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The SLO3 sperm-specific potassium channel plays a vital role in male fertility

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Abstract

Here we show a unique example of male infertility conferred by a gene knock-out of the sperm-specific, pH-dependent SLO3 potassium channel. In striking contrast to wild-type sperm which undergo membrane hyperpolarization during capacitation, we found that *SLO3* mutant sperm undergo membrane depolarization. Several defects in *SLO3* mutant sperm are evident under capacitating conditions, including impaired motility, a bent “hairpin” shape, and failure to undergo the acrosome reaction (AR). The failure of AR is rescued by valinomycin which hyperpolarizes mutant sperm. Thus SLO3 is the principal potassium channel responsible for capacitation-induced hyperpolarization, and membrane hyperpolarization is crucial to the AR.

Keywords

SLO3; capacitation; sperm; acrosome reaction

Introduction

The SLO3 potassium channel is a closely related paralogue of the high conductance calcium-activated potassium channel, SLO1. Notably, however, SLO3 is expressed only in mammalian testes [1,2] while SLO1 is widely expressed in many tissues and in many phyla, including invertebrates [3]. Furthermore, SLO3 channels are activated by alkalinization and lack the calcium sensors present in SLO1 channels [1,3]. Mammalian sperm are unable to fertilize eggs immediately after ejaculation. They acquire fertilization capacity after residing for a certain time in the female genital tract through a process called capacitation. Capacitation is associated with an increase in intracellular pH (pHi)[4,5] and hyperpolarization of the sperm plasma membrane [6;7]. SLO3 channels are only found in mammals, the only class of vertebrate animals requiring capacitation prior to fertilization. This, in addition to its localization in

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spermatocytes and its activation by intracellular alkalinization, make the SLO3 potassium channel a likely candidate to account for the hyperpolarization that occurs during capacitation. NH_4Cl induced increases in sperm pHi in patch clamp experiments have been shown to activate two major conductances, an outward K^+ conductance postulated to be carried by SLO3 channels [8,9] (also called Ksper [9]), and an inward conductance permitting Ca^{2+} entry carried by CatSper cation selective channels [10]. Here we show that *SLO3* mutant sperm subjected to conditions normally used to capacitate sperm, undergo membrane depolarization, in contrast to wild-type (*wt)* sperm which undergo membrane hyperpolarization when subjected to the same conditions. The membrane depolarization seen in *SLO3* mutant sperm could reflect a slight increase in the activity of the pH-dependent CatSper channel [10,11] which is not offset by the increase in SLO3 K^+ channel activity. Surprisingly, sperm from the *SLO3* homozygous knock-out are deficient in the acrosome reaction (AR) even when exposed to A23187, an ionophore which directly permits calcium entry obviating the need to activate other Ca^{2+} channels. However, we observe that the mutant deficiency in the AR is largely rescued by the application of the K^+ ionophore valinomycin in the presence of A23187. This suggests that, in addition to a rise in $[Ca^{2+}]$ in the cytosol, the AR may require an additional voltage-dependent process. These results clearly demonstrate that the SLO3 channel is a key player in the fertilization process and no other channel seems to be able to undertake its function.

Material and Methods

SLO3 knockout mouse

The *SLO3* (*Kcnu1*) null mutation was synthesized by removal of the first two coding exons of the *Kcnu1* gene (TG0050 TIGM). This removed the initiation codon and DNA sequence encoding the first and partial second membrane spanning domains. The next possible downstream site of translational initiation would, if used, result in the synthesis of a short out-of-frame polypeptide.

Animals

All procedures described herein were reviewed and approved by the Animals studies Committee of Washington University (St Louis) and were performed in accord with the NIH Guiding Principles of the care and use of laboratory animals.

Testis total membrane preparation and Western blot analysis

Testis from knockout (−/−), heterozygous (+/−) and *wt* (+/+) mice were processed in a Teflon/glass homogenizer in 250 mM sucrose, 10 mM HEPES, pH 7.40 plus protease inhibitor cocktail (0.01 U/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml benzamidine, 1 μg/ml antipain, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, 1 mM PMSF). Suspensions were spun down at 10,000 g at 4 °C for 1 min; the supernatants were spun down 200,000 g at 4 °C for 45 min. Pellets were then dispersed by 1 % SDS and protein measured using BCA protein assay kit (Pierce, Rockfold, IL). 100 micrograms of total membrane proteins were treated 15 min at 60 °C in loading buffer plus 50 mM DTT and then loaded on a 7.5 % PAGE-SDS gel and subsequently transferred to a nitrocellulose membrane. The Western blot was probed with anti-monoclonal antibody (NeuroMab Anti-SLO3, clone N2/16) (2.8 ug/ml in 5 % low fat milk, PBS, 0.5 % Tween 20) and IRDye 680 donkey anti-mouse IgG (LI-COR Biosciences, Lincoln, NE) as secondary antibody (1 in 5000 dilution). Band intensities were measured using an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

Assay for Acrosome reaction

Caudal epididymal sperm were collected in HS medium (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 MgSO4, 20 HEPES, 5 glucose, 10 lactic acid, 1 Na-pyruvate, supplemented with 15 mM of

$NaHCO_3$ and 5 mg/ml of bovine serum albumin at 37 °C. The swim up method [12] was used to separate sperm with > 90% motility. The sperm suspension was incubated for 10 min and the top 500 μl, was separated and capacitated by incubating at 37 °C for 40 min using the technique of Visconti et al [13]. AR was induced after capacitation in a 50 μl aliquot by 15 μM Ca^{2+} ionosphore A23187 and incubating at 37°C. The percentage of AR was determined 30 min later using Coomassie Blue staining [14]. At least 200 sperm were assayed for each experimental condition to calculate the percentage of AR.

Measurement of membrane potential

Mature sperm from caudal epididymides were capacitated *in vitro* as described above. After 40 min of incubation, the potential-sensitive dye 3,3′-dipropylthiocabocyanine iodine (DiSC3) was added to a final concentration of 1 μM. Fluorescence was monitored with a Varian Cary Eclipse Fluorescence Spectrophotometer at 620/670 nm excitation/emission wavelength pair [15]. Mithocondrial membrane potential was dissipated with 500 nM carbonyl cyanide m-chorophenylhydrazone (CCCP). Cell hyperpolarization decreases the dye fluorescence. Recordings were initiated after reaching steady-state fluorescence (1–3 min) and were converted to membrane potential as described previously [14].

Sperm motility analysis

Computer assisted sperm analysis (CASA) was performed to determine sperm motility characteristics. Sperm from three pairs of mature *wt* and *SLO3* mutant mice of approximately four months of age were incubated in HS medium: and motility parameters were assessed using the HTM-IVOS Vs12 Integrated Visual Optical System (Hamilton-Thorne Research Danvers, MA, USA).

Electrophysiology

Whole cell currents recordings from testicular sperm were performed essentially as described [8]. The internal pipette solution contained in mM: 130 K-methansulphonate, 8 KCl, 20 KF, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 EGTA 2 HEPES, pH 6 with NaOH. The external solution contained: 118 mM Na-methanesulfonate, 8 NaCl, 2.5 $CaCl_2$, 2 KSO_4 1 $MgCl_2$ 10 HEPES, and supplemented with glucose to achieve an osmolarity of 290 mOsm. Currents recorded with an Axopatch 200A amplifier, filtered at 2–5 kHz (4-pole Bessel filter), were digitized at 5–10 kHz using a PC equipped with a DigiData 1200 (Axon). Data capture and analysis were performed with pCLAMP software (Axon, Molecular Devices, Palo Alto, CA) and Origin 6 (Microcal Software, Northampton MA).

In vitro fertilization

Oocytes were recovered from superovulated female mice 13 hs after 10 Units of hCG (human chorionic gonadotropin) injection. Sperm were collected from cauda epididymides of wt and *SLO3* mutant mice and capacitated *in vitro* at 37 °C for 1 hr. Capacitated sperm at a concentration of approximately 10^5 sperm/ml were co-incubated with eggs for 6 hrs and unbound sperm were subsequently washed away. After 24 hrs incubation the embryos were observed under light microscopy. The development of two-cell stage was considered successful fertilization. Some sperm were incubated with zona pellucida (ZP)-free eggs. The ZP-free eggs were prepared as follows. Oocytes were treated with hyaluronidase (10 mg/ml) for 10 min and then washed with media. Cumulus-free oocytes were transferred into acidic Tyrode's solution (Sigma) for 30 s to dissolve the ZP. The oocytes were then washed twice and incubated with sperm.

Results

The *SLO3* knockout mouse is infertile

We confirmed the absence of the SLO3 protein by Western blot analysis of testes from homozygous null *SLO3*$^{-}$ mutants which showed no detectable SLO3 protein (Fig. 1). Western blots of heterozygous *SLO3*$^{+}$/*SLO3*$^{-}$ animals showed approximately half the level of protein. Homozygous null *SLO3*$^{-}$ mutant males were infertile. Matings of 8 homozygous *SLO3*$^{-}$ males with multiple *wt* females (2 females per male) produced no offspring in a period of approximately four months, while 9 out of 10 homozygous mutant females mated to *wt* males produced offspring during the same period. Heterozygous *SLO3*$^{+}$/*SLO3*$^{-}$ animals of both sexes were fertile; 17/18 heterozygous males produced offspring; 24/25 heterozygous females produced offspring. The genotypes of offspring produced by intercrossing heterozygous (+/−) males and females exhibited roughly Mendelian proportions (46 +/+; 102 +/−; 41 −/−) which suggest that the mutation did not affect embryonic development. The presence of vaginal plugs noted in females mated to *SLO3* homozygous mutant males suggested normal mating behavior in mutant males. Body and testes weight were not significantly different between *wt* and mutant *SLO3* males. Neither *SLO3* homozygous mutant adult mice nor sperm showed any morphological abnormalities, except for the fact that, after the capacitation incubating period, 70% of mutant sperm showed flagellar angularity between the midpiece and the principal piece, compared to only 30% of the *wt* sperm (n = 4 animals; and > 200 sperm/animal). Indeed, many of the mutant sperm had a “hairpin” configuration in which the midpiece was folded back 180° and aligned along the principal piece (see online supplementary videos). Such abnormalities are known to be associated with sperm having deficient osmoregulation and volume control [16], a condition not unexpected to result from the mutant ablation of a major ion channel.

SLO3 mutant sperm depolarize during capacitation

Intrinsic to the maturation of spermatozoa is the process of capacitation which includes a pHi raise [4,5] and an accompanying membrane hyperpolarization [6]. In our initial characterization of the SLO3 potassium channel [1,2] we noted that because of its pH sensitivity, the SLO3 channel would be a good candidate to alter sperm membrane potential during capacitation. More recent studies showing that there is a pH-sensitive potassium current in sperm [8,9] that hyperpolarizes these cells upon exposure to external NH_4Cl [9] have supported this hypothesis. However, none of these studies provide conclusive evidence that the SLO3 potassium channel is responsible for the pH-dependent potassium current. More importantly, since these experiments were not done under conditions normally used to capacitate sperm, (these experiments were conducted with more immature stages of the sperm: corpus epididymes and testicular sperm where the patch clamp experiments are accessible) there is no evidence that the SLO3 potassium channel plays a critical role during capacitation. A rigorous test of the hypothesis that the SLO3 potassium channel is responsible for membrane hyperpolarization during capacitation would be the observation that caudal epididymal sperm from *SLO3* null mutant fail to undergo membrane hyperpolarization when exposed to physiological conditions that are well known to capacitate sperm of *wt* animals *in vitro*. Thus, we measured the membrane potentials of both *wt* and *SLO3* null mutant sperm, before and after capacitation. *In vitro* capacitation was undertaken by incubating caudal epididymal sperm at 37°C for 1hr in HS medium (methods) supplemented with 15 mM $NaHCO_3$, and 5mg/ml of bovine serum albumin. Membrane potential was measured using the potential-sensitive dye 3,3′-dipropylthiocabocyanine iodine as described (see methods). The results of these measurements were striking; unlike *wt* sperm, *SLO3* null mutant sperm failed to hyperpolarize their membrane potential after capacitation. Indeed, they showed a depolarization (Fig. 2). Notably, before capacitation no significant difference was evident between the membrane potential of the mutant and *wt* sperm. Only after the *in vitro* capacitation process occurred did differences become evident. This suggests that capacitation conditions trigger a membrane

conductance that hyperpolarizes the sperm. Although different ion channels have been claimed as candidates to produce such hyperpolarization [14,17,18], we found that the removal of SLO3 channels alone is sufficient to remove the hyperpolarization. The membrane depolarization seen in *SLO3* mutant sperm after capacitation may be the consequence of a slight increase in the activity of the pH-dependent CatSper channel [10,11]

SLO3 mutant sperm do not have pH-sensitive potassium currents

By whole cell voltage clamp experiments of mutant and *wt* sperm we showed that the *SLO3* gene encodes the potassium channels responsible for the pH-sensitive current present in testicular sperm. To demonstrate this we used the technique of Kirichock et al, 2006 [11]. In these experiments we used testicular mouse sperm, which are less mature than epididymal sperm and have a larger cytoplasmic droplet. As undertaken previously [8,9], internal alkalinization was achieved by the addition of NH_4Cl. The results of these experiments show that, in contrast to *wt* sperm, *SLO3* null mutant sperm lack the alkalinization-induced increase in outward current (Fig. 3). In addition there was a qualitative difference noted between *wt* and mutant; outward currents in *wt* sperm had a higher noise level suggestive of the presence of high conductance channels, that was not observed in the outward currents recorded from *SLO3* null mutant sperm.

SLO3 mutant sperm have impaired progressive motility and acrosome reaction (AR)

As expected, *SLO3* null mutant sperm, which undergo abnormal depolarization after capacitation, were markedly deficient in progressive motility relative to sperm from *wt* animals (Fig. 4). Perhaps the most unexpected result was the observation that sperm from the *SLO3* homozygous knockout are deficient in the AR when treated with the calcium ionophore A23187 which should bypass the need to activate other Ca^{2+} channels. A23187 potently elicits the AR in *wt* sperm (Fig. 5). These results are consistent with our *in vitro* fertilization results which show that even after removal of the ZP, the mutant sperm are unable to fertilize the eggs (see fig 6). However, we have noted that the mutant AR phenotype is substantially rescued by the inclusion of the K^+ ionophore valinomycin which is able to substitute for the *SLO3* mutant deficit in potassium ion conductance and result in membrane hyperpolarization in SLO3 mutant sperm.

Discussion

In our initial characterization of the SLO3 potassium channel [1,2] we noted that the pH sensitivity of the SLO3 channel made it a good candidate to alter sperm membrane potential during capacitation. More recent studies have supported this hypothesis [8,9]. However, those experiments were not done under conditions normally used to capacitate sperm and several ion channels have been claimed as candidates to produce such hyperpolarization [14,17,18]. However, we found that the genetic removal of the *SLO3* gene alone is sufficient to completely remove hyperpolarization in capacitating conditions, and indeed, produce a slight depolarization instead which might be the consequence of an increase in the activity of the pH-dependent CatSper channel [10,11,19] or the activation of high-V-dependent Ca^{2+} channels present in sperm [20,21]. As a consequence of the depolarization seen in *SLO3* mutant mouse sperm, the Ca^{2+} driving force would be reduced and consequently calcium influx into the flagellum will be reduced impairing flagellar motility, thus explaining the abnormalities of motility we see in these mutants [10,19,22,23].

The most unanticipated defect, however, is the deficiency in the AR when *SLO3* mutant sperm are exposed to the Ca^{2+} ionophore A23187. Although control of membrane potential has been hypothesized to be a key step in calcium entry [24,25], the addition of A23187 should bypass the need for calcium entry through any native calcium plasma membrane channel. Yet *SLO3*

mutant sperm still fail to achieve exocytosis under these conditions. The fact that valinomycine rescue the AR phenotype seems to suggest the presence of an additional membrane potential dependent process in addition to calcium ion entry. One possible candidate is the novel mammalian sperm specific Na^+/H^+ exchanger, sNHE, which contains a putative voltage sensor [26]. Overall our results highlight the involvement of SLO3 channels in multiple mechanisms of male fertility and may have revealed the presence of an essential voltage-sensitive process not previously known. These results also suggest several compelling reasons to consider the SLO3 channel an attractive pharmacological target for male contraception.

Legend to SI video files: File designation **mutant HR.avi** indicates sperm of SLO3 homozygous knockout mutant; **wt HR.avi** indicates wild-type sperm. Sperm were collected from cauda epididymides of wild-type and SLO3 −/− mutant mice and capacitated *in vitro* at 37 °C for 1 hr as indicated in Materials and Methods. Mutant sperm show a high percentage of flagellar angularity between the midpiece and the principal piece (see text). In addition, hyperactivation was not evident in mutant sperm. Each of the video files contains 30 frames (15 FPS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Schreiber M, Wei A, Yuan A, Gaut J, Saito M, Salkoff L. Slo3, a novel pH-sensitive K^+ channel from mammalian spermatocytes. J Biol Chem 1998;273:3509–3516. [PubMed: 9452476]
2. Santi CM, Butler A, Kuhn J, Wei A, Salkoff L. Bovine and mouse SLO3 K^+ channels: Evolutionary divergence points to a RCK1 region of critical function. J Biol Chem 2009;284(32):21589–98. [PubMed: 19473978]
3. Salkoff L, Butler A, Ferreira G, Santi CM, Wei A. High conductance Potassium Channels of the *Slo* Family. Nature Reviews Neuroscience 2006;5:921–931.
4. Vredenburgh-Wilberg WL, Parrish JJ. Intracellular pH of bovine sperm increases during capacitation. Mol Reprod Biol 1995;40 (4):490–502.
5. Zeng Y, Oberdorf JA, Florman HM. pH regulation in mouse sperm: identification of Na+, Cl−, and HCO3− dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. Dev Biol 1996;173:510–520. [PubMed: 8606009]
6. Zeng Y, Clark EN, Florman HM. Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev Biol 1995;71(2):554–63. [PubMed: 7556936]
7. Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM. 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 1999;96(12):6757–62. [PubMed: 10359785]
8. Martínez-López P, Santi CM, Treviño CL, Ocampo-Gutiérrez AY, Acevedo JJ, Alisio A, Salkoff LB, Darszon A. Mouse sperm K+ currents stimulated by pH and cAMP possibly encoded by Slo3 channels. BBRC 2009;381:204–209. [PubMed: 19338774]
9. Navarro B, Kirichok Y, Clapham DE. KSper, a pH-sensitive K^+ current that controls sperm. Proc Natl Acad Sci U S A 2007;104(18):7688–92. [PubMed: 17460039]

10. Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham DE. A sperm ion channel required for sperm motility and male fertility. Nature 2001;413:603–609. [PubMed: 11595941]
11. Kirichok Y, Navarro B, Clapham DE. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. Nature 2006;439(7077):737–40. [PubMed: 16467839]
12. Henkel RR, Schill WB. Sperm preparation for ART. Reprod Biol Endocrinol 2003 Nov 14;1:108. Review. [PubMed: 14617368]
13. Visconti PE, Stewart-Savage J, Blasco A, Battaglia L, Miranda P, Kopf GS, Tezón JG. Roles of bicarbonate, cAMP, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. Biol Reprod 1999;61(1):76–84. [PubMed: 10377034]
14. Muñoz-Garay C, De la Vega-Beltrán JL, Delgado R, Labarca P, Felix R, Darszon A. Inwardly rectifying K(+) channels in spermatogenic cells: functional expression and implication in sperm capacitation. Dev Biol 2001;234(1):261–74. [PubMed: 11356034]
15. Espinosa F, Darszon A. Mouse sperm membrane potential: changes induced by Ca2+ FEBS Lett 1995;372(1):119–25. [PubMed: 7556631]
16. Cooper TG, Yeung C, Wagenfeld A, Nieschlag E, Poutanen M, Huhtaniemian I, d Sipilä P. Mouse models of infertility due to swollen spermatozoa. Mol Cell Endocrinol 2004;216 (1–2):55–63. [PubMed: 15109745]
17. Acevedo JJ, Mendoza-Lujambio I, de la Vega-Beltrán JL, Treviño CL, Felix R, Darszon A. KATP channels in mouse spermatogenic cells and sperm, and their role in capacitation. Dev Biol 2006 Jan 15;289(2):395–405. [PubMed: 16343479]
18. Hernández-González EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, López-González I, Demarco I, Wertheimer E, Darszon A, Visconti PE. Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyperpolarization in mouse sperm. J Biol Chem 2006;281(9):5623–33. [PubMed: 16407190]
19. Quill TA, Ren D, Clapham DE, Garbers DL. A voltage-gated ion channel expressed specifically in spermatozoa. Proc Natl Acad Sci USA 2001;98:12527–12531. [PubMed: 11675491]
20. Wennemuth G, Westenbroek RE, Xu T, Hille B, Babcock DF. Cav2.2 and Cav2.3 (N-and R-type) Ca^{2+} channels in depolarization-evoked entry of Ca^{2+} into mouse sperm. J Biol Chem 2000;275:21210–21217. [PubMed: 10791962]
21. Babcock DF, Pfeiffer DR. Independent elevations of cytosolic Ca^{2+} and pH of mammalian sperm by voltage dependent and pH-sensitive mechanisms. J Biol Chem 1987;262:15041–15047. [PubMed: 3667622]
22. Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF. CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. Proc Natl Acad Sci U S A 2003;100(25):14864–8. [PubMed: 14657352]
23. Marquez, Suarez. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca influx. Biol Reprod 2007;76:660–665. [PubMed: 17182893]
24. Florman HM, Corron ME, Kim TDH, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. Dev Biol 1992;152:304–314. [PubMed: 1379559]
25. Florman HM. Sequential focal and global elevations of sperm intracellular Ca2+ are initiated by the zona pellucida during acrosomal exocytosis. Dev Biol 1994;165:152–164. [PubMed: 8088433]
26. Wang D, King SM, Quill TA, Doolitle LK, Garbers DL. A new sperm-specific N^+/H^+ exchanger required for sperm motility and fertility. Nat Cell Biol 2003;5:1117–1122. [PubMed: 14634667]

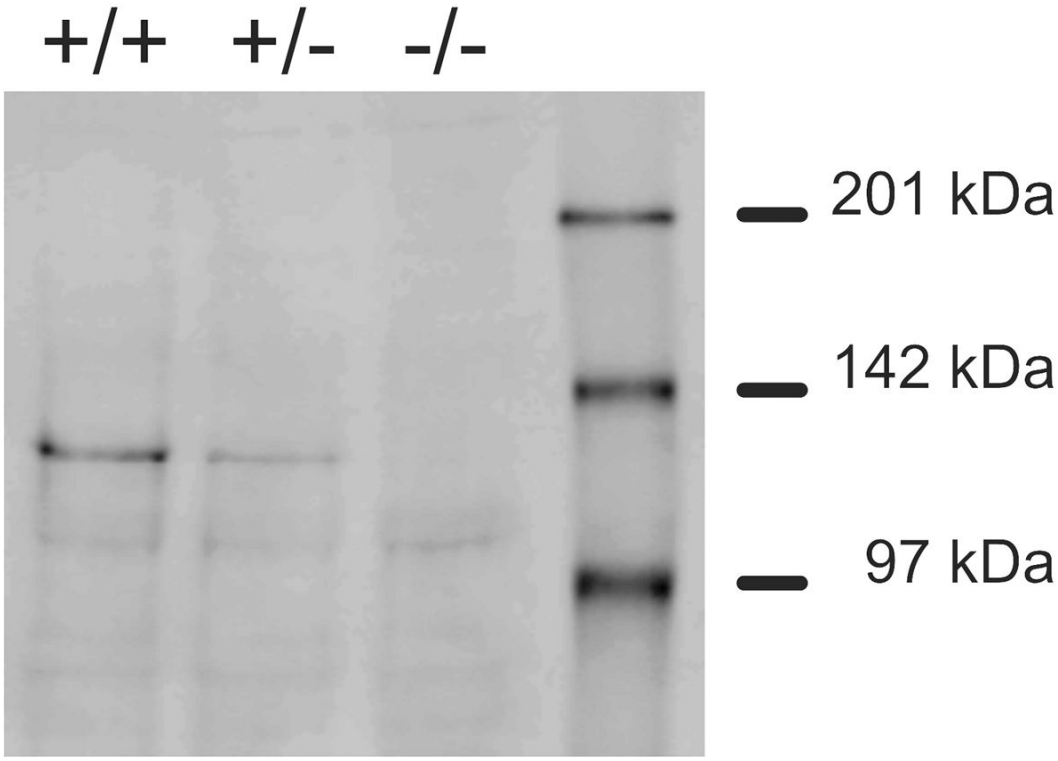

Fig 1. SLO3 channel is absent in mutant mice as illustrated by western blot analysis

Equal amounts of membrane protein from testis of wild type (*wt*)(+/+), heterozygous mutant (+/−) and homozygous mutant (−/−) mice were stained with anti-SLO3 antibody (methods). The SLO3 protein was undetectable in homozygous mutants (−/−) and present in approximately half the *wt* amount in heterozygous (+/−) mice.

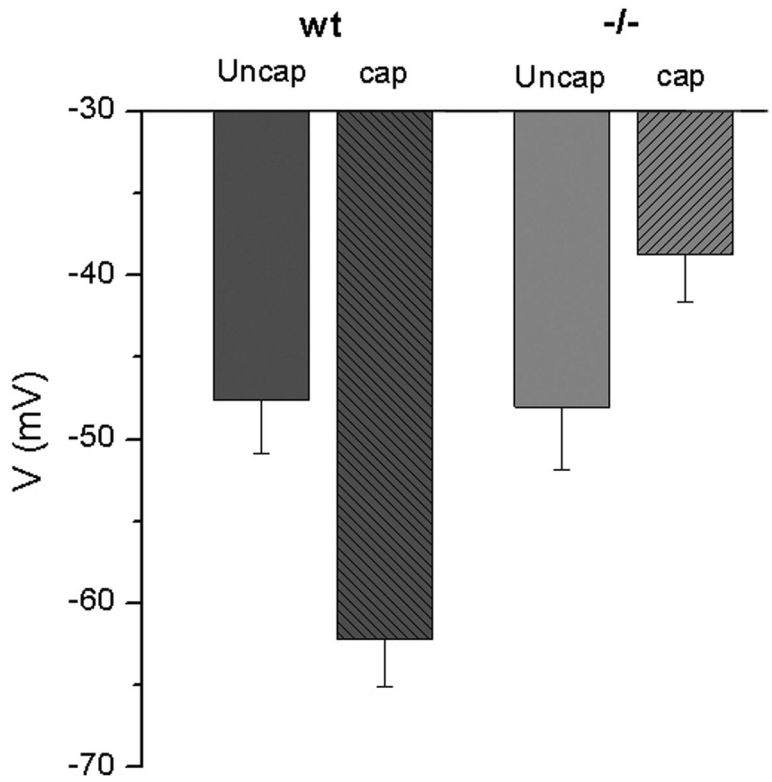

Fig 2. *SLO3* mutant sperm lack capacitation induced hyperpolarization and, in contrast to *wt*, depolarize after capacitation

Membrane potentials before and after *in vitro* capacitation are illustrated for *wt* and *SLO3* homozygous mutant (−/−) sperm (error bars show SEM). Sperm membrane potential was measured using the fluorescence dye DiSC3 as described in methods. Uncapacitated *wt* sperm have a membrane potential value of −47 ± 3.3 mV and hyperpolarize to −62.2 ± 2.9 mV (n =4) after capacitation. Uncapacitated *SLO3* mutant sperm have a membrane potential of −48 mV ± 3.9 mV and depolarize during capacitation to −38.8 ± 2.8 mV (n = 6). Membrane potential differences between *wt* and mutant sperm are significant for all pairwise comparisons at the 0.05 % level (paired t-test).

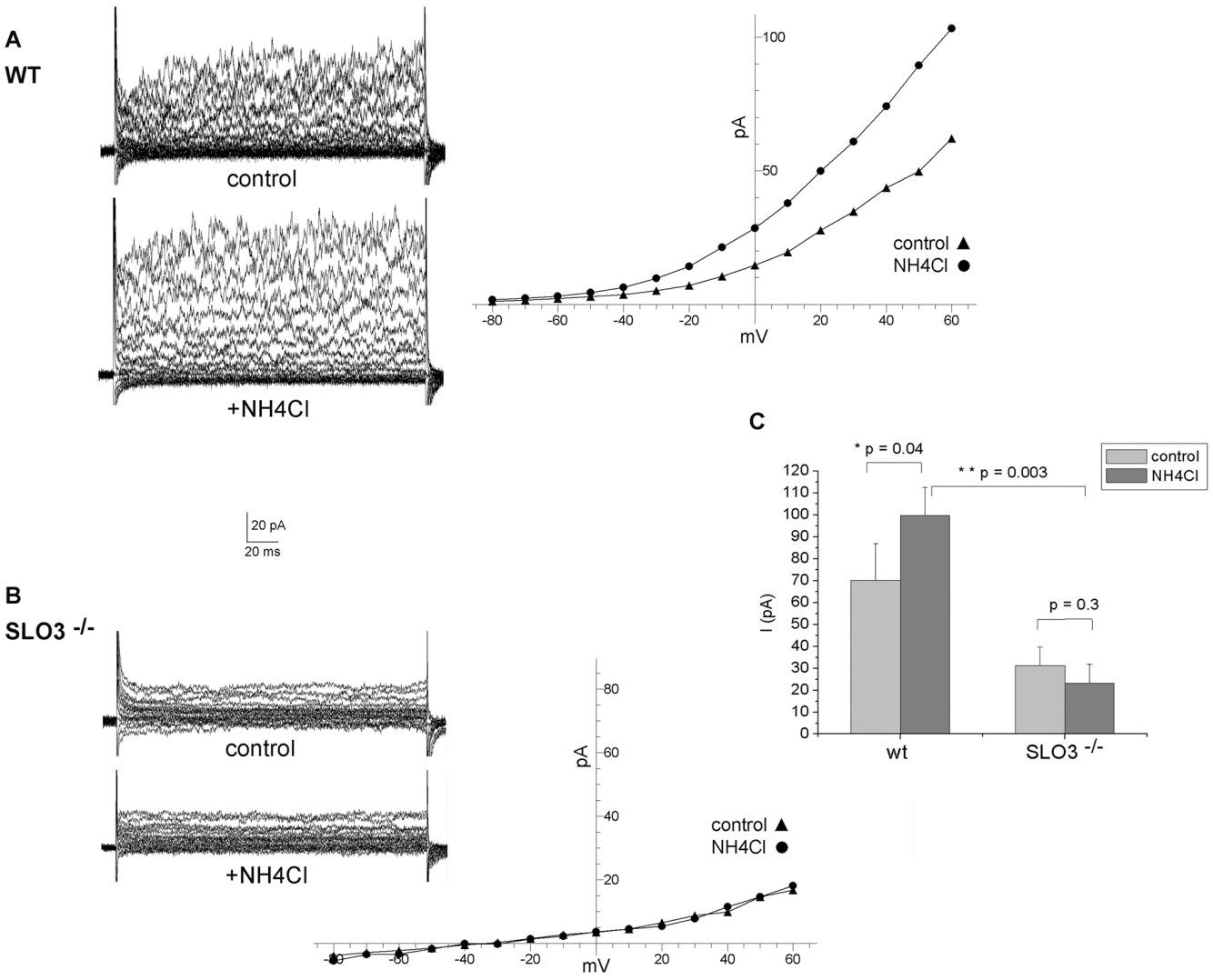

Fig 3. Mutant *SLO3* sperm lack pH sensitive K^+ currents

Representative whole cell voltage clamp recordings and current/voltage relationships from testicular sperm are shown for *wt* (A) and *SLO3* mutant sperm (B). Whole cell currents were evoked by 10 mV voltage steps from −80 to +60 mV at a holding potential of −50 mV. Currents are shown in control conditions and after bath application of 40 mM NH_4Cl. In control experiments with *wt* sperm we noted that the relative increase in outward current amplitude after alkalization with NH_4Cl was somewhat smaller than that reported in a previous paper (Navarro et al, 2007). However this may be due to the different stages of sperm that were analyzed; in our study testicular sperm were used, while in the previous study, more mature epididymal sperm were used. The bar graphs in (C) show a comparison of outward current amplitudes evoked at +60 mV for *wt* (n=4) and mutant *SLO3* sperm (n=4), before (control) and after (NH_4Cl) exposure to 40 mM NH_4Cl.

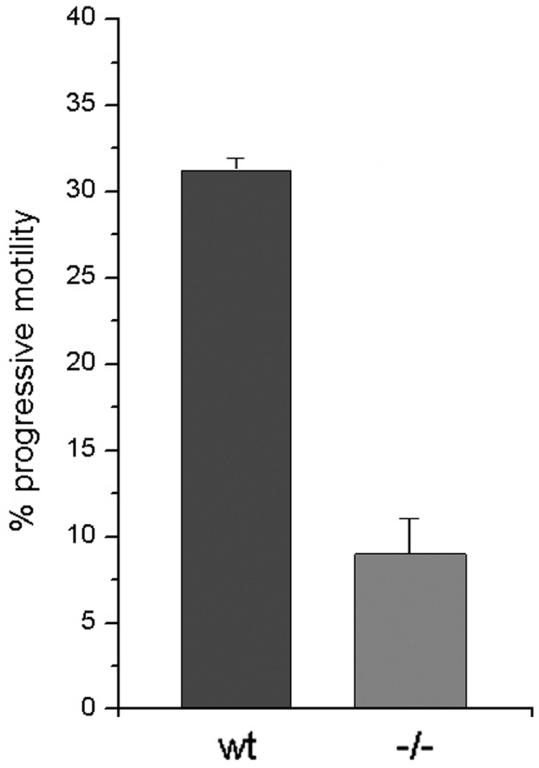

Fig 4. Mutant *SLO3* sperm display less progressive motility

Percentage of progressive motility is shown for *wt* and mutant *SLO3* $^{-/-}$ sperm. Sperm showing progressive motility were counted as a percentage of total sperm using computer assisted sperm analysis (CASA). While 31.3 ± 0.7 % of *wt* sperm showed progressive motility, only 9 ± 2.1 % of mutant sperm showed progressive motility for both *wt* (n=3) and mutant (n=3) animals; approximately 1000 sperm were counted for each animal). Differences were significant ($p < 0.05$).

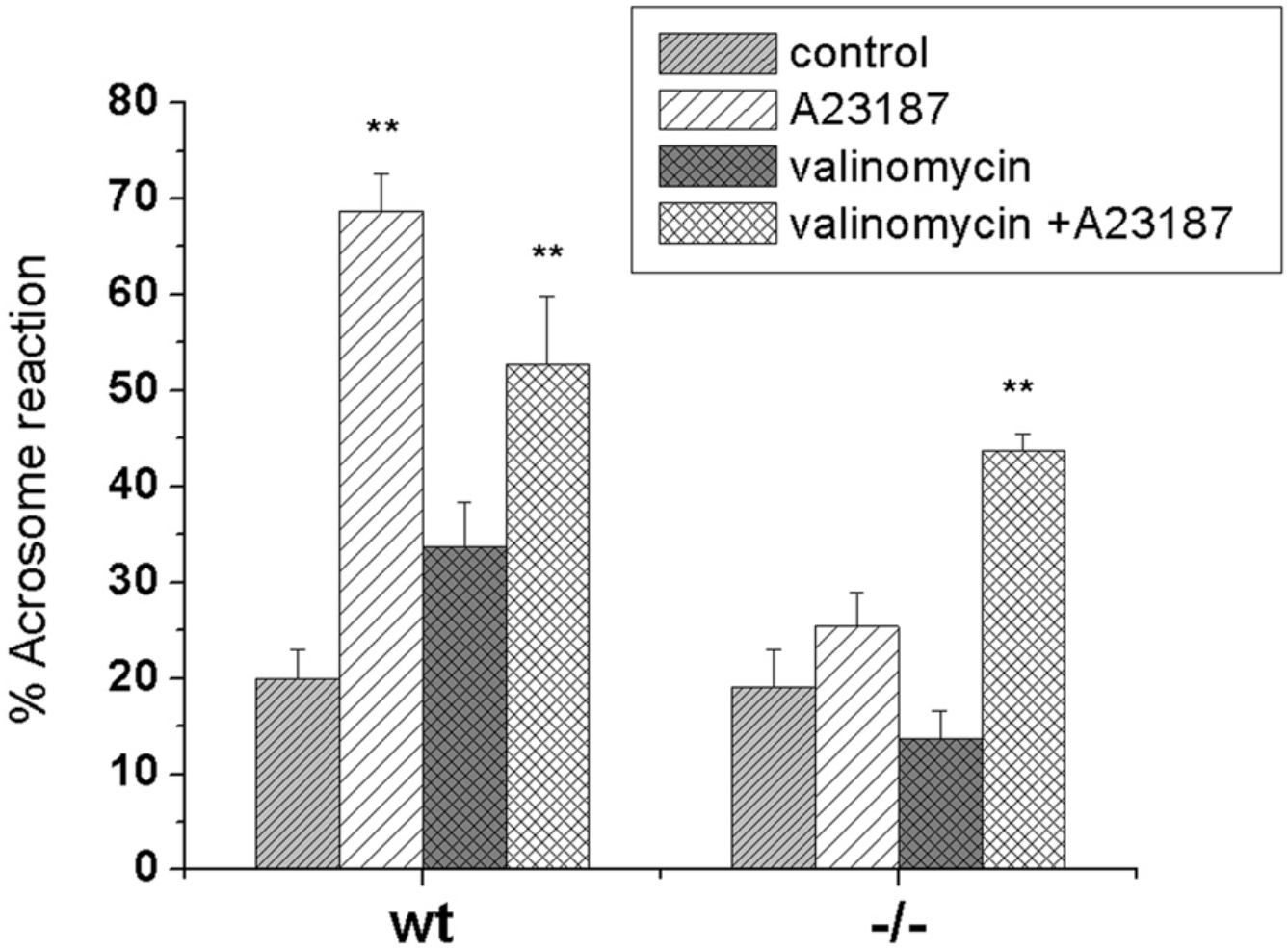

Fig 5. Acrosome reaction (AR) induced by A23187 is impaired in *SLO3* mutant sperm, & rescued by valinomycin

The percentage of spontaneous AR is similar in wild-type (WT) and *SLO3* mutant sperm after exposure to capacitating conditions. However, the addition of A23187 greatly increases AR in WT ($p \ll .001$) but has no significant effect on *SLO3* mutant sperm ($p=0.09$). The addition of valinomycin, however, to A23187 greatly increases the AR reaction in *SLO3* mutant sperm ($p<0.02$). 200 sperm for each condition were counted as acrosome reacted (no staining in the acrosomal region) or as acrosome intact (dark blue staining over the acrosomal region). [Numbers of animals for each condition: WT spontaneous=13; WT A23187=9; WT valinomycin=3; WT A23187+valinomycin=3; *SLO3* mutant (SLO3): SLO3 spontaneous=11; SLO3 A23187=8; SLO3 valinomycin=3; SLO3 valinomycin=A23187=3]

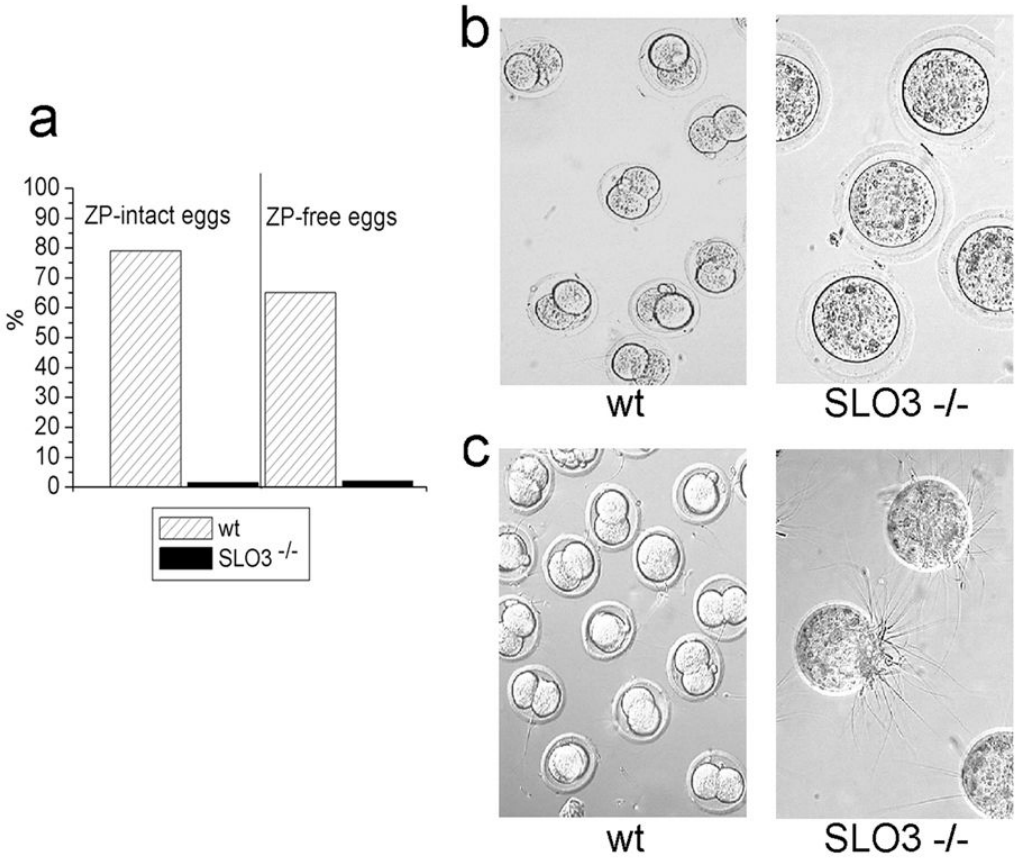

Fig 6. Sperm from *SLO3* mutants are not capable of fertilization under *in vitro* conditions

Results of *in vitro fertilization* (IVF) experiments performed with *wt(*n = 2 animals) and *SLO3* mutant sperm from 2 animals **a**. % of two stage cell embryos observed after IVF using *wt* or *SLO3* mutant sperm. Capacitated *wt* and mutant *SLO3* mutant sperm were incubated with superovulated oocytes with ZP intact from C57BL/6 wt mice (145 and 134 eggs respectively), and with the ZP removed (94 and 88 eggs respectively). The percentage (respectively for *wt* and *SLO3* mutant sperm) of two-stage embryos was: 79% (114/145) and 1.5% (2/134) respectively in intact-ZP eggs, and 65% (61/94) and 2% (2/88) in ZP-free eggs. **b**. representative micrographs of intact-ZP eggs after 24 hs of fertilization with wt or *SLO3* mutant sperm. **c**. representative micrographs of eggs with ZP removed after 24 hrs of fertilization with wt or mutant *SLO3* sperm.