TRPV4 is the temperature-sensitive ion channel of human sperm

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Abstract

Ion channels control human sperm fertilizing ability by triggering hyperactivated motility, which is regulated by membrane potential, intracellular pH, and cytosolic calcium. Previous studies unraveled three essential ion channels that regulate these parameters: 1) the Ca\textsuperscript{2+} channel CatSper, 2) the K\textsuperscript{+} channel KSper, and 3) the H\textsuperscript{+} channel Hv1. However, the molecular identity of the sperm Na\textsuperscript{+} conductance that mediates initial membrane depolarization and, thus, triggers downstream signaling events is yet to be defined. Here, we functionally characterize DSper, the Depolarizing Channel of Sperm, as the temperature-activated channel TRPV4. It is functionally expressed at both mRNA and protein levels, while other temperature-sensitive TRPV channels are not functional in human sperm. DSper currents are activated by warm temperatures and mediate cation conductance, that shares a pharmacological profile reminiscent of TRPV4. Together, these results suggest that TRPV4 activation triggers initial membrane depolarization, facilitating both CatSper and Hv1 gating and, consequently, sperm hyperactivation.
Introduction

The ability of human spermatozoa to navigate the female reproductive tract and eventually locate and fertilize the egg is essential for reproduction [1]. To accomplish these goals, a spermatozoon must sense the environment and adapt its motility, which is controlled in part by ATP production and flagellar ion homeostasis [2]. Of vital importance is the transition from symmetrical basal tail bending into “hyperactivated motility” – an asymmetrical, high-amplitude, whip-like beating pattern of the flagellum - that enables sperm to overcome the egg’s protective vestments [3]–[8]. The steroid hormone progesterone (P4) acts as a major trigger of human sperm hyperactivation [9], [10]. P4 is released by cumulus cells surrounding the egg [11] and causes a robust elevation of human sperm cytoplasmic [Ca^{2+}] via the principal Ca^{2+} channel of sperm, CatSper (EC_{50}= 7.7 ± 1.8 nM) [12]–[14]. The steroid acts via its non-genomic receptor ABHD2, a serine hydrolase that, upon P4 binding, releases inhibition of human CatSper [15]. The Ca^{2+} influx produced from the opening of CatSper channels is a necessary milestone in the process of fertilization and initiates hyperactivated motility [7], [16]–[23].

CatSper channels exhibit weak voltage-dependency with half-maximal activation at V_{1/2\text{human CatSper}} = +70 mV for capacitated sperm cells [12]. This parameter (V_{1/2}) reflects a certain membrane voltage condition under which 50% of CatSper channels are in the open state. Given this unusually high V_{1/2}, only a small fraction of human CatSper channels are open at physiological-relevant membrane potentials. P4 has been shown to potentiate CatSper activity by shifting V_{1/2} to more negative values (V_{1/2\text{human CatSper}} = +30 mV with 500 nM P4 for capacitated sperm cells [12]). However, CatSper still requires both additional intracellular alkalization and significant membrane depolarization to function properly [12], [24]. The proton channel Hv1 was revealed as one of the potential regulators of intracellular pH (pH_i) in human spermatozoa [25], [26]. By mediating unidirectional flow of protons to the extracellular environment, Hv1 represents an important component in the CatSper activation cascade, but it also induces membrane hyperpolarization by exporting positive charges out of the cell. Hv1 is a voltage-gated channel and depends on membrane depolarization to be activated [27], [28]. Therefore, both CatSper and Hv1 must rely on yet unidentified depolarizing
ion channels. P4 was shown to inhibit the K\(^+\) channel of human sperm KSper (IC\(_{50}\)=7.5 ± 1.3 \(\mu\)M [29], [30]) making KSper inhibition one of the potential origins for membrane depolarization. However, efficient KSper inhibition requires P4 concentrations in the \(\mu\)M range, which are only present in close vicinity of the egg. Sperm hyperactivation, however, occurs in the fallopian tubes, where P4 concentrations are not sufficient to block KSper [31]. Hence, the current model is missing a fourth member – the "Depolarizing Channel of Sperm" (DSper) [32]. Activation of this hypothetical DSper would induce long-lasting membrane depolarization and provide the necessary positive net charge influx for CatSper/Hv1 activity. Despite its central role, the molecular identity of DSper yet remains elusive.

The goal of this work was to characterize DSper and resolve its molecular identity in human spermatozoa. Using whole-cell voltage-clamp measurements, we recorded a novel non-CatSper conductance in both capacitated and noncapacitated spermatozoa. This unidentified, nonselective cation conductance exhibited outward rectification and pronounced temperature sensitivity in a range that matches the temperature spectrum of TRPV4 activity. Moreover, the pharmacological profile of DSper bears resemblance to TRPV4. Based on our electrophysiological, biochemical and immunocytochemical data, we thus conclude that the molecular identity of DSper is TRPV4.

Results

A novel non-CatSper conductance of human sperm cells

As many calcium channels, CatSper conducts monovalent ions, such as Cs\(^+\) and Na\(^+\) in the absence of divalent cations from the extracellular solution (divalent free; DVF) [12], [33]. CatSper is also permeable to Ca\(^{2+}\) and Ba\(^{2+}\), but it cannot conduct Mg\(^{2+}\) (Figure 1 – Figure Supplement 1; Table 2 in Fig. 1 – Source Data 1. In presence of extracellular Mg\(^{2+}\) the CatSper pore is blocked, resulting in the inhibition of monovalent CatSper currents (\(I_{\text{CatSper}}\)) (Figure 1 – Figure Supplement 1; Table 2 in Fig 1 -Source data 1).

In whole-cell voltage-clamp recordings from human ejaculated spermatozoa, we consistently observed residual currents when \(I_{\text{CatSper}}\) was blocked with 1 mM
extracellular Mg$^{2+}$ (Fig. 1 A, B). Cs$^+$ inward and outward currents elicited under DVF condition (black traces and bars) were larger than currents recorded in the presence of Mg$^{2+}$ (red traces and bars) (Fig. 1 A-C; Table 1 in Fig. 1 – Source data 1). This phenomenon was observed in both noncapacitated and capacitated spermatozoa, respectively. Notably, capacitated cells generally showed increased current densities under both conditions (Fig. 1 C). The data suggests that the remaining conductance is a novel non-CatSper conductance via the yet to be identified DSper ion channel. DSper currents were potentiated during capacitation (Fig. 1 C; Table 1 in Fig. 1 – Source data 1) and exhibited outward rectification, though, DSper currents recorded from capacitated cells were notably less rectifying (Fig. 1 A, B). This DSper component is unlikely a remnant of an increased leak current since the cells returned to their initial “baseline” current after returning to initial (HS) bath solution (Figure 1 – Figure Supplement 2). Cation influx is the physiologically relevant entity to be analyzed as it represents channel activity under physiological relevant conditions and ensures membrane depolarization. Therefore, we preferentially analyzed DSper inward currents elicited by the change of membrane potential from 0 mV to -80 mV. To rule out ‘contamination’ of putative $I_{DSper}$ with remaining $I_{CatSper}$, we next tested whether 1 mM Mg$^{2+}$ is sufficient to completely block $I_{CatSper}$ and selectively isolate DSper currents. The CatSper inhibitor NNC 55-0396 [12], [13] did not elicit any additional inhibitory effect on $I_{DSper}$ (Fig. 1 D-F; Table 1 in Fig. 1 – Source data 1), confirming efficient CatSper pore block by Mg$^{2+}$. These findings corroborate our hypothesis that a novel CatSper-independent cation conductance could provide additional depolarization under physiological conditions. To isolate $I_{DSper}$, we performed all following experiments in presence of both Mg$^{2+}$ and NNC 55-0396.

**Human sperm DSper current exhibits temperature sensitivity**

We next aimed to investigate mechanism(s) of DSper activation. Previous work had focused on various DSper candidates, one being ATP-activated P2X channels. Navarro *et al.* showed functional expression of P2X2 in mouse spermatozoa [34]. However, human spermatozoa appear to be insensitive to extracellular ATP [35]. De Toni *et al.* suggested that human spermatozoa perform thermotaxis mediated by a member of the thermosensitive transient receptor potential vanilloid channel family, TRPV1 [36],
supporting their claim by immunocytochemistry and Ca^{2+} imaging. By contrast, Kumar et al. detected TRPV4 expression in human spermatozoa using immunocytochemistry and calcium imaging [37]. To date, several temperature-sensitive ion channels and specific transporters have been reported in mammalian sperm [37]–[39]. However, functional characterization of a temperature-activated cation conductance via direct methods, such as electrophysiology, has not been performed in human sperm yet. Since the functional expression of a thermosensitive TRP ion channel in human spermatozoa is currently under debate, and their cation permeability renders many of them DSper candidates, we investigated the impact of temperature on DSper activity. As shown in Figure 2 A-C, elevating temperature profoundly increased I_{DSper}. We observed a temperature-induced potentiation of both inward and outward currents in noncapacitated, as well as capacitated human spermatozoa (Fig. 2A-B; Table 3 in Fig. 2 – Source data 1). A temperature ramp from 23°C to 37°C potentiated I_{DSper} inward currents by factors of 2.7 ± 0.5 for noncapacitated cells and 2.0 ± 0.2 for capacitated cells, respectively (Q_{10,noncapacitated}=1.76, Q_{10, capacitated}=1.65 for caesium inward currents). Half-maximal activation was achieved at T_{1/2} = 34°C (noncapacitated) and T_{1/2} = 31°C (capacitated) (Fig. 2D). Moreover, the temperature-induced potentiation effect was reversible for both noncapacitated and capacitated cells (Figure 2E). We hence conclude that the observed phenomenon is not a temperature-induced loss of the seal and compromised membrane stability and that DSper is indeed temperature-activated.

**DSper conducts Na^{+}**

Since Na^{+} is the major extracellular ion in the female reproductive tract ([Na^{+}] = 140 – 150 mM [40]), Na^{+} is a likely source for membrane depolarization. We therefore investigated whether DSper has the capacity to conduct Na^{+}. As indicated in Figure 3 A, a similar outward rectifying I_{DSper} was recorded when extracellular Cs^{+} was replaced with equimolar concentrations of Na^{+}. I_{DSper} inward Na^{+} currents were entirely CatSper-independent, since NNC 55-0396 had no significant inhibitory effect (Fig. 3 B, C; Table 4 in Fig. 3 – Source data 1). In presence of both 1 mM Mg^{2+} and 1 μM NNC 55-0396, I_{DSper} was still reversibly activated by warm temperatures (Fig. 3 D, E; Table 4 in Fig. 3 – Source data 1) with a 4.1 ± 0.5 -fold increase for inward sodium currents from 22°C to 37°C, which is notably larger than the fold-increase as observed for cesium currents.
Half-maximum activation was at $T_{1/2} = 34^\circ C$, comparable to previously analyzed values for temperature-activated Cs$^+$ currents, however sodium conductance via DSper produced a larger $Q_{10\text{ noncapacitated}} = 2.30$. Together, these electrophysiological data indicate that DSper shares characteristic hallmarks with thermosensitive TRPV channels [41]. We thus proceeded to define which TRPV channel(s) is involved.

**DSper is represented by the cation channel TRPV4**

Based on the observed $I_{\text{DSper}}$ temperature spectrum (Fig. 2D and 3E), candidate channels could be TRPV3, TRPM3 or TRPV4 [42]–[45]. We have ruled out TRPV2 involvement, since TRPV2 has an unusually steep activation threshold of above $53^\circ C$ [46]. In addition, TRPV1 was previously proposed as a mediator of human sperm thermotaxis [36]. To discriminate between these channels, we tested potential effects of corresponding selective agonists – carvacrol [47] for TRPV3, RN1747 [48] for TRPV4, capsaicin [49] for TRPV1, and pregnenolone sulfate [50] for TRPM3. Employing either electrophysiological, or Ca$^{2+}$ imaging recordings, only the TRPV4 agonist RN1747 elicited an effect. In detail, application of 10 $\mu$M RN1747 ($EC_{50} = 0.77 \mu$M [48]) potentiated DSper outward currents significantly (Fig. 4 A, B; Table 5 in Fig. 4 – Source data 1) for noncapacitated human sperm. In contrast, no effects were observed by 1 $\mu$M or 10 $\mu$M capsaicin ($EC_{50} = 711.9 \text{nM}$ [49]) (Figure 4 – Figure Supplement 1, Tables 6-8 in Fig. 4 – Source data 1). In order to confirm TRPV1 absence, we repeated our capsaicin experiments with 30 $\mu$M PI$_{4,5}$P$_2$ inside, to account for a possible loss of capsaicin sensitivity due to potential depletion of endogenous PI$_{4,5}$P$_2$ during whole-cell recording [51], [52]. However, no change in DSper inward- and outward currents was observed. Using Ca$^{2+}$ imaging in fluo-4/AM-loaded sperm, we next recorded fluorescence changes in the flagellar principle piece while stimulating human sperm with either 10 $\mu$M capsaicin or 500 $\mu$M carvacrol (Figure 4 – Figure Supplement 1; Tables 7 and 8 in Fig. 4 – Source data 1). Neither the TRPV1 nor the TRPV3 agonist did elicit any rise in cytosolic calcium levels. We thus conclude that human spermatozoa do not express functional TRPV1 or TRPV3 channels. TRPM3 channels also exhibit temperature sensitivity between ambient warm to hot, which resembles the range observed for DSper [53]. Therefore, we have tested the possibility of TRPM3...
involvement in l_DSper generation by applying the TRPM3 agonist pregnenolone sulfate (PS) (Figure 4 – Figure Supplement 1E; Table 9 in Fig. 4 – Source data 1). Application of 10 µM PS did not result in any change of the basal DSper current, confirming the absence of functional TRPM3 in human spermatozoa. Taking together, our results indicate that the temperature-activated cation channel TRPV4 is likely to be functionally expressed and provides membrane depolarization in human sperm.

Interestingly, additional pharmacological investigation of DSper revealed that both TRPV4-specific antagonists, HC067047 and RN1734 [48], [54], prevented temperature activation of DSper, confirming that DSper pharmacology matches that of TRPV4 (Figure 5A-C; Table 10 in Fig. 5 – Source data 1). Since both inhibitors are dissolved in ethanol, we performed a vehicle control to exclude any inhibitory effect of ethanol on temperature activation. Indeed, the same vehicle concentration (0.1% ethanol) failed to inhibit DSper temperature activation and yielded results comparable to control conditions (Figure 5 – Figure Supplement 1; Table 11 in Fig. 5 – Source data 1).

Supporting our functional data, TRPV4 was detected in human sperm on both mRNA and protein levels. Reverse transcriptase PCR performed with mRNA isolated from swim-up purified spermatozoa, followed by an amplification of full-length TRPV4 (Figure 5 – Figure Supplement 2A) detected a band at the expected size but was absent in negative controls (no reverse transcriptase and no template). Sequencing the isolated PCR product of that specific band (dotted square), yielded the full-length sequence of TRPV4 isoform A (2620 bp, 98 kDa, Q9ERZ8). Moreover, the presence of TRPV4 protein was confirmed by western blotting (Figure 5 – Figure Supplement 2B). Immunoreactive bands were detected at ~115 kDa in extracts from human testicular tissue (1), capacitated (2) and noncapacitated (3) spermatozoa (Figure 5 – Figure Supplement 2B). Immunostaining with anti-hTRPV4 specific antibodies (Figure 5 – Figure Supplement 2C) yielded an immunopositive signal in the acrosome and flagellum. Finally, when TRPV4 was cloned from human sperm mRNA extracts and recombinantly expressed in HEK293 cells (Fig. 6A), a band of similar molecular weight could be detected by western blotting (Fig. 6B). Moreover, TRPV4 cloned from human sperm mRNA recapitulates DSper temperature sensitivity (Fig. 6 D-E; Table 12 in Fig. 6).
– Source data 1), as well as activation by the selective TRPV4 agonist RN1747 (Figure 6F-G; Table 12 in Fig. 6 – Source data 1), indicating that TRPV4 cloned from human sperm cells indeed assembles to a functional channel.

Discussion

Sperm transition to hyperactivated motility is essential for fertilization. Hyperactivation provides the propulsion force required to penetrate through viscous luminal fluids of the female reproductive tract and protective vestments of the egg. The CatSper channel is a key player in the transition to hyperactivated motility [55]. However, proper CatSper function requires three concurrent activation mechanisms: 1) membrane depolarization [12], [24], 2) intracellular alkalization [12], [24], and for primate CatSper specifically 3) abundance of progesterone [12]–[14], [56]. While the two latter mechanisms have been described in detail, the source of membrane depolarization remained puzzling.

In human spermatozoa, K⁺, Ca²⁺, Cl⁻, and H⁺ conductances have been described [12]–[14], [25], [26], [29], [57]–[61]. However, the Na⁺ conductance of sperm remained unknown. Upon ejaculation, mammalian spermatozoa are exposed to increased [Na⁺] (~30 mM in cauda epididymis versus 100–150 mM in seminal plasma). In the female reproductive tract, Na⁺ levels are similar to those in serum (140–150 mM) [40]. Hence, Na⁺ is ideally suited to provide a depolarizing charge upon sperm deposit into the female reproductive tract.

Here, we record a novel CatSper-independent cation conductance that exhibits outward rectification as well as potentiation upon capacitation. We propose that this novel conductance is carried by the hypothetical “Depolarizing Channel of Sperm” DSper and provides the necessary cation influx for membrane depolarization. DSper is activated by warm temperatures between 22 °C and 37 °C (Fig. 2, 3 D-E) which makes the protein thermoresponsive to physiologically relevant temperatures (34.4°C in the epididymis [62], 37 °C body core temperature at the site of fertilization). Previous studies showed
that capacitated rabbit and human sperm cells have an inherent temperature sensing
ability [63], which could be an additional driving force to guide male gametes from the
reservoir towards the warmer fertilization site. It is thus very likely, that human
spermatozoa express a temperature-activated ion channel, which operates in the
described temperature range and enables thermotaxis. Another potential role for this
channel is to serve as a sensor for the initiation of human sperm capacitation. During
maturation in the female reproductive tract, human sperm are exposed to elevated
temperature, especially before and during ovulation, which is correlated with an
increase in basal body temperature by 1 °C. As spermatozoa are able to survive in the
female reproductive tract for several days by binding to the ciliated epithelia of the
fallopian tubes, they must eventually undergo hyperactivation to detach [64].
Accordingly, CatSper-deficient spermatozoa that cannot hyperactivate are not able to
ascend the fallopian tubes [65]. In order for hyperactivation to occur, spermatozoa must
be fully capacitated – a process that takes approximately 5 hours in humans, and
requires sperm exposure to bicarbonate, albumin, and elevated temperature. While
sperm capacitation can be achieved in vitro, exposure to 37°C is an absolute
requirement. Therefore, the presence of a temperature-sensitive sperm ion channel
could serve as the potential sensor for the onset of capacitation and might ensure
sperm final maturation in the female reproductive tract.

The temperature response profile of DSper conforms with previously reported
temperature sensitivity of TRPV4 [41], [43], [44]. Moreover, we observed \( I_{DSper} \)
potentiation by the selective TRPV4 agonist RN1747 (Fig. 4), as well as decreased
temperature-sensitivity upon stimulation with TRPV4 selective inhibitors HC067047 and
RN1734 (Fig. 5). It should be noted that the absence of a significant inward current via
TRPV4 in presence of both extracellular \( Mg^{2+} \) and \( Ca^{2+} \), such as in HS solution (Fig.1A-
B), results from competition for the channel pore between the divalent and monovalent
ions. It has been reported that extracellular \( Ca^{2+} \) inhibits TRPV4 monovalent
conductance [66].

The temperature coefficient \( Q_{10} \) reflects the temperature dependence of the membrane
current and has been reported to be between 9 and 19 for recombinantly expressed
TRPV4 [43], [44]. However, endogenously expressed TRPV4 channels recorded from aorta endothelial cells [44] exhibit less steep temperature dependence, which resembles the Q_{10} of sperm TRPV4 (Q_{10, sodium} = 2.30, noncapacitated sperm). It is possible that different lipid environments or additional channel modifications are responsible for such differences.

It is also possible that sperm cells possess more than one type of temperature-regulated ion channel. The biphasic inhibition of DSper with TRPV4-selective antagonists (Figure 5) does not result in complete current inhibition, particularly in the temperate range between 24°C and 32°C. This may suggest an additional, non-TRPV4 conductance. The molecular nature of such additional conductance(s) could be either temperature-dependent release of NNC inhibition on CatSper or perhaps the presence of other yet undiscovered temperature-sensitive ion channel(s). Interestingly, according to one published report [39], murine TRPV4 regulates sperm thermotaxis. However, TRPV4-deficient male mice are fertile which may indicate either presence of an additional temperature sensor or a compensatory mechanism.

According to our model (Fig. 7), human spermatozoa are exposed to an increase in both temperature and [Na^+] upon deposit to the female reproductive tract. TRPV4-mediated Na^+ influx induces membrane depolarization, which in turn activates both Hv1 and CatSper. H^+ efflux through Hv1 promotes intracellular alkalization and thus enhanced CatSper activation. Approaching the egg, sperm is exposed to P4 and the endocannabinoid anandamide (AEA), both secreted by cumulus cells [67], [68]. P4 binding to ABHD2 releases CatSper inhibition [15] while AEA was shown to activate Hv1 [25]. The resulting opening of CatSper generates a Ca^{2+} influx along the flagellum and serves as the trigger for hyperactivation. P4 not only potentiates CatSper, it also inhibits KSper-mediated hyperpolarization, which gives the CatSper activation cascade an additional impulse [29].

Using a CatSper2-deficient infertile patient, no remaining cation current was recordable, when both Hv1 and KSper were blocked [14]. However, these recordings were performed in a condition where ATP was absent from the pipette solution. According to Phelps et al. intracellular ATP binding to the N-terminal ankyrin repeat domain of
TRPV4 has a profound sensitizing effect [69], [70], which is a feature of the TRPV ankyrin repeats and is shared between TRPV1 and TRPV4 [70]. Indeed, addition of 4 mM ATP to the pipette solution, allowed us to consistently record TRPV4 activity from fertile human sperm.

Our data suggests that TRPV4 activity is increased upon capacitation. Since capacitation encompasses changes in the phosphorylation state of many proteins [71], and TRPV4 requires tyrosine phosphorylation to function properly [72], it is likely that TRPV4 phosphorylation is required. It would also explain, why only capacitated human spermatozoa appear to be thermotactically responsive [63]. Interestingly, we also observed different $I_{\text{DSPer}}$ kinetics (i.e., less outward rectification) after capacitation. This finding could also be the result of phosphorylation, modified lipid composition or even formation of TRPV4/X heteromers upon capacitation. These aspects will be addressed in future studies.

Selective anti-hTRPV4 antibodies located TRPV4 at the flagellum and acrosome of human sperm (Figure 5 – Figure Supplement 2C). The localization of TRPV4 in the acrosome region should be evaluated critically since this compartment is highly antigenic and attracts antibodies in general [73]. However, TRPV4 appears to be distributed in the sperm flagellum. The principal piece of the sperm tail is also the compartment where CatSper and Hv1 reside [25], [55], bringing those three interdependent ion channels in close proximity to each other.

TRPV4 – more precisely its hyperfunction - might underlie the aversive effect of increased scrotal temperatures on sperm production and epididymal preservation. As proposed by Bedford et al., increased scrotal temperatures when clothed contribute substantially to the inferior quality of human ejaculate [74]. By contrast, TRPV4 might represent an attractive target for male fertility control, since TRPV4 is likely to lie upstream in the signaling cascades leading to sperm hyperactivation and can be heterologously expressed for high-throughput functional studies.
### Materials and methods

#### Table 1: Key Resources Table

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*Human sperm cells.*

A total of 5 healthy male volunteers were recruited to this study, which was conducted with approval of the Committee on Human Research at the University of California, Berkeley (protocol 10-01747, IRB reliance #151). Informed consent was obtained from all participants. Ejaculates were obtained by masturbation and spermatozoa were purified following the swim-up protocol as previously described [12]. In-vitro capacitation
was accomplished by 4-hour incubation in 20 % Fetal bovine serum, 25 mM NaHCO₃ in HTF buffer [75] at 37 °C and 5 % CO₂.

**Reagents**

RN1747, HC067047 and RN1734 were purchased from Tocris Bioscience (Bristol, UK), NNC 55-0396 was purchased from R&D systems (Minneapolis, USA), capsaicin from Cayman Chemical (Ann Arbor, USA), fluo-4/AM is from Invitrogen (Thermo Fisher Scientific, Carlsbad, USA) and all other compounds were obtained from Sigma (St. Louis, USA). NNC 55-0396 stock solution was dissolved in water, HC067047 and RN1734 were dissolved in EtOH, while RN1747 and PS were dissolved in DMSO.

**Electrophysiology**

For electrophysiological recordings, only the ultra-pure upper 1 ml of the swim-up fraction was used. Single cells were visualized with an inverse microscope (Olympus IX71) equipped with a differential interference contrast, a 60 x Objective (Olympus UPlanSApo, water immersion, 1.2 NA, ∞/0.13-0.21/FN26.5) and a 1.6 magnification changer. An AXOPATCH 200B amplifier and an Axon™ Digidata 1550A digitizer (both Molecular Devices, Sunnyvale, CA, USA) with integrated Humbug noise eliminator was used for data acquisition. Hardware was controlled with the Clampex 10.5 software (Molecular Devices). We monitored and compensated offset voltages and pipette capacitance \( C_{\text{fast}} \). Gigaohm seals were established at the cytoplasmic droplet of highly motile cells in standard high saline buffer (“HS” in mM: 135 NaCl, 20 HEPES, 10 lactic acid, 5 glucose, 5 KCl, 2 CaCl₂, 1 MgSO₄, 1 sodium pyruvate, pH 7.4 adjusted with NaOH, 320 mOsm/l) [33], [12]. The patch pipette was filled with 140 mM CsMeSO₃, 20 mM HEPES, 10 mM BAPTA, 4 mM NaATP, 1 mM CsCl (pH 7.4 adjusted with CsOH, 330 mOsm/l). For recordings from capacitated spermatozoa, BAPTA was substituted for 5 mM EGTA and 1 mM EDTA. We confirmed that changing of the chelator composition had no effect on DSper current amplitudes in noncapacitated cells. Transition into whole-cell mode was achieved by applying voltage pulses (499–700 mV, 1-5 ms, \( V_{\text{hold}} = 0 \) mV) and simultaneous suction. After establishment of the whole-cell configuration, inward and outward currents were elicited via 0.2 Hz stimulation with voltage ramps (-80 mV to +80 mV in 850 ms, \( V_{\text{hold}} = 0 \) mV, total 1000 ms/ramp). Data was not corrected for
liquid junction potential changes. To ensure stable recording conditions, only cells with baseline currents (in HS solution) ≤ 10 pA at -80 mV were used for experiments. Under “HS” condition, CatSper and DSper currents were considered to be minimal, thus any remaining baseline current represented the cells leak current. During whole-cell voltage-clamp experiments, the cells were continuously perfused with varying bath solutions utilizing a gravity-driven perfusion system. If not stated otherwise, electrophysiological experiments were performed at 22°C. Temperature of the bath solution was controlled and monitored with an automatic temperature control (TC-324B, Warner Instrument Corporation, Hamden, CT, USA). Both, CatSper and DSper currents were recorded under symmetric conditions for the major permeant ion. Under these conditions, the bath solution was divalent free (“DVF”) containing (in mM) 140 CsMeSO$_3$, 20 HEPES, 1 EDTA, and pH 7.4 was adjusted with CsOH, 320 mOsm/l. To isolate DSper conductances, monovalent currents through CatSper channels were inhibited by supplementing the DVF solution with 1 mM Mg$^{2+}$ [21]. Experiments with different bath solutions were performed on the same cell. Signals were sampled at 10 kHz and low-pass filtered at 1 kHz (Bessel filter; 80 dB/decade). Pipette resistance ranged from 9 – 15 MΩ, access resistance was 21–100 MΩ, membrane resistance ≥ 1.5 GΩ. Membrane capacitance was 0.8-1.3 pF and served as a proxy for the cell surface area and thus for normalization of current amplitudes (i.e., current density). Capacitance artifacts were graphically removed. Statistical analysis was done with Clampfit 10.3 (Molecular Devices, Sunnyvale, CA, USA), OriginPro 8.6 (OriginLab Corp., Northampton, MA, USA) and Microsoft Excel 2016 (Redmond, WA, USA). Statistical data are presented as mean ± standard error of the mean (SEM), and (n) indicates the number of recorded cells. Statistical significance was determined with unpaired t-tests.

Temperature dependency for cesium and sodium inward currents was fitted using the Boltzmann equation $y = A2 + (A1 - A2)/(1 + \exp((x - x0)/dx))$ with parameters as indicated in Table 2.

Table 2: Fitting parameters
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<th>cell type</th>
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<th>A2</th>
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<th>dx</th>
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</thead>
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<td>5.19918</td>
<td>34.1</td>
<td>3.6</td>
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</table>

The temperature coefficient $Q_{10}$ reflects the temperature dependence of the membrane current and was obtained using the van't Hoff equation: $Q_{10} = (I_2/I_1)^{10/(T_2-T_1)}$ where $I_n$ are the corresponding current amplitudes at the lower ($T_1$) and higher temperatures ($T_2$) in °C. Here, we analyzed current amplitudes at 22 °C and 37 °C.

**Calcium Imaging**

All calcium imaging experiments were performed in HS solution. Prior to fluorescence recording, swim-up purified human spermatozoa were bulk loaded with 9 µM fluo-4/AM (dissolved in DMSO) and 0.05% Pluronic (dissolved in DMSO) in HS solution for 30 min at room temperature. Cells were then washed with dye-free HS solution and allowed to adhere to glass imaging chambers (World Precision Instruments, Sarasota, USA) for 1 min. Via continuous bath perfusion, the attached spermatozoa were presented with alternating extracellular conditions (HS +/- agonist/antagonist). Fluorescence was recorded at 1 Hz, 100 ms exposure time over a total time frame as indicated. Imaging was performed using a Spectra X light engine (Lumencore, Beaverton, USA) and a Hamamatsu ORCA-ER CCD camera. Fluorescence change over time was determined as $\Delta F/F_0$ where $\Delta F$ is the change in fluorescence intensity ($F - F_0$) and $F_0$ is the baseline intensity as calculated by averaging the fluorescence signal of the first 20 s in HS solution. Regions of interest (ROI) were restricted to the flagellar principal piece of each cell by manual selection in ImageJ (Java, Redwood Shores, CA, USA). Statistical data are presented as mean ± standard error of the mean (SEM), and (n) indicates the number of recorded cells.

**Immunocytochemistry**

Purified spermatozoa were plated onto 20-mm coverslips in HS and allowed to attach for 20 min. The cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min and washed twice with PBS. Additional fixation was performed with 100% ice-cold methanol.
for 1 min with two washing steps in PBS. Cells were blocked and permeabilized by 1-hour incubation in PBS supplemented with 5 % immunoglobulin-γ (IgG)–free BSA and 0.1 % Triton X-100. Immunostaining was performed in the same blocking solution. Cells were incubated with primary antibodies (rabbit polyclonal αTRPV4, 1:100, abcam ab39260) overnight at 4°C. After extensive washing in PBS, secondary antibodies (mouse monoclonal αRabbit-DyLight™488, 1:1000, Jackson 211-482-171) were added for 45 min at room temperature. After vigorous washing, cells were mounted with ProLong Gold Antifade with DAPI reagent (Life Technologies, Carlsbad, CA) and imaged with a confocal microscope.

**RT-PCR and cloning**

Total donor-specific RNA was extracted from purified spermatozoa with a QIAGEN RNAeasy mini kit followed by complementary DNA synthesis with a Phusion RT-PCR kit (Finnzymes, MA, USA). The donor-specific translated region of TRPV4 (cDNA) was amplified with the primers forward 5'-ACAGATATCACCATGGCGGATTCCAGCG -3' and reverse 5'-AACACAGCGGCGCTAGAGCGGCGGTATC-3' and was subcloned into a pTracer-CMV2 vector (Invitrogen) using the restriction sites: EcoRV and NotI. TRPV4 identity was sequence verified. HEK293 (ATCC CRL-1573) cells were transfected during passages 2 to 15 using a standard lipofectamine protocol (Invitrogen). Transfected cells were identified as green fluorescent and successful transfection was verified via both western blotting and electrophysiology. The cell lines was not tested for mycoplasma and is not on the list of commonly misidentified cell lines maintained by the International Cell Line Authentication Committee: [http://iclac.org/wp-content/uploads/Cross-Contaminations-v8_0.pdf](http://iclac.org/wp-content/uploads/Cross-Contaminations-v8_0.pdf).

**Immunoblotting**

The highly motile sperm fraction was separated from other somatic cells (mainly white blood cells, immature germ cells, and epithelial cells) by density gradient consisting of 90% and 50% isotonic Isolate (Irvine Scientific, CA) solution diluted in HS solution with the addition of protease inhibitors (Roche). Protease inhibitors were used throughout the whole procedure. After centrifugation at 300 g for 30 min at 24 °C, the sperm pellet at the bottom of the 90% layer was collected, diluted ten times, and washed in HS by
centrifugation at 2000 g for 20 min. Cells were examined by phase-contrast microscopy for motility and counted before centrifugation. Contamination of the pure sperm fraction by other cell types was minimal, with less than 0.2% of somatic cells, which was below the protein detection threshold for immunoblotting applications. The pellet was subjected to osmotic shock by a 5 min incubation in 0.5x HS solution, the addition of 10 mM EDTA and 10mM dithiothreitol (DTT) for 10 min, and sonication in a water bath at 25 °C for 5 min. Osmolarity was adjusted by addition of 10x phosphate-buffered saline (PBS). Laemmli sample buffer (5x) was added to a final 1x concentration, and the DTT concentration was adjusted to 20 mM. An additional 5 min sonication and boiling at 100 °C for 5 min were performed. The total crude cell lysate was loaded onto a 4%–20% gradient Tris-HCl Criterion SDS-PAGE (BioRad) with 500,000 sperm cells/well.

TRPV4- and empty vector-transfected HEK293 cells were lysed in 2x Laemmli sample buffer and subjected to SDS-PAGE. Ten thousand cells per well were loaded onto SDS-PAGE. After transfer to polyvinylidene fluoride membranes, blots were blocked in 0.1% PBS-Tween20 with 3% IgG-free BSA for 15 min and incubated with primary antibodies overnight at 4 °C. Blots were probed with rabbit anti-b-tubulin antibodies (Abcam), mouse monoclonal anti-actin C4 antibodies (Abcam), or anti-TRPV4 antibodies (Abcam). After subsequent washing and incubation with secondary horseradish peroxidase-conjugated antibodies (Abcam), membranes were developed with an ECL SuperSignal West Pico kit (Pierce).

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Competing interests
The authors declare that no competing interests exist.

Figure legends

**Figure 1**: Electrophysiological recordings reveal a novel non-CatSper conductance.

**Figure 1 – Figure Supplement 1**: Human CatSper conducts Ca$^{2+}$ and Ba$^{2+}$ but not Mg$^{2+}$

**Figure 1 – Figure Supplement 2**: DSper currents are recorded under stable conditions.

**Figure 2**: DSper is activated by warm temperatures.

**Figure 3**: DSper conducts sodium.

**Figure 4**: DSper is activated by the TRPV4 agonist RN1747.

**Figure 5**: TRPV4 inhibitors RN1734 and HC067047 antagonize human DSper activity.

**Figure 5 – Figure Supplement 1**: EtOH vehicle control.

**Figure 5 – Figure Supplement 2**: TRPV4 can be detected on protein and mRNA level.

**Figure 6**: Sperm TRPV4 assembles to a functional channel when recombinantly expressed in HEK293 cells.

**Figure 7**: Interdependency of ion channel complexes in the sperm flagellum.

Source data file legends

**Figure 1-source data 1**. Table 1: Inward and Outward DSper currents. Table 2: Divalent CatSper inward currents

**Figure 2-source data 1**. Table 3: DSper inward currents as a function of temperature

**Figure 3-source data 1**. Table 4: DSper sodium inward currents

**Figure 4-source data 1**. Table 5: DSper inward currents potentiated by RN1747. Table 6: DSper currents after stimulation with Capsaicin. Table 7: Calcium imaging
(stimulation with 10 μM capsaicin after 20 seconds). Table 8: Calcium imaging (stimulation with 500 μM carvacrol after 20 s). Table 9: Pregnonolone sulfate effect on \( I_{DSper} \).

**Figure 5-source data 1.** Table 10: DSper’s temperature-sensitivity in presence of TRPV4 inhibitors RN1734 and HC067047. Table 11: EtOH vehicle control.

**Figure 6-source data 1.** Table 12: TRPV4 activity recombinantly expressed in HEK293 cells.
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Fig. 4 - Supplementary Fig. 1:
TRPV1, TRPV3 and TRPM3 are not functionally expressed in human spermatozoa
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**Fig. 5 - Supplementary Fig. 1: EtOH vehicle control**

**Panel A**: Graph showing the effects of 0.1% EtOH on the current-voltage relationship at 22 °C and 34 °C. The current is measured at 200 ms and 25 pA.

**Panel B**: Bar graph showing the fold change in current with 4 mM ATP, 140 mM 1 mM Mg, 150 mM Cs, and 1 μM NNC at 22 °C and 34 °C for control and 0.1% EtOH conditions. The data is indicated as (5) and (3) for respective conditions.
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