SLO3 K⁺ Channels Control Calcium Entry through CATSPER Channels in Sperm*

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Background: SLO3 and CATSPER are two sperm-specific ion channels.

Results: SLO3 K⁺ channels control Ca²⁺ entry through CATSPER channels.

Conclusion: SLO3 control of CATSPER channel activity involves an intermediary step in which SLO3-dependent hyperpolarization may elicit internal alkalization via a voltage-dependent mechanism.

Significance: Understanding the control of Ca²⁺ entry in sperm is crucial to understanding fertility; this study also reveals an unusual role for a K⁺ channel.

Here we show how a sperm-specific potassium channel (SLO3) controls Ca²⁺ entry into sperm through a sperm-specific Ca²⁺ channel, CATSPER, in a totally unanticipated manner. The genetic deletion of either of those channels confers male infertility in mice. During sperm capacitation SLO3 hyperpolarizes the sperm, whereas CATSPER allows Ca²⁺ entry. These two channels may be functionally connected, but it had not been demonstrated that SLO3-dependent hyperpolarization is required for Ca²⁺ entry through CATSPER channels, nor has a functional mechanism linking the two channels been shown. In this study we show that Ca²⁺ entry through CATSPER channels is deficient in Slō3 mutant sperm lacking hyperpolarization; we also present evidence supporting the hypothesis that SLO3 channels activate CATSPER channels indirectly by promoting a rise in intracellular pH through a voltage-dependent mechanism. This mechanism may work through a Na⁺/H⁺ exchanger (sNHE) and/or a bicarbonate transporter, which utilizes the inward driving force of the Na⁺ gradient, rendering it intrinsically voltage-dependent. In addition, the sperm-specific Na⁺/H⁺ exchanger (sNHE) possesses a putative voltage sensor that might be activated by membrane hyperpolarization, thus increasing the voltage sensitivity of internal alkalization.

To fertilize the egg, the sperm must undergo a process known as capacitation that takes place in the female genital tract but can also be accomplished in vitro by incubating the sperm in defined conditions (1, 2). Capacitation involves a complex series of molecular events that include: protein tyrosine phosphorylation (3), an increase in intracellular pH (pHᵢ) (4–6), an increase in K⁺ permeability (7–11) a hyperpolarization of the plasma membrane (7–11), and an increase in intracellular Ca²⁺ (12–14). As a result of capacitation, sperm acquire a special form of motility known as hyperactivation and the ability to undergo a regulated acrosome reaction. Two major ion channels become active in mouse sperm during capacitation, the SLO3 K⁺ channel and the CATSPER Ca²⁺ channel. Knock-out mutations of either of these channel genes confers male infertility, indicating that both of these channels play a vital role in sperm physiology (15–22). It has been proposed that the elevation of Ca²⁺ necessary to hyperactivate the sperm is primarily driven by the concerted interplay of these two ion channel types (16, 18, 22). However, the functional relationship between the two channels has not yet been demonstrated. Both of these channels are activated by intracellular alkalization, and both are voltage-activated and open upon depolarization (15, 20). Although voltage-sensitive, sperm-specific SLO3 channels can contribute to the resting potential (9) SLO3 channels have relatively high conductance (15), and the opening of only a relatively small number of SLO3 channels at resting potential in response to intracellular alkalization is sufficient to hyperpolarize the sperm membrane (9). We and others have shown that activation of the SLO3 K⁺ channel is the principal mechanism whereby the sperm plasma membrane hyperpolarizes during capacitation (16–18). Supporting this are experiments showing that sperm from the knock-out strain of Slō3 (Slō3⁻/⁻) lack the hyperpolarization that occurs during capacitation and remain depolarized during the process (17, 18). The events that lead to the activation of SLO3 channels and membrane hyperpolarization are complex and varied, but generally, external conditions favorable for capacitation promote internal alkalization (5, 6). Indeed, we have found that sperm encountering an external alkaline environment alone is sufficient to activate SLO3 channels (9), probably by an increase in pHᵢ. The mechanisms that govern pHᵢ in sperm are complex and might differ between species. In sea urchin sperm it has been proposed that membrane hyperpolarization raises intracellular

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pH, probably through a Na\(^{+}\)/H\(^{+}\) exchange (NHE)\(^2\) mechanism (23–25). In mammalian sperm, pH is known to be an important factor regulating initial sperm motility (26–29), capacitation (4–6), and hyperactivation (30–32). Both the acid load during sperm storage and the subsequent alkaline shift after release are required for fertility. During their transit through the female genital tract sperm encounter increasing external pH and higher concentrations of HCO\(_3\)\(^{-}\), and both HCO\(_3\)\(^{-}\) and H\(^{+}\) transporters are considered to play a key role in capacitation and sperm hyperactivation (33). Several types of these transporters have been described in mammalian sperm including the Na\(^{+}\)/H\(^{+}\) exchanger (34–37), a Na\(^{+}\)/Cl\(^{-}\)/HCO\(_3\)\(^{-}\) transporter (4), and a Na\(^{+}\)/HCO\(_3\)\(^{-}\) transporter (38), all of which have been proposed to participate in sperm alkalization during capacitation, and all of which use the energy stored in the Na\(^{+}\) gradient. Transport mechanisms that depend on the stored energy of the Na\(^{+}\) ion gradient rather than ATP are intrinsically voltage-dependent because they rely on the inwardly directed driving force of Na\(^{+}\), which increases with hyperpolarization. In addition, in mouse sperm, a different form of NHE has been described (sperm-specific Na\(^{+}\)/H\(^{+}\) exchanger (sNHE)) that possesses a putative voltage sensor domain that could possibly promote activation of Na\(^{+}\)/H\(^{+}\) exchange upon membrane hyperpolarization (35–37).

It has also been proposed that linear increases observed in pH following increases in extracellular pH might be due to a membrane proton permeability present in several mammalian species (29, 39). In human sperm the proton channel Hv1 has been proposed to play a key role in sperm alkalinization (40). This channel is activated by the combination of the pH gradient and membrane depolarization. In addition, it is proposed that Hv1 may also be activated by the removal of zinc during sperm passage through the female genital tract and by encountering anandamide during sperm penetration through the cumulus oophorus (40, 41). In contrast, in mouse sperm the Hv1 channel may not play as significant a role in the control of intracellular pH. Based on these facts it was proposed that human and mouse sperm control their intracellular pH in different ways (40). However, human sperm also have sNHE that can contribute to sperm alkalinization as well (34). This could be a significant factor as sperm exit the cauda epididymis, where [Na\(^{+}\)] is <25 mM and pH is <7, into the higher [Na\(^{+}\)] and slightly more alkaline conditions present in most of the female reproductive tract (42). As sperm enter this new environment, the driving force of the Na\(^{+}\) gradient increases, which may facilitate proton efflux through sNHE, and hence pH, alkalinization. Thus, it is possible that human sperm may utilize two mechanisms for proton efflux, one of which is shared with mouse. Details of the relative importance of Hv1 and sNHE in human sperm are yet to be established.

In this study we propose a model by which changes in pH produced by different mechanisms may integrate the activity of the two sperm-specific ion channels, SLO3 and CATSPER, to produce an influx of Ca\(^{2+}\) ions. The process may unfold when sperm encounter HCO\(_3\)\(^{-}\) and a more alkaline environment in the female genital tract, both of which may initiate an increase in pH. This may then be responsible for the initial activation of SLO3 channels leading to hyperpolarization, which in turn leads to a further rise in pH via the effect of hyperpolarization on one of the voltage-sensitive mechanisms described above. The increasing alkalinity may then be sufficient to activate CATSPER channels, allowing Ca\(^{2+}\) influx either incrementally at hyperpolarized voltages or in a bolus upon depolarization. Depolarization can occur experimentally by the application of high extracellular K\(^{+}\) or by a more physiological source such as zona pellucida (ZP) (43). The data we present in this study are consistent with this model. We show that SLO3\(^{-/}\) mutant sperm, which lack capacitation-associated hyperpolarization, are also deficient in Ca\(^{2+}\) entry through CATSPER channels. Furthermore, we show that the mutant phenotype of deficient Ca\(^{2+}\) entry can be rescued in SLO3\(^{-/}\) mutant sperm by two different strategies: 1) artificial hyperpolarization using the K\(^{+}\)-specific ionophore valinomycin, and 2) increasing extracellular pH, which results in an increase in intracellular pH. A plausible hypothesis then is that hyperpolarization per se, which increases the inward driving force on Na\(^{+}\), is contributing to intracellular alkalinization, possibly by augmenting the efficiency of H\(^{+}\) export by NHE and/or bicarbonate transport. Supporting this model are experiments we present showing that the treatment of sperm with valinomycin to achieve hyperpolarization in itself elevates internal pH.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Valinomycin (catalog number V0627) and dimethyl sulfoxide (DMSO) (catalog number D8418) were purchased from Sigma. Ionomycin (catalog number 407950) was obtained from Calbiochem (Darmstadt, Germany). Fluo-4 (catalog number F-14201), Pluronic F-127 (catalog number P3000MP), and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; catalog number B-1151) were purchased from Life Technologies.

**Animals**—All procedures described herein were reviewed and approved by the Animals Studies Committee of Washington University (St. Louis, MO) and were performed in accord with the National Institutes of Health Guiding Principles of the care and use of laboratory animals. SLO3 knock-out mice were synthesized by removal of the first two coding exons of the Kcnul gene (TG0050 TIGM). This removed the initiation codon and DNA sequence encoding the first and partial second membrane-spanning domains (17). CatSper1 knock-out mice were obtained from The Jackson Laboratory. CatSper knock-out mice were synthesized by replacing the second exon encoding the first putative transmembrane domain, with an IRES-LacZ sequence followed by a neomycin resistance gene in 129S4/SvJae-derived 11 ES cells (19).

**Sperm Preparation**—Caudal epididymal sperm were collected from ~90-day-old C57BL/6 male breeders from wild-type, SLO3\(^{-/}\) mutant, and CatSper1\(^{-/}\) mutant mice. Minced cauda epididymis from each animal were placed in HS medium (in mM): 135 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgSO\(_4\), 20 HEPES, 5 glucose, 10 lactic acid, 1 Na\(^{+}\) pyruvate in pH 7.4 or as indicated. The swim-up procedure was employed to separate the motile sperm.
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FIGURE 1. Simultaneous recordings of [Ca²⁺]i changes in the head and in the flagellum in a single mouse spermatozoa in response to 50 mM external KCl (pH 8.5) followed by 5 µM ionomycin. A, simultaneous recording shows fluorescent responses in the flagellum and the head, when stimulated with 50 mM KCl, pH 8.5; however, the response is larger and slightly more sustained in the head. B, fluorescent responses in the flagellum and the head of sperm were normalized to better illustrate a tail-to-head propagation of the response stimulated by 50 mM KCl; note that, as reported previously (46), the onset of the fluorescent change in the flagellum (black trace) slightly precedes the onset of the change in the head (red trace). A similar delay was not observed when the Ca²⁺ ionophore ionomycin was used to trigger [Ca²⁺]i, at the end of the experiments. Similar results were reported by Xia et al. (46).

fraction of the sperm sample. To capacitate the sperm, we supplemented HS medium with 5 mg/ml BSA and 15 mM NaHCO₃ and incubated for 60 min at 37 °C. For some experiments, we used solubilized ZP from homogenized ovaries of 60-day-old virgin females obtained according to Ref. 44.

Ca²⁺ Imaging—Motile cells were incubated with 2 µM Fluo-4 AM and 0.05% Pluronic F-127 in HS non-capacitated medium (lacking BSA or NaHCO₃) for 30 min at 37 °C. Immediately after, sperm were washed once at 1,500 rpm during 5 min and were resuspended in HS non-capacitated media. Once loaded, sperm were attached to laminin (1 mg/ml)-coated coverslips and incubated 15 min to permit attachment. For capacitation, attached sperm were incubated in complete media (5 mg/ml BSA and 15 mM NaHCO₃) for 60 min. Non-capacitated sperm were incubated with 15 mM NaHCO₃ for 10 min before the recordings (45). Sperm were incubated with HS alone for control experiments or with 1 µM valinomycin for 15 min. A local perfusion device with an estimate exchange time of <0.5 s applied various test solutions, and recordings were started at least 2 min prior to the addition of 50 mM KCl, solubilized ZP, or changes in external pH. Ionomycin (5 µM) was added at the end of the recordings as a control stimulus. A DG4 combination light source/excitation filter wheel switcher (Sutter Instruments, Novato, CA) with a 175-W Xenon lamp was used to generate the excitation at 488 nm. A 63X oil objective on an inverted microscope (Zeiss Axiosvert 200) was used for imaging. Emissions bandwidth (515–565 nm) were band-pass-filtered (Lambda 10-2 emission filter wheel switcher; Sutter Instruments, Novato, CA), and images were collected with a Cascade 512B CCD camera (Photometrics, Tucson, AZ) (30 ms every 2 s). Online control and data collection were done using SlideBook 5.0 software (Intelligent Imaging Innovations, Boulder, CO). Images were analyzed using ImageJ software (version 1.48, National Institute of Health) and Origin 6 (MicroCal Software, Northampton MA). The Fluo-4 [Ca²⁺]i changes are presented as (F – F₀₀)/F₀₀ after background subtraction.

All the imaging experiments were done at room temperature. Motile sperm that were attached to the coverslip mainly by the head were used for the analysis. Images were obtained from the head region of the sperm. It has been previously shown (46) that although CATSPER channels are located in the principal piece of the flagellum, calcium influx through CATSPER channels propagates from the sperm tail to the head within seconds. Our results shown in Fig. 1 confirm these findings and show a similar [Ca²⁺]i increase in the flagellum and the head of an individual sperm upon alkaline depolarization. Both regions of the sperm responded in a similar way, but with a larger and delayed response in the head. Because Ca²⁺ responses are larger in the head, and laminin principally attached sperm at the head while the flagellum remained motile, we conducted our Ca²⁺ measurements in sperm heads. Cells with peak [Ca²⁺]i changes of >10% relative to the ionomycin changes were counted as responsive.

pH Imaging—Motile cells were loaded with 0.5 µM BCECF-AM (BCECF with acetoxymethyl ester) in non-capacitated medium during 10 min at 37 °C. Cells were washed once at 1,500 rpm during 5 min and resuspended in non-capacitated media without BCECF. For the recordings, cells were attached to laminin (1 mg/ml)-coated coverslips and were excited with a stroboscopic LED-based fluorescence illumination system as described previously (47) with 1-ms light excitation pulses, recording one image every 2 s. Fluorescence was captured with CoolSNAP (Photometrics). Images were obtained from the sperm head and analyzed using ImageJ software version 1.48v (National Institutes of Health) and Origin 6 (MicroCal Software). The BCECF pH₁ changes are presented as (F – F₀₀)/F₀₀ after background subtraction.

RESULTS

 Increases in Internal Ca²⁺ Can Be Evoked by Alkaline Depolarization in Both Wild-type and Slo3−/− Mutant Sperm—Previous studies have shown that alkaline depolarization (high KCl at pH 8.5) triggers an increase in intracellular Ca²⁺ in non-capacitated sperm (45). CATSPER channels are opened by both depolarization and intracellular alkaline pH₁ (20), and it has been shown that depolarization in an extracellular alkaline medium produces intracellular calcium ([Ca²⁺]i) increases.
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through CATSPER channels (21, 46). This is apparently due to the combination of depolarization and the increase in pH, that results from a change in extracellular pH (29). This increase in \([\text{Ca}^{2+}]\), triggered by external alkaline depolarization is absent in sperm from \textit{CatSper} \(-/-\) mutant knock-out mice (21, 46). To determine whether this internal rise in \([\text{Ca}^{2+}]\) in response to alkaline depolarization also occurred in Slo3 knock-out mice (Slo3 \(-/-\)), we undertook a series of similar experiments in both Slo3 \(-/-\) mice and wild-type mice as a control. In these experiments a similar increase in \([\text{Ca}^{2+}]\), was found in virtually 100% of sperm from both Slo3 \(-/-\) mice and wild-type mice (Fig. 2A). Thus, in alkaline depolarizing conditions, there seems to be no dependence of CATSPER channels on the activity of SLO3 channels. This indicates that either CATSPER channels are not dependent on the activity of SLO3 channels or the conditions of alkaline depolarization bypass the need for SLO3 channels. The following experiments suggest that this latter interpretation is correct and that the activity of SLO3 channels is essential for the internal rise of intracellular \([\text{Ca}^{2+}]\) during sperm capacitation.
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Increases in \([\text{Ca}^{2+}]\), Depend on SLO3 Channel Activity after Capacitation—It has previously been shown that alkaline depolarization triggers \([\text{Ca}^{2+}]\) entry through CATSPER channels (19, 21), and it was proposed that alkaline depolarization causes an increase in pH, (29, 48). This experiment is reproduced in Fig. 2A. To show the dependence of the rise in internal \([\text{Ca}^{2+}]\) on high extracellular K⁺, similar experiments were undertaken with high extracellular K⁺ applied at neutral pH. In these experiments a rise in \([\text{Ca}^{2+}]\) was not seen in most (>94%) sperm from either wild-type or Slo3⁻/⁻ mice (Fig. 2B). As in Fig. 2A, these experiments were undertaken in non-capacitated sperm where internal pH is reported to be low (−6.5 (4, 5)).

After being subjected to capacitating conditions, however, it is known that internal pH becomes more alkaline in wild-type sperm (4–6). Hence, after being subject to capacitating conditions, sperm from both wild-type and Slo3⁻/⁻ mice were again treated with high extracellular K⁺ applied this time at neutral pH. In these experiments (Fig. 2C) wild-type and Slo3⁻/⁻ mutant sperm reacted differently; ~60% of wild-type sperm reacted with a robust increase in \([\text{Ca}^{2+}]\), whereas only about 2% of Slo3⁻/⁻ mutant sperm responded. Additionally, these few mutant respondents showed a \([\text{Ca}^{2+}]\) increase, which was about half that of wild-type. (Actual values and statistics for these and other experiments can be found in the figure legends, and Table 1 provides additional information.) The fact that only ~60% and not 100% of wild-type sperm reacted with an increase in \([\text{Ca}^{2+}]\), in response to high external KCl or ZP may be related to the fact that, in a population of sperm subjected to capacitated conditions, not all the sperm achieve a capacitated state (10, 49).

Thus, SLO3 channels appear to play a key role in the activation of CATSPER channels. As a control to verify that \([\text{Ca}^{2+}]\) entry in capacitated wild-type sperm subjected to KCl depolarization at neutral pH was through CATSPER channels, we repeated the same experiment as shown in Fig. 2C with sperm from the CatSper knock-out mutant (CatSper⁻/⁻) and observed a negative result (Fig. 3B). Thus, there is a failure of \([\text{Ca}^{2+}]\) entry in both Slo3⁻/⁻ mutant sperm (Fig. 2C, right) and CatSper⁻/⁻ mutant sperm (Fig. 3B) stimulated in the same way. This result suggests a functional relationship between the SLO3 and CATSPER ion channels. In Fig. 2C (right) there appears to be a failure of SLO3 to facilitate \([\text{Ca}^{2+}]\) entry through CATSPER channels, whereas in Fig. 3B the CATSPER channel is simply not present.

One explanation for the result in Fig. 2C could be that Slo3⁻/⁻ sperm fail to undergo internal alkalization when subjected to capacitating conditions. However, we (9, 11, 17) and other laboratories (16, 18) have shown that a primary role of SLO3 channels is to evoke sperm membrane hyperpolarization when exposed to capacitating conditions. Thus, membrane hyperpolarization could be a factor that itself substitutes for an increase in intracellular pH, or SLO3-dependent membrane hyperpolarization could be involved in the mechanism by which internal alkalization occurs during capacitation. To test whether membrane hyperpolarization is an essential step, we conducted experiments to evoke membrane hyperpolarization in a manner that bypasses the activation of SLO3 channels. These experiments are shown in Fig. 2D where membrane hyperpolarization was achieved by pretreatment of both non-capacitated wild-type and Slo3⁻/⁻ sperm with the K⁺ ionophore valinomycin, which is known to hyperpolarize the sperm cell membrane. The results of these experiments (Fig. 2D) were similar to those in Fig. 2A, where an increase in \([\text{Ca}^{2+}]\), was found in ~60% of sperm from both Slo3⁻/⁻ mice and wild-type mice. The similarity of responses in Fig. 2A, where elevated pH appears to be a key factor, and Fig. 2D, where a period of hyperpolarization appears to be a relevant factor, suggests that either elevated pH or membrane hyperpolarization can substitute for...

**TABLE 1**

|                     | % of responsive cells | Response amplitude* |
|---------------------|-----------------------|---------------------|
| Wild-type NC-K8.6   | 96.6 ± 1.9 (85/88) (4 mice) | 26.2 ± 1.2 |
| Wild-type NC-KCl    | 5.95 ± 1.8 (10/168) (6 mice) | 14.5 ± 0.96 |
| Wild-type Cap-KCl   | 61.3 ± 4.5 (73/119) (7 mice) | 29.9 ± 1.76 |
| Wild-type NC-volatile-KCl | 65.5 ± 4.5 (74/133) (7 mice) | 32.8 ± 2.15 |
| Wild-type NC-ZP     | 4.6 ± 2.2 (4/87) (4 mice) | 15.0 ± 2.9 |
| Wild-type Cap-ZP    | 66.7 ± 5.7 (36/69) (4 mice) | 32.8 ± 3.3 |
| Wild-type NC-volatile-ZP | 56 ± 5.9 (40/72) (3 mice) | 47.4 ± 4.7 |
| Slo3⁻/⁻ NC-K8.6    | 99 ± 1.9 (96/98) (3 mice) | 35.5 ± 1.3 |
| Slo3⁻/⁻ NC-KCl     | 2.8 ± 1.4 (4/143) (6 mice) | 13.95 ± 1.3 |
| Slo3⁻/⁻ Cap-KCl    | 1.72 ± 1.2 (2/116) (6 mice) | 15.7 ± 3.2 |
| Slo3⁻/⁻ NC-volatile-KCl | 62.5 ± 4.4 (74/120) (5 mice) | 47.2 ± 2.7 |
| Slo3⁻/⁻ NC-ZP      | 3.2 ± 1.8 (3/95) (5 mice) | 11.7 ± 1.65 |
| Slo3⁻/⁻ Cap-ZP     | 15.8 ± 3.4 (20/118) (5 mice) | 25 ± 3.1 |
| Slo3⁻/⁻ NC-volatile-ZP | 81 ± 3.6 (98/121) (4 mice) | 38.6 ± 2.39 |

*Response amplitude is [\text{Ca}^{2+}] increase relative to the ionomycin-induced [\text{Ca}^{2+}] increase.

**FIGURE 3.** \([\text{Ca}^{2+}]\) increases evoked by application of 50 mM KCl in capacitated WT sperm, or in non-capacitated sperm pretreated with valinomycin, are due to \([\text{Ca}^{2+}]\) influx through CATSPER channels. A, WT capacitated positive control from Fig. 2C.  
B, capacitated CatSper⁻/⁻ cells show no \([\text{Ca}^{2+}]\) response. Mean [\text{Ca}^{2+}], traces of n = 5 representative capacitated CatSper⁻/⁻ sperm. As the figure indicates, in both [\text{Ca}^{2+}], increases were observed in n = 119 CatSper⁻/⁻ cells tested (0/119 sperm cells, 3 mice), C, WT non-capacitated positive control treated with 1 μM valinomycin from Fig. 1D. D, non-capacitated CatSper⁻/⁻ mutant cells treated with valinomycin show no \([\text{Ca}^{2+}]\) response. Mean [\text{Ca}^{2+}], traces of n = 5 representative CatSper⁻/⁻ sperm pretreated with 1 μM valinomycin are shown. A tiny fraction (3.2%) of CatSper⁻/⁻ sperm = 3/63 sperm cells, 3 mice) showed an unexplained non-representative significant increase in [\text{Ca}^{2+}].
one another; on the other hand membrane hyperpolarization may be a functional step required for the intracellular rise in pH. We show in a following section that this latter suggestion appears to be correct.

As a control to substantiate that Ca\(^{2+}\) entry in these experiments is through CATSPER channels, we showed that CatSper\(^{-/-}\) mutant sperm cannot be rescued by membrane hyperpolarization achieved by pretreatment with the K\(^{+}\) ionophore valinomycin (Fig. 3D). This failure of hyperpolarization to rescue sperm lacking the CATSPER channel suggests that hyperpolarization acts by facilitating the activity of CATSPER channels.

An Increase in [Ca\(^{2+}\)]\(_{i}\) in Response to ZP Application Is Also Deficient in Slo3\(^{-/-}\) Mutant Sperm—The ZP is a glycoprotein membrane surrounding the plasma membrane of an oocyte. It has been shown that ZP, as well as high external KCl, can trigger sperm membrane depolarization (43). It has also been shown that ZP triggers calcium influx through CATSPER channels (50). Thus, to investigate whether ZP would have a different effect on Slo3\(^{-/-}\) mutant sperm relative to wild-type sperm with regard to Ca\(^{2+}\) entry, we undertook similar experiments with ZP as we had done with high external KCl (51). First we applied ZP at neutral pH to both non-capacitated wild-type and Slo3\(^{-/-}\) mutant sperm as had been done with KCl in Fig. 2B. The results of these experiments (Fig. 4A) were identical to those in Fig. 2B; a rise in [Ca\(^{2+}\)]\(_{i}\) was not seen in sperm from either wild-type or Slo3\(^{-/-}\) mice (Fig. 4A). However, after being subjected to capacitating conditions, sperm from wild-type and Slo3\(^{-/-}\) mice reacted differently with ZP. ZP triggers an increase in [Ca\(^{2+}\)]\(_{i}\), in 66.7 ± 5.7% (46/69 sperm cells, 4 mice) of WT sperm, whereas only 15.8 ± 3.4% (20/118 sperm cells, 5 mice) of capacitated Slo3\(^{-/-}\) mutant sperm respond. The mean [Ca\(^{2+}\)]\(_{i}\) traces are shown for n = 13 representative WT sperm cells and n = 27 representative Slo3\(^{-/-}\) sperm cells. Error bars = S.E.

FIGURE 4. Intracellular Ca\(^{2+}\) Increases in Response to ZP. A, the application of ZP does not induce [Ca\(^{2+}\)]\(_{i}\), increases in either WT or Slo3\(^{-/-}\) non-capacitated sperm. 95.4 ± 2.2% of WT sperm (83/87 sperm cells, 4 mice), and 96.8 ± 1.8% of Slo3\(^{-/-}\) mutant sperm (92/95 sperm cells, 5 mice), did not show a significant [Ca\(^{2+}\)]\(_{i}\) increase in response to ZP. The mean [Ca\(^{2+}\)]\(_{i}\) traces are from n = 17 representative WT cells (left panel) and n = 18 representative Slo3\(^{-/-}\) sperm cells (right panel). Iono, ionomycin. Error bars = S.E. B, ZP induces an increase in [Ca\(^{2+}\)]\(_{i}\), in WT capacitated sperm but not in Slo3\(^{-/-}\) mutant capacitated sperm. ZP triggers an increase in [Ca\(^{2+}\)]\(_{i}\), in 66.7 ± 5.7% (46/69 sperm cells, 4 mice) of WT sperm, whereas only 15.8 ± 3.4% (20/118 sperm cells, 5 mice) of capacitated Slo3\(^{-/-}\) mutant sperm respond. The mean [Ca\(^{2+}\)]\(_{i}\) traces are from n = 7 representative WT cells and n = 9 representative Slo3\(^{-/-}\) cells. Error bars = S.E. C, pretreatment with the K\(^{+}\) ionophore valinomycin rescues the Ca\(^{2+}\) responses. The application of 50 mM KCl (pH 7.4) in non-capacitated WT sperm pretreated with 1 \(\mu M\) valinomycin induces a [Ca\(^{2+}\)]\(_{i}\) increase in 56 ± 5.9% (40/72 sperm cells, 3 mice) and also induces an increase in [Ca\(^{2+}\)]\(_{i}\), in 81 ± 3.6% of the Slo3\(^{-/-}\) mutant cells (98/121 sperm cells, 4 mice). Mean [Ca\(^{2+}\)]\(_{i}\) traces are shown for n = 13 representative WT sperm cells and n = 27 representative Slo3\(^{-/-}\) sperm cells. Error bars = S.E.
mycin (Fig. 4C); sperm so treated where then subjected to ZP stimulation. In these experiments a similar robust increase in internal [Ca\(^{2+}\)] was seen in sperm from both Slo3\(^{-/-}\) mice and wild-type mice.

Both Fig. 2 and Fig. 4 suggest the same conclusion: a period of hyperpolarization prior to depolarization is important for Ca\(^{2+}\) ion entry through CATSPER channels. The hyperpolarization can arise either from artificially applied conditions or from a physiological source; the application of the K\(^+\) ionophore, valinomycin, to impose hyperpolarization is equally effective as when hyperpolarization results from the activation of SLO3 channels during capacitation. This result presents a conundrum because the evidence suggests that Ca\(^{2+}\) is entering through CATSPER channels, and there is no evidence that hyperpolarization directly activates or facilitates CATSPER channels. On the other hand hyperpolarization may be acting indirectly to enable CATSPER channel activity, possibly by facilitating intracellular alkalization. This is a plausible hypothesis because sodium-hydrogen exchangers (Na\(^+/\)H\(^+\) exchangers), which regulate intracellular pH, are ATP-independent and rely on inwardly directed Na\(^+\) ion electrochemical driving force to transport protons out of the cell. Hence, the system is inherently voltage-dependent and facilitated by hyperpolarization, which increases the driving force on Na\(^+\) ion.

One way to test whether the increased inward driving force on Na\(^+\) ion is a key factor that comes into play during membrane hyperpolarization is to eliminate it. We previously showed (9, 52) that sperm undergo membrane hyperpolarization when external Na\(^+\) ion is reduced (and replaced with choline). In this condition sperm membrane hyperpolarization occurs while simultaneously eliminating the inward driving force on Na\(^+\). Thus, we undertook the experiments to test the effectiveness of reducing external Na\(^+\) in evoking a rise in [Ca\(^{2+}\)], (Fig. 5). As we had done in Fig. 2D where we hyperpolarized the sperm membrane with valinomycin, we then depolarized the cells by applying high external K\(^+\). However, unlike the results when cells were hyperpolarized with valinomycin, cell depolarization with high K\(^+\) did not result in any detectable rise in [Ca\(^{2+}\)], in any of the cells tested (n = 30 for wild-type sperm, n = 27 for Slo3\(^{-/-}\) mutant sperm). This result suggested that the inward driving force of Na\(^+\) could be a key factor during hyperpolarization and adds weight to the hypothesis that sperm membrane hyperpolarization may facilitate intracellular alkalization, which in turn would facilitate the activation of CATSPER channels. Finally, we also show that the treatment of sperm with valinomycin to achieve hyperpolarization in itself elevates intracellular pH (Fig. 6). Hyperpolarization with valinomycin, unlike hyperpolarization by lowering external Na\(^+\), leaves the driving force of Na\(^+\) undiminished, and we previously showed that it effectively promotes Ca\(^{2+}\) entry (Figs. 2D and 4C). It follows that if hyperpolarization promotes Ca\(^{2+}\) entry through CATSPER channels and the mechanism by which this occurs is through the increased Na\(^+\) driving force facilitating Na\(^+\)/H\(^+\) exchange and/or bicarbonate transport, then valinomycin should promote internal alkalinity. Fig. 6 shows that this appears to be the case.

DISCUSSION

There are at least two examples in the literature where pH, may be regulated by K\(^+\) channels. In sea urchin sperm, membrane hyperpolarization raises intracellular pH, probably through a voltage-sensitive NHE mechanism (23–25). In this instance, as in the example we are now reporting, the K\(^+\) channel type involved appears to have acquired unusual properties as a result of evolutionary pressure unique to reproductive physiology. On the other hand, an example of K\(^+\) channel involvement in the control of pH in somatic rather than reproductive physiology employs K\(^+\) channel types found in many somatic tissues (53). In this example where hydrochloric acid is produced in parietal cells of the gastric glands, a proton pump couples the outward movement of H\(^+\) to the inward movement of K\(^+\). To maintain the activity of the pump, K\(^+\) is recirculated over the apical membrane via KCNQ1/KCNE2 K\(^+\) channels. In our current study we show a significant role for both hyperpolarization and internal alkalization on Ca\(^{2+}\) entry into sperm and suggest a mechanism of interplay between the two physiological processes. Interestingly, a similar role of hyperpolarization in Ca\(^{2+}\) influx shown here for mouse sperm has also been reported for sea urchin sperm (25); in addition, the importance
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of internal alkalization on Ca²⁺ entry was also reported for sea urchin sperm (54).

It has been previously shown by our group and others that SLO3 K⁺ channels are key players in sperm physiology. The SLO3 knock-out mice are male infertile and show deficits both in hyperactivated motility and in the acrosome reaction (17, 18), both of which require Ca²⁺ entry. Slo3⁻/⁻ mutant sperm fail to hyperpolarize during the capacitation process (17, 18). It has been suggested that hyperpolarization is probably necessary for the activation of CATSPER channels and that these two channels work together during capacitation to confer sperm hyperactivated motility (16), but the functional relationship between SLO3 and CATSPER channels has not been shown until now. In this study we established that the hyperpolarization produced by SLO3 channel activation during capacitation is crucial for Ca²⁺ influx through CATSPER channels. Our results indicate that the influx of Ca²⁺ through CATSPER channels into the cell requires a period of membrane hyperpolarization prior to membrane depolarization induced by high external KCl or ZP. However, CATSPER channels are Ca²⁺-permeable channels weakly activated by depolarization and strongly activated by intracellular alkalization and do not show voltage-dependent inactivation (20). Thus, the requirement for hyperpolarization prior to CATSPER channel activation is not obvious, nor is the relationship between SLO3 channel activation and the resulting hyperpolarization, and the activation of CATSPER channels and Ca²⁺ influx is straightforward. It was previously suggested that hyperpolarization might increase Ca²⁺ influx through CATSPER channels due to an increase in Ca²⁺ driving force (16, 18, 22). However, this appears to be irrelevant in the current experiments because we are testing Ca²⁺ entry upon depolarization.

In experiments to reveal the role of SLO3 channels in activating CATSPER channels, we show that the Ca²⁺ increase that fails to occur in capacitated Slo3⁻/⁻ mutant sperm in response to high KCl can be restored by two different experimental treatments: 1) by increasing extracellular pH, and 2) by hyperpolarizing the sperm plasma membrane with the K⁺ ionophore valinomycin. It was previously shown in wild-type sperm that the application of high external KCl in the presence of high external pH can trigger Ca²⁺ responses through CATSPER channels, possibly by an increase in pH (21, 48, 55). Our results showing that Ca²⁺ responses can also be obtained in Slo3⁻/⁻ sperm under these conditions suggest that external alkalinity can bypass the need for hyperpolarization by increasing pHₗ.

On the other hand hyperpolarization of the sperm plasma membrane by a K⁺ ionophore (valinomycin) can also rescue the Ca²⁺ responses in Slo3⁻/⁻ sperm. One possible conclusion from these experiments is that the necessary period of hyperpolarization prior to depolarization is indirectly rather than directly affecting CATSPER channels. Taking all these results into consideration, a plausible unifying principle underlying these phenomena could be that the high external pH and/or high concentrations of HCO₃⁻ that sperm encounter in the female genital tract could trigger an initial change in pHₗ and activation of SLO3 channels; the resulting membrane hyperpolarization, which increases the Na⁺ inward driving force, may then, in turn, further increase pHₗ by increasing the activity of the Na⁺/H⁺ exchanger or other Na⁺-dependent mechanisms controlling intracellular pH. This can result in a positive feedback loop as the increase in pHₗ further activates SLO3 channels, leading to further hyperpolarization. Supporting this model are several mechanisms present in sperm that may confer voltage dependence to sperm intracellular alkalization. An sNHE was identified in mice that contains a putative voltage sensor domain that theoretically could confer direct voltage sensitivity to proton export (35–37). sNHE null mice are infertile and show defects both in hyperactivated motility and in the acrosome reaction (37). The Na⁺ dependence of sNHE and the HCO₃⁻ transport systems, which are powered by the energy stored in the inward Na⁺/H⁺ ion gradient, furthermore suggests indirect mechanisms by which internal alkalization can be voltage-sensitive (4, 33, 38).

Our results showing that the application of valinomycin induces a pHₗ increase in 50% of wild-type sperm tested supports the hypothesis that intracellular pHₗ is voltage-dependent. This further supports the model that SLO3-elicted hyperpolarization and the elevation of pHₗ might work hand-

![FIGURE 6. pH increase in response to the addition of the K⁺ ionophore valinomycin. Results of two separate experiments from different mice are shown. A, mean representative pH traces of n = 6 wild-type sperm in the presence of 1 μM valinomycin and 10 mM NH₄Cl. B, mean representative traces of n = 4 wild-type sperm. 10/20 sperm tested responded to the application of valinomycin with an increase in fluorescence.](image-url)
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in-hand with one factor augmenting the other in a positive feedback loop. The control of pH, and activation of CATSPER channels might be different in human sperm where the proton channel Hv1 has been proposed to be responsible for human sperm intracellular alkalization (40). A H+ current carried by Hv1 was characterized using the whole-cell patch clamp technique and has been proposed as the major mechanism to produce intracellular alkalization in human sperm. This channel is activated in sperm by membrane depolarization and by alkaline extracellular pH and micromolar concentrations of anandamide (40). Although these currents are prominent in human sperm, their physiological relevance is not completely established yet. The Hv1 currents are activated when sperm membrane potential is set to values close to or greater than 0 mV, and it is not yet known whether sperm can generate these potentials under physiological conditions. It has been proposed that an as yet unidentified ligand-gated cation channel may provide such depolarization (41). Whether such a mechanism for depolarization exists in human sperm remains to be explored. It was recently shown by our group that the human sperm membrane potential does not depolarize during capacitation, as might be required for activation of Hv1, but instead hyperpolarizes (56). Nevertheless, the augmentation of Hv1 channel activity by anandamide and alkaline extracellular pH may be key factors in the physiological relevance of this channel and require further study. Hence, despite elegant patch clamp studies clearly suggesting the ability of Hv1 to function as an acid extrusion mechanism in human sperm, it is too early to say the extent to which mouse and human sperm differ with regard to the control of intracellular pH. It is worth noting that human sperm, like murine sperm, also have an sNHE transporter that, like murine sNHE, also contains a putative voltage sensor (34, 37, 57). Because the patch clamp technique cannot detect transmembrane transport mechanisms, such as non-electrogenic transporters, it is conceivable that other mechanisms similar to those in mouse (like the Na+/H+ exchanger) contribute to human sperm acid extrusion, pH regulation, and CATSPER activation in human sperm. Finally, the principal K+ channel active in human sperm is a subject of debate, with one group presenting evidence that the Ca2+-dependent SLO1 channel is the principle player in human sperm (58), whereas a second group, writing in the same journal shortly afterward, presented evidence that it is the sperm-specific SLO3 channel, as in mouse sperm (59). Because of the fundamental differences between these two channels, verification that it is SLO1, SLO3, or both is necessary to better understand the physiology of human sperm.

In summary, we have shown that SLO3 channels are essential for Ca2+ entry through CATSPER channels during the capacitation process and that the mechanism of CATSPER activation appears to be indirect and may involve a voltage-dependent change in intracellular pH. Conceivably, because SLO3 channels are themselves activated by alkaline pH, they may go hand-in-hand with the sNHE to increase pH, functioning together as a positive feedback system. Significantly, both of these factors (an intracellular rise in pH and membrane hyperpolarization) are known intracellular changes that occur in sperm during capacitation (4–11). Our earlier findings, that sperm encountering an alkaline external environment, as exists in the female genital tract, can activate SLO3 channels (9) may have revealed the trigger for this positive feedback cascade.

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REFERENCES

1. Chang, M. C. (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168, 697–698
2. Austin, C. R. (1951) Observations on the penetration of the sperm in the mammalian egg. Aust. J. Sci. Res. B 4, 581–596
3. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121, 1139–1150
4. Zeng, X., Oberdorf, J. A., and Florman, H. M. (1996) pH regulation in mouse sperm: identification of Na+/H+, Cl–, and HCO3– dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. Dev. Biol. 173, 510–520
5. Vredenburgh-Wilberg, W. L. and Parrish, J. J. (1995) Intracellular pH of bovine sperm increases during capacitation. Mol. Reprod. Dev. 40, 490–502
6. Breitbart, H. (2003) Signaling pathways in sperm capacitation and acrosome reaction. Cell. Mol. Biol. 49, 321–327
7. Zeng, Y., Clark, E. N., and Florman, H. M. (1995) Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev. Biol. 171, 554–563
8. Santi, C. M., Orta, G., Salkoff, L., Visconti, P. E., Darson, A., and Treviño, C. L. (2013) K+ and Cl– channels and transporters in sperm function. Curr. Top. Dev. Biol. 102, 385–421
9. Chávez, J. C., de la Vega-Beltrán, J. L., Escoffier, J., Visconti, P. E., Treviño, C. L., Darson, A., Salkoff, L., and Santi, C. M. (2013) Ion permeabilities in mouse sperm reveal an external trigger for SLO3-dependent hyperpolarization. PLoS One 8, e60578
10. Arnout, C., Kazam, I. G., Visconti, P. E., Kopf, G. S., Villaz, M., and Florman, H. M. (1999) Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc. Natl. Acad. Sci. U.S.A. 96, 6757–6762
11. Muñoz-Garay, C., De la Vega-Beltrán, J. L., Delgado, R., Labarca, P., Felix, R., and Darson, A. (2001) Inwardly rectifying K+ channels in spermatogenic cells: functional expression and implication in sperm capacitation. Dev. Biol. 234, 261–274
12. DasGupta, S., Mills, C. L., and Fraser, L. R. (1993) Ca2+-related changes in the capacitation state of human spermatozoa assessed by a chlorotetracycline fluorescence assay. J. Reprod. Fertil. 99, 135–143
13. Baldi, E., Casano, R., Falsetti, C., Krausz, C., Maggi, M., and Forti, G. (1991) Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J. Androl. 12, 323–330
14. Suarez, S. S., Varosi, S. M., and Dai, X. (1993) Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. Proc. Natl. Acad. Sci. U.S.A. 90, 4660–4664
15. Schreiber, M., Wei, A., Yuan, A., Gauth, J., Saito, M., and Salkoff, L. (1998) Slo3, a novel pH-sensitive K+ channel from mammalian spermatoocytes. J Biol Chem 273, 3509–3516
16. Navarro, B., Kirschok, Y., and Clapham, D. E. (2007) KSper, a pH-sensitive K+ channel from mammalian spermatocytes. J Biol Chem 273, 3509–3516
17. Santi, C. M., Martínez-López, P., de la Vega-Beltrán, J. L., Butler, A., Alisio, A., Darson, A., and Salkoff, L. (2010) The Slo3 sperm-specific potassium channel plays a vital role in male fertility. FEBS Lett. 584, 1041–1046
18. Zeng, X. H., Yang, C., Kim, S. T., Lingle, C. J., and Xia, X. M. (2011) Deletion of the Slo3 gene abolishes alkalization-activated K+ current in mouse spermatozoa. Proc. Natl. Acad. Sci. U.S.A. 108, 5879–5884
19. Ren, D., Navarro, B., Perez, G., Jackson, A. C., Hsu, S., Shi, Q., Tilly, J. L., and Clapham, D. E. (2001) A sperm ion channel required for sperm motility and male fertility. Nature 413, 603–609

20. Kirichok, Y., Navarro, B., and Clapham, D. E. (2006) Whole-cell patch clamp measurements of spermatozoa reveal an alkaline-activated Ca\(^{2+}\) channel. Nature 439, 737–740

21. Carlson, A. E., Westenbroek, R. E., Quill, T., Ren, D., Clapham, D. E., Hille, B., Garbers, D. L., and Babcock, D. F. (2003) CatSper required for evoked Ca\(^{2+}\) entry and control of flagellar function in sperm. Proc. Natl. Acad. Sci. U.S.A. 100, 14864–14868

22. Zeng, X. H., Navarro, B., Xia, X. M., Clapham, D. E., and Lingle, C. I. (2013) Simultaneous knockout of Slo3 and CatSper1 abolishes all alkalization- and voltage-activated current in mouse spermatozoa. J. Gen. Physiol. 142, 305–313

23. Lee, H. C., and Garbers, D. L. (1986) Modulation of the voltage-sensitive Na\(^+/\)H\(^+\) exchange in sea urchin spermatozoa through membrane potential changes induced by the egg peptide speract. J. Biol. Chem. 261, 16026–16032

24. González-Martínez, M., and Darzsón, A. (1987) A fast transient hyperpolarization occurs during the sea urchin sperm acrosome reaction induced by egg jelly. FEBS Lett. 218, 247–250

25. González-Martínez, M. T., Guerero, A., Morales, E., de La Torre, L., and Darzsón, A. (1992) A depolarization can trigger Ca\(^{2+}\) uptake and the acrosome reaction when preceded by a hyperpolarization in L. pictus sea urchin sperm. Dev. Biol. 150, 193–202

26. Accot, T. S., and Carr, D. W. (1984) Inhibition of bovine spermatozoa by caudal epididymal fluid II. Interaction of pH and a quiescence factor. Biol. Reprod. 30, 926–935

27. Carr, D. W., and Accot, T. S. (1989) Intracellular pH regulates bovine sperm motility and protein phosphorylation. Biol. Reprod. 41, 907–920

28. Giroux-Widemann, V., Jouanet, P., Pignot-Paintrand, I., and Feneux, D. (1991) Effects of pH on the reactivation of human spermatozoa demembranated with Triton X-100. Mol. Reprod. Dev. 29, 157–162

29. Hamamah, S., and Gatti, J. L. (1998) Role of the ionic environment and internal pH on sperm activity. Hum. Reprod. 13, Suppl. 4, 20–30

30. Ho, H. C., Granish, K. A., and Suarez, S. S. (2007) Bovine sperm hyperactivation is necessary and sufficient to prepare sperm for the acrosome reaction. J. Biol. Chem. 282, 15041–15047

31. Marquez, B., and Suarez, S. S. (2007) Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca\(^{2+}\) influx. Biol. Reprod. 76, 660–665

32. Suarez, S. S. (2008) Control of hyperactivation in sperm. Hum. Reprod. Update 14, 647–657

33. Nishigaki, T., José, O., González-Cota, A. L., Romero, F., Treviño, C. L., and Darzsón, A. (2014) Intracellular pH in sperm physiology. Biochem. Biophys. Res. Commun. 450, 1149–1158

34. García, M. A., and Meizel, S. (1999) Regulation of intracellular pH in capacitated human spermatozoa by a Na\(^+/\)H\(^+\) exchanger. Mol. Reprod. Dev. 52, 189–195

35. Lishko, P. V., Botchkina, I. L., Fedorenko, A., and Kirichok, Y. (2010) Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. Cell 140, 327–337

36. Xia, J., Reigada, D., Mitchell, C. H., and Ren, D. (2007) CATSPER channel-mediated Ca\(^{2+}\) entry into mouse sperm triggers a tail-to-head propagation. Biol. Reprod. 77, 551–559

37. Nishigaki, T., Wood, C. D., Shiba, K., Baba, S. A., and Darzsón, A. (2006) Stroboscopic illumination using light-emitting diodes reduces phototoxicity in fluorescence cell imaging. Biotechniques 41, 191–197

38. Babcock, D. F., and Pfeiffer, D. R. (1987) Independent elevation of cytosolic [Ca\(^{2+}\)] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. J. Biol. Chem. 262, 15041–15047

39. Nishigaki, T., Wood, C. D., Shiba, K., Baba, S. A., and Darzsón, A. (2006) Stroboscopic illumination using light-emitting diodes reduces phototoxicity in fluorescence cell imaging. Biotechniques 41, 191–197

40. Ward, C. R., Kopf, G. S., and Storey, B. T. (1994) Solubilization and partial purification of sperm motility channels. Mol. Reprod. Dev. 38, 250–256

41. García-Soto, J., González-Martínez, M., de De la Torre, L., and Darszon, A. (2003) Slo1 is the principal potassium channel of human spermatozoa. Mol. Reprod. Dev. 63, 80–87

42. García-Soto, J., González-Martínez, M., de De la Torre, L., and Darszon, A. (2003) SLC9/NHE gene family, Aspects Med. Biol. 236–251

43. García-Soto, J., González-Martínez, M., de De la Torre, L., and Darszon, A. (2003) SLC9/NHE gene family, Aspects Med. Biol. 236–251