

Exocytotic Insertion of Calcium Channels Constrains Compensatory Endocytosis to Sites of Exocytosis

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Abstract. Proteins inserted into the cell surface by exocytosis are thought to be retrieved by compensatory endocytosis, suggesting that retrieval requires granule proteins. In sea urchin eggs, calcium influx through P-type calcium channels is required for retrieval, and the large size of sea urchin secretory granules permits the direct observation of retrieval. Here we demonstrate that retrieval is limited to sites of prior exocytosis. We tested whether channel distribution can account for the localization of retrieval at exocytotic sites. We

find that P-channels reside on secretory granules before fertilization, and are translocated to the egg surface by exocytosis. Our study provides strong evidence that the transitory insertion of P-type calcium channels in the surface membrane plays an obligatory role in the mechanism coupling exocytosis and compensatory endocytosis.

Key words: agatoxin • conotoxin • P-type calcium channels • sea urchins • microscopy

Introduction

Upon fertilization, calcium release from internal stores triggers sea urchin egg cortical granule exocytosis (Epel, 1977; Hagiwara and Jaffe, 1979; Shen, 1995). The physiological role of cortical granule exocytosis is to prevent polyspermy (Epel, 1977), and to erect a physical barrier around the egg that protects it during the early stages of development (Miyake and McNeil, 1998). These functions require extensive secretion across the entire egg surface. The amount of exocytosis is so extensive that the addition of cortical granule membranes to the cell surface doubles the surface area of the egg (Jaffe et al., 1978; Schroeder, 1979). Fortunately, as is observed in many other cells (Nordmann et al., 1974; Thomas et al., 1990; Parsons et al., 1994; Rosenboom and Lindau, 1994; Artalejo et al., 1995; Smith and Betz, 1996; Smith and Neher, 1997; Engisch and Nowycky, 1998), cortical granule exocytosis in sea urchin eggs is followed by endocytotic membrane retrieval that compensates for the increase in cell surface area (Whalley et al., 1995). In the sea urchin egg fertilization initiates the depolarization of the membrane potential from -70 mV to 0 mV (Jaffe and Robinson, 1978) which opens ω -agatoxin sensitive (P-type) voltage-gated calcium channels (Vogel et al., 1999). Calcium influx through these channels in turn triggers compensatory endocytosis (Vogel et al., 1999). As is the case in most other cell types, other aspects

of compensatory endocytosis in sea urchin eggs are poorly understood. In many cells it is believed that prior exocytosis is required for compensatory endocytosis because specific retrieval of granule membrane proteins has been observed (Phillips et al., 1983; Patzak et al., 1984; Thilo, 1985; Patzak and Winkler, 1986; Pow and Morris, 1991; Nordmann and Artault, 1992). However, the mechanism that directs specific retrieval of granule membrane components is not known (Warren and Mellman, 1999). We show that in sea urchin eggs compensatory endocytosis requires prior exocytosis, and that granule proteins are selectively retrieved. We also show that the dependence of compensatory endocytosis on prior exocytosis arises, at least in part, from the subcellular localization of P-type calcium channels in the membranes of the secretory granules. Thus, exocytosis inserts calcium channels into a depolarized plasma membrane, and calcium influx through these channels triggers selective retrieval of the cortical granule membrane in which it resides.

Materials and Methods

Obtaining and Handling Gametes

Sea urchins (*Strongylocentrotus purpuratus*) were obtained from Marinus and maintained in artificial seawater (ASW)¹ in marine aquaria. ASW is

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¹Abbreviations used in this paper: ASW, artificial seawater; LEMS, Lambert-Eaton myasthenic syndrome; SVM, subcortical vesicular structures; TVM, translucent vesicles.

routinely prepared by dissolving Instant Ocean (Aquarium Systems) per the manufacturer's directions and adjusting the osmolarity to $1,000 \pm 10$ mOsm and pH to 8.0. For experiments ASW was prepared using the formula of the Marine Biological Laboratory: 425 mM NaCl, 9 mM KCl, 9.3 mM CaCl₂, 19.9 mM MgCl₂, 25.5 mM MgSO₄, and 2.1 mM NaHCO₃. Eggs and sperm were obtained as previously described (Vogel et al., 1999).

Imaging Focal Exocytosis and Endocytosis

Sea urchin eggs were attached to a polylysine-treated coverslip as previously described (Vogel et al., 1999). A Zeiss Axiophot microscope with a cooled CCD camera (Photometrics, Sensys) was used to image focal elevation of the egg fertilization envelope and focal uptake of tetramethylrhodamine dextran using a 40×1.0 NA objective. Focal fertilization envelope elevation was imaged using differential interference contrast (DIC) optics and a rhodamine filter set was used to image tetramethylrhodamine dextran fluorescence. DIC and fluorescence images were superimposed with Adobe Photoshop. All figures were prepared with Adobe Photoshop and Adobe Illustrator.

Membrane Retrieval Assay

A fluid phase uptake assay that measures the amount of tetramethylrhodamine dextran (mol. wt. 3000) was used to quantitate the percent retrieval as previously described (Vogel et al., 1999).

Labeling Eggs with Alexa Maleimide

The vitelline membrane of eggs in suspension was removed by treatment with 10 mM dithiothreitol and 50 mM glycine in artificial seawater (ASW) at pH 9.1 ± 0.1 for 10 min at $9 \pm 2^\circ\text{C}$ (Epel et al., 1970). Next, eggs were washed three times in ASW and incubated with ASW containing $4 \mu\text{M}$ Alexa 488 maleimide (Molecular Probes) prepared from a 10-mM stock of the dye in DMSO. Eggs were washed three times with ASW to remove dye that had not covalently attached to the egg surface.

Labeling Eggs with Concanavalin A

The vitelline membrane was removed from eggs as described above and eggs were attached to polylysine-treated coverslips (0.1 mg/ml) and placed into a perfusion chamber. Eggs were perfused with ASW containing a green fluorescent conjugate of concanavalin A and Oregon green 488 ($2 \mu\text{g}/\text{ml}$; Molecular Probes) for 15 min. Free concanavalin A was removed with three ASW washes. In some experiments newly exposed concanavalin A binding sites on activated eggs were labeled with the red fluorescent conjugate of concanavalin A and Texas red ($2 \mu\text{g}/\text{ml}$; Molecular Probes).

Labeling Eggs with Octadecylrhodamine

Eggs were labeled with the lipidic fluorescent dye octadecylrhodamine (R18; Molecular Probes) by perfusion with ASW containing $20 \mu\text{M}$ R18 (prepared freshly from a 10-mM stock solution in ethanol) for five minutes. Nonincorporated R18 micelles were removed by three washes with ASW.

Confocal Microscopy

A Molecular Dynamics confocal microscope (Multiprobe 2001) with an argon-krypton laser was used for all confocal laser scanning imaging. For imaging Alexa 488 fluorescence or Oregon green fluorescence (green channel) with either tetramethylrhodamine dextran, Alexa 594, or Texas red fluorescence (red channel) we used the 488- and 568-nm excitation laser lines, a 530 ± 15 -nm filter (green channel) and a 590-nm longpass emission filter (red channel), and a Nikon 60×1.2 NA PlanApo objective on an inverted microscope. We used the 568-nm excitation laser line and a 590-nm longpass emission filter to image octadecylrhodamine and tetramethylrhodamine fluorescence.

Agatoxin Immunofluorescence Microscopy

Anti-agatoxin-specific antibody was purchased from Calbiochem. The outer vitelline membrane was removed to allow access of antibodies and antibody conjugates to the surface of the plasma membrane using the protocol of Epel (Epel et al., 1970). Unfertilized eggs and calcium ionophore-

activated eggs ($50 \mu\text{M}$, A23187) were incubated with 100 nM ω -agatoxin-IVA (Calbiochem) for 7 min, washed, and then fixed with 4% formaldehyde in ASW for 20 min. In control experiments agatoxin was omitted. Following fixation all samples were washed in ASW and perfused with a 1:1,000 dilution of normal goat IgG in ASW to block nonspecific antibody binding. Next the samples were incubated for 1 h at RT in ASW supplemented with 1% BSA and a 1:500 dilution of a rabbit anti-agatoxin antibody (Calbiochem). Finally, after several washes in ASW supplemented with 1% BSA, the samples were incubated with a 1:2,000 dilution of a goat anti-rabbit IgG conjugated to tetramethylrhodamine for 1 h, washed, and visualized by confocal microscopy.

Immunogold Electron Microscopy

Eggs were fixed for 1 h at 4°C in PKME buffer (50 mM Pipes, pH 6.7, 425 mM KCl, 10 mM MgCl₂, 5 mM EGTA, and 1 mM benzamidine) containing 1% glutaraldehyde and 0.2% picric acid to provide both adequate morphologic detail and antigen reactivity. Eggs were dehydrated with a graded ethanol series (10 min each in 70, 80, and 85% and three times 10 min in 90%) then infiltrated twice for 30 min with one part 90% ethanol: two parts LR White resin (Electron Microscopy Sciences), and then overnight in 100% LR White resin. The following morning the eggs were embedded in gelatin capsules containing fresh LR White resin and cured for 48 h at 65°C . Thin sections (700\AA) were cut and mounted on nickel grids. Immunostaining was begun within 4 h of cutting the sections and the procedure followed has been previously described (Smith and Jarett, 1993). In brief, sections were blocked overnight at 4°C in TBS (50 mM Tris-HCl and 145 mM NaCl), pH 7.4, containing 1% bovine serum. Sections were then transferred to primary antibody diluted in TBS containing 0.1% BSA for 2 h at room temperature. The production of affinity-purified P-type (BC- α_{1A}) and L-type (BC- α_{1D}) calcium channel-specific antibodies have been described previously (White et al., 1998). BC- α_{1A} and anti-agatoxin (Calbiochem) were used at 1:2,000 dilution. Controls for BC- α_{1A} included substituting 1:2,000 preimmune IgG, 1:2,000 preabsorbed IgG for BC- α_{1A} and omission of primary antibody. Controls for anti-agatoxin included substituting normal rabbit IgG for anti-agatoxin, omission of primary antibody, and staining of eggs with anti-agatoxin antibody that had not been incubated with agatoxin-TK. After incubation with primary antibody or controls the sections were washed four times with TBS containing 1% BSA and once in PBS (10 mM sodium phosphate buffer, 145 mM NaCl, pH 7.4) with 1% BSA. The washed sections were incubated with 15-nm diam colloidal gold-labeled anti-rabbit IgG for 1 h at room temperature. (The anti-rabbit IgG was obtained from Sigma.) The colloidal gold and antibody complex were prepared as described previously (Smith and Jarett, 1993). The sections were washed four times with PBS and 1% BSA and two times with deionized water. The tissue was counterstained with 2% aqueous uranyl acetate for 3 min. The stained sections were examined in a JEOL 1010 transmission electron microscope and digital images were recorded with Advanced Microscopy Techniques Corp software (Kodak Megaplus 6.1I camera).

Quantitative Analysis of the Distribution of Colloidal Gold Particles

Six blocks of fixed eggs from each incubation condition were embedded for each experiment. Thin sections were cut from each of the six blocks and duplicate grids from three blocks stained on two separate days. 25–40 randomly selected images were recorded at high magnification (25–40,000 \times) for each experimental condition. No more than two images were recorded of any given egg. The distribution of gold particles on cellular membrane domains, e.g., the microvilli, plasma membranes, subcortical vesicles, cortical granule membrane, clear translucent cytoplasmic vesicles, and yolk granule membranes, was determined by counting gold particles associated with those membranes and measuring the length of membrane included in the image whether or not it was labeled with gold. The results were expressed as particles per micron of membrane. The distribution of gold particles on various cellular organelles, e.g., cortical granule core, yolk granule core, mitochondria, and cytoplasm, was similarly determined except the surface area of those structures was determined and the results expressed as particles/ μ^2 .

Immunoblotting

Membranes were prepared from isolated sea urchin cortical granules using the protocol of Vater and Jackson (Vater and Jackson, 1989) and sep-

arated by 7.5% SDS-PAGE. Proteins were electroblotted onto nitrocellulose and blocked with TBS supplemented with 1% BSA and 0.05% Tween-20. All blots were incubated at 1 μ g/ml of antisera. Peptide preincubation was with a twofold molar excess of either P-type immune peptide or L-type immune peptide (White et al., 1998). Immunoreactivity was detected with a goat anti-rabbit antisera conjugated to alkaline phosphatase using a ProtoBlot[®] detection kit (Promega).

Quantitative Morphometry

Quantitative analysis of the effects of fertilization and/or agatoxin on the number of cortical granules and subcortical vesicles was performed on 20 low magnification (10,000 \times) prints from each of two experiments for each condition. Cortical granules were identified by their proximity to the plasma membrane and characteristic electron-dense luminal inclusion. Subcortical vesicles were identified by their proximity to and often continuity with the plasma membrane and the lack of electron-dense luminal inclusion, while translucent vesicles were identified as vesicular structures located more than 1 μ m from the plasma membrane.

Results

Imaging the Specificity of Compensatory Endocytosis

Exocytosis inserts granule proteins and lipids into the cell surface. Coupled endocytotic membrane retrieval removes membrane from the cell surface, but in sea urchins it is not known if the retrieved membrane is comprised of components whose origin is solely the cortical granule membrane, components from the plasma membrane, or a mixture of components from both sources. We devised an imaging strategy to directly test if plasma membrane components are specifically retrieved by compensatory retrieval. Egg membrane surface proteins were labeled with Alexa 488 maleimide before fertilization (Fig. 1 a). The plasma membrane components labeled by this reagent (membrane proteins with free sulfhydryl groups) were not internalized by endocytotic membrane retrieval (i.e., they did not colocalize with the fluid phase marker tetramethylrhodamine dextran; see Fig. 1 a, B) upon activation with calcium ionophore. Occasionally, we did observe a limited amount of fluorescent plasma membrane marker internalization. However, internalized Alexa 488 did not colocalize with the fluid phase marker. Presumably under certain circumstances Alexa 488 maleimide can be transported into the egg by a separate mechanism. In control experiments, we left Alexa 488 maleimide on after fertilization and confirmed that under these conditions some Alexa dye does colocalize in the same intracellular inclusions as the fluid phase marker (Fig. 1 a, C). Colocalization with the fluid phase marker suggests that Alexa 488 maleimide labels both plasma membrane proteins as well as components tightly associated with the membrane surface. Upon membrane retrieval, some of these labeled components extend into the lumen of these intracellular inclusions. A similar labeling pattern was observed with the fluorescent membrane probe FM1-43 in neuroendocrine cells (Angleton et al., 1999). Plasma membrane components prelabeled with a green fluorescent conjugate of concanavalin A were not retrieved by compensatory endocytosis, while a red conjugate applied after egg activation was retrieved (Fig. 1 b). It is known that concanavalin A binds to glucosyls attached to the egg plasma membrane, as well as with components on the granule membrane (Veron and Shapiro, 1977; Veron et al., 1977). Because the binding

specificity of maleimide and concanavalin A are quite different, and because these reagents will bind to a broad spectrum of membrane proteins, we conclude that the majority of proteins retrieved by compensatory endocytosis did not originate in the plasma membrane.

Compared with membrane proteins, the low mass of membrane lipids and their limited interaction with other cellular components tend to endow them with a greater lateral mobility in the membrane. We wished to determine if a plasma membrane lipid marker would also be excluded from retrieval by compensatory endocytosis. Such an exclusion would be no trivial matter. It is known that cortical granule-plasma membrane fusion forms a mosaic surface comprised of granule and plasma membrane domains (Schroeder, 1979). Granule exocytosis randomly inserts enough granule membrane into the egg surface to double its surface area (Jaffe et al., 1978). Because endocytotic membrane retrieval occurs over a 15-min time course (Whalley et al., 1995), and because the diffusion coefficients of lipids in the sea urchin plasma membrane are on order 10^{-9} cm²/s (Wolf et al., 1981), endocytotic membrane retrieval should randomly retrieve roughly half of the lipids originally found in the plasma membrane (if lipids are free to diffuse between plasma membrane and granule domains). When eggs were labeled with the fluorescent lipid marker octadecyl rhodamine (R18), washed, and then activated, R18 was indeed observed in both the membranes retrieved by endocytosis and on the cell surface (Fig. 1 c). Thus, it appears that unlike most plasma membrane proteins, a plasma membrane lipid marker mixes freely with cortical granule membrane lipids upon membrane fusion. Similar conditions might explain why some cortical granule components are not retrieved after fertilization (Conner et al., 1997).

Are proteins inserted into the plasma membrane by cortical granule exocytosis specifically retrieved by compensatory endocytosis? Experiments suggest that cortical granule membrane proteins are specifically retrieved because protein components labeled on the surface with Alexa 488 maleimide (Fig. 1 a) or concanavalin A (Fig. 1 b) after fertilization and cortical granule exocytosis were translocated into the egg. To test if components inserted into the egg surface after fertilization are retrieved we took advantage of our ability to trap cortical granule components in the cell surface (Vogel et al., 1999). Eggs were labeled with Alexa 488 maleimide, washed, activated with calcium ionophore, and placed in calcium-free sea water to arrest endocytosis. Next, cortical granule membrane proteins inserted into the egg surface were labeled with Alexa 594 maleimide, washed, and imaged by confocal microscopy (Fig. 2 A). We observed eggs whose plasma membrane were labeled in patches of red (Alexa 594), green (Alexa 488), and yellow (where the Alexa 488 and 594 signals overlapped). After as much as 15 min after activation, endocytotic retrieval was initiated by depolarization in normal sea water (9.3 mM CaCl₂) containing 50 mM potassium (Vogel et al., 1999). Subsequent confocal microscopy of these eggs (Fig. 2 B) revealed a dramatic segregation of the two markers. Alexa 594-labeled membranes (the red cortical granule marker) were internalized, while the vast majority of Alexa 488-labeled membrane (the green plasma membrane marker) remained on the egg

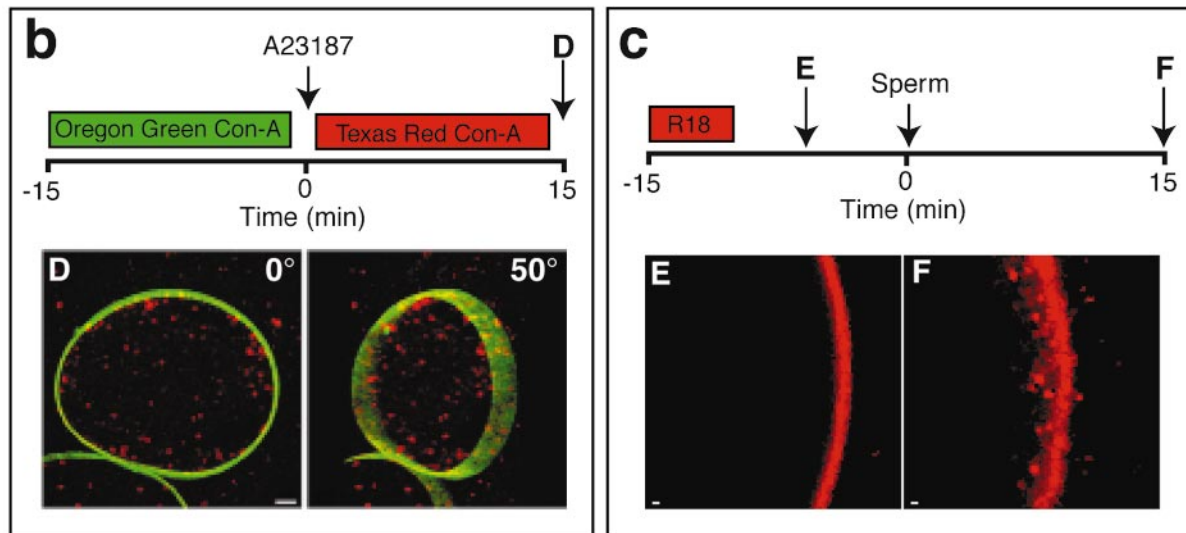
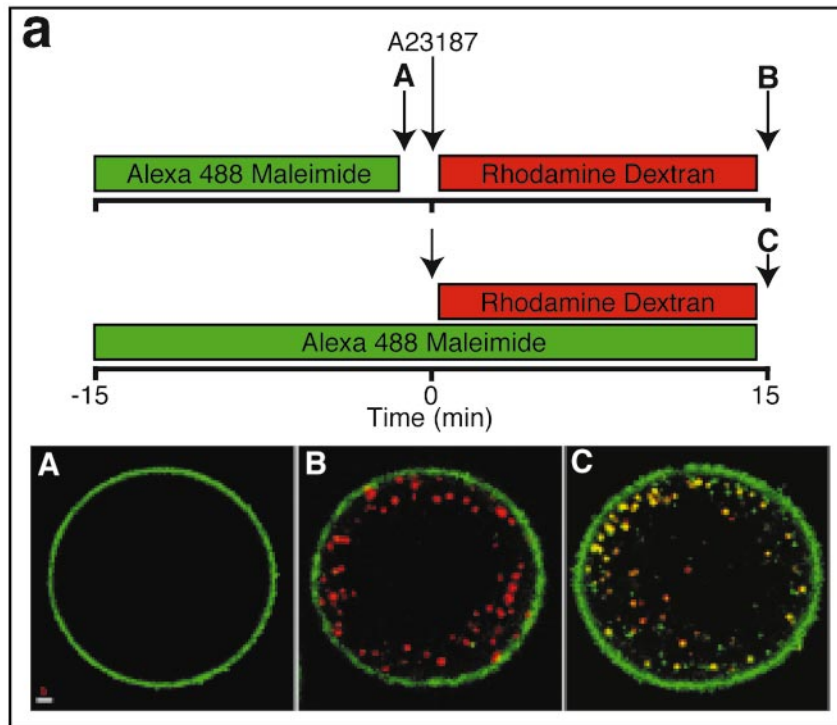


Figure 1. Compensatory endocytosis excludes retrieval of plasma membrane proteins but not lipids. (a) The vitelline membrane of eggs in suspension was removed, surface proteins were labeled with Alexa 488 maleimide, and then imaged by confocal microscopy (see A). Next, eggs were activated with 25 μM A23187 and 30 μM tetramethylrhodamine dextran was added as a fluid phase marker of endocytosis. After 15 min the eggs were washed three times in ASW and green (Alexa 488) and red (tetramethylrhodamine) fluorescence was imaged (B). In control experiments the eggs were treated as in the first experiment except Alexa 488 maleimide was not removed before egg activation (see bottom time line). Eggs were imaged 15 min after activation with calcium ionophore (C). All pictures in a are representative micrographs, $n = 8$, from five different egg preparations. (b) The vitelline membrane was removed and surface proteins were labeled with a green fluorescent conjugate of concanavalin A and Oregon green 488 (2 $\mu\text{g}/\text{ml}$). Next, eggs were activated with A23187 and perfused with a red fluorescent conjugate of concanavalin A and Texas red to label any new exposed concanavalin A binding sites. After a 15-min incubation, the activated eggs were washed three times with ASW and imaged by confocal microscopy (D). A Z-axis series of 15 images spaced 1 μm apart was used in conjunction with a look-through algorithm to generate a three-dimensional rendition of the egg viewed from directly above (0°) or after being rotated by 50° . Pictures in b are representative micrographs, $n = 9$, from nine different egg preparations. (c) Eggs were labeled with the lipidic fluorescent dye octadecylrhodamine and imaged by confocal microscopy (see E). Eggs were activated with a 1:1,000 dilution of sperm and the same egg was imaged again 15 min later (see F). Note the formation of fluorescent intracellular inclusions and a few elongated microvilli extending out from the surface. All pictures in c are representative micrographs, $n = 11$, from five different egg preparations. Bars: (A) 5 μm ; (D) 10 μm ; (E and F) 1 μm .

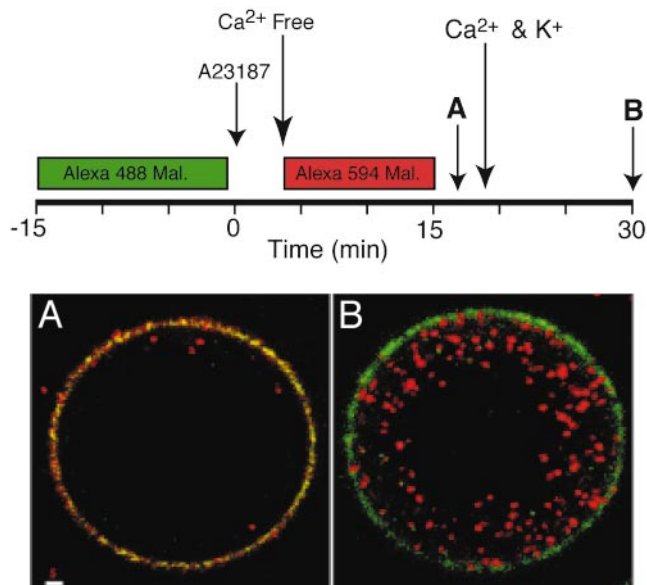


Figure 2. Compensatory endocytosis selectively retrieves cortical granule membrane proteins. The vitelline membranes of eggs in suspension were removed. Next, eggs were washed three times in ASW and incubated with ASW containing Alexa 488 maleimide to label-free sulfhydryl groups on the egg surface. Eggs were then washed three times with ASW to remove dye that had not covalently attached. Eggs were activated with 25 μ M A23187 and 3 min later placed into calcium-free ASW to trap cortical granule membranes in the cell surface. Newly introduced sulfhydryl groups on the egg surface were now labeled with Alexa 594 maleimide (4 μ M in ASW). After three washes with ASW to remove nonincorporated Alexa 594, the Alexa 488 (green) and Alexa 594 (red) fluorescence was imaged by confocal microscopy (A). The two indicators were primarily distributed on the surface of the egg. Membrane depolarization and the subsequent compensatory endocytosis was triggered by the addition of 9.3 mM calcium chloride and 50 mM potassium chloride as indicated on the protocol time line. Subsequent imaging revealed a dramatic segregation of the two fluorescent indicators (B). All pictures are representative micrographs, $n = 5$, from five different egg preparations. Bar, (A) 5 μ m.

surface. Thus, depolarization triggered specific retrieval of membranes inserted into the egg surface long after fertilization (Fig. 2).

Exocytosis Is Required for Compensatory Endocytosis

By definition, compensatory endocytosis is preceded by exocytosis, and in the sea urchin egg exocytosis is the physiologically relevant trigger for membrane retrieval. Nonetheless, the mechanism of compensatory endocytosis may not inherently require prior exocytosis. For example, retrieval might be triggered by a plasma-membrane flaccidity sensor, or by the binding of secreted ligands to cell surface receptors. Activation of the hypothetical cell surface sensors would then activate a signaling pathway to initiate membrane retrieval. If such a coupling mechanism was operant in the sea urchin egg, one could imagine that activation of the appropriate signaling pathway might trigger retrieval even in the absence of exocytotic activity. Clearly, it is important to test if exocytosis is required for retrieval.

Specific retrieval of granule components suggests that prior exocytosis is required for compensatory endocytosis. If prior cortical granule exocytosis is an absolute requirement for compensatory endocytosis, (a) membrane retrieval should occur only at sites where exocytosis has occurred, and (b) depolarization-triggered gating of P-type calcium channels should only trigger retrieval when granule components are present on the cell surface. We exploited the ability to trigger exocytosis focally (Lawson et al., 1978; Chambers and Hinkley, 1979) to test the first prediction. We used focal application of a calcium ionophore to trigger exocytosis focally (rather than globally). Eggs were placed in sea water containing the fluid phase marker tetramethylrhodamine dextran and the calcium ionophore A23187 was focally applied to the egg with a micropipette. This resulted in local cortical granule exocytosis manifested by focal elevation of the fertilization envelope. When eggs were subsequently perfused with sea water to remove extracellular fluid phase marker and imaged by fluorescent microscopy, large red fluorescent inclusions were observed only at the sites where cortical granule exocytosis had occurred (Fig. 3). Other eggs in the field were not activated and had no fluorescent inclusions. Fluid phase uptake evoked in this manner was completely blocked by ω -agatoxin TK (which blocks the calcium influx required for endocytotic membrane retrieval [Vogel et al., 1999]), yet focal elevation of the fertilization envelope was still observed (data not shown). It is known that ionophore-mediated calcium influx does not rescue retrieval activity in agatoxin-treated cells in normal seawater (with 9.3 mM calcium; Vogel et al., 1999). Calcium ionophores did rescue retrieval activity when the extracellular calcium concentration was elevated to >12 mM (Vogel et al., 1999). Therefore, in this experiment where we use normal sea water, focal cortical granule exocytosis was triggered by ionophore-mediated calcium influx, while focal membrane retrieval was triggered by P-channel-mediated calcium influx. Since the depolarization required to open channels is presumed to be uniform across the egg surface,

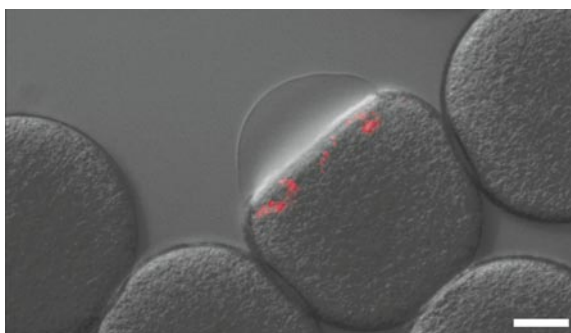


Figure 3. Compensatory endocytosis occurs at sites where exocytosis has occurred. Sea urchin eggs were attached to a polylysine-treated coverslip as previously described (Vogel et al., 1999). Eggs were incubated in artificial sea water (ASW) containing 100 μ M tetramethylrhodamine dextran (3,000 mol wt; Molecular Probes) and activated by focal application of the calcium ionophore A23187 (50 μ M) with a micropipette. After partial elevation of the fertilization envelope was observed, the eggs were washed in sea water to remove exogenous tetramethylrhodamine dextran. Bar, 20 μ m.

these observations are consistent with the notion that cortical granule exocytosis is required for subsequent compensatory endocytosis.

Furthermore, depolarization-triggered gating of P-type calcium channels should only trigger retrieval when granule components are present on the cell surface. To test this, endocytotic membrane retrieval was triggered 15 min after fertilization (by depolarizing eggs with 50 mM extracellular potassium in the presence of the normal extracellular calcium concentration) either in eggs perfused with calcium-free seawater after fertilization, or in eggs maintained in normal seawater. Placing eggs in calcium-free seawater after fertilization trapped cortical granule membranes in the cell surface (Vogel et al., 1999), while eggs maintained in normal seawater completed membrane retrieval within 15 min (Whalley et al., 1995). Retrieval was monitored by the uptake of a fluid phase marker, tetramethylrhodamine dextran, as previously described (Whalley et al., 1995; Vogel et al., 1999). We found that endocytotic membrane retrieval was only triggered by elevated extracellular potassium when cortical granule membranes had been previously trapped in the cell surface (Fig. 4). Depolarization triggered retrieval 15 min after fertilization was blocked by treatment with ω -agatoxin TK (data not shown). Excess retrieval was not observed when the normal time course of membrane retrieval was allowed to transpire before high potassium depolarization (Fig. 4). These data suggest that granule exocytosis and calcium influx through P-type channels are both required for compensatory endocytosis. We cannot, however, rule out the possibility that retrieval removes P-channels from the cell surface. If this were the case, depolarization would fail to trigger further endocytosis because the voltage-sensitive calcium channel would no longer be present in the plasma membrane. Indeed, upon reflection it occurred to us that P-type calcium channels might reside exclusively in the membranes of cortical granules. Such a limited localization would then constrain membrane retrieval to sites of exocytosis, and compensatory endocytosis would remove P-channels from the cell surface.

P-Type Calcium Channels Are Absent from the Cell Surface of the Unfertilized Egg

To test if P-type calcium channels might reside in the cortical granule membrane (as opposed to the plasma membrane) before fertilization we took advantage of the fact that ω -conotoxin MVIIC is a functionally irreversible inhibitor of P-type channels (McDonough et al., 1996), whereas cadmium is a readily reversible inhibitor. If the channels are present in the plasma membrane before fertilization, application of ω -conotoxin MVIIC should block them and prevent endocytosis (Vogel et al., 1999). However, if they are inserted into the plasma membrane by exocytosis it should be possible to block them only after the cortical granules have fused. Virtually every cortical granule fuses with the plasma membrane by 3 min after fertilization (Mohri and Hamaguchi, 1990; Vogel et al., 1996). We treated eggs before and after fertilization with either cadmium, or with ω -conotoxin MVIIC (Fig. 5). We found that a 5-min pretreatment (green bars) with either cadmium or ω -conotoxin MVIIC followed by washing

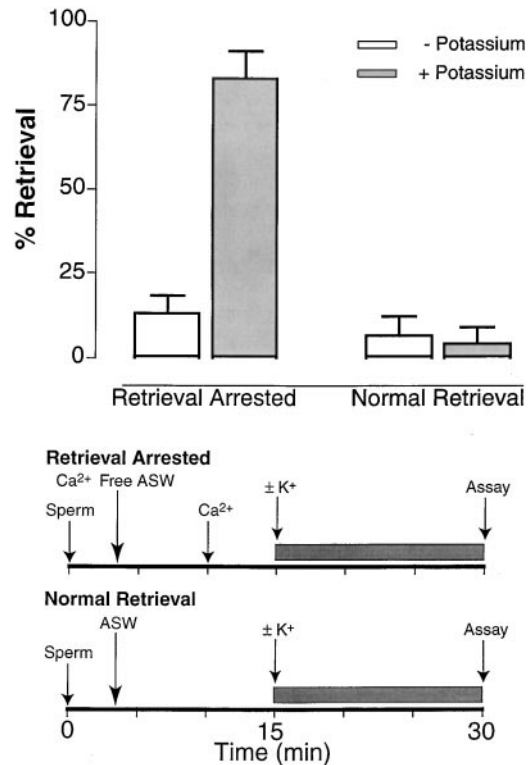


Figure 4. Depolarization triggers compensatory retrieval only when granule components are present on the egg surface. Eggs were activated with a 1:1,000 dilution of sperm and washed with either calcium-free artificial sea water (see Retrieval Arrested time line) or normal ASW (see Normal Retrieval time line) 3 min after addition of sperm. The normal extracellular calcium concentration (9.3 mM) was restored 10 min after sperm addition for the Retrieval Arrested eggs. Note: Extracellular calcium addition at $t = 10$ min does not trigger retrieval because the egg membrane potential at that time is hyperpolarized thus keeping P-type calcium channels closed (Vogel et al., 1999). 15 min after fertilization eggs were either treated with 50 mM potassium chloride to depolarize the egg membrane potential or mock treated. The fluid phase uptake marker (100 μ M tetramethylrhodamine dextran) was also added at 15 min to monitor endocytotic retrieval (black bar in time line). At $t = 30$ min eggs were washed and percent retrieval was measured. The depolarization-induced membrane retrieval was measured (+ Potassium; grey bars) and compared with eggs that were not depolarized (- Potassium; white bars) for eggs placed in calcium-free ASW (Retrieval Arrested) and eggs that had been allowed to undergo their normal retrieval reactions in normal ASW (Normal Retrieval). All points are mean \pm SD, $n = 6$.

away unbound inhibitor did not affect membrane retrieval (Fig. 5). In contrast, treatment with ω -conotoxin MVIIC at fertilization and washing 3 min after fertilization (yellow bars) completely blocked endocytotic membrane retrieval. This same protocol with cadmium did not affect membrane retrieval (Fig. 5), as expected if the cadmium block of calcium channels is reversible and membrane retrieval recovers following the transient block. These results also confirm that the ω -conotoxin MVIIC block of calcium channels is functionally irreversible in this system. The fact that a 3-min incubation with ω -conotoxin MVIIC following fertilization completely inhibited membrane retrieval

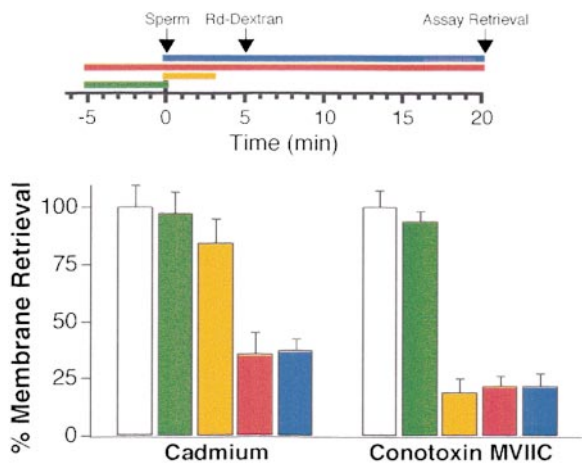


Figure 5. P-type channels are inaccessible to toxin before fertilization. Eggs were fertilized in artificial seawater and 100 μM tetramethylrhodamine dextran was added at 5 min after fertilization and net tetramethylrhodamine dextran uptake was determined after a 15-min incubation. All points are normalized to a positive control in the absence of any inhibitor (white bars). Before fertilization some eggs were preincubated with either 500 μM cadmium or 5 μM conotoxin MVIIC for 5 min and then washed to remove free inhibitor (green bars). These eggs were then fertilized and net membrane retrieval was determined. Other eggs in parallel were fertilized in the presence of either cadmium or conotoxin and after three min the eggs were washed to remove free inhibitor (yellow bars). Note the complete inhibition with the irreversible inhibitor, conotoxin. Finally, some eggs were fertilized in the presence of either cadmium or conotoxin with preincubation (red bars) and without preincubation (blue bars), and net membrane retrieval was determined after 15 min. 5 μM conotoxin MVIIC had no effect on sperm-induced fertilization envelope elevation (Vogel et al., 1999). All points are mean \pm SD, $n = 6$ normalized relative to the control.

suggests that a 5-min preincubation with toxin before fertilization gave ample time for toxin binding. Even a 15-min preincubation with ω -agatoxin TK, another irreversible P-channel inhibitor, failed to inhibit membrane retrieval ($89.1 \pm 5.2\%$ retrieval; mean \pm SEM, $n = 6$), while ω -aga-

toxin TK treatment between 0 and 3 min resulted in an almost complete inhibition of membrane retrieval ($9.7 \pm 1.9\%$ retrieval; mean \pm SEM, $n = 6$). Thus, if ω -conotoxin MVIIC or ω -agatoxin TK-sensitive channels had been present before fertilization, they should have been blocked by toxin pretreatment. We conclude that the P-type channels required for membrane retrieval are either absent from the egg surface before fertilization or toxin binding is uncoupled from channel gating.

Immunolocalization of ω -agatoxin was used to determine if toxin does bind to channels on the surface of the unfertilized egg and whether there is an increase in the number of ω -agatoxin binding sites after fertilization, as expected if the channels are present on cortical granules. We used a polyclonal antibody that recognizes both free ω -agatoxin and the toxin when it is bound to the P-type channel (Calbiochem). Confocal microscopy was used to image the surface fluorescence of unfertilized and fertilized eggs treated with ω -agatoxin, the anti-agatoxin antibody, and a fluorescent secondary antibody. We found that there was a dramatic increase in the surface labeling of the egg following egg activation (Fig. 6 B). Immunogold electron microscopy with this antibody confirmed that there was no specific labeling in the egg before activation (Fig. 7, A and C), but agatoxin binding sites were observed on the membranes of subcortical vesicular structures (SVM) and on the membranes of translucent vesicles (TVM) of fertilized eggs (Fig. 7, B and D). Specific binding was not observed on microvilli or the flat portions of the plasma membrane. We did observe nonspecific binding of gold particles to the core contents of cortical granules and yolk granules (data not shown). Because the density of gold particles on these structures was the same when probed with anti-agatoxin IgG, nonimmune IgG, and when IgG was omitted completely, we conclude that this binding is not specific for agatoxin. Low levels (<2 gold particles/ μm^2) of nonspecific labeling were also occasionally observed on mitochondria and cytoplasm (data not shown). Our findings are consistent with the hypothesis that new ω -agatoxin binding sites are inserted into the surface by cortical granule exocytosis after egg activation.

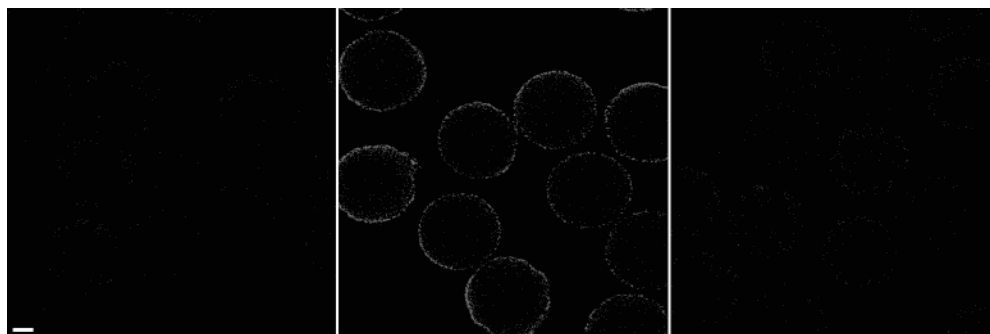


Figure 6. Egg activation increases the number of agatoxin binding sites on the egg surface. (A) Unfertilized eggs were incubated with 100 nM ω -agatoxin-IVA for 7 min, washed, and then fixed. (B) Eggs were activated in ASW containing 50 μM A23187 and 100 nM agatoxin-IVA for 7 min before fixation. (C) Eggs were treated as those in B but agatoxin was absent. After fixation all samples

were washed, blocked, and incubated in a 1:500 dilution of a rabbit anti-agatoxin antibody. Next, samples were incubated with a 1:2,000 dilution of a goat anti-rabbit IgG conjugated to tetramethylrhodamine, washed, and visualized by confocal microscopy. Note that there was virtually no fluorescence observed in unfertilized eggs or in fertilized eggs that were not treated with agatoxin. All pictures are representative micrographs, $n = 4$, from four different egg preparations, excluding eggs damaged by excessive DTT and/or ionophore treatment. Bar, 20 μm .

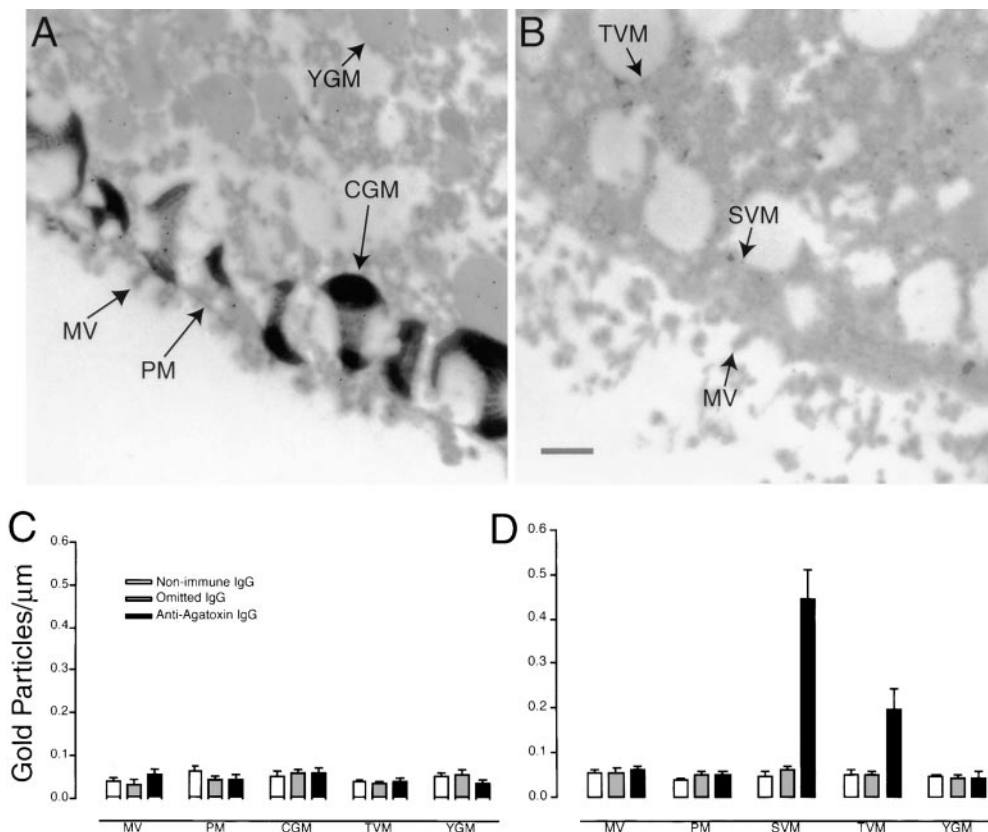


Figure 7. Immunogold electron microscopy of ω -agatoxin binding sites. (A and B) Representative micrographs of sea urchin egg thin sections of unfertilized (A), and 15 min post-fertilized eggs (B) that had been pre-treated with ω -agatoxin TK, washed, fixed, and incubated successively with a rabbit anti-agatoxin antibody, and then with a goat anti-rabbit IgG coupled to 15-nm gold particles. The black arrows indicate examples of the membrane structures analyzed: MV, microvilli; CGM, cortical granule membrane; PM, plasma membrane; TVM, translucent vesicle membrane; SVM, subcortical vesicle membrane; and YGM, yolk granule membrane. Note that a typical gold particle can be observed at the tip of the arrow marked SVM in B. Bar, 0.5 μ m. (C and D) Gold particle density per micron of membrane in unfertilized (C) and 15 min post-fertilization eggs

(D) for the different sea urchin egg membrane structures when treated with nonimmune IgG (white bars), no IgG (grey bars), or anti-agatoxin IgG (black bars). All points are mean \pm SD, $n = 4$ experiments with 40 micrographs analyzed in each experiment.

P-Type Calcium Channels Reside in Cortical Granule Membranes before Fertilization

Antibodies specific for either the *Aplysia* P-type or L-type calcium channel (White et al., 1998) were used to probe immunoblots of isolated cortical granule membranes (Fig. 8 A). We found that the P-type-specific antibody (BC- α_{1A}) specifically bound to components on the sea urchin cortical granule membranes. The two major granule membrane components observed on immunoblots had molecular masses of 168.8 ± 1.3 (mean \pm SEM, $n = 6$) and 135.0 ± 2.0 kD ($n = 4$). These components were not observed on immunoblots probed with the *Aplysia* L-type calcium channel-specific antibody (BC- α_{1D}), preimmune sera, or when *Aplysia* P-type calcium channel peptides were prebound to the BC- α_{1A} . We determined the distribution of P-type calcium channels by quantifying the amount of BC- α_{1A} binding (compared with both preimmune IgG and preabsorbed BC- α_{1A}) using immunogold electron microscopy. As mentioned above, nonspecific binding of gold particles to the core contents of cortical granules and yolk granules was observed (data not shown). Cortical granule membranes were the only cellular component of unfertilized eggs that displayed specific labeling (at a density of 0.57 ± 0.07 gold particles/ μ m of membrane cross-section, mean \pm SD, $n = 4$) (Fig. 8, B and D). Specific binding was not observed on microvilli, plasma membrane, or yolk granule membrane. 15 min after fertilization most cortical

granules had disappeared due to cortical granule exocytosis. Now specific labeling with BC- α_{1A} was observed in subcortical vesicles near the egg surface (0.53 ± 0.06 particles/ μ m), and in the membranes of the electron translucent vesicles deeper in the cytoplasm (0.51 ± 0.03 particles/ μ m; Fig. 8 C). Cortical granule membranes, subcortical vesicle membranes, and translucent vesicle membranes were the only structures specifically labeled in these experiments, the density of gold particle labeling was similar between these structures, and the vesicles bounded by these membranes were approximately the same size. These findings suggest that cortical granules fuse with the plasma membrane to form subcortical vesicles and these structures are retrieved into the cytoplasm to form translucent vesicles.

To confirm that the subcortical structures labeled by BC- α_{1A} and by the agatoxin-specific sera represent a transient intermediate structure of the exocytosis-endocytosis pathway, we measured the number of cortical granules and subcortical vesicles observed in electron micrographs of unfertilized eggs, and in fertilized eggs at 5 and 15 min after fertilization. In both control and agatoxin-treated eggs the cortical granules should disappear upon fertilization. Furthermore, the number of subcortical vesicles which appear upon fertilization in agatoxin-treated eggs (at 5 and 15 min post fertilization) should be approximately equivalent to the number of cortical granules in un-

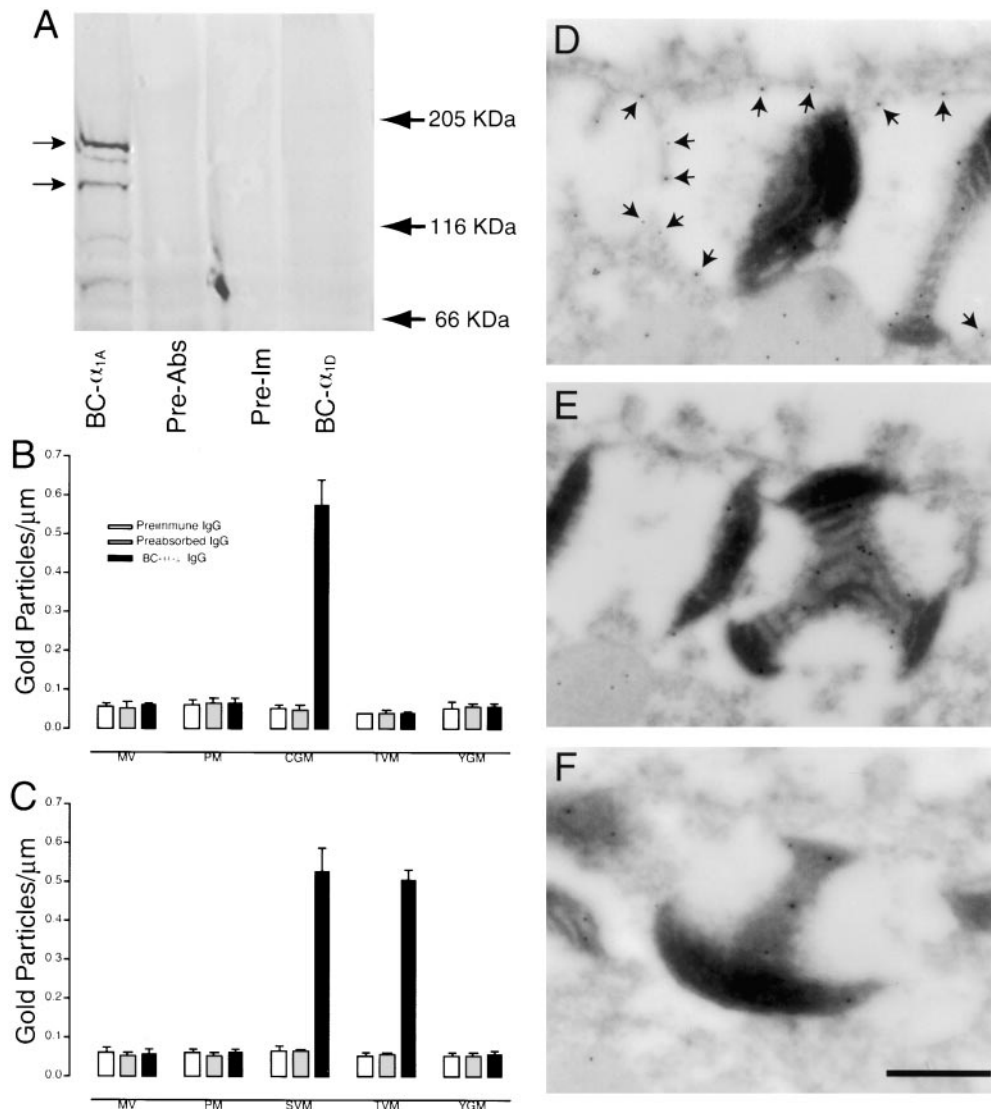


Figure 8. Immunolocalization of sea urchin egg P-type calcium channels. (A) Membranes were prepared from isolated sea urchin cortical granules and separated on a 7.5% SDS-PAGE. Proteins were electroblotted onto nitrocellulose, blocked and incubated with 1 μ g/ml of: *Aplysia* P-type-specific affinity-purified rabbit antisera, BC- α_{1A} ; L-type-specific antisera, BC- α_{1D} ; BC- α_{1A} preincubated with a twofold excess of immune peptide, Pre-Ab; and the preimmune sera of the rabbit which produced BC- α_{1A} , Pre-Im. Immunoreactivity was detected with a goat anti-rabbit antisera conjugated to alkaline phosphatase. Arrows on left show major components unique to the P-type-specific antisera. Arrows on right are size markers. (B and C) Gold particle density per micron of membrane in unfertilized (B) and 15 min post-fertilization eggs (C) for the different sea urchin egg membrane structures (MV, microvilli; CGM, cortical granule membrane; PM, plasma membrane; TVM, translucent vesicle membrane; SVM, subcortical vesicle membrane; and YGM, yolk granule membrane) when treated with preimmune IgG (white bars), preabsorbed BC- α_{1A} (grey bars), or BC- α_{1A} (black bars). All

points are mean \pm SD, $n = 4$ experiments with 40 micrographs analyzed in each experiment. (D-F) Representative micrographs of unfertilized sea urchin egg thin sections treated with BC- α_{1A} (D), preimmune sera (E), or BC- α_{1A} preabsorbed with immune peptide (F), and then treated with a goat anti-rabbit IgG coupled to 15-nm gold particles. The black arrows indicate gold particles on the cortical granule surface. Note: The nonspecific binding of gold particles over yolk and cortical granule contents in all three micrographs. Bar, 0.5 μ m.

fertilized eggs because membrane retrieval is inhibited by agatoxin. In contrast, the number of subcortical vesicles observed at 5 and 15 min after fertilization in control eggs should decrease as these structures are internalized by compensatory endocytosis and should be transported into the cytoplasm. These dynamic predictions were observed in our micrographs (Fig. 9). In agatoxin-treated eggs the number of cortical granules (37.2 ± 3.8 structures/micrograph, mean \pm SD, $n = 40$) observed in unfertilized eggs and the number of subcortical vesicles at 5 (34.5 ± 5.1 structures/micrograph) and 15 min after fertilization (30.6 ± 4.7 structures/micrograph) were similar. In control eggs the number of subcortical vesicles observed 5 min after fertilization (12.9 ± 2.5 structures/micrograph) was less than the number of cortical granules in unfertilized eggs (36.4 ± 4.1 structures/micrograph), and the number of

subcortical vesicles declined with time (compare 5 and 15 min after fertilization in Fig. 9 A).

Discussion

Little is known about the mechanism of compensatory endocytosis and how it is triggered by exocytotic activity. Specific retrieval of granule membrane proteins has been observed in chromaffin cells (Phillips et al., 1983; Patzak et al., 1984; Patzak and Winkler, 1986), mast cells (Thilo, 1985), and in the neurosecretory nerve endings of the neurohypophysis (Pow and Morris, 1991; Nordmann and Artault, 1992), and these findings suggest that granule components are required for retrieval. Here we show in the sea urchin egg that the mechanism of compensatory endocytosis discriminates against proteins associated with the

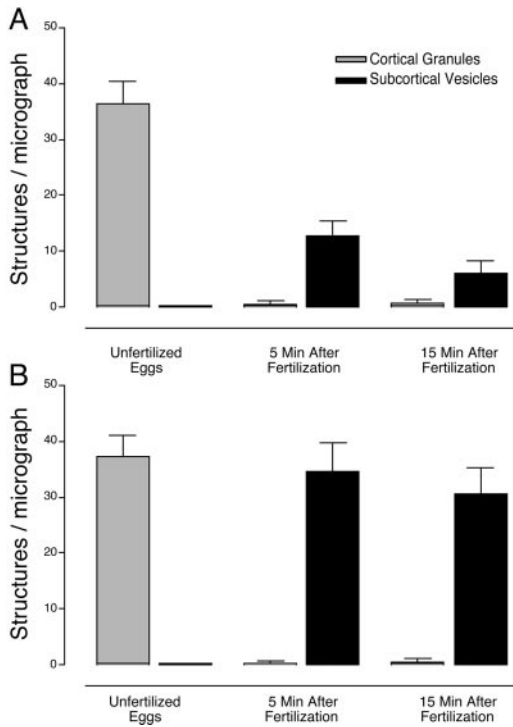


Figure 9. Subcortical vesicles are a transient compartment of the exocytosis–endocytosis cycle. The average number of cortical granules (grey bars) and subcortical vesicles (black bars) per micrograph was determined in unfertilized eggs, and at 5 and 15 min after fertilization in the absence (A) or presence (B) of ω -agatoxin. All points are mean \pm SD, from 40 micrographs.

plasma membrane (Fig. 1), selects for proteins associated with the granule (Fig. 2), but does not bar the retrieval of lipids from the plasma membrane (Fig. 1). Furthermore, we show that compensatory endocytosis only occurs at sites where exocytosis has occurred (Fig. 3). The spatial specificity of compensatory endocytosis can be used to limit the types of mechanisms that can be considered for this process.

In sea urchin eggs depolarization opens ω -agatoxin-sensitive channels and calcium influx through these channels triggers compensatory endocytosis (Vogel et al., 1999). It was reasonable to speculate that depolarization of the cell membrane potential couples exocytosis and compensatory endocytosis by opening plasma membrane resident P-channels. This simple coupling mechanism must now be rejected because a cell, particularly a round cell like an egg, is isopotential, and therefore either depolarized or not. Focal retrieval cannot be explained by this mechanism since focal depolarization is not possible. Furthermore, depolarization did not trigger additional membrane retrieval when compensatory endocytosis had been allowed to run its normal course in fertilized eggs (Fig. 4), nor did depolarization trigger either cortical granule exocytosis (Schmidt et al., 1982) or endocytosis (Vogel et al., 1999) in unfertilized eggs, despite the fact that high extracellular potassium is known to depolarize the egg membrane potential (Jaffe and Robinson, 1978), and stimulate a verapamil-sensitive calcium influx (Schmidt et al., 1982). Thus,

while depolarization is required to open voltage-gated calcium channels, depolarization alone cannot explain the coupling of exocytosis and compensatory endocytosis. Apparently, prior cortical granule exocytosis is also required. Clearly, a full understanding of the coupling of cortical granule exocytosis and endocytotic membrane retrieval requires the elucidation of the mechanism of egg depolarization upon fertilization.

What mechanism can then account for focal retrieval at exocytotic sites? We speculated that prior exocytosis is required for compensatory endocytosis because cortical granule membrane components are critical for the retrieval mechanism. We hypothesized that the active components which respond to depolarization of the cell, the P-type calcium channels, are themselves limited to exocytotic sites by virtue of a cellular localization in secretory granule membranes. If this were the case, depolarization and prior granule exocytosis would be required for retrieval, and retrieval would be limited to exocytotic sites. Focal application of calcium ionophore would trigger focal cortical granule exocytosis and a focal insertion of P-type calcium channels into the cell surface. These newly inserted P-channels would respond to a depolarization of the membrane potential by opening and thus mediate a local Ca^{2+} influx. The spatially limited rise in calcium concentration would trigger endocytosis only at this site. Voltage-gated calcium channels have been found in other secretory granule membranes, and are known to be transiently inserted into the surface as a result of exocytosis (Bokvist et al., 1995; Passafaro et al., 1996). In the bag cell neurons of *Aplysia*, P-type calcium channels reside on intracellular vesicles and may be inserted into the plasma membrane upon activation of protein kinase C (White et al., 1998). While the functional significance of these vesicular calcium channels has not been determined in these systems, in *Aplysia* bag cells it was noted that the internal sites of P-channel immunoreactivity were associated with membrane recycling sites (White et al., 1998).

Four lines of evidence argue that P-type channels do in fact reside in cortical granule membranes before fertilization and are translocated into the cell surface by cortical granule exocytosis. First, ω -conotoxin MVIIC and ω -agatoxin TK, specific and essentially irreversible inhibitors of P-type calcium channels (and of compensatory endocytosis), failed to inhibit retrieval when eggs were pretreated with the toxins before triggering cortical granule exocytosis. In contrast, these toxins effectively blocked retrieval when transiently applied to the egg during cortical granule exocytosis (0–3 min after fertilization). Application of a reversible inhibitor of P-type channels, cadmium, during this same short time period failed to block retrieval. Presumably there was enough calcium influx after removing cadmium to support complete retrieval.

Second, ω -agatoxin did not bind to the surface of unfertilized eggs, but did bind to the surface of fertilized eggs (Figs. 6 and 7). It is unlikely that ω -agatoxin binding in our experiments, or the binding of secondary and tertiary detection reagents, was caused by nonspecific adhesion to granule contents deposited on the cell surface by exocytosis because omitting ω -agatoxin from our labeling protocol prevented cell surface labeling (Fig. 6 C), as did omitting the anti-agatoxin antibody or substituting normal rabbit

sera (Fig. 7 D). Furthermore, there was no change in the amount of ω -agatoxin binding detected on microvilli after fertilization (Fig. 7). Microvilli would be surrounded by the secreted contents of cortical granules.

Third, cortical granule membrane components have an epitope that is recognized by a P-type channel-specific affinity-purified antibody, BC- α_{1A} (Fig. 8 A). These components were not recognized by an L-type channel specific affinity-purified antibody, BC- α_{1D} , or when preimmune sera from the rabbit that produced the BC- α_{1A} antibody were used. The specificity of BC- α_{1A} was further demonstrated by its inability to bind to these granule components when it was prebound to its immunogenic peptide (Fig. 8 A). In contrast, there was no competitive inhibition of binding when the BC- α_{1A} antibody was preincubated with an L-type calcium channel peptide (data not shown). We always observed multiple immunoreactive components when cortical granule membranes were probed with BC- α_{1A} in immunoblots. The relative abundance of these components was variable between membrane preparations, and there was a shift toward lower molecular mass species when frozen membrane samples were reprobated. It is well known that calcium channel subunits are labile (Conley and Brammar, 1999), yet inclusion of protease inhibitors did not prevent the appearance of multiple components. Nonetheless, the molecular mass of the largest component, 169 kD, is an appropriate size for an α_{1A} calcium channel subunit (Conley and Brammar, 1999), and this and the lower molecular mass components recognized by BC- α_{1A} did not appear in our negative controls.

Finally, the only site of specific gold particle labeling with BC- α_{1A} in the unfertilized egg was on the membranes of cortical granules (Fig. 8 B). Specific labeling was not observed on the plasma membrane, or on any other intracellular organelle, and the number of gold particles measured per μm of cortical granule membrane was over five times that observed on the plasma membrane, or on the cortical granule membrane when preabsorbed BC- α_{1A} or preimmune sera was used. Upon fertilization, gold particle labeling with BC- α_{1A} is observed on the membranes of subcortical vesicles and on translucent vesicle membranes.

Subcortical vesicles and translucent vesicles are both electron-translucent, and in cross-section appear approximately the same size as cortical granules (see Fig. 7). Many subcortical translucent vesicles appear under the plasma membrane in fertilized eggs (Whalley et al., 1995). Because cortical granules in the unfertilized egg are essentially the only large vesicles observed within 1 μm of the cell surface, we defined subcortical vesicles as the electron-translucent vesicles and membrane invaginations that are located within 1 μm of the cell surface (primarily observed in the fertilized egg). It is not known if all of these structures are contiguous with the plasma membrane because serial sections were not reconstructed in this study. Translucent vesicles, in contrast, are located deeper in the cytoplasm. While the distinction between these two structures might be artificial, we felt it was necessary to describe them as two different structures because some large electron-translucent vesicles are observed in both unfertilized and fertilized egg, and because many subcortical vesicles might in reality be subcortical invaginations. Nonetheless, the density of gold particles observed on subcortical

vesicle membranes and translucent vesicle membranes was identical to the density observed on the membranes of cortical granules (~ 0.5 particles/ μm) when BC- α_{1A} was used as a probe, suggesting that these labeled structures are all manifestations of the cortical granule membrane at different stages of the exocytosis-endocytosis cycle, and that the P-channels density in these membranes have not been diluted by mixing with plasma-membrane components. This interpretation was further substantiated by counting the average number of cortical granules and subcortical vesicles observed on comparable electron micrographs (Fig. 9). When compensatory retrieval was inhibited with ω -agatoxin we found that the number of subcortical vesicles at both 5 and 15 min after fertilization was comparable with the number of cortical granules observed in the unfertilized egg. In contrast, when endocytosis was allowed to proceed (i.e., no toxins) the number of subcortical vesicles per micrograph was less than the number of cortical granules in the unfertilized egg, and this number declined with time.

Under normal physiological conditions exocytotic sites are distributed evenly over the entire surface of the egg, and cortical granule exocytosis is temporally skewed over a time course of a minute or more (Vogel et al., 1996). Why, then, do sea urchin eggs have such an intricate mechanism to limit P-channels to exocytotic sites? If eggs use a ubiquitous calcium-activated retrieval mechanism, selective retrieval of granule membrane proteins could be generated by selectively exposing granule proteins to elevated calcium. Such a mechanism for specific retrieval requires the containment of intracellular calcium in micro-domains immediately under patches of inserted cortical granule membrane. Indeed, attempts to image the P-channel-mediated calcium influx into eggs have failed (Vogel et al., 1999), suggesting that the calcium influx is limited to regions below the resolution of light microscopy ($\sim 0.2 \mu\text{m}$). Furthermore, without calcium containment to the region under cortical granule membrane patches, focal calcium influx through P-type calcium channels would initiate a chain reaction of adjoining cortical granule exocytosis and thus further channel insertion. Ultimately a single exocytotic event would trigger exocytotic events across the entire egg surface. Since a chain reaction is not observed (see Fig. 3), the calcium influx through P-channels that triggers compensatory retrieval is likely to be limited to micro-domains situated immediately under individual surface resident cortical granule membrane patches. If eggs use a ubiquitous calcium-activated retrieval mechanism, we would expect to see a greater amount of membrane retrieval with ionophore-mediated influx (compared with P-channel-mediated influx) because ionophore is likely to be randomly distributed across the egg surface. The amount of membrane retrieval triggered by ionophore-mediated calcium influx, however, was identical to the amount of retrieval triggered by calcium influx through P-type calcium channels (Vogel et al., 1999). Thus, selective exposure to elevated calcium alone is not sufficient to explain the selectivity of retrieval for granule components. Distinct patches of granule membrane were observed on the cell surface 15 min after egg activation when membrane retrieval was arrested (Fig. 2 A), and the gold particle membrane density observed with the BC- α_{1A} antibody

was the same in cortical granule membranes, subcortical vesicle membranes and translucent vesicle membranes (Fig. 8). For these reasons, we suggest that P-channels, as well as the majority of proteins associated with the cortical granule membrane remain associated in membrane rafts (Brown and London, 1998; Lafont et al., 1998, 1999; Cheong et al., 1999), and are not free to mix with plasma-membrane proteins during their short residency on the cell surface.

We do not know if a mechanism involving exocytotic insertion of calcium channels is involved in coupling exocytosis and endocytotic membrane retrieval in other cells. Clearly, regulation of the exocytotic delivery of channels and their retrieval from the cell surface will regulate channel surface density and thus their activity (Strong et al., 1987; Katsura et al., 1997; Marinelli et al., 1997; Lissin et al., 1999; Shi et al., 1999). Chromaffin cells treated with Lambert-Eaton myasthenic syndrome (LEMS) antibodies, many of which primarily bind to P/Q type calcium channels, showed a decrease in depolarization evoked calcium influx, as well as a decrease in the membrane capacitance phases typically interpreted as exocytosis, and endocytotic membrane retrieval (Engisch et al., 1999). While this might suggest a role for P/Q type channels in exocytosis-endocytosis coupling in chromaffin cells, other methods that reduce calcium influx, but may not involve P/Q type channels, also interfere with compensatory endocytosis (Engisch and Nowycky, 1998). In neurons there is also a tight coupling of exocytosis and membrane retrieval (Heuser and Reese, 1981; Ramaswami et al., 1994; Henkel and Betz, 1995). The fact that P-type calcium channels are found in the plasma membrane of neurons but are absent from the plasma membrane of unfertilized eggs might suggest that exocytosis-endocytosis coupling in neurons use a different coupling mechanism. Presumably, P-type channels in synaptic membranes are inserted into the cell surface by vesicle fusion. It is not known if these vesicles are synaptic vesicles, nor is it known if P-type channels are retrieved and/or recycled. If so, the large potentiation of neuromuscular transmission observed with LEMS could be explained by an increase in the number of P/Q type channels trapped on the presynaptic surface membrane if retrieval is inhibited by LEMS antibodies. Perhaps the biggest difference between the sea urchin egg and the synapse vis-a-vis exocytosis-endocytosis coupling is the duration of membrane depolarizing in response to an action potential. At synapses, the membrane potential remain depolarized for only milliseconds, while in eggs, depolarization lasts for minutes. The short depolarization period at synapses might not allow sufficient time to trigger all fusion competent secretory vesicles to fuse, nor need it allow for sufficient time to retrieve all previously inserted membranes. Thus, it is possible that a short depolarization might trigger exocytotic insertion of calcium channels and endocytotic removal of previously inserted channels concurrently, while a long depolarization could allow for both the insertion and retrieval of the same calcium channels in a single cycle. Consistent with the insertion of calcium channels upon synaptic vesicle fusion, at the chick calyx synaptic terminal a distinct inward current was observed coincident with vesicle discharge (Stanley, 1993). If the mechanism that we propose couples exocytosis and membrane re-

trieval in the egg is operant at synaptic terminals this mechanism might also participate in activity-dependent synaptic recovery after transmission (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998).

In conclusion, we have shown that cortical granule exocytosis is required for compensatory endocytosis and compensatory endocytosis is only observed at sites of prior exocytosis. Three types of experiments (toxin inhibition of retrieval activity, anti-agatoxin immunolocalization, and anti-P-channel immunolocalization) all argue that P-type calcium channels are absent from the egg surface before fertilization, but appear on the cell surface immediately after egg activation. Two types of experiments (immunoblotting and immunogold electron microscopy) directly argue that P-type channels reside on cortical granule membranes before fertilization. Furthermore, it is known that cortical granules fuse with the plasma membrane upon fertilization, and that the only way that an integral membrane protein like a voltage-gated calcium channel could be inserted into the cell surface is by exocytotic membrane fusion. Thus, we conclude that the calcium influx required for compensatory endocytosis in the sea urchin egg can only occur at exocytotic sites because these are the only sites on the egg surface that have these voltage-gated channels.

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