The CatSper channel controls chemosensation in sea urchin sperm

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Abstract

Sperm guidance is controlled by chemical and physical cues. In many species, Ca2+ bursts in the flagellum govern navigation to the egg. In Arbacia punctulata, a model system of sperm chemotaxis, a cGMP signaling pathway controls these Ca2+ bursts. The underlying Ca2+ channel and its mechanisms of activation are unknown. Here, we identify CatSper Ca2+ channels in the flagellum of A. punctulata sperm. We show that CatSper mediates the chemotactant-evoked Ca2+ influx and controls chemotactic steering; a concomitant alkalinization serves as a highly cooperative mechanism that enables CatSper to transduce periodic voltage changes into Ca2+ bursts. Our results reveal intriguing phylogenetic commonalities but also variations between marine invertebrates and mammals regarding the function and control of CatSper. The variations probably reflect functional and mechanistic adaptations that evolved during the transition from external to internal fertilization.

Keywords CatSper; Ca2+ signaling; chemotaxis; sperm

Subject Categories Development & Differentiation; Membrane & Intracellular Transport

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Introduction

The intracellular Ca2+ concentration ([Ca2+]i) coordinates several sperm functions required for fertilization (Ho & Suarez, 2001; Eisenbach & Giojalas, 2006; Florman et al, 2008; Kaupp et al, 2008; Publicover et al, 2008). In particular, Ca2+ controls the beat of the flagellum and, thereby, the swimming behavior. In mice and humans, the sperm-specific Ca2+ channel CatSper (cation channel of sperm) represents the principal pathway for Ca2+ entry into the flagellum (Quill et al, 2001; Ren et al, 2001; Kirichok et al, 2006; Lishko et al, 2010). Targeted disruption of CatSper in mice impairs sperm motility (Qi et al, 2007), and CatSper—/- sperm fail to traverse the oviduct (Ho et al, 2009; Miki & Clapham, 2013; Chung et al, 2014) and to penetrate the egg coat (Ren et al, 2001)—deficits that cause male infertility (Quill et al, 2001; Ren et al, 2001; Qi et al, 2007). Similarly, mutations in human CatSper genes cause infertility in men (Avénarius et al, 2009; Hildebrand et al, 2010).

CatSper has been proposed to serve as a polymodal sensor that integrates diverse chemical and physical cues (Brenker et al, 2012; Miki & Clapham, 2013; Tavares et al, 2013; Schiffer et al, 2014): In general, CatSper is activated at depolarized membrane potentials (Vm) and at alkaline intracellular pH (pHi) (Kirichok et al, 2006; Lishko et al, 2010, 2011; Strünker et al, 2011). However, the interplay between Vm and pHi to control CatSper during fertilization is unknown. In human sperm, hormones in the seminal fluid and the oviduct, i.e. prostaglandins and progesterone, directly activate CatSper (Lishko et al, 2011; Strünker et al, 2011; Brenker et al, 2012; Smith et al, 2013) and, thereby, affect sperm motility (Aitken & Kelly, 1985; Alasmari et al, 2013). Progesterone has been implicated in human sperm chemotaxis (Oren-Benaroya et al, 2008; Publicover et al, 2008; Teves et al, 2009); yet, in vivo, neither sperm chemotaxis nor the physiological role of these hormones during fertilization has been definitely established (Baldi et al, 2009). This is due to the demanding challenge to experimentally emulate the complex chemical, hydrodynamic, and topographical landscape of
the female genital tract (Suarez & Pacey, 2006; Suarez, 2008; Kirkman-Brown & Smith, 2011; Miki & Clapham, 2013).

In contrast, many aquatic species, in particular marine invertebrates, release their gametes into the ambient water; consequently, gametes of broadcast spawners can be studied under close to native conditions. For 100 years, sperm of marine invertebrates have served as a powerful model of fertilization research (Kaupp, 2012). It is well established that in the aquatic habitat, sperm are guided to the egg by chemotaxis. A case in point is sea urchin sperm. In sea urchin sperm, a cGMP signaling pathway generates Ca2+ bursts in the flagellum that coordinate chemotactic steering (Böhmer et al., 2005; Wood et al., 2005; Darszon et al., 2008; Kaupp et al., 2008; Guerrero et al., 2010a,b; Alvarez et al., 2012). Important components and cellular events of this signaling pathway have been identified (reviewed in Darszon et al., 2008; Kaupp et al., 2008; Alvarez et al., 2014). Briefly, the chemoattractant activates a receptor guanylyl cyclase (GC) and, thereby, stimulates rapid cGMP synthesis (reviewed in Darszon et al., 2008; Kaupp et al., 2008; Beltrán et al., 2006; Galindo et al., 2007; Bönigk et al., 2009). The ensuing hyperpolarization (Cook & Babcock, 1993; Reynaud et al., 1993; Beltrán et al., 1996; Strünker et al., 2006) activates a sperm-specific voltage-dependent Na+/H+ exchanger (nNHE) (Lee, 1984a; Lee, 1984b; Lee & Garbers, 1986), mediating a rapid rise of pH; (Nishigaki et al., 2001; Solzin et al., 2004) and, eventually, opens voltage-gated Ca2+ channels (Gonzáles-Martínez et al., 1992; Beltrán et al., 1996; Nishigaki et al., 2001; Kaupp et al., 2003; Strünker et al., 2006). However, to date, the molecular identity of the Ca2+ channel and its mechanism of activation have been elusive.

CatSper genes exist in many metazoan genomes, including aquatic animals (Cai & Clapham, 2008), yet the expression and function of CatSper in non-mammalian species are unknown. Here, we show that CatSper represents the long-sought Ca2+ channel of the chemotactic signaling pathway in sperm of the sea urchin A. punctulata. CatSper mediates the chemoattractant-induced Ca2+ bursts and controls chemotactic steering. We unveil the intimate, allosteric relationship between pH; and Vm for CatSper activation: A minute chemoattractant-induced increase of pH; enables CatSper in a highly cooperative fashion to open during a subsequent depolarization. The pH;-induced shift of the voltage dependence of CatSper activation enables sperm to transduce periodic Vm changes into periodic Ca2+ bursts during sperm navigation on periodic paths in a chemoattractant gradient. We reveal intriguing commonalities and variations in the function and molecular makeup of chemosensory signaling pathways in sperm from mammals and marine invertebrates. Although different in design, these pathways share the CatSper channel as a key component of Ca2+ signaling.

Results

CatSper is expressed in the flagellum of Arbacia punctulata sperm

From a cDNA library of A. punctulata testis, we cloned four cDNAs encoding pore-forming CatSper subunits (ApCatSper 1–4) (Fig 1A, Supplementary Fig S1). Each ApCatSper subunit harbors six transmembrane segments (S1–S6), a voltage sensor in S4, and a pore loop between S5 and S6 (Fig 1A, Supplementary Fig S1). The pore loops carry the signature sequence of Ca2+, and CatSper channels (Fig 1B, upper panel) (Navarro et al., 2008). Similar to other voltage-gated channels, the S4 segments of all four ApCatSper subunits carry six to seven positively charged residues (Fig 1B, lower panel). The intracellular N- or C-termini of ApCatSper 1, 2, and 4, but not of ApCatSper 3, carry coiled-coil domains (Fig 1A, Supplementary Fig S1) that were proposed to mediate heterotetramerization in mammalian CatSper (Lobley et al., 2003). The overall homology of ApCatSper subunits with their mammalian CatSper ortholog is low (25–35%).

To localize ApCatSper subunits in sperm, we raised monoclonal antibodies against ApCatSper 2 and 3 (Supplementary Fig S1). Hemagglutinin (HA)-tagged ApCatSper 2 and 3 subunits were heterologously expressed in Chinese hamster ovary (CHO) cells. In Western blots, an anti-HA antibody labeled polypeptides with apparent molecular weights (Mr) of 66.5 ± 3.1 kDa (ApCatSper 2, n = 24) and 41.6 ± 2.1 kDa (ApCatSper 3, n = 9). The same polypeptides were recognized by the monoclonal anti-ApCatSper 2 and 3 antibodies in transfected CHO cells and in sperm (Fig 1C), demonstrating that ApCatSper 2 and 3 are expressed in A. punctulata sperm. In immunocytochemistry, the anti-ApCatSper 3 antibody stained the flagellum (Fig 1D, left). The staining pattern of the receptor GC (Fig 1D, middle), the CNGK channel (Fig 1D, right), and ApCatSper 3 overlapped, showing that CatSper colocalizes with components of the chemotactic signaling pathway. Mass spectrometry confirmed the presence of ApCatSper 1–4 in the flagellum: In protein preparations from purified flagella, we identified proteolytic peptides for all four ApCatSper subunits (Supplementary Fig S1, Supplementary Table S1); the peptides covered 5–25% of the respective protein sequences (Supplementary Table S1). Moreover, in the A. punctulata genome and testis transcriptome (to be published), we identified a gene encoding the accessory subunit CatSper δ (Chung et al., 2011) and mRNAs encoding CatSper β (Liu et al., 2007) and CatSper γ (Wang et al., 2009) (Supplementary Fig S1, Fig 1A). In purified flagella, we identified proteolytic peptides of the predicted accessory subunits (Supplementary Table S1). We conclude that ApCatSper 1–4 and ApCatSper β, δ, and γ are expressed in sperm and are located in the flagellum.

We immunoprecipitated ApCatSper 2 and ApCatSper 3, using the respective anti-ApCatSper antibodies. Analysis of the co-immunoprecipitates by Western blotting (Fig 1E) and mass spectrometry (Fig 1F, Supplementary Table S2) indicates that ApCatSper 1–4, β, δ, and γ interact to form a protein complex. Therefore, we propose that the architecture of the CatSper channel is similar in sea urchins and mammals. Unfortunately, like their mammalian counterparts (Ren et al., 2001), heterologously expressed ApCatSper subunits did not yield functional channels.

Intracellular pH and membrane voltage control Ca2+ influx in sea urchin sperm

Using a stopped-flow apparatus and fluorescent probes for Ca2+, Vm, and pH, we studied the role of CatSper in intact A. punctulata sperm. Ammonium chloride (NH4Cl) evoked a rapid and sustained intracellular alkalization (Supplementary Fig S2) that stimulated a Ca2+ increase (Fig 2A). At low NH4Cl concentrations (≤ 3 mM),
Ca\(^{2+}\) signals slowly reached a plateau; at higher concentrations (\(\geq 10\) mM), NH\(_4\)Cl evoked rapid, oscillatory Ca\(^{2+}\) responses (Fig. 2A). Mixing of sperm with both NH\(_4\)Cl and EGTA, which lowers extracellular [Ca\(^{2+}\)] to \(\leq 400\) nM, abolished the Ca\(^{2+}\), but not the pH\(_i\) response (Supplementary Fig S2), demonstrating that alkalization stimulates Ca\(^{2+}\) influx.
Two distinct CatSper inhibitors, MDL12330A (MDL) (Brener et al., 2012) and mibebradil (Strünker et al., 2011), suppressed the alkaline-evoked Ca\(^{2+}\) signal (Fig 2B and C, Supplementary Fig S2); the constants of half-maximal inhibition (K\(_{i}\)) were 15.6 ± 3.3 μM (MDL) and 20.7 ± 5.1 μM (mibebradil) (n = 4) (Fig 2B and C, Supplementary Fig S2). Sperm were mixed simultaneously with NH\(_4\)Cl and the inhibitors, and the time course of inhibition probably reflects the time required for the drug to reach the blocking site; we did not test whether drug action reached steady state within the recording time.

The drugs inhibit CatSper-mediated Ca\(^{2+}\) signals in human sperm with similar potency (Strünker et al., 2011; Brener et al., 2012). We conclude that in sea urchin sperm, similar to mouse and human sperm, CatSper mediates alkaline-evoked Ca\(^{2+}\) influx. Because MDL and mibebradil are not selective for CatSper, we cannot exclude that the sperm might harbor additional, so far unknown Ca\(^{2+}\)-permeable channels that are also activated at alkaline pH and inhibited by both drugs.

We determined the pH sensitivity of the alkaline-induced Ca\(^{2+}\) influx using the “pH\(_i\) pseudo-null-point” method (Eisner et al., 1989; Chow et al., 1995; Bond & Varley, 2005; Swietach et al., 2010) that allows clamping of pH\(_i\) to fixed values and calibration of the pH indicator BCECF. Key is a set of pH\(_i\)-clamp solutions composed of a weak acid (butyric acid, BA) and a weak base (trimethylamine, TMA) at different molar ratios (see Materials and Methods). TMA and BA freely equilibrate across the membrane and, at sufficiently high concentrations (see Materials and Methods), establish a defined pH\(_i\) that is set by the acid/base ratio (Chow et al., 1996).

Mixing of sperm with a pH 7.2-clamp solution changed pH\(_i\) only slightly, suggesting a resting pH (pH\(_{rest}\)) of about 7.2 (Fig 3A). Mixing with pH\(_i\)-clamp solutions < 7.2 and > 7.2 evoked acidification and alkalization, respectively, that was stable after 4–5 s and persisted for at least 14 s (Fig 3A). The changes in AR/R of BCECF fluorescence were linearly related to pH\(_i\)-clamp values (Fig 3B); interpolation yielded a pH\(_{rest}\) of 7.16 ± 0.04 (Fig 3B; n = 7). Similar pH\(_{rest}\) values of sea urchin sperm were determined by other methods (Babcock et al., 1992; Guerrero et al., 1998). Moreover, the calibration allowed rescaling of the data in Fig 3A to absolute pH\(_i\) values (inset in Fig 3B).

Figure 3C shows the time course of Ca\(^{2+}\) responses in sperm mixed with different pH\(_i\)-clamp solutions. Plotting the amplitude of the Ca\(^{2+}\) signals versus the respective pH\(_i\)-clamp values disclosed an exceptionally steep dose–response relation with a pH\(_{thr}\) of 7.47 ± 0.01 and a Hill coefficient of 10.8 ± 2.2 (Fig 3D, n = 4). From the time course of the changes in pH\(_i\) and Ca\(^{2+}\), we reconstructed the threshold pH\(_i\) (pH\(_{thr}\)) at which the Ca\(^{2+}\) influx commenced (Fig 3E). For example, using the pH\(_i\) 7.6-clamp solution, the Ca\(^{2+}\) signal was observed after a latency of ≈ 200 ms (Fig 3E, dotted black line), at which the pH\(_i\) of sperm had increased to ≈ 7.3 (Fig 3E, dotted red line), i.e. pH\(_{thr}\) for Ca\(^{2+}\) influx. We determined pH\(_{thr}\) for the entire range of pH\(_i\)-clamp solutions (Fig 3F). The latency of the Ca\(^{2+}\) influx decreased with increasing pH\(_i\)-clamp values (Fig 3F, black), because the alkalization proceeded on a faster time scale (Fig 3A and inset of Fig 3B).

However, pH\(_{thr}\) was largely independent of the rate and magnitude of the pH\(_i\) increase (Fig 3F, red). The invariant pH\(_{thr}\) for the alkaline-induced Ca\(^{2+}\) influx and its exceptionally steep, switch-like dose–response relation suggest that intracellular alkalization sensitizes CatSper to open during depolarization.

We also wondered whether depolarization evokes a Ca\(^{2+}\) increase. In fact, rapid elevation of the extracellular K\(^+\) concentration ([K\(^+\)]\(_o\)) to ≥ 30 mM evoked a transient Ca\(^{2+}\) signal (Fig 4A), whose amplitude was graded with [K\(^+\)]\(_o\). MDL inhibited Ca\(^{2+}\) signals evoked by 80 mM and 160 mM K\(^+\) with a K\(_i\) of 38.8 ± 7.5 μM and 29.2 ± 11.2 μM (n = 3), respectively (Fig 4B–D); the Ca\(^{2+}\) signals were also suppressed by mibebradil (Supplementary Fig S2). We conclude that CatSper also supports depolarization-evoked Ca\(^{2+}\) influx in sea urchin sperm.

We examined the relationship between pH\(_{thr}\) and V\(_{m}\). To manipulate the resting potential (V\(_{rest}\)), sperm were incubated at different [K\(^+\)]\(_o\). In standard artificial sea water (ASW, 9 mM [K\(^+\)]\(_o\), V\(_{rest}\) was −51.9 ± 2 mV (Fig 4E, n = 6); sperm were hyperpolarized and depolarized to −54.9 ± 2.2 mV and −26.3 ± 4.2 mV, respectively, at low (3 mM) and high (191 mM) [K\(^+\)]\(_o\) (Fig 4F, n = 3); V\(_{rest}\) was determined by the [K\(^+\)]\(_o\) null-point method (Strünker et al., 2006; see also Materials and Methods), assuming an intracellular K\(^+\) concentration of 423 mM. Probing cells with different pH\(_i\)-clamp solutions and analyzing the time course of pH\(_i\) and Ca\(^{2+}\) signals...
influx via CatSper by pH i and membrane voltage is closely
values (Fig 4E and F, Supplementary Fig S3); a linear fit of the data
revealed pH thr for different
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Figure 3. Determination of the threshold pHi for alkaline-evoked Ca2+ influx.

A Changes in pHi evoked by mixing with pHi-clamp solutions (see explanation in the text); sperm were loaded with the pHi indicator BCECF. ΔR/R (%) indicates the change in the BCECF fluorescence emission ratio (ΔR = R_{pH}\text{clamp} - R_{basal}) with respect to the basal ratio (R, mean of the first 3–5 data points).
B Steady-state change (at t = 14 s) of BCECF fluorescence for the pHi signals shown in (A). The intercept of the fitted straight line with the x-axis yields the resting pHi.
C The slope of the straight line yields the threshold pHi. Inset: calibrated changes in pHi evoked by various pHi-clamp solutions.
D Steady-state change (at t = 14 s) of BCECF fluorescence for the Ca2+ signals shown in (B, inset) and (C), respectively. The threshold pHi for CatSper activation was deduced from the latency of the Ca2+ signal.

We studied the control of Ca2+ influx by pH i and membrane voltage is closely intertwined.

Chemoattractant-induced ΔpHi and ΔVm orchestrate Ca2+ influx in sea urchin sperm

We studied the control of Ca2+ influx by pH i and Vm in sperm stimulated with either the chemoattractant resact or the intracellular messenger cGMP. Ca2+ signals evoked by photorelease of resact from caged resact were abolished by MDL (K i = 6.4 ± 1 μM) and mibebradil (9.5 ± 1.6 μM) (n = 3) (Fig 5A, C, and D, Supplementary Fig S3). Both drugs also abolished Ca2+ signals evoked by intracellular photorelease of cGMP from caged cGMP (MDL: K i = 3.9 ± 0.4 μM; mibebradil: K i = 25.2 ± 3.7 μM) (n = 3) (Fig 5B–D, Supplementary Fig S4). The potency of MDL to inhibit the resact- and cGMP-evoked Ca2+ responses was about 2- to 3-fold higher than the potency to inhibit Ca2+ signals evoked by alkalization or depolarization. However, sperm were first pre-incubated for about 5 s before resact or cGMP was released. This short pre-incubation might enhance the potency. It is however unclear, why mibebradil inhibited the resact- and cGMP-induced Ca2+ responses with about 3-fold different potencies. Altogether, we conclude that CatSper mediates the chemoattractant- and cGMP-induced Ca2+ influx.

Resact and cGMP stimulate a transient hyperpolarization, and the Ca2+ influx commences shortly after the hyperpolarization peaks (Strünker et al, 2006). Moreover, resact and cGMP also evoke a rapid pHi increase (Fig 6D, Supplementary Fig S5) (Darson et al, 2008). Given the steep pHi sensitivity of the alkaline-evoked Ca2+ influx, we scrutinized the hypothesis that the pHi increase allosterically shifts the voltage dependence of CatSper to more negative Vm values. A prerequisite for such a mechanism is that the onset of ΔpHi precedes the onset of the Ca2+ signal. Therefore, we determined the exact timing and sequence of signaling events for resact concentrations ranging from 500 fM to 25 nM (Fig 6A, Supplementary Fig S6). For all concentrations, the sequence of cellular events...
**Figure 4.** The threshold pH for alkaline-evoked Ca$^{2+}$ influx is controlled by $V_m$.

A Depolarization-evoked Ca$^{2+}$ signals in sperm mixed with ASW containing high KCl concentrations.
B Ca$^{2+}$ signals evoked by mixing of sperm with 80 mM KCl and the CatSper inhibitor MDL12330A.
C Ca$^{2+}$ signals evoked by mixing of sperm with 160 mM KCl and MDL12330A.
D Dose–response relation for the Ca$^{2+}$ signals shown in (B, C) at $t = 1–2$ s.
E Threshold pH for Ca$^{2+}$ signals evoked by pH-clamp solutions in sperm bathed in ASW containing low (3 mM), high (191 mM), and normal (9 mM) KCl (mean ± SD; n ≥ 3); data for 9 mM KCl are from Fig 3F.
F Resting pH and resting $V_m$ in sperm bathed in ASW containing low (3 mM), high (191 mM), and normal (9 mM) KCl (black) (mean ± SD; n ≥ 3). Mean threshold pH for CatSper activation at different membrane potentials (red); mean threshold pH was derived from data shown in (E).

**Figure 5.** CatSper inhibitors abolish chemoattractant- and cGMP-induced Ca$^{2+}$ influx.

A Ca$^{2+}$ signals in sperm evoked by photorelease (at $t = 0$) of resact from caged resact in the presence of the CatSper inhibitor MDL12330A.
B Ca$^{2+}$ signals evoked by intracellular photorelease (at $t = 0$) of cGMP in sperm loaded with caged cGMP in the presence of the CatSper inhibitor MDL12330A.
C Normalized dose–response relation for inhibition of the resact- and cGMP-induced Ca$^{2+}$ signals shown in (A, B) ($K_i = 6.2$ and 4.3 μM, respectively).
D Normalized dose–response relation for inhibition of the resact- and cGMP-induced Ca$^{2+}$ signals shown in Supplementary Fig S4 by the CatSper inhibitor mibefradil ($K_i = 7.7$ and 20.9 μM, respectively).
was as follows: first sperm hyperpolarized, then the cytosol alkalized, and finally, Ca\(^{2+}\) commenced to rise (Fig 6B and C, Supplementary Fig S6). Furthermore, the pH\(_i\) increase evoked by intracellular photorelease of cGMP also preceded the onset of the Ca\(^{2+}\) signal (Fig 6D) (Darszon et al, 2008). These results are consistent with the notion that the resact-induced alkalization enables activation of CatSper channels upon depolarization.

From the latency of the Ca\(^{2+}\) signal at different resact concentrations, we identified pairs of voltage threshold (V\(_{\text{thr}}\)) and pH\(_{\text{thr}}\) at which the Ca\(^{2+}\) influx commenced (Fig 6E–G). For example, using 1 nM resact, Ca\(^{2+}\) influx commenced at V\(_{\text{thr}}\) of −71 ± 3 mV and at pH\(_{\text{thr}}\) of 7.36 ± 0.004 (Fig 6E–G, n = 3). For resact concentrations from 10 pM to 25 nM, the respective V\(_{\text{thr}}\) versus pH\(_{\text{thr}}\) pairs displayed an inverse, linear relationship (Fig 6G), i.e. with increasing resact concentrations, the Ca\(^{2+}\) influx commenced at more negative V\(_{\text{thr}}\) and at more alkaline pH\(_{\text{thr}}\) (Supplementary Fig S7). The slope of the straight line fitted to the data yielded a \(\Delta V_{\text{thr}}/\Delta \text{pH}\) ratio of 75 mV. This result underscores the intimate relationship between pH\(_i\) and V\(_m\) for CatSper activation, which requires that changes in pH\(_i\) and V\(_m\) proceed in precise chronology. In mouse sperm, a change of pH\(_i\) from 6 to 7 shifts the voltage dependence of CatSper activation by about −70 mV (Kirichok et al, 2006), indicating that the pH\(_i\) sensitivity of mammalian and sea urchin CatSper is similar. In summary, our experiments indicate that the resact-induced alkalization is key to the Ca\(^{2+}\) influx via CatSper.
We attempted to prevent the resact-induced alkalization by incubating sperm with the membrane-permeant pH buffer imidazole. Imidazole ≤ 20 mM attenuated the resact-induced alkalization in a dose-dependent fashion; at 30 mM imidazole, the alkalization was abolished (Supplementary Fig S8A). Concomitantly, the Ca^{2+} response was abolished as well (Supplementary Fig S8B), suggesting that the alkalization is required for CatSper activation. However, we observed that imidazole also strongly reduced the initial hyperpolarization (Supplementary Fig S8D and E), demonstrating that the drug is not suited to study signaling in sea urchin sperm. We wondered whether incubation with a physiological pH buffer like bicarbonate enters the sperm. However, the resact-induced alkalization was similar in the absence and presence of 10 and 30 mM HCO_3^- (Supplementary Fig S8F).

**CatSper controls chemotaxis of sperm**

Finally, we tested whether CatSper controls chemotactic steering of sperm. In a shallow observation chamber under a dark-field microscope, sperm were bathed in caged resact (Kaupp et al., 2003; Böhmer et al., 2005; Alvarez et al., 2012). A resact gradient was established by photolysis of caged resact in the center of the recording chamber (Fig 7A). After the flash, sperm accumulated in the irradiated area, indicated by a decrease in sperm dispersion in the field of view, whereas the surrounding area became depleted of sperm (Fig 7, Supplementary Movies S1 and S2; control); MDL and mibefradil abolished the resact-induced accumulation of sperm (Fig 7, MDL12330A; Supplementary Movies S1 and S2). We conclude that the chemoattractant-induced Ca^{2+} influx via CatSper controls navigation of sperm in a resact gradient.

**Discussion**

Although CatSper has been discovered more than a decade ago and CatSper genes are present in many phyla, knowledge about CatSper channels originates exclusively from studies of human and mouse sperm. We show that CatSper constitutes the long-sought Ca^{2+} channel that controls chemotaxis in sea urchin sperm. Moreover, we unravel in quantitative terms the interplay between pH_i and V_m to control Ca^{2+} influx via CatSper in intact sperm.

At rest, V_{rest} of CatSper activation is slightly more positive than V_{rest} and the channel is closed. The chemoattractant-induced hyperpolarization (Fig 8B, black arrow) evokes a rapid intracellular alkalization via the sNHE exchanger that harbors a classic voltage-sensor motif, which probably mediates the voltage dependence (Wang et al., 2003; Nomura & Vacquier, 2006). The alkalization shifts the voltage dependence of CatSper by as much as 30 mV to more negative values (Fig 8B, blue arrow) and, thereby, enables CatSper to open during the subsequent depolarization brought about by hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels (Fig 8B, red arrow) (Gauss et al., 1998; Galindo et al., 2005). The high cooperativity of the allosteric pH control serves as a sensitive mechanism that allows gating of CatSper within the operational voltage range set by V_{rest} (about –50 mV) and the reversal potential of the CNGK channel (about –95 mV).

In a chemical gradient, sperm are periodically stimulated with the angular frequency of circular swimming of ~1 Hz (Böhmer et al., 2005). The periodic stimulation can be emulated by repetitive (1 Hz) photorelease of cGMP; each cGMP pulse evokes a V_m and Ca^{2+} response of similar amplitude (Kashikar et al., 2012). We gained further insight into the interplay between pH_i and Ca^{2+} responses by studying pH_i signals evoked by repetitive photorelease of cGMP. Whereas the first flash produced a large increase of pH_i, subsequent flashes evoked only a small or no further alkalization (Fig 8C). Thus, once alkalization shifted the voltage dependence of CatSper to the permissive voltage range, channel gating is controlled by voltage only. This indicates that ApH_i is the mechanism that enables sperm to transduce periodic V_m changes into periodic Ca^{2+} changes during sperm navigation on periodic paths in a chemoattractant gradient (Kashikar et al., 2012).

Here, we reveal intriguing commonalities and differences between signaling pathways in sperm from mammals and marine invertebrates. Although distinct in many respects, signaling pathways share both CatSper and the sNHE exchanger (Fig 8A), suggesting that a voltage-induced change in pH_i and a pH-induced activation of CatSper are evolutionary conserved signaling events. A mechanism reminiscent of that controlling CatSper in sea urchins has recently been proposed for mouse sperm (Chavez et al., 2014). Hyperpolarization by Slo3, the principal K^+ channel in mouse (Santi et al., 2010; Zeng et al., 2011, 2013) and human sperm (Brenker et al., 2014), is required for CatSper to open upon depolarization. The hyperpolarization might involve alkalization, probably mediated by sNHE (Chavez et al., 2014). These events seem to control sperm capacitation, a maturation process inside the female genital tract. At first sight, these capacitation events are reminiscent of the hyperpolarization-induced events during chemotaxis in sea urchin sperm. However, the time scales are entirely different: Mammalian sperm capacitation proceeds in minutes to hours, whereas chemotactic signaling happens within subseconds. Finally, the
physiological trigger for Slo3 activation and the precise interplay between $V_m$ and pH$_i$ to control mammalian CatSper are unknown. In conclusion, the allosteric mechanism of CatSper activation by pH$_i$ and $V_m$ in sea urchins provides a blueprint for studies in mammalian sperm.

On a final note, a difference between chemosensation in sea urchin and human sperm might concern the interplay of CatSper and K$^+$ channels (Fig 8A). In sea urchin, the opening of CNGK first activates sNHE and eventually CatSper. Thus, CNGK is located upstream of CatSper on the excitatory limb of the signaling pathway (Fig 8A). By contrast, in human sperm, Slo3 is activated by Ca$^{2+}$ (Brenker et al., 2014), and a Ca$^{2+}$-induced hyperpolarization might curtail the progesterone-induced Ca$^{2+}$ influx via CatSper. Thus, Slo3 is placed downstream of CatSper on the recovery limb of signaling (Fig 8A). This variation of signaling motifs might reflect a phylogenetic adaptation to the direct activation of human CatSper by extracellular ligands such as progesterone.

In conclusion, CatSper constitutes a crucial component of Ca$^{2+}$ entry employed by diverse signaling pathways. In general, CatSper might serve as a versatile polymodal sensor that integrates multiple stimuli such as pH$_i$, female factors, and membrane voltage. Depending on the species, CatSper employs either intracellular alkalization or ligand binding to allosterically shift its voltage dependence to the permissive range of membrane potentials. We envisage that variations or combinations of these two mechanisms control CatSper in other species.

**Materials and Methods**

**Arbacia punctulata sperm**

Collection of dry sperm and composition of artificial seawater (ASW) was as described previously (Kaupp et al., 2003; Strümpfel et al., 2006; Kashikar et al., 2012). In brief, about 0.5 ml of 0.5 M KCl was injected into the body cavity to evoke spawning. Spawned sperm (dry sperm) were collected using a Pasteur pipette and stored on ice.

**Cloning of ApCatSper subunits**

For cloning of ApCatSper 2 and ApCatSper 3, fragments of partial clones from orthologous Strongylocentrotus purpuratus SpCatSper 2 and 3 (kindly provided by D. Ren, University of Pennsylvania, Philadelphia, USA) were amplified. For cloning of ApCatSper 1 and ApCatSper 4, we compared the sequences of mammalian CatSper 1 and 4 subunits with predicted messenger RNA (mRNA) sequences from the S. purpuratus genome project, and we designed primers to amplify fragments of SpCatSper 1 and SpCatSper 4 from a cDNA.
library of S. purpuratus testis. The SpCatSper 1–4 fragments were used as probes to screen random-primed cDNA libraries of A. punctulata testis under low-stringency conditions. Overlapping ApCatSper partial clones were combined to yield full-length clones; missing sequence information at the 5’- and 3’-end was completed by RACE-PCR (Frohman et al., 1988). PCRs, the construction and screening of cDNA libraries, subcloning, and sequencing of cDNA were performed according to standard protocols. The ApCatSper 2 and 3 clones were fused with the coding sequence for a C-terminal hemagglutinin tag (HA-tag) and cloned into the mammalian expression vector pcDNA3.1+ (Invitrogen).

**Antibodies**

The antibodies directed against the GC (GCN3D12) and the CN GK (AP47C9) were described previously (Bönigk et al., 2009; Pichlo et al., 2014). A monoclonal antibody from rat (RKKE4F6) was directed against the C-terminus (amino acids 297–317) of ApCatSper 3. Another monoclonal antibody from rat (APCS28G4) was directed against the N-terminus of ApCatSper 2 (amino acids 42–58). The rat anti-HA antibody was from Roche Applied Science. Secondary antibodies were used as follows: goat anti-rat-HRP antibody (Dianova); goat anti-rat-IRDye800cw antibody (LI-COR); donkey anti-rat-Cy3 (Dianova) and goat anti-rat-Alexa488 (Life Technologies).

**Immunocytochemistry**

Sperm were immobilized on SuperFrost Plus microscope slides (Menzel) and fixed for 5 min with 4% paraformaldehyde. After preincubation with 0.5% Triton X-100 and 5% chemiblocker (Millipore) in 0.1 M phosphate buffer (pH 7.4), sperm were incubated for 1 h with antibodies RKKE4F6 or AP47C9 (undiluted in the presence of 0.5% Triton X-100), or GCN3D12 (1:100 diluted in the presence of 0.5% Triton X-100) and visualized with the donkey anti-rat-Cy3 (RKKE4F6, GCN3D12) or goat anti-rat-Alexa488 (AP47C9) antibodies.

**Western blotting**

CHO cells transiently transfected with ApCatSper 2 or ApCatSper 3 were resuspended in phosphate-buffered saline (PBS) containing (in mM) 137 NaCl, 2.7 KCl, 6.5 Na2HPO4, 1.5 KH2PO4, pH 7.4, and the protease inhibitor Complete (Roche). Total protein content was determined by using the BCA Assay kit (Pierce). Ten μg of total protein was used in the Western blot analysis. Membrane proteins from A. punctulata sperm were prepared as previously described (Mengerink & Vacquier, 2004). Ten μg of membrane proteins was used in the Western blot analysis. Proteins were separated by 10% polyacrylamide gel electrophoresis (SDS-PAGE) and blotted, and the membranes were probed with RKKE4F6 (undiluted), APCS28G4 (dilution 1:100), or rat anti-HA (dilution 1:1,000). The goat anti-rat-HRP antibody (dilution 1:5,000) was used to visualize protein bands by a chemoluminescence detection kit; chemoluminescence was detected via a CCD-imaging system (LAS-3000; Fuji) and the membranes were probed with RKKE4F6 (undiluted), or GCN3D12 (1:100 diluted in the presence of 0.5% Triton X-100), or GCN3D12 (1:100 diluted in the presence of 0.5% Triton X-100) and visualized with the donkey anti-rat-Cy3 (RKKE4F6, GCN3D12) or goat anti-rat-Alexa488 (AP47C9) antibodies.

**Mass spectrometry of proteins from A. punctulata flagella**

Sperm flagella and heads were separated as described (Mengerink & Vacquier, 2004; Strünk et al., 2006) with some modifications: Dry sperm was diluted (1:25) in ASW pH 7.8 and centrifuged (200 g, 7 min) to sediment coelomocytes. The supernatant was centrifuged (3,000 g, 15 min) to sediment sperm. The sperm pellet was diluted in ASW pH 7.8 with protease inhibitor Complete (Roche) (1:10 dilution). The sperm suspension was sheared ~20 times with a 24-G needle and centrifuged (800 g, 10 min) to sediment intact sperm and sperm heads. The purity of flagella preparations was checked by phase-contrast microscopy. Shearing and subsequent centrifugation was repeated several times until pure flagella samples were obtained. All steps were performed on ice. Flagella were lysed by several “freeze/thaw” cycles and sonification steps in buffer containing (in mM): 25 HEPES pH 7.5, 10 NaCl, 2 EGTA, and protease inhibitor cocktails (Roche Applied Science and Sigma). Membranes were sedimented by ultracentrifugation (100,000 g, 30 min, 4°C) and washed twice with 0.1 M (NH4)2CO3. After another ultracentrifugation step, membrane pellets were resuspended, sonicated, and processed by tryptic in-solution digestion (sequencing grade modified trypsin, Promega) in a methanol and NH4HCO3 buffer (Fischer et al., 2006). After removal of membranes by ultracentrifugation, samples were desalted using Spec PT C18 AR tips (Varian). Both MudPIT (2D) with seven salt steps and one-dimensional (1D) analysis were performed on an LTQ Orbitrap Velos (Thermo Fisher Scientific) according to Fränzel et al. (2010) and Trötschel et al. (2012). All database searches were performed using SEQUEST algorithm, embedded in Proteome DiscovererTM (Rev. 1.2.0.208 or Rev. 1.4.0.288, Thermo Fischer Scientific). Searches were done by using both an A. punctulata protein database derived from testis transcriptome and sperm genome sequencing (to be published) and an NCBI protein database for S. purpuratus proteins, in which the S. purpuratus protein sequences for the CatSper subunits 1, 2, 3, 4, GC, and CN GK were replaced by the respective A. punctulata sequences. Tryptic peptides with ≤ 2 missed cleavages were accepted. Oxidation of methionine was permitted as variable modification. The mass tolerance for precursor ions was set to 6 ppm; the mass tolerance for fragment ions was set to 0.8 amu. For search result filtering, a false discovery rate (FDR) of < 1% was applied, and ≥ 2 peptides per protein as well as peptides with search result rank 1 were required.

**Co-immunoprecipitation**

The monoclonal rat anti-ApCatSper 2 and anti-ApCatSper 3 antibodies APCS28G4 and RKKE4F6, respectively, were immobilized on Protein G Sepharose 4 Fast Flow (GE Healthcare). Arbacia punctulata dry sperm were suspended in lysis buffer containing in mM: 140 NaCl, 1 EDTA, 1% n-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 10 Tris—HCl (pH 7.6), and protease inhibitor cocktail (Sigma). The suspension (total lystate) was centrifuged for 10 min at 10,000 × g, and the total protein content of the supernatant, containing cytosolic and solubilized membrane proteins, was determined by a BCA Assay kit (Pierce). For co-immunoprecipitation, proteins (input) were pre-incubated with fresh Protein G resin end-over-end for 30 min at 4°C. The suspension was briefly centrifuged
(0.5 min, 200 × g, 4°C), and the supernatant was added to the respective antibody-coupled resin, incubated end-over-end overnight at 4°C, and centrifuged to remove the supernatant (flow through). The resin was subsequently washed five times with lysis buffer; finally, co-immunoprecipitated proteins were eluted with 1× SDS–PAGE sample buffer (2% [w/v] SDS, 50 mM Tris, 12.5% glyc erin, 1% 2-mercaptoethanol, 0.01% bromphenol blue). For Western blot analysis, proteins were separated by 10% SDS–PAGE and blot ted, and membranes were probed with either the anti-ApCatSper 2 or anti-ApCatSper 3 (both undiluted) antibody and visualized, using the Odyssey Imaging System (LI-COR).

For mass spectrometry analysis, (co-)immunoprecipitated proteins were separated by 10% SDS–PAGE. Gels were stained with colloidal Coomassie, containing 0.08% (w/v) Coomassie G-250, 1.6% (v/v) phosphoric acid, 8% (w/v) ammonium sulfate, and 20% (v/v) methanol, destained with 1% (v/v) acetic acid, and cut into 10 slices. Proteins in the slices were processed by tryptic in-gel digestion and analyzed by protein mass spectrometry.

**Measurement of changes in intracellular Ca**2+ **concentration, pH, and membrane voltage**

We measured changes in [Ca**2+**], pH, and V**m** in a rapid-mixing device (SFM-400; BioLogic) in the stopped-flow mode. The changes in [Ca**2+**], pH, and V**m** were measured with the Ca**2+** indicator Fluo-4-AM, the pH indicator BCECF-AM, and the voltage-sensitive indicator di-8-ANEPPS (Molecular Probes), respectively (Solzin et al., 2004; Strünker et al., 2006; Bönigk et al., 2009; Kashikar et al., 2012). Dry sperm were suspended 1:6 (vol/vol) in loading buffer containing ASW and the indicator in the absence (BCECF-AM) or presence (Fluo-4-AM, di-8-ANEPPS) of 0.5% Pluronic F127 (Sigma-Aldrich or Molecular Probes). After incubation (for 45–120 min with Fluo-4-AM, 10–15 min for BCECF-AM, or 5 min for di-8-ANEPPS) at 17°C, the sample was diluted 1:20 to 1:200 with ASW. Sperm were allowed to equilibrate in the new medium for 5 min. In the stopped-flow device, the sperm suspension was rapidly mixed 1:1 (vol/vol) with the respective stimulus. Concentrations of inhibitors or ligands are given as final concentrations after mixing. Fluorescence was excited by a 150-W Xe lamp (LSB521; LOT Oriel) or a SpectraX Light Engine (Lumencor). Emission was recorded by photomultiplier modules (H9656-20; Hamamatsu Photonics). The signal was amplified and filtered through a voltage amplifier (DLPVA-100-B-S; Femto Messtechnik). Data acquisition was performed with a data acquisition panel (PCI-6221; National Instruments) and Bio-Kine software (BioLogic). For Ca**2+** and V**m** recordings, the excitation light was passed through either an ET490/20 nm (Chroma Technology) (Xe lamp) or a BrightLine 475/28-nm filter (Semrock) (SpectraX Light Engine). For pH**m** measurements, the excitation light was passed through a BrightLine 452/45-nm filter (Semrock). For Ca**2+** measurements, the emitted light was passed through a BrightLine 536/40 filter (Semrock). Ca**2+** signals represent the average of at least two recordings and are depicted as the percent change in fluorescence (ΔF/F**0**) with respect to the mean of the first 5–10 data points before the onset of the signal (F**0**). The control (ASW) ΔF/F**0** signal was subtracted from the NH**4**Cl-, pH**m**-clamp, resact-, or cGMP-induced signals. The V**m** signals were recorded in the ratiometric dual-emission mode. The filters in front of the two photomultipliers were BrightLine 536/40 nm and BrightLine 628/40 (Semrock). The BioLogic software was used to record fluorescence in the dual-emission mode. The V**m** signals represent the ratio F536/F628 (R). The control (ASW) R signal was subtracted from the resact- or cGMP-induced signals. The mean R of the first 5–10 data points before the onset of the changes in fluorescence was set to 0, yielding AR. The V**m** signals represent the average of at least three recordings and were digitally smoothed with five-point average smoothing. The changes in di-8-ANEPPS fluorescence were calibrated to yield V**m** values (mV) by mixing sperm with both resact (2 nM) and various [K**+]o (Strünker et al., 2006). With increasing [K**+]o, the amplitude of the resact-induced hyperpolarization decreases and, eventually, sperm depolarized. Plotting the resact-evoked AR versus [K**+]o allows interpolation of the [K**+]o at which resact does not change V**m**. At this [K**+]o null-point, the Nernst potential of K**+** equaled V**m** before stimulation. We calculated the respective Nernst potential, assuming an intracellular K**+** concentration of 423 mM. Moreover, AR is linearly related to [K**+]o, which allows to determine ΔK**+**/mV. Determination of V**m** and calibration of AR into mV was performed for each set of experiments. BCECF fluorescence was recorded in a dual-emission mode using BrightLine 494/20-nm and BrightLine 540/10-nm filters (Semrock). The pH**m** signals represent the ratio of F494/540, represent the average of at least two recordings, and are depicted as the percent of the relative change in ratio (ΔR/R) with respect to the mean of the first 5–10 data points before the onset of the signal. The control (ASW) signal was subtracted from the NH**4**Cl-, pH**m**-clamp-, resact-, or cGMP-induced signals.

The calibration procedure for BCECF fluorescence to yield pH**m** by the pH**m**-null-point method is described in the section and below; pH**m** calibration was performed for each set of experiments. The pH**m**-null-point solutions were prepared according to the following equation: pH**m**-null = pH**rest** – 0.5 log ([TMA]/[BA]); pH**m** = extracellular pH (7.8) (Eisner et al., 1989), wherein [TMA] indicates the concentration of trimethylamine and [BA] that of butyric acid. According to this equation, each [TMA]/[BA] ratio defines a new pH**m** or pH**m**-null-point. When a cell with a resting pH**m** (pH**rest**) is placed in a pH**m**-null-point solution, it will not change its pH**m** when the pH**m**-null-point solution matches pH**rest**. If the null-point is more alkaline than pH**rest**, the cell will alkalize; if the null-point is more acidic, then the cell will acidify. Monitoring the changes in pH**m** after mixing sperm with various null-point solutions allows interpolating pH**rest**. When the pH**m**-null-point does not match pH**rest**, the absolute concentrations of acid and base determine to what extent the pH**m** of a cell will change (Chow et al., 1996). The higher the concentrations of the acid/base mixture, the more the pH**m** will be shifted toward the pH**m**-null-point. At saturation, the newly established pH**m** matches the pH**m**-null-point solution; thus, the cell is clamped to a new pH**m**. Therefore, we refer to this saturating pH**m**-null-point solution as pH**m**-clamp solution. Finally, the time course of pH**m** determines the time window for which this pH**m**-clamp concept holds. To ensure that in A. punctulata sperm, the pH**m** was indeed clamped to the pH**m**-null-point for several tens of seconds, we determined for each pH**m**-null-point solution, i.e. for each TMA/BA ratio, the molar concentrations of TMA and BA required to produce saturating changes in pH**m** (Supplementary Fig S9). The pH**m**-clamp solutions that clamped pH**m** in A. punctulata sperm to pH**m**-null all contained 60 mM BA, whereas the TMA concentration was varied to yield the respective pH**m**-null; for example, for the pH**m**-null 7.0 solution, we used 1.5071 mM TMA/60 mM BA, for the pH**m**-null 7.2 solution, 3.7857 mM TMA/60 mM BA, etc.
Addition of TMA and BA increased the osmolarity of the ASW by < 13%.

Caged compounds and flash photolysis

DEACM-caged cGMP and DMNB-caged resact were obtained from V. Hagen (Leibniz-Institut für Molekulare Pharmakologie, Berlin) (Hagen et al., 2003; Kaupp et al., 2003). For Ca\textsuperscript{2+} recordings, sperm were diluted 1:6 in loading buffer (ASW) containing Fluo-4-AM and 30 μM DEACM-caged cGMP for ≥ 45 min (Kaupp et al., 2003). For pH\textsubscript{i} recordings, sperm were incubated first with 30 μM DEACM-caged cGMP for ≥ 40 min followed by incubation for another 10–15 min with BCECF-AM. After loading, sperm were diluted 1:20 to 1:200 for stopped-flow experiments. For experiments with DMNB-caged resact, sperm were first loaded with Fluo-4-AM; after loading, the sample was diluted 1:20 to 1:200 with ASW containing 1 μM DMNB-caged resact. Sperm were allowed to equilibrate in the new medium for 5 min. In the stopped-flow device, the sperm suspension was rapidly mixed 1:1 (vol/vol) with ASW (control) or the respective inhibitors. About 2–5 s after mixing, caged cGMP and caged resact were photolyzed by a UV flash (~ 1 ms) from a Xenon flash lamp (JML-C2; Rapp OptoElectronic). The UV flash was passed through a bandpass 295- to 395-nm interference filter (Rapp Opto Electronic) and delivered by a liquid light guide to the cuvette (FC-15; BioLogic) of the stopped-flow device.

Sperm chemotaxis

Sperm accumulation in a resact gradient was studied as described with some modifications (Alvarez et al., 2012; Hirohashi et al., 2012). In brief, sperm swimming in a recording chamber (150 μm depth) were imaged using a microscope (IX71; Olympus) equipped with a 10× objective (UPlanSApo; NA 0.4; Olympus). Stroboscopic dark-field illumination (2 ms pulses) was achieved using a white LED (K2 star; Luxeon), a custom-made housing, and a pulse generator. Images were bandpass-filtered (HQ520/40; Chroma) and LED (K2 star; Luxeon), a custom-made housing, and a pulse generator. The sperm suspension was rapidly mixed 1:1 (vol/vol) with ASW (control) or the respective inhibitors. About 2–5 s after mixing, caged cGMP and caged resact were photolyzed by a UV flash (~ 1 ms) from a Xenon flash lamp (JML-C2; Rapp OptoElectronic). The UV flash was passed through a bandpass 295- to 395-nm interference filter (Rapp Opto Electronic) and delivered by a liquid light guide to the cuvette (FC-15; BioLogic) of the stopped-flow device.

Data analysis

The data obtained from the stopped-flow recordings were analyzed using Prism 5 (GraphPad Software) and OriginPro 8.1G SR3 (OriginLab Corporation). All data are given as mean ± standard deviation.

Supplementary information for this article is available online:

http://emboj.embopress.org

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Author contributions

RS and TS conceived the project. RS, MF, WB, LA, CT, AP, AM, NG, PP, NDK, EK, JJ, BT, HK, DF, FW, UBK, and TS designed and performed experiments. TS, RS, and UBK wrote the manuscript. All authors revised the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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