

# Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation

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**ABSTRACT** Sperm adhesion to egg zonae pellucidae initiates sperm acrosome reactions, an exocytotic event that is an early step during fertilization. Previously, it was suggested that zona pellucida-evoked  $\text{Ca}^{2+}$  entry into sperm through low voltage-activated  $\text{Ca}^{2+}$  channels is an essential step in acrosome reactions, based on the inhibitory effects of  $\text{Ca}^{2+}$  channel antagonists. However, analysis of this channel is limited by the inability to apply electrophysiological methods directly to sperm. In this report, optical methods of determining membrane potential and internal  $\text{Ca}^{2+}$  levels were used to demonstrate that (i) contact with zonae pellucidae activates a transient  $\text{Ca}^{2+}$  response in sperm that has a time course and antagonist sensitivity anticipated of low voltage-activated  $\text{Ca}^{2+}$  channels; (ii) these channels are unavailable for opening in uncapacitated sperm because of voltage-dependent, steady state inactivation; (iii) membrane hyperpolarization during sperm capacitation is sufficient to recruit channels into a closed state, from which they are available for opening during fertilization; and (iv) channel conductance state may be a factor in determining the efficacy with which channel antagonists inhibit fertilization. This study provides evidence for the activation of sperm  $\text{Ca}^{2+}$  channels during gamete adhesion and offers a mechanism that may account for aspects of the regulation of sperm fertility during capacitation through the control of channel availability. Finally, these results suggest that channel conductance state may be a central feature in the design of channel antagonists that inhibit sperm function.

The acrosome is a secretory vesicle that is present in the apical region of sperm of many animal species, including all mammals. The release of vesicle contents occurs through an exocytotic process known as the acrosome reaction during the early stages of fertilization and must be completed in order for sperm to engage in the later events of fertilization, including gamete fusion. In mammals, acrosome reactions are controlled by both indirect and direct mechanisms. First, sperm must undergo a preliminary activation phase, designated "capacitation," which regulates the efficiency of exocytosis. Although the essential role of capacitation in fertilization is generally accepted, the molecular mechanisms that underlie this regulatory process are not well understood. Second, exocytosis by capacitated sperm is initiated during sperm-egg contact by ZP3, a component of the egg's zona pellucida (ZP) extracellular matrix (1–3).

Three lines of evidence suggest that  $\text{Ca}^{2+}$  entry through voltage-sensitive channels mediates ZP3 signal transduction. (i) ZP3 produces elevations of sperm intracellular  $\text{Ca}^{2+}$

( $\text{Ca}^{2+}_i$ ), as reported by ion-selective fluorescent probes. (ii) ZP3-evoked  $\text{Ca}^{2+}_i$  responses and acrosome reactions are inhibited by  $\text{Ca}^{2+}$  channel antagonists. (iii) Under certain circumstances, depolarization of sperm membrane potential ( $V_M$ ) also produces  $\text{Ca}^{2+}_i$  elevations and acrosome reaction, and these are, again, inhibited by  $\text{Ca}^{2+}$  channel antagonists (2, 3). These channels may account for the reported male contraceptive effects of 1,4-dihydropyridine-type  $\text{Ca}^{2+}$  channel antagonists (4, 5).

Characterization of  $\text{Ca}^{2+}$  currents in sperm is complicated by difficulties in applying either molecular genetic methods or patch clamp techniques directly to these cells. An alternative approach has been to examine channels in testicular spermatogenic cells, a developmental precursor stage that synthesizes proteins for later use in the translationally quiescent sperm. Low voltage-activated (LVA) T-type channels are detected in rodent spermatogenic cells by using the patch clamp technique (6–12). Moreover, the close correspondence between the effects of channel antagonists on the LVA  $\text{Ca}^{2+}$  currents of mouse spermatogenic cells and on the ZP3-evoked  $\text{Ca}^{2+}_i$  response of mouse sperm suggests that these channels mediate  $\text{Ca}^{2+}$  influx during acrosome reactions (6). However, LVA currents inactivate rapidly and do not generally produce the protracted and delayed  $\text{Ca}^{2+}_i$  responses that are observed in sperm. This may reflect the fact that earlier studies of ZP3-dependent  $\text{Ca}^{2+}_i$  responses lacked the temporal resolution to detect LVA channels. An understanding of the role of LVA currents during fertilization requires direct analysis of their activation in sperm.

Here, we use fluorescent probe methods to determine  $\text{Ca}^{2+}_i$  and  $V_M$  in single mouse sperm. These studies demonstrate that ZP3 activates a  $\text{Ca}^{2+}$  influx into sperm. This influx produces a  $\text{Ca}^{2+}_i$  elevation that has both a time course and a susceptibility to inhibition by channel antagonists that is anticipated of LVA currents. In addition, we demonstrate that the availability of these channels for opening is controlled during sperm capacitation. This latter form of regulation may contribute to the coordination of exocytosis with gamete contact, as well as providing new targets for channel-based antifertility agents.

## MATERIALS AND METHODS

**Biological Preparations.** Sperm and spermatogenic cells were obtained from CD-1 mice from caudae epididymides and by manual trituration of testicular slices, respectively (6). The

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: ZP, zona pellucida; LVA, low voltage-activated; CTC, chlortetracycline; di8-ANEPPS, 4-[2-[6-(diethylamino)-2 naphthalenyl]ethenyl]-1-(3-sulfopropyl)].

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fraction of motile sperm was determined by visual inspection within 20 min, and preparations with <75% motile cells were discarded. Sperm were capacitated *in vitro* during a 120-min incubation (37°C) in medium HBCM, containing (in mM): NaCl (94), KCl (4.78), MgCl<sub>2</sub> (2.1), CaCl<sub>2</sub> (1.71), KH<sub>2</sub>PO<sub>4</sub> (1.19), NaHCO<sub>3</sub> (10), Hepes [10 (pH 7.4 with NaOH)], sodium lactate (26), sodium pyruvate (1.19), glucose (5.56), and BSA (2% wt/vol) (13). Noncapacitating incubations were carried out in a low BSA (0.1% wt/vol)-HBCM medium (13). Phosphate salts were removed during experiments with La<sup>3+</sup>.

Capacitation was assayed (i) in single sperm, by chlortetracycline (CTC) fluorescence emission ( $\lambda_{EX}$ , 395 nm;  $\lambda_{EM}$ , 510 nm) (13, 14) and by determining the ability to undergo ZP3-dependent exocytosis (13); and (ii) in populations by monitoring tyrosine phosphorylation of sperm proteins (15, 16). Acrosome reactions were assayed by Coomassie blue staining (17). Cumulus-free, ZP-intact eggs were fertilized during 4-hr incubations in 100- $\mu$ l drops of HBCM containing 10<sup>5</sup> sperm/ml, a concentration at which  $\approx$ 75% of eggs are fertilized within 4 hr and at which the extent of fertilization depends on the concentration of functional sperm. Fertilization was assayed by the presence of a sperm head within egg cytoplasm (18).

ZP were obtained by manual dissection from germinal vesicle-intact, follicular oocytes. ZP2 and ZP3 were purified by SDS/PAGE (19). Soluble ZP extracts were prepared by heating (80°C, 60 min).

**Electrophysiological Methods.** Spermatogenic cells were attached to Cell-Tak-coated plastic culture dishes and were perfused with a solution containing (in mM): NaCl (100), KCl (5), CaCl<sub>2</sub> (10), MgCl<sub>2</sub> (1), tetraethylammonium-Cl (26), sodium lactate (6), Hepes [10 (pH 7.4 with NaOH)], and glucose (3.3). Ca<sup>2+</sup> currents were recorded by using hard glass (Gardner 7052; 2–10 megaohms of pipette resistance), Sylgard-coated pipettes by means of the whole cell-configuration of the patch clamp method (20). The pipette internal solution contained (in mM): cesium glutamate (120), MgCl<sub>2</sub> (5), tetraethylammonium-Cl (20), Mg<sub>2</sub>ATP (3), CsEGTA (10), Hepes [10 (pH 7.0 with CsOH)], and glucose (5). Currents were obtained by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), corrected for leakage and capacitance currents, filtered at 3 kHz, digitized every 250 msec, and analyzed by using Biopatch (Biologic, Grenoble, France) as described (6, 8, 9). Voltage protocols and drug treatment regimens are described in *Results*.

**Fluorescent Probe Methods.** Sperm (10<sup>7</sup> per ml) were labeled with dyes (Molecular Probes) by incubation (30 min, 37°C) in a 0.01% BSA-supplemented medium containing either 3  $\mu$ M of the acetoxymethyl-ester conjugates of mag-fura 2 or fura 2 or 1  $\mu$ M di8-ANEPPS 4-[2-[6-(dioctylamino)-2-naphthalenyl] ethenyl]-1-(3-sulfolpropyl) (Molecular Probes). Sperm were immobilized on Cell-Tak-coated glass coverslips and were maintained at 37°C on a microscope stage in medium HBCM. Excitation illumination from a DG4 Optical Switch (Sutter Instruments, Novato, CA) was selected by using the following filter combinations: for fura 2 or mag-fura 2, 340 nm and 380 nm; for di8-ANEPPS, 440 nm and 530 nm. Emission (510 nm) from fura 2 or mag-fura 2 was restricted to single sperm with a field iris and was directed to a photomultiplier tube. Di8-ANEPPS emission (610 nm) from groups of sperm was digitized with a GenIVSys-intensified Dage 72 CCD camera (Dage-MTI) and was analyzed in single cells by using Axon Instruments WORKBENCH software (7).

Ca<sup>2+</sup> values were estimated by using the algorithm of Grynkiewicz *et al.* (21), using  $K_d$  values for Ca<sup>2+</sup>:dye complexes of 224 nM for fura 2 (21) and 44  $\mu$ M for mag-fura 2 (22). Di8-ANEPPS signals were calibrated in the range -100–0 mV, with standard curves obtained from fluorescent emission of voltage-clamped round spermatids. The relationship between  $V_M$  and dye emission is approximated by the expression

$V_M = 1.002 + (0.00099 * [F_{440}/F_{530}])$ , in which  $[F_{440}/F_{530}]$  is the ratio of fluorescent emission after excitation at the indicated energies. A similar relationship was described for these styryl probes (23). BSA alters the spectral characteristics of di8-ANEPPS. This protein is essential for mouse sperm capacitation (13, 24) but may be omitted from spermatogenic cell cultures. BSA was therefore removed from the bath by superfusion before the determination of  $V_M$  in immobilized sperm.

## RESULTS

**ZP3-Dependent Ca<sup>2+</sup> Transients in Sperm.** Previously, it was demonstrated that the major component of the Ca<sup>2+</sup><sub>i</sub> response to ZP3 requires minutes to develop (6, 25) and is too slow to reflect ionic influx through LVA channels (26). However, the fluorescence detector systems used in those studies were limited by a temporal resolution of >15 sec. To determine whether this delayed Ca<sup>2+</sup><sub>i</sub> response phase is preceded by more rapid events, we examined the early stages of ZP3 signal transduction in single sperm by using a detector system with a temporal resolution, as estimated by Nyquist's criterion, of 2–4 msec.

Ca<sup>2+</sup><sub>i</sub> was monitored with mag-fura 2 and with fura 2, which are low- and high-affinity, Ca<sup>2+</sup>-selective fluorescent probes, respectively. Ca<sup>2+</sup><sub>i</sub> of capacitated sperm was below the level of accurate detection with mag-fura 2 but was determined to be  $0.154 \pm 0.023 \mu$ M ( $n = 31$  cells) by using fura 2. Addition of ZP glycoprotein extracts (40  $\mu$ g/ml; 4 ng of protein/ZP) produced transient Ca<sup>2+</sup><sub>i</sub> elevations that were resolved by mag-fura 2 in 73% (19/26) of cells (Fig. 1A and B). Similar responses were noted in 65% (24/37) of cells treated with purified ZP3 from an equivalent number of ZP (10  $\mu$ g/ml; 1 ng ZP3/ZP; Fig. 1B). The presence of nonresponsive cells is consistent with observations that only 40–80% of mouse sperm respond to ZP3 (13, 18, 27) and may reflect inefficient capacitation *in vitro*. Acrosome reactions occurred within 15 min in 93% (40/43) of cells exhibiting ZP/ZP3-evoked transients but occurred in only 15% (3/20) of cells in which transients were not detected.

Sperm were treated in control experiments with proteins (BSA, fetuin, mouse ZP2) that did not promote acrosome reactions. Such treatments failed to initiate Ca<sup>2+</sup><sub>i</sub> transients (0/37 cells), although Ca<sup>2+</sup><sub>i</sub> levels rose linearly to  $0.209 \pm 0.027 \mu$ M during the 10-min treatment period (Fig. 1B). Thus, Ca<sup>2+</sup><sub>i</sub> transients are a specific response to ZP3 stimulation.

The ZP3-dependent Ca<sup>2+</sup><sub>i</sub> transient had the following salient characteristics. (i) It occurred after a time lag (mean =  $9 \pm 4$  sec,  $n = 43$ ) that reflects, in part, ZP3 diffusion to sperm. (ii) Ca<sup>2+</sup><sub>i</sub> increased from 10 to 90% of peak values in  $37 \pm 11$  msec, and the time course was approximated by a single exponential expression ( $\tau = 11.4 \pm 2.3$  msec). The rise time reflects Ca<sup>2+</sup> influx into and diffusion from the small internal volume of sperm (10<sup>-14</sup> liters) (30), where significant changes in Ca<sup>2+</sup><sub>i</sub> may be achieved by a small number of activated channels. (iii) Peak Ca<sup>2+</sup><sub>i</sub> values of  $9.2 \pm 1.3 \mu$ M were resolved. (iv) Ca<sup>2+</sup><sub>i</sub> levels returned to basal levels within  $179 \pm 23$  msec, with the initial portion of the relaxation curve approximated by a single exponential expression ( $\tau = 47.2 \pm 6.8$  msec). (v) A single transient was observed in 85% (34/40) of responsive sperm, with the remaining cells exhibiting 2–4 spikes of similar magnitude and time course during a 1- to 4-sec interval. The activation time course of this transient is similar to that of the LVA Ca<sup>2+</sup> current (6, 8, 9, 11, 12, 26).

To determine whether the ZP3-dependent Ca<sup>2+</sup><sub>i</sub> transient requires influx of extracellular Ca<sup>2+</sup>, experiments were carried out in media supplemented with either Cd<sup>2+</sup> or La<sup>3+</sup>. These cations block Ca<sup>2+</sup> entry through a variety of transport mechanisms, including voltage-sensitive channels. La<sup>3+</sup> and Cd<sup>2+</sup> inhibited peak Ca<sup>2+</sup><sub>i</sub> responses during transients with IC<sub>50</sub> values of  $171 \pm 63$  and  $652 \pm 134 \mu$ M, respectively (Fig. 1C).

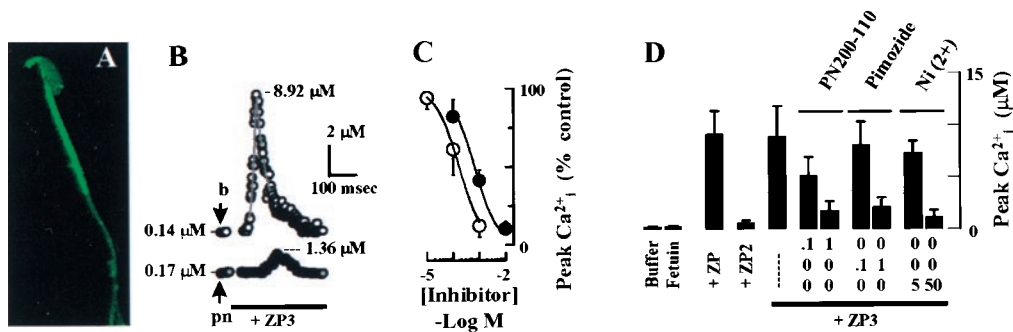


FIG. 1. ZP3 produces  $\text{Ca}^{2+}_i$  transients that are detected with mag-fura2. (A) Dye ( $\lambda_{\text{EX}}, 360 \text{ nm}$ ;  $\lambda_{\text{EM}}, 510 \text{ nm}$ ) distributes throughout sperm and permits ratiometric image acquisition from all regions. (B)  $\text{Ca}^{2+}_i$  responses to ZP3 treatment in the absence ( $\circ$ ) and the presence ( $\bullet$ ) of PN200-110. Cells were treated with buffer (b) or with  $1 \mu\text{M}$  PN200-110 (pn) for 10 min before addition of ZP3 ( $10 \mu\text{g}/\text{ml}$ ; bar). The rising phase of the ZP3-dependent  $\text{Ca}^{2+}_i$  response in the absence of channel antagonists is approximated by the expression  $\text{Ca}^{2+}_i = e^{t/\tau}$ , where  $t$  and  $\tau$  represent the time and the time constant, respectively (—). In this cell,  $\tau = 12.7 \text{ msec}$ . The initial portion of the relaxation trace is approximated by the expression  $\text{Ca}^{2+}_i = ae^{-t/\tau}$ , where  $a$  designates the maximal response (---) and where  $a = 9.11 \mu\text{M}$  and  $\tau = 40.9 \text{ msec}$ . (C) Peak  $\text{Ca}^{2+}_i$  responses to ZP ( $40 \mu\text{g}/\text{ml}$ ) or to ZP3 ( $10 \mu\text{g}/\text{ml}$ ) are inhibited by  $\text{La}^{3+}$  ( $\circ$ ) and by  $\text{Cd}^{2+}$  ( $\bullet$ ). Data points represent the means ( $\pm$ SD) of observations on 4–7 different cells and are approximated by the expression  $R = (100 * \text{IC}_{50}) / (\text{IC}_{50} + D)$ .  $R$  is observed response;  $D$  is antagonist concentration; and  $\text{IC}_{50}$  is the drug concentration producing 50% inhibition. Derived  $\text{IC}_{50}$  values were  $\text{La}^{3+}$ ,  $171 \pm 63 \mu\text{M}$ ; and  $\text{Cd}^{2+}$ ,  $652 \pm 134 \mu\text{M}$ . Results using ZP3 and ZP are indistinguishable and are pooled for presentation. (D) ZP3-dependent  $\text{Ca}^{2+}_i$  transients are inhibited by LVA current antagonists. Pimozide was added at 15 min of a 120-min incubation in a capacitating medium whereas PN200-110 and  $\text{Ni}^{2+}$  were added at 90 min. After incubation for 120 min, sperm were treated with ZP3. Data represent peak  $\text{Ca}^{2+}_i$  values (mean  $\pm$  SD). The fraction of cells in each treatment group that exhibit transients is indicated. Total cells were: buffer, 12; fetuin ( $40 \mu\text{g}/\text{ml}$ ), 14; ZP ( $40 \mu\text{g}/\text{ml}$ ), 26; ZP2 ( $40 \mu\text{g}/\text{ml}$ ), 14; ZP3 ( $10 \mu\text{g}/\text{ml}$ ), 37; +PN200-110 ( $0.1 \mu\text{M}$ ), 9; +PN200-110 ( $1 \mu\text{M}$ ), 11; +pimozide ( $0.1 \mu\text{M}$ ), 12; +pimozide ( $1 \mu\text{M}$ ), 16; + $\text{Ni}^{2+}$  ( $5 \mu\text{M}$ ), 8; + $\text{Ni}^{2+}$  ( $50 \mu\text{M}$ ), 7.

The relationship of the sperm  $\text{Ca}^{2+}_i$  transient that is detected by optical methods and the spermatogenic cell LVA current detected by patch clamp methods was examined in experiments using channel antagonists. Peak  $\text{Ca}^{2+}_i$  levels during ZP3-dependent transients were inhibited in a concentration-dependent manner by PN200-110, a 1,4-dihydropyridine; by pimozide, a diphenylbutylpiperidine; and by  $\text{Ni}^{2+}$  (Fig. 1B and D). The inhibitory efficacy of these agents is similar to that reported previously for the inhibition of both the LVA  $\text{Ca}^{2+}$  current of somatic cells (28–32) and spermatogenic cells (6, 9, 11), as well as for the inhibition of ZP-initiated acrosome reactions (6). These observations demonstrate that ZP3 promotes  $\text{Ca}^{2+}_i$  transients that are coupled to induction of acrosome reactions and that have the anticipated time course and inhibitor sensitivity of LVA  $\text{Ca}^{2+}$  currents.

**V<sub>M</sub> Regulation During Sperm Capacitation.** LVA  $\text{Ca}^{2+}$  currents exhibit voltage-dependent, steady-state inactivation (26, 32). In spermatogenic cells, the threshold and half-maximal voltages for inactivation were  $-80$  and  $-72 \text{ mV}$ , respectively, and currents were not readily activated from holding potentials more positive than  $-60 \text{ mV}$  (6). To assess the availability of LVA channels for activation during ZP3 stimulation, it is necessary to determine sperm  $V_M$ .

$V_M$  was determined from the fluorescence emission of di8-ANEPPS (23, 33). The population-averaged  $V_M$ , derived from values for single cells as reported by this probe, was  $-25.7 \pm 6.6 \text{ mV}$  after 5 min of incubation in capacitating medium (range =  $-45$  to  $-11 \text{ mV}$ ;  $n = 75$ ) and  $-63.4 \pm 17.1 \text{ mV}$  by 90–120 min (range at 120 min,  $-92$  to  $-32 \text{ mV}$ ; Fig. 2A,  $\blacksquare$ ). These estimates from immobilized sperm agree with previous data from motile populations (34–36) suggesting that  $V_M$  is not perturbed by immobilization. In contrast, sperm failed to hyperpolarize during incubation in a 0.1% BSA medium that does not promote efficient capacitation:  $V_M$  was  $-23.6 \pm 4.7 \text{ mV}$  after a 5-min incubation and decreased to only  $-35.3 \pm 6.8 \text{ mV}$  after 120 min (Fig. 2A,  $\square$ ;  $n = 64$ ).

The relationship between hyperpolarization and capacitation was examined in greater detail by monitoring  $V_M$  during a 120-min incubation in a capacitating medium. Sperm were subsequently challenged with ZP extracts or with purified ZP3. These preparations induce acrosome reactions only in capacitated sperm (13). In these experiments, 57% (43/75) of sperm acrosome reacted in response to ZP/ZP3 stimulation, and  $V_M$

of these cells was  $-78.7 \pm 10.2 \text{ mV}$  (range =  $-52$  to  $-92 \text{ mV}$ ;  $n = 43$ ) at 120 min (Fig. 2B,  $\bullet$ ). In contrast,  $V_M$  of the group that failed to acrosome react was  $-43.2 \pm 6.8 \text{ mV}$  (range =  $-32$  to  $-57 \text{ mV}$ ;  $n = 32$ ; Fig. 2B,  $\circ$ ).

These experiments used immobilized sperm, permitting the reconstruction of time-dependent alterations in  $V_M$  in individual cells during incubation. Sperm were grouped according to whether they underwent acrosome reactions when challenged with ZP3 after 120 min of incubation (Fig. 2B,  $\bullet$ ) or failed to do so ( $\circ$ ). Fig. 2B shows a time course for changes in  $V_M$  during incubation. Sperm from both populations had a narrow and overlapping distribution of  $V_M$  values after 5 min of incubation in capacitating medium (Fig. 2B,  $\bullet$ ,  $-25.4 \pm 7.1 \text{ mV}$ ;  $\circ$ ,  $-25.9 \pm 5.9 \text{ mV}$ ). However, sperm segregated into two populations with different  $V_M$  ranges during further incubation: Cells that capacitated by 120 min in this assay system hyperpolarized by  $>50 \text{ mV}$  (Fig. 2B,  $\bullet$ ) whereas cells that failed to capacitate hyperpolarized by  $<20 \text{ mV}$  (Fig. 2B).

The progress of sperm capacitation also was determined by using CTC fluorescence emission. Fig. 2C shows that 61% of sperm capacitated during a 120-min incubation in medium HBCM ( $\blacktriangle$ ). The time course of capacitation, as assessed in this independent assay, parallels membrane hyperpolarization (Fig. 2A and B). In contrast, only 21% of sperm exhibited the CTC fluorescence labeling pattern associated with capacitation during incubation in a low-BSA medium (Fig. 2C,  $\triangle$ ). This limited response parallels the weak hyperpolarization of sperm  $V_M$  that was noted during incubation in the low-BSA medium (Fig. 2A,  $\square$ ).

In addition, we determined the effects of incubation under capacitating conditions on ZP/ZP3-dependent  $\text{Ca}^{2+}_i$  transients. In these experiments, sperm were incubated for 5–120 min in a capacitating medium and subsequently were stimulated with either ZP extracts or with purified ZP3 (Fig. 2D,  $\blacktriangledown$ ).  $\text{Ca}^{2+}_i$  transients were not observed in sperm during the first 30 min of incubation but were apparent after prolonged incubation. A maximal effect, in which  $>60\%$  of sperm exhibited transient responses, was observed by 90 min of incubation ( $t_{1/2} \approx 70 \text{ min}$ ). In contrast,  $\text{Ca}^{2+}_i$  transients were observed in  $<20\%$  of sperm that were incubated for similar time periods in a medium that does not support capacitation (Fig. 2D,  $\triangledown$ ). These experiments demonstrate that ZP3-evoked  $\text{Ca}^{2+}_i$  transients only occur in capacitated sperm populations. Moreover,



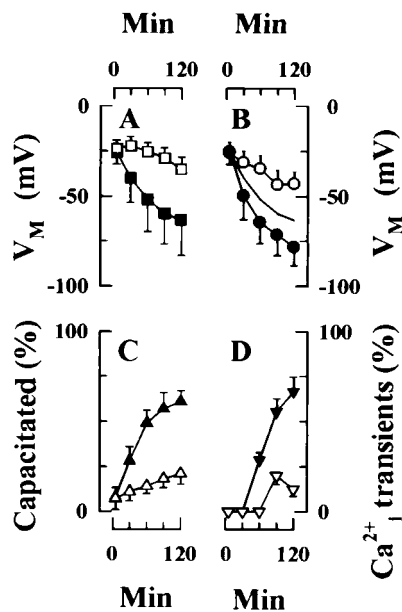


FIG. 2. Capacitation results in membrane hyperpolarization. (A) Hyperpolarization of sperm populations during capacitation.  $V_M$  values of immobilized sperm were calculated from di8-ANEPPS emission of single cells during a 120-min incubation under conditions that do (■, 75 cells) or do not support capacitation (□, 61 cells). Data are the mean ( $\pm$ SD) of 5–8 different experiments and entail determinations from the indicated number of cells. (B) Membrane hyperpolarization is associated with capacitation. Capacitation state of individual sperm was determined by ability of sperm to initiate exocytosis during a subsequent challenge with ZP. Data from 75 cells were summed to assemble population  $V_M$  values (—; redrawn from A), as well as from the subpopulations that are (●) or are not (○) capacitated by 120 min. Strong hyperpolarization is restricted to the subpopulation of sperm that capacitate. (C) The time course of capacitation was determined by CTC fluorescence emission. Motile sperm were incubated in a capacitating medium (▲) or in a medium that does not capacitate mouse sperm efficiently (△). Data are the mean  $\pm$  SD of triplicate experiments, with 200 sperm assayed/time point in each experiment. (D) Sperm develop the ability to generate ZP/ZP3-dependent  $Ca^{2+}_i$  transients during capacitation. Sperm were incubated for the indicated times either in a capacitating medium (▼) or in a noncapacitating medium (▽), as described in C and were stimulated with either ZP (40  $\mu$ g/ml) or ZP3 (10  $\mu$ g/ml), and  $Ca^{2+}_i$  responses were recorded. Data represents mean ( $\pm$ SD) of observations on 3–8 cells, with ZP- and ZP3-stimulated responses pooled for presentation.

this response and membrane hyperpolarization developed during incubation with similar time courses.

To determine whether hyperpolarization is necessary for exocytosis, sperm were incubated under capacitating conditions, and  $V_M$  was adjusted to  $-46.7 \pm 7.2$  mV (range =  $-29$  to  $-54$  mV;  $n = 41$ ) with 30 mM  $K^+_o$ . As shown in Table 1,

Table 1. Effect of  $K^+_o$  on the ZP-dependent acrosome reaction

	Percent of acrosome-reacted sperm	
	30 mM $K^+_o$	3 mM $K^+_o$
+Buffer	15 (10/68)	12 (5/43)
+Ionomycin	71 (76/107)	84 (70/83)
+ZP	20 (17/83)	57 (43/75)

Sperm were incubated in medium HBCM for 120 min; medium  $K^+_o$  was adjusted to the indicated values; sperm were treated with equal volumes of buffer, of ionomycin (5  $\mu$ M final concentration), or of ZP (40  $\mu$ g/ml final protein concentration); and the fraction of acrosome reacted cells was determined after an additional 30-min incubation. Data represent the percent of acrosome-reacted cells as well as the number of acrosome reacted and total sperm examined.

hyperpolarization was required for the induction of acrosome reactions by ZP.

**Effects of Sperm  $V_M$  on Ca Channel Antagonist Action.** Thus,  $V_M$  attains values more negative than  $-75$  mV during capacitation, as measured fluorometrically. This value would be sufficient to relieve steady-state, voltage-dependent inactivation of LVA  $Ca^{2+}$  currents (6, 26). This suggests that LVA channels shift during capacitation from an inactivated state to a closed state that can be subsequently activated during ZP3-evoked depolarization. This hypothesis was tested by using pimozide, an agent that previously was shown to inhibit the inactivated state of spermatogenic cell LVA currents with high affinity but to block the closed state of this channel with lower affinity (9). Sperm were treated with pimozide (0.001–50  $\mu$ M) either after 15 or 90 min of incubation. Two aspects of sperm function that depend on LVA channel activity were measured: ZP-dependent acrosome reactions and fertilization.

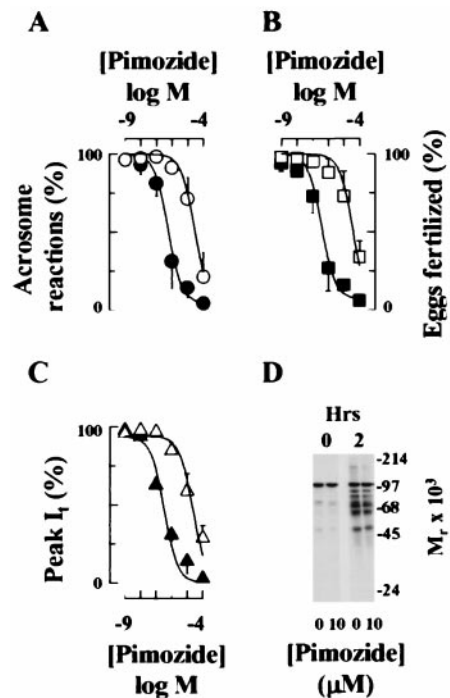


FIG. 3. Effects of capacitation state on the inhibition of ZP3-dependent exocytosis and fertilization by  $Ca^{2+}$  channel antagonists. (A and B) Pimozide was added to sperm either at 15 min (●, ■) or at 90 min (○, □) of a 120-min incubation in a capacitating medium. Sperm subsequently were assayed for the ability to undergo ZP-dependent exocytosis (A, ● and ○) or fertilize eggs *in vitro* (B, ■ and □). Data are the results (mean  $\pm$  SD) of 2–3 separate experiments in which each experiment determined the acrosome reaction status of 200 sperm or the fertilization status of 31–56 eggs. (C) Inhibition of LVA current of a pachytene spermatocyte by pimozide. Germ cells were treated with pimozide at  $-80$  mV (△) or to  $-45$  mV (▲), when channels are predominantly in the closed and inactivated states, respectively.  $V_M$  subsequently was returned to holding potential ( $-90$  mV), and  $Ca^{2+}$  currents were measured during steps to a  $-20$ -mV test potential. Drug was present during prepulse and test pulse intervals. (A–C) Data were corrected for background levels of exocytosis, fertilization, or leak currents and then were approximated by the expression  $R = (100 * IC_{50}) / (IC_{50} + D)$ . These terms are defined in Fig. 1. Values in the absence of pimozide were background exocytosis,  $23 \pm 8\%$ ; exocytosis in ZP-treated sperm,  $74 \pm 9\%$ ; fertilization,  $71 \pm 8\%$ .  $IC_{50}$  values are provided in the text. (D) Pimozide does not inhibit capacitation. Capacitation of sperm populations was assayed by protein tyrosine phosphorylation after a 120-min incubation in capacitating medium. Lanes: 1, uncapacitated sperm, 0 min; 2, uncapacitated sperm, + pimozide, 0 min; 3, sperm incubated for 120 min in capacitating medium; 4, sperm incubated 120 min in capacitating medium containing pimozide.

Exocytosis and fertilization were inhibited with  $IC_{50}$  values of  $0.53 \pm 0.02$  and  $0.38 \pm 0.03 \mu\text{M}$ , respectively, when pimozone was introduced after 15 min of incubation in capacitating medium, (Fig. 3A and B). This efficacy is similar to that for the inhibition of the inactivated state of the LVA current in spermatogenic cells ( $IC_{50} = 0.72 \pm 0.13 \mu\text{M}$ ; Fig. 3C) (9). However, when pimozone was added at 90 min of incubation, it inhibited exocytosis and fertilization with  $IC_{50}$  values of  $28.7 \pm 1.6$  and  $14.1 \pm 3.6 \mu\text{M}$ , respectively (Fig. 3A and B). These values are similar to that with which it inhibits the closed state of the spermatogenic cell LVA current ( $IC_{50} = 14.0 \pm 2.1 \mu\text{M}$ ; Fig. 3C).

This reduced efficacy was not the indirect effect of an inhibition of capacitation, as demonstrated by control studies in which capacitation was determined by CTC fluorescence (data not shown) or by sperm protein tyrosine phosphorylation (Fig. 3D). Additional control experiments addressed the effects of the duration of drug treatment on efficacy. Sperm were incubated in medium HBCM for 90 min, at which time capacitation was maximal (Fig. 2C), and then were treated with pimozone for 105 min. In this protocol, pimozone acted as a low-efficacy inhibitor of the acrosome reactions and fertilization, with  $IC_{50}$  values of  $11.4 \pm 4.2$  and  $8.9 \pm 2.5 \mu\text{M}$ , respectively. Thus, the higher efficacy of inhibition by pimozone when this agent was added at early times during capacitation is not caused by the duration of drug treatment. These data support the hypothesis that LVA channels are predominantly in the inactivated state in uncapacitated sperm but do not remain predominantly in this state after capacitation. Moreover, conductance state-specific antagonists may be selected that are highly efficacious antagonists of LVA channels in uncapacitated sperm.

## DISCUSSION

The two central observations of this study are that (i) transient  $Ca^{2+}_i$  elevations occur during the initial seconds of sperm stimulation by ZP3; and (ii) membrane hyperpolarization during capacitation regulates the ability of sperm to generate transient  $Ca^{2+}_i$  elevations.  $Ca^{2+}_i$  is a mediator of the acrosome reaction that is initiated during contact with eggs. Previously, an essential role of sperm LVA currents in ZP3 signal transduction had been deduced, based on the presence of these currents in spermatogenic cells (6, 10, 11) and on the effects of LVA channel antagonists on acrosome reactions (6). However, it had not been possible to detect LVA currents in sperm because patch clamp recordings are not readily obtained from these cells and because attempts to monitor  $Ca^{2+}_i$  responses associated with LVA channel activity were limited previously by the low temporal resolution ( $\geq 15$  sec) of fluorescence detector systems (7).

We used an optical detector with an estimated time resolution of 2–4 msec to resolve a  $Ca^{2+}_i$  transient during the first seconds of ZP3 stimulation. This transient has the kinetic and pharmacological features anticipated of LVA currents. First, the time courses for activation and inactivation were similar. The time constants for  $Ca^{2+}_i$  transient activation and inactivation, as determined optically, were 10–15 msec and 40–50 msec, respectively. Similar values are observed for LVA currents of rodent spermatogenic cells and of somatic cells by using whole cell-patch clamp methods (6, 26, 31, 32, 37, 38).

Second, structurally unrelated classes of  $Ca^{2+}$  channel antagonists had similar inhibitory effects on the spermatogenic cell LVA current and on the sperm  $Ca^{2+}_i$  transient. For example, pimozone and  $Ni^{2+}$  inhibited peak  $Ca^{2+}_i$  responses to ZP3 with  $IC_{50}$  values of 0.1–1 and 5–50  $\mu\text{M}$  (Fig. 1B), respectively, and these same agents inhibited spermatogenic cell LVA currents with  $IC_{50}$  values of 0.46 and 34  $\mu\text{M}$ , respectively (6, 9). The 1,4-dihydropyridines inhibit LVA currents from many cells, although a wide range of efficacies

are reported. In spermatogenic cells (6, 9), as well in several neuronal preparations and in atrial myocytes (28, 29, 39–41), LVA currents are inhibited by these drugs with  $IC_{50}$  values  $\leq 1 \mu\text{M}$ . The 1,4-dihydropyridine, PN200–110, inhibited both peak  $Ca^{2+}_i$  responses in sperm and LVA current in spermatogenic cells with similar efficacies ( $IC_{50}$  values of 0.1–1 and 0.04  $\mu\text{M}$ , respectively). Finally, the  $Ca^{2+}_i$  transient was more sensitive to inhibition by  $Ni^{2+}$  than by  $Cd^{2+}$ , a characteristic shared by LVA currents but not by most other classes of voltage-sensitive  $Ca^{2+}$  channels (26, 31, 42, 43). These shared kinetic and pharmacological features strongly suggest that the ZP3-evoked  $Ca^{2+}_i$  transient reflects the activation of LVA currents during sperm contact with the ZP.

The rapid  $Ca^{2+}_i$  transients in response to ZP3 stimulation that are documented here were not observed in previous reports. That failure may be attributed to the lower time resolution of the detector systems used in those earlier studies. Instead, we and others had recorded  $Ca^{2+}_i$  responses to ZP3 that developed with a slower time course and were of lower peak amplitude (6, 25, 44–46). This delayed response is inhibited by LVA current antagonists and likely reflects the activation of a downstream  $Ca^{2+}_i$  regulatory mechanism that depends on the LVA current. One proposed mechanism includes the release of sequestered  $Ca^{2+}$  from intracellular stores through an IP<sub>3</sub> receptor pathway (47) and is compatible with the well established modulation of that channel by cytosolic  $Ca^{2+}$  (48, 49).

A signature feature of LVA currents is a low threshold for voltage-dependent inactivation. These currents inactivate at holding potentials between  $-80$  and  $-60$  mV, and currents cannot readily be activated from more positive holding potentials (6, 26, 50, 51). This inactivation range has two implications.

First, it suggests that sperm must maintain a hyperpolarized  $V_M$  during the early stages of interaction with the ZP. Previous  $V_M$  determinations were based on population-averaged signals obtained from sperm suspensions, and the derived values were sufficiently depolarized so as to impose complete voltage-dependent inactivation on LVA currents (7, 34–36). It was observed in those studies that membrane hyperpolarization accompanies capacitation, but the final  $V_M$  was only  $\approx -55$  mV (36) and hence was not sufficient to relieve inactivation. However, the interpretation of those data was complicated by observations that only a portion of sperm populations capacitate *in vitro* (1). In the present study,  $V_M$  of single sperm is estimated and is correlated with capacitation state. These studies confirm that  $V_M$  of uncapacitated sperm is relatively depolarized. Sperm that capacitate *in vitro*, as assessed by both signal transduction-based and fluorescent probe assays, exhibit strong hyperpolarization to a value that is sufficient to relieve voltage-dependent inactivation. In contrast, sperm that fail to capacitate during these incubations exhibit a weak hyperpolarization that will not remove inactivation.

Sperm have only a single secretory vesicle, and exocytosis must be coordinated with egg contact for efficient fertilization (1, 3). The present study supports a mechanism in which the ability of sperm to undergo ZP3-dependent acrosome reactions is regulated by the availability of LVA channels for opening and in which that availability is controlled during capacitation by  $V_M$ . Specifically, membrane depolarization of uncapacitated sperm, with the attendant voltage-dependent inactivation of LVA currents, provides a means of suppressing premature secretion until the completion of capacitation-associated hyperpolarization, at which time sperm have arrived near the site of fertilization and are primed for ZP3-dependent activation. Previous studies indicate that this hyperpolarization is mediated by an increased contribution of  $K^+$  permeability to sperm  $V_M$  (36). In this regard, a large number of biochemical modifications have been described within sperm during capacitation (1, 52), yet the role of such events in the acquisition

of fertility remains poorly understood. The present study represents a case in which a molecular mechanism can account for certain aspects of capacitation.

The recognition of the voltage-dependent inactivation of sperm LVA currents, and the regulation of conductance states during capacitation, also has implications with regard to the design of channel-based antifertility agents. Antagonists that bind to the inactivated state with high affinity, such as pimozone (9), are likely to be most effective when  $V_M$  is relatively depolarized, as occurs in uncapacitated sperm and also may occur during sperm storage within the male (53). Pimozone also inhibits sperm function after capacitation, albeit with lower efficacy. This may be attributable to the fact that transitions between conductance states are stochastic and occur to some extent at all  $V_M$  values (26). In contrast, agents that inhibit conductance by binding to the closed state of germ cell LVA channels with high affinity, such as 1,4-dihydropyridines (9), are expected to inhibit fertilization with high efficacy only after capacitation. The significance of these observations lies not in the specific feasibility of pimozone for such use but, rather, in the demonstration that LVA channel conductance state is regulated as sperm prepare for fertilization and can provide new targets for channel-based antagonists.

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1. Yanagimachi, R. (1994) in *Mammalian Fertilization*, eds. Knobil, E. & Neill, J. D. (Raven, New York), pp. 189–317.
2. Wassarman, P. M. & Florman, H. M. (1997) in *Handbook of Physiology*, eds. Hoffman, J. F. & Jamieson, J. (Oxford Univ Press, New York), Vol. 14, pp. 885–938.
3. Wassarman, P. M. (1999) *Cell* **95**, 176–183.
4. Benoff, S., Cooper, G. W., Hurley, I., Mandel, F. S., Rosenfeld, D. L., Scholl, G. M., Gilbert, B. R. & Hershlag, A. (1994) *Fertil. Steril.* **62**, 606–617.
5. Hershlag, A., Cooper, G. W. & Benoff, S. (1995) *Hum. Reprod.* **10**, 599–606.
6. Arnoult, C., Cardullo, R. A., Lemos, J. R. & Florman, H. M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13004–13009.
7. Arnoult, C., Zeng, Y. & Florman, H. M. (1996) *J. Cell Biol.* **134**, 637–645.
8. Arnoult, C., Lemos, J. R. & Florman, H. M. (1997) *EMBO J.* **16**, 1593–1599.
9. Arnoult, C., Villaz, M. & Florman, H. M. (1998) *Mol. Pharmacol.* **53**, 1104–1111.
10. Hagiwara, S. & Kawa, K. (1984) *J. Physiol.* **356**, 135–149.
11. Lievano, A., Santi, C. M., Serrano, J., Trevino, C. L., Bellve, A. R., Hernandez-Cruz, A. & Darszon, A. (1996) *FEBS Lett.* **388**, 150–154.
12. Santi, C. M., Darszon, A. & Hernandez-Cruz, A. (1996) *Am. J. Physiol.* **271**, C1583–C1593.
13. Ward, C. R. & Storey, B. T. (1984) *Dev. Biol.* **104**, 287–296.
14. Saling, P. M. & Storey, B. T. (1979) *J. Cell Biol.* **83**, 544–555.
15. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P. & Kopf, G. S. (1995) *Development (Cambridge, U.K.)* **121**, 1139–1150.
16. Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P. & Kopf, G. S. (1995) *Development (Cambridge, U.K.)* **121**, 1129–1137.
17. Miller, D. J., Gong, X. & Shur, B. D. (1993) *Development (Cambridge, U.K.)* **118**, 1279–1289.
18. Florman, H. M. & Storey, B. T. (1982) *Dev. Biol.* **91**, 121–130.
19. Bleil, J. D. & Wassarman, P. M. (1980) *Cell* **20**, 873–882.
20. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391**, 85–100.
21. Grynkiwicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450.
22. Konishi, M., Hollingworth, S., Harkins, A. B. & Baylor, S. M. (1991) *J. Gen. Physiol.* **97**, 271–301.
23. Montana, V., Farkas, D. L. & Loew, L. M. (1989) *Biochemistry* **28**, 4536–4539.
24. Go, K. J. & Wolf, D. P. (1985) *Biol. Reprod.* **32**, 145–152.
25. Florman, H. M., Tombes, R. M., First, N. L. & Babcock, D. F. (1990) *Dev. Biol.* **135**, 133–146.
26. Hille, B. (1992) *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA), pp. 1–594.
27. Bleil, J. D. & Wassarman, P. M. (1983) *Dev. Biol.* **95**, 317–324.
28. Akaike, N., Kostyuk, P. G. & Osipchuk, Y. V. (1989) *J. Physiol.* **412**, 181–195.
29. Cohen, C. J. & McCarthy, R. T. (1987) *J. Physiol.* **387**, 195–225.
30. Eneart, J. J., Biagi, B. A., Day, R. N., Sheu, S.-S. & Maurer, R. A. (1990) *J. Biol. Chem.* **265**, 16373–16379.
31. Herrington, J. & Lingle, C. J. (1992) *J. Neurophysiol.* **68**, 213–232.
32. Huguenard, J. R. (1996) *Annu. Rev. Physiol.* **58**, 329–348.
33. Bedlack, R. S., Wei, M.-D. & Loew, L. M. (1992) *Neuron* **9**, 393–403.
34. Babcock, D. F. & Pfeiffer, D. R. (1987) *J. Biol. Chem.* **262**, 15041–15047.
35. Espinosa, F. & Darszon, A. (1995) *FEBS Lett.* **372**, 119–125.
36. Zeng, Y., Clark, E. N. & Florman, H. M. (1995) *Dev. Biol.* **171**, 554–563.
37. Alvarez, J. L. & Vassort, G. (1992) *J. Gen. Physiol.* **100**, 519–545.
38. Coulter, D. A., Huguenard, J. R. & Prince, D. A. (1989) *J. Physiol.* **414**, 587–604.
39. Akaike, N., Kanaide, H., Kuga, T., Nakamura, M., Sadoshima, J.-I. & Tomoike, H. (1989) *J. Physiol.* **416**, 141–160.
40. Richard, E. A. & Lisman, J. E. (1992) *Nature (London)* **356**, 336–338.
41. Takahashi, K. & Akaike, N. (1991) *J. Pharmacol. Exp. Ther.* **256**, 169–175.
42. Bourinet, E., Zamponi, G. W., Stea, A., Soong, T. W., Lewis, B. A., Jones, L. P., Yue, D. T. & Snutch, T. P. (1996) *J. Neurosci.* **16**, 4983–4993.
43. Ertel, S. I. & Ertel, E. A. (1997) *Trends Pharmacol. Sci.* **18**, 37–42.
44. Bailey, J. L. & Storey, B. T. (1994) *Mol. Reprod. Dev.* **39**, 297–308.
45. Florman, H. M. (1994) *Dev. Biol.* **165**, 152–164.
46. Storey, B. T., Hourani, C. L. & Kim, J. B. (1992) *Mol. Reprod. Dev.* **32**, 41–50.
47. Walensky, L. D. & Snyder, S. H. (1995) *J. Cell Biol.* **130**, 857–869.
48. Bezprozvanny, I., Watras, J. & Ehrlich, B. E. (1991) *Nature (London)* **351**, 751–754.
49. Finch, E. A., Turner, T. J. & Goldin, S. M. (1991) *Science* **252**, 443–446.
50. Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R. & Fox, A. P. (1988) *Trends Neurosci.* **11**, 431–438.
51. Tsien, R. W., Lipscombe, D., Madison, D., Bley, K. & Fox, A. (1995) *Trends Neurosci.* **18**, 52–54.
52. Ward, C. R. & Kopf, G. S. (1993) *Dev. Biol.* **158**, 9–34.
53. Hinton, B. T. & Palladino, M. A. (1995) *Microsc. Res. Tech.* **30**, 67–81.