 A shows example traces of the total number of individual exocytotic events plotted against time at different  $\text{Ca}^{2+}$  concentrations (Fig. 3 A). The rate of exocytosis was obtained from the slope of the fitted time course at half-maximum exocytosis. The specific rate of exocytosis was calculated by taking the rate of exocytosis and dividing it by the area occupied by exocytotic events. The time constant of exocytotic events was also derived from the fitted curve. Three distinct groups formed when the specific rate was plotted against the time constant in double log scales (Fig. 3 B). For cells that resealed after wounding ( $\text{Ca}^{2+} = 10$  mM and  $\text{Ca}^{2+} = 2$  mM), we observed a strong correlation between the specific rate and the time constant (Spearman test two-tailed  $P < 0.001$  for all resealed eggs and  $P < 0.01$  for cells in NSW). The fitted line in double log scales has a slope near  $-1$ , indicating a near inverse-proportional relationship between these two parameters, and therefore between resealing time and local exocytosis. This is consistent with the qualitative observation that rapid local exocytosis leads to fast resealing which in turn terminates exocytosis. The averaged specific rate of exocytosis was highly dependent on extracellular  $\text{Ca}^{2+}$  (Fig. 3 C), in a manner consistent with the calcium dependence of membrane resealing (Steinhardt et al., 1994).

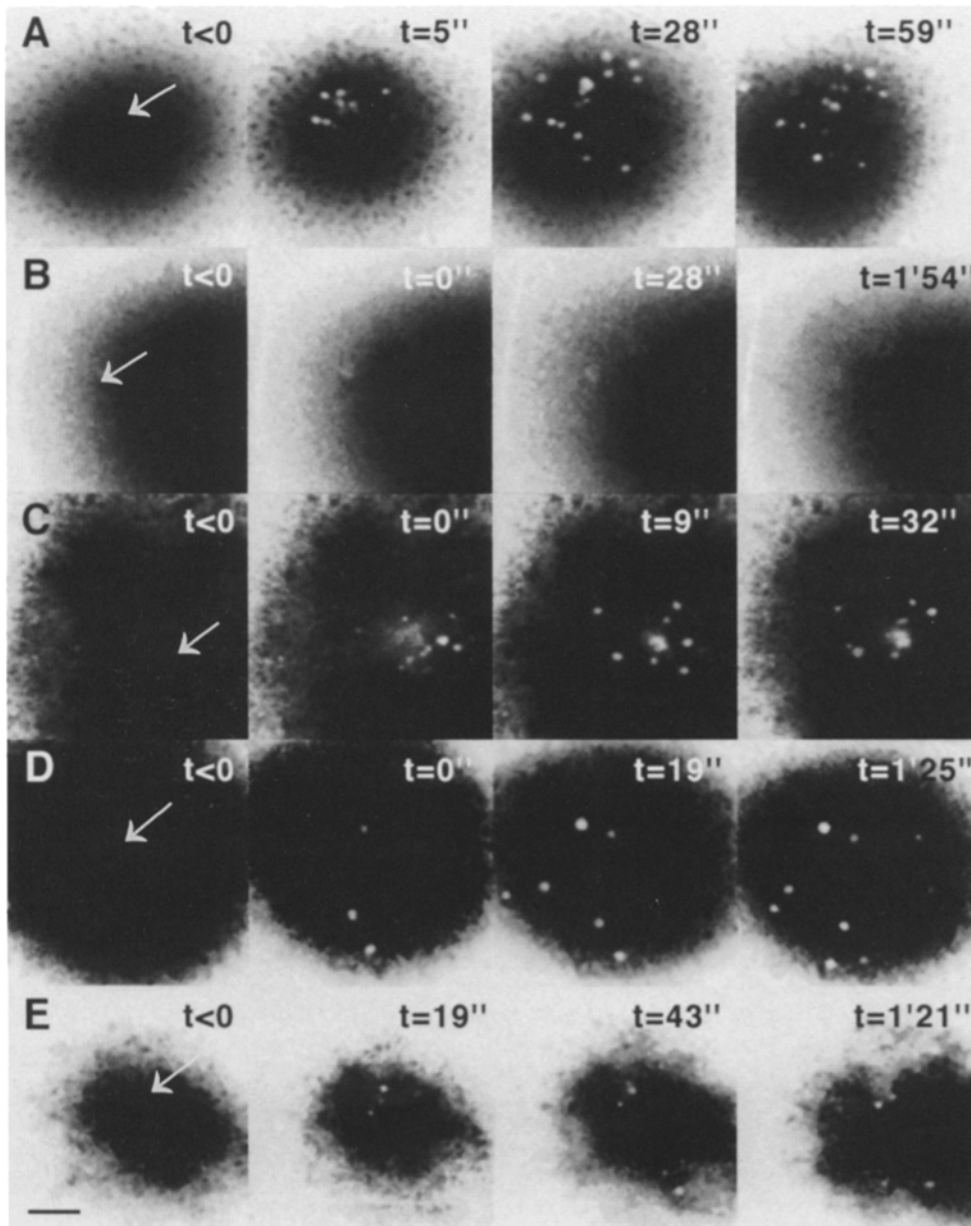
#### Calcium-regulated Exocytosis in Early Sea Urchin Embryos Is Induced by Wounding and Plays a Key Role in Plasma Membrane Resealing

The previously reported effects of neurotoxins on membrane resealing suggested the existence of calcium-regulated exocytosis in early sea urchin embryos. However, exocytosis had never been observed in embryos. Since exocytotic vesicles in other preparations are too small to be resolved optically, we were pleasantly surprised to be able to record distinct images of exocytosis after laser wounding on the plasma membrane of early sea urchin embryos (Fig. 4).

This pattern (Fig. 4 A) was in many aspects similar to that of cortical vesicle exocytosis in unfertilized eggs during membrane resealing. The appearance of bright disks began within 5 s near the wound site and usually ceased by  $\sim 15$ – $30$  s. The sizes of the disks appeared to be slightly smaller and more variable in size than those of cortical vesicles. Each disk emerged quite abruptly (virtually no intermediate state can be captured at a scanning rate of 1.5 s/frame), consistent with the characteristics of fast exocytosis

range of data under different conditions. Data for 2 and 10 mM  $[\text{Ca}^{2+}]_0$ , where cells resealed after wounding was fitted with the power equation (best fit at  $y = 0.351x^{-0.923}$ ) and gave a straight line in double-log scales.  $\square$ ,  $\text{Ca}^{2+} = 0.4$  mM;  $\triangle$ ,  $\text{Ca}^{2+} = 2.0$  mM;  $\circ$ ,  $\text{Ca}^{2+} = 10$  mM. (C) Average of specific rates at different calcium conditions. Bars are standard errors.





**Figure 4.**  $\text{Ca}^{2+}$ -regulated exocytosis detected during membrane resealing in early sea urchin embryos. Laser wounding induced an exocytosis pattern in 4–8-cell stage embryos similar to that of cortical vesicle exocytosis in unfertilized eggs. This exocytosis was absent without extracellular calcium and could be inhibited by injection of proteolytic neurotoxins. Because most (if not all) cortical vesicles had exocytosed during fertilization (Vogel et al., 1991; Whitaker, 1994), the pattern observed here reveals a novel form of calcium-regulated exocytosis. (A) Intact cell in NSW. A burst of exocytosis around the wound site is clearly seen. (B) Intact cell in zero calcium ASW. No apparent exocytosis is seen. (C) Control buffer-injected cell in NSW. Similar exocytosis pattern as in uninjected cell. (D) Tctx-injected cell in NSW. Exocytosis still occurred but was slower and more diffuse spatially compared with control cells. The cell failed to reseal. (E) BNT-A LC injected cell in NSW. Typical slow and diffuse exocytosis in toxin-injected cells. The cell shape change was common in toxin-injected cells after wounding. Bar, 5  $\mu\text{m}$ .

rather than slow endocytosis. About a third of the disks lasted for only a few seconds, presumably disappearing after the exocytosed membrane flattened, and new disks appear. To verify the calcium dependence of this exocytosis, we wounded the embryos in calcium-free ASW (Fig. 4 B). No exocytotic pattern occurred under such conditions and the injured cells appeared dead or dying in  $\sim 20$ –30 min. If NSW was added back quickly, cells wounded in calcium-free ASW could induce the usual exocytotic pattern (data not shown).

#### **Additional Tests of Neurotoxins on Plasma Membrane Resealing in Embryos**

In our previous experiments (Steinhardt et al., 1994), we showed that BNT-B and BNT-A could block membrane resealing in fibroblasts and in early embryos of sea urchin, *L. pictus*, but not in unfertilized sea urchin eggs. Since both

toxins are proteases selectively targeting vesicle docking/fusion proteins synaptobrevin and SNAP-25, respectively (Schiavo et al., 1992, 1993; Binz et al., 1994), we proposed that a similar protein complex might be responsible for membrane resealing. In the present study, we extended the previous characterization of resealing by investigating the effects of BNT-C1 which cleaves syntaxin (Blasi et al., 1993), a plasma membrane component of the docking/fusion complex (Bennett et al., 1992), tetanus toxin (Tctx) which cleaves VAMP/synaptobrevin (Schiavo et al., 1992), as does BNT-B and the effects of purified light chain of BNT-A (BNT-A LC), the proteolytic subunit of BNT-A, which cleaves SNAP-25 (Mochida et al., 1989; Dayanithi et al., 1990; de Paiva and Dolly, 1990). We also were able to make use of more recent knowledge of the cleavage sites for SNAP-25 and syntaxin to perform additional controls. In fertilized eggs and early embryos, resealing after puncture by micropipette was completely blocked by all these

Table I. Inhibition of Plasma Membrane Resealing in Early Sea Urchin Embryos by Proteolytic Toxins

Reagents injected	Percentage resealing			
	0-45 min	46-90 min	91-135 min	136-180 min
Tetanus toxin	92% n = 13	17% n = 23	0% n = 16	0% n = 2
BNT-A	100% n = 3	0% n = 5	0% n = 8	0% n = 8
BNT-A + SNAP-25 peptide	100% n = 3	100% n = 6	71% n = 7	0% n = 7
BNT-A + 19 aa peptide	80% n = 5	0% n = 5	0% n = 8	0% n = 7
BNT-A LC	80% n = 5	20% n = 10	0% n = 5	—
BNT-C1	87% n = 15	7% n = 29	0% n = 15	0% n = 7
BNT-C1 + syntaxin peptide	80% n = 10	100% n = 7	86% n = 7	0% n = 7
BNT-C1 + VAMP/ synaptobrevin peptide	100% n = 3	0% n = 6	0% n = 8	0% n = 7

Cells were injected with different reagents and assayed for resealing by wounding with glass micropipette during different time periods after injection. All of the three toxins tested inhibited resealing in fertilized eggs. SNAP-25 peptide and syntaxin peptide contained cleavage sites of the corresponding toxins and delayed the effect of these toxins. VAMP/synaptobrevin peptide and 19-amino acid CaM kinase peptide (19 aa peptide) were used as controls. Although VAMP/synaptobrevin peptide delayed the effect of BNT-B (Steinhardt et al., 1994), it did not delay the effect of BNT-C1.

toxins ~1 h after injection (Table I). Coinjection of corresponding substrate peptides that contained the toxin cleavage sites (Schiavo et al., 1992, 1993; Blasi et al., 1993; Binz et al., 1994) delayed the block of resealing while nonsubstrate peptides had no effect, showing that the toxins inhibited membrane resealing specifically through their selective proteolytic activity. Purified BNT-A LC inhibited resealing similarly as the whole toxin.

#### Tests of Neurotoxins on Exocytosis in Embryos

With the confocal microscope, we examined the effect of neurotoxin on exocytosis using BNT-A LC and Tetx (Fig. 4, D and E). Surprisingly, we still detected some exocytotic disks in embryos injected with enough Tetx to block resealing when tested by micropipette puncture (Table I). However, in contrast to the rapid and localized exocytotic pattern in control cells (Fig. 4, A and C), the spatial and temporal pattern of exocytosis in toxin-injected sea urchin embryos was slower and more diffuse. Occasionally, a normal-like exocytotic pattern was also observed in cells that were injected with toxin (one case out of 18 experiments done in 4-8-cell stage embryos 2-3 h after injection (Fig. 5 B), and more frequently (7 out of 14 experiments) in experiments done 4-6 h after injection of toxin). Eventually, toxins were rendered less effective in blocking exocytosis and resealing in the rapid-developing embryos, something we had not observed before since micropipette punctures were technically very difficult at these later stages.

Quantification of these experiments shows clear inhibitory effects of these neurotoxins on exocytosis (Fig. 5). In addition, similar to the results in unfertilized eggs, uninjected and control buffer injected embryos also showed strong correlation between the specific rate of exocytosis and the time constant (Fig. 5 B, Spearman test two-tailed  $P$  value < 0.01). The slope of the fitted line in double log scales is also near -1, indicating that faster local exocytosis promotes resealing, ending the exocytotic burst as calcium influx ceases. Data from the toxin injected cells deviated significantly from the fitted line based on the data from the control cases. This might be due to the partial depletion of exocytosis-competent vesicle pools by the toxins, elevated intracellular  $Ca^{2+}$  that speeded up exocytosis of remaining vesicles, contribution to resealing from smaller vesicles that escaped confocal imaging, and/or additional

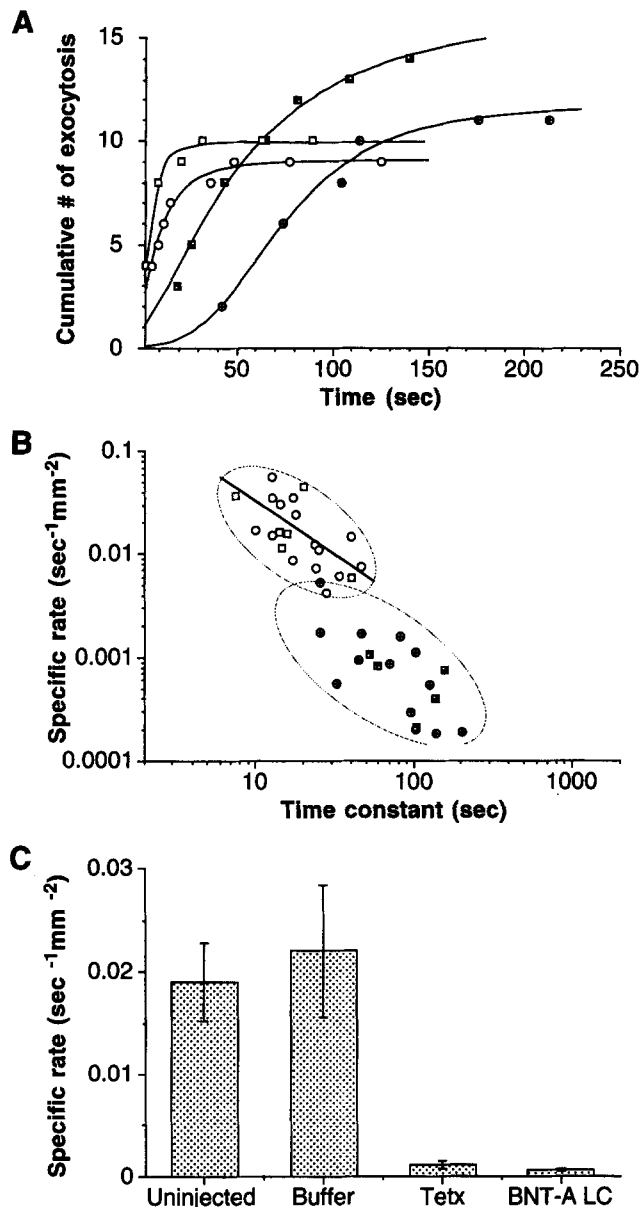
mechanisms such as cytoskeleton dynamics that contribute to the resealing process.

#### Undocking Cortical Vesicles with Stachyose Reversibly Inhibits Exocytosis and Membrane Resealing in Unfertilized Sea Urchin Eggs

Although we could interfere with membrane resealing in fibroblasts and sea urchin embryos using several specific reagents, resealing in unfertilized sea urchin eggs was not affected by most of them (Steinhardt et al., 1994). Therefore, we had no clear loss-of-function test for unfertilized egg (i.e., if exocytosis is indeed required, resealing should fail even in unfertilized eggs once we blocked exocytosis). The ability of stachyose to reversibly undock cortical vesicles has now allowed us to examine the role of exocytosis in the resealing of unfertilized sea urchin eggs.

Injection of the fluorescent dye calcium green dextran into unfertilized sea urchin eggs showed the clear pattern of docked cortical vesicles (Fig. 1 G). Using the same technique, we were also able to directly investigate the undocking and redocking of cortical vesicles in vivo. In sea water that contains 0.4 M stachyose (vesicles were not undocked at this concentration [Chandler et al., 1989]), the docking pattern of cortical vesicles was not significantly different from the normal docking pattern (Fig. 6 A). However, the pattern was substantially changed in 0.8 M stachyose (Fig. 6 B). The disappearance of dark disks indicates lack of vesicles near the membrane region, consistent with EM evidence that they have been undocked by this level of stachyose (Chandler et al., 1989). After the stachyose concentration is reduced, the normal docking pattern recovers (Fig. 6 C), indicating that vesicles have redocked to the membrane.

The ability to exocytose these cortical vesicles corresponded precisely with their docking status (Fig. 7). 0.4 M stachyose had only minor effects on the exocytosis in the eggs after laser wounding (Fig. 7 A). However, in 0.8 M stachyose solution, exocytosis was virtually absent and the wound persisted (Fig. 7 B). After stachyose was washed out, the wound-induced exocytosis pattern recovered completely (Fig. 7 C), showing the excellent reversibility of this undocking operation. Similar effects were seen when eggs were tested for resealing by micropuncture (Table II). All eggs resealed normally in the conditions where confocally

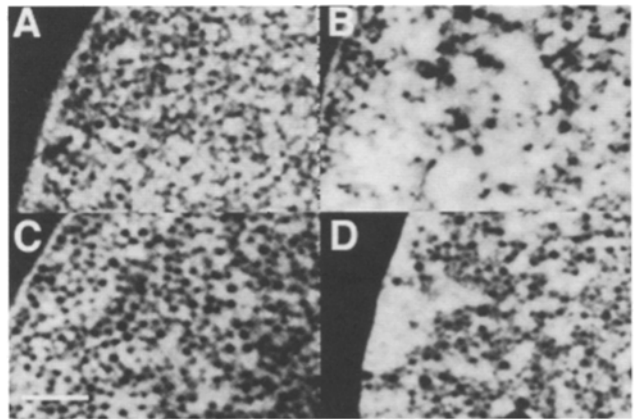


**Figure 5.** Quantification of wound-induced exocytosis in early sea urchin embryos. (A) Sample traces of exocytosis in sea urchin embryos under different conditions. (B) Scatter graph summarizes all images of exocytosis in sea urchin eggs under different conditions. The ellipses indicate the range of data under normal (uninjected and buffer-injected) or inhibitory (toxin-injected) conditions. Data from normal conditions were fitted using the power equation (best fit  $y = 0.292x^{-0.997}$ ) to give a straight line in double-log scale. ○, uninjected; □, buffer control; ⊕, Tetx; ⊞, BNT-ALC. (C) Average of specific rates of exocytosis under different conditions. Bars indicate standard errors.

visualized exocytosis was normal. None could reseal when exocytosis was blocked in 0.8 M stachyose. The inhibition of resealing by 0.8 M stachyose was also completely reversible when stachyose was diluted to  $\leq 0.4$  M.

**Transient Undocking of Cortical Vesicles Allows BNT-C1 and Tetx to Inhibit Resealing in Unfertilized Sea Urchin Eggs**

We have shown that resealing in unfertilized eggs was not

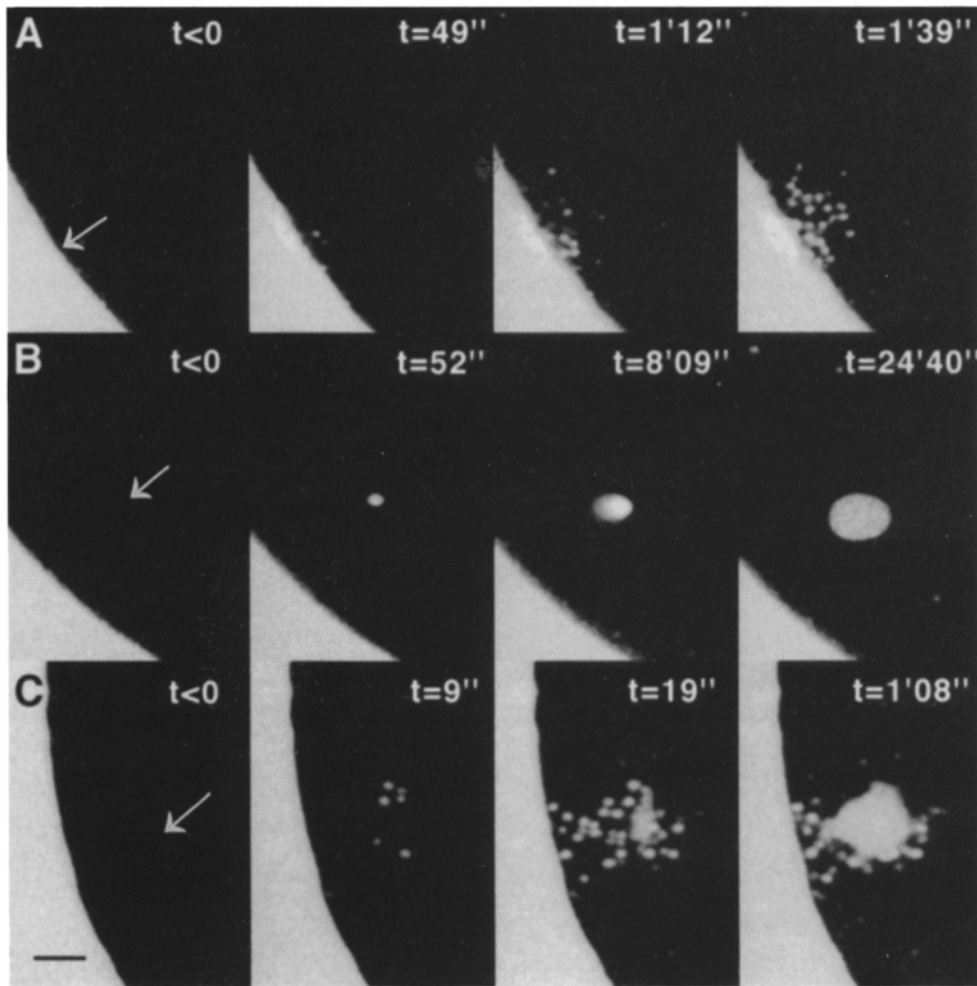


**Figure 6.** Docking of cortical vesicles in unfertilized sea urchin egg and the effect of stachyose. Cells were injected with calcium green dextran and confocal images were obtained by scanning just inside the cell surface for calcium green fluorescence. Dark disks with relatively uniform morphology show submembrane docked cortical vesicles. (A) Cell in 0.4 M stachyose. Granule morphology is affected, but the general pattern of docking remains similar to that of intact eggs (see Fig. 1 G). Treatment time is not critical, this image was taken  $\sim 1$  h after changing into stachyose SW. (B) Cell in 0.8 M stachyose. Very few vesicles remain docked. Most of the region is devoid of vesicles. Treatment time is not critical, this image was taken  $\sim 1$  h after changing into stachyose SW. Some of the big dark disks near the center of the image may be projections of endoplasmic reticulum since they continued indefinitely as we focused down into the interior. (C) Cell was treated with 30-min 0.8 M stachyose SW pulse. This image was taken  $\sim 20$  min after stachyose SW was replaced with NSW. The cell has recovered its normal morphology. The normal pattern of docked cortical vesicles also recovered. (D) Cell injected with Tetx and treated the same as in C. The cell had recovered its normal morphology when imaged. More than 50% of the region has recovered its normal docking pattern.

affected by injection of any of the neurotoxins we tested (Steinhardt et al., 1994). We assumed this was because docked cortical vesicles were already competent to exocytose and their docking/fusion proteins were not accessible to enzymatic digestion by these toxins. This is consistent with *in vitro* results showing that synaptobrevin and SNAP-25 are protected against proteolysis by Tetx or BNT-A when they form a complex with syntaxin (Hayashi et al., 1994; Pellegrini et al., 1994). However, we could not exclude the possibility that the protein isoforms on cortical vesicles were not cleavable by the toxins, or less likely, that the resealing mechanism in unfertilized eggs might be different. By undocking the vesicles with stachyose in the presence of injected toxins, we were able to examine this issue in more detail.

We injected Tetx, BNT-A, or BNT-C1 into unfertilized sea urchin eggs as well as some early embryos. Parallel tests of resealing in embryos were done to confirm that the toxins were working as expected. After  $\sim 1$  h, when the injected embryos began to fail after wounding, we treated the unfertilized eggs with 0.8 M stachyose for 30 min, and then returned them back to normal sea water to test for resealing. Eggs preinjected with BNT-C1 or Tetx failed to reseal after this transient stachyose exposure (Table III). Apparently, undocking of cortical vesicles exposed the toxin binding/cleavage sites of these proteins. However,





**Figure 7.** High levels of stachyose reversibly inhibit wounding-induced exocytosis of cortical vesicles in unfertilized sea urchin eggs. (A) Egg in 0.4 M stachyose. Exocytosis pattern is similar to that in normal conditions except that the rate is obviously lower and that the expansion of secreted material from vesicles was partially inhibited by the elevated osmolarity. The cell resealed in  $\sim 1.5$  min. (B) Egg in 0.8 M stachyose. Bright fluorescence blob at the center of each image indicates slow but persistent dye leakage through the wound. Very few exocytotic disks can be seen. (C) Egg was treated with 30-min 0.8 M stachyose pulse. Stachyose had been washed away before imaging. Exocytosis is very similar to that in untreated cells both in spatial and in temporal patterns.

unfertilized eggs injected with BNT-A and exposed to 0.8 M stachyose for 30 min, still resealed normally (Table III) although BNT-A inhibited resealing of the fertilized embryos in the parallel experiments. This result would be consistent with the interpretation that the BNT-A binding/cleavage site on SNAP-25 is protected by binding to syntaxin even after vesicles were undocked by stachyose.

#### **Transient Undocking of Cortical Vesicles Allows Tetx to Inhibit Exocytosis in Unfertilized Sea Urchin Eggs**

Fig. 6 D shows a typical example of the redocking of cortical vesicles in an egg that was injected with Tetx and treated with 30-min stachyose (0.8 M) pulse ( $n = 4$ ). Although the number of “redocked” vesicles appeared smaller than that in eggs without toxin injection (Fig. 6 C), it was apparent that a significant portion of vesicles had morphologically redocked at the membrane. However, when these eggs were wounded with a laser beam, these vesicles were unable to exocytose (Fig. 8 B). In contrast, cortical vesicles in unfertilized eggs injected with Tetx, but not treated with stachyose, exocytosed normally (Fig. 8 A). These results were perfectly consistent with the resealing test results, suggesting that the synaptobrevin/VAMP-like protein in sea urchin eggs plays a relatively minor role in the morphologically defined docking of cortical vesicles, but is essential for their fusion in exocytosis.

## **Discussion**

### **A Dynamic View of Plasma Membrane Resealing**

In the present study, we used confocal microscopy to visualize individual  $Ca^{2+}$ -regulated exocytotic events during membrane resealing. When a sea urchin egg was wounded with a laser beam, rapid and localized exocytosis followed calcium influx at the wound site. Similar wounding-triggered exocytosis patterns were also seen in early sea urchin embryos, which required new vesicles to dock and exocytose.

**Table II. Effect on Plasma Membrane Healing of the Addition of a Tetrasaccharide, Stachyose, to the Sea Water Surrounding Unfertilized Eggs of *L. pictus***

Treatment	Total time of stachyose treatment before wounding	Percent resealing	<i>n</i>
In 0.4 M stachyose	5–95 min	100	10
In 0.8 M stachyose	5–35 min	0	7
Transfer into 0.4 M stachyose after 40 min in 0.8 M solution	45–71 min	100	16

Cells resealed normally in sea water containing 0.4 M stachyose. However, 0.8 M stachyose inhibited resealing. This inhibition was completely reversed when cells were returned to noninhibitory conditions (e.g., 0.4 M stachyose or normal sea water).

**Table III. Effect of Proteolytic Neurotoxins on Membrane Resealing in Unfertilized Eggs of *L. pictus***

Toxin tested	Percent resealing and number of experiments			
	Toxin injection only		Toxin injection + 30 min stachyose treatment	
Tetx	100	n = 9	0	n = 10
BNT-C1	100	n = 12	0	n = 11
BNT-A	100	n = 15	100	n = 10

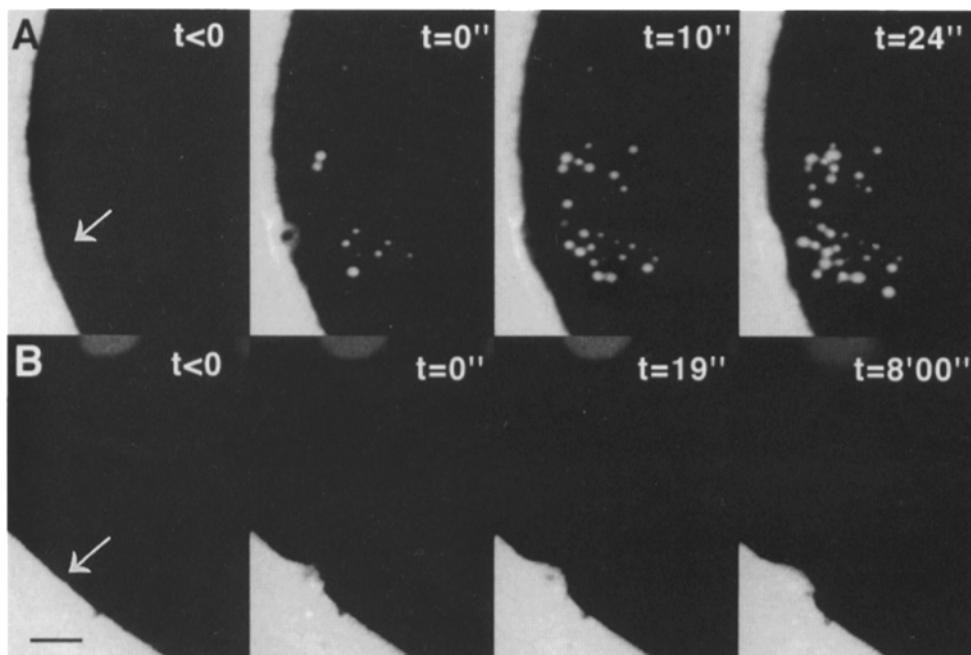
The transient stachyose treatment was done by exposing injected cells to 0.8 M stachyose (0.53 g/ml sea water) for 30 min after, in parallel experiments, toxin-injected fertilized eggs began to lose resealing ability. The unfertilized eggs were tested for resealing ability after stachyose was washed away.

tose upon calcium influx, since the cortical vesicles had previously fused after fertilization. Quantitative analysis of the spatial and temporal dynamics of wound-triggered exocytosis in both unfertilized eggs and in early embryos showed that the rate of local  $Ca^{2+}$ -triggered exocytosis is strongly correlated with the kinetics of resealing. The inhibitory action of the clostridial neurotoxins on membrane resealing has now been directly shown to be a block of the exocytotic process in this preparation. We have good evidence that this inhibitory action results from selective proteolytic action on members of the SNARE complex as coinjection of corresponding substrate peptides that contained the toxin cleavage sites for synaptobrevin, SNAP-25, and syntaxin matched appropriately with specific delays of the block of resealing. We also studied the resealing and exocytosis in unfertilized sea urchin egg with the ability to reversibly undock cortical vesicles with stachyose. Both exocytosis and resealing are reversibly inhibited when cortical vesicles are undocked. In addition, we have also shown that Tetx and BNT-C1 can inhibit resealing and exocytosis

in unfertilized eggs when their cortical vesicles are transiently undocked by stachyose. These results have demonstrated that resealing in unfertilized eggs is not an exception from the same exocytotic mechanism.

Our results, and those of the companion paper by Miyake and McNeil (1995), suggest that a ubiquitous form of a  $Ca^{2+}$ -regulated exocytosis is a required, and possibly a rate-limiting step, for plasma membrane resealing. This mechanism may finally provide the explanation for the description of the protective calcium-requiring formation of membrane, the surface precipitation reaction described nearly six decades ago (Heilbrunn, 1937).

Studies on synthetic membranes and on RBC ghosts have shown that lipid bilayers have the ability to reseal their ruptures spontaneously (Hoffman et al., 1960; Bodemann and Passow, 1972; Billah et al., 1977; Chang and Reese, 1990; Hoffman, 1992), presumably driven by the thermodynamic force that favors a continuous sheet of amphiphilic lipid bilayers in water. However, this passive resealing is not likely to be enough for living cells to survive since it is too slow (time constant ranges from 30 minutes to hours [Billah et al., 1977]) unless the lesion is extremely small (Chang and Reese, 1990). In contrast, living cells normally reseal their wounds within 10 s (Steinhardt et al., 1994). Moreover, resealing in living cells requires extracellular  $Ca^{2+}$  and is antagonized by  $Mg^{2+}$  (Steinhardt et al., 1994), while the passive resealing in RBC ghosts happens without  $Ca^{2+}$  and can be facilitated by  $Mg^{2+}$  (Bodemann and Passow, 1972). We speculate that in living cells, plasma membrane surface tension and the interaction with dynamic elements of cytoskeleton may prevent a membrane wound from being closed without additional material, or make such a "closure" movement too slow for cell survival.



**Figure 8.** Effect of Tetx on cortical vesicle exocytosis in unfertilized sea urchin eggs. Tetx injection did not affect wounding-induced exocytosis of cortical vesicles in otherwise intact unfertilized eggs. The combination of Tetx injection and transient undocking by stachyose irreversibly blocked vesicle exocytosis. (A) Exocytosis of cortical vesicles in unfertilized eggs injected with Tetx. (B) Inhibition of exocytosis by Tetx in unfertilized eggs that have been treated with 30-min stachyose pulse (0.8 M). Wounding and imaging were done after stachyose had been washed away, and cortical vesicle docking pattern had partially recovered. Continuous dye leakage through the wound indicates lack of resealing. This cell visually lysed ~30 min after wounding. In contrast, cells without Tetx injection exocytosed and resealed normally after this treatment (see Fig. 7 C).

With the rapid exocytosis triggered by the calcium influx through the wound, however, relatively large amounts of fresh material from the fused vesicles adds into the plasma membrane near the wound site, therefore helping alleviate surface tension and other constraints on the local membrane.

### *A Ubiquitous Form of Calcium-regulated Exocytosis*

Although calcium-regulated exocytosis in neurons has been well-characterized (Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994), it is generally assumed to exist only in certain specialized cell types, while it has been thought that most cells can only exocytose in a calcium-independent "constitutive" way. However, in concert with some recent works suggesting calcium-dependent exocytosis in muscle cells (Dan and Poo, 1992) and in fibroblasts (Girod et al., 1995), our imaging experiments in sea urchin embryos have provided direct evidence for calcium-regulated exocytosis in these nonsecretory cells. Since membrane resealing has been observed in all cell types tested, our results imply the existence of a ubiquitous form of calcium-regulated exocytosis in a much larger variety of eucaryotic cells.

This ubiquitous calcium-regulated exocytotic mechanism might be a phylogenetically primitive form of more specialized forms of calcium-regulated exocytosis such as neurotransmission. Membrane disruptions would have been a common challenge for the survival of many eucaryotic cells and rapid calcium-triggered exocytosis may have been selected early as a successful strategy of membrane resealing. Slight modifications of this basic mechanism for rapidly adding new material into plasma membrane for repair could have provided the basis for the evolutionary development of diverse exocytotic and endocytotic mechanisms both for regulated secretion and for remodeling of the plasma membrane surface.

### *Conserved Molecular Mechanism*

The molecular mechanism of this calcium-regulated exocytosis appears to have a highly conserved framework. The fact that Clostridial neurotoxins can inhibit membrane resealing indicates that proteins homologous to VAMP/synaptobrevin, syntaxin, and SNAP-25 mediate the exocytosis that is required in resealing. This is consistent with the finding of cellubrevin (McMahon et al., 1993), a protein homologous to VAMP/synaptobrevin that exists in many cell types, and the studies suggesting that cellubrevin is not specialized for constitutive exocytosis (Chilcote et al., 1995). Our imaging experiments further directly demonstrated the involvement of conserved SNAP-25 and VAMP-like proteins in this ubiquitous form of regulated exocytosis. We would also have liked to directly examine the effects of BNT-C1 on exocytosis with our imaging system. We would have expected to see similar inhibition as seen with Tetx and BNT-A because BNT-C1 also inhibits resealing. Unfortunately, unlike the other two toxins, the pure protein form of BNT-C1 or its light chain was not available to us. The crude toxin we used in the resealing experiments appeared to have some complicated effects on cell morphology that made it impossible to do the more demanding imaging experiments on the four-cell stage of the embryo.

In vitro experiments have shown that VAMP/synaptobrevin, syntaxin, and SNAP-25 can form a stable protein complex resistant to the proteolytic attack from neurotoxins (Hayashi et al., 1994; Pellegrini et al., 1994). Our experiments on resealing and exocytosis in unfertilized sea urchin eggs provide in vivo evidence for the formation and functional integrity of such a complex. Furthermore, similar to the case in nerve terminals, where Tetx inhibits exocytosis of synaptic vesicles but does not inhibit their docking (Hunt et al., 1994), sea urchin cortical vesicles could redock, although to a lesser extent, but could not exocytose after Tetx cleavage. These results indicate that neurotoxins may attack VAMP/synaptobrevin, syntaxin, SNAP-25 and their homologous proteins before vesicle docking and although VAMP/synaptobrevin and its homologue may not be essential for the morphologically defined docking, they are essential for vesicle fusion.

The membrane resealing process has provided a simple assay to investigate the conserved mechanisms of calcium-regulated exocytosis. This system has several advantages. Since membrane resealing can be easily observed in any cell system, regulated exocytosis can be studied in any cell, including those that are easily manipulated at the molecular level. In sea urchin eggs, individual exocytotic events can be directly monitored with the confocal microscope. With these advantages, in vivo experiments may be able to be designed to dissect the roles of individual proteins in docking and fusion.

The low level of intracellular free calcium and its tight control by intracellular buffers and transporters, makes calcium influx an ideal signal to trigger and focus the membrane repair process. At synapses, voltage-regulated calcium influx appears to play regulatory roles at multiple steps in exocytosis (Neher and Zucker, 1993). It is reasonable to assume that calcium may regulate more than one step in the more ubiquitous form of calcium-regulated exocytosis described here. In the special case of unfertilized eggs, since the cortical vesicles have already docked on the plasma membrane before calcium elevation, calcium obviously triggers a downstream fusion event that could be mediated by one or more specialized calcium sensor molecules. In neural tissue exocytosis is extremely rapid and calcium sensitivity may be linked to forms of synaptotagmin adapted just for submillisecond fusion events in neurotransmission (DeBello et al., 1993; Geppert et al., 1994; Heidelberger et al., 1994; Schwarz, 1994). More recently other homologues of synaptotagmin have been found in nonneural tissue (Li et al., 1995). Many steps including vesicle recruiting, docking, fusion, and the resulting endocytosis could be targets for calcium. This may be reflected in the higher calcium thresholds for resealing in embryos, whose vesicles must be recruited and docked before fusion (Steinhardt et al., 1994).

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