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 (Avenarius 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 (V_m) and at alkaline intracellular pH (pH_i) (Kirichok et al, 2006; Lishko et al, 2010, 2011; Strünker et al, 2011). However, the interplay between V_m and pH_i 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 Ca\(^{2+}\) 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 (Dangott & Garbers, 1984; Bentley et al., 1986, 1988; Shimomura & Garbers, 1986; Dangott et al., 1989; Kaupp et al., 2003). cGMP opens K\(^{+}\)-selective cyclic nucleotide-gated (CNGK) channels (Strünker 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 (sNHE) (Lee, 1984a; Lee, 1984b; Lee & Garbers, 1986), mediating a rapid rise of pH\(_{i}\) (Nishigaki et al., 2001; Solzin et al., 2004) and, eventually, opens voltage-gated Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) channel of the chemotactic signaling pathway in sperm of the sea urchin *A. punctulata*. CatSper mediates the chemoattractant-induced Ca\(^{2+}\) bursts and controls chemotactic steering. We unveil the intimate, allosteric relationship between pH\(_{i}\) and V\(_{m}\) for CatSper activation: A minute chemoattractant-induced increase of pH\(_{i}\) enables CatSper in a highly cooperative fashion to open during a subsequent depolarization. The pH\(_{i}\)-induced shift of the voltage dependence of CatSper activation enables sperm to transduce periodic V\(_{m}\) changes into periodic Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(_{\text{a}}\), 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 (Lobelley 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 (M\(_{r}\)) 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 proteotypic 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* 3, using the respective protein sequences (Supplementary Table S1). In purified flagella, we identified proteotypic 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 Ca\(^{2+}\) influx in sea urchin sperm**

Using a stopped-flow apparatus and fluorescent probes for Ca\(^{2+}\), V\(_{m}\), and pH, we studied the role of CatSper in intact *A. punctulata* sperm. Ammonium chloride (NH\(_{4}\)Cl) evoked a rapid and sustained intracellular alkalization (Supplementary Fig S2) that stimulated a Ca\(^{2+}\) increase (Fig 2A). At low NH\(_{4}\)Cl concentrations (≤ 3 mM),...
Ca^2+ signals slowly reached a plateau; at higher concentrations (≥ 10 mM), NH₄Cl evoked rapid, oscillatory Ca^2+ responses (Fig 2A). Mixing of sperm with both NH₄Cl and EGTA, which lowers extracellular [Ca^{2+}] to ≤ 400 nM, abolished the Ca^2+ signal. However, not the pH response (Supplementary Fig S2), demonstrating that alkalization stimulates Ca^2+ influx.

Figure 1. Features of ApCatSper 1–4, β, γ, and δ and localization of CatSper in A. punctulata sperm.  
A Predicted membrane topology and predicted molecular weight of ApCatSper 1–4 cloned from A. punctulata testis, and of ApCatSper β, γ, and δ. A gene encoding ApCatSper β was identified in the A. punctulata genome; transcripts encoding ApCatSper β and ApCatSper γ were identified in the A. punctulata transcriptome. S1 to S6, transmembrane segments; +, positively charged amino acids in S4; gray cylinder, coiled-coil domain. 
B Upper panel, alignment of pore regions of ApCatSper 1–4. Amino acids of the Ca^{2+} selectivity-filter motif are highlighted. Lower panel, alignment of S4 voltage-sensor segments of ApCatSper 1–4 and of D. melanogaster ShakerB Kv channel. Positively charged amino acids are highlighted. Numbers indicate start and end position of amino acids of the selected region. 
C Western blots of total protein of CHO cells transfected with ApCatSper 2 or 3, non-transfected control cells (−), and A. punctulata sperm (Sp). The Western blots were probed with anti-HA, anti-ApCatSper 2, or anti-ApCatSper 3 antibodies. Arrows indicate bands representing ApCatSper 2 and 3. 
D Immunocytochemical analysis of sperm stained with anti-ApCatSper 3, anti-GC, or anti-CNGK antibodies; superposition of images obtained by fluorescence and bright-field microscopy; scale bar = 10 μm. The DNA was stained with DAPI (blue). 
E Western blot analysis of co-immunoprecipitation (IP) of A. punctulata sperm proteins. The input (I), flow through (FT), washes (W1–5), and the eluate (E) of the IP using the anti-ApCatSper 2 antibody were probed with the anti-ApCatSper 3 antibody (upper panel) and vice versa (lower panel). ApCatSper 3 and ApCatSper 2 were co-immunoprecipitated with the anti-ApCatSper 3 antibody (upper panel) and anti-ApCatSper 3 antibody (lower panel), respectively. 
F Analysis by mass spectrometry of immunoprecipitated proteins. ApCatSper 1–4, β, γ, and δ were identified in the immunoprecipitates obtained with both the anti-ApCatSper 2 and anti-ApCatSper 3 antibodies. The number of tryptic peptides identified and the respective sequence coverage are given.

Source data are available online for this figure.
Two distinct CatSper inhibitors, MDL12330A (MDL) (Brenker et al., 2012) and mibebradil (Strü¨nker et al., 2011), suppressed the alkaline-evoked Ca2+ 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 NH4Cl 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 Ca2+ signals in human sperm with similar potency (Strü¨nker et al., 2011; Brenker et al., 2012). We conclude that in sea urchin sperm, similar to mouse and human sperm, CatSper mediates alkaline-evoked Ca2+ influx. Because MDL and mibebradil are not selective for CatSper, we cannot exclude that the sperm might harbor additional, so far unknown Ca2+-permeable channels that are also activated at alkaline pH and inhibited by both drugs.

We determined the pH_i sensitivity of the alkaline-induced Ca2+ influx using the “pH_i pseudo-null-point” method (Eisner et al., 1989; Chow et al., 1996; 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_i 7.2-clamp solution changed pH_i only slightly, suggesting a resting pH_i (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).

We also determined the pH_i sensitivity of the alkaline-induced Ca2+ influx using the “pH_i pseudo-null-point” method (Eisner et al., 1989; Chow et al., 1996; 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).

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Figure 3C shows the time course of Ca2+ responses in sperm mixed with different pH_i-clamp solutions. Plotting the amplitude of the Ca2+ signals versus the respective pH_i-clamp values disclosed an exceptionally steep dose–response relation with a pH_rest 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 Ca2+, we reconstructed the threshold pH_i (pH_rest) at which the Ca2+ influx commenced (Fig 3E). For example, using the pH_i 7.6-clamp solution, the Ca2+ 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_rest for Ca2+ influx. We determined pH_rest for the entire range of pH_i-clamp solutions (Fig 3F). The latency of the Ca2+ 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_rest was largely independent of the rate and magnitude of the pH_i increase (Fig 3F, red). The invariant pH_rest for the alkaline-induced Ca2+ 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 Ca2+ increase. In fact, rapid elevation of the extracellular K+ concentration ([K+]o) to ≥ 30 mM evoked a transient Ca2+ signal (Fig 4A), whose amplitude was graded with [K+]o. MDL inhibited Ca2+ signals evoked by 80 mM and 160 mM K+ with a K_i of 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 Ca2+ signals were also suppressed by mibebradil (Supplementary Fig S2). We conclude that CatSper also supports depolarization-evoked Ca2+ influx in sea urchin sperm.

We examined the relationship between pH_rest 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 Ca2+ signals...
revealed pH_rest for different V_rest values (Fig 4E, Supplementary Fig S3). At hyperpolarized V_rest, the pH_rest for Ca^2+ influx was shifted to more alkaline pH values (Fig 4E, Supplementary Fig S3); when V_rest was depolarized, the pH_rest was shifted to more acidic pH values (Fig 4E and F, Supplementary Fig S3); a linear fit of the data yielded a slope of ΔpH_rest/10 mV of 0.07 ± 0.004 (standard error of the fit) (Fig 4F, red line). In addition, pH_rest was more acidic at depolarized V_rest (Fig 4F). We conclude that the control of Ca^2+ influx via CatSper by pH_i and membrane voltage is closely intertwined.

**Chemoattractant-induced ΔpH_i and ΔV_m orchestrate Ca^2+ influx in sea urchin sperm**

We studied the control of Ca^2+ influx by pH_i and V_m in sperm stimulated with either the chemoattractant resact or the intracellular messenger cGMP. Ca^2+ 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 S4). Both drugs also abolished Ca^2+ 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 Ca^2+ responses was about 2- to 3-fold higher than the potency to inhibit Ca^2+ 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 Ca^2+ responses with about 3-fold different potencies. Altogether, we conclude that CatSper mediates the chemoattractant- and cGMP-induced Ca^2+ influx.

Resact and cGMP stimulate a transient hyperpolarization, and the Ca^2+ influx commences shortly after the hyperpolarization peaks (Strünker et al., 2006). Moreover, resact and cGMP also evoke a rapid pH_i increase (Fig 6D, Supplementary Fig S5) (Darzon et al., 2008). Given the steep pH_i sensitivity of the alkaline-evoked Ca^2+ influx, we scrutinized the hypothesis that the pH_i increase allosterically shifts the voltage dependence of CatSper to more negative V_m values. A prerequisite for such a mechanism is that the onset of ΔpH_i precedes the onset of the Ca^2+ 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

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**Figure 3. Determination of the threshold pH_i for alkaline-evoked Ca^{2+} influx.**

A Changes in pH_i evoked by mixing with pH-clamp solutions (see explanation in the text); sperm were loaded with the pH_i indicator BCECF. ΔR/R (%) indicates the change in the BCECF fluorescence emission ratio (ΔR = R_F - R_B) 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 pH_i signals shown in (A). The intercept of the fitted straight line with the x-axis yields the resting pH_i; the slope of the straight line yields the ΔR/R (%) × ΔpH_i⁻¹. Inset: calibrated changes in pH_i evoked by various pH-clamp solutions. C Ca^2+ signals evoked by mixing of sperm with pH-clamp solutions. D Dose-response relation for the Ca^2+ response (black) evoked by mixing of sperm with a pH-clamp solution; depicted are, on an extended time scale, the first 500 ms of the respective pH_i increase and Ca^2+ signal shown in (B, inset) and (C), respectively. The threshold pH_i for CatSper activation was deduced from the latency of the Ca^2+ signal. E Calibrated pH_i increase (red) and respective Ca^2+ signal shown in (B, inset) and (C), respectively. The threshold pH_i for CatSper activation was deduced from the latency of the Ca^2+ signal.
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) (Ki = 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 mibebradil (Ki = 7.7 and 20.9 μM, respectively).
was as follows: first sperm hyperpolarized, then the cytosol alkalized, and finally, Ca\textsuperscript{2+} commenced to rise (Fig 6B and C, Supplementary Fig S6). Furthermore, the pH\textsubscript{i} increase evoked by intracellular photorelease of cGMP also preceded the onset of the Ca\textsuperscript{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\textsuperscript{2+} signal at different resact concentrations, we identified pairs of voltage threshold (V\textsubscript{thr}) and pH\textsubscript{thr} at which the Ca\textsuperscript{2+} influx commenced (Fig 6E–G). For example, using 1 nM resact, Ca\textsuperscript{2+} influx commenced at V\textsubscript{thr} of –71 ± 3 mV and at pH\textsubscript{thr} of 7.36 ± 0.004 (Fig 6E–G, n = 3). For resact concentrations from 10 pM to 25 nM, the respective V\textsubscript{thr} versus pH\textsubscript{thr} pairs displayed an inverse, linear relationship (Fig 6G), i.e. with increasing resact concentrations, the Ca\textsuperscript{2+} influx commenced at more negative V\textsubscript{thr} and at more alkaline pH\textsubscript{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\textsubscript{i} and V\textsubscript{m} for CatSper activation, which requires that changes in pH\textsubscript{i} and V\textsubscript{m} proceed in precise chronology. In mouse sperm, a change of pH\textsubscript{i} from 6 to 7 shifts the voltage dependence of CatSper activation by about –70 mV (Kirichok et al, 2006), indicating that the pH\textsubscript{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\textsuperscript{2+} influx via CatSper.

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**Figure 6.** The chemoattractant-induced Ca\textsuperscript{2+} influx is orchestrated by changes in pH\textsubscript{i} and V\textsubscript{m}.

- **A** Calibrated V\textsubscript{m} (left) and pH\textsubscript{i} (right) changes and Ca\textsuperscript{2+} signals (middle) evoked by resact.
- **B** Normalized V\textsubscript{m}, pH\textsubscript{i}, and Ca\textsuperscript{2+} signals evoked by 10 pM resact; the first 600 ms after mixing are shown. The hyperpolarization precedes the pH\textsubscript{i} increase, whereas the pH\textsubscript{i} increase precedes the Ca\textsuperscript{2+} increase.
- **C** Latency of the V\textsubscript{m}, the pH\textsubscript{i}, and the Ca\textsuperscript{2+} signals evoked by various resact concentrations (mean ± SD; n = 3).
- **D** Normalized pH\textsubscript{i} and Ca\textsuperscript{2+} signals evoked by photorelease of cGMP in sperm loaded with caged cGMP.
- **E** Calibrated V\textsubscript{m} and Ca\textsuperscript{2+} signals evoked by 1 nM resact; the threshold voltage (V\textsubscript{thr}) for the Ca\textsuperscript{2+} influx was deduced from the latency of the Ca\textsuperscript{2+} signal.
- **F** Calibrated pH\textsubscript{i} and Ca\textsuperscript{2+} signals evoked by 1 nM resact; the threshold pH\textsubscript{thr} at which the Ca\textsuperscript{2+} influx commences was deduced from the latency of the Ca\textsuperscript{2+} signal.
- **G** Linear relationship between pH\textsubscript{thr} and V\textsubscript{thr} for activation of Ca\textsuperscript{2+} influx by various resact concentrations (data derived from Supplementary Fig S6; mean ± SD; n ≥ 3).
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 (HCO\(_3\)) prevents the pH responses—assuming that HCO\(_3\) 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, \(V_{\text{rest}}\) of CatSper activation is slightly more positive than \(V_m\), 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 \(V_{\text{rest}}\) (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 chemotactic 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\(_i\)-induced 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 \(V_{\text{rest}}\) (about −50 mV) and the reversal potential of the CNGK channel (about −95 mV).

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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; Strümpfer 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 CNGK (AP47C9) were described previously (Böningk *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% SDS–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; chemiluminescence was detected via a CCD-imaging system (LAS-3000; Fuji (CHO proteins) or by hyperfilms (GE Healthcare) (sperm proteins). The goat anti-rat-IRDye800cw antibody (1:20,000) was used to visualize sperm protein bands via the Odyssey Imaging System (LI-COR).

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

Sperm flagella and heads were separated as described (Mengerink & Vacquier, 2004; Strünger *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 Franzel *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 Fisher 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 CNGK 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 lysate) 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; Strücker 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 pad (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_4Cl-, pH_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 536/628 (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 (Strücker 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_rest 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 ΔR/mV. Determination of V_rest 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 (AR/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_4Cl-, pH_clamp-, resact-, or cGMP-induced signals.

The calibration procedure for BCECF fluorescence to yield pH_m by the pH-null-point method is described in the result section and below; pH_m calibration was performed for each set of experiments. The pH-null-point solutions were prepared according to the following equation: pH_null = pH_o – 0.5 log ([TMA]/[BA]); pH_o = 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_null or pH_steady null-point. When a cell with a resting pH_m (pH_rest) is placed in a pH_null-point solution, it will not change its pH_m when the pH-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-null-point does not match pH_rest, the absolute concentrations of acid and base determine to what extent the pH of a cell will change (Chow et al., 1996): The higher the concentrations of acid/base mixture, the more the pH_rest will be shifted toward the pH-null-point. At saturation, the newly established pH_m matches the pH-null-point; thus, the cell is clamped to a new pH_m. Therefore, we refer to this saturating pH-null-point solution as pH_clamp solution. Finally, the time course of pH_m determines the time window for which this pH_clamp concept holds. To ensure that in A. punctulata sperm, the pH_m was indeed clamped to the pH-null-point for several tens of seconds, we determined for each pH-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_clamp solutions that clamped pH_m in A. punctulata sperm to pH_null all contained 60 mM BA, whereas the TMA concentration was varied to yield the respective pH_null; for example, for the pH_null 7.0 solution, we used 1.5071 mM TMA/60 mM BA, for the pH_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<sup>2+</sup> 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<sub>i</sub> 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 new medium for 5 min. In the stopped-flow device, the sperm accumulation in a resact gradient was studied as described with some modifications (Alvarez et al, 2012; Hirohashi et al, 2012; Hirohashi et al, 2013). In brief, sperm swimming in a recording chamber (150 µm depth) were imaged using a microscope (IX71; Olympus) equipped with a 10× objective (UPPlanSapo; 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. Images were bandpass-filtered (HQ520/40; Chroma) and 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. Images were bandpass-filtered (HQ520/40; Chroma) and LED (K2 star; Luxeon), a custom-made housing, and a pulse generator.

Sperm chemotaxis

Sperm accumulation in a resact gradient was studied as described with some modifications (Alvarez et al, 2012; Hirohashi et al, 2013). In brief, sperm swimming in a recording chamber (150 µm depth) were imaged using a microscope (IX71; Olympus) equipped with a 10× objective (UPPlanSapo; 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 acquired at 20 Hz, using an electron-multiplying charge-coupled device camera (DU-897D; Andor). Sperm were suspended at about 2–5 × 10<sup>3</sup> cells/ml in ASW containing caged resact (50 nM; control) or caged resact and MDL 12330A (10 µM) or milbefradil (60 µM). Resact was released by 400-ms UV flashes (LED M365L2-C1; Thorlabs) with a Gaussian profile of σ = 178 µm width, coupled to the microscope using a beam splitter (495 nm cutoff, BrightLine; Semrock). The light power delivered to the sample was 1.7 mW. The sperm distribution around the center of the illuminated area was quantified by the relative changes of the weighted standard distance (Alvarez et al, 2012; Hirohashi et al, 2013). Only points within a distance ≤ 2σ to the center of the UV flash were considered. The uncaging gradient was quantified by imaging fluorescein (10 µM) with the same UV light source and optical components.

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://embj.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.

References

Babcock DF, Bosma MM, Battaglia DE, Darszon A (1992) Early persistent activation of sperm K<sup>+</sup> channels by the egg peptide speract. Proc Natl Acad Sci USA 89: 6001–6005


Ho K, Woff CA, Suarez SS (2009) CatSper-null mutant spermatozoa are unable to ascend beyond the oviductal reservoir. Reprod Fertil Dev 21: 345 – 350


Kaupp UB (2012) 100 years of sperm chemotaxis. J Gen Physiol 140: 583 – 586


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common interaction domain and evidence for expression in testis. Reprod Biol Endocrinol 1: 53


