A genetically targeted sensor reveals spatial and temporal dynamics of acrosomal calcium and sperm acrosome exocytosis

Roy Cohen, Chinatsu Mukai, Jacquelyn L. Nelson, Shoshana S. Zenilman, Danielle M. Sosnicki, and Alexander J. Travis

From the 1Baker Institute for Animal Health, College of Veterinary Medicine, and 2Department of Public and Ecosystem Health, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

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Secretion of the acrosome, a single vesicle located rostrally in the head of a mammalian sperm, through a process known as “acrosome exocytosis” (AE), is essential for fertilization. However, the mechanisms leading to and regulating this complex process are controversial. In particular, poor understanding of Ca2+ dynamics between sperm subcellular compartments and regulation of membrane fusion mechanisms have led to competing models of AE. Here, we developed a transgenic mouse expressing an Acrosome-targeted Sensor for Exocytosis (AcroSensE) to investigate the spatial and temporal Ca2+ dynamics in AE in live sperm. AcroSensE combines a genetically encoded Ca2+ indicator (GCaMP) fused with an mCherry indicator to spatiotemporally resolve acrosomal Ca2+ ion flux (1–3) and in need of new tools to understand the regulation and process of exocytosis.

The “acrosome reaction” model involves receptors on the sperm membrane being triggered by physical contact with ligands on the zona pellucida (ZP), resulting in exocytosis of the acrosome, the sperm’s single exocytotic vesicle. This long-dominant model defines two states or conditions for sperm—acrosome-intact or acrosome-reacted. In this model, it is thought that creating pores between the plasma membrane and outer acrosomal membrane (OAM) would result in loss of acrosomal contents in a manner analogous to popping a water balloon. Almost 2 decades of data challenge this thinking, suggesting a more complex, multistep model of “acrosome exocytosis” (AE). However, the acrosome reaction model has proven remarkably persistent, due both to its simplicity and lack of precise understanding of the steps involved in AE. The multistep AE model is based on numerous lines of evidence from different laboratories, including that putative receptors for the ZP are found in the acrosomal matrix (1) and then exposed on the sperm surface (2); that AE occurs during transit through the cumulus cells prior to the sperm reaching the ZP (3); that contact with the ZP is insufficient to induce AE in sperm expressing GFP in their acrosome (AcrGFP sperm), even several hours after binding (4); that the majority of sperm have undergone AE even before reaching the cumulus mass (5); and that only ~5% of the sperm traversing the ampulla are acrosome-intact (6). However, there are contradictions even within these reports, and the field remains highly controversial (7) and in need of new tools to understand the regulation and process of exocytosis.

A critical knowledge gap in understanding AE results from challenges in studying sperm Ca2+ dynamics. There are multiple factors underlying this, including (A) sperm are transcriptionally and translationally inactive with no culture systems available to produce/manipulate mature sperm; (B) mature sperm are motile and difficult to image; (C) across mammalian species, only a subset of sperm are actually capable of fertilizing (8–14) and these have different Ca2+ dynamics (15); (D) sperm only acquire the ability to fertilize after undergoing functional maturation in a complex process known as “capacitation” involving multiple changes in membrane lipids such as removal of sterols, polarization state, and ion flux (16–18); (E) sperm membranes are remarkably temperature sensitive with phase transitions occurring between physiological and room temperatures (19, 20); and (F) there is scant cytoplasmic space between the plasma and OAMs, making it difficult to discern differences in Ca2+ dynamics within cytoplasmic versus acrosomal compartments without a compartmentalized marker.

We previously described how removal of cholesterol and focal enrichments of G~M~ play a regulatory role in enabling AE through at least 2 complementary pathways in the plasma membrane overlying the acrosome (acrosomal plasma membrane [APM]). First, sterol efflux enables proteolytic activation
of phospholipase B, which cleaves both tails of a phospholipid molecule (21, 22), changing local membrane curvature to support point fusion formation. Second, sterol efflux enables G<sub>M1</sub> regulation of the Ca<sub>v</sub>2.3 voltage-gated Ca<sup>2+</sup> channel, giving rise to Ca<sup>2+</sup> transients that facilitate AE in response to subsequent Ca<sup>2+</sup> waves in the sperm’s head (23). Along with these roles, sterol efflux also exerts broad regulatory functions, such as facilitating intracellular alkalization through activation of the sperm proton channel Hv1 in human sperm (24) or sperm-specific sodium hydrogen exchanger in murine sperm (25) and plasma membrane hyperpolarization through activation of the SLO3 potassium channel (26). Both alkalization and hyperpolarization are critical steps for capacitation and AE to occur (27, 28).

As a complement to these membrane lipid-regulated pathways, progesterone (P4) is also a critical regulator of sperm function. Released by cumulus cells and encountered by sperm in the vicinity of the egg, P4 has been reported to stimulate multiple events in sperm including stimulation of sperm in the vicinity of the egg, P4 has been reported to stimulate multiple events in sperm including stimulation of sperm-speciﬁc sodium hydrogen exchanger in murine sperm (29) and hyperactivation and facilitation of the SLO3 potassium channel (26). Both alkalization and hyperpolarization are critical steps for capacitation and AE to occur (27, 28).

Production and validation of the AcroSensE mouse model

To generate the AcroSensE plasmid, we replaced the GFP sequence in the pAcr3-EGFP backbone plasmid (39) with a cassette comprising the low-affinity GCaMP3 (42) and mCherry in fusion (Fig. 1A). The mCherry and GCaMP3 were coupled by a 23-aa linker (flexible alanine–proline repeat linker (43)). Transgenic founders were obtained by injecting the linearized AcroSensE plasmid into the pronucleus of 100+ fertilized embryos obtained by mating C57BL6/J and CBA F1 hybrids (Stem Cell and Transgenic Core Facility). Transgenic founders were backcrossed to C57BL/6 mice. Insertion of the target construct in the transgenic founders was verified by genomic PCR, using primers targeting the GCaMP3 region of the AcroSensE construct. To validate expression of the transgene, we first determined the expression levels of AcroSensE in various tissues. Figure 1B shows the results of RT-PCR analysis of AcroSensE demonstrating the highest transcript levels in the liver and testes. However, immunoblots of protein extracts using an antibody against the GFP backbone of the GCaMP3 (1 ng/μl, Abcam, Cambridge, MA) demonstrated that the AcroSensE protein was expressed predominantly in testes (Fig. 1C). Moreover, we were not able to detect expression of the AcroSensE construct at the protein level in any tissue but sperm using fluorescence microscopy (detected via the mCherry fluorescence; data not shown).

Resolution of intermediate MF steps during AE

Sperm from AcroSensE mice were collected as previously described (23). Following incubation for 30 to 60 min at 37 °C in the presence or absence of 3 mM CD and 10 mM bicarbonate as indicated, sperm were placed on a polylysine-coated coverslide dish (MatTek Corp) and imaged on a Zeiss LSM 510, with a controlled delivery system for local administration of ionophore A23187, G<sub>M1</sub>, CD, or P4. Preincubation of populations of sperm under capacitating conditions was performed with some stimuli (CD with/without bicarbonate) to mimic the physiological timeline when sterol efflux would occur relative to the effects of those other molecules (e.g., G<sub>M1</sub> and P4). To stimulate exocytosis in single cells, a short (10 s) puff of stimulant was applied near an individual sperm via...
micropipette while recording the changes in GCaMP3 and mCherry fluorescence intensities at image rates of up to 5 frames per second for each wavelength. Concentrations are indicated for the solution in the micropipette, as specified in the Experimental procedures section.

To understand baseline effects and aid interpretation, we quantified the percentage of w.t. B6 sperm undergoing AE as a result of initial incubation under noncapacitating and capacitating conditions (Fig. S1). Using Coomassie staining, we found that 21.7% of the cells underwent AE in response to a 30-min incubation (at 37°C) with 3 mM CD or 24.6% of cells with 25 μM GM1 (data from 3 mice each, counting at least 180 cells in each condition). Under noncapacitating control conditions (no CD or GM1), 10.7% of sperm underwent AE. After 90 min, 36.3% of sperm underwent AE in response to GM1, 45.2% in response to CD, and 47.1% in response to both CD and GM1. At this time point, the value for noncapacitating conditions also increased to 25%. These percentages are similar to our prior observations (23). Because the sperm that responded to the preincubation by undergoing MF and AE would have lost both their GCaMP3 and mCherry signals prior to encountering the other stimuli in our experimental approach, they would not be observed and quantified in the live imaging experiments. That is, any sperm undergoing spontaneous AE or AE in response to preincubation did not contribute to the subpopulations showing acrosomal Ca2+ rise (ACR) and MF upon stimulation with various reagents mentioned in the following part of the article.

After preincubation for 30 min, the AcroSensE sperm were then imaged. In each trial, sperm from multiple mice were pooled, and typically only 1 to 2 of the experimental conditions described here were tested per trial to ensure that length of preincubation would not be a confounding factor in interpreting results from different conditions. Under these experimental conditions, we observed several steps in the process leading to AE, consistent with ACR, and full MF leading to loss of the mCherry fluorescence and therefore full AE. In some cells, we saw traces similar to prespike foot (PSF) events as frequently observed when using amperometry to study exocytosis; however, these were on a much slower time course than when observed in other cells using amperometry. Although temporally these PSF-like events were the earliest events we observed using AcroSensE fluorescence, they were observed infrequently and only under specific circumstances. Therefore, we will first describe the more common ACR and MF responses to the different treatment conditions.

Figure 2 provides traces demonstrating either representative changes in the GCaMP3 and mCherry fluorescence intensities following various stimulations or changes shown in specific subpopulations of sperm deserving special mention (Fig. 2, A and D; note that population data are summarized in Fig. 3, B and C). Following the addition of ionophore A23187, one subpopulation of sperm demonstrated rapid ACR which was typically quickly followed by MF as evidenced by loss of mCherry signal (Fig. 2A, left panel; 40% of responding sperm).
In contrast, the remaining 60% of sperm responding to A23187 showed a slow, gradual ACR that did not result in AE within the time frame of image acquisition (until AE occurred or up to 8 min, Fig. 2A right panel).

Stimuli resulting in alterations in membrane lipids (i.e., sterol efflux mediated by addition of CD and sterol efflux coupled with exogenous G_{M1}, Fig. 2, B and C) typically resulted in a lower proportion of cells responding, but more uniformity in having a rapid ACR, followed quickly by MF in most of the responding sperm. The notable exception was the case of CD stimulation in the absence of extracellular Ca^{2+} (CD + EGTA), in which no ACR was observed. Interestingly, although the EGTA sufficiently reduced Ca^{2+} in this condition such that there was no detectable ACR (Figs. 2D), 17.1% of sperm still underwent exocytosis (shown here in Fig. 2D by loss of mCherry fluorescence; traces representing the majority of sperm that did not undergo MF and AE are not shown; see also Fig. 3B and discussion in the later part of the article).
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Both low and high P4 conditions (with and without bicarbonate) induced ACR in varying percentages of sperm, with several differences seen in patterns of Ca\(^{2+}\) rise (more on spatial and temporal dynamics in figures). Excepting the CD+EGTA treatment, under all other conditions in which MF and AE were observed, a rise in GCaMP3 signal preceded the loss of mCherry fluorescence. These results combine to suggest that ACR resulted from extracellular Ca\(^{2+}\) entry into the acrosome and occurred prior to exocytosis of the acrosomal contents. This temporal pattern is illustrated in Figure 3A and further discussed.

Figure 3A provides a schematic representation of the typical observations for the AcroSensE sperm, in which time (X axis) is expanded relative to Figure 2, to highlight that the response to stimulation initiates with an increase in the GCaMP3 fluorescence intensity (reflecting ACR), followed by the loss of the mCherry and then loss of the GCaMP3 fluorescent signals. Figure 3, B and C summarize population data of the ACR and MF responses of cells to the various stimulations. In response to the addition of ionophore (A23187) following preincubation with 3 mM CD and/or 10 mM bicarb (n = 73). All concentrations identical to those in Figure 2.

In contrast, adding exogenous G\(_{\text{M1}}\) (following preincubation with CD) to the sperm’s plasma membrane, or induction of sterol efflux by addition of CD alone (with none added during preincubation), resulted in a lower percentage of cells responding with ACR than that responding to A23187 (31.9% and 27.7%, respectively, Fig. 3B). However, the vast majority of these responding cells also exhibited MF/AE (Fig. 3, B and C; G\(_{\text{M1}}\) + CD = 83.1% of ACR-positive cells underwent MF/AE; CD = 91.3% of ACR-positive cells underwent MF/AE). Note that the value of 25.3% of sperm undergoing MF in response to single-cell application of CD without prior exposure during preincubation is very close to what we saw when preincubating populations of sperm with CD as noted earlier, giving us confidence that exposure to this mediator of sterol efflux under these conditions of single-cell application had similar effectiveness regardless of the method of exposure. Recall that because preincubation with CD occurred first, and then the G\(_{\text{M1}}\) was added, those cells responding to G\(_{\text{M1}}\) represent a subpopulation that had not already responded to CD-mediated sterol efflux alone within the preincubation time frame. The combined percentage of sperm exhibiting MF in response to modulation of these two membrane lipids was therefore approximately 45% (calculated as the sum of 24.6% responding to CD alone and 26.5% of the remaining 75%, which is almost identical to prior results for exposure to both stimuli (23)). This reinforces the complementary nature of the multiple membrane lipid–dependent pathways that promote AE, but the combined results suggest that the total pool of AE-capable sperm is roughly 45%–50% regardless of the membrane lipid stimulus.

Comparison of responses under the other conditions revealed very different percentages of sperm experiencing ACR (Fig. 3B) but roughly comparable transition rates to that seen with A23187 (A23187: 40.0%, Low P4: 42.7%, Hi P4: 55.2%, Hi P4 + Bicarb: 58.1%; Fig. 3C). However, keep in mind that of sperm that responded to A23187 quickly, 89.4% of sperm with fast ACR and 18% with slow ACR did progress into full MF).

Spatial characterization of the steps leading to AE

Live cell microscopy of AcroSensE sperm enabled us to determine the spatial characteristics of ACR and MF events. As
seen in Figure 4, an increase in the fluorescent signal coming from the GCaMP3 was used to localize the initiation site of the intensity change associated with ACR, whereas the initiation site for AE was noted by the initial site of mCherry signal loss. Changes in fluorescence in both wavelengths were used to track the directionality and progression of Ca\(^{2+}\) rise and AE.

A summary of the findings for the spatial characteristics of ACR formation is provided in Figure 5A. We observed 4 main patterns of progression: (1) diffuse—in which the signal initiated evenly over the entire apical acrosome area (D), (2) top to bottom—initiating from a rostral position (“top” position, near the perforatorium [tip]) and progressing caudally (“bottom” position, near the subacrosomal ring) toward the post-acrosomal region (T > B), (3) center outward—initiating at the center and propagating in opposite directions to the top and bottom of the apical acrosome (C > TB), and (4) bottom to top—progression rostrally from near the subacrosomal ring to the top (B > T). Following A23187 and various P4 stimulations, the plurality of responding cells showed diffuse patterns of Ca\(^{2+}\) rise throughout the acrosome. In sperm following the addition of exogenous GM1 or induction of sterol efflux by CD, this pattern was observed less frequently. Particularly, addition of GM1 or CD to cells resulted in increased incidence of signals initiating from the top or top/center (Fig. 5A). Initiation of ACR from the bottom of the acrosome near the subacrosomal ring was consistently lower under all conditions (16% of sperm when averaged across all conditions).

For MF (Fig. 5B), as determined by the loss of the mCherry signal, spatial patterns were similar with the distinction that instead of a signal propagating from the center to the ends as seen for ACR (C > TB pattern), we observed MF initiating from both ends and moving toward the center (bottom and top toward the center, TB > C). In cells incubated with bicarbonate during preincubation, we observed higher rates of B > T patterns of loss of the mCherry signal (>50% of responding cells for high P4 + CD + bicarbonate, Fig. 5B). In contrast, higher incidence of T > B were observed when P4 concentration (+CD) was low (Fig. 5B). All patterns were observed relatively equally upon GM1 stimulation, while addition of CD alone resulted in slightly less than half of the sperm demonstrating a diffuse pattern of release of the AcroSensE during AE (Fig. 5B).

Next we examined the percentage of cells transitioning from ACR to full MF and AE as a function of the initiation site of the rise in the AcroSensE fluorescence intensity (Fig. 5C). Interestingly, upon A23187 stimulation, ACR transformed into AE almost exclusively if the ACR events initiated at the center of the sperm head (C > TB). Under all conditions, cells were less likely to demonstrate progression from ACR to MF if the ACR events were diffusely localized or had a bottom-to-top progression. Addition of exogenous GM1 to the cells had a higher chance to generate MF (80% of cells) if the ACR events initiated at the center or the top of the sperm head, but not at the bottom (20% of cells). In the case of CD and low P4 with

Figure 4. Representative images of AcroSensE sperm undergoing different spatial patterns of acrosomal Ca\(^{2+}\) rise (ACR), membrane fusion (MF), and acrosomal exocytosis (AE). A, visualization of bottom-to-top spatial progression of ACR and MF following addition of high P4 (+bicarb + CD) to AcroSensE sperm. The initial basal signal of the GCaMP3 is low, while maximal mCherry intensity is detected throughout the apical acrosome. Following stimulation, an increase in the GCaMP3 signal is detected near the bottom of the plasma membrane overlying the acrosome (arrow 1). The green signal then propagated rostrally (arrow 2), while a decrease in the red signal was observed near the same point of origin (arrow a) and then propagated in the same direction (arrow b). There was complete loss of both red and green signals in this cell, consistent with full MF and AE. B, visualization of top-to-bottom spatial progression of ACR and bottom-to-top progression of MF in the same sperm cell following addition of High P4 (+bicarb + CD). Before stimulation, the basal signal of the GCaMP3 is low, while maximal mCherry intensity is detected. Following stimulation, an increase in the GCaMP3 signal was detected near the top of the sperm head (arrow 1). The green signal propagated caudally toward the bottom of the sperm head (arrow 2), whereas a decrease in the red signal was observed at the bottom of the APM as soon as the green fluorescence intensity increased in that location (arrow a). The decrease in the red signal propagated rostrally toward the top of the sperm head (arrow b), resulting in a complete loss of both red and green signals. For both A and B, time is provided in seconds measured after the start of application of the stimulus, next to each frame. AcroSensE, Acrosome-targeted Sensor for Exocytosis; APM, acrosomal plasma membrane; CD, 2-hydroxypropyl β-cyclodextrin.
CD and bicarbonate, at least 50% of the sperm undergoing AE demonstrated ACR initiating rostrally (at the top of the sperm head) and moving downward toward the subacrosomal ring (CD: 51.5%, Low P4: 50%).

Finally, we looked at the spatial correlation between the initiation sites of ACR and MF. Figure 5D provides the percentage of cells in each experimental condition where the ACR and MF initiated at the same location. These data show that AE can initiate independently from the original site of ACR, but a stronger spatial link between that site and MF was associated in membrane lipid–mediated signaling.

### Temporal characterization of ACR events

Next we examined key temporal characteristics of ACR as they occurred under various conditions (Fig. 6). Strikingly, we found substantial differences in the onset time of ACR depending on whether the stimulation was mediated by membrane lipids or P4. ACRs were significantly faster to appear following high P4 in sperm that had previously been capacitated with CD and bicarbonate (18.6 ± 3 s, Fig. 6A).
Conversely, ACRs were slowest to initiate in sperm stimulated with exogenous GM1 (231 ± 31 s), more than 12 times slower. The trend of onset times between the different conditions was consistent with the values observed for time to peak, in which GM1-mediated ACR was the slowest and P4 was the fastest (Fig. 6B).

Surprisingly, these trends were followed in reverse regarding the total rise time (rise duration), in which longer start times and times to peak for CD and GM1 (Fig. 6A and B) corresponded with shorter rise durations (Fig. 6C). The rise duration (Fig. 6C) indicates the timeframe in which the ACR either reached its peak value or diminished by the occurrence of AE. Comparable results were found for GM1 (18.7 ± 5.4 s) and CD stimulation (25.1 ± 12.4 s, Fig. 6C), whereas P4 demonstrated approximately 2-fold longer time-to-peak duration (Low P4+bicarb+CD: 54.4 ± 8.6 s, Hi P4+CD: 43.9 ± 5.1 s, Hi P4+bicarb+CD: 41.4 ± 6.3 s). Compared to all other conditions, the average time-to-peak duration was significantly longer following A23187 stimulation.

Significant differences of almost 2-fold were also observed between the various conditions for the GCaMP3 signal when looking at the rise amplitude (ΔF). Figure 6D summarizes the increase in the GCaMP3 fluorescence signal, which corresponds to the net change in the acrosomal Ca²⁺ concentration during ACR. Our data show that for A23187 (576 ± 20 ΔF) and High P4 with bicarbonate (577 ± 32 ΔF), the total Ca²⁺ rise in the acrosome was almost twofold higher than for CD (304 ± 32 ΔF, p < 0.001), or High P4 without bicarbonate during capacitation (352 ± 0.13 ΔF, p < 0.001), or Low P4 with bicarbonate (297 ± 44 ΔF, p < 0.001). GCaMP3 signal increase in response to GM1 was only marginally lower than the ionophore A23187 (p < 0.0081) or high P4 with bicarbonate (454 ± 43 ΔF).

Conversely, the kinetic rates of GCaMP3 Ca²⁺ signal rise (slope, ΔF/sec) were the highest following GM1 and CD stimulations (137 ± 25 ΔF/sec, and 124 ± 19 ΔF/sec, respectively), with other conditions being approximately two-fold slower (Fig. 6E). However, there was more cell-to-cell variation in this parameter reducing the statistical significance of many differences.

Remarkably, the kinetic rate of GCaMP3 fluorescent signal rise was directly associated with the ability of the stimulated sperm to undergo MF and AE. Figure 6F breaks down the slope (ΔF/sec) for the sperm in each condition that either underwent membrane fusion and those that did not. The data clearly show that although there was particularly high variance when stimulating with P4, in all conditions, cells that successfully transitioned from ACR to MF had significantly higher rates of GCaMP3 signal increase (i.e., faster rates of Ca²⁺ influx into the acrosome).

**Identification and characterization of PSF-like events during AE**

Unexpectedly, the AcroSensE sperm revealed what is typically a subtle intermediate step in the process of MF and cellular exocytosis (Fig. 7). These events preceded the major Ca²⁺ rise (ACR), and while demonstrating much slower
timescale, their occurrence and profile are reminiscent of PSF-like events observed in a variety of secretory cells when measured with amperometric methods using carbon fiber electrodes (44). **Figure 7A** provides two types of representative traces of PSF-like events detected in AcroSensE sperm, following stimulation with A23187 or high P4 (after incubation with CD and bicarbonate). The PSF-like events were defined as a Ca^{2+} signal rise that preceded the major ACR event and which demonstrated reduced amplitude and kinetics (i.e., slope) in comparison to that ACR event. **Figure 7A** shows the application of these criteria for determining a PSF event, where the GCaMP3 signal rises above the baseline but demonstrates a distinctive dissimilar rise rate compared to the ACR. In addition, the PSF-like events were significantly smaller (4–7 fold) in amplitude than the ACR, depending on the condition, and resulted in a biphasic increase. Together, these two criteria were used to define these events. Except for sperm incubated in medium depleted of extracellular Ca^{2+} (EGTA in medium), PSF-like events were detected under all conditions, suggesting that these miniature elevations in the GCaMP3 signal were dependent on influx of extracellular Ca^{2+} into the acrosome. Nevertheless, considerably higher incidence of PSF-like events was observed in cells stimulated by A23187 (PSF detected in 15.1% of responding cells; *p* ≤ 0.024 versus GM1, CD and Low P4) and in cells stimulated with high P4 following capacitation in the presence of CD and bicarbonate (detected in 23.3% of responding cells; *p* ≤ 0.003 for GM1, CD and Low P4 and High P4, **Fig. 7B**).

Although their manifestation in response to high P4 stimulation was lower in the absence of bicarbonate, PSF-like event duration in this condition was roughly 3 times longer (42.5 ± 4.6 s) than that of low P4 or high P4 with bicarbonate (16.4 ± 1.6, and 13.1 ± 1 s, respectively; **Fig. 7C**). The amplitude values of the PSF-like events (as measured from the baseline cell

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**Figure 7. Summary of prespike foot (PSF)-like events preceding ACR.** A, representative traces of PSF-like events following stimulations with A23187 (left) or high P4+bicarbonate+CD (right panel). The shaded area (green) is provided to highlight the PSF-like event. Arrows indicate the duration (D) and amplitude (A) above the baseline GCaMP3 signal, as measured for the analysis provided in (C) and (D). B, summary of PSF-like event occurrence (as the percentage of total cells) under various conditions. C, average duration of the PSF-like events, as measured from the initial rise of signal to the beginning of the main Ca^{2+} rise (see left panel in (A)). D, average amplitude of the fluorescence intensity increase during the PSF-like event. ACR, acrosomal Ca^{2+} rise.
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fluorescence to the amplitude of where the foot transformed into a typical ACR slope) were independent of the duration parameters, demonstrating the highest values in cells stimulated with high P4 with bicarbonate and in cells stimulated with ionophore A23187 (Fig. 7D). Nonetheless, by definition, the average amplitude was markedly lower than the amplitude of ACR (4–7 fold), indicating the limited amount of Ca2+ ions able to enter the acrosome via the PSF-like events.

Characterization of MF dynamics in the AcroSensE sperm

In mice expressing GFP in the acrosome, loss of fluorescence results from loss of acrosomal matrix contents due to MF events (45). As shown in the aforementioned figures, we used a similar strategy to relate loss of mCherry fluorescence and MF events and full AE. In Figure 8A, we schematically show several analyses of the loss of the mCherry signal we performed to determine whether they differed with various forms of stimulation. First, we determined the onset of MF events following the various stimulations. As shown in Figure 8B, MF initiated nearly 2.5 times faster in response to high P4 in sperm that were capacitated with CD and bicarbonate versus those that were incubated only with CD (60 ± 11 and 149 ± 16 s, respectively, p = 4.9 × 10^{-5} Fig. 8B). In contrast, following the addition of G_{M1} or A23187, cells demonstrated the longest delay to the onset of the MF responses (238 ± 38 s and 190 ± 29 s, respectively, Fig. 8B). Note that the trends of start times between the different conditions were comparable for ACR and MF.

Next, we determined the duration of MF events, as calculated from the time frame of the start of mCherry signal loss to complete loss of signal, consistent with the completion of AE. Interestingly, cells stimulated with CD under zero Ca2+ conditions demonstrated the shortest duration times of the mCherry signal dispersal from the acrosome (CD-EGTA, 5 ± 0.6 s). On the other hand, stimulation of cells with P4 resulted in the longest duration periods regardless of the presence or absence of bicarbonate during capacitation (29.41 ± 2.69 and 24.87 ± 3.60 s, respectively; Fig. 8C). All other conditions demonstrated similar times for the diffusion of the AcroSensE out of the acrosome ranging from 11 to 15 s on average.

As a single fusion protein comprising both the GCaMP3 and mCherry elements, the AcroSensE construct also allowed us to look at the delay time between ACR and full MF. Figure 8D summarizes the ACR–MF time interval, as calculated from the time between the onset of the ACR (start of increase in GCaMP3 signal) and the initiation of MF (when the mCherry signal starts to decrease). The ACR–MF time interval

Figure 8. Characterization of membrane fusion (MF) events under various conditions. Statistical differences of p < 0.05 are noted by different letters. A, a schematic illustration of typical MF proceeding until full AE as detected from the loss of mCherry fluorescence in the acrosome and the various parameters used in the analysis provided in (B–F). B, the average time in which MF initiated, as measured from the time of stimulation to the start of loss of the mCherry signal. C, the average duration of the mCherry fluorescence loss until it reached its minimum intensity. D, the average delay between the onset of the ACR (initial rise in green GCaMP3 signal) and the initiation of MF (mCherry signal loss). Compared to all other conditions, the delay between ACR and MF was the shortest for CD, with p values of 0.06 (A23187), 0.03 (G_{M1}), 0.01 (Low P4), 0.0003 (Hi P4), and 0.0005 (High P4 + bicarb). E, the average change in the mCherry signal during acrosome exocytosis (∆F), normalized to the baseline fluorescence intensity (F0). F, the rate of signal loss over time as calculated from the slope of the mCherry fluorescence decay (as measured from the initiation of signal loss). ACR, acrosomal Ca2+ rise; AE, acrosome exocytosis; CD, CD, 2-hydroxypropyl β-cyclodextrin.
showed little variability among the various conditions, except for cells stimulated with CD where the delay between Ca\(^{2+}\) rise and MF was considerably shorter than all other conditions (6.1 ± 3 s). Importantly, no significant differences were observed for the total amount of mCherry fluorescent signal lost as demonstrated by the ΔF/F₀ analysis provided in Figure 8E, suggesting that under all conditions tested here, once AE occurred, the loss of protein content (matrix) from the acrosome vesicle was to a similar extent. Conversely, the rates of protein diffusion out of the acrosome during AE were dependent on the stimulation, where P4 signaling resulted in slower protein release rates and lipid modulation of the sperm or addition of A23187 induced generally faster rates of protein diffusion out of the matrix within the acrosome vesicle (Fig. 8F).

Discussion

Sperm from the AcroSensE mice demonstrated spatiotemporal relationships between Ca\(^{2+}\) dynamics in the compartment of the acrosome and MF between the plasma membrane and OAM. Differences in response to varied stimuli in turn revealed important insights into the mechanisms and pathways leading to AE, enabling us to generate a working model (shown below) that summarizes current understanding.

Mechanistic findings

Working through the findings as they would occur temporally during AE, data presented here show absolute reliance on extracellular Ca\(^{2+}\) for ACR. Interestingly, some exocytosis was observed under (nonphysiologic) Ca\(^{2+}\)-depleted conditions. These were the only conditions under which MF occurred without ACR. This observation could be explained by some combination of two known mechanisms that are not physiologically relevant: EGTA causes membrane ruffling/perturbation (46), and Ca\(^{2+}\)-independent PLA₂ is shown to be responsible for spontaneous (unstimulated) AE (47).

Based on the current understanding that most sperm undergo AE before they reach the zona pellucida (which had been the main stimulus in the acrosome reaction model) (6, 7, 48), we focused our attention on stimuli that sperm would encounter in the vicinity of the egg (progesterone from cumulus cells), that would mimic stimuli found in the oviduct in a reductionist, controlled way (CD as a chemically defined mediator of sterol efflux replacing effects of oviductal albumin or high-density lipoproteins), or stimuli that exist on sperm but are released from inhibition provided by the binding of seminal plasma proteins (G₅M₂). The methodological soundness of delivering these stimuli via puffing by micropipette was supported by the similarity of results with puffed CD to those seen when we preincubated sperm with CD in the medium (Fig. 51). Also, current results with CD and G₅M₂ closely matched those when incubating sperm in media in prior studies (23). One interesting finding in making these comparisons within and between studies is that with membrane lipid–stimulated exocytosis, it appears that a subpopulation of roughly 45 to 50% of sperm can maximally be triggered to undergo AE. This finding is consistent with a wealth of data across species that only a subset of sperm can undergo physiologically relevant capacitation and AE.

How does extracellular Ca\(^{2+}\) increase acrosomal Ca\(^{2+}\)?

Our data suggest that PSF-like events and ACR are most likely mediated by FP formation between the APM and OAM, as evidenced via the increase in GCaMP3 signal mediated by Ca\(^{2+}\) influx into the acrosome. A limited number of FPs, FPs of a transient nature, and/or FPs of very small diameter could all account for the PSF-like events. As the number/size of FPs would increase, this would lead to more rapid ACR, and simultaneously the increase in MF events would be predicted to result in quicker AE. This relationship would account for our finding that the rate of ACR in sperm undergoing MF/AE was higher than that in cells that did not demonstrate MF (Fig. 6F). However, mechanisms for Ca\(^{2+}\) entry into the acrosome other than FPs must also be considered:

1. GCaMP3 signal increase was a result of Ca\(^{2+}\) elevation in the acrosome generated by mobilization from other internal stores. Mobilization of Ca\(^{2+}\) ions from one of the sperm’s internal stores (i.e., mitochondria or redundant nuclear envelope [RNE] (49)) into the acrosome would result in an increase in the acrosome Ca\(^{2+}\) concentration, without involvement of Ca\(^{2+}\) influx via FPs from the medium. However, this alternative can be ruled out by data showing that GCaMP3 signals were absent when stimulating cells in Ca\(^{2+}\)-depleted medium (EGTA), demonstrating the dependence of the acrosomal GCaMP3 signal on extracellular Ca\(^{2+}\). Moreover, this alternative is also spatially incompatible with our observations, as both the putative Ca\(^{2+}\) stores in the RNE and mitochondria are located in the connecting piece/midpiece of the sperm, whereas we frequently observed high occurrence of ACR initiating at the top (apical tip) or center of the sperm (Fig. 5A).

2. The ACR was mediated via transporters and/or channels localized on the OAM. In this alternative model, Ca\(^{2+}\) entry to the acrosome occurs in a two-step process, where cytosolic Ca\(^{2+}\) increase (via channels/transporters on the APM or release from RNE/mitochondria stores) is followed by Ca\(^{2+}\) entry into the acrosome through either a channel or a transporter on the OAM. Indeed, several proteins have been reported to be involved in acrosome Ca\(^{2+}\) mobilization including Ip3R (50) and TPC1/TPC2 (NAADP-gated intracellular Ca\(^{2+}\)/Na⁺ channels (51, 52)). Because of its baseline high Ca\(^{2+}\) concentration, additional influx into the acrosome of a mature sperm must occur against the electrochemical gradient, requiring expenditure of energy. Two mechanisms that could function in this capacity have been reported to enable Ca\(^{2+}\) influx into the acrosome. First, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase 2 (SERCA2) has been shown to be expressed in mammalian sperm (53) and regulate Ca\(^{2+}\) uptake into the acrosome (41). There are conflicting data regarding SERCA activity in mature sperm, with functional effects from inhibition by...
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thapsigargin tending to occur only at high concentrations. Furthermore, studies in HeLa cells showed that intracellular alkalization inhibits SERCA (54), suggesting that in capacitated sperm, these pumps would demonstrate lower activity. Alkalization of the acrosome itself has been observed during capacitation and is also observed throughout the cytoplasm. Second, the secretory pathway Ca\(^{2+}\) ATPases (SPCAs) have been reported to function in mammalian sperm (49). Although SERCA or SPCA pump activation under some conditions could result in ACR, this rise would be predicted to be independent from extracellular Ca\(^{2+}\) [e.g., refer to figure 2 in the study by Williams and Ford (55)]. In addition, in human sperm, the SPCA clearly localized to the midpiece/connecting piece, and not the acrosome. Together with our data using EGTA, these transporters seem unlikely to explain ACR events in the AcroSensE sperm. In addition, based on reports of Ca\(^{2+}\) clearance from mouse sperm (which involves all the available Ca\(^{2+}\) transport mechanisms (56)), it is clear that the theoretical velocity and rate of Ca\(^{2+}\) influx into the ER as mediated by this channel are slower than we observe during ACR. Yet another candidate for mobilizing Ca\(^{2+}\) into the acrosome is the stromal interaction molecule (STIM)–Orai channel complex involved in store-operated Ca\(^{2+}\) entry (49, 57–59). Indeed, STIM and Orai proteins were documented in human (49) and mouse sperm (60) by Western blot and immunocytochemistry. Using cell-penetrating peptides attached to a protein domain that stimulates STIM–Orai activity, Morris et al (61) were able to induce store-operated Ca\(^{2+}\) activity at the neck of human sperm, downstream of CatSper stimulation by progesterone. Thus, these findings strongly suggest that as with SPCA, this store-operated Ca\(^{2+}\) entry complex is spatially separated from our observations of Ca\(^{2+}\) increase located over the acrosome/apical-acrosome during ACR.

3. Change in acrosomal pH positively modulates the GCaMP fluorescence intensity.

According to this alternative explanation, pH changes in the acrosome would increase GCaMP3 fluorescence, mimicking a true ACR and leading us to interpret the results as FP formation. Before capacitation, the acrosomal pH is maintained at acidic levels via several mechanisms (including V-ATPase, sodium–proton exchanger (NHE), and Cl\(^{-}/\text{HCO}_3^-\) exchanger (62)). During capacitation, the acrosomal pH was shown to gradually increase over the incubation period of 120 minutes from pH 5.3 ± 0.1 to pH 6.2 ± 0.3 (63). Thus, the pH increase in the acrosome is occurring too slowly to explain the rapid changes in GCaMP3 fluorescence intensity observed for the AcroSensE sperm during FP formation. Moreover, it is also important to note that the GCaMP Ca\(^{2+}\) indicator is relatively stable in the physiological pH range that has been reported for the acrosome (64), and therefore, abrupt fluorescence intensity changes as seen during our recordings are highly unlikely to be a result of pH fluctuations.

4. Release of sequestered Ca\(^{2+}\) from Ca\(^{2+}\)-binding proteins.

Several types of Ca\(^{2+}\)-binding proteins have been detected in spermatozoa, including calmodulin, calreticulin, CABS1, and glucose-regulated protein 78 (BiP), although the latter demonstrates only low Ca\(^{2+}\)-binding capacity (65). These proteins have been reported to be localized throughout the sperm head and flagellum, but little is known about Ca\(^{2+}\)-binding protein functions in the acrosome. Ca\(^{2+}\)-binding proteins typically release bound Ca\(^{2+}\) ions upon depletion of a store, which would not be observable with the AcroSensE mice.

How does ACR trigger MF/AE?

Our results showed clearly that it was the rate of ACR, and not how long it takes to induce it or the absolute amounts of Ca\(^{2+}\) brought into the acrosome, that was most clearly linked with ability to undergo MF/AE (Fig. 6). This was seen with both the nonphysiological ionophore A23187, in which slow rise led to very high GCaMP3 fluorescence but relatively few cells showing MF, as well as with the physiologically relevant stimuli. At least two separate mechanisms could account for the existence of these subpopulations. First, the sperm exhibiting slow rise in response to A23187 might have had that increase mediated by different processes (e.g., A23187 permeating through APM and then the OAM, which would result in Ca\(^{2+}\) leaking into the acrosome), which were not coupled to mechanisms promoting MF/AE. Second, this subpopulation of cells might have had other differences that decoupled acrosomal Ca\(^{2+}\) concentrations and MF/AE. For example, differences in membrane lipid composition such as in local sterol abundance could render them less fusogenic. Regardless, the retention of mCherry signal showed that AE was not triggered in these cells. Under all conditions, the rate of ACR was much higher in sperm that underwent MF/AE than in sperm that did not (Fig. 6F). Whether the rate of ACR itself regulates MF such as through stimulation of MF machinery (i.e., SNARE proteins) or whether a faster rate of ACR results from pre-existing membrane differences that would have made them more fusogenic cannot be determined by these correlations and will be a topic of future study.

Interestingly, the AcroSensE sperm also revealed that the spatial location of the initiation site of ACR and initiation site of MF tended to vary with the stimuli (Figs. 5 and S2). This finding is of importance because it suggests that the stimuli exerted their effects through different, functionally redundant mechanisms that reach a common endpoint. However, the existence of different patterns in response to a single stimulus is evident that there is not a single initiation site or spatial pathway for propagation of ACR or for MF that is unique for each stimulus. A biological explanation for such differences could be that plasma membrane proteins that mediate sterol efflux (such as ATP-binding cassette transporters) or act as receptors for P4 are enriched in certain membrane subdomains, but these are distributed throughout the plasma membrane over the apical acrosome. Biophysical factors such as local differences in membrane fluidity and distances between phospholipid head groups in the highly curved apical acrosome could lead to differences in ease of integration of lipids such as G\(_{\text{M1}}\), or the removal of sterols. The architecture
of the sperm head and localization of microdomains highly enriched in sterols can also lead to mechanical resistance to fusogenicity (66).

Mechanistically, some differences in the patterns and kinetics of ACR can be explained by the hypothesized mechanisms of action for each stimulus. For example, G_M1 took the longest time to initiate ACR and time to peak (Fig. 6, A and B). This would make sense if this sphingolipid acted as previously proposed, by inserting into the plasma membrane and then causing a focal enrichment, leading to a transient that would then locally prime MF machinery, facilitating AE in response to a subsequent Ca^{2+} wave (23). In contrast, CD had a shorter time to ACR and the shortest delay between initiation of ACR and MF (Fig. 8D). This would be consistent with a mechanism of action involving activation of PLB, which would cause immediate local changes in membrane curvature through its hydrolysis of both fatty acid tails at the sn-1 and sn-2 sites, leading to MF events (22). Both of these stimuli function by affecting the local membrane lipid environment, and it is noteworthy that both had the highest transition rates (the percentage of sperm that exhibited ACR progressing to MF/AE; Fig. 3C). If broken out by subpopulation, those sperm responding to A23187 with a fast ACR had a similar transition percentage to G_M1 and CD as noted earlier in the study.

**Findings lead to a model for AE**

Across all stimulation conditions excepting the small subset of cells responding nonphysiologically in the presence of EGTA, ACR was required for, and preceded, MF and AE. Of critical importance in trying to resolve existing controversy over the process of AE, the AcroSensE model enabled moderately fast imaging with high resolution. Spatiotemporal analysis of rise in GCaMP3 signal and loss of mCherry fluorescence surprisingly showed a lack of correlation between the sites of initiation of ACR and MF. G_M1 and CD showed the highest levels of correlation, but even these were below 50%. This very simple finding provides compelling evidence against the acrosome reaction model in which fusion between the plasma and OAMs results in explosive exocytosis akin to popping a water balloon.

Rather, we observed that different stimuli were more likely to initiate ACR in different parts of the apical acrosome and therefore have that rise in Ca^{2+} propagate in different directions (Figs. 5, and S3). These data support a model in which FP s can be initially formed over the apical acrosome in different places. From that initiation site, elevated Ca^{2+} is soon found throughout the acrosomal matrix, and the presence of FPs large enough to allow outward diffusion of the AcroSensE protein can begin to occur at a distance within the apical acrosome.

The most parsimonious explanation for all our findings combined is shown as a model in Figure 9. In this model, we feature the MF events that result in changes in AcroSensE fluorescence. More complexity can of course be added in terms of MF machinery, plasma membrane channels, membrane microdomains, etc. In the model, transitory MFs can occur leading to the small increases in acrosomal Ca^{2+} that appear similar to either small FPs or small FPs are formed as a result of the same stimuli. The small size of the FPs is implied by the fact that the increase in GCaMP3 is seen before any mCherry is lost, suggesting that small molecules such as Ca^{2+} can enter before something the size of mCherry in fusion with the GCaMP3 (approx. 75 kDa) can leak out. Over time, the number of FPs increases in response to the one or more stimuli, and eventually, they coalesce to make hybrid vesicles resulting in full AE. Note that although our data (Fig. 8E) show loss of mCherry signal occurring to the same degree under all conditions, both the length of time over which it is lost (Fig. 8C) and the rate at which it is lost (Fig. 8F) differ between the membrane lipids and A23187 and the progesterone treatments. Given that the fusion construct is a soluble protein, this difference in the rate of loss likely reflects differences in the size and/or number of FPs through which the protein would diffuse out.

Overall, the combined data provided by the AcroSensE mice clearly support a regulated, multistep model of AE instead of the prior dogma of the acrosome reaction. Although AE is a common endpoint, different stimuli were found to induce it with different spatial and temporal dynamics underscoring functional redundancy in mechanisms for this critical step, which is essential for the transmission of life.

**Experimental procedures**

**Reagents and animals**

All reagents were purchased from Sigma, unless otherwise noted. Male and female B6129SF/J mice were purchased from Jackson Laboratories. All animal procedures were performed under the guidelines and approved by the Institutional Animal Care and Use Committee at Cornell University.

**Acr-GCaMP3-mCherry plasmid construction**

Plasmid construction was performed by BlueSky BioServices. The pAcr3-EGFP backbone plasmid was a gift from Dr Masahito Ikawa (Osaka University). The GCaMP3-expressing vector (#63885) and mCherry were obtained from Addgene. The mCherry was first inserted downstream of the GCaMP3, and then the GCaMP3–mCherry cassette was inserted into the Acr-EGFP plasmid (pBluescript II SK+), which had had the GFP portion excised using EcoRI. The full construct contained a 2.4-kb mouse genomic region carrying the acrosin gene with the sequences of a signal peptide and an N-terminal peptide (KDNTT). The plasmid was verified by sequencing using the following primers: GTGGAGCATTGTGAGGTCACAG and GAGCTTGCCGGTGAGTGCAGATG.

**Transgenic mice**

Acr-GCaMP3-mCherry transgenic mice were generated by microinjection of gel-purified linearized DNA into a pronucleus of 100+ fertilized embryos collected from superovulated mice using standard techniques. Embryos for injection were obtained by mating (C57BL6/J and CBA) F1 hybrids. Transgenic founders were backcrossed to C57BL/6J mice. Primers for genotyping and RT-PCR were GTGGAGCATTGTAGAGTCAG and
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Figure 9. Schematic illustration of hypothesized changes in AcroSensE fluorescence, Ca²⁺ flux, and membrane fusion (MF) events between the plasma membrane overlying the acrosome (APM) and outer acrosomal membrane (OAM) of the sperm head. Prespike foot (PSF)-like events occur as a result of transitory membrane fusion, either before or leading to more stable—but still small—fusion pores (FPs). These small focal fusion events enable Ca²⁺ influx into the acrosome. Full-membrane fusion leading to the loss of mCherry signal and the loss of acrosomal contents occurs as a result of coalescence of FPs. In this model, the dimensions of the FPs initially allow only for influx of small molecules into the acrosome’s lumen, including extracellular Ca²⁺ ions that can bind to the GCaMP3, resulting in an increase in the “green” fluorescence intensity (ACR). Progression of the FP into full-membrane fusion events results in much larger openings resulting from the loss of hybrid APM/OAM membrane vesicles, allowing the AcroSensE protein to diffuse out of the acrosome lumen into the extracellular space, resulting in a decrease in both the “red” and “green” fluorescence intensities. ACR, acrosomal Ca²⁺ rise; APM, acrosomal plasma membrane; OAM, outer acrosomal membrane.

GAGCTTGCCGTTGTGCAGATG. Immunoblotting was performed on tissues collected from the AcroSensE mice including the brain, heart, muscle, testis, and liver. Protein was quantified, and equal amounts of protein were loaded for SDS-PAGE. Blots were blocked with 10% milk overnight at 4 °C. The primary rabbit GFP antibody (Abcam) was diluted 1:2500 in Tween Tris buffered saline to 2.5 μg/μL and incubated for 2 h at room temperature. Anti-rabbit IgG linked to horse radish peroxidase was used as a secondary antibody (Species-Specific F(ab')2 fragment from donkey; GE Healthcare) and was diluted 1:3000 in Tween Tris buffered saline and incubated at 4 °C overnight.

Live sperm Ca²⁺ imaging

For murine sperm, a modified Whitten’s medium (MW; 22 mM Hepes, 1.2 mM MgCl₂, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemi-Ca²⁺ salt, pH 7.35; (67)) was used for all incubations. Glucose (5.5 mM), NaHCO₃ (10 mM), and CD (3 mM) were supplemented as needed. Cauda epididymal murine sperm collection was performed at 37 °C overnight.

For microscopy, sperm were plated on poly-D-lysine–coated 35-mm coverslip dishes (MatTek Corp), and 3 ml of warm MW base media supplemented with 10 mM CaCl₂ was added. The dish with the sperm was then mounted on a Zeiss 510 microscope with a heated 100X oil immersion objective (37 °C). GCaMP3 was excited using the 488-nm line of a krypton/argon laser and viewed with a 505– to 550-nm BP filter. The mCherry was excited using the 555-nm line of a HeNe laser and viewed with a 575– to 615-nm BP filter. Selected cells were approached with a ~5-μm-diameter pulled-glass capillary, prefilled with stimulating solution, and positioned within 100 μm from the sperm head. The stimulating solutions were made at 5X the normal concentration (e.g., G_M1 = 125 μM) to compensate for diffusion in the volume/spaces between the capillary and the cell. Sperm cells were imaged at 2 to 4 Hz while applying a puff of 10 s/5 psi from the pipette, controlled by a Picospritzer III (FMI Medical Instruments). Unless otherwise stated, all data are presented as mean ± SEM. For each of the experiments, the number of cells analyzed (n) is presented in the text or legend. Data were processed and plotted using Origin 8 (OriginLab) and Excel (Microsoft). Statistical comparisons (Student’s t test or χ²) were performed using Excel or JMP Statistical Software and are provided in Figs. S2–S7.

Image analysis

Offline image analysis was conducted using Zeiss LSM image analysis software and ImageJ (National Institutes of Health). Changes in fluorescence (F) were normalized by the initial fluorescence (F₀) and are expressed as ΔF.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supporting information—This article contains supporting information.

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S. S. Z. data acquisition and interpretation; C. M., J. L. N., and A. J. T. discussions; D. M. S. critical discussion and manuscript preparation; A. J. T. interpretation of results; A. J. T. manuscript preparation.

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Conflict of interest—A. J. T. discloses that he is a founder and an officer of Androvia LifeSciences, LLC, a biotechnology company investigating solutions for male infertility, in which he holds a minor equity stake. There are no conflicts with the current work. All other coauthors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ACR, acrosomal Ca2+ rise; AcroSensE, Acrosome-targeted Sensor for Exocytosis; AE, acrosome exocytosis; APM, acrosomal plasma membrane; CD, 2-hydroxypropyl-β-cyclodextrin; FP, fusion pore; MF, membrane fusion; OAM, outer acrosomal membrane; PSF, prespike foot; RNE, redundant nuclear envelope; SERCA, sarcoplasmic/endoplasmic reticulum Ca2+-ATPase; SPCA, secretory pathway Ca2+-ATPase; STIM, stromal interaction molecule; ZP, zona pellucida.

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