5-4-2000

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Citation: Mol Biol Cell. 2000 May;11(5):1571-84. Link to article on publisher’s website

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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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Submitted December 7, 1999; Revised February 15, 2000; Accepted February 24, 2000

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 \(10\) \(\mu\)M 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 (Espino et al., 1999), and the associated currents were detected by whole cell patch clamp methods (Hagiwara and Kawa, 1984; Arnoult et al., 1996a, 1996b; 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\(^{2+}\) 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 re-
actions occurred after a plateau was established. The sus-
tained response was inhibited by the addition of LVA Ca2+
channel antagonists before ZP3. However, both the slow
activation time course of the sustained Ca2+ response and its
failure to inactivate during prolonged ZP3 stimulation were
consistent with a direct role of LVA channels (Hille, 1992).
It was instead proposed that the transient phase of Ca2+
entry through LVA channels was an obligatory early event
leading to downstream generation of sustained Ca2+ re-
sponses through a second mechanism (Walensky and Smy-
der, 1995; Arnoult et al., 1999).

Given the central role of Ca2+ responses in fertilization,
we examined the sustained response phase in greater detail.
We report that the sustained phase is mediated by Ca2+
entry through Ca2+ store depletion–activated channels.
These channels are present in a wide range of cells, where
they function both to refill internal Ca2+ stores and to par-
ticipate 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 Ca2+
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 cauda epididymi-
des, respectively. Sperm were capacitated in vitro, as described
previously (Arnoult et al., 1996a). Acrosome reactions were assessed
during Ca2+-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 pre-
parations were indistinguishable, consistent with previous observa-
tions 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 ex-
tracts 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

Ca2+ 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 Ca2+ 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).

Ca2+, 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 incuba-
tions with 1 µM BAPTA-AM (N,N’-[1,2-ethanediylbis(oxy-2,1-phe-

Extracellular dye was removed by superfusion, and coverslips
were mounted on a heated microscope stage. Excitation illumina-
tion 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-cou-
ded device camera (Dage MTI, Michigan City, IN), and data were
analyzed with Axon Workbench software (Axon Instruments). Ex-
citation wavelength pairs of 340 and 380 nm were used for Ca2+-i
measuring fertilization.

RESULTS

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 sys-
tem, based on Nyquist’s criterion (Inoue and Spring, 1997), was 2–8 s.
The transient phase of the Ca2+ response, which activated and
inactivated within ~250 msec (Arnoult et al., 1999), is not resolved
here.

Molecular Biology of the Cell

Chromatography 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 Ca2+, Increase

Ca2+, in mouse sperm that were incubated under capacitat-
ing 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 remain-
ing cells exhibited Ca2++, 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).
those sperm in which sustained Ca\textsuperscript{2+} 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\textsuperscript{2+} 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\textsuperscript{2+} response was required for acrosome reactions.

**Role of Intracellular Ca\textsuperscript{2+} Pools in ZP3 Signal Transduction**

It was proposed that ZP3 promotes Ca\textsuperscript{2+} efflux from an intracellular pool during the initiation of acrosome reactions (Walensky and Snyder, 1995). To determine whether the mobilization of Ca\textsuperscript{2+} from intracellular stores was sufficient to initiate exocytosis, we examined the effects of Ca\textsuperscript{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\textsuperscript{2+} medium. However, when ionomycin treatments were carried out in a medium containing 5 mM extracellular BAPTA (BAPTA\textsubscript{o}; calculated Ca\textsuperscript{2+} < 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\textsuperscript{2+} ionophore, A23187 (our unpublished results). These agents readily release Ca\textsuperscript{2+} from intracellular stores, including those of sperm (Babcock et al., 1976). These observations demonstrate that such Ca\textsuperscript{2+} release from intracellular stores is not sufficient to drive acrosome reactions.

**ZP3-activated Ca\textsuperscript{2+} Influx Mechanisms in Sperm**

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

To determine whether Ca\textsuperscript{2+}\textsubscript{o} was required specifically during the sustained response or acted indirectly by inhibiting the earlier, transient Ca\textsuperscript{2+} entry through LVA channels, we took advantage of the distinctive time courses of the transient and sustained responses: the sustained response (Figure 2A), this represents an 81% inhibition. The remaining sperm exhibited either increases of <25 nM Ca\textsuperscript{2+} (30 of 47, 64%;

The salient characteristics of this Ca\textsuperscript{2+} response are: 1) a delay of 1.3 ± 0.4 min (range, 0.5–2.8 min) before detectable Ca\textsuperscript{2+} 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\textsuperscript{2+} values either remain increased or decline slowly (<10 nM/min) in the presence of ZP3. This represents the sustained Ca\textsuperscript{2+} phase of ZP3 signaling. The earlier, transient Ca\textsuperscript{2+} response mediated by LVA channels (Arnault et al., 1999) was not resolved here.

Acrosome reactions occurred in 89% (109 of 122) of sperm exhibiting sustained Ca\textsuperscript{2+} 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\textsuperscript{2+} increases yet failed to acquire some react, as was observed previously (Arnault et al., 1996a,b). This may reflect inefficient sperm capacitation, with ZP3 signal transduction blocked at a site downstream of the sustained Ca\textsuperscript{2+} response (Yanagimachi, 1994; Florman et al., 1999). In contrast, acrosome reactions were detected in only 8% (4 of 55) of sperm in which sustained Ca\textsuperscript{2+} 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\textsuperscript{2+} 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\textsuperscript{2+} response was required for acrosome reactions.

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To determine whether Ca\textsuperscript{2+}\textsubscript{o} was required specifically during the sustained response or acted indirectly by inhibiting the earlier, transient Ca\textsuperscript{2+} entry through LVA channels, we took advantage of the distinctive time courses of the transient and sustained responses: the sustained response (Figure 2A), this represents an 81% inhibition. The remaining sperm exhibited either increases of <25 nM Ca\textsuperscript{2+} (30 of 47, 64%;
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 BAPTAo. 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 \(\mu M\) 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 \(\pm\) 5 \(\mu M\) (C) 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 \(\mu M\); 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 \(\pm\) 21 \(\mu M\); Figure 4, F).

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 \(\mu M\) \(\alpha\)-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 \(\mu M\) PN200-110, 50 \(\mu M\) verapamil) also inhibited the sustained phase of Ca\(^{2+}\)\(_i\) 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\(^{2+}\)\(_i\) 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
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\(^{2+}\) 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\(^{2+}\), 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

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**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 \(\mu\)g/ml) was added as indicated (bars). Times of 10 \(\mu\)M 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 \(\mu\)g/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).

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**Figure 4.** Ni\(^{2+}\) inhibits Ca\(^{2+}\) responses to ZP3. Ni\(^{2+}\) was added either 2 min before (○) or 15 s after ZP3 (●). Response rates (\(\Delta\text{Ca}^{2+}/\Delta t\)) 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\(^{2+}\), response by 50%, respectively. Calculated values of IC\(_{50}\) were 26 \(\mu\)M for Ni\(^{2+}\) before ZP3 (black line) and 481 \(\mu\)M 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 \(\mu\)g/ml ZP3 (●), with 1 mM Ni\(^{2+}\) added 15 s after ZP3 (●), or with Ni\(^{2+}\) added 2 min before ZP3 (○).
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 \(\mu\)M) 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 \(\mu\)M 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\(^{2+}\) increase (1.9–7.4 min). In contrast, 0.1 \(\mu\)M thapsigargin had no detectable effects on sperm Ca\(^{2+}\)\(_i\).

The efficacy of thapsigargin here (IC\(_{50}\) = 0.77 \(\mu\)M) is lower than that typically observed in somatic cells but is similar to values reported in gametes (rat sperm, 0.5 \(\mu\)M [Walensky and Snyder, 1995]; mouse eggs, >1 \(\mu\)M [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 \(\mu\)M) 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 min).

**Figure 5.** Thapsigargin activates Ca\(^{2+}\) influx. Fura 2–loaded sperm were treated with 10 \(\mu\)M 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 \(\mu\)M La\(^{3+}\). (F) Sperm were incubated in <30 nM Ca\(^{2+}\)\(_o\) containing 10 \(\mu\)M Mn\(^{2+}\). Thapsigargin activated Mn\(^{2+}\) influx in fura 2/BAPTA\(_o\)-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.
4.5-fold, to 0.082 M

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

ZP3 and thapsigargin promote increases of sperm Ca^{2+}, 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 mM Mn^{2+}, medium was 0.018 ± 0.011 [F_r/F_i] × min^{-1}, and this quenching rate was increased by 4.5-fold, to 0.082 ± 0.023 [F_r/F_i] × min^{-1} (p < 0.001; n = 17), by the addition of 10 mM thapsigargin. ZP3 (10 μg/ml) also stimulated the quenching rate from leak levels to 0.038 ± 0.014 [F_r/F_i] × 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_r/F_i] × 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 mM thapsigargin in a Ca^{2+},-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+} increases that occurred after the readdition of Ca^{2+}, 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, D).

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+} increase of 91 ± 12 nmol/min (n = 31) in DMSO-treated control cells; this rate was reduced by 81% to 17 ± 8 nmol/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+} 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+}, 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+}, 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+}, 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+}, 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_r/F_i] × min^{-1} to 0.051 ± 0.017 [F_r/F_i] × min^{-1}, by 1 μM AN1043 (n = 9); it was further reduced by 73%, to 0.025 ± 0.009 [F_r/F_i] × min^{-1}.
Figure 6. Thapsigargin and ZP3 activate a similar cation permeation pathway in sperm. Fura 2–loaded sperm were incubated in a 30 nM Ca\textsuperscript{2+} medium, and Ca\textsuperscript{2+}-independent dye fluorescence was monitored as described in Figure 3. Mn\textsuperscript{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\textsubscript{x} / F\textsubscript{i}] / [t\textsubscript{x} / t\textsubscript{i}], where F\textsubscript{x} and F\textsubscript{i} are the fluorescence emission at x min [t\textsubscript{x}] and at the initial time [t\textsubscript{i}], respectively) were determined for individual sperm during a 2-min incubation in 1 μM Mn\textsuperscript{2+} and designated R\textsubscript{L} (leak rate). Quenching rates during experimental treatment (R\textsubscript{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\textsubscript{X} / R\textsubscript{L}) and are the mean of observations on 12–39 sperm. (B) A fluorescence trace showing the effects of thapsigargin and ZP3 on Mn\textsuperscript{2+}-dependent quenching. Data from this and other cells were used to construct panel A. (C) Ni\textsuperscript{2+} inhibits thapsigargin-induced Ca\textsuperscript{2+} responses. Thapsigargin triggered Ca\textsuperscript{2+} increases upon addition of Ca\textsuperscript{2+}. Addition of Ni\textsuperscript{2+} 15 s after Ca\textsuperscript{2+} inhibited this response in a concentration-dependent manner (>). Data were fit to the expression described in Figure 4, and the derived IC\textsubscript{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\textsuperscript{2+} 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\textsuperscript{2+} responses in the absence (>) and in the presence (>) of 1 μM Ni\textsuperscript{2+}.
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 Ca2+ 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 Ca2+ influx into mouse sperm, that this influx is due to the opening of a Ca2+ store depletion-activated Ca2+ entry mechanism, and that the Ca2+ entering through this pathway controls the progress of acrosome reactions. A late phase of Ca2+ influx during ZP3 signal transduction was identified based on observations that the sustained ionic response required Ca2+ entry, was attenuated by late addition of Ca2+ entry antagonists, and was associated with Mn2+ influx.

The permeation pathway mediating Ca2+ entry during the sustained phase is distinct from the LVA Ca2+ channel that is activated during the first seconds of ZP3 signaling. LVA Ca2+ 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 Ca2+ channel was inhibited by Ni2+ and nifedipine (IC50 = 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 Ni2+ (IC50 = 481 μM; Figure 4) and nifedipine (<10% inhibition by 10 μM nifedipine) but was more sensitive to inhibition by AN1043 (IC50 = 0.8 μM; Figure 8). Thus, a second Ca2+ influx mechanism was activated during the later phases of ZP3 signaling and mediated the sustained Ca2+ response.

A question arises concerning the relationship between the activation of LVA Ca2+ currents and of the late phase of Ca2+ entry. LVA Ca2+ channel antagonists, when applied before ZP3, inhibited both pathways with efficacies that were similar to those with which they inhibited LVA Ca2+ currents in spermatogenic cells (Arnoult et al., 1996a; present study). In contrast, when antagonists were added after ZP3-induced LVA Ca2+ currents had inactivated, they either failed to block sustained Ca2+ responses (>10% inhibition with nifedipine and fluspirilene) or did so with efficacies that did not reflect action on LVA currents (Ni2+). Thus, early activation of LVA Ca2+ channels was required for the later, sustained phase of Ca2+ entry. The precise role of transient Ca2+ entry in the generation of sustained Ca2+ responses is poorly understood. Yet, simple models in which Ca2+ 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 Ca2+ entry (Figure 3). More complex models can be envisioned.

A second question concerns the identification of the sustained Ca2+ influx pathway. Several lines of evidence suggest that influx occurs through the Ca2+ store–operated channel. Ca2+ channels that are activated by depletion of intracellular Ca2+ stores have been well characterized in nonexcitable cells (Parekh and Penner, 1997) and may also

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**Figure 7.** AN1043 inhibits the thapsigargin-dependent Ca2+ regulator of sperm but not the LVA channel. (A) The Ca2+ permeation pathway of sperm was activated by treatment with 10 μM thapsigargin (tp), followed by the addition of 1.7 mM Ca2+-w. AN1043 (10 μM) or DMSO was added 15 s after Ca2+-w. Initial and final Ca2+ 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 Ca2+ entry, 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 IC50 value was 0.7 ± 0.2 μM. (C) Effects of AN1043 on the LVA current of mouse pachytene spermatocyte. Peak whole cell Ca2+ 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.
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 = (ARi - AR0)/(AR0), where ARi and AR0 are the acrosome reaction levels before ZP/ZP3 addition and at i min. AR0 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 × [(ARAN30 - AR0)/(AR30B - AR0)], where AR0, ARAN30, and AR30B 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 IC50 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 Ca2+ response that is activated by ZP/ZP3. Ca2+ 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 (ΔCa2+/Δt) 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 (ΔCa2+/Δt) rather than acrosome reaction values. The derived IC50 value for inhibition of the Ca2+ 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 Ca2+ entry mechanism in sperm was suggested from studies of uncapacitated human sperm (Blackmore, 1995) 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 Ca2+ metabolism (Fraser et al., 1995), it was necessary to determine whether this mechanism also operated in capacitated sperm. We observed that thapsigargin activated a Ca2+ influx mechanism in capacitated sperm populations that was dependent on Ca2+ entry inhibitors, and was associated with cation influx. The thapsigargin-induced release of Ca2+ from internal stores could promote Ca2+ influx either through a store depletion-activated channel or through a Ca2+-activated cation channel (Parekh and Penner, 1997). However, the role of a Ca2+-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 Ca2+ (Figure 5F). Thus, thapsigargin activated a Ca2+ influx pathway into capacitated sperm with the anticipated characteristics of a Ca2+ store depletion-activated channel.

The thapsigargin-evoked permeation pathway shares several features with the pathway that mediates the late phase of ZP3-dependent Ca2+ entry. Both pathways were insensitive to nifedipine and fluspirilen but were inhibited by Ni2+ and AN1043. These latter two reagents cannot be considered highly selective, yet they inhibited both the Ca2+ entry mechanism that is promoted by thapsigargin and that activated during the late phase of ZP3 signaling with similar efficacies: the IC50 values were, respectively, 517 ± 29 μM and 481 ± 22 μM for Ni2+ 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 Ca2+ current. Thus, Ca2+-entry pathways in sperm can be differentiated with Ni2+ 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 Ca2+ entry through a store depletion-activated channel.

In this regard, the presence of internal Ca2+ stores in sperm has been questioned, based on the absence of endoplasmic reticulum. However, acrosomal membranes contain both a Ca2+-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 Ca2+ 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 Ca2+, 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 Ca2+ in somatic cells (Petersen, 1996).

Store-operated Ca2+ channels were initially presumed to function chiefly to facilitate refilling of intracellular Ca2+ stores (Putney, 1977), but they are now understood to participate in signal transduction. In some cells, influx through this pathway maintains Ca2+ levels in intracellular stores that may otherwise be depleted during production of repetitive Ca2+ spikes (Putney, 1986; Takemura and Putney, 1989). More recently, it was suggested that these channels can generate Ca2+ signals directly. For example, store-operated Ca2+ 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 Ca2+ 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 Ca2+ channels activate during the first seconds of ZP3 signaling, likely in response to a more rapid membrane depolarization (Florman, 1994), and generate a transient Ca2+ 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 Ca2+ 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 Ca2+ release from intracellular stores of digitonin-permeabilized sperm (Walensky and Snyder, 1995), it is likely that such Ca2+ pool mobilization is an element of ZP3 signal transduction. Mobilization of stored Ca2+ has not been detected directly, possibly because of the small size of mouse acrosomes and a limited understanding of sperm Ca2+ buffering. It is tempting to speculate that transient Ca2+ increases induced by ZP3 or thapsigargin in the absence of Ca2+o (Figures 2A and 5B and C) represent pool emptying. In any case, Ca2+ was required for the production of sustained Ca2+ responses (Figure 2) and acrosome reactions by ZP3 and for either the production of Ca2+ responses or secretion by Ca2+ ionophores or thapsigargin (Blackmore, 1995; Meizel and Turner, 1993; Spungin and Breitbart, 1996; present study). These observations strongly suggest that Ca2+ 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 Ca2+ conductance through this pathway. For example, ZP3 produced transient increases of sperm internal pH that develop with the same time course as sustained Ca2+responses (Arnoult et al., 1996b), and the store-operated Ca2+ channel of mouse spermatogenic cells is controlled by internal pH (Santi et al., 1998). In any case, the
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 \(\text{Ca}^{2+}\) response and acrosome reaction may reflect the sustained activation of store-operated \(\text{Ca}^{2+}\) entry. It is interesting to speculate that the acrosome reaction requires sustained \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) stores are expected to act in a synergistic manner with ZP3, whereas agents that inhibit such depletion or otherwise prevent \(\text{Ca}^{2+}\) entry through store-operated pathways may act as noncompetitive antagonists of ZP3-dependent acrosome reactions and of fertilization.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (grants HD 32177 and GM 56479 to H.M.F. and grant AR 44066 to R.A.S.) by the Centre National de la Recherche Scientifique (C.A.);
and by Consejo Nacional de Ciencia y Tecnología, Dirección General de Asuntos para el Personal Académico, Howard Hughes Medical Institute, and the International Center for Genetic Engineering and Biotechnology (A.D.).

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