Calcium influx is required for endocytotic membrane retrieval

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ABSTRACT Cells use endocytotic membrane retrieval to compensate for excess surface membrane after exocytosis. Retrieval is thought to be calcium-dependent, but the source of this calcium is not known. We found that, in sea urchin eggs, endocytotic membrane retrieval required extracellular calcium. Inhibitors of P-type calcium channels—cadmium, ω-conotoxin MVIIIC, ω-agatoxin IVa, and ω-agatoxin TK—blocked membrane retrieval; selective inhibitors of N-type and L-type channels did not. Treatment with calcium ionophores overcame agatoxin inhibition in a calcium-dependent manner. Cadmium blocked membrane retrieval when applied during the first 5 minutes after fertilization, the period when the membrane potential is depolarized. We conclude that calcium influx through ω-agatoxin-sensitive channels plays a key role in signaling for endocytotic membrane retrieval.

On fertilization, the intracellular stores of sea urchin eggs release calcium into the cytoplasm, which in turn triggers the exocytosis of ~15,000 docked secretory granules. The secretion of proteinaceous cortical granule contents is responsible for the elevation of the fertilization envelope, whose physiological role is the prevention of polyspermy (1, 2). One potentially adverse effect of exocytotic release is the addition of vesicular membrane to a cell’s plasma membrane. Propitiously, a burst of exocytotic activity is often followed by a burst of endocytotic activity (3–5). In sea urchin eggs, cortical granule exocytosis is followed by retrieval of surface membrane by an endocytotic mechanism into large intracellular vesicles (6). The magnitude of this endocytotic reaction quantitatively compensates for the excess membrane added to the surface. Other than an apparent requirement for prior exocytosis, the specific molecular components that initiate endocytotic membrane retrieval are not known.

Endocytotic membrane retrieval in some cells is thought to be calcium-dependent (7–13). At the frog neuromuscular junction, extracellular calcium is required for endocytotic membrane retrieval (14). At the Drosophila neuromuscular junction, extracellular calcium is required before a step blocked by mutations in dynamin (15). In contrast, high extracellular calcium inhibits endocytosis in retinal bipolar cells (16), and in cultured hippocampal neurons membrane retrieval is thought to be calcium-independent (17, 18). In most secretory cells, exocytosis requires calcium influx (19), and the time course of exocytosis and endocytosis overlaps (20, 21). These factors complicate analysis of the dependence of membrane retrieval on calcium influx. In the sea urchin egg, fertilization envelope elevation is the direct result of cortical granule exocytosis (22, 23). Calcium influx is not required for cortical granule exocytosis (24–26); fertilization envelope elevation has been observed in calcium-free seawater (27). Furthermore, exocytosis and membrane retrieval can be temporally resolved in this system (6, 28).

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Similar measurements were taken at different time points to determine TMR-Dex endocytosis, and incubations were performed at room temperature. All samples were centrifuged at 300 × g for 30 seconds, and the egg pellets were resuspended and washed three times in 10 ml of PKME at 4°C. The final egg pellets were solubilized in 1 ml of 2.5% Triton X-100. Nonsolubilized material was removed by centrifugation at 4,000 × g for 5 min. TMR-Dex endocytosis was determined in a microtiter plate reader (Cytosensor II, PerSeptive Biosystems, Framingham, MA) by using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Protein concentration was determined by using the micro BCA assay method (Pierce), and samples were read in a SpectraMax microplate spectrophotometer (Molecular Devices). Background or nonspecific absorption of TMR-Dex determined in the samples taken immediately after the addition of TMR-Dex was subtracted from all other values to yield a result corresponding to stimulated, time-dependent TMR-Dex uptake normalized to the protein concentration, which we refer to as “membrane retrieval.” Membrane retrieval normalized to the maximal retrieval in ASW is referred to as % membrane retrieval.

**Calcium Imaging.** Eggs (*L. pictus*) were attached to poly(Lysine)-treated glass coverslips (0.1 mg/ml for 1–2 min) and mounted in a microscope chamber. Eggs were injected with the fluorescent calcium indicator calcium green dextran (200 μM in 450 mM KCl; Molecular Probes) by using a model 5246 transjector unit coupled to a model 5171 micromanipulator (Eppendorf). We estimate from the diameter of the injection bolus that the final concentration of indicator was between 4 and 10 μM. Calcium green fluorescence was imaged on a Zeiss Axiowert microscope by using a ×40 1.3 numerical aperture objective. The microscope was equipped with a Photometrics (Tuscon, AZ) cooled charge-coupled device PXL camera. Data acquisition and analysis was controlled by RAITOOL software from Inovision (Raleigh, NC) running on a Silicon Graphics O2 workstation. For fertilization, eggs were incubated with ASW containing a 1:1,000 dilution of sperm.

**Microscopy.** A Zeiss Axiophot microscope with a cooled charge-coupled device camera (Photometrics, Sensys) was used to image elevation of the egg fertilization envelope and uptake of TMR-Dex by using a ×40 1.0 numerical aperture objective.

**RESULTS**

Eggs were suspended in ASW and fertilized. After exocytosis was complete (3 min; refs. 28 and 29), the eggs were incubated for 15 minutes in DC-ASW (calcium concentration ranged from 0 to 9.3 mM) containing 100 μM tetramethylrhodamine dextran. Eggs were then washed four times with PKME and assayed for fluorescent dextran uptake, a fluid-phase marker for endocytic membrane retrieval in eggs (6). The calcium concentration of normal seawater is 9.3 mM. We observed maximal dextran uptake when the extracellular calcium concentration was ≥1 mM (Fig. 1). Below 21 μM calcium, the majority of membrane retrieval was blocked (>80%). The residual retrieval that still occurred in CF-ASW might have been triggered by calcium liberated from intracellular stores during the first 3 minutes of fertilization. At 114 μM extracellular calcium (pCa = –3.945 ± 0.073, mean ± SEM), we observed 50% of retrieval activity, and at between 21 μM and 1 mM extracellular calcium, we observed a linear dependence of retrieval on pCao. Thus, extracellular calcium is required for most of the observed endocytic membrane retrieval, and membrane retrieval has a steep dependence on the extracellular calcium concentration.

Because a major route of calcium influx into cells is through voltage-gated calcium channels, we tested the hypothesis that voltage-gated calcium channels provide the calcium signal required for membrane retrieval. Cadmium, a nonselective blocker of voltage-gated calcium channels, inhibited membrane retrieval (at 500 μM) to the same extent as removal of extracellular calcium (Fig. 2A). The IC50 for inhibiting membrane retrieval was 38.3 μM. Voltage-gated calcium channels should only allow calcium influx when the membrane potential of the cell depolarizes. It is known that the membrane potential of sea urchin eggs drops from approximately −70 mV to 0 mV within seconds of fertilization and then slowly hyperpolarizes to below its initial value (30). Because the membrane potential remains above −40 mV for only ~5 minutes, the calcium influx that triggers membrane retrieval should only happen during this initial period. We tested this prediction by adding cadmium at various times after fertilization and measuring membrane retrieval. We found that addition of cadmium 3 and 5 min after fertilization resulted in a significant inhibition of membrane retrieval (by one-way ANOVA, P < 0.001) whereas addition of cadmium at 7 or 10 minutes did not significantly inhibit retrieval (P > 0.05) (Fig. 2B). Fertilization-envelope elevation and egg division was observed in eggs fertilized in the presence of 500 μM cadmium (data not shown).

Even though cadmium only inhibited retrieval when applied during the first 5 minutes, retrieval itself continued for 15 minutes (6) (Fig. 2B). This persistence might reflect a prolonged elevation of intracellular calcium concentration. Alternatively, it might result from an independent activation of downstream components. It is known that, in sea urchin eggs, the intracellular calcium concentration increases after fertilization and remains elevated for ~5 min (31) (Fig. 2C), suggesting that the persistence of retrieval activity is not caused by the persistent elevation of bulk calcium. This increase is known to be caused by the release of calcium from intracellular stores (31). It is possible that there is a persistent spatially restricted calcium signal that is beyond the limits of detection of the techniques obtainable here. Indeed, when cadmium was applied to eggs 1 min after activation, we failed to observe any change in the bulk calcium signal (Fig. 2C), even though we know that membrane retrieval is substantially inhibited by cadmium. Imaging of the calcium signal also failed to reveal any observable spatial change in calcium associated with the perfusion of cadmium (data not shown).

**FIG. 1.** Extracellular calcium is required for endocytotic membrane retrieval. Eggs were suspended in ASW and fertilized with sperm. After 3 minutes, the eggs were incubated for 15 minutes in DC-ASW containing the indicated concentration of free calcium and 100 μM TMR-Dex (M = 3,000). Eggs were then washed 4 times with PKME and assayed for fluorescent dextran uptake. All data points are mean ± SD, n = 6.
Several types of voltage-gated calcium channels can be pharmacologically distinguished, including N-, P-, Q-, L-, R-, and T-type channels. ω-Conotoxin MVIIC, a peptide blocker of N-, P-, and Q-type calcium channels (but not L-, R-, or T-type channels) inhibited membrane retrieval (IC₅₀ = 3.5 μM; Fig. 3). In contrast, nifedipine, which selectively blocks L-type calcium channels, did not inhibit retrieval (Fig. 3). The peptide ω-agatoxin IVA inhibited membrane retrieval at a concentration that is selective for P-type calcium channels (IC₅₀ = 7.3 nM), whereas ω-conotoxin GVIA, which selectively blocks N-type calcium channels, did not (Fig. 3). The ability of specific inhibitors to block endocytotic membrane retrieval at the appropriate concentrations strongly suggests that calcium influx through calcium channels similar to mammalian P-type channels (32–34) is utilized for endocytotic membrane retrieval. P-type channels are high-threshold voltage-gated calcium channels that show little inactivation (35, 36) and could thus mediate the 5-minute window of calcium influx (Fig. 2B).

We verified that a P-type calcium channel was involved in calcium influx by showing that ω-agatoxin TK, a synthetic P-type specific blocker, also inhibited membrane retrieval (Fig. 3). If the only effect of this toxin was to inhibit the calcium influx required for membrane retrieval, then retrieval should be rescued by application of calcium ionophores. Furthermore, the rescue should depend on the extracellular calcium concentration. Indeed, ω-agatoxin TK blockage of retrieval could be completely reversed by the addition of calcium ionophores (Fig. 4A), and the rescue of retrieval activity with ionophore depended on the extracellular calcium concentration (Fig. 4B). To rule out the possibility that ω-agatoxin TK was simply inhibiting cortical granule exocytosis, we verified that normal fertilization envelope elevation, the direct result of cortical granule exocytosis, occurred in the presence of toxin (Fig. 5).

Fig. 2. Cadmium blocks the calcium influx required for membrane retrieval. (A) Cadmium inhibits endocytotic membrane retrieval. Eggs were suspended in ASW and fertilized with sperm. After 3 minutes, the eggs were incubated for 15 minutes in ASW containing the indicated concentration of cadmium and 100 μM TMR-Dex. Eggs were then washed 4 times with ASW and assayed for fluorescent dextran uptake. All data points are mean ± SD (n = 6), normalized to a zero cadmium control. (B) To determine when calcium influx is required for triggering membrane retrieval, eggs were suspended in ASW containing 100 μM TMR-Dex and fertilized. At the indicated times (black arrows) after the addition of sperm, cadmium was added to a final concentration of 500 μM, and the eggs were incubated for up to 30 minutes. Eggs were then washed 4 times with ASW and assayed for fluorescent dextran uptake. All points are mean ± SD (n = 6), normalized relative to the control. (C) Cadmium does not alter the calcium signal from whole eggs. Eggs injected with the calcium indicator calcium green dextran were fertilized at t = 1 min. At t = 2 min, cadmium in ASW was added to a final concentration of 500 μM (●, mean whole-cell fluorescence ±SD, n = 18 cells), or ASW was added as a control (○; mean ± SD, n = 11 cells).

Fig. 3. The pharmacological characterization of the calcium influx required for membrane retrieval. Eggs were suspended in ASW and fertilized with sperm. After 3 minutes the eggs were incubated for 15 minutes in ASW containing the indicated concentration of ω-conotoxin MVIIC, an inhibitor of N-, P-, and Q-type voltage-activated calcium channels (○); all data points are mean ± SD, n = 3 except 0 μM was n = 6); nifedipine, an inhibitor of L-type voltage-activated calcium channels (●; all points are mean ± SD, n = 3); ω-conotoxin GVIA, a specific inhibitor of N-type voltage-activated calcium channels (●; all data points are mean ± SD, n = 6); ω-agatoxin TK, a selective inhibitor of P-type voltage-activated calcium channels (●; all data points are mean ± SD, n = 6 except 2.5 and 5 nM which were n = 3) and ω-agatoxin TK, a selective inhibitor of P-type voltage-activated calcium channels in this concentration range (●; all data points are mean ± SD, n = 6 except 2.5 and 5 nM which were n = 3) and ω-agatoxin TK, a selective inhibitor of P-type voltage-activated calcium channels in this concentration range (open triangles; all data points are mean ± SD, n = 3). The insert in B shows the ω-agatoxin IVA and ω-agatoxin TK dose–response curves on a logarithmic scale. All data points were normalized to controls lacking inhibitors.
Despite the complete inhibition of membrane retrieval, as indicated by the absence of fluorescent inclusions, fertilization envelope elevation was not inhibited by treatment with \(-\text{aga-}
\text{toxin TK.}
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Because membrane retrieval is substantially inhibited in CF-ASW, our expectation was that granule membrane components would be trapped in the cell surface under this condition. We reasoned that because the membrane potential hyperpolarizes by 5 minutes after fertilization, retrieval of trapped granule components should only occur if extracellular calcium is present and if the membrane potential is artificially depolarized. Eggs were activated with sperm and placed and maintained in CF-ASW. We found that addition of either calcium or depolarization by addition of potassium at 10 minutes after fertilization failed to trigger retrieval (Fig. 6). However, addition of calcium and potassium simultaneously did trigger retrieval.

\[\text{v-agatoxin TK blocked this uptake (Fig. 6).}\]

**DISCUSSION**

We found that 80% of endocytotic membrane retrieval in sea urchin eggs required extracellular calcium. Cadmium and structurally unrelated peptide toxins all inhibited retrieval to the same extent as removal of calcium. Cadmium blocked membrane retrieval only during the period when the plasma membrane is depolarized. Agatoxin block of membrane retrieval was overcome by addition of calcium ionophores in a calcium-dependent manner. We cannot escape the conclusion that calcium influx through agatoxin-sensitive calcium channels is required for membrane retrieval.

Three lines of evidence argue that the calcium dependence of endocytotic membrane retrieval is distinct from the calcium

**FIG. 6.** Depolarization triggers membrane retrieval by opening agatoxin-sensitive channels. Eggs were fertilized and placed into CF-ASW 3 minutes after fertilization. Ten minutes after fertilization, the eggs were placed into CF-ASW containing 100 \(\mu\)M TMR-Dex and supplemented with 9.3 mM CaCl\(_2\) (+Ca), 50 mM KCl (+K), 9.3 mM CaCl\(_2\) + 50 mM KCl (+Ca + K), or 9.3 mM CaCl\(_2\) + 50 mM KCl + 50 nM \(\alpha\)-agatoxin TK (+Ca + K + AgaTK). The amount of subsequent membrane retrieval was assayed 15 minutes after the addition of tetramethylrhodamine \((t = 25\) min). All points are normalized to TMR-Dex uptake in sperm-activated eggs in ASW (between \(t = 3\) and 18 minutes) represents 100% retrieval, and TMR-Dex uptake in sperm-activated eggs in CF-ASW (between \(t = 3\) and 18 minutes) represents 0% retrieval. All data points are mean ± SD \(n = 6\).

**FIG. 5.** \(\alpha\)-Agatoxin TK inhibits membrane retrieval but not cortical granule exocytosis. Sea urchin eggs were incubated in ASW containing 100 \(\mu\)M TMR-Dex, either with (C and D) or without (A and B) 50 nM \(\alpha\)-agatoxin TK. Eggs were fertilized by the addition of sperm. After 15 minutes, the eggs were washed in ASW to remove extracellular TMR-Dex. Eggs were imaged on a Zeiss Axiophot microscope by using differential interference contrast optics (A and C) to visualize the elevation of the fertilization envelope and with a rhodamine filter set to visualize fluorescent inclusions (B and D). (Bar = 30 \(\mu\)m.)
dependence of exocytosis. First, the time period where calcium is required for exocytosis is shorter than the time period where it is required for endocytosis. Calcium-triggered cortical granule exocytosis is complete by 3 minutes after fertilization (28, 29), whereas membrane retrieval required calcium influx over a 5-minute period after fertilization and is complete by 15 minutes after fertilization. Second, treatment with ω-agatoxin TK did not inhibit cortical granule exocytosis but did inhibit endocytotic membrane retrieval. Finally, both 9.3 and 15 mM extracellular calcium supported cortical granule exocytosis after ionophore application on agatoxin-treated eggs, but only 15 mM extracellular calcium supported membrane retrieval. We therefore conclude that endocytotic membrane retrieval has a calcium-dependent step distinct from the calcium dependence of exocytosis.

Little is known about how endocytotic membrane retrieval is coupled to exocytosis. The observation that application of calcium ionophore triggered exocytosis, but not membrane retrieval, in toxin-treated cells (in ASW with 9.3 mM calcium) demonstrates that exocytosis alone is not sufficient to trigger retrieval; an increased calcium influx also is required. Membrane retrieval was triggered by ionophore in agatoxin-treated eggs only when the extracellular calcium concentration was >12 mM. This would be expected if membrane retrieval requires calcium concentrations higher than those capable of triggering exocytosis. Alternatively, the site of action for calcium to trigger membrane retrieval might be deeper in the cytoplasm than the site that triggers exocytosis. Both explanations support the contention that the calcium dependence of endocytotic membrane retrieval is distinct from the role of calcium in exocytosis.

It is somewhat surprising that calcium influx is required for membrane retrieval in fertilized sea urchin eggs, because cytoplasmic calcium is already quite elevated because of release from intracellular stores (31). In fact, our calcium imaging experiments suggest that virtually the entire volume-averaged cytoplasmic calcium signal is derived from intracellular stores, rather than from influx. One possible explanation of this finding is that membrane retrieval requires a higher local calcium concentration than the average achieved in the cytoplasm. If this is the case, detection of this signal may necessitate the use of membrane-bound indicators with low affinity for calcium. A similar approach by using mutant aequorins was successful for imaging the calcium microdomain responsible for triggering exocytosis at the squid giant synapse (37).

Cadmium is a rapid extracellular blocker of calcium influx through voltage-gated calcium channels (38). Application of cadmium during a 5-minute period after fertilization arrested most, but not all, of the subsequent membrane retrieval. Application of cadmium after 5 minutes had no significant effect on the subsequent retrieval, despite the fact that at 5 minutes, only 50% of membrane retrieval has occurred. The inability of cadmium to inhibit after the first 5 minutes would be expected if calcium influx only occurs when the plasma membrane potential is depolarized. The membrane potential of sea urchin eggs remains depolarized during the first 5 minute after fertilization (30). The calcium influx occurring during the first 5 minutes after fertilization was capable of supporting membrane retrieval over an additional 10 minutes (see time course of membrane retrieval in Fig. 2). The underlying mechanism of this persistent activation of the retrieval process is not known. Persistent activation of a retrieval mechanism might arise from a persistent elevation of calcium in microdomains involved in membrane retrieval. Alternatively, a transient elevation of the calcium concentration might activate proteins involved in retrieval, even after the calcium concentration has returned to baseline levels (39). The fact that the calcium concentration returns to baseline levels by 5 minutes after fertilization (31) (Fig. 2C) argues that persistent activation of membrane retrieval is not caused by persistent elevation of the bulk cytoplasmic calcium concentration. We cannot, however, eliminate the possibility that a microdomain of high calcium concentration evaded detection in these experiments. Regardless, the interpretation of experiments investigating the calcium dependence of membrane retrieval in other systems will be complicated if the underlying mechanism can be persistently activated by calcium, as we have shown for sea urchin eggs.

ω-Agatoxin IVA and ω-agatoxin TK, selective antagonists of P- and Q-type calcium channels, blocked endocytotic membrane retrieval. Specific inhibitors of calcium influx through N-type channels and L-type channels did not. P- and Q-type calcium channels can be distinguished by their sensitivity to ω-agatoxin IVA (32–34). P-type calcium channels can be blocked by <10 nM ω-agatoxin IVA, whereas Q-type channels require >10 nM toxin for inhibition. We found that 50% of endocytotic membrane retrieval was inhibited by 7.3 nM ω-agatoxin IVA, a concentration indicative of the involvement of P-type channels. Treatment with calcium ionophores reversed agatoxin blocked cells in a calcium-dependent manner. This argues against the possibility that in the sea urchin there is another, as-yet-undefined target for ω-agatoxin TK and supports the hypothesis that calcium influx through P-type channels is required for membrane retrieval in sea urchin eggs. Nonetheless, we are reluctant to define the target of agatoxin in sea urchin eggs as P-type channels. Compared with mammalian calcium channels, little is known about the structure or functional properties of echinoderm calcium channels. Calcium currents have been observed in sea urchin eggs (30). High extracellular potassium depolarizes the egg membrane potential (40) and stimulates a verapamil-sensitive calcium uptake in unfertilized eggs (27) but does not trigger cortical granule exocytosis (27) or endocytotic membrane retrieval (data not shown).

We conclude that voltage-gated calcium channels (possibly P-type calcium channels), in addition to their role in signaling for exocytosis at synapses (41–45), are required for endocytotic membrane retrieval after fertilization. This result reveals a new functional role for this class of ion channel and predicts that membrane retrieval will be voltage-dependent, emphasizing the need to assay endocytosis under conditions that do not perturb membrane potential (46, 47). Finally, this result also suggests that in addition to providing a fast block to polyspermy (48) and facilitating sperm entry into the cell (49), the fertilization potential (30) functions to gate calcium channels and trigger endocytosis.

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