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Patching plasma membrane disruptions with cytoplasmic membrane

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SUMMARY

Vesicle-vesicle fusion initiated in cell cytoplasm by high Ca^{2+} can rapidly erect large membrane boundaries. These might be used as a 'patch' for resealing plasma membrane disruptions. Three central predictions of this 'patch' hypothesis are here established in sea urchin eggs. First, we show that surface markers for plasma membrane protein and lipid are initially absent over disruption sites after resealing is complete. Second, we demonstrate that resealing capacity is strongly dependent upon local availability of fusion competent cytoplasmic organelles, specifically the reserve or yolk granule. Lastly, we demonstrate that the reserve granule is capable of rapid ($t_{1/2}$ <1 second), Ca^{2+}-regulated (high threshold) fusion capable of erecting large (>1000 μm^2), continuous membrane boundaries. Production of patch vesicles for resealing may proceed by an 'emergency' fusion mechanism distinct from that utilized for the much slower, highly regulated, cytosol-requiring organelle-organelle fusion events typical of constitutive membrane trafficking pathways.

Key words: Plasma membrane, Disruption, Resealing, Fusion, Homotypic

INTRODUCTION

Plasma membrane disruption (PMD) occurs not only in the laboratory, when for example a cell is impaled by a microneedle, but also in nature. PMD injury is a widespread, common and normal cell event in many mechanically active mammalian tissues that scales with imposed load (McNeil, 1993). This suggested the existence of a multiplicity of undiscovered adaptations that might allow cells to survive or respond to PMD. Resealing is one such predicted adaptation, since, in its absence, cell death is the rapid and invariant outcome.

Indeed the biological importance of resealing is probably reflected in the diversity and complexity of described resealing mechanisms. Small disruptions, on the nanometer scale, are thought to be resealed passively as a result of thermodynamically driven lipid interactions. Erythrocytes are capable of resealing on this scale, such as occurs during 'reversible' electroporation (Benz and Zimmermann, 1981) but fail to reseal, or reseal very slowly (hours), larger disruptions, induced for example by higher, 'irreversible' electroporating voltages (Serpersu et al., 1985). Larger disruptions, in the micron diameter range such as are typical of microneedle punctures, are rapidly (seconds) resealed by nucleated animal cells, but resealing on this scale requires exocytotic addition of internal membrane to the cell surface (Bi et al., 1995; Miyake and McNeil, 1995). Such additions may promote resealing, in part, by reducing plasma membrane surface tension (Togo et al., 1999). For even larger disruptions (>10 μm^2) resealing is proposed to occur by a 'patch' mechanism that we will describe below (Terasaki et al., 1997). Cells lacking a sufficiently abundant supply of vesicles for local fusion-mediated construction of these patches, or for exocytosis-mediated reduction of membrane surface tension, may create them locally by initiating endocytosis at PMD sites (Eddleman et al., 1997, 1998). Even in cells rich in internal membrane, such as fibroblasts and sea urchin eggs, active transport of vesicles to the disruption site may be used to ensure a supply sufficient for resealing purposes (Bi et al., 1997; Steinhardt et al., 1994). Giant cells, such as the *Xenopus* egg, display contractile 'purse strings' in the peri-disruption cell cortex composed of actin-based cytoskeleton (Bement et al., 1999). These 'purse strings' may aid in restoration of cortex integrity.

We propose that the mechanism used for resealing large (>10 μm^2 in extent) plasma membrane disruptions is analogous to that used by a mechanic to patch a hole in a tire (Terasaki et al., 1997). According to this 'patch' hypothesis, influx of Ca^{2+} into cytosol through the disruption site down an ~10^4-fold concentration gradient creates a peri-disruption zone of cytosolic calcium elevation. Directed vesicle movement concentrates intracellular membrane in the peri-disruption space. The elevated Ca^{2+} present there induces accumulated vesicles to fuse with one another, forming a larger vesicle or vesicles that rapidly fill up the peri-disruption space. These we call 'patch' vesicles, because, in the final step in this model, we propose that these large vesicles are added, as a kind of 'patch', to the plasma membrane by heterotypic fusion events.

This event completes resealing. The patch hypothesis is, at present, uniquely capable of explaining how large plasma membrane disruptions, many hundreds or thousands of square microns in extent, are resealed within seconds, and how therefore cells are able to survive this injury.

Lacking were direct tests of several key predictions of the patch hypothesis. The first of these predictions is that a domain of internal membrane, spanning the PMD site, will rapidly be added to the cell surface. To test this prediction, we have developed methods for visualizing living cell surface membrane protein and lipid dynamics during the occurrence of large PMDs. The second untested prediction is that in the local absence of this fusion competent vesicle compartment, resealing will fail. To test this prediction, we have developed a method for creating PMD after stratification in situ of the egg's vesicular compartments. The last untested prediction is that homotyptic fusion of the requisite organelle compartment will be rapid, extensive, and Ca^{2+}-regulated but with a high threshold. To test this prediction, we have developed a method for isolating reserve granules, and for reconstituting and characterizing homotypic fusion in vitro.

MATERIALS AND METHODS

Gametes

Starfish (*Asterina miniata*) were obtained from Marinus Inc. (Longbeach, CA), and sea urchins from Jennifer Jackson (*Lytechinus variegatus*; Duke University Marine Laboratory, Beufort, NC) or from Marinus Inc. (*Stronglyocentratus purpuratus*). They were maintained in running natural sea water (NSW) at the Marine Biological Laboratory (Woods Hole, MA) or in artificial sea water (ASW) in aquaria at the Medical College of Georgia (Augusta, GA) or University of Connecticut (Farmington, CT). Starfish gametes were accessed using a small sample corer (Fine Science Tools Inc., Foster City, CA). Sea urchin eggs were spawned from single or multiple gonads by injection of 0.5 M KCl. Sperm were stored until use at 4°C without dilution in sea water.

Exovate formation

For these exovate and the DI labeling experiments (below), egg envelopes had to be removed. *Lytechinus* eggs were dejellied by several NSW rinses and a small aliquot of sperm (1-2 μl) was added to eggs (~0.1 ml, wet-packed, or less) suspended in a small volume of NSW (no more than 0.3 ml). One minute later, the sperm/egg mixture was diluted 20-fold with 1.0 M glycine, and the eggs were agitated gently by repeated tube inversion over a minute interval. Finally, eggs were gently centrifuged (100 ***g***, 1 minute), resuspended in NSW, and washed (sedimentation under gravity) several further times in NSW before use in experiments.

Exovates (Rappaport, 1976) were formed by creating PMDs while simultaneously imposing a positive mechanical pressure on the egg. A chamber for accomplishing this consisted of a coverslip (22 mm square, No. 1) mounted on a slide and supported on one end only with a single piece of double-stick tape ('Scotch' brand). Eggs pipetted into the tape-raised end of this chamber are drawn by capillary action towards its narrower end. During this passage, the eggs suffer shear-induced PMD as they are dragged along and compressed by the chamber's narrowing side walls.

Triton-induced PMD

Lytechinus eggs, fertilized and de-enveloped as described above, were held in a microscope chamber previously described (Terasaki and Jaffe, 1993). Microneedle tips were positioned (micromanipulator; Narshige Scientific Instruments Laboratory, Tokyo, Japan) within ~5 μm of the surface of egg targets, and then ~22 pl of 3% Triton X-100 detergent dissolved in sea water was expelled from the tip onto the surface of the egg target to induce a PMD.

Microscopy

For confocal microscopy, an upright microscope (Axioskop; Carl Zeiss Inc., Thornwood, NY) was coupled with a scanning confocal microscope (MRC 600; Bio-Rad Laboratories, Cambridge, MA). For most experiments, images were saved directly to hard disk, but in the experiment shown in Fig. 3, the microscope was set to continuous scan, and each image was recorded on an optical memory disk recorder (Panasonic TQ-3038F) by use of a custom made trigger circuit (described at http://terasaki.uchc.edu/trigger.html).

For conventional transmitted and fluorescence microscopy, an upright microscope (Axioskop; Carl Zeiss Inc., Thornwood, NY) was coupled to a video camera ('Sensys', Photometrics Ltd, Tuscon, AZ) under the control of image acquisition and analysis software (IPLab Spectrum, Signal Analytics Corp., Vienna, VA).

Electron microscopy of isolated granules was performed as previously described (Terasaki et al., 1997).

DiI labeling and photobleaching

Five μl of a DiIC18(3) (Molecular Probes, Eugene OR) stock solution (2.5 mg/ml in ethanol) was added to 50 μl sea water containing fertilized de-enveloped *Lytechinus* eggs. Eggs were then swirled briefly, and rapidly (<5 seconds post dye addition) diluted 20-fold with ASW. Eggs were allowed to settle under gravity, and then washed several more times under gravity sedimentation in ASW before use in experiments.

For photobleaching, a ×40 plan neofluar NA 1.3 lens was used. The cells were imaged with the confocal microscope at the zoom 2 setting and bleached with full intensity laser light at the zoom 12 setting for 10× 1 second scans. The cells were then imaged at zoom 2 to monitor redistribution of fluorescence after the photobleaching.

Egg stratification and quantitation of PMD survival

Stronglyocentratus eggs, used because the heavy and light egg fragments of this species are readily distinguished from one another in transmitted light micrographs, and because stratification generates a large proportion of these fragments relative to intact eggs, were spun at 12,000 ***g*** in a microfuge (Beckman) onto a cushion of 25% Percoll (v/v; ASW diluent) until a majority had been split into light and heavy fragment halves (10-15 minutes). PMDs were then induced in the population by taking it up into and expelling it from a 1 ml syringe fitted with a 20-gauge needle (Becton-Dickinson; Clarke and McNeil, 1994). Samples of this population were taken prior to the first (controls) and after each full syringe stroke, FM1-43 dye (stock 1 mM in ethanol; working concentration, 2 μM) was added to each, and egg samples were then mounted for microscopy under a coverslip supported by double stick tape at each corner. Alternatively, PMDs were induced in populations adhered (15 minutes, 1 ***g***) to tissue culture grade plastic (6-well Costar dishes) coated with poly-L-lysine (0.1%, 10 minutes) by scraping them from this substratum with a rubber policeman (McNeil et al., 1984). Controls, that were not adhered to the plastic or scraped, and the scraped eggs, were then stained with FM1-43 dye before mounting for microscopy as just described.

Images (transmitted and fluorescence) of microscope fields containing each of the above generated populations were obtained at random and scored for the presence of intact (e.g. surface FM1-43 fluorescence only) oblong, whole eggs, and 'light' and 'heavy' fragments.

Reserve granule isolation and reconstitution of fusion in vitro

Stronglyocentratus eggs (~1-5 ml, wet-packed, the product usually of one or two highly productive spawns), used because of the large yeild per spawn, were dejellied by several natural sea water (ASW) washes, resuspended in 1-5 ml of a Ca^{2+}-free, K^{+}-based buffer (PKME) (Pipes,

50 mM, KCl, 425 mM, $MgCl_2$, 10 mM, EGTA, 5 mM, pH 6.7) containing a cocktail of protease inhibitors (aprotinin, TPCK, pepstatin A, trypsin protease inhibitor and benzamidine), and homogenized on ice by 8-10 passages through a 23-gauge needle. The crude homogenate was then layered on top of 50% Percoll and centrifuged (Ti 70.1 rotor) for 45 minutes at 20,000 RPM. The yellow-colored reserve granule compartment stratified in the approximate center of the gradient formed, below an upper band consisting of the endoplasmic reticulum, and above 4-5 additional bands representing heavier organelles. A second Percoll gradient fractionation was then conducted to increase purity. The granules, which tend to be present as clumps of 5-50 vesicles, could at this point be used in fusion assays (see below) or could be stored for 3-5 days on ice for later assays of fusion. After this interval of storage, there was a distinct 'rundown' of fusion activity.

Semi-quantitative assay of yolk fusion

Fusion reduces visible light scattering/absorption by a factor of 2- to 4-fold (Vogel and Zimmerman, 1992). Upon fusion, granule contents apparently expand (see Results) to fill the volume defined by the merged membranes. It is likely this dilution of granule contents that accounts for this decrease in light scatter. Fusion could therefore be measured easily, accurately and economically by placing granule samples in a standard 96-well microtiter plate and then monitoring visible light absorption in a plate reader (Spectra MAX 250, Molecular Devices, Sunnyvale, CA). Generally, we added 100 μl of a reserve granule suspension (~0.1-0.2 OD units density) per well and then initiated fusion by the addition of 100 μl of EGTA-buffered Ca^{2+}. EGTA buffers were calibrated with a Ca^{2+}-electrode to yield upon dilution into PKME buffer a range (1 μM-10 mM) of free Ca^{2+} ion concentrations. The fusion events initiated by Ca^{2+} addition proceed to completion during the short interval required for placing the plate in the reader and entering the requisite computer commands.

Estimation of fusion kinetics

Time lapse sequences of reserve granule homotypic fusion were visualized by differential interference contrast (DIC) optics on a Zeiss Axiovert 135 microscope coupled to a Phtometrics PXL camera. Uniblitz electronic shutters and a Lodl filter wheel were used to regulate transmitted light and epiport UV excitation. The timing sequence of these components were controlled by Inovision software running on a Silicon Graphics O2 computer. Reserve granules were centrifuged onto glass coverslips (Chestkov et al., 1998) and placed in a buffer containing caged calcium (1 mM DM-nitrophen, 500 μM $CaCl_2$) as previously described (Shafi et al., 1994). DIC images were obtained at 1 Hz and UV photolysis of DM-nitrophen was initiated by opening a shutter on the epifluorescence port of the microscope to irradiate the sample through a 360 nm band-pass filter. The Ca^{2+} is predicted to rise from from <1 μM before u.v. photolysis to ~500 μM after complete u.v. photolysis.

Light scattered by granules as a function of time after Ca^{2+} addition was measured in a fluorometer (excitation = 495 nm; emission = 495 nm; SPF-500 SLM Aminco) whose signal output was registered on a chart recorder. Granules (100 μl) were added to a sample cuvette placed in the fluorometer and recording begun. Fusion was then initiated by the rapid, syringe-mediated injection 900 μl of EGTA-buffered saline into the sample cuvette.

Measurement of granule and patch vesicle pH

A stock solution of fluorescein-rhodamine dextran (10 kDa) was made at 10 mg/ml in ASW. To determine the pH dependence of this reagent, it was diluted to 0.2 mg/ml in ASW solutions buffered at 0.3 pH unit intervals from 4.0 to 8.2 with either 20 mM sodium acetate, 20 mM MES, or 20 mM Hepes and the fluorescence ratio measured from paired confocal images of buffered solutions contained between a slide and coverslip.

For measurements of patch vesicle pH, starfish oocytes, selected because their large size and high responsiveness to SW injections facilitated the production of large 'patch' vesicles, were injected with 60 picoliters (2% volume injection) of 0.2 mg/ml fluorescein-rhodamine dextran in ASW (pH 8). Confocal, paired fluorescein and rhodamine images were then collected sequentially over time (for up to 6 hours) after injection, and the fluoresecence intensity ratio (fluorescein/rhodamine) measured from these paired images.

Solutions and reagents

Marine Biological Laboratory ASW contains 9.25 mM Ca^{2+}, 48.4 mM Mg^{2+}, 425 mM Na^{2+}, 9.0 mM K^+, 25.5 SO_4^-, 489 Cl^-; for 'Ca^{2+}-free' sea water (CFSW) the Ca^{2+} component is omitted. Intracellular buffer contained 350 mM potassium glutamate, 350 mM glycine, 3 mM $MgCl_2$, 0.57 mM $CaCl_2$, 10 mM EGTA, 20 mM Hepes, pH 6.7. All fluorescent reagents were obtained from Molecular Probes Inc. (Eugene, OR).

RESULTS

Proteins of the native plasma membrane labeled by conA are not utilized in forming a resealing membrane over a disruption site

When PMDs were induced in fertilized, de-enveloped eggs

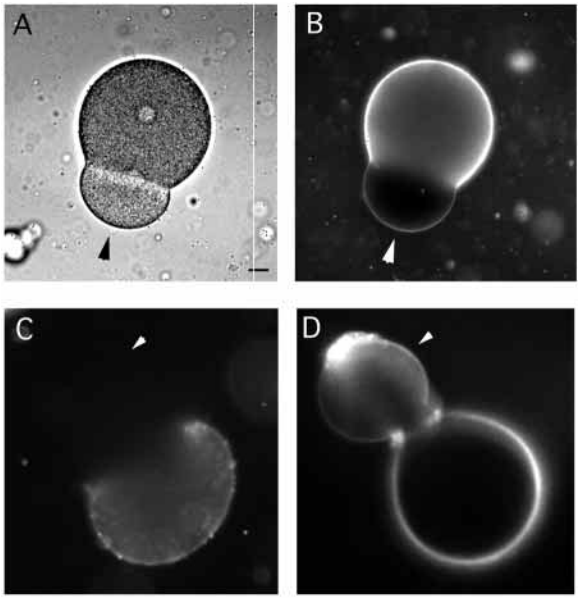

Fig. 1. The membrane that rapidly forms over sea urchin egg exovate domains does not incorporate pre-existent surface con A label. (A) Exovates (arrowhead) form when a PMD is made while the cell is held under a positive pressure, and cytoplasm herniates out of the PMD site. (B) The cell in A was immersed in the lipidic dye, FM1-43 (1 μM), and then imaged by fluorescence microscopy 15 seconds after exovate formation. The exovate surface is stained but internal membranes are entirely devoid of stain. Complete resealing over the large exovate domain shown here (r ~50 μm, area ~4000 μm^2) was therefore a rapid event that occurred before it was technically feasible to obtain a fluorescence image of the cell. (C) Another egg was stained on its surface with Oregon green con A (0.1 mg/ml; 15 minutes) before inducing exovate formation, and then this image was acquired 180 seconds later. The exovate that formed on this egg is entirely unstained with this label. (D) The same egg (as in C) stained 30 seconds later by immersion in FM1-43. The location of the exovate structure, that did not stain with con A, is now evident from the surface staining with FM1-43 (arrowhead). Bar, 100 μm.

maintained under a positive pressure between a slide and coverslip, exovates formed (Fig. 1A). Exovates, as has previously been described, consist of extrusions of cytoplasm through a PMD (Rappaport, 1976). Exovate formation is survived by fertilized eggs, some of which divide normally after exovate formation. Exovates are studied here because they comprise large domains of cytoplasm exposed to the external environment that require, for the egg's survival, formation of an extensive domain of resealing membrane.

When examined 15 seconds after formation, exovate surface domains were clearly labeled with FM1-43, a lipidic, membrane bilayer selective dye (Cochilla et al., 1999; Fig. 1B). Thus, in less than 15 seconds, membrane appears over the large (~4000 μm^2) exovate surface. In these experiments, the exovate was formed in the presence of FM1-43, which is water soluble and partitions rapidly between polar and non-polar environments, yet no staining of internal membranes was observed. Thus, a continuous coverage of membrane is also rapidly errected, e.g. resealing is rapidly complete, since otherwise dye entry through any remaining openings would have stained internal membranes.

Eggs incubated at 4°C in fluorescent con A became uniformly labeled on their surface (B. Babikov and S. S. Vogel, unpublished result). Labeled eggs, if allowed to warm to room temperature, internalized this fluorescence (not shown), confirming that the con A had labeled plasma membrane components, presumably membrane glycoproteins (Sharon and Lis, 1990). When exovates were formed in eggs pre-labeled on their surfaces with con A, this dye, by contrast with the FM1-43 distribution just described, was absent for >15 minutes from exovate surface domains (Fig. 1C). Secondary labeling, after imaging of con A labeling, with FM1-43 clearly revealed the presence of an exovate surface membrane on the Con A labeled eggs, showing that the con A treatment was not inhibiting membrane replacement over the exovate surface (Fig. 1D). Thus the membrane used for resealing exovates does not incorporate or involve components of the egg surface that are labeled by con A.

Lipid in native plasma membrane is also not utilized in forming a resealing membrane over a disruption site

The con A depleted surface domain over the exovate could be explained as: (1) a spread or 'flow' of lipid components from the surrounding plasma membrane across the disruption site that left behind more slowly diffusing or immobile components such as con A labeled glycoproteins; or (2) an addition of internal, unlabeled membrane to the disruption site. The prediction of the first of these alternatives is that if a fluorescent lipid marker is placed in the plasma membrane before disruption, then the appearance of this marker over the PMD site will be temporally co-incident with the resealing event itself. This prediction was tested by labeling the egg plasma

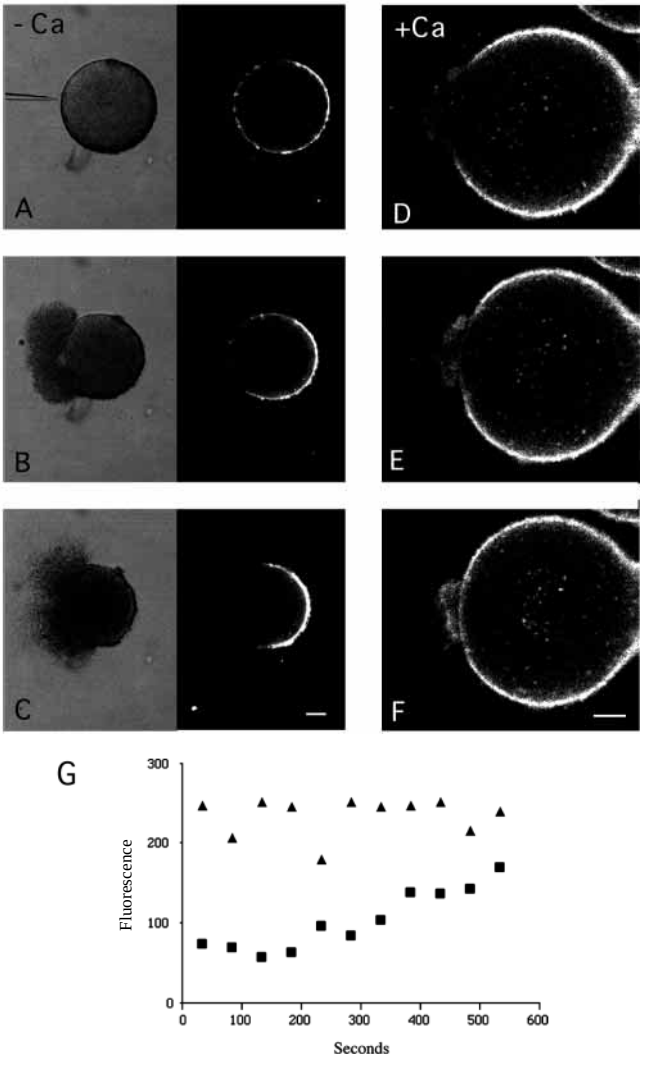

Fig. 2. Recovery of plasma membrane DiI fluorescence over sites of detergent generated PMD. (A) A microneedle containing Triton X-100 is positioned next to an egg stained on its surface with DiI and held in Ca^{2+}-free sea water. Resealing cannot occur in this medium, and so any PMD created will be readily demonstrated by leakage of cytoplasm. (B) At 10 seconds after detergent extrusion from the micropipette onto the egg surface, a large domain of surface is now devoid of DiI fluorescence, and cytoplasm is spilling out the cell in the region of this non-fluorescent surface domain. (C) At 330 seconds after detergent extrusion, loss of cytoplasm continues and there is no evidence of recovery of DiI fluorescence over the PMD site. (D) DiI fluorescence at 120 seconds after PMD production by detergent in the presence of NSW, containing ~10 mM Ca^{2+}. Resealing is permitted and complete (see Fig. 3) under this condition. However, no DiI fluorescence is evident over the PMD site at this time point. (E) At 250 seconds, return of DiI fluorescence over the PMD site begins to become evident. (F) At 420 seconds, return of DiI fluorescence is apparently approaching completion. (G) Quantitation of fluorescence recovery over the disruption site in NSW. An image analysis measurement tool 'line' was placed over the center of the PMD site present in the above image sequence (perpendicular to the tangent of membrane curvature at this point). The peak of DiI fluorescence measured along this measurement line was then recorded at various intervals in the complete time lapse sequence. Recovery of PMD site fluorescence (squares) above the initial level immediately after PMD creation is not evident quantitatively until ~200 seconds after PMD creation, probably because a residue of fluorescence debris remained after the detergent injury. A $t_{1/2}$ of fluorescence recovery over the disruption site of ~350 seconds is indicated by this data. The fluorescence measured from an undisturbed portion of the plasma membrane is shown for comparison (triangles). Bars, 100 μm.

membrane before PMD induction with DiI, an amphipathic, long-chain, irreversible bilayer dye (Axelrod, 1979), and then recording the time course of fluorescence recovery over the PMD site. For this purpose, a new method for large PMD production had to be developed as a substitute for the mechanically-based 'rip-off' technique previously employed (Terasaki et al., 1997).

To make large, well-defined PMDs in the absence of gross movement of the egg surface at the point of PMD-induction, detergent (Triton X-100) was extruded from a pipette onto the surface of eggs. That this maneuver produced a large PMD (~50 μm diameter) was demonstrated by carrying it out in CFSW (minus Ca^{2+}). Under this condition, which inhibits resealing, cytoplasm flowed out of a large DI negative gap in the cell surface present at the site of Triton-X exposure (Fig. 2A-C). In ASW (plus Ca^{2+}), resealing of these triton-induced PMDs was found to be complete within 3-5 seconds, as expected based on previous studies (Terasaki et al., 1997). Thus, the localized rise in cytosolic Ca^{2+} concentration produced by the triton-induced PMD began to decline at ~3 seconds after PMD, indicating completion of membrane barrier formation at this time point (Fig. 3A-F). As was the case when large PMD were created by the 'rip-off' technique, this rapid resealing allowed the egg to survive this large PMD injury. The Ca^{2+} rise initiated locally by the PMD did not spread across the egg, which could usually be fertilized successfully (Fig. 3G) and then undergo cleavage (Fig. 3H).

In comparison with the rapid kinetics of resealing, return of DiI fluorescence over the PMD site was a much slower process (Fig. 2D-F): quantitative measurement indicates a $t_{1/2}$ for DiI recovery of ~350 seconds over PMD sites (Fig. 2G). To confirm that the DiI was mobile in the sea urchin egg membrane, and therefore informative about membrane lipid dynamics, we photobleached surface domains of DiI labeled eggs. Fluorescence recovery of ~80% was observed over the bleach site (not shown) and quantitation of this event yielded an estimate for a diffusion constant for DiI of 1.3×10^{-8} cm^2/second, a value close to those previously reported for other lipids in egg plasma membrane (Wolf et al., 1981).

Thus, while a membrane domain rapidly (<15 seconds in exovates, <5 seconds in detergent-induced PMDs) forms over a large PMD site, this domain does not, at these time points, contain markers of plasma membrane protein or lipid components.

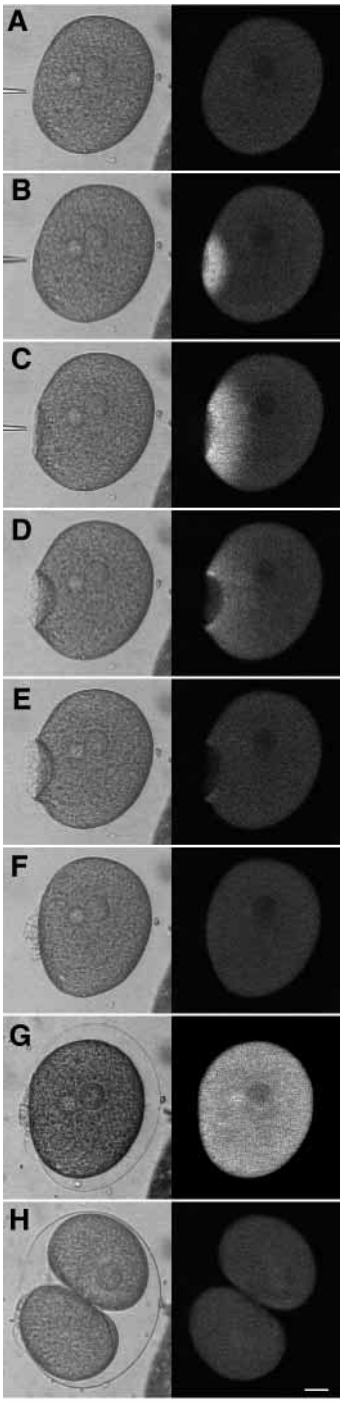

Fig. 3. Resealing of the Triton-induced PMD is a rapid event. A sea urchin egg was injected with calcium green dextran as an indicator for intracellular Ca^{2+}, and then was wounded by local detergent application. The top six image pairs shown were obtained at (A) 0 seconds, (B) 0.5 seconds, (C) 3 seconds, (D) 11 seconds, (E) 42 seconds and (F) 4 minutes 30 seconds. A large crater like PMD in the cell surface is accompanied by a large increase in local intracellular Ca^{2+}. Completion of resealing to the point of prevention of further Ca^{2+} entry, evident as declining intracellular Ca^{2+} concentration at the center of the disruption site, was evident quantitatively as soon as 3 seconds after triton-injury. (G) Sperm were added ~22 minutes after wounding; this image pair shows the Ca^{2+} increase at fertilization and normal fertilization envelope elevation except at the wound site. (H) Normal cleavage at 1 hour 30 minutes. Bar, 20 μm.

Cytoplasmic vesicles are required for resealing: functional and physiological evidence implicating the reserve granule

Eggs were centrifuged onto a cushion of Percoll until their organelles became stratified (Harvey, 1956). This treatment reshapes eggs into oblong structures, and, if prolonged, can split them into 'light' (ER rich) and 'heavy' (reserve granule rich) fragment halves (Fig. 4A,B). Both fragment halves contain 'docked' cortical granules, and probably different proportions of mitochondria and other organelles. Next, the stratified eggs, and accompanying light and heavy fragments, were subject to PMD-inducing shear forces by taking them up into and expelling them from a syringe (Clarke and McNeil, 1994). Before the first syringe stroke (control eggs) and after each of 8 strokes, eggs and egg fragments were plated on poly-l-lysine coated coverslips for staining with FM dye and microscopic examination. Digital images of eggs viewed by transmitted and fluorescence microscopy were obtained at random on each coverslip. These images were then scored for the number of transmitted light profiles representing: (1) intact (no staining of internal structures with the FM dye) stratified eggs, e.g. oblong bodies consisting of a relatively transparent and opaque end; (2) intact heavy end fragments, e.g. spherical fragments that are relatively opaque and intact (no FM staining internal structures); and (3) light end fragments, e.g. spherical fragments that are relatively transparent and intact (no internal FM staining).

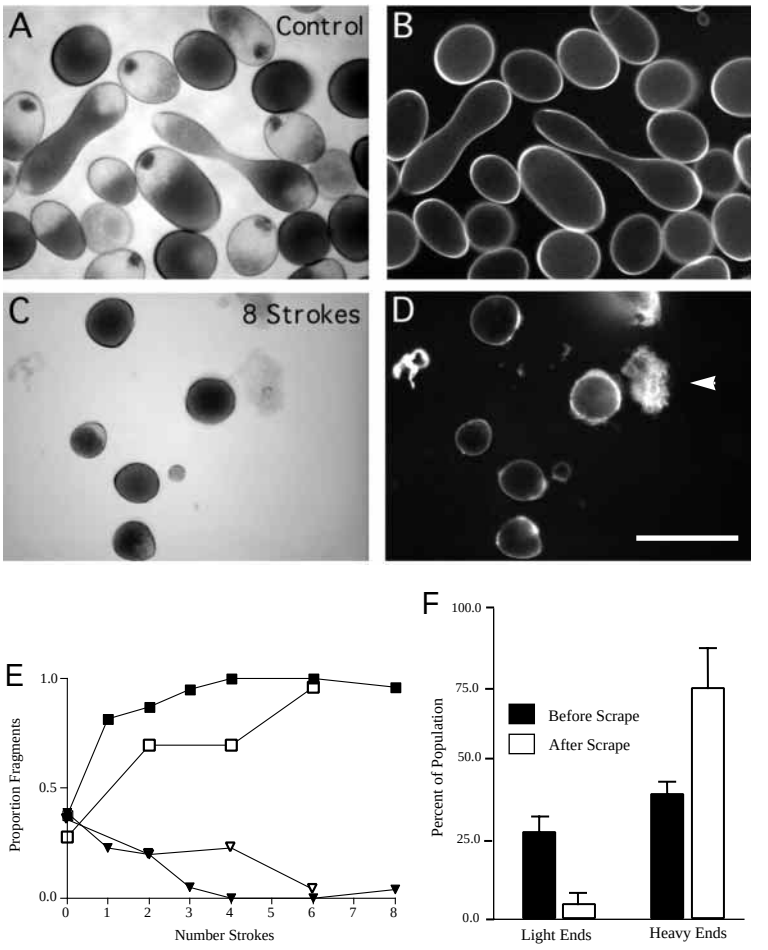

Fig. 4. Functional evidence that egg organelles, probably the reserve granules, are required for resealing. (A) Eggs were stratified by centrifugation into oblong bodies having easily identifiable 'heavy' (H) and 'light' (L) ends. Under the sedimentation conditions used, most eggs were split into heavy (h) and light (l) fragments. (B) The same stratified population, stained with FM1-43. All of the eggs and fragments are intact, as indicated by the staining of their delimiting membranes only: internal membranes are unstained. (C) After syringe injury (8 uptakes and expulsions through a 20-gauge needle), only heavy fragments remain. (D) Surface but not internal membrane staining with FM dye reveals that the surviving heavy fragments maintain a surface diffusion barrier after PMD-induction by syringing, whereas presumptive unsealed light fragments (arrowhead) did not. (E) Quantitation of differential survival of syringe injury by heavy and light fragments. Shown is the proportion of intact heavy (open and closed squares) and light (open and closed triangles) fragments as a function of number of syringe strokes applied to the stratified egg population. Each data point (from two separate experiments) represents counts of 40-50 eggs/fragments. The two-tailed p value derived from linear regression analysis of the heavy end data is 0.0119, considered statistically signifigant. (F) Quantitation of differential survival by heavy and light fragments of scrape injury. Shown is the mean (and s.e.m.) of four experiments (four spawns) in which a total of 138 (control) and 75 (scraped) eggs/egg fragments were scored. There is a signifigant reduction in the percentage of light fragments after scraping (two-tailed Student's P=0.0222). Bar, 100 μm.

With increasing syringe strokes, e.g. increasing imposition of shear stress and PMD injury (Clarke and McNeil, 1994), the proportion of heavy end fragments increased in the population until by 4-6 strokes virtually all of the fragments remaining were heavy ends (Fig. 4C-E). When egg fragments were subject to PMD by a separate method, scraping fragments off of an adherent substratum (McNeil et al., 1984), a similar loss of the light fragments was observed (Fig. 4F). Thus, the light ends of stratified eggs, which are lacking in reserve granules, do not reseal.

We next attempted to confirm that the patch vesicles formed in vivo in response to Ca^{2+} are derived from reserve granules. We reasoned that if reserve granules were acidic organelles, then so too would be the patch vesicles formed as a product of their homotypic fusion. We knew that a cytoplasmic 'patch' vesicle could be formed in intact egg cytoplasm by microinjection of calcium-containing sea water (Terasaki et al., 1997). Therefore, we employed fluorescent pH indicators to measure the pH of these two compartments. Granule pH was assessed qualitatively by incubating eggs in lysosensor green, which accumulates in a membrane impermeant, fluorescent form preferentially in acidic cell compartments (Cousin and Nicholls, 1997). Confocal microscopy of such eggs revealed that a widely distributed, large compartment consisting of ~1 μm diameter spherical vesicles was labeled with this dye (Fig. 5A). Confocal imaging of eggs co-labeled with Nile red (Fig. 5B), a dye that selectively labels the reserve granule compartment (Harvey, 1956) and does not label cortical granules in confocal microscope images (data not shown), revealed that the patterns of labeling with these two probes were identical (Fig. 5, compare A and B). This is strong evidence that the reserve granule is an acidic compartment.

To measure the pH of the lumina of the 'patch' vesicle, hypothesized to be formed via reserve granule-granule homotypic fusion, we injected SW containing a fluorescein-rhodamine co-conjugate of dextran. Fluorescein fluorescence is pH sensitive, while rhodamine fluorescence is not, so the fluorescence ratio can be used to monitor intracellular pH by reference to an in vitro calibration curve (Fig. 5C; McNeil et al., 1983). The internal 'patch' vesicle was acidic (pH ~5) at the first possible measurement point, ~15 seconds after its formation, and became increasingly more acidic during the next ~5 hours thereafter, reaching a minimum of pH ~4 (Fig. 5D). This is strong evidence that the SW injection induced 'patch' vesicle is derived in part from an acidic compartment. In conjunction with the above demonstration that the reserve granules constitute the predominant acidic compartment in the egg, this is further, physiological evidence for reserve granule involvement in patch formation.

Reserve granules fuse homotypically in vitro with characteristics expected of a process leading to patch formation

Reserve granules with the capacity for homotypic and plasma

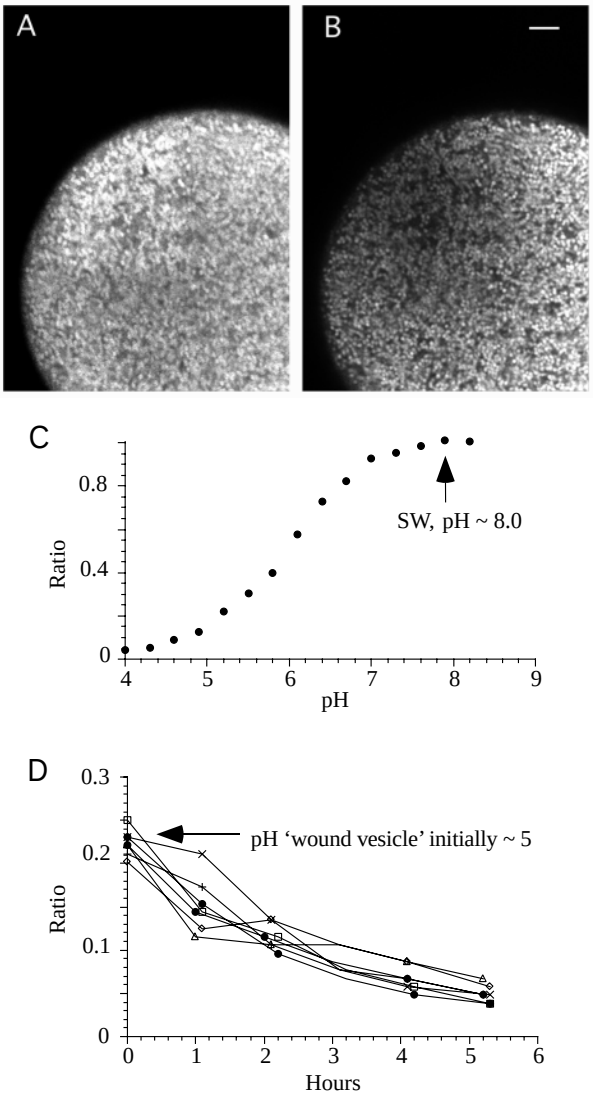

Fig. 5. Estimation of reserve granule and patch vesicle pH. (A) Image of fluorescence derived from egg stained with the acid selective dye, lysosensor green (1 μM from 1 mM DMSO stock; 30 minutes). (B) Same cell co-stained with nile red (2 μg/ml from a 2 mg/ml stock in DMSO). There is an apparently complete co-incidence of staining. (C) 10 kDa dextran conjugated with fluorescein and rhodamine was used to monitor pH. The ratio of fluorescein to rhodamine fluorescence was determined in SW buffered at different pH. The curve shows a pK of ~6. (D) Sea water containing fluorescein / rhodamine dextran was injected into starfish oocytes. The first measurements of the ratio are already relatively low, and are followed by a slow further decrease. Each set of points connected by a line represents a different oocyte. Bar, 10 μm.

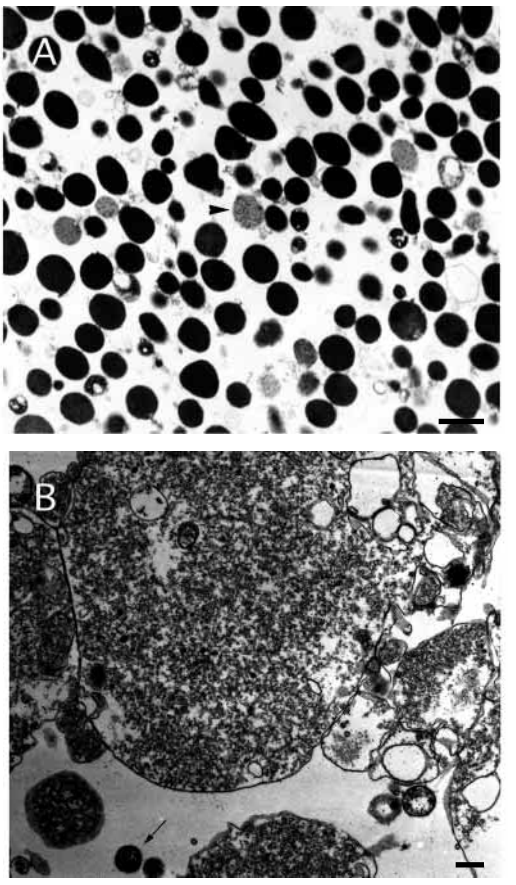

Fig. 6. Electron micrograph of the reserve granule isolate. (A) In the absence of Ca^{2+}, greater than 95% of the profiles present in the population can be identified as reserve granules. Cortical granules (arrowhead) are the most frequent contaminant. (B) Ca^{2+} addition dramatically alters the morphology of the granules as isolated. Large, membrane bounded structures, filled with a less dense content predominate over individual granule profiles (arrowhead). Bar, 1 μm.

membrane fusion had previously been isolated by free flow electrophoresis (Chestkov et al., 1998). As this was not a method available to us, we developed an alternative, Percoll-gradient based protocol for the isolation of fusion competent vesicles. Electron microscopic analysis revealed that >95% of the profiles in the isolate have the distinctive morphology of the reserve (or yolk) granule (Fig. 6A).

As previously reported, fusion of the reserve granules did not require added cytosol (Chestkov et al., 1998). Addition of Ca^{2+} alone to the isolated vesicle fraction was sufficient to trigger fusion. Fusion could be quantitated in a plate reader assay: fusion reduces the amount of light scattered by a population of granules by ~50%. As shown in Fig. 6B, fusion reduces the concentration of granule content, presumably due to expansion of this content into the larger potential volume of the fusion product. It seems likely that this decrease in content concentration explains the measurable fusion-based decline in light scatter. A typical Ca^{2+} dose-response curve generated by quantitation of light scatter is shown in Fig. 7. The threshold and half maximal concentration for fusion were in the supramicromolar range. Cytoplasm depleted of granules did not display Ca^{2+}-triggered fusion, as assessed visually or by the light scattering assay (data not shown).

In order to gain a first approximation of the kinetics of egg reserve granule fusion, two approaches were taken. In the first, granules were imaged while bathed in DM-nitrophen caged Ca^{2+}. Fusion events, in which individual vesicles clearly had merged into larger vesicles, were observed as soon as 2 seconds after initiation of Ca^{2+} release by u.v. exposure (Fig. 8) and the reaction was essentially complete within 20 seconds. In a second approach, a large volume of Ca^{2+} was injected into a

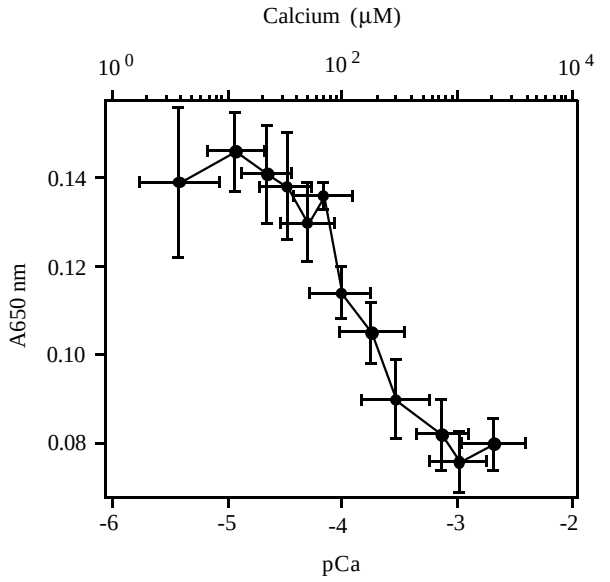

Fig. 7. Granule fusion measured in the plate reader assay as a function of increasing free Ca^{2+} ion concentration. Error bars represent the standard deviation of the mean for fusion measured as a change in sample absorbance, and the free Ca^{2+} ion concentration measured using a Ca^{2+} electrode.

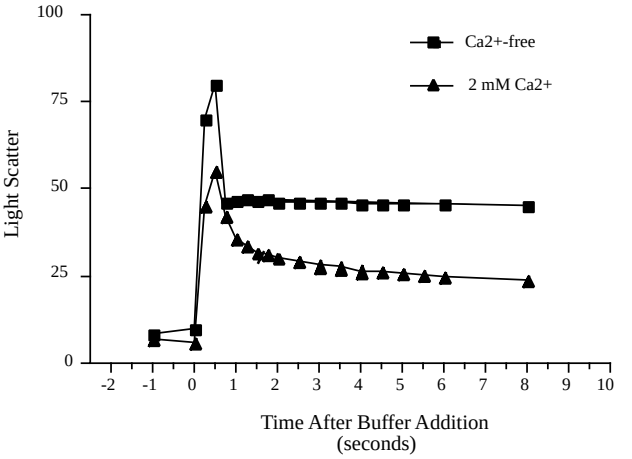

Fig. 9. Granule fusion kinetics upon rapid mixing with Ca^{2+}. A tenfold excess of Ca^{2+} containing buffer (2 mM, triangles) or Ca^{2+}-free buffer (squares) was injected into a cuvette containing reserve granules. The light scattered by these mixtures was recorded over time after Ca^{2+} injection (t=0). Upon initial buffer injection, there is an artifactual 'overshooting' spike in scatter, of <0.5 seconds in duration, due probably to injection-mediated introduction of air bubbles into the cuvette. A decline in the scatter of the Ca^{2+} injection sample relative to the Ca^{2+}-free control is evident by 0.5 seconds, and the half time for scatter decay in the Ca^{2+} injection sample is ~1 second.

smaller volume of reserve granules held in a cuvette while the amount of light scattered was measured. The half time for the reduction in light scatter measured by this method was ~1 second (Fig. 9).

Lastly, we assessed the capacity for formation of large vesicles, capable of spanning large PMD with a continuous membrane permeability barrier. Granules clumped together by centrifugation (Fig. 10A) and then contained between a coverslip and slide were perfused with maximally fusogenic Ca^{2+} while imaging. Some of the products of fusion obtained in this way were more than 100 μm in length (Fig. 10B). When the fusion was carried out the presence of fluorescein dextran, this permeability tracer became trapped in the large fusion product vesicles and was retained there for several hours after all exogenous dextran had been washed away, confirming that a continuous membrane barrier had been erected (Fig. 10C). Moreover, these large 'patch' vesicles displayed a continuous electron dense border when viewed by electron microscopy, consistent with their enclosure by a membrane barrier (Fig. 6B).

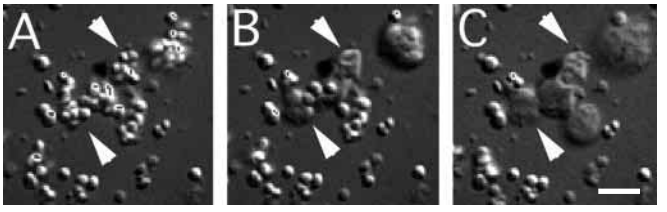

Fig. 8. Granule fusion kinetics upon release of caged Ca^{2+}. Shown are Nomarski images of granules (A) before and at intervals of (B) 2 seconds and (C) 20 seconds after initiation of Ca^{2+} release by ultraviolet illumination. Two clumps of individual granules (arrowheads) have fused to form large vesicles at the 2 seconds time point, and fusion is apparently complete by the 20 seconds time point. Bar, 5 μm.

DISCUSSION

Based on micromanipulation experiments utilizing intact sea urchin eggs, we previously showed that cytoplasm alone, when challenged with the external environment, could erect a continuous membrane-based permeability barrier (Terasaki et al., 1997). This capacity was explainable if Ca^{2+} was capable of inducing a rapid, homotypic vesicle-vesicle fusion reaction in egg cytoplasm. This reaction of cytoplasm upon exposure to potentially toxic (Trump and Berezesky, 1996) extracellular Ca^{2+} ions could be viewed as a protective one. Moreover, it seemed possible that an identical protective response might occur when cytoplasm was similarly exposed to extracellular medium during a PMD event. We therefore proposed that vesicle-vesicle fusion occurring at a PMD site might form a disruption breaching 'patch' of membrane barrier. A patch of this kind could then be joined to the plasma membrane by exocytotic fusion events, completing resealing. A more detailed view of this process (summarized in Fig. 11) is that sudden exposure of cytoplasm to high Ca^{2+} causes rapid, chaotic and multiple fusions of granule vesicles with each other and with the plasma membrane, until by chance a continuous barrier forms which stops the entry of extracellular Ca^{2+}.

Several predicted elements of the patch hypothesis, in addition to those just mentioned, were indeed already established (reviewed by McNeil and Steinhardt, 1997). First, Ca^{2+} was known to be required for resealing (Heilbrunn, 1930). Exocytosis, moreover, had been shown to accompany, in time and space, resealing events (Bi et al., 1995; Miyake

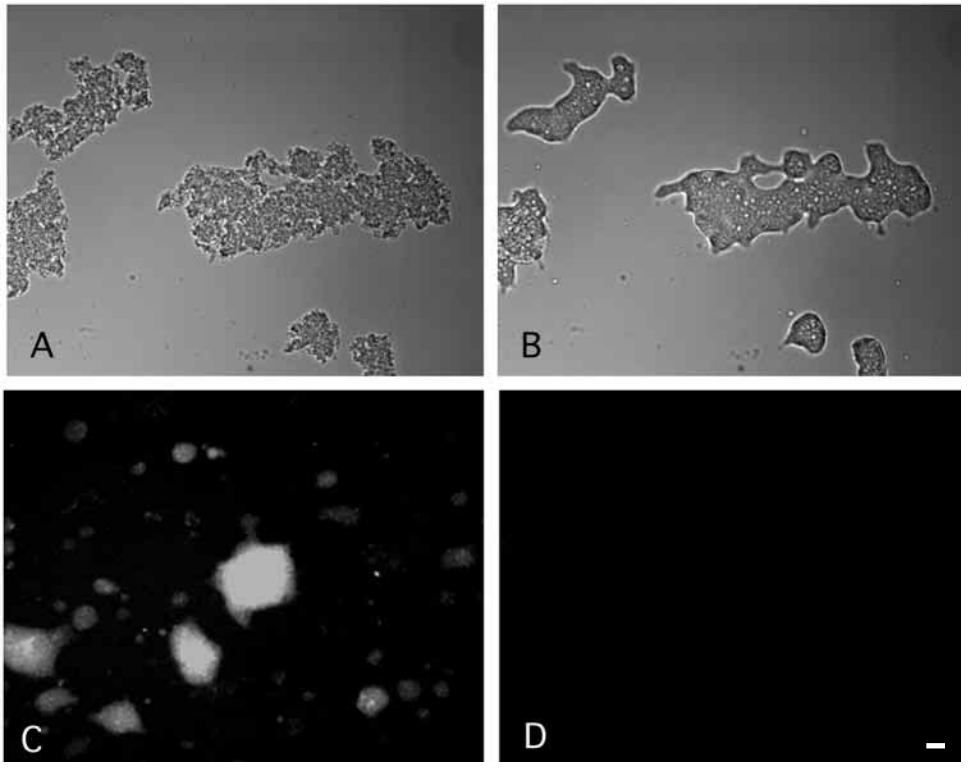

Fig. 10. Fusion of large granule clusters to form large patch vesicles. (A) Granules pelleted in a microfuge (10,000 rpm, 5 minutes) were present in large clusters after mild resuspension and mounting between a slide and coverslip for microscope viewing. (B) Upon Ca^{2+} addition, individual granules are no longer resolvable. Instead, large vesicle-like structures, with smooth, refractile outlines, some greater than a 100 μm in length, were present. (C) When Ca^{2+} was added to vesicle clumps in the presence of fluorescein dextran (1 mg/ml; 10,000 M_r), and then the preparation was washed thoroughly with plain buffer, the resultant patch could be seen to trap and retain (for many hours) fluorescein dextran. (D) Control granules, incubated in the fluorescein dextran before (shown) or after (not shown) but not during fusion were unlabeled. Bar, 10 μm.

and McNeil, 1995). The reserve or yolk granule of the egg was implicated as a key component in this cytoplasmic reaction (Terasaki et al., 1997). Large vesicles were moreover observed to form near sites of large plasma membrane disruptions (Miyake et al., 1995; Terasaki et al., 1997). Reserve granules were known to be capable of Ca^{2+}-induced fusion (Chestkov et al., 1998). Finally, SNARE proteins, known to be required for other secretory (exocytotic) events, appeared similarly to be required for resealing, since disruption by toxins blocked resealing (Steinhardt et al., 1994). However, several key elements of the hypothesis lacked direct experimental support.

There was, in the first place, no evidence for the predicted surface deposit of a domain of internal membrane across the disruption site. Here we show that after a membrane barrier has been erected over a disruption site, e.g. resealing is complete, protein and lipid components normally distributed uniformly over the cell surface remain absent at PMD sites. This establishes, by a process of elimination, that internal membrane

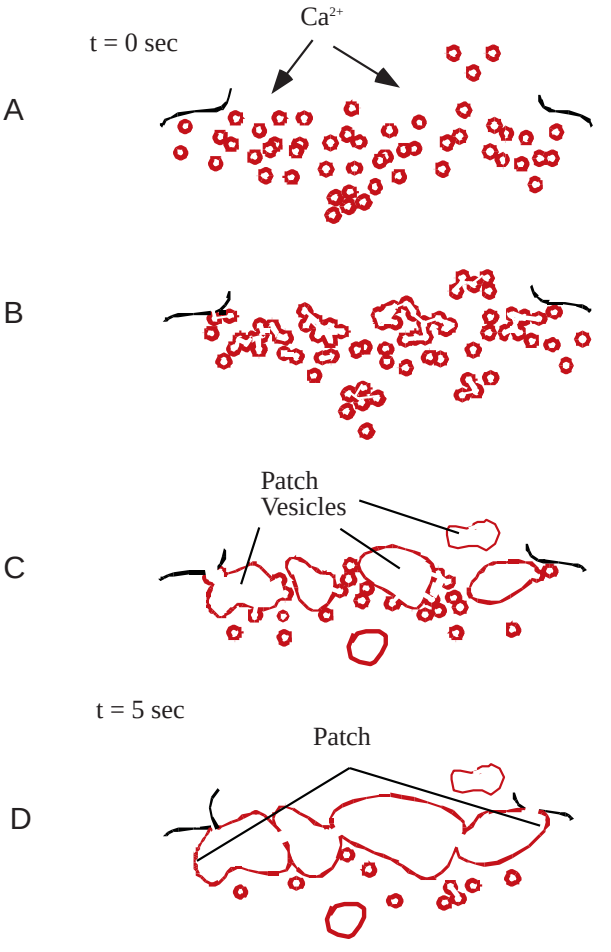

Fig. 11. The patch hypothesis. (A) Ca^{2+} enters cytosol through the PMD. (B) Vesicle-vesicle fusion events are rapidly initiated, in a chaotic fashion, throughout the resultant zone of elevated intracellular Ca^{2+}. (C) Fusion of native and enlarged vesicles with one another continues. Some of these large products of fusion will ultimately become incorporated into the plasma membrane as a resealing 'patch', others will not, and these can frequently be observed in the peri-disruption cytoplasm, and outside the cell at the PMD site, after resealing is completed. (D) Exocytosis couples the patch vesicle(s) to surface membrane, completing resealing. This exocytotic event, though depicted here as the terminal event in a sequence, may be occurring in parallel with the vesicle-vesicle fusion events depicted above. In any case, addition of a 'patch' derived from internal membrane to the plasma membrane, completes resealing.

is applied to such sites, since it is the only unlabeled source available.

The major alternative hypothesis is that resealing is mediated by rapid 'flow' of native, pre-disruption plasma membrane over the disruption site. This explanation requires, in the first place, availability of surface membrane for this purpose. Although sufficient (>1000 μm^2 for the disruptions studied here) surface area might be present in the form of plasma membrane evaginations (microvilli), spread of bilayer over the PMD site from this source would require that it be freed from the physical constraint imposed by microvillar cytoskeletal elements. There is no indication from scanning electron microscopy that rapid (<5 seconds) microvillar disruption/dissolution occurs in eggs that have suffered PMD (P. McNeil, unpublished data), nor are micovilli known to be capable of dissolution on this time scale. More importantly, if lipid 'flow' across the detergent generated PMD site were responsible for resealing, then recovery of DiI fluorescence over a PMD site would occur on the same time scale as resealing. In fact, DiI recovery occurred on a minutes time scale, resealing on a seconds time scale. Thus, these separate considerations strongly reinforce the conclusion that the source of membrane used for resealing of large PMD is derived from the cytoplasm, not the cell surface.

A second general prediction of the patch hypothesis, addressed here, is that resealing capacity will be dependent on local availability of cytoplasmic vesicles. This is clearly shown to be the case: the two classes of egg fragment produced by centrifugation were not equally capable of resealing. Since it is unlikely that the centrifugational conditions (12,000 ***g***, 15 minutes) used altered soluble component distribution in the egg, it follows that vesicle composition was the key alteration at the disruption site.

The stratification experiment also provides direct evidence that the cytoplasmic vesicle required for resealing is the reserve granule. Only the heavy ends of stratified eggs, containing the great majority of the egg's reserve granule population, survived multiple PMD inducing events. Both ends of the stratified egg contain docked cortical granules, and can support fertilization, so this granule, which fuses with the plasma membrane upon fertilization in a Ca^{2+}-dependent fashion, is ruled out as sufficient for resealing. The observation here and elsewhere (Steinhardt et al., 1994) that fertilized eggs are capable of resealing is further evidence that cortical granules are not required, since after fertilization cortical granules are no longer present in the egg cortex. Indeed, vesicles that are pre-docked with the plasma membrane, such as the cortical granule, may in general be of little use in resealing: such vesicles will be destroyed along with associated plasma membrane during a PMD event, and so will not be present where needed, e.g. in peri-disruption cytoplasm where patch formation leads to resealing.

Finally, we demonstrate here that the cytoplasmic 'patch' vesicle produced by sea water injection is immediately acidic, and that the major acidic compartment in the egg is comprised of reserve granules. Since the sea water injection was made deep in egg cytoplasm, ruling out cortical granule involvement, this pH measurement data further implicates the reserve granule in Ca^{2+}-initiated patch vesicle formation and hence resealing.

Previous work too had suggested the importance of the reserve granule, or yolk granules, in resealing. First, a reserve granule selective stain (Nile Red) was observed to label the large, fusion-derived vesicles induced by sea water injections (Terasaki et al., 1997). Second, cytoplasm from the heavy but not the light end of stratified eggs was found to be capable of forming a membrane barrier when ejected from a pipette tip (Terasaki et al., 1997). Finally, Heilbrunn (1958) states that the surface precipitation reaction, the process he identified with successful repair of surface tears, occurred only in the heavy end of the stratified egg. Functional, structural and physiological data are therefore now available with which to identify the reserve granule as a key component of patch vesicle formation and egg resealing.

A third general prediction of the patch hypothesis is that the vesicle population required for resealing, the reserve granules, will exhibit fusion properties compatible with a role in resealing. A recent study had shown that isolated reserve granules were capable of Ca^{2+}-triggered, homotypic fusion, and of heterotypic fusion with the plasma membrane (Chestkov et al., 1998). We further demonstrate here that isolated reserve granules exhibit three resealing compatible fusion properties, e.g. high calcium threshold, rapid kinetics and impressive capacity to form large membrane barriers.

The expectation of a high Ca^{2+} threshold for fusion derives from the high (~1 mM) resealing requirement for extracellular Ca^{2+} (Steinhardt et al., 1994). We show here that reserve granule fusion is half maximal at ~100 μM of free Ca^{2+}. Of course, numerous egg responses, such as elevation of the fertilization envelope (Steinhardt et al., 1977), utilize Ca^{2+} as a second messenger. Therefore, the function of a high threshold for resealing-based fusion could be to prevent inappropriate and wasteful 'patch' vesicle formation during non-emergency egg responses. Indeed, the maximal level of intracellular free Ca^{2+} concentration reached envelope elevation is apparently well below the Ca^{2+} optimum for patch vesicle formation (Steinhardt et al., 1977).

Resealing of large PMDs is a rapid, 'emergency' reaction, hence the second expected property, that granule fusion will be an unusually rapid homotypic fusion process. We estimate from imaging of fusion after caged calcium release and light scatter measurements that Ca^{2+}-triggered reserve granule fusion occurs on a second or possibly even sub-second time scale. Clearly, fusion of reserve granules in vitro is much faster (by one or more orders of magnitude) than most other homotypic or heterotypic organelle-organelle fusion events (Mayer et al., 1996; Nichols et al., 1997; Ungermann et al., 1998). Shuttling of membrane or cargo from one cell compartment to another in highly targeted, constitutive pathways, the function served in most organelle-organelle fusion systems studied to date, may not require a comparably rapid fusion machine. Resealing-based fusion differs in a second, and perhaps related, aspect from other homotypic and heterotypic organelle fusion systems: it lacks a requirement for cytosolic protein components, and for ATP or GTP. If the time course, many minutes in vitro, over which these cytosolic 'priming' factors are required to act reflects biological reality, then it follows that a much more rapid fusion process, such as that occurring during resealing, has to utilize an alternative mechanism. Resealing-based fusion likely uses a pre-assembled fusion machinery, but one that does not result in vesicle 'docking' before the fusion stimulus is present. It is therefore unlike cortical granules in the sea urchin egg or

synaptic vesicles in the neuron, which by virtue of a partially pre-assembled fusion machinery are intimately associated with their fusion partner, e.g. docked with the plasma membrane, before the fusion stimulus is received. In sum, its unusual characteristics suggest that the molecular components of resealing-based, or 'emergency' fusion, may differ from those discovered in other described systems.

A third characteristic expected of resealing-based vesicle-vesicle fusion is that it have the capacity to create large, continuous, and therefore large-disruption spanning, membrane domains. Survivable disruptions can be hundreds or even thousands of square microns in extent. We now have documented that the patch vesicles formed in vitro by homotypic fusion of reserve granules can be quite large (>1000 μm^2 in extent) and that these vesicular structures are surrounded by a continuous membrane boundary.

What, if any, is the functional homologue in other cells of the sea urchin's reserve granule compartment? There are of course many non-docked vesicle populations in most cell types, but available evidence implicates only one at present, the endosome/lysosome compartment. The endosome/lysosome compartment of endothelial cells undergoes PMD-induced exocytosis (Miyake and McNeil, 1995). Endosomes/lysosomes are moreover induced by Ca^{2+} to fuse homotypically in vivo (Rodriguez et al., 1997) and in vitro (Myorga et al., 1994). In fact, Ca^{2+}-activated endosome fusion in vitro resembles that of the reserve granule system in its lack of a requirement for cytosol or ATP (Mayorga et al., 1994). The reserve granule may, like the yolk granule of the mosquito or *Xenopus*, arise during egg development by endocytotic events (Raikhel, 1987; Wallace et al., 1983). Certainly, it contains acid hydrolases characteristic of lysosomes (Armant et al., 1986) and, as we show here, is an acidic compartment, like endosomes/lysososmes. It is therefore an intriguing possibility that endosomes/lysosomes are the functional homologue of the reserve granule in cells lacking this organelle.

In conclusion, we have provided strong evidence that a 'patch' of membrane derived from the cytoplasm is inserted into egg plasma membrane at sites of large disruptions. The source of the membrane for this patch is the reserve granule, since local absence of this organelle prohibits resealing. Fusion of reserve granules in vitro possesses many of the characteristics expected of fusion leading to patch formation in vivo: it is rapid, can create extensive membrane boundaries and is Ca^{2+}-triggered but with a high threshold. The simple, quantitatable fusion reconstitution system described here should provide a powerful tool for defining the molecular components of resealing, which may be distinct, based on the fusion properties described here, from those used in other organelle fusion reactions.

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