The Intraacrosomal Calcium Pool Plays a Direct Role in Acrosomal Exocytosis*

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The acrosome reaction is a unique type of regulated exocytosis. The single secretory granule of the sperm fuses at multiple points with the overlying plasma membrane. In the past few years we have characterized several aspects of this process using streptolysin O-permeabilized human spermatozoa. Here we show that Rab3A triggers acrosomal exocytosis in the virtual absence of calcium in the cytosolic compartment. Interestingly, exocytosis is blocked when calcium is depleted from intracellular stores. By using a membrane-permeant fluorescent calcium probe, we observed that the acrosome actually behaves as a calcium store. Depleting calcium from this compartment by using a light-sensitive chelator prevents secretion promoted by Rab3A. UV inactivation of the chelator restores exocytosis. Rab3A-triggered exocytosis is blocked by calcium pump and inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium channel inhibitors. Calcium measurements inside and outside the acrosome showed that Rab3A promotes a calcium efflux from the granule. Interestingly, release of calcium through IP_3-sensitive calcium channels was necessary even when exocytosis was initiated by increasing free calcium in the extracellular compartment in both permeabilized and intact spermatozoa. Our results show that a calcium efflux from the acrosome through IP_3-sensitive channels is necessary downstream Rab3A activation during the membrane fusion process leading to acrosomal exocytosis.

As in other regulated secretory events, calcium plays a central role in acrosome reaction (1). Recent results from different laboratories suggest that a first transient cytosolic calcium increase (probably mediated by T-type calcium channels (2)) leads to a second sustained increase of cytosolic calcium that is necessary for the acrosome reaction (3–9). Although the connection between the two events is not completely clear, the current hypothesis is that the first calcium increase causes the activation of a phospholipase C (PLC).1 There are several isoforms of PLC in the spermatozoa (10, 11). PLCδ4, in particular, has been implicated in the early events of the acrosome reaction (12). Active PLC would produce inositol 1,4,5-trisphosphate (IP_3) that would open IP_3-sensitive calcium channels in the membrane of intracellular stores. The emptying of these stores would trigger the opening of store-operated calcium (SOC) channels in the plasma membrane causing a second and sustained calcium increase that would trigger acrosomal exocytosis (1, 7). Although direct proof for the nature of the calcium stores involved in this mechanism is lacking, several lines of evidence point to the acrosome. This secretory granule possesses a calcium pump (9) and IP_3-sensitive calcium channels (5, 11, 13).

A rise in cytoplasmic calcium levels triggers exocytosis in most cell types, including neurotransmitter and hormone-secreting cells (14). Several calcium-binding proteins have been implicated in the membrane fusion mechanism underlying exocytosis. These include the synaptotagmins, a family of transmembrane proteins that have been postulated to be the calcium sensors responsible for triggering exocytosis (15). The calcium binding domains of synaptotagmins interact with members of the SNARE complex (16). SNAREs are key proteins involved in the docking of membranes that are going to fuse (17). Another family of proteins involved in membrane fusion are the Rabs, small GTPases that upon activation by exchanging GDP for GTP, cause the formation of large aggregates of proteins that contribute bringing together the membranes participating in fusion (18). In most membrane fusion events, both Rab-mediated tethering and SNARE-dependent docking are necessary steps for fusion to proceed.

In previous reports, we have shown that calcium can trigger acrosomal exocytosis in streptolysin O (SLO)-permeabilized human sperm cells (19). The same effect can be obtained by adding GTP-bound Rab3A, a member of the Rab family present

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1 The abbreviations used are: PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; SNARE, soluble NSF attachment protein receptors; SOC, store-operated calcium; 2-APB, 2-aminoethoxy-diphenylborate; SLO, streptolysin O; GMP gamete preparation medium; CPA, cyclopiazonic acid; AM, acetoxymethyl ester.

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in acrosomal membranes (19–22). In this report we show that the acrosome is a calcium store and that a calcium efflux from this compartment through IP₃-sensitive channels is a necessary event for Rab3A-triggered acrosomal exocytosis. The results indicate that acrosomal calcium is important not only for opening SOC channels but also for a later step related to the membrane fusion process leading to acrosomal exocytosis.

EXPERIMENTAL PROCEDURES

Reagents—Fura2, EGTA-AM, O-nitrophenyl EGTA-AM (EGTA-NP), BAPTA, BAPTA-AM, caged Br-2A3187, and calcium sponge S were purchased from Molecular Probes (Eugene, OR). Fluo-3-AM, xestospongin C, cyclopiazonic acid and 2-aminoethoxy-diphenylborate (2-APB) were from Calbiochem (La Jolla, CA). SLO was obtained from Murex (Dartford, UK). Gamete preparation medium (GPM, Serono, Madrid, Spain) was used as culture medium. All other reagents were from Sigma or from ICN Biochemicals, Inc. (Aurora, OH). Plasmids encoding human Rab3A and the light chain of botulinum neurotoxin E (BoNT/E) were generously provided by Dr. P. D. Stahl (Washington University, St. Louis, MO) and Dr. T. Binz (Medizinische Hochschule Hannover, Hannover, Germany), respectively.

Acrosome Reaction in Intact and Permeabilized Spermatozoa—Human semen samples were obtained from normal healthy donors. Highly motile sperm were recovered following swim-up separation for 1 h in GPM, 37 °C in an atmosphere of 5% CO₂/95% air. Cell concentration was then adjusted to 5–10 × 10⁶/ml, and incubation proceeded for at least 2 h under conditions that support capacitation (GPM, 37 °C, 5% CO₂/95% air). In some experiments, spermatozoa without further treatment were used to study thapsigargin-induced acrosome reaction. In other experiments, sperm were permeabilized as described (19) and resuspended in 250 mM sucrose, 20 mM Hepes-K, 0.5 mM EGTA, pH 7 (SB). Acrosomal status was evaluated by staining with fluorescein isothiocyanate-coupled Pisum sativum as described (23). At least 200 cells were scored using a Nikon microscope equipped with epifluorescence optics. Negative (no stimulation) and positive (calcium in permeabilized spermatozoa and A23187 in intact cells) controls were included in all experiments. For each experiment, the data were normalized by subtracting the number of reacted spermatozoa in the negative control (range 18–30%) from all values and expressing the resulting values as a percentage of the acrosome reaction observed in the positive control (range 30–50%).

Recombinant proteines—Recombinant Rab3A was expressed in Escherichia coli and purified by following standard procedures (19). Rab3A was prenylated in vitro as described (19). Just before use, aliquots of the prenylated protein were loaded with the nonhydrolyzable nucleotide (SB). Acrosomal status was evaluated by staining with fluorescein isothiocyanate-coupled Pisum sativum as described (23). At least 200 cells were scored using a Nikon microscope equipped with epifluorescence optics. Negative (no stimulation) and positive (calcium in permeabilized spermatozoa and A23187 in intact cells) controls were included in all experiments. For each experiment, the data were normalized by subtracting the number of reacted spermatozoa in the negative control (range 18–30%) from all values and expressing the resulting values as a percentage of the acrosome reaction observed in the positive control (range 30–50%).

Fluo3 Imaging of Intracellular Calcium Stores—SLO-permeabilized sperm were incubated for 30 min at 37 °C in the presence of 10 μM Fluo3-AM. The cells were then washed with SB at the same temperature and immobilized on glutaraldehyde-coated glass coverslips. Fluorescence was recorded in an inverted Eclipse TE300 Nikon microscope equipped with a CCD Hamamatsu Orca 100 camera operated with Meta Morph software (Universal Imaging Corp.). Time lapse images were taken every 30–60 s with B-2A filters (excitation 450–490, barrier 515) and processed with MetaMorph and Paint Shop Pro (Jasc Software, Inc., Eden Prairie, MN).

Determination of Free Calcium with Fura2—To measure calcium release from SLO-permeabilized spermatozoa, the cells were resuspended in SB buffer without EGTA. The free calcium concentration in this buffer was equilibrated with calcium sponge S to minimize contaminant calcium ions in the system. Sperm were treated for 15 min at 37 °C with 10 μM BoNT/E followed by 2 μM membrane-impermeant Fura2. The samples were then transferred to the cuvette of an Aminco SLM 8000 spectrophluorometer, and fluorescence at 510 nm (emission) was monitored exciting at 340 nm and 380 nm. At the appropriate times, 300 nM GTP-γS-loaded Rab3A was added to the medium. As a negative control, the Rab3A buffer, having the same composition as the Rab3A preparation but lacking the protein, was used in some experiments. At the end of the incubation, dye fluorescence at zero and saturating calcium concentrations were obtained by adding 5 mM EGTA or 5 mM BAPTA. Subsequently, the samples were incubated for a further 15 min at 37 °C in the absence of any stimulus (control), in the presence of 0.5 mM CaCl₂ (Ca), or 300 nM recombinant Rab3A loaded with GTP-γS (Rab). Free calcium concentrations were measured by using the Fura2 340/380 fluorescence ratio. When calcium was added to the media, the free concentration was estimated using the freeware Sliders program (www.stanford.edu/~cpattom/maxc.html, Chris Patton, Stanford University). Acrosomal exocytosis was evaluated by lectin binding. The data represent the mean ± S.E. of at least three independent experiments.

RESULTS

Rab3A-triggered Acrosomal Exocytosis Does Not Require Cytosolic Calcium—Addition of micromolar concentrations of calcium triggers acrosomal exocytosis in permeabilized spermatozoa (Fig. 1A). The same effect is observed without calcium addition when recombinant Rab3A in the GTP-bound form is included in the assay. Under the latter condition, free calcium concentration in the buffer, which contains 0.5 mM EGTA, is in the 100 nM range (Fig. 1B). When 5 mM EGTA was added to the medium, the free calcium concentration dropped to less than 10 nM (Fig. 1B). At these very low calcium concentrations, there was still a significant percentage of sperm cells that underwent exocytosis in response to Rab3A (Fig. 1A). BAPTA is another calcium chelator with similar affinity for the ion but with a faster binding rate. In the acrosomal exocytosis assay, BAPTA diminished calcium concentration below the sensitivity of the method used to determine this divalent ion. However, it did not inhibit acrosomal exocytosis more efficiently than EGTA (Fig. 1, A and B). Taken together, these data show that active Rab3A can trigger acrosomal exocytosis in the virtual absence of calcium in the medium.

Rab3A-triggered Acrosomal Exocytosis Requires Calcium From Intracellular Stores—To assess whether calcium from an intracellular reservoir has a role in Rab3A-triggered acrosomal exocytosis, calcium from membrane-bound stores was depleted by pretreating with the ionophore A23187 in the presence of 5 mM EGTA. This treatment significantly inhibited acrosomal exocytosis (Fig. 2). Even more inhibition was observed after chelating calcium in the lumen of intracellular stores with BAPTA-AM or EGTA-AM, two membrane-permeant chelating agents that accumulate in membrane-bound compartments (Fig. 2). EGTA-NP, a photosensitive EGTA-AM analog, was used to demonstrate that the effect was reversible. In the dark, the membrane-bound chelator was active, and exocytosis was inhibited (Fig. 2). Upon illumination, a treatment that decreases the affinity of EGTA-NP for calcium about 10,000-fold, Rab3A-stimulated exocytosis was recovered (Fig. 2). These results indicate that depletion of calcium from intracellular stores prevents acrosomal exocytosis.

Labeling of the Intraacrosomal Calcium Pool by Fluo3-AM—Despite the fact that several reports postulate the existence of

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**Fig. 1.** Rab3A triggers acrosomal exocytosis at very low calcium concentrations. A, permeabilized spermatozoa were incubated for 15 min at 37 °C in SB supplemented, when indicated, with 5 mM EGTA or 5 mM BAPTA. Subsequently, the samples were incubated for a further 15 min at 37 °C in the absence of any stimulus (control), in the presence of 0.5 mM CaCl₂ (Ca), or 300 nM recombinant Rab3A loaded with GTP-γS (Rab). B, free calcium concentrations were measured by using the Fura2 340/380 fluorescence ratio. When calcium was added to the media, the free concentration was estimated using the freeware Sliders program (www.stanford.edu/~cpattom/maxc.html, Chris Patton, Stanford University). Acrosomal exocytosis was evaluated by lectin binding. The data represent the mean ± S.E. of at least three independent experiments.
an intraacrosomal calcium reservoir, this pool has never been visualized. Permeabilized spermatozoa are an ideal system to assess the existence and regulation of such a store. When permeabilized sperm cells were incubated with Fluor3-AM, the acrosomal region of the spermatozoa showed a very distinct labeling (Fig. 3A, 0s). The fluorescence pattern in permeabilized spermatozoa was different from that observed in intact cells where the entire cell was fluorescent (data not shown). By using time lapse microscopy of single cells, we observed that addition of membrane-impermeant EGTA did not significantly decrease intraacrosomal calcium (Fig. 3A, 60s). In contrast, BAPTA-AM depleted the acrosomal pool (Fig. 3A, 180s and onward). Although Fluor3-AM also labeled the midpiece, this signal was virtually insensitive to BAPTA-AM (Fig. 2A). The relative changes in fluorescence in the acrosome and midpiece of the two spermatozoa shown in Fig. 3A are plotted in Fig. 3B. Similar results were observed when the experiment was repeated in other sperm batches (Fig. 3C). Intraacrosomal calcium was also efficiently depleted by the combination of EGTA plus the calcium ionophore Br-A23187 (Fig. 3C). This combination caused a modest but statistically significant decrease in the midpiece fluorescence.

The images demonstrate that the acrosome is a calcium reservoir. They also show that the experimental conditions in Fig. 2 that prevent acrosomal exocytosis efficiently deplete intraacrosomal calcium. This finding is consistent with the hypothesis that intraacrosomal calcium is important for acrosomal content release.

The Activity of a Cyclopiazonic Acid- and Thapsigargin-sensitive Calcium Pump is Required for Rab3A-triggered Exocytosis—If the intraacrosomal calcium pool is important for exocytosis, the activity of the Ca$^{2+}$-ATPase pump, responsible for maintaining intracellular calcium stores, should be necessary for acrosomal exocytosis. In agreement with this hypothesis, thapsigargin and cyclopiazonic acid, two inhibitors of this pump, inhibited Rab3A-triggered exocytosis (Fig. 4A). It is worth noticing that although ATP is not added to the system, we have previously shown that permeabilized sperm cells produce enough ATP to sustain ATP-dependent processes (22).

If calcium is pumped by an active mechanism, the effect of BAPTA-AM should be reversible provided that the sperm are incubated under conditions that permit the calcium pump to overcome the chelating capacity of the BAPTA present in the acrosome. To test this hypothesis, the cells were first incubated at 20 °C with BAPTA-AM. Subsequently, sperm cells were incubated at the same temperature in the presence of 10 μM free calcium to allow the pump to compensate for the effect of intraacrosomal BAPTA. At 20 °C, no acrosomal exocytosis is expected to occur even in the presence of calcium. Finally, an excess of impermeant EGTA was added to chelate the calcium remaining in the buffer, and the cells were transferred to 37 °C with Rab3A added to initiate exocytosis. Under these conditions, the effect of BAPTA-AM was overridden (Fig. 4B). The recovery was sensitive to calcium pump inhibitors as would be expected if the pump were involved in the inward transport of calcium. When Rab3A was not added during the incubation at 37 °C, no exocytosis was observed, ruling out the possibility that calcium had triggered exocytosis during the 20 °C incubation (Fig. 4B). When EGTA was added before calcium, no exocytosis was observed even in the presence of Rab3A indicating

FIG. 2. Rab3A triggered acrosomal exocytosis requires calcium from intracellular stores. Permeabilized spermatozoa were incubated for 15 min at 37 °C with SB buffer supplemented, when indicated, with the following compounds: 5 mM EGTA + 10 μM A23187 (EGTA + Iono), 10 μM BAPTA-AM (BAPTA-AM), 10 μM EGTA-AM (EGTA-AM), 10 μM photosensitive EGTA-AM (EGTA-NP). When the photosensitive EGTA was used, the experiment was performed protected from light. The samples were further incubated for 15 min at 37 °C with no addition (cntr), in the presence of 10 μM free calcium (Ca), or 300 μM recombinant Rab3A loaded with GTPγS (Rab). When required, EGTA-NP was inactivated by illuminating the sample with a UV lamp for 2 min prior to the addition of Rab3A (Rab + h). Acrosomal exocytosis was evaluated by lectin binding. The data represent the mean ± S.E. of at least three independent experiments.

FIG. 3. Visualization of the acrosomal calcium store in permeabilized spermatozoa. Permeabilized sperm cells were incubated for 30 min at 37 °C with 10 μM Fluor3-AM. Cells were then washed in SB buffer, and the fluorescence was recorded in an inverted microscope. At appropriate times, 10 μM BAPTA-AM (B-AM) or 5 mM EGTA, or 5 mM EGTA + 10 μM Br-A23187 (EGTA + I) were added to the medium, and the fluorescence was recorded up to 10 min. A, Fluor3 fluorescence images of sperm cells. A signal is clearly observed in the acrosome and midpiece of two spermatozoa. No changes are observed upon addition of 5 mM EGTA (60s). In contrast, the acrosomal signal starts fading after the addition of 10 μM BAPTA-AM (120s). Notice that the fluorescence in the midpiece was only slightly affected by BAPTA-AM. Bar, 10 μm. B, fluorescence in the acrosomes (solid lines) and midpiece (dotted lines) of the images shown in A was processed by using the MetaMorph software, expressed as a percentage of the starting signal and plotted as a function of time. C, fluorescence in the acrosome and midpiece from 10 to 17 cells were processed as in B and expressed as a percentage of the starting signal (mean ± S.E.).
that the presence of calcium during the preincubation was necessary to overcome BAPTA-AM inhibition (Fig. 4B).

Another prediction is that if the acrosomal calcium store is replenished by a mechanism that does not require the pumping of calcium from the medium, then the exocytosis should not be sensitive to inhibitors of the pump. In fact, when intraacrosomal free calcium was increased by photo-inhibition of intracapsular EGTA-NP, exocytosis proceeded even in the presence of cyclopiazonic acid (Fig. 4C). These results are consistent with a role for an acrosomal calcium pump sensitive to thapsigargin and cyclopiazonic acid in the mechanism of Rab3A activation of exocytosis.

A Calcium Channel, Blocked by IP3-sensitive Calcium Channel Inhibitors, Is Required for Rab3A-dependent Acrosomal Exocytosis—We have shown that an acrosomal calcium store is necessary for the Rab3A effect on acrosomal exocytosis. We then assessed whether the process requires the release of calcium from the acrosomal store through calcium channels. Rab3A-dependent acrosomal exocytosis was sensitive to lanthanum, a general calcium channel blocker, and to xestospongin C and 2-APB, two inhibitors of IP3-sensitive calcium channels (Fig. 5A). In contrast, the process was not affected by nifedipine and verapamil, two inhibitors of voltage-dependent calcium channels. As expected, lanthanum, xestospongin C and 2-APB inhibited the process under conditions where the intraacrosomal calcium pool was replenished either by preincubation with calcium (Fig. 5B) or by photoinhibition of intracellular EGTA-NP (Fig. 5C).

These observations indicate that a release of calcium from the acrosomal store is necessary for Rab3A-stimulated acrosomal exocytosis. Therefore, we should be able to overcome the inhibitory effect of calcium channels inhibitors by using a calcium ionophore. We took advantage of a photo-activatable calcium ionophore to test this hypothesis. Permeabilized sperm cells were incubated in the presence of xestospongin C, to inhibit calcium channels, and with a photo-activatable calcium ionophore in the dark (inactive ionophore). The cells were then incubated with Rab3A. According to the hypothesis, under these conditions, the exocytic process should be arrested at a step that requires a calcium efflux from the acrosome. The calcium ionophore was then activated by a short exposure to the appropriate light source, and the incubation continued for a few minutes. The results indicate that an efflux of calcium ions after Rab3A activation overcomes the inhibition of calcium channels (Fig. 5D).

Rab3A Triggers the Release of Calcium from the Acrosome—If Rab3A activation of acrosomal exocytosis requires the opening of calcium channels, a calcium efflux from the acro-
some into the incubation medium would be expected upon addition of Rab3A. To test this prediction, changes in intraacrosomal calcium were measured in BoNT/E-treated spermatozoa after addition of Rab3A loaded with GTPyS. Sperm were treated with the toxin to prevent acrosomal exocytosis (26) that would cause the release of intraacrosomal calcium. Addition of Rab3A significantly decreased calcium in the acrosome (Fig. 6, A and B) as assessed by single cell imaging under conditions where exocytosis was blocked (Fig. 6C). The effect was sensitive to xestospongin C and 2-APB (data not shown). The calcium released by Rab3A addition caused a measurable increase in calcium in the medium (Fig. 6, D and E). In conclusion, the experiments shown in Fig. 6 indicate that Rab3A facilitates the release of calcium from the acrosomal reservoir.

Calcium-triggered Acrosomal Exocytosis in Intact and Permeabilized Spermatozoa Requires Calcium Release from Intraacellular Stores—We have presented consistent evidence that Rab3A-triggered acrosomal exocytosis requires the release of calcium from the acrosome. In a more physiological scenario, endogenous Rab3A is probably activated as part of the events initiated by the sustained cytosolic calcium increase caused by the opening of SOC channels. This means that the calcium efflux would be necessary even when calcium is present outside the acrosome. Consistent with this hypothesis, calcium-triggered acrosomal exocytosis was abrogated by chelating the acrosomal calcium pool or by inhibiting IP3-sensitive calcium channels (Fig. 7A).

In intact spermatozoa, thapsigargin triggers exocytosis by opening SOC channels. If IP3-sensitive calcium channels are required after SOC channels are opened, thapsigargin-stimulated acrosome reaction should be inhibited by xestospongin C and 2-APB. To directly assess this possibility, nonpermeabilized sperm cells were treated with thapsigargin in the presence or absence of these inhibitors. As shown in Fig. 7B, thapsigargin promoted exocytosis, an effect that was hampered by addition of IP3-sensitive calcium channels inhibitors. A possible shortcoming for this approach is that these inhibitors could interfere with the effect of thapsigargin on SOC channels by preventing calcium efflux from intracellular stores. To address this possibility, sperm were incubated with thapsigargin in the absence of calcium in the medium, a condition that opens SOC channels but does not trigger acrosomal exocytosis. Subsequently, IP3-sensitive calcium channels inhibitors were added, and finally the free calcium concentration in the medium was increased. As shown in Fig. 7C, the inhibitors abrogated exocytosis even when added after SOC channels were open and calcium could permeate into the spermatozoa. These results suggest that acrosome reaction requires a release of calcium from the acrosome through IP3-sensitive calcium channels at a step downstream of SOC channels activation.

**DISCUSSION**

The acrosomal granule has been postulated to be a calcium store for several years. A thapsigargin-sensitive calcium pump...
(9) and IP$_3$-sensitive calcium channels (5, 11, 13) are present in the acrosomal membrane. Moreover, it has been shown that digitonin-permeabilized sperm cells can accumulate calcium in the presence of a mitochondria uncoupler, a process that has been ascribed to the acrosome (5). However, direct visualization of the acrosomal pool in its regulation was lacking. The opening of SLO pores in the plasma membrane of sperm cells allowed us to load intracellular membrane-bound compartments, including the acrosome, with AM derivatives. This procedure permitted us to visualize directly the presence of a calcium pool inside the acrosome and to monitor the changes in the acrosomal pool under different experimental conditions that affect acrosomal exocytosis. Our results show a direct correlation between disappearance of the acrosomal calcium pool and inhibition of acrosomal exocytosis. In contrast, changes in the midpiece calcium store were less significant under the same conditions. We speculate that the free calcium concentration inside the mitochondria may be more resistant to depletion due to the presence of putative calcium-releasing sources such as calcium-protein complexes or calcium-phosphate precipitates. Whatever the reason for the midpiece resistance to calcium depletion, the results are consistent with the hypothesis that the acrosome is the calcium store necessary for acrosomal exocytosis.

It has been proposed that a putative acrosomal calcium store has an important role in the acrosome reaction. A decrease in this pool is supposed to mediate the opening of SOC channels in the plasma membrane causing a sustained calcium increase at the acrosomal membrane. Moreover, it has been shown that the depletion due to the presence of putative calcium-releasing compounds, including the acrosome, with AM derivatives. This procedure permitted us to visualize directly the presence of a calcium pool inside the acrosome and to monitor the changes in the acrosomal pool under different experimental conditions that affect acrosomal exocytosis. Our results show a direct correlation between disappearance of the acrosomal calcium pool and inhibition of acrosomal exocytosis. In contrast, changes in the midpiece calcium store were less significant under the same conditions. We speculate that the free calcium concentration inside the mitochondria may be more resistant to depletion due to the presence of putative calcium-releasing sources such as calcium-protein complexes or calcium-phosphate precipitates. Whatever the reason for the midpiece resistance to calcium depletion, the results are consistent with the hypothesis that the acrosome is the calcium store necessary for acrosomal exocytosis.

Therefore, acrosomal exocytosis may occur long before the acrosomal calcium store has been depleted to a point that precludes membrane fusion.

According to our results, activation of Rab3A triggers acrosomal exocytosis in a process that requires the opening of IP$_3$-sensitive calcium channels. These channels have been implicated in the efflux of calcium necessary for the fusion of vesicles during nuclear envelope assembly (27) and in the release of calcium from secretory granules (28). The molecular processes connecting Rab3A and IP$_3$-sensitive calcium channels are at present unknown. There are some reports that peptides from the effector domain of Rab3 increase IP$_3$ levels in several cell types, although direct evidence that the effect is mediated by a Rab3-dependent mechanism is missing (29). The assembly of the fusion complex initiated by activation of Rab3A could encompass IP$_3$-sensitive calcium channels that may mediate the calcium efflux necessary for acrosomal exocytosis, as it has been postulated for voltage-operated calcium channels in neurotransmitter release (30, 31).

Why would a calcium efflux from the acrosome be necessary for exocytosis? A direct role for intravesicular calcium in fusion has been proposed in several other transport events (32), including the secretion of exocytic granules (33–35). During the fusion process, membranes that are going to fuse are maintained in close proximity creating a partially isolated environment where the calcium concentration may be strongly altered by the local action of calcium pumps or the opening of calcium channels. More experiments are necessary to characterize the molecular mechanism underlying acrosomal exocytosis that is activated by the efflux of calcium from the acrosome.

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