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Ca^{2+} Entry through Store-operated Channels in Mouse Sperm Is Initiated by Egg ZP3 and Drives the Acrosome Reaction

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Fertilization occurs after the completion of the sperm acrosome reaction, a secretory event that is triggered during gamete adhesion. ZP3, an egg zona pellucida glycoprotein, produces a sustained increase of the internal Ca^{2+} concentration in mouse sperm, leading to acrosome reactions. Here we show that the sustained Ca^{2+} concentration increase is due to the persistent activation of a Ca^{2+} influx mechanism during the late stages of ZP3 signal transduction. These cells also possess a Ca^{2+} store depletion–activated Ca^{2+} entry pathway that is open after treatment with thapsigargin. Thapsigargin and ZP3 activate the same Ca^{2+} permeation mechanism, as demonstrated by fluorescence quenching experiments and by channel antagonists. These studies show that ZP3 generates a sustained Ca^{2+} influx through a store depletion–operated pathway and that this drives the exocytotic acrosome reaction.

INTRODUCTION

Alterations of intracellular Ca^{2+} activity (Ca^{2+}_i) mediate information transmission from many extracellular signals to internal response systems. The activation of membrane receptors can stimulate Ca^{2+} transport through a variety of pathways, including mobilization from internal stores through inositol trisphosphate (IP3) receptor or ryanodine receptor channels or by influx through plasma membrane channels. The mechanisms that provide for spatial and temporal regulation of Ca^{2+}_i are essential for the fine control of cellular function (Berridge, 1997).

This is well illustrated in sperm, in which Ca^{2+}_i is an essential mediator of the acrosome reaction, a secretory event that is completed by sperm of many animal species at an early stage of fertilization (Darszon *et al.*, 1999; Florman *et al.*, 1999). In mammals, secretion is triggered during gamete contact by ZP3, a glycoprotein component of the egg's extracellular matrix, or zona pellucida (ZP). Sperm penetration of the ZP and the later events of fertilization require the prior completion of acrosome reactions (Wassarman, 1999).

Efforts to determine the mechanisms by which Ca^{2+}_i is regulated by ZP3 redoubled with the recognition that inhibition of Ca^{2+}_i responses arrests gamete interaction in vitro (Florman *et al.*, 1989, 1992); these mechanisms may account for the reported human contraceptive effects of certain Ca^{2+} channel antagonists (Benoff *et al.*, 1994; Hershlag *et al.*, 1995). Two phases of ZP3-evoked Ca^{2+}_i responses were resolved with the use of ion-selective fluorescent probes. First, a transient Ca^{2+}_i increase occurred during the first seconds of ZP3 signaling. Ca^{2+}_i increased within 40–50 msec to ~10 μM and subsequently relaxed to resting values within the next 200 msec (Arnoult *et al.*, 1999). The transient response mechanism had the anticipated characteristics of a low-voltage-activated (LVA) T-type Ca^{2+} channel, including a similar time course of activation and inhibitor sensitivity (Arnoult *et al.*, 1999). LVA Ca^{2+} channel genes are expressed during rodent spermatogenesis (Espinosa *et al.*, 1999), and the associated currents were detected by whole cell patch clamp methods (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996a, 1997, 1998; Liévano *et al.*, 1996; Santi *et al.*, 1996). Finally, channel activation was associated with acrosome reactions (Arnoult *et al.*, 1996a). It is thus likely that transient Ca^{2+}_i responses are mediated by ZP3-dependent Ca^{2+} influx through LVA channels.

The transient response was followed by a second phase in which increased Ca^{2+}_i levels were sustained for the duration of ZP3 stimulation. The sustained Ca^{2+}_i response developed slowly, requiring many seconds to several minutes to reach

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maximal levels (Florman *et al.*, 1989; Lee and Storey, 1989; Bailey and Storey, 1994; Florman, 1994; Arnoult *et al.*, 1996a,b; Shirakawa and Miyazaki, 1999), and acrosome reactions occurred after a plateau was established. The sustained response was inhibited by the addition of LVA Ca^{2+} channel antagonists before ZP3. However, both the slow activation time course of the sustained $Ca^{2+}{}_i$ phase and its failure to inactivate during prolonged ZP3 stimulation were inconsistent with a direct role of LVA channels (Hille, 1992). It was instead proposed that the transient phase of Ca^{2+} entry through LVA channels was an obligatory early event leading to downstream generation of sustained $Ca^{2+}{}_i$ responses through a second mechanism (Walensky and Snyder, 1995; Arnoult *et al.*, 1999).

Given the central role of $Ca^{2+}{}_i$ responses in fertilization, we examined the sustained response phase in greater detail. We report that the sustained phase is mediated by Ca^{2+} entry through Ca^{2+} store depletion–activated channels. These channels are present in a wide range of cells, where they function both to refill internal Ca^{2+} stores and to participate in signal transduction processes (Berridge, 1995, 1997; Parekh and Penner, 1997). Moreover, these channels are present in mammalian sperm (Blackmore, 1993; Santi *et al.*, 1998), although their physiological role has not been determined. We have found that the store-operated Ca^{2+} channels of sperm remain functional after capacitation and play an essential role by driving the acrosome reaction during fertilization.

MATERIALS AND METHODS

Biological Preparations

Spermatogenic cells and sperm were obtained from CD-1 mice (12–16 wk old; Charles River Laboratories, Wilmington, MA) by manual trituration of testicular slices and from caudae epididymides, respectively. Sperm were capacitated in vitro, as described previously (Arnoult *et al.*, 1996a). Acrosome reactions were assessed during $Ca^{2+}{}_i$ determinations with the use of differential interference contrast optics, in which acrosome-intact cells exhibit a prominent ridge in the equatorial region of the sperm head and acrosome-reacted sperm lack this ridge (Bleil and Wassarman, 1986). These results were confirmed at the end of sperm incubations with the use of a Coomassie blue staining method (Thaler and Cardullo, 1995). The fraction of motile sperm was assessed before capacitation by visual examination, and preparations with <70% motility were discarded.

Initial observations were carried out with the use of ZP extracts (40 μg/ml) that contained ZP3 and were confirmed with the use of purified ZP3 preparations. The responses to these two agonist preparations were indistinguishable, consistent with previous observations that ZP3 accounts for all of the agonist activity in the ZP (Bleil and Wassarman, 1983). Consequently, results obtained with these two agonist preparations are pooled for presentation.

ZPs were obtained from germinal vesicle-intact follicular oocytes and from two-cell embryos after fertilization in vivo. Soluble extracts were prepared by heating (60°C, 60 min). ZP2 and ZP3 were resolved by SDS-PAGE and isolated by electrophoretic elution from gel slices (Bleil and Wassarman, 1983; Arnoult *et al.*, 1996b).

Electrophysiological Methods

Ca^{2+} currents were recorded from spermatogenic cells with the use of the whole cell configuration of the patch clamp method, exactly as described (Arnoult *et al.*, 1996a). Peak LVA Ca^{2+} currents were measured during 100 msec of depolarization from holding potential (−90 mV) to a test potential of −30 mV. Currents were recorded with the use of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Data were sampled at 10 kHz, filtered at 3 kHz, corrected for leak and capacitance currents, and analyzed with Biopatch (Biologic, Grenoble, France).

$Ca^{2+}{}_i$ Determinations

Sperm were immobilized on Cell-Tak–coated glass coverslips, transferred to a 0.1% BSA medium, and passively loaded with dye during a 15-min incubation with 1 μM fura 2-AM and a further 15-min incubation to permit probe deesterification, exactly as described previously (Arnoult *et al.* 1996a,b). Intracellular BAPTA was loaded during similar incubations with 1 μM BAPTA-AM (*N*,*N*′-[1,2-ethanediylbis(oxy-2,1-phenylene)] bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]] bis[(acetyloxy)-methyl]ester).

Extracellular dye was removed by superfusion, and coverslips were mounted on a heated microscope stage. Excitation illumination from a 75-W Xe arc lamp was directed through a 60× PlanApo objective (numerical aperture 1.3), fluorescent images were digitized with the use of a GenIVSys-intensified Dage 72 charge-coupled device camera (Dage MTI, Michigan City, IN), and data were analyzed with Axon Workbench software (Axon Instruments). Excitation wavelength pairs of 340 and 380 nm were used for $Ca^{2+}{}_i$ determination, whereas 360-nm illumination was used for Mn^{2+}-quenching studies. $Ca^{2+}{}_i$ values were determined with the use of the algorithm of Grynkiewicz and coworkers (1985), assuming a dissociation constant for intracellular fura 2:Ca^{2+} complexes of 225 nM. Readers are cautioned that this value has not yet been confirmed specifically in sperm.

The small cytosolic volumes of sperm, and the associated low-fluorescence signals, necessitated integration of dye signals for 1–4 s in typical imaging experiments. Temporal resolution of this system, based on Nyquist's criterion (Inoue and Spring, 1997), was 2–8 s. The transient phase of the $Ca^{2+}{}_i$ response, which activated and inactivated within ~250 msec (Arnoult *et al.*, 1999), is not resolved here.

Chemicals and Reagents

AN1043 was the generous gift of Athena Neurosciences (South San Francisco, CA). Conotoxins and agatoxins were provided by Dr. Jose Lemos (University of Massachusetts). Other reagents were obtained from the following sources: nifedipine (Calbiochem, La Jolla, CA); thapsigargin, ionomycin, A-23187, fura 2-AM, BAPTA, and BAPTA-AM (Molecular Probes, Eugene, OR); Cell-Tak (Becton-Dickinson, Franklin Lakes, NJ); all other chemicals and reagents (Sigma, St. Louis, MO).

Statistical Analysis

All data were analyzed by Student's *t* test.

RESULTS

ZP3 Evokes a Sustained $Ca^{2+}{}_i$ Increase

$Ca^{2+}{}_i$ in mouse sperm that were incubated under capacitating conditions was 159 ± 21 nM (mean ± SD; range, 133–193 nM; n = 177), as reported with fura 2. ZP3 (10 μg/ml, ~120 nM) increased this value to 396 ± 24 nM (range, 334–488 nM) in 69% (122 of 177) of the cells (Figure 1A). The remaining cells exhibited $Ca^{2+}{}_i$ alterations of only ±20 nM. The presence of a nonresponsive group is consistent with other observations that only 40–80% of mouse sperm respond to ZP3 and may reflect inefficient sperm capacitation in vitro (Arnoult *et al.*, 1999).

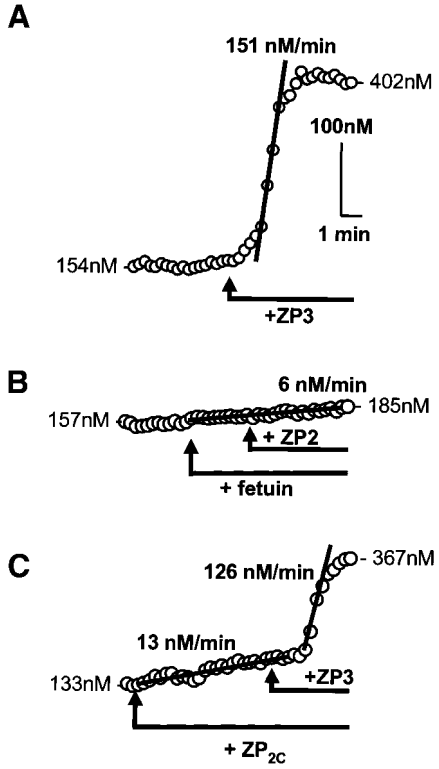

Figure 1. ZP3 produces a sustained $Ca^{2+}{}_i$ increase in capacitated mouse sperm. (A) Resting $Ca^{2+}{}_i$ values were determined before the addition of ZP3 (10 μg/ml). Similar results were obtained with ZP extracts containing ZP3 (40 μg/ml ZP; our unpublished results). Maximal $Ca^{2+}{}_i$ response rates ($\Delta Ca^{2+}{}_i/\Delta t$) were determined from linear fits of the slopes of time courses (straight line) and corrected for agonist-independent rates. Rate values and the initial and final calculated $Ca^{2+}{}_i$ values are displayed here and in subsequent figures. (B) Control glycoproteins (10 μg/ml fetuin and mouse ZP2) failed to initiate robust $Ca^{2+}{}_i$ responses. (C) Robust $Ca^{2+}{}_i$ responses were initiated by ZP3 from oocytes but not by ZP from two-cell embryos (ZP_{2C}; 10 μg/ml). Data are representative of 122 cells treated with ZP3 and 9–34 cells treated with control glycoproteins.

The salient characteristics of this $Ca^{2+}{}_i$ response are: 1) a delay of 1.3 ± 0.4 min (range, 0.5–2.8 min) before detectable $Ca^{2+}{}_i$ increases; 2) an increase from 10 to 90% of peak values in 1.9 ± 0.4 min (range, 0.8–3.4 min), with a maximal rate of 176 ± 29 nM/min; and 3) $Ca^{2+}{}_i$ values either remain increased or decline slowly (<10 nM/min) in the presence of ZP3. This represents the sustained $Ca^{2+}{}_i$ phase of ZP3 signaling. The earlier, transient $Ca^{2+}{}_i$ response mediated by LVA channels (Arnoult *et al.*, 1999) was not resolved here.

Acrosome reactions occurred in 89% (109 of 122) of sperm exhibiting sustained $Ca^{2+}{}_i$ responses and were detected at 9.2 ± 4.1 min (range, 5.7–24.4 min) after addition of ZP3. A small fraction of sperm (13 of 122, 11%) generated sustained $Ca^{2+}{}_i$ increases yet failed to acrosome react, as was observed previously (Arnoult *et al.*, 1996a,b). This may reflect inefficient sperm capacitation, with ZP3 signal transduction blocked at a site downstream of the sustained $Ca^{2+}{}_i$ response (Yanagimachi, 1994; Florman *et al.*, 1999). In contrast, acrosome reactions were detected in only 8% (4 of 55) of those sperm in which sustained $Ca^{2+}{}_i$ increases were not detected. In control experiments, sperm were treated with 10 μg/ml solutions of three different glycoproteins that fail to initiate acrosome reactions: fetuin, oocyte ZP2, and ZP glycoproteins from two-cell embryos (Bleil and Wassarman, 1983). These glycoproteins generated linear $Ca^{2+}{}_i$ increases of 8 ± 5 nM/min (Figure 1, B and C) but failed to trigger the sustained responses that were produced by oocyte ZP3. These observations, like previous studies that used antagonists of ZP3 signal transduction (Florman *et al.*, 1999), demonstrated that the sustained $Ca^{2+}{}_i$ response was required for acrosome reactions.

Role of Intracellular Ca^{2+} Pools in ZP3 Signal Transduction

It was proposed that ZP3 promotes Ca^{2+} efflux from an intracellular pool during the initiation of acrosome reactions (Walensky and Snyder, 1995). To determine whether the mobilization of Ca^{2+} from intracellular stores was sufficient to initiate exocytosis, we examined the effects of Ca^{2+} ionophores on acrosome reactions. Ionomycin (5 μM) increased the occurrence of acrosome reactions from 23 ± 5% to 52 ± 7% ($p < 0.001$) during 60-min incubations in a 1.7 mM Ca^{2+} medium. However, when ionomycin treatments were carried out in a medium containing 5 mM extracellular BAPTA ($BAPTA_o$; calculated $Ca^{2+}{}_o < 30$ nM), acrosome reactions increased only from 19 ± 4% in untreated populations to 25 ± 6% ($n = 3$; $p > 0.1$). Similar results were obtained with a second Ca^{2+} ionophore, A23187 (our unpublished results). These agents readily release Ca^{2+} from intracellular stores, including those of sperm (Babcock *et al.*, 1976). These observations demonstrate that such Ca^{2+} release from intracellular stores is not sufficient to drive acrosome reactions.

ZP3-activated Ca^{2+} Influx Mechanisms in Sperm

These results suggest that Ca^{2+} influx may play an essential role in the generation of ZP3-dependent $Ca^{2+}{}_i$ increases and acrosome reactions. This hypothesis was examined in experiments in $Ca^{2+}{}_o$-depleted medium. The reduction of $Ca^{2+}{}_o$ to <30 nM by $BATPA_o$ before the addition of ZP3 inhibited the sustained $Ca^{2+}{}_i$ response in 100% (18 of 18) of sperm that were examined (Figure 2A).

To determine whether $Ca^{2+}{}_o$ was required specifically during the sustained response or acted indirectly by inhibiting the earlier, transient Ca^{2+} entry through LVA channels, we took advantage of the distinctive time courses of the transient and sustained responses: the sustained response occurred after a delay of ≥0.5 min, whereas the transient phase was completed within several seconds. Consequently, Ca^{2+} entry in the sustained response phase can be examined selectively by the application of $Ca^{2+}{}_o$ chelators 15 s after the addition of ZP3, when the transient phase response has been completed. A similar protocol was used previously to determine the role of Ca^{2+} channels in the echinoderm acrosome reaction (Guerrero and Darszon, 1989).

When $BAPTA_o$ was added 15 s after ZP3, sustained $Ca^{2+}{}_i$ increases were resolved in 13% (6 of 47) of sperm. Compared with parallel incubations in the absence of BAPTA, in which 69% of sperm exhibited a sustained $Ca^{2+}{}_i$ response (Figure 1A), this represents an 81% inhibition. The remaining sperm exhibited either increases of <25 nM $Ca^{2+}{}_i$ (30 of 47, 64%;

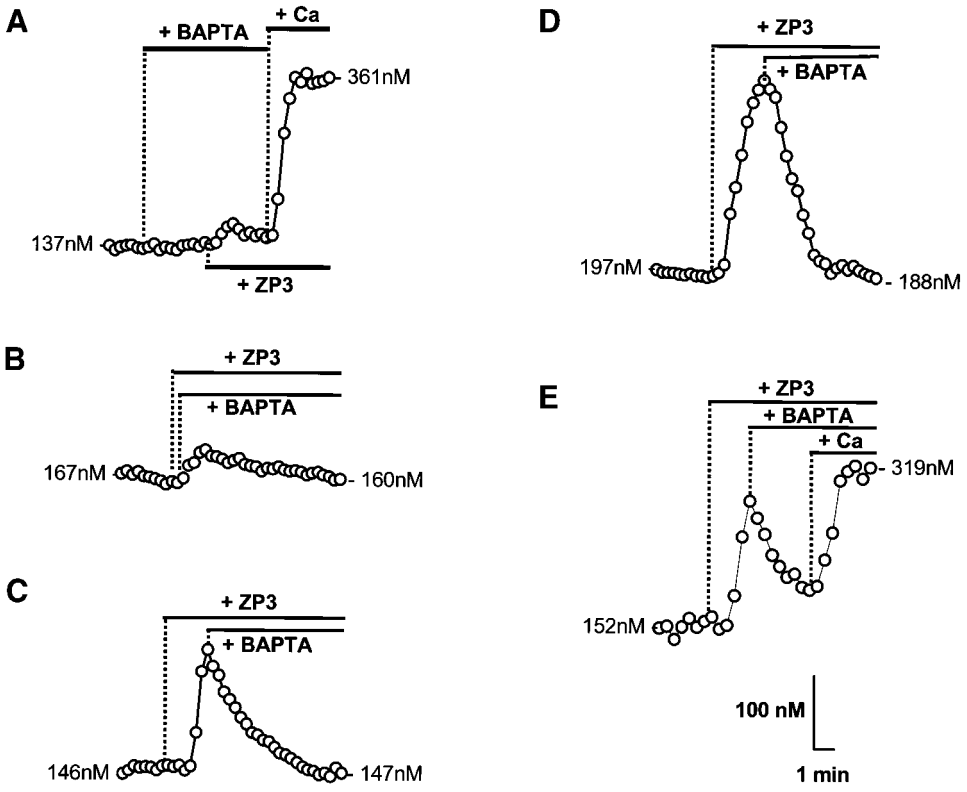

Figure 2. Sustained $Ca^{2+}{}_i$ increases after ZP3 treatment require $Ca^{2+}{}_o$. Sperm were loaded with fura 2 and incubated in a 1.7 mM Ca^{2+} medium. BAPTA and Ca^{2+} (5 mM) additions served to alternate $Ca^{2+}{}_o$ between >30 nM and 1.7 mM, respectively. ZP3 (10 μg/ml) was added as indicated. Traces are representative of 13–47 cells in these treatment groups. Similar results were obtained with the use of ZP extracts.

Figure 2B) or had no detectable response (11 of 47, 23%). In a second series of experiments, sustained responses were initiated during a 2- to 3-min treatment with ZP3 before the addition of $BAPTA_o$. Chelation of $Ca^{2+}{}_o$ curtailed these responses, with $Ca^{2+}{}_i$ levels returning to basal values in 79% (19 of 24) of sperm that had initiated sustained responses (Figure 2, C and D). Such effects were reversible, and sustained $Ca^{2+}{}_i$ responses were restored in 85% (11 of 13) of sperm after the readdition of $Ca^{2+}{}_o$ in the persistent presence of ZP3 (Figure 2E). Thus, sustained $Ca^{2+}{}_i$ responses required the continual presence of $Ca^{2+}{}_o$.

We then measured Mn^{2+} influx during the sustained phase of ZP3 signaling by means of fura 2 quenching (Grynkiewicz *et al.*, 1985). Mn^{2+} is conducted through many Ca^{2+} transport pathways and so provides an indirect indication of Ca^{2+} permeation (Merritt *et al.*, 1989). Mn^{2+} entry is detected with the use of the Ca^{2+}-independent emission of fura 2 (excitation wavelength, 360 nm); hence, these signals were not complicated by Ca^{2+} release from intracellular stores.

ZP3 did not alter the Ca^{2+}-independent fluorescence of fura 2 in the absence of $Mn^{2+}{}_o$ (Figure 3A, upper trace), indicating that dye emission was not otherwise quenched during stimulation. However, rapid quenching occurred when Mn^{2+} was added between 0.25 and 3 min after ZP3 (Figure 3A), but not when sperm were treated with either ZP2 (Figure 3B) or fetuin (our unpublished results). These results were consistent with those of other experiments in which ZP3 failed to produce a sustained increase of $Ca^{2+}{}_i$ in the presence of 100 μM La^{3+}, a nonselective Ca^{2+} entry blocker (our unpublished results), and suggest the activation of a persistent Ca^{2+} influx mechanism.

Ni^{2+} blocks LVA Ca^{2+} channels (Hille, 1992; Arnoult *et al.*, 1996a) and permitted us to determine the relationship between the persistent influx pathway and the LVA Ca^{2+} channel that was activated during the first seconds of ZP3 signaling (Arnoult *et al.*, 1999). Ni^{2+} was added to sperm either 1 min before ZP3 or 15 s after ZP3. As shown in Figure 4, Ni^{2+} inhibited the sustained $Ca^{2+}{}_i$ increase with an IC_{50} of 26 ± 5 μM (○) when added before ZP3, an efficacy similar to that with which it inhibited the LVA Ca^{2+} currents of mouse spermatogenic cells, as assessed in whole cell patch clamp studies (IC_{50} = 34 μM; Arnoult *et al.*, 1996a). In contrast, this efficacy was reduced by 18-fold when Ni^{2+} was added 15 s after ZP3 (IC_{50} = 481 ± 21 μM; Figure 4, ●).

Antagonists were also used to determine whether the late phase of Ca^{2+} entry was mediated by other voltage-sensitive Ca^{2+} channels. The rate of $Ca^{2+}{}_i$ increase and the maximal $Ca^{2+}{}_i$ value attained during the sustained response phase were inhibited by <10% when blockers of N-type channels (1 μM ω-conotoxin GVIA) or P/Q-type channels (200 nM agatoxin IVA) were added either 2 min before ZP3 or 15 s after ZP3. Inhibitors of L-type Ca^{2+} channels (1 μM PN200-110, 50 μM verapamil) also inhibited the sustained phase of Ca^{2+} entry by <10% when added 15 s after ZP3. However, when these latter agents were added 2 min before ZP3, they inhibited the sustained phase of Ca^{2+} entry by >50%. This can be attributed to their ability to block permeation through LVA T-type channels (Arnoult *et al.*, 1996a, 1998; Liévano *et al.*, 1996; Santi *et al.*, 1996). These results, together with the

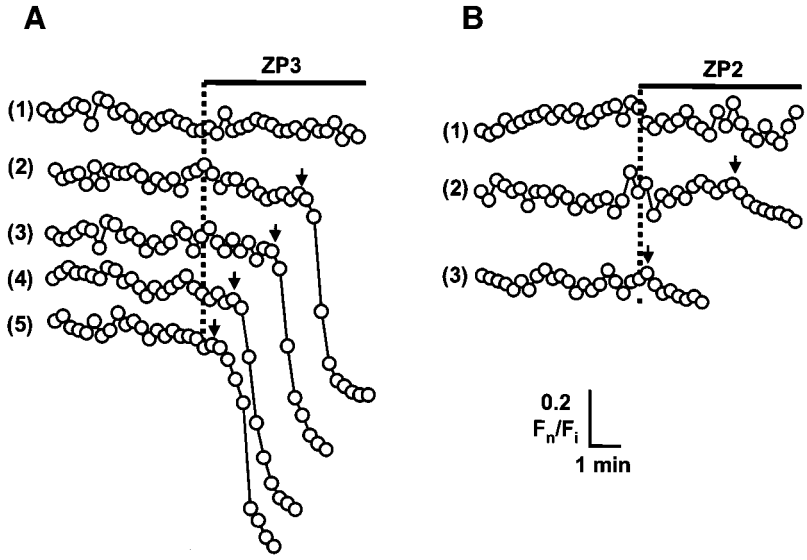

Figure 3. Mn^{2+} influx is activated by ZP3 but not by ZP2. Sperm were loaded with fura 2 and incubated in a Ca^{2+}-depleted medium. Mn^{2+} influx was monitored by the quenching of fura 2 fluorescence (excitation wavelength, 360 nm). ZP3 or ZP2 (10 μg/ml) was added as indicated (bars). Times of 10 μM Mn^{2+} addition are indicated (arrows in traces A2–A5 and B2 and B3). Relative fluorescence emission data (F_n/F_i) are presented, where F_n and F_i are emission at *n* min and at initial time, respectively. (A) ZP3 produces a sustained Mn^{2+} influx. Similar results were obtained with the use of ZP extracts containing ZP3 (40 μg/ml). (B) ZP2 does not activate a Mn^{2+} influx pathway. Traces are from 8 different cells and are typical of responses in 11 cells treated with buffer (traces A1 and B1), 37 cells treated with ZP3 or ZP solutions (traces A2–A5), and 21 cells treated with ZP2 (traces B2 and B3).

time course study results described in Figure 2, suggest that the late phase of Ca^{2+} uptake by sperm, which is required for the sustained $Ca^{2+}{}_i$ response to ZP3, is through a pathway that is different from both the LVA channel and other types of voltage-sensitive Ca^{2+} channels.

Store-operated Ca^{2+} Influx Mechanisms in Capacitated Sperm

Mammalian sperm must complete a process of functional maturation, or capacitation, to fertilize eggs (Yanagimachi, 1994). A $Ca^{2+}{}_i$ regulatory mechanism with the anticipated characteristics of a store-operated channel has been detected in uncapacitated sperm (Blackmore, 1993; Santi *et al.*, 1998). However, it is not known whether this permeation mechanism remains active after capacitation. A series of experiments was carried out to characterize this pathway in capacitated sperm.

Ca^{2+} influx into stores is mediated by the sarcoplasmic/endoplasmic reticular Ca^{2+} ATPase, whereas efflux of sequestered Ca^{2+} occurred through Ca^{2+}-release channels or

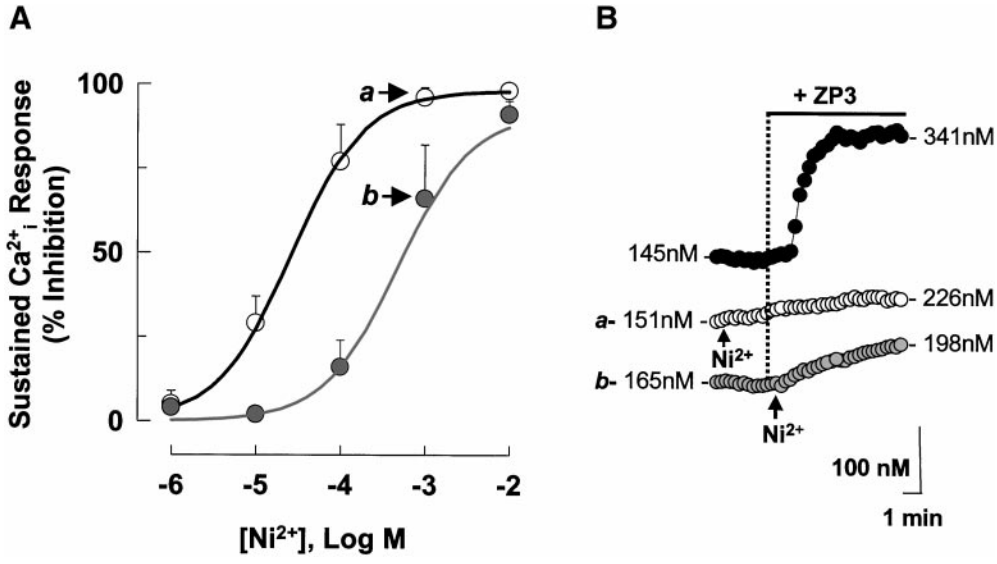

Figure 4. Ni^{2+} inhibits $Ca^{2+}{}_i$ responses to ZP3. Ni^{2+} was added either 2 min before (○) or 15 s after ZP3 (◍). Response rates ($\Delta Ca^{2+}{}_i/\Delta t$) were determined as described (Figure 1). (A) Data are means ± SD of observations on 7–22 cells and are fit to the expression $I = (100 \times IC_{50})/(IC_{50} + C)$, where I is the inhibition of response rates in the presence Ni^{2+} and C and IC_{50} are the specific Ni^{2+} concentration and the concentration that inhibits $Ca^{2+}{}_i$ response by 50%, respectively. Calculated values of IC_{50} were 26 μM for Ni^{2+} before ZP3 (black line) and 481 μM for Ni^{2+} after ZP3 (gray line). Letters (a and b) designate points that are illustrated by traces in B. (B) Montage of traces showing sperm treatment with 10 μg/ml ZP3 (●), with 1 mM Ni^{2+} added 15 s after ZP3 (◍), or with Ni^{2+} added 2 min before ZP3 (○).

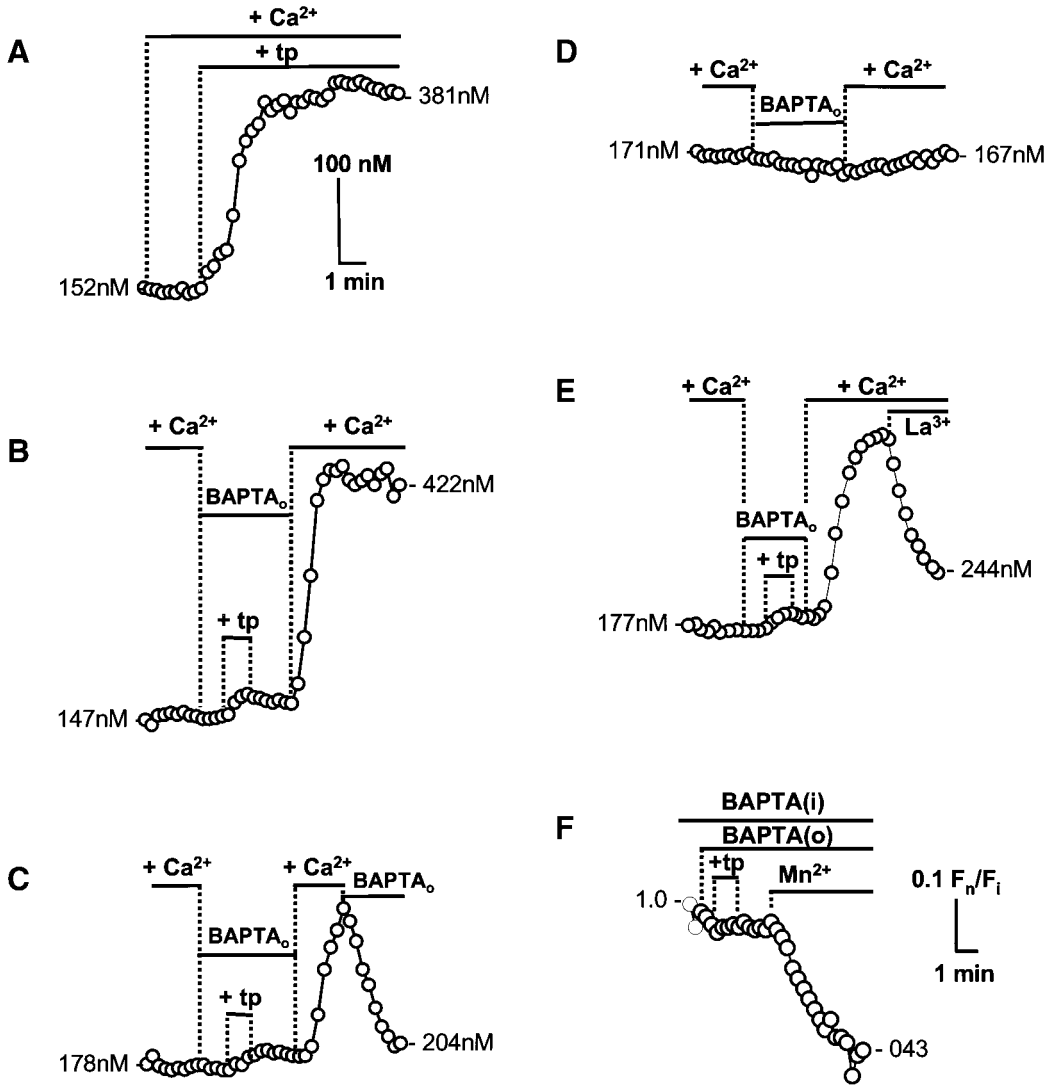

Figure 5. Thapsigargin activates Ca^{2+} influx. Fura 2–loaded sperm were treated with 10 μM thapsigargin (tp) in a <30 nM Ca^{2+} medium, and 1.7 mM Ca^{2+}_o was added. (A–E) Sustained increases of Ca^{2+}_i were observed in the presence of Ca^{2+}_o and were curtailed by 5 mM $BAPTA_o$ or 100 μM La^{3+}. (F) Sperm were incubated in <30 nM Ca^{2+}_o containing 10 μM Mn^{2+}_o. Thapsigargin activated Mn^{2+} influx in fura 2/$BAPTA_i$-loaded sperm, resulting in quenching of fura 2 fluorescence. Fluorescence quenching data are expressed as the value F_n/F_i, as described in Figure 3. Representative traces were chosen from the following numbers of cells: (A) 71, (B) 49, (C) 44, (D) 19, (E) 17, and (F) 9.

by leak pathways. To determine whether store-operated channels were present, sperm were treated with thapsigargin, a sesquiterpene lactone that irreversibly inhibits the sarcoplasmic/endoplasmic reticular Ca^{2+} ATPase system (Thastrup *et al.*, 1989) and results in a gradual efflux of Ca^{2+} through leak pathways (Berridge, 1995; Parekh and Penner, 1997).

Thapsigargin (10 μM) produced an increase in Ca^{2+}_i of capacitated sperm incubated in a Ca^{2+}_o medium (Figure 5A). The peak Ca^{2+}_i response was 411 ± 36 nM (range, 328–466 nM) and was detected in 87% (62 of 71) of cells observed. Responses of similar magnitude (396 ± 22 nM; range, 341–455 nM) were produced by 1 μM thapsigargin, although these occurred in a smaller fraction of cells (59%, 17 of 29 sperm) and with a highly variable delay between drug application and Ca^{2+}_i increase (1.9–7.4 min). In contrast, 0.1 μM thapsigargin had no detectable effects on sperm Ca^{2+}_i. The efficacy of thapsigargin here (IC_{50} = 0.77 μM) is lower than that typically observed in somatic cells but is similar to values reported in gametes (rat sperm, 0.5 μM [Walensky and Snyder, 1995]; mouse eggs, >1 μM [Kline and Kline, 1992]).

The sustained Ca^{2+}_i increase produced by thapsigargin was dependent on Ca^{2+}_o (Figure 5B). Treatment of sperm with this drug (10 μM) in a 5 mM $BAPTA_o$ medium (Ca^{2+}_o < 30 nM) produced only a small, transient Ca^{2+}_i increase; in 92% (45 of 49) of sperm, Ca^{2+}_i values increased by 16 ± 7 nM and returned to basal levels in 1.4 ± 0.5 min (ranges, 6–26

nM and 0.4–2.2 min, respectively; Figure 5, B and C). Sustained increases were not produced until 1.7 mM $Ca^{2+}{}_o$ was restored (Figure 5B) and required the continued presence of $Ca^{2+}{}_o$ (Figure 5C). Control experiments demonstrated that sustained $Ca^{2+}{}_i$ responses were not an indirect effect of $Ca^{2+}{}_o$ chelation but rather required the presence of thapsigargin (Figure 5D). To determine whether the regulation of $Ca^{2+}{}_i$ by $Ca^{2+}{}_o$ was due to ion influx, a series of experiments was carried out with Ca^{2+} entry antagonists. Addition of either 100 μM La^{3+} (Figure 5E) or 1 mM Ni^{2+} (our unpublished results) curtailed the sustained increase of $Ca^{2+}{}_i$ that was produced by thapsigargin.

Intracellular BAPTA ($BAPTA_i$) was next used to determine whether the Ca^{2+} entry that was evoked by thapsigargin depended on $Ca^{2+}{}_i$. influx. Sperm were loaded with $BAPTA_i$ and fura 2 in a <30 nM $Ca^{2+}{}_o$ medium (5 mM $BAPTA_o$) containing 10 μM Mn^{2+} and treated with 10 μM thapsigargin, and the activation of a cation permeation pathway was monitored by Mn^{2+}-dependent quenching of fura 2 fluorescence. Under these conditions, the small, transient increases of $Ca^{2+}{}_i$ that were produced by thapsigargin (Figure 5, B, C, and E) were attenuated by $BAPTA_i$ (our unpublished results). Nevertheless, Figure 5F shows that a cation permeation pathway was activated by thapsigargin even in the absence of detectable increases of $Ca^{2+}{}_i$. Similar responses were observed in eight of nine cells. Together, these results suggest that capacitated mouse sperm have a Ca^{2+} influx mechanism that is regulated by an intracellular Ca^{2+} store.

Activation of Store-operated Ca^{2+} Influx into Sperm by ZP3

ZP3 and thapsigargin promote increases of sperm $Ca^{2+}{}_i$ that share a requirement for Ca^{2+} influx and lead to acrosome reactions. To determine if a common mechanism was activated during these two treatment protocols, we first examined whether the responses were additive.

The monitoring of Mn^{2+} influx, and the consequent quenching of fura 2 fluorescence, permitted us to detect the activation of cation entry pathways in the absence of acrosome reactions. As shown in Figure 6A, the leak rate of quenching in a 1 μM $Mn^{2+}{}_o$ medium was 0.018 ± 0.011 $[F_x/F_i] \times min^{-1}$, and this quenching rate was increased by 4.5-fold, to 0.082 ± 0.023 $[F_o/F_i] \times min^{-1}$ ($p < 0.001$; $n = 17$), by the addition of 10 μM thapsigargin. ZP3 (10 μg/ml) also stimulated the quenching rate from leak levels to 0.038 ± 0.014 $[F_o/F_i] \times min^{-1}$ ($p < 0.002$). However, the sequential application of these two agents did not increase the quenching rate above that produced by thapsigargin alone (Figure 6, A and B). These results suggest that thapsigargin and ZP3 activate a similar cation permeation pathway but that thapsigargin produced a maximal stimulation and ZP3 had a submaximal effect. The alternative argument, that unknown processes limit the maximal rate of fluorescence quenching, is unlikely, because greater rates are produced by the Mn^{2+}-transporting ionophore Br-A23187 (0.227 ± 0.031 $[F_o/F_i] \times min^{-1}$; $n = 16$).

This relationship was examined in greater detail by determining the effects of Ni^{2+} on the store-operated mechanism. In these experiments, sperm were treated with 10 μM thapsigargin in a $Ca^{2+}{}_o$-depleted medium to dissipate internal Ca^{2+} stores and thereby activate store-operated Ca^{2+} influx. However, as shown in Figure 6C, the $Ca^{2+}{}_i$ increases that occurred after the readdition of $Ca^{2+}{}_o$, and that are produced through store-operated Ca^{2+} influx in control sperm, were inhibited in a concentration-dependent manner by Ni^{2+} (IC_{50} = 517 ± 19 μM). The inhibitory efficacy is similar to that with which Ni^{2+} inhibits the persistent, ZP3-dependent Ca^{2+} influx mechanism (IC_{50} = 481 ± 21 μM; Figure 4, ◍).

We also examined the effects of 1,4-dihydropyridines on ZP3 signal transduction and on store-operated Ca^{2+} entry. In skeletal muscle of *mdx* mice, AN1043 inhibited a Ca^{2+} entry mechanism that may be a store-operated Ca^{2+} channel but had no detectable effect on the L-type Ca^{2+} channels (Hopf *et al.*, 1996). Figure 7A shows that AN1043 is an effective inhibitor of the store-operated permeation pathway of sperm. In these experiments, the thapsigargin-activated mechanism produced an initial rate of $Ca^{2+}{}_i$ increase of 91 ± 12 nM/min ($n = 31$) in DMSO-treated control cells; this rate was reduced by 81% to 17 ± 8 nM/min ($n = 23$) as a consequence of treatment with 10 μM AN1043 (IC_{50} = 0.7 ± 0.2 μM; Figure 7B). This inhibitory action was rapid, and maximal effects were observed within 10–15 s after drug addition (our unpublished results).

Some 1,4-dihydropyridines inhibit LVA currents (Arnoult *et al.*, 1996a, 1998; Liévano *et al.*, 1996; Santi *et al.*, 1996), and it was necessary to determine whether such an indirect effect of AN1043 might account for its inhibition of sustained $Ca^{2+}{}_i$ responses in sperm. Therefore, we examined the effects of 10 μM AN1043 on the LVA Ca^{2+} currents of spermatogenic cells with the use of the whole cell patch clamp method. A slow, partial inhibition was observed in which LVA currents were inhibited by <20%, and 5 min was required for maximal inhibition (Figure 7C).

AN1043 treatment, therefore, was initiated either 5 min before the addition of ZP3, when it is expected to inhibit both the transient and the sustained $Ca^{2+}{}_i$ regulators, or 15 s after ZP3 addition, in which case it would selectively block the sustained mechanism. Figure 8A shows that acrosome reactions were inhibited by >80% when sperm were treated with AN1043 for 5 min before the addition of ZP3. Similar effects were observed with nifedipine, a related 1,4-dihydropyridine that inhibited spermatogenic cell LVA Ca^{2+} channels (Arnoult *et al.*, 1996a, 1998) but had no effect on the putative store-operated pathway of *mdx* mice (Hopf *et al.*, 1996). In contrast, when drugs were added 15 s after ZP3, AN1043 produced a concentration-dependent inhibition of the acrosome reaction (IC_{50} = 1.1 ± 0.2 μM; Figure 8, A and B), whereas nifedipine had no effect. It is unlikely that AN1043 was acting indirectly at a site downstream of the $Ca^{2+}{}_i$ response, because acrosome reactions are triggered in AN1043-treated sperm by 5 μM ionomycin, the Ca^{2+} ionophore (our unpublished results).

AN1043, when added 15 s after ZP3, also reduced the sustained increase of $Ca^{2+}{}_i$ in a concentration-dependent manner (IC_{50} = 0.8 ± 0.1 μM; Figure 8C). A series of fluorescence quenching experiments was carried out with 1 μM $Mn^{2+}{}_o$ to determine whether the mechanism of dihydropyridine action was through an inhibition of cation influx. The thapsigargin-evoked rate of fluorescence quenching was reduced by 45%, from 0.092 ± 0.020 $[F_o/F_i] \times min^{-1}$ to 0.051 ± 0.017 $[F_o/F_i] \times min^{-1}$, by 1 μM AN1043 ($n = 9$); it was further reduced by 73%, to 0.025 ± 0.009 $[F_o/F_i] \times$

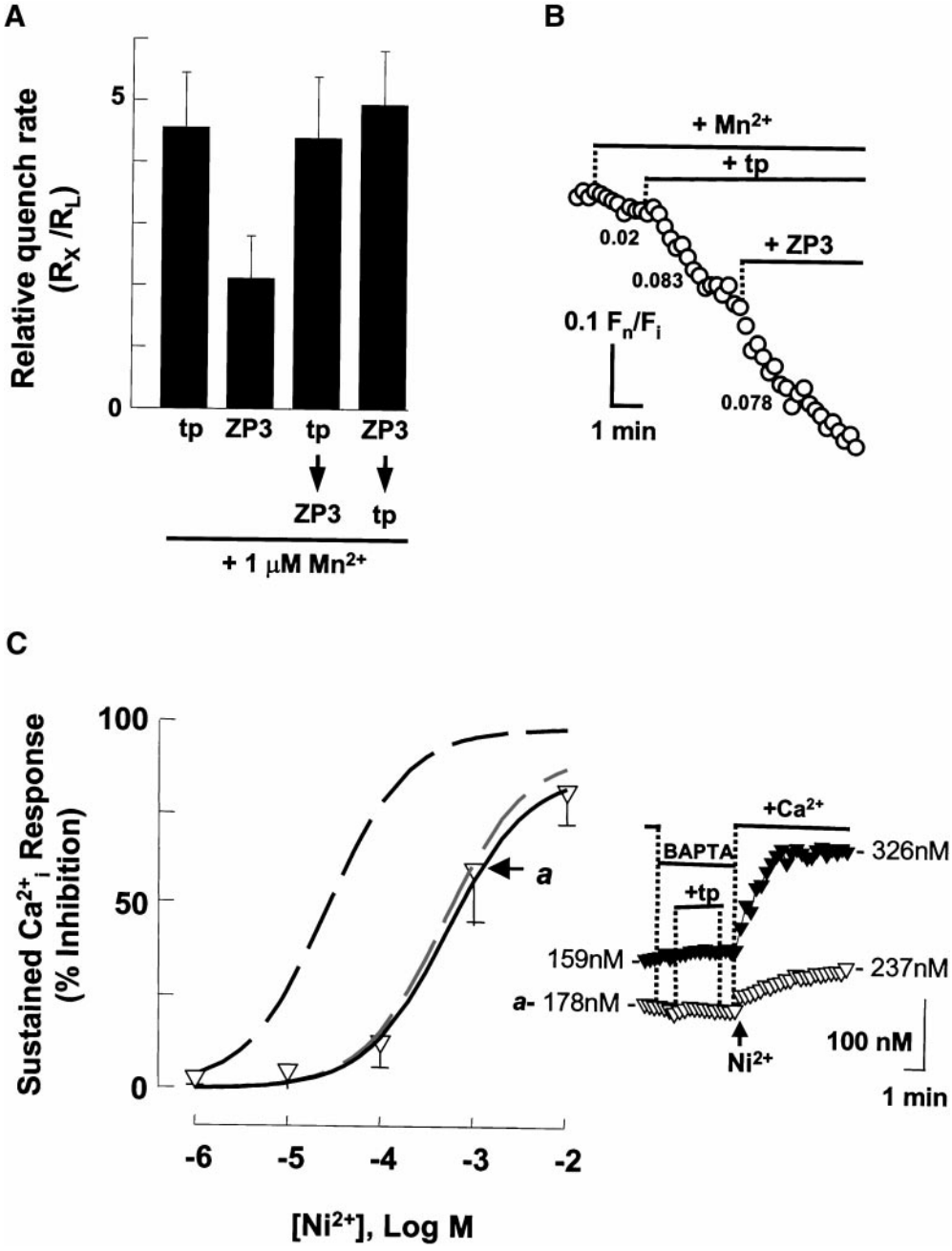

Figure 6. Thapsigargin and ZP3 activate a similar cation permeation pathway in sperm. Fura 2–loaded sperm were incubated in a <30 nM $Ca^{2+}{}_o$ medium, and Ca^{2+}-independent dye fluorescence was monitored as described in Figure 3. Mn^{2+} (1 μM), thapsigargin (tp; 10 μM) and ZP3 (10 μg/ml) were added (dashed lines), and fluorescence quenching rates were determined by the slopes of these traces. (A) Quenching rates ($[F_x/F_i]/[t_x/t_i]$, where F_x and F_i are the fluorescence emission at x min [t_x] and at the initial time [t_i], respectively) were determined for individual sperm during a 2-min incubation in 1 μM Mn^{2+} and designated R_L (leak rate). Quenching rates during experimental treatment (R_X) were determined during a 10-min incubation with thapsigargin or with ZP3 or by the addition of one reagent followed 3 min later by the second reagent. Results are expressed as the relative quench rate (R_X/R_L) and are the mean of observations on 12–39 sperm. (B) A fluorescence trace showing the effects of thapsigargin and ZP3 on Mn^{2+}-dependent quenching. Data from this and other cells were used to construct panel A. (C) $Ni^{2+}{}_o$ inhibits thapsigargin-induced $Ca^{2+}{}_i$ responses. Thapsigargin triggered $Ca^{2+}{}_i$ increases upon addition of $Ca^{2+}{}_o$. Addition of Ni^{2+} 15 s after $Ca^{2+}{}_o$ inhibited this response in a concentration-dependent manner (▽). Data were fit to the expression described in Figure 4, and the derived IC_{50} value was 517 μM. For comparison, fit curves for the inhibition of the early (black dashed line) and late (gray dashed line) phases of ZP3-evoked $Ca^{2+}{}_i$ responses are redrawn from Figure 4A. The letter (a) designates the point that is used in the traces to the right. These traces are from two sperm in which thapsigargin triggered $Ca^{2+}{}_i$ responses in the absence (▼) and in the presence (▽) of 1 μM. $Ni^{2+}{}_o$.

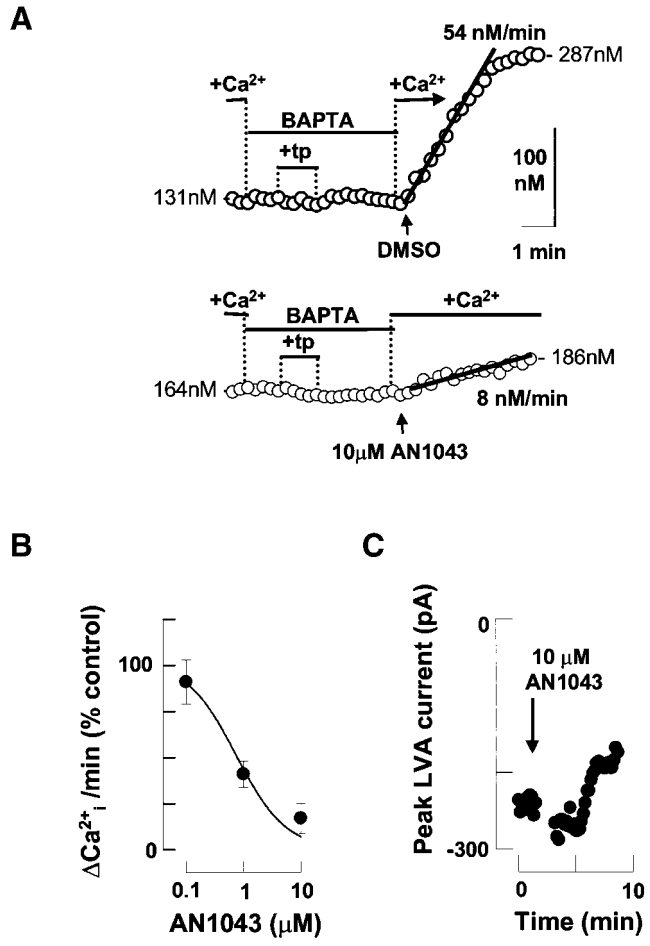

Figure 7. AN1043 inhibits the thapsigargin-dependent $Ca^{2+}{}_i$ regulator of sperm but not the LVA channel. (A) The Ca^{2+} permeation pathway of sperm was activated by treatment with 10 μM thapsigargin (tp), followed by the addition of 1.7 mM $Ca^{2+}{}_o$. AN1043 (10 μM) or DMSO was added 15 s after $Ca^{2+}{}_o$. Initial and final $Ca^{2+}{}_i$ values are displayed, as are the maximum response rates. Traces are representative of observations on 31 cells treated with control medium and 23 cells treated with AN1043. (B) Dose-response relationship for the inhibition of sustained $Ca^{2+}{}_i$ by AN1043. Data points are means (±SD) of observations on 16–23 cells in AN1043 treatment groups and were normalized to a population of 31 cells that were treated with thapsigargin in the absence of AN1043. Data are fit to the same expression as in Figure 4, where the derived IC_{50} value was 0.7 ± 0.2 μM. (C) Effects of AN1043 on the LVA current of mouse pachytene spermatocyte. Peak whole cell Ca^{2+} currents were recorded after test depolarizations to −30 mV from a holding potential of −90 mV. AN1043 (10 μM) was added as indicated. AN1043 reduced the peak current by 20% from an initial value of 245 pA. Data are representative of current recordings on six cells.

min^{-1}, by 10 μM AN1043 (n = 7). Similarly, the ZP3-triggered quenching rate was reduced by 56%, from 1.03 ± 0.018 $[F_o/F_i] \times min^{-1}$ to 0.046 ± 0.021 $[F_o/F_i] \times min^{-1}$, by 1 μM AN1043 (n = 6); it was further reduced by 81%, to 0.020 ± 0.011 $[F_o/F_i] \times min^{-1}$, by 10 μM AN1043 (n = 13). The derived IC_{50} values for inhibition of thapsigargin- and ZP3-dependent fluorescence quenching were 0.9 ± 0.2 μM and 0.9 ± 0.3 μM, respectively.

We also examined the effects of two LVA current antagonists (10 μM nifedipine and 10 μM fluspirilene; Arnoult *et al.*, 1998), which had no effect on the thapsigargin-activated Ca^{2+} entry pathway. These agents inhibited the sustained response by <10% when added to sperm 15 s after ZP3 (our unpublished results).

DISCUSSION

The central conclusions of this study are that egg ZP3 activates a late stage of Ca^{2+} influx into mouse sperm, that this influx is due to the opening of a Ca^{2+} store depletion–activated Ca^{2+} entry mechanism, and that the Ca^{2+} entering through this pathway controls the progress of acrosome reactions. A late phase of Ca^{2+} influx during ZP3 signal transduction was identified based on observations that the sustained ionic response required $Ca^{2+}{}_o$, was attenuated by late addition of Ca^{2+} entry antagonists, and was associated with Mn^{2+} influx.

The permeation pathway mediating Ca^{2+} entry during the sustained phase is distinct from the LVA Ca^{2+} channel that is activated during the first seconds of ZP3 signaling. LVA Ca^{2+} channels have been characterized in spermatogenic cells by patch clamp techniques (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996a; Liévano *et al.*, 1996; Santi *et al.*, 1996) and in sperm with the use of optical methods (Arnoult *et al.*, 1999). The LVA Ca^{2+} channel was inhibited by Ni^{2+} and nifedipine (IC_{50} = 34 and 0.4 μM, respectively; Arnoult *et al.*, 1996a, 1998) but was only partially blocked by 10 μM AN1043, whereas the late phase of influx was less sensitive to inhibition by Ni^{2+} (IC_{50} = 481 μM; Figure 4) and nifedipine (<10% inhibition by 10 μM nifedipine) but was more sensitive to inhibition by AN1043 (IC_{50} = 0.8 μM; Figure 8). Thus, a second Ca^{2+} influx mechanism was activated during the later phases of ZP3 signaling and mediated the sustained $Ca^{2+}{}_i$ response.

A question arises concerning the relationship between the activation of LVA Ca^{2+} currents and of the late phase of Ca^{2+} entry. LVA Ca^{2+} channel antagonists, when applied before ZP3, inhibited both pathways with efficacies that were similar to those with which they inhibited LVA Ca^{2+} currents in spermatogenic cells (Arnoult *et al.*, 1996a; present study). In contrast, when antagonists were added after ZP3-induced LVA Ca^{2+} currents had inactivated, they either failed to block sustained $Ca^{2+}{}_i$ responses (>10% inhibition with nifedipine and fluspirilene) or did so with efficacies that did not reflect action on LVA currents (Ni^{2+}). Thus, early activation of LVA Ca^{2+} channels was required for the later, sustained phase of Ca^{2+} entry. The precise role of transient Ca^{2+} entry in the generation of sustained $Ca^{2+}{}_i$ responses is poorly understood. Yet, simple models in which Ca^{2+} entry during the transient phase is required to open the sustained channel can be rejected, because fura 2–quenching experiments demonstrated the opening of that latter channel even in the absence of $Ca^{2+}{}_o$ (Figure 3). More complex models can be envisioned.

A second question concerns the identification of the sustained Ca^{2+} influx pathway. Several lines of evidence suggest that influx occurs through the Ca^{2+} store–operated channel. Ca^{2+} channels that are activated by depletion of intracellular Ca^{2+} stores have been well characterized in nonexcitable cells (Parekh and Penner, 1997) and may also

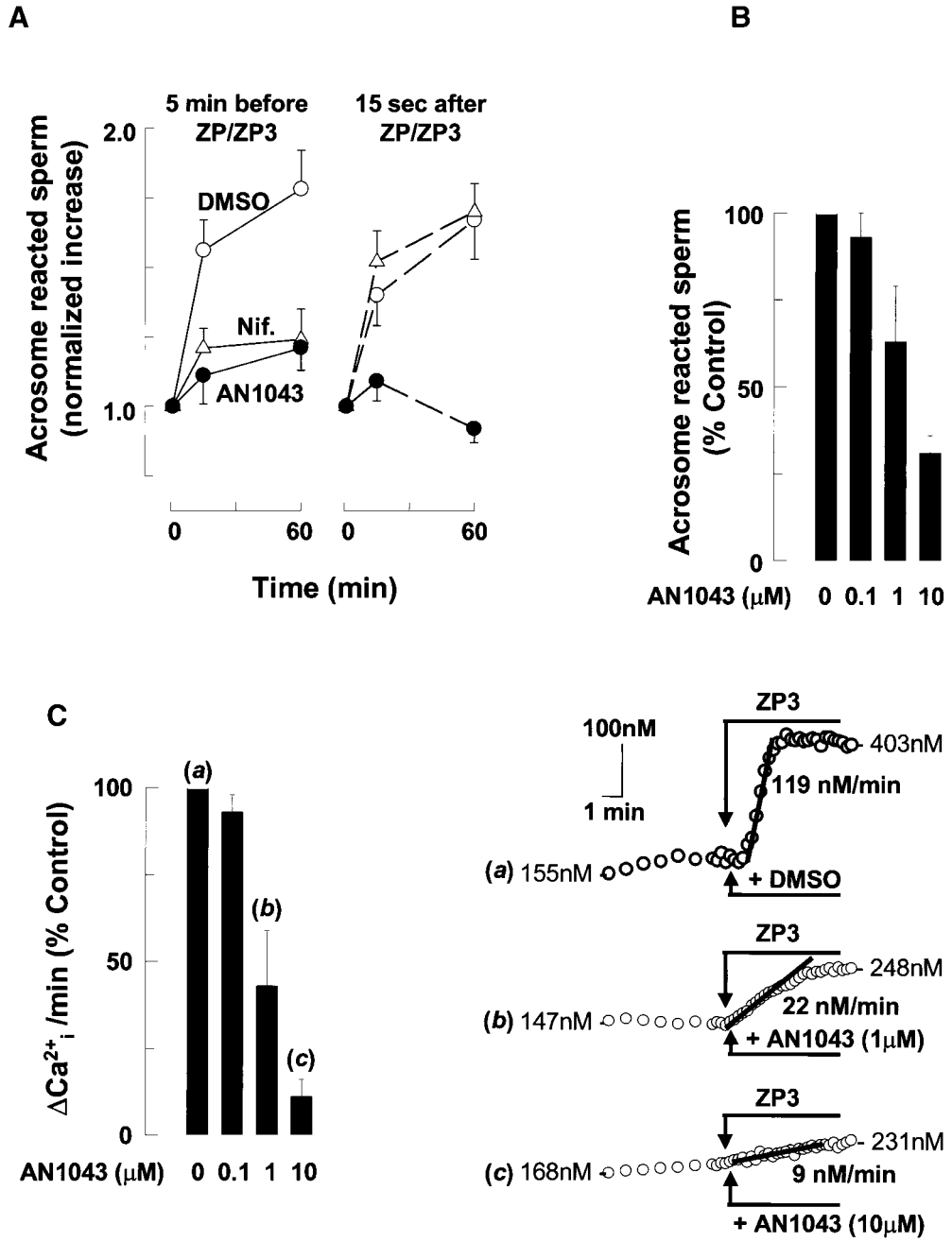

Figure 8. AN1043 inhibits ZP3 signal transduction. (A) Sperm were treated with 10 μM AN1043 (●), 10 μM nifedipine (△), or DMSO vehicle (○) added either 5 min before (left) or 15 s after (right) the addition of 10 μg/ml ZP3 or 40 μg/ml ZP extracts. Acrosome reactions were determined during a 60-min incubation, and data were normalized according to the expression $AR = (AR_i - AR_0)/(AR_0)$, where AR_0 and AR_i are the acrosome reaction levels before ZP/ZP3 addition and at *i* min. AR_0 was 21 ± 8 and 17 ± 5% when drugs were added before or after agonists, respectively. (B) Dose-response relationship for the inhibition of acrosome reactions after the addition of AN1043 15 s after ZP/ZP3. Acrosome reactions were determined 30 min after the addition of ZP/ZP3. Results are normalized according to the expression $AR = 100 \times [(AR_{AN30} - AR_0)/(AR_{30B} - AR_0)]$, where AR_0, AR_{AN30}, and AR_{30B} are the levels of acrosome reactions at 0 min, at 30 min in the presence of AN1043, and at 30 min in the absence of AN1043, respectively. The derived IC_{50} for inhibition of the acrosome reaction was 1.1 ± 0.2 μM. Data in A and B represent the means (±SD) of triplicate experiments, with 200 sperm examined at each time or concentration point in each experiment. (C) AN1043 inhibits the sustained $Ca^{2+}{}_i$ response that is activated by ZP/ZP3. $Ca^{2+}{}_i$ was monitored after the addition of either 10 μg/ml ZP3 or 40 μg/ml ZP. AN1043 or DMSO was added 15 s after the agonist. Response rates ($\Delta Ca^{2+}{}_i/\Delta t$) were determined as described in Figure 1. (Left) Pooled response (means ± SD) of observations on 12–47 cells. Data were normalized according to the same expression as in B, in which data reflect rates ($\Delta Ca^{2+}/\Delta t$) rather than acrosome reaction values. The derived IC_{50} value for inhibition of the $Ca^{2+}{}_i$ response was 0.8 ± 0.1 μM. Letters above the bars refer to individual traces shown on the right. (Right) Representative traces for sperm treated with 0 μM (a), 1 μM (b), or 10 μM (c) AN1043.

operate in excitable cells (Zhu *et al.*, 1996; Garcia and Schilling, 1997; Philipp *et al.*, 1998; Fomina and Nowycky, 1999; Li *et al.*, 1999; Liman *et al.*, 1999). The presence of a store-operated Ca^{2+} entry mechanism in sperm was suggested from studies of uncapacitated human sperm (Blackmore, 1993) and spermatogenic cells and testicular sperm of mouse (Santi *et al.*, 1998). Because mammalian sperm require capacitation to express fertilizing ability (Yanagimachi, 1994), and because capacitation may include alterations in Ca^{2+} metabolism (Fraser *et al.*, 1995), it was necessary to determine whether this mechanism also operated in capacitated sperm. We observed that thapsigargin activated a Ca^{2+} influx mechanism in capacitated sperm populations that was dependent on Ca^{2+}_o, was blocked by Ca^{2+} entry inhibitors, and was associated with cation influx. The thapsigargin-induced release of Ca^{2+} from internal stores could promote Ca^{2+} influx either through a store depletion–activated channel or through a Ca^{2+}_i-activated cation channel (Parekh and Penner, 1997). However, the role of a Ca^{2+}_i-gated channel in ZP3 signaling is unlikely, because the sustained entry mechanism into sperm was activated by thapsigargin even when internal chelators prevented increases of Ca^{2+}_i (Figure 5F). Thus, thapsigargin activated a Ca^{2+} influx pathway into capacitated sperm with the anticipated characteristics of a Ca^{2+} store depletion–activated channel.

The thapsigargin-evoked permeation pathway shares several features with the pathway that mediates the late phase of ZP3-dependent Ca^{2+} entry. Both pathways were insensitive to nifedipine and fluspirilene but were inhibited by Ni^{2+} and AN1043. These latter two reagents cannot be considered highly selective, yet they inhibited both the Ca^{2+} entry mechanism that is promoted by thapsigargin and that activated during the late phase of ZP3 signaling with similar efficacies: the IC_{50} values were, respectively, 517 ± 29 μM and 481 ± 22 μM for Ni^{2+} and 0.7 ± 0.2 μM and 0.8 ± 0.1 μM for AN1043. Moreover, as noted previously, these reagents have markedly different effects on the sperm LVA Ca^{2+} current. Thus, Ca^{2+} entry pathways in sperm can be differentiated with Ni^{2+} and AN1043. Finally, the cation influx rate evoked by a maximal dose of thapsigargin was not enhanced by the subsequent addition of ZP3 (Figure 6). This lack of synergy suggests that these agents work through a common mechanism. Interestingly, when the sequence of treatment was reversed, thapsigargin enhanced the influx rate above the ZP3-evoked rate and the final rate never again exceeded that produced by thapsigargin alone. It is interesting to speculate that this finding reflects a more localized action of ZP3. These observations strongly suggest that ZP3 promoted a late phase of Ca^{2+} entry through a store depletion–activated channel.

In this regard, the presence of internal Ca^{2+} stores in sperm has been questioned, based on the absence of endoplasmic reticulum. However, acrosomal membranes contain both a Ca^{2+}-ATPase (Gordon, 1973; Gordon *et al.*, 1978) and IP3 receptors (Walensky and Snyder, 1995; Zapata *et al.*, 1997; Trevino *et al.*, 1998). Moreover, a thapsigargin-sensitive Ca^{2+} pump was identified in bovine sperm membranes and tentatively localized to the acrosome (Spungin and Breitbart, 1996). It is likely, therefore, that the acrosome functions as an internal store of releasable Ca^{2+}, as was first suggested by Walensky and Snyder (1995), and as the target for thapsigargin action. Similarly, secretory granules may serve as pools of mobilizable Ca^{2+} in somatic cells (Petersen, 1996).

Store-operated Ca^{2+} channels were initially presumed to function chiefly to facilitate refilling of intracellular Ca^{2+} stores (Putney, 1977), but they are now understood to participate in signal transduction. In some cells, influx through this pathway maintains Ca^{2+} levels in intracellular stores that may otherwise be depleted during production of repetitive Ca^{2+}_i spikes (Putney, 1986; Takemura and Putney, 1989). More recently, it was suggested that these channels can generate Ca^{2+} signals directly. For example, store-operated Ca^{2+} influx is an essential signal for T lymphocyte activation by antigen (Zweifach and Lewis 1993; Fanger *et al.*, 1995) and may also permit regulation of signal-transducing elements (Parekh and Penner, 1997; Fagan *et al.*, 1998).

A model for the control of sperm Ca^{2+}_i during fertilization can be proposed based on these and other results (Figure 9). The early events of ZP3 signal transduction may consist of the generation of two second messengers: 1) LVA Ca^{2+} channels activate during the first seconds of ZP3 signaling, likely in response to a more rapid membrane depolarization (Florman, 1994), and generate a transient Ca^{2+} influx (Arnoult *et al.*, 1999); and 2) phospholipase C is activated, leading to the production of IP3 (Roldan *et al.*, 1994; Tomes *et al.*, 1996). An intermediate stage of ZP3 signaling consisted of activation of the IP3 receptor, leading to depletion of intracellular Ca^{2+} stores. Given the facts that ZP3 stimulated IP3 production in sperm, that IP3 receptors were present in acrosomal membranes (Walensky and Snyder, 1995; Zapata *et al.*, 1997; Trevino *et al.*, 1998), and that IP3 evoked Ca^{2+} release from intracellular stores of digitonin-permeabilized sperm (Walensky and Snyder, 1995), it is likely that such Ca^{2+} pool mobilization is an element of ZP3 signal transduction. Mobilization of stored Ca^{2+} has not been detected directly, possibly because of the small size of mouse acrosomes and a limited understanding of sperm Ca^{2+}_i buffering. It is tempting to speculate that transient Ca^{2+}_i increases induced by ZP3 or thapsigargin in the absence of Ca^{2+}_o (Figures 2A and 5, B and C) represent pool emptying. In any case, Ca^{2+}_o was required for the production of sustained Ca^{2+}_i responses (Figure 2) and acrosome reactions by ZP3 and for either the production of Ca^{2+}_i responses or secretion by Ca^{2+} ionophores or thapsigargin (Blackmore, 1993; Meizel and Turner, 1993; Spungin and Breitbart, 1996; present study). These observations strongly suggest that Ca^{2+} release from intracellular stores is not sufficient to drive acrosome reactions.

The final stage of ZP3 signal transduction consists of the generation of gating signals that couple store depletion to the opening of store-operated permeation pathways in the plasma membrane. This gating signal is not well characterized and may entail generation of soluble messengers, a mechanical coupling mechanism, or a secretion-based mechanism (Berridge, 1995; Parekh and Penner, 1997; Patterson *et al.*, 1999; Yao *et al.*, 1999). Other ZP3-dependent processes may further modulate Ca^{2+} conductance through this pathway. For example, ZP3 produced transient increases of sperm internal pH that develop with the same time course as sustained Ca^{2+}_i responses (Arnoult *et al.*, 1996b), and the store-operated Ca^{2+} channel of mouse spermatogenic cells is controlled by internal pH (Santi *et al.*, 1998). In any case, the

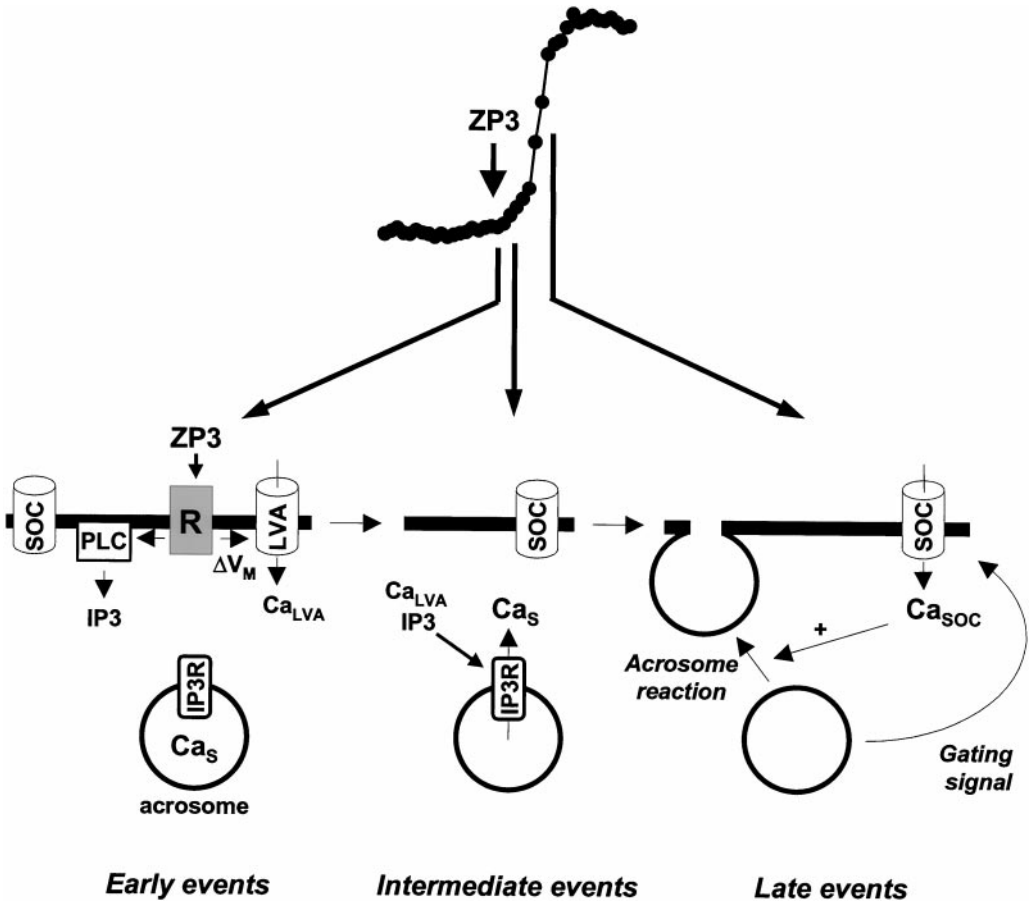

Figure 9. Model of the ionic events of ZP3 signal transduction. (Top) ZP3 increases sperm $Ca^{2+}{}_i$. (Bottom) This response may be due to the sequential activation of three Ca^{2+} channels. (Early events) ZP3 activation of a receptor (R) results in: 1) membrane depolarization (ΔV_M) and the subsequent Ca^{2+} influx (Ca_{LVA}) through LVA Ca^{2+} channels; and 2) the activation of phospholipase C (PLC), leading to the generation of IP3. (Intermediate events) IP3 activates IP3 receptors (IP3R) in the acrosomal membrane, leading to an efflux of Ca^{2+} from this internal store (Ca_S) into the cytosol. (Late events) Depletion of intracellular Ca^{2+} stores produces a signal (gating signal) that opens a store-operated Ca^{2+} channel (SOC) in the plasma membrane. SOC activation also requires Ca_{LVA} and may be further regulated by other ZP3-dependent events, such as increased internal pH. The resultant Ca^{2+} influx (Ca_{SOC}) drives acrosome reactions.

Ca^{2+} that acts as a signal for secretion then enters and drives the final stages of the acrosome reaction.

The protracted time course of the sustained $Ca^{2+}{}_i$ response and acrosome reaction may reflect the sustained activation of store-operated Ca^{2+} entry. It is interesting to speculate that the acrosome reaction requires sustained Ca^{2+} influx, such as is provided by store-operated channels, to prevent precocious activation and loss of sperm function in cells with a single secretory granule. A similar need may underlie the use of store-operated channels to drive gene expression during T cell activation (Crabtree, 1999). In contrast, the delay before the generation of sustained $Ca^{2+}{}_i$ responses, which is >30 s in mouse and bovine sperm (Florman *et al.*, 1989; Lee and Storey, 1989; Bailey and Storey, 1994; Arnoult *et al.*, 1996a) but may be only several seconds in hamster sperm (Shirakawa and Miyazaki, 1999), may provide a measure of the rates of upstream processes such as Ca^{2+} store depletion.

One benefit of this model is that it may account for the reported presence of additional acrosome reaction–inducing agonists. For example, progesterone, which also promotes secretion, activates phospholipase C (Thomas and Meizel, 1989) and may thus deplete sperm Ca^{2+} stores and activate the same store-operated channel. The convergence of these pathways is consistent with reports that progesterone and ZP3 activate distinct upstream signaling elements (Tesarik *et al.*, 1993; Murase and Roldan, 1996) and yet act cooperatively in driving secretion (Roldan *et al.*, 1994). More generally, agents that deplete internal Ca^{2+} stores are expected to act in a synergistic manner with ZP3, whereas agents that inhibit such depletion or otherwise prevent Ca^{2+} entry through store-operated pathways may act as noncompetitive antagonists of ZP3-dependent acrosome reactions and of fertilization.

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