A selective inhibitor of the sperm-specific potassium channel SLO3 impairs human sperm function

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To fertilize an oocyte, the membrane potential of both mouse and human sperm must hyperpolarize (become more negative inside). Determining the molecular mechanisms underlying this hyperpolarization is vital for developing new contraceptive methods and detecting causes of idiopathic male infertility. In mouse sperm, hyperpolarization is caused by activation of the sperm-specific potassium (K^+) channel SLO3 [C. M. Santi *et al.*, *FEBS Lett.* 584, 1041–1046 (2010)]. In human sperm, it has long been unclear whether hyperpolarization depends on SLO3 or the ubiquitous K^+ channel SLO1 [N. Mannowetz, N. M. Naidoo, S. A. S. Choo, J. F. Smith, P. V. Lishko, *Elife* 2, e01009 (2013), C. Brenker *et al.*, *Elife* 3, e01438 (2014), and S. A. Mansell, S. J. Publicover, C. L. R. Barratt, S. M. Wilson, *Mol. Hum. Reprod.* 20, 392–408 (2014)]. In this work, we identified the first selective inhibitor for human SLO3—VU0546110—and showed that it completely blocked heterologous SLO3 currents and endogenous K^+ currents in human sperm. This compound also prevented sperm from hyperpolarizing and undergoing hyperactivated motility and induced acrosome reaction, which are necessary to fertilize an egg. We conclude that SLO3 is the sole K^+ channel responsible for hyperpolarization and significantly contributes to the fertilizing ability of human sperm. Moreover, SLO3 is a good candidate for contraceptive development, and mutation of this gene is a possible cause of idiopathic male infertility.

human sperm | ion channels | capacitation | SLO3 | drug discovery

Infertility affects 10 to 15% of couples worldwide (1), spurring widespread use of in vitro fertilization (IVF), which has been used to conceive over 6.5 million babies. A key finding that made these births possible was Chang and Austin's (2, 3) demonstration that the ability of sperm to fertilize an oocyte depends on factors present in the female genital tract. These factors trigger the activation of intracellular signals resulting in sperm "capacitation." The capacitation causes a sperm to become hyperactive and prepared to undergo the acrosome reaction, allowing it to bind to and fuse with an oocyte (4).

A conserved mechanism underlying capacitation appears to be plasma membrane hyperpolarization (in which the membrane becomes more negatively charged on the inside) in response to a signal from an egg (e.g., sea urchins ref. 5) or female genital tract (e.g., mice and cows refs. 6 and 7). For example, in murine and bovine sperm, lack of hyperpolarization is associated with failure to undergo the acrosome reaction (7). Additionally, an increase in membrane potassium (K^+) efflux is essential for sperm capacitation in mice (8). As in other species, human sperm hyperpolarize under capacitating conditions (9), and failure to maintain a sufficiently negative sperm membrane potential is a common feature in men with subfertility and is associated with low fertilization rates in IVF (10). Consistent with this finding, hyperpolarization of human sperm positively correlates with IVF success (11). However, the mechanisms responsible for human sperm membrane hyperpolarization have not been fully defined.

In mice, the hyperpolarization associated with sperm capacitation is caused by a pH-sensitive K^+ current (mKSper) carried by the sperm-specific K^+ channel SLO3 (KCNU1). This conclusion is supported by the findings that sperm from *Slo3* knockout mice lack mKSper do not hyperpolarize during capacitation. Furthermore, *Slo3* knockout mice are unable to undergo hyperactivation or respond to acrosome reaction induction but have otherwise normal motility and spontaneous acrosome reaction rates (8, 12). As a result, male *Slo3* knockout mice are completely infertile. Like mouse sperm, human sperm express SLO3 channels, hyperpolarize during capacitation, and have a physiologically regulated K^+ current (hKSper) (13, 14). However, whether SLO3 is responsible for hKSper has been unclear for two reasons.

First, the initial examination of hKSper revealed that this current was activated by an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) and was not as sensitive to

Significance

To fertilize an egg, both mouse and human sperm must undergo membrane hyperpolarization. In mouse sperm, hyperpolarization is caused by activation of the sperm-specific potassium (K^+) channel SLO3. In human sperm, it is unclear whether hyperpolarization depends on SLO3 or the ubiquitous K^+ channel SLO1, as no selective SLO3 inhibitors or SLO3 mutations have been identified. Here, we identified a selective SLO3 inhibitor and demonstrated that it blocked human sperm K^+ currents, hyperpolarization, and downstream processes essential for fertilization. Therefore, SLO3 has a key role in human male fertility, SLO3 inhibitors have contraceptive potential, and SLO3 mutations could cause some cases of idiopathic male infertility.

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intracellular pH (pH_i) as mKSper (13). However, a later report showed that human SLO3 is sensitive to both $[Ca^{2+}]_i$ and pH_i and could therefore carry hKSper (14–16). Second, inhibitor studies have been inconclusive. For example, hKSper was shown to be inhibited by non-selective SLO inhibitors including quinidine, clofilium, and the hormone progesterone (13, 14, 16). hKSper was also inhibited by the canonically SLO1-selective inhibitors charybdotoxin, slotoxin, and paxilline (13, 17). Another SLO1 inhibitor, Iberiotoxin, inhibited or had no effect on hKSper in different recording conditions (13, 14). Importantly, the selectivity of these compounds was primarily based on the selectivity observed between mouse SLO3 and SLO1 (BK, KCNMA1) and may not reflect the selectivity between human SLO3 and SLO1 (18). Human SLO3 expressed in CHO cells was sensitive to charybdotoxin, iberiotoxin, and slotoxin, whereas results with clofilium and TEA have been inconclusive (9, 19). To date, no compounds have been identified that specifically inhibit human SLO3 and not SLO1. Our goals here were to identify a compound that sensitively and specifically blocks SLO3 and use it to conclusively determine whether or not SLO3 is responsible for hKSper and human sperm hyperpolarization.

Results

A High-Throughput Screen to Identify Inhibitors of hSLO3.
We modeled the SLO3 screening assay on those we and others have used for several K^+ channels and transporters (20–23). The assay is built around a stably transfected, monoclonal HEK293 cell line that expresses human SLO3 and its regulatory subunit γ2 (also known as LRRC52). Stably transfected HEK293-SLO3/γ2 cells express robust, voltage-dependent, outwardly rectifying K^+ currents that are completely inhibited by quinidine. These currents

are absent in non-transfected HEK293 cells and are modulated by pH_i and $[Ca^{2+}]_i$ as expected for SLO3 (*SI Appendix*, Fig. S1).

After validating the cell line, we established a thallium (Tl^+) flux assay for SLO3 that was suitable for high-throughput screening (22). The assay uses the fluorescent indicator dye Thallos (21) to report the inward movement of Tl^+ through expressed SLO3 channels (Fig. 1*A*). We found that SLO3 channels were mostly closed in HEK293 cells and consequently conducted Tl^+ too poorly to enable a high-throughput screen. We therefore screened several putative openers and found that the SLO1 activator NS11021 also activates SLO3 at high concentrations (Fig. 1*B*). Thus, in our screen of 50,240 compounds, we included NS11021 in each well containing test compounds. Fig. 1*C* shows a scatter plot of data from a representative 384-well plate for which the mean Z' score was 0.65. Test compounds were defined as inhibitors if they caused over 75% inhibition or caused 50% inhibition with a Z' score of at least –2. A summary of the screening data is shown in Fig. 1*D*. We selected 1,273 putative inhibitors to retest in the Tl^+ flux assay, of which 53 were confirmed to inhibit human SLO3.

VU0546110 Potently Inhibits Human SLO3 Expressed in HEK293 Cells.
Based on its initial potency for SLO3 inhibition, one of the most promising compounds identified in the high-throughput screen was VU0546110 (Fig. 2*A*). To confirm the ability of this compound to inhibit SLO3, we performed whole-cell patch-clamp experiments on HEK293 cells that stably expressed human SLO3 channels and the γ2 accessory subunit (Fig. 2*B*). The human SLO3 current was almost completely inhibited at 10 µM VU0546110 (Fig. 2*C*). To eliminate possible effects of VU0546110 on currents from channels endogenously expressed in HEK293 cells, we treated the cells with the non-selective K^+ channel inhibitor quinidine (19). For each concentration of VU0546110 tested,
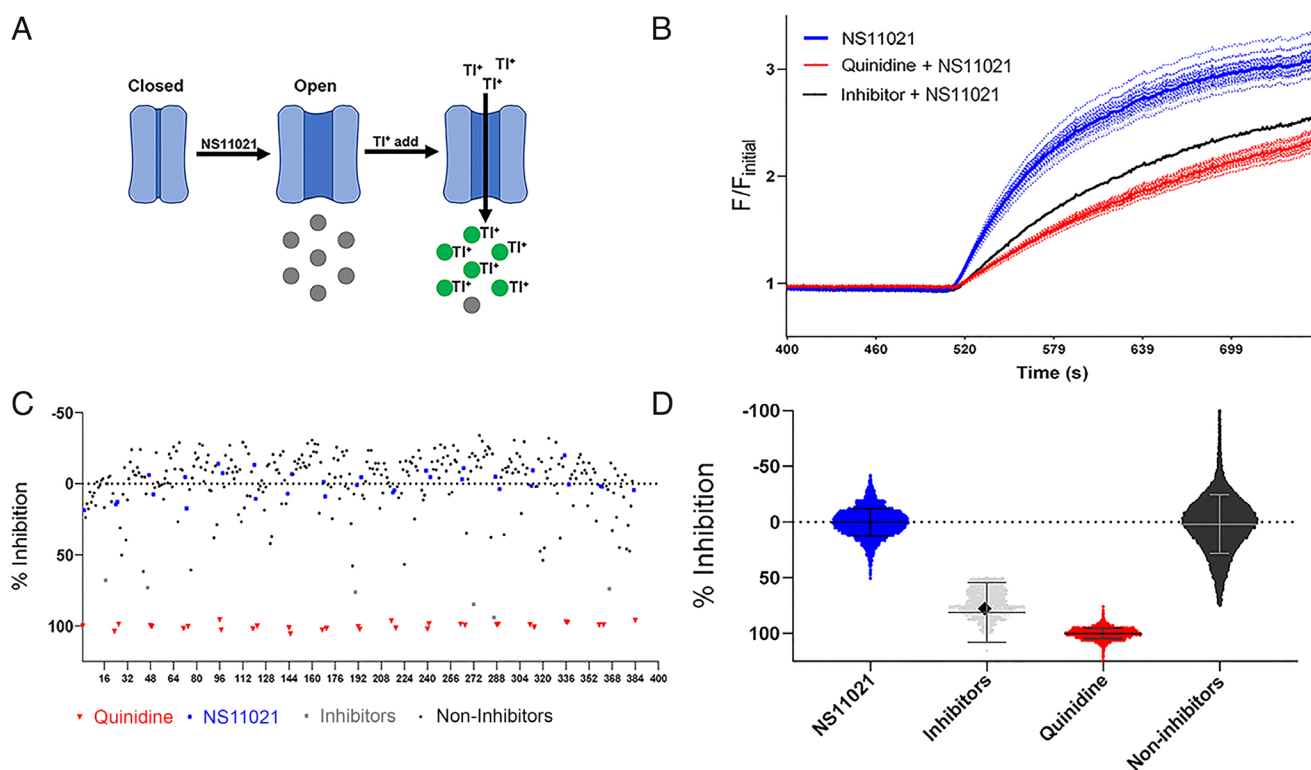

Fig. 1. High-Throughput Screen for SLO3 Inhibitors. (*A*) Schematic of thallium (Tl^+) flux assay. HEK293 cells expressing SLO3 and γ2 were loaded with the dye Thallos, and SLO3 was activated by adding the non-selective K^+ channel activator NS11021. Tl^+ was then added, causing an increase in fluorescence as it passed through SLO3 channels and bound to Thallos. (*B*) Representative mean responses of SLO3 to NS11021 (n = 32, blue traces), quinidine (n = 32, red traces), and a compound defined as an inhibitor (n = 1, black trace) in a 384-well plate (Z' = 0.65). (*C*) Representative data from a 384-well screen plate. Dots are colored to indicate the types of compounds. (*D*) Total data from 50,240 small molecules screened, plotted as % inhibition. Bars indicate SD. Black ◆ indicates VU0546110.
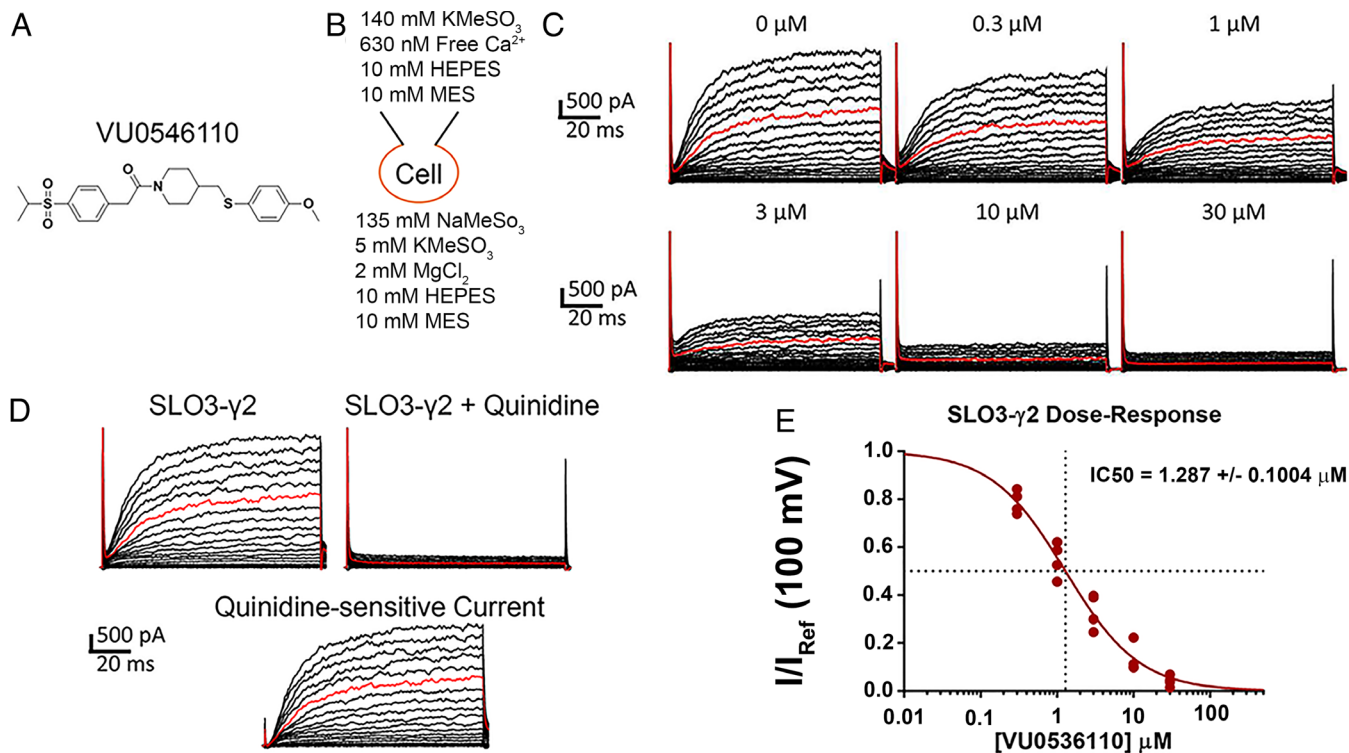
Fig. 2. VU0546110 Inhibits SLO3-γ2 Channels in HEK293 Cells. (*A*) Chemical structure of VU0546110. (*B*) Diagram of whole-cell patch-clamp configuration and intra- and extracellular ionic compositions. (*C*) Representative recordings of whole-cell currents at indicated VU0546110 concentrations. Currents at +100 mV are highlighted in red. (*D*) Representative trace of SLO3-γ2 currents in the presence and absence of 10 μM quinidine. Quinidine-sensitive currents were derived by subtracting currents in the presence of quinidine from control currents. (*E*) Currents at +100 mV were normalized to the current with no VU0546110 (1) and 10 μM quinidine (0) and fit with a modified Hill equation to derive the IC50.

we subtracted the currents that remained in the presence of 10 μM quinidine (Fig. 2*D*). We then measured the steady-state current evoked at +100 mV potential, normalized the values to those obtained in the absence of VU0546110, and generated a dose–response curve, which we fitted with the Hill Equation (Fig. 2*E*). We determined that VU0546110 inhibited SLO3 currents with an IC50 of 1.287 ± 0.1004 μM.

As they traverse through the male and female reproductive tracts, sperm experience environments with different pH. Thus, we wanted to determine whether the effects of VU0546110 depended on external pH. *SI Appendix,* Fig. S2*A* shows that VU0546110 was equally effective in inhibiting SLO3 at external pH 7.2 and 8.0. Because the activity of SLO3 is also dependent on $[Ca^{2+}]_i$, we tested the effect of VU0546110 at 32 nM and 100 μM free Ca^{2+} (*SI Appendix,* Fig. S2*B*) and found that VU0546110 inhibited the SLO3 current equivalently at both $[Ca^{2+}]_i$ values. These results indicate that VU0546110 potently inhibits heterologously expressed SLO3 at physiological ranges of pH and $[Ca^{2+}]_i$.

VU0546110 Selectively Inhibits Human SLO3 over Human SLO1. To determine whether VU0546110 was selective for SLO3 over SLO1, we stably expressed SLO1 in HEK293 cells and measured the dose–response of SLO1 currents to VU0546110 (Fig. 3*A*). The IC50 for SLO1 was 59.80 ± 14.47 μM, more than 40-fold higher than the IC50 of VU0546110 for SLO3 (Fig. 3 *B* and *E*). VU0546110 had low potency for SLO1 at free $[Ca^{2+}]_i$ between 32 nM and 100 μM (*SI Appendix,* Fig. S3).

In our experiments with SLO3, the channel was stably expressed with the γ2 subunit, as this subunit is expressed in the testis, is required for male fertility in mice, and is necessary to achieve stable expression of SLO3 (24–26). To test the possibility that VU0546110 exerted its effect on γ2, we generated HEK293 cells

expressing SLO1 and γ2 and tested various doses of the compound, revealing an IC50 of over 120 μM (Fig. 3 *C* and *E*). We next tested the β4 subunit, which is expressed in the testis (26, 27), sperm, and brain and functionally interacts with both SLO1 and SLO3 (27, 28). β4 can alter pharmacological interactions of SLO1 channels, reducing their sensitivity to iberiotoxin and charybdotoxin (29). We co-expressed β4 with SLO1 and generated dose-response curves of inhibition. VU0546110 did not inhibit SLO1 expressed with β4 at any concentration up to 50 μM (Fig. 3 *D* and *E*). Together, these results indicate that VU0546110 is highly selective for SLO3 over SLO1 at both low and high $[Ca^{2+}]_i$ and that it interacts directly with the SLO3 α subunit and not auxiliary subunits that might interact with either SLO3 or SLO1.

VU0546110 Blocks the K⁺ Current in Human Sperm (hKSper). Given that VU0546110 specifically inhibited SLO3, we could use it to determine whether or not hKSper currents are carried by SLO3 channels. To do so, we used whole-cell patch-clamp electrophysiology to measure the effect of VU0546110 on hKSper currents in human sperm at a range of $[Ca^{2+}]_i$ (Fig. 4*A*). Currents were evoked by a voltage ramp from –100 to +100 mV (Fig. 4*B*). VU0546110 inhibited hKSper currents at all $[Ca^{2+}]_i$ tested (Fig. 4*C*), and the VU0546110-resistant currents did not significantly differ between 0 and 50 μM Ca^{2+} (Fig. 4*D*). The VU0546110-resistant currents were less than 10 pA at +100 mV, substantially lower than the size of the current expected for the high-conductance SLO1 channels at that membrane potential. Moreover, the concentration of VU0546110 used, 10 μM, was substantially lower than its IC50 for SLO1 (59.80 μM). After washing the sperm to remove VU0546110, hKSper currents recovered (*SI Appendix,* Fig. S4). Together, these data indicate that hKSper is carried exclusively by SLO3 channels.
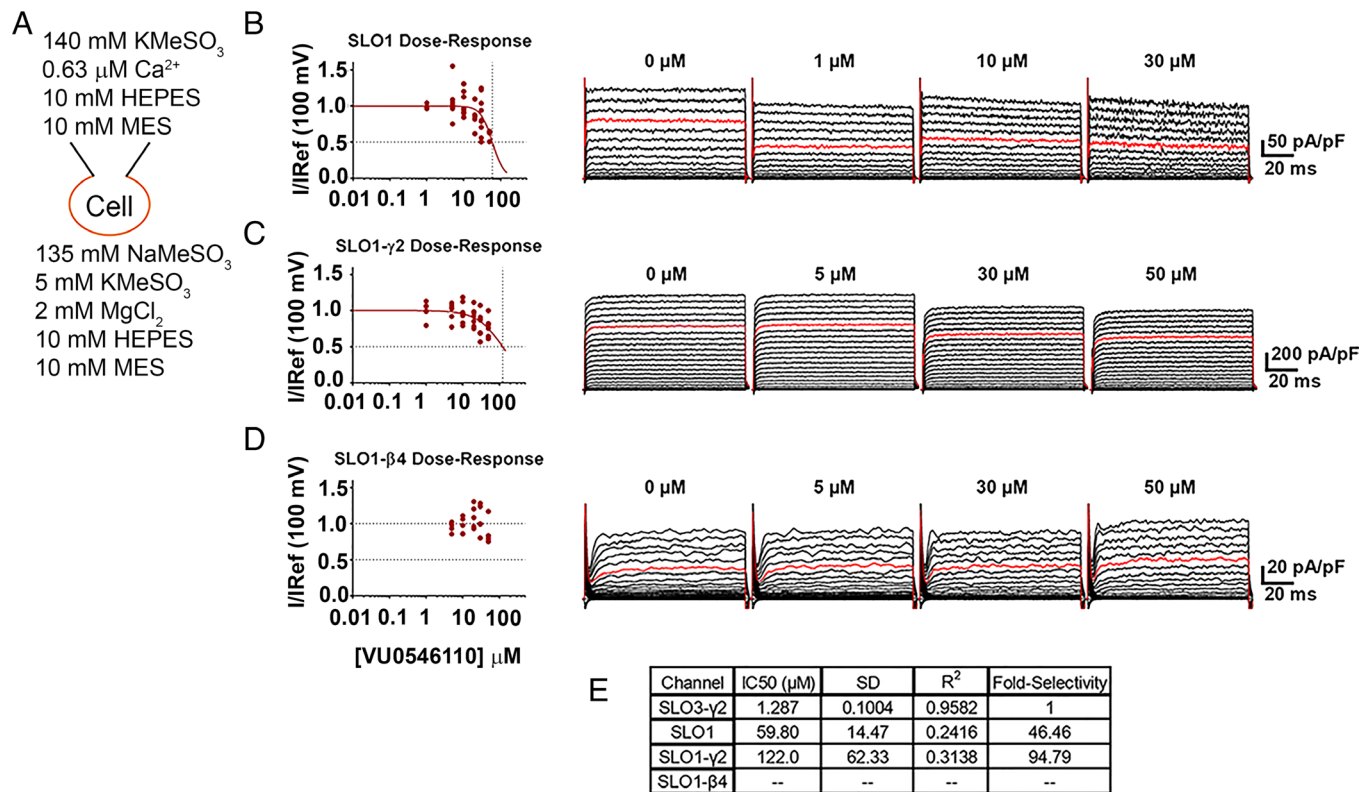
Channel	IC50 (µM)	SD	R^2	Fold-Selectivity
SLO3-γ2	1.287	0.1004	0.9582	1
SLO1	59.80	14.47	0.2416	46.46
SLO1-γ2	122.0	62.33	0.3138	94.79
SLO1-β4	--	--	--	--

Fig. 3. VU0546110 Does Not Inhibit SLO1 Channels. (*A*) Whole-cell patch-clamp schematic and intra- and extracellular solutions. (*A, C, D*) Nonlinear regressions and representative traces of VU0546110 inhibition of K+ currents in HEK293 cells expressing (*B*) SLO1, (*C*) SLO1 and γ2, or (*D*) SLO1 and β4. (*E*) IC50s calculated with the Hill equation.—indicates that no inhibition occurred, and the data failed to fit the Hill equation.

VU0546110 Inhibits Human Sperm Hyperpolarization. We previously showed that incubating mouse sperm at high external pH led to an increase in intracellular pH and hyperpolarization (30). Neither of these effects occurred in sperm from *Slo3* knockout mice. Human sperm also hyperpolarize when incubated at high external pH (9, 11). To determine whether SLO3 was required for human sperm hyperpolarization induced by increased external pH, we incubated sperm at pH 5.8 and pH 8 in the absence and presence of VU0546110. To measure changes in membrane potential, we loaded sperm with the voltage-sensitive dye DiSC$_3$(5) and performed spectrophotometry. Because DiSC$_3$(5) distributes across membranes according to the membrane potential, changes in DiSC$_3$(5) fluorescence are directly proportional to changes in membrane potential and can be used to measure membrane potential in sperm populations (11, 31, 32). As expected, human sperm incubated at pH 8.0 had more hyperpolarized membrane potential than sperm at pH 5.8 (Fig. 5). The incubation with VU0546110 at pH 8.0 eliminated this hyperpolarization. Specifically, sperm incubated at pH 8.0 in the presence of VU0546110 had a similar membrane potential as those incubated at pH 5.8 with ($P > 0.9999$) or without ($P > 0.9999$) VU0546110. We also tested the SLO1 inhibitors paxilline and iberiotoxin at concentrations that blocked heterologous SLO1 currents but not heterologous SLO3 currents (*SI Appendix*, Fig. S5) and found that they did not prevent sperm hyperpolarization at pH 8.0 (Fig. 5). These results indicate that SLO3, not SLO1, is responsible for human sperm hyperpolarization induced by high external pH.

VU0546110 Inhibits the Induced Acrosome Reaction and Hyperactivated Motility in Human Sperm. As a consequence of capacitation, sperm undergo two changes that are essential for fertilization, the acrosome reaction and hyperactivated motility

(33, 34). To determine whether VU0546110 prevented these events, we first measured the effect of 2.5 µM VU0546110 on the acrosome reaction induced by the addition of the Ca^{2+} ionophore A23187 or progesterone (35). As has been previously reported, 9 to 11% of human sperm underwent induced acrosome reaction in control conditions. VU0546110 significantly reduced the percentage of reacted sperm induced by A23187 or progesterone without significantly affecting the spontaneous acrosome reaction. (Fig. 6 *A–C*). The spontaneous acrosome reaction rate was 49.92% ± 13.56% with DMSO and 54.22% ± 15.77% with 2.5 µM VU0546110 ($P = 0.1080$, n = 17).

Next, to assess the effect of VU0546110 on hyperactivated motility, we incubated sperm in capacitating conditions for 1 h and then incubated them for 1 min in increasing concentrations of VU0546110. The percent of hyperactivated sperm was significantly reduced by VU0546110 concentrations greater than or equal to 0.5 µM (Fig. 6*D*), whereas 10 µM VU0546110 inhibited hyperactivated motility by 70% it only inhibited total motility by 22% (*SI Appendix*, Fig. S6B). When sperm were incubated for 1 h in capacitating media with VU0546110, the compound had no effect on sperm motility at any concentration tested (*SI Appendix*, Fig. S6 *A* and *C*). Sperm viability was not affected at any concentration of VU0546110 tested (*SI Appendix*, Fig. S6B). Together, these results indicate that human SLO3 is necessary for two key steps following human sperm capacitation—acrosome reaction and hyperactivation.

Discussion

Using our selective inhibitor of human SLO3, VU0546110, we present here three lines of evidence that this channel is responsible for the main K+ current in human sperm and has a significant role
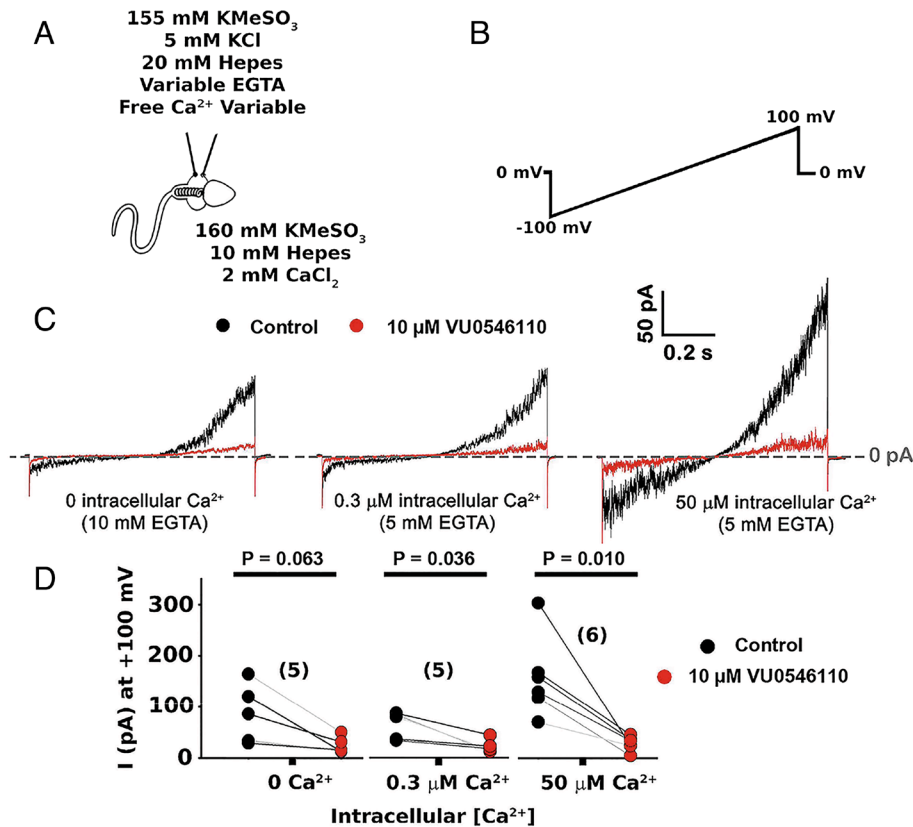
Fig. 4. VU0546110 Blocks KSper Currents in Human Spermatozoa. (*A*) Schematic of whole-cell sperm patch-clamp configuration and intra- and extracellular solutions. (*B*) Whole-cell currents were evoked with a voltage ramp from −100 to +100 mV from a holding potential of 0 mV. (*C*) Representative current traces from sperm without (black) and with (red) 10 μM VU0546110 at 0, 0.3, and 50 μM intracellular free $[Ca^{2+}]_i$. (*D*) Quantification of currents obtained at +100 mV at different $[Ca^{2+}]_i$ in indicated conditions compared with a paired *t* test. A one-way ANOVA test was performed to evaluate significance of difference between currents in the presence of 10 μM VU0546110 in the three intracellular calcium concentrations ($P = 0.679$). Numbers of cells tested for each condition are indicated in parentheses.

in human sperm capacitation. First, we showed that SLO3 is the sole K^+ channel responsible for the calcium-activated hKSper currents. hKSper currents were completely inhibited by 10 μM VU0546110 independently of the intracellular calcium concentration. In contrast, 10 μM VU0546110 had almost no effect on SLO1 currents. Second, we showed that, as in mouse sperm, SLO3 is required for the human sperm hyperpolarization associated with increased external pH. Conversely, the SLO1 inhibitors iberiotoxin and paxilline had no effect on human sperm hyperpolarization at concentrations that completely inhibited human SLO1 but not human SLO3. Third, we showed that inhibition of SLO3 by VU0546110 inhibited the two hallmarks of sperm capacitation: hyperactivated motility, which is required for sperm to pass through the female reproductive tract (34), and the acrosome reaction, which is necessary for sperm to penetrate the outer layers of the egg and for exposure of sperm sites that will fuse with the egg (36, 37).

We observed a clearer effect of SLO1 inhibition on acrosomal responsiveness than on hyperactivated motility which is not unexpected as previous studies in mice and humans have found that SLO3 has a more pronounced role in preparing sperm to undergo acrosomal exocytosis (7, 8, 38). Furthermore, the effect we observed was on the induced acrosome reaction rate which is known to be important for human male fertility (39–41). We saw an inhibitory effect of VU0546110 on sperm hyperactivation when we applied the drug for 1 min after 1 h of sperm incubation in capacitating media. However, VU0546110 did not affect hyperactivated motility when incubated with sperm for 1 h under

capacitating conditions. This finding implies that the inhibitory effect of the drug on hyperactivation is acute and decreases over time. One possible explanation is that the effective drug concentration might decrease over time by interaction with components of the capacitating media or by metabolization. This putative decrease in effective drug concentration does not seem to interfere with the inhibitory action of VU0546110 on the acrosome
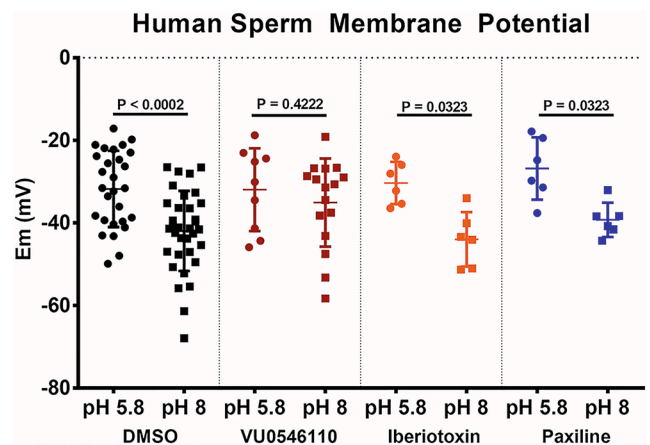

Fig. 5. SLO3, not SLO1 channels, are required for human sperm hyperpolarization. Human sperm membrane potential (Em) at pH 5.8 and 8.0 in the presence of DMSO (black), 2.5 μM VU0546110 (red), 100 nM iberiotoxin (orange), or 100 nM paxilline (blue). *P* values determined by Kruskal–Wallis one-way ANOVA.
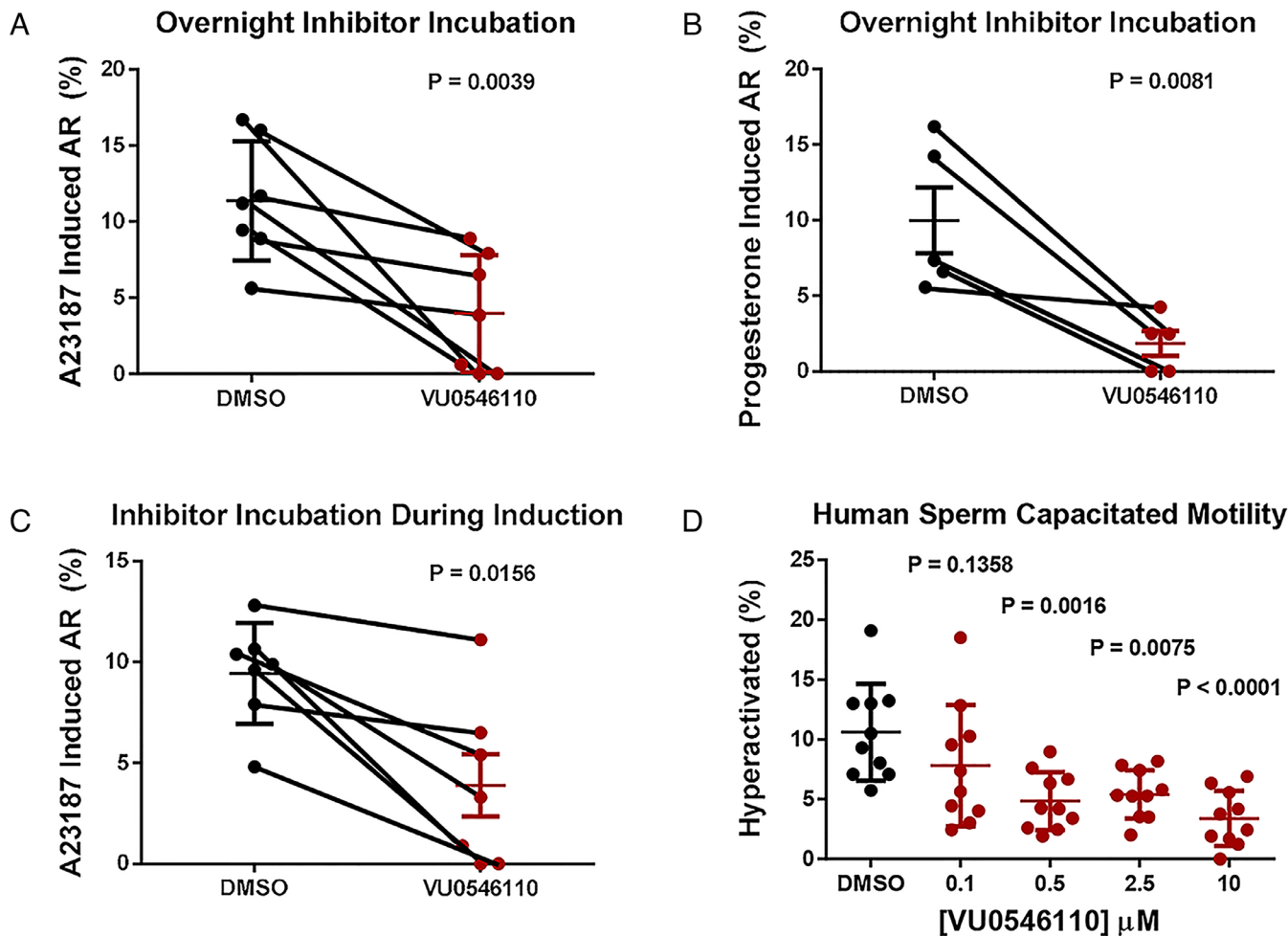
Fig. 6. VU0546110 prevents induced acrosome reaction and hyperactivated motility. Acrosome reaction rate induced by 10 μM (*A, C*) Ca^{2+} ionophore A23187 or (*B*) 10 μM progesterone. Sperm were incubated with 2.5 μM VU0546110 (red) or equal volume DMSO vehicle (black) (*A, B*) during overnight incubation in capacitating media or (*C*) during 30-min incubation with A23187. *P* values calculated by Wilcoxon matched-pairs signed-rank test. Black and red dots connected by a line were from the same donor. (*D*) Hyperactivated motility of sperm incubated in capacitating conditions for 1 h and exposed to increasing concentrations of VU0546110 for 1 min (n = 10). *P* values determined by unpaired *t* test. Lines connect data points from the same donor in different conditions.

reaction, which has been characterized as a process highly sensitive to changes in sperm membrane potential (7, 42).

One reason the channel responsible for the main K$^+$ current in human sperm (hKSper) has been unclear is that this current is activated by calcium (13). Given that mouse SLO1 is sensitive to [Ca^{2+}]$_i$ but mouse SLO3 is not, investigators initially concluded that SLO1 was responsible for the K$^+$ current in human sperm. However, later studies from Brenker et al. and Geng et al. revealed that human SLO3 channels expressed in heterologous systems were sensitive to both [Ca^{2+}]$_i$ and pH$_i$ (14, 15). This difference in Ca^{2+} sensitivity between human and mouse SLO3 is not surprising, as genes involved in sperm production and function evolve much faster than genes not involved in reproduction (43). For example, a comparison of amino acid sequences from several species revealed much greater structural divergence in SLO3 than in SLO1 (44).

Another reason for confusion about the channel responsible for the K$^+$ current in human sperm was that pharmacological studies of hKsper currents yielded contradictory results. Mannowetz et al. reported that 100 nM iberiotoxin, a classical SLO1 inhibitor, inhibited hKSper (13). In contrast, Brenker et al. showed that iberiotoxin did not inhibit hKSper at all (14). Part of this discrepancy could be explained by differences in the recording conditions used in the experiments. Adding more complexity to this problem, the degree of iberiotoxin inhibition seemed to differ for mouse

and human SLO channels. Whereas iberiotoxin inhibited mouse SLO1 with a K$_d$ of 0.5 ± 0.3 nM, and mouse SLO3 was insensitive to 100 nM iberiotoxin (18), the drug appears less selective for human SLO1 vs. SLO3. We found that iberiotoxin was 18-fold selective for human SLO1 over human SLO3 but that 100 nM iberiotoxin could inhibit 30% of SLO3 activity in our stable HEK-293 cell line. This result is similar to what Sanchez Carranza et al. reported for human SLO3 channels expressed in Chinese hamster ovary cells (19).

A final reason for the lack of clarity regarding the K$^+$ current in human sperm was the paucity of identified human mutations. A recent publication identified two infertile males carrying homozygous variants of the KCNU1 (*Slo3*) gene (38). One of these patients had an abnormal splicing isoform that resulted in a truncation in SLO3, and another had a predicted loss-of-function point mutation in which a highly conserved histidine was replaced by an arginine at position 715 (p.his715arg). Sperm from the patient carrying the p.his715arg mutation failed to hyperpolarize and to undergo an induced acrosome reaction. Hyperactivated motility was not measured. Furthermore, sperm from both patients failed to fertilize eggs in vitro, though sperm from both patients successfully fertilized eggs via intracytoplasmic sperm injection. The p/his715arg mutation resulted in infertility when knocked-in to a mouse model. The mutant mouse sperm did not

undergo membrane hyperpolarization under capacitating conditions, had impaired acrosome reaction, and failed to fertilize in vitro. These data are consistent with our prediction that loss of SLO3 function prevents the capacitation of human sperm.

Our results have two potential long-term translational outcomes: First, because VU0546110 inhibits human sperm hyperpolarization, hyperactivation, and acrosome reaction, it will likely prevent human sperm from fertilizing eggs. Consistent with this idea, we previously showed that human sperm hyperpolarize during capacitation and that this hyperpolarization correlates with success in IVF (10, 11, 45). Thus, drugs based on VU0546110 could be developed as contraceptives. Importantly, SLO3 is only expressed in sperm, so a SLO3-specific inhibitor would have minimal off-target effects. Second, because our data indicate that SLO3 is required for fertility, mutations in the *SLO3* gene could explain infertility in some of the 25% of infertile male patients with normospermic infertility, defined as normal sperm number, morphology, and linear motility. Future work will be directed at achieving these translational outcomes.

Materials and Methods

Cell Lines. HEK293 cells were transfected with bicistronic vector pBUD-CE4.1 containing the genes of interest and a gene conferring antibiotic resistance. Cells were exposed to gradients of selection antibiotics, and optimal growth conditions for the stable lines were as follows: SLO3 and γ2 (SLO3-γ2) supplemented with 700 μg/mL Zeocin (Invitrogen); human SLO1 and β1 (SLO1-β1) with 1 mg/mL G418 (Sigma) and 3 μg/mL puromycin (Corning, Thermo Fischer); and human SLO1 and β4 (SLO1-β4) with 250 μg/mL zeocin and 3 μg/mL puromycin. All cell lines were grown in DMEM media (Gibco, Thermo Fischer) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin (Millipore Sigma), and selection antibiotics. To generate cells expressing SLO1 and γ2, stable SLO1-expressing HEK293 cells were transiently transfected with expression vectors for γ2 and GFP in the presence of lipofectamine (Invitrogen) for 5 h. The cells were then washed and incubated in supplemented DMEM media for 48 h before use. Patch-clamp electrophysiology was performed only on cells expressing GFP.

Tl$^+$ Flux Assay. A high-throughput Tl$^+$ screening assay was performed as described in the literature with modifications (20, 21). Briefly, HEK293 cells stably expressing SLO3 and γ2 were plated in clear-bottomed, 384-well plates at a density of 20,000 cells per well and cultured overnight in HEPES-buffered Hank's Balanced Salt Solution (HBSS, Gibco). Cells were then loaded with 2.5 μg/mL Thallos (ION Biosciences), washed, incubated in HBSS, and then moved to a Bravo liquid handling instrument where DMSO (solvent control, Sigma) or quinidine (control blocker, Sigma) was added to every other well of the outer two columns. This "checkerboard" was included so that a Z′ statistic could be calculated for every 384-well plate to ensure the assay performed optimally during a screen. The average Z′ calculated for the 157 plates used in the 50,240-compound screen was 0.5, which indicates the assay was robust and reproducible enough to ensure high-confidence hit picking. The remaining 320 wells were used for compound screening. Small molecules from the Vanderbilt Institute of Chemical Biology library, which are cryopreserved as anhydrous DMSO stock solutions, were diluted in HBSS to a screening concentration of 10 μM immediately before use. Each well received 100 μM NS11021 to activate SLO3 and one compound from the small-molecule library. Thallium-induced fluorescence recorded in the presence of 100 μM NS11021 was used as the baseline in the inhibitor screen; each compound was tested only once in the primary screen. The cells were incubated with compounds for 8 min, and Thallos fluorescence at 536/40 nm (482/35 nm excitation) was collected at 1 Hz before and after the simultaneous addition of Tl$^+$ stimulus buffer (6 mM Tl$_2$SO$_4$) to every well of the plate. In the primary screen, compounds were ranked based on their Z′- and B-score, and compounds exceeding 75% inhibition or 50% inhibition with a Z′ score of at least -2 were retested in the Tl$^+$ flux assay.

Patch-Clamp Electrophysiology. Currents in HEK293 cells were measured in the whole-cell patch-clamp configuration with an Axopatch 200B amplifier, digitized with a Digidata 1440a, and interfaced with Clampex 10.6.0.13 (Molecular

Devices). Recording solutions were prepared as previously described (15, 46). Growth media was aspirated and replaced with external recording solution (135 mM NaMeSO$_3$, 5 mM KMeSO$_3$, 2 mM MgCl$_2$, 10 mM HEPES, 10 mM MES, pH 7.2). An isolated cell was located on an Axiovert 200 microscope (Zeiss), and a gigaohm seal was formed with a borosilicate pipette with resistance between 3 and 6 MΩ and filled with internal solution (140 mM KMeSO$_3$, 10 mM HEPES, 10 mM MES, 1 mM EGTA, pH 7). The seal was then broken by applying negative pressure. Cells were kept at a holding potential of −60 mV for 50 ms, then tested with a stepped voltage protocol from −80 to +150 mV in 10 mV increments for 100 ms, then dropped to −40 mV for 50 ms with 2.5 s between sweeps. This protocol was used to record currents present with increasing the concentration of VU0546110 or 3 μL/mL vehicle (DMSO) in the bath solution. Individual cells were tested with between 1 and 5 concentrations of VU0546110 or until the patch was lost. Currents were allowed to stabilize before initial recording and after perfusion solution was changed before recording. Inside-out patch-clamp recordings were performed similarly. Pipette resistance was typically 0.8 to 2 MΩ after fire-polishing and filled with pipette solution containing (in mM): 140 KMeSO$_3$, 20 KOH, 2 MgCl$_2$, 10 HEPES, pH 7.0. The macro-patch was then perfused with solutions containing (in mM): 0 Ca^{2+} pH 5.8 (140 KMeSO$_3$, 20 KOH, 5 mM EGTA, 10 HEPES, pH 5.8); 50 Ca^{2+} pH 5.8 (140 KMeSO$_3$, 20 KOH, 50 μM CaCl$_2$, 10 HEPES, pH 5.8); 0 Ca^{2+} pH 8 (140 KMeSO$_3$, 20 KOH, 5 mM EGTA, 10 HEPES, pH 8); 50 Ca^{2+} pH 8 (140 KMeSO$_3$, 20 KOH, 50 μM CaCl$_2$, 10 HEPES, pH 8). Currents were evoked with a voltage ramp from −100 mV to +200 mV from a holding potential of 0 mV.

Patch-Clamp Data Analysis. Data were analyzed with Clampfit 10.6.2.2 (Molecular Devices). Currents in each cell were normalized to the currents evoked at +100 mV in the absence of inhibitor. Quinidine was added at the end of each experiment in cells expressing SLO3 to remove quinidine-insensitive currents that were likely carried by other K$^+$ channels endogenously expressed in HEK293 cells. This quinidine-insensitive current was then subtracted from all recordings. To ensure that is subtraction did not affect calculated IC50s, data were recalculated without the subtraction. The addition of quinidine after perfusion of VU0546110, paxilline, or iberiotoxin, resulted in a less than threefold change in IC50 (*SI Appendix*, Fig. S7). The currents at each concentration were plotted with Prism 6.1 (GraphPad Software Inc.) or OriginPro 7.5 (OriginLab Corporation) and fit with the Hill Equation (47): $Y=1/(1+[IC50/X]^{HillSlope})$, where X is the concentration of inhibitor and Y is the current evoked at +100 mV. This function was used to calculate the IC50, the concentration at which the current at +100 mV was reduced to half the uninhibited value.

Sperm Preparation. Human sperm samples were obtained by masturbation from de-identified patients after 3 to 5 d of abstinence. The Washington University Fertility and Reproductive Medicine Center confirmed that they met normal semen parameters according to the World Health Organization (≥15 million sperm per mL, ≥40% total motility, ≥32% progressive motility) (48). Samples were allowed to liquefy for 1 h at room temperature. Sperm were purified by swim-up method as previously described (11, 46). Briefly, within 2 h of production, sperm were allowed to swim up in non-commercial human tubular fluid medium (HTF; 98 mM NaCl, 4.7 mM KCl, 0.4 mM KH$_2$PO$_4$, 2 mM CaCl$_2$, 0.2 mM MgSO$_4$, 20 mM HEPES, 3 mM glucose, 21 mM lactic acid, 0.3 mM sodium pyruvate, pH adjusted to 7.4 with NaOH, 285 mmol/kg osmolarity measured with a VAPRO® Vapor Pressure Osmometer 5,600, ELITechGroup, Belgium) for 1 h at 37 °C without CO$_2$.

Sperm Patch-Clamp. Sperm patch-clamp was performed as follows (17, 46). Briefly, sperm from swim-up were placed on a glass coverslip in an RC-22 chamber (Warner Instruments). Gigaohm seals were formed on the cytoplasmic droplet located at the neck of the sperm with a borosilicate pipette fire polished to a resistance of 12 to 15 MΩ in high saline (HS) solution (130 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM glucose, 1 mM sodium pyruvate, 10 mM lactic acid, and 20 mM HEPES, pH 7.4 adjusted with NaOH). Pipettes contained sperm internal recording solution (155 mM KOH, 5 mM KCl, 10 mM BAPTA, 20 mM HEPES, 115 mM MeSO$_3$, pH adjusted to 7.4 with HMeSO$_3$). Internal free Ca^{2+} concentration was controlled by adding CaCl$_2$ and EGTA in concentrations calculated with Ca-EGTA Calculator v1.3 (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/index.html). The seal was broken by applying negative pressure and a 1 ms pulse of 350 to 600 mV. After breaking-in, cells were perfused with a symmetrical external K$^+$ recording solution (160 mM KMeSO$_3$,

10 mM HEPES, 2 mM $CaCl_2$, pH 7.4). Osmolarities of bath and pipette solutions were approximately 320 mmol/kg and 335 mmol/kg respectively. Cells were kept at a holding potential of 0 mV for 50 ms, then tested with a ramped voltage protocol from −100 to +100 mV. Currents were allowed to stabilize, and then 10 μM VU0546110 was added to the perfusion.

Spectrophotometry Measurements of Sperm Membrane Potential. Membrane potential was measured as previously described with minor alterations (30). Briefly, 6 to 7.5 million sperm obtained after swim-up were transferred to gently stirring recording cuvettes and diluted to 1.5 mL. Sperm were then briefly incubated with 1 μM $DiSC_3(5)$ (Sigma) and inhibitor or DMSO. Fluorescence was recorded with a Cary Eclipse spectrophotometer (Agilent) at 620/670 nm excitation/emission wavelength. Once fluorescence reached a steady state value, calibration was performed by adding 1 μM Valinomycin (Sigma) followed by sequential additions of KCl to final concentrations of 7.2 mM, 11.7 mM, 20.7 mM, and 38.7 mM external K^+. These values were then used to calculate the K^+ equilibrium potential according to the Nernst equation with a reference $[K^+]_i$ of 120 mM (49). This corresponded to theoretical membrane potentials of −87.04 mV, −75.79 mV, −61.69 mV, −45.72 mV, and −35.94 mV. The sperm membrane potential was then determined by linearly interpolating the theoretical membrane potential vs. the fluorescence of each trace.

Computer-Assisted Sperm Analysis and Viability Measurement. Human sperm collected by swim-up were incubated in capacitating media for 1 h. After this incubation hyperactivated motility was measured as previously described (11). Briefly, 3 μL sperm suspension was loaded in a 20-micron Leja standard count four-chamber slide. Motility was measured with a HTR-CEROS II v.1.7 (Hamilton-Thorne Research). Sperm were classified as hyperactivated if they met the following criteria: curvilinear velocity (VCL) >150 μm/s, lateral head displacement (ALH) >7.0 μm, and linearity coefficient (LIN) <50% (50). For acute inhibition experiments, 0.1, 0.5, 2.5, or 10 μM VU0546110 or equal volume DMSO (0.5%) was added for 1 min prior to recording motility. For inhibitor incubation experiments, VU0546110 or DMSO was added during the 1 h incubation in capacitating media. Sperm viability was measured by leaving the samples in an incubator overnight at 37 °C and 5% CO_2. 20 nM/mL Hoechst 33342 (Cayman Chemical Company) was added for 2 to 5 min before recording fluorescence intensity using a Flow Cytometer Aurora 4L 16V-14B-10YG-8R using the V3 or 458 nm filter. At least 20,000 cells were recorded and a cutoff for live and dead cells was determined by treatment with 3 to 6% sodium hypochlorite for 2 to 5 min.

Acrosome Reaction Fluorescent Imaging. Human sperm acrosome reaction was measured as previously described (11). Briefly, sperm purified by swim-up were incubated in capacitating media (HTF supplemented with 25 mM HCO_3 and 5 mg/mL BSA) for 18 to 20 h. The acrosome reaction was induced by adding 10 μM calcium ionophore A23187 (Sigma), 10 μM progesterone (Sigma) or DMSO control for 30 min, then sperm were fixed with 3% paraformaldehyde in Tris at 4 °C. Sperm were then plated on slides and stained with FITC-labeled Pisum sativum agglutinin (Millipore Sigma, St. Louis). Fluorescence was imaged with an EVOS FL Cell Imaging System epifluorescence microscope (Thermo Fischer Scientific) at 20× or 40× magnification. Sperm with bright, uniformly stained acrosomes were counted as intact, and sperm with equatorial or no staining were counted as reacted. 2.5 μM VU0546110 or equal volume DMSO were added immediately after swim-up for overnight incubation or during A23187 addition. The induced acrosomal reaction rate was determined by subtracting the percent of reacted sperm induced with DMSO from the percentage induced with A23187 or progesterone.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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