A family of hyperpolarization-activated channels selective for protons


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Edited by Ehud Y. Isacoff, University of California, Berkeley, CA, and approved April 10, 2020 (received for review January 25, 2020)

Proton (H\(^+\)) channels are special: They select protons against other ions that are up to a millionfold more abundant. Only a few proton channels have been identified so far. Here, we identify a family of voltage-gated “pacemaker” channels, HCNL1, that are exquisitely selective for protons. HCNL1 activates during hyperpolarization and conducts protons into the cytosol. Surprisingly, protons permeate through the channel’s voltage-sensing domain, whereas the pore domain is nonfunctional. Key to proton permeation is a methionine residue that interrupts the series of regularly spaced arginine residues in the S4 voltage sensor. HCNL1 forms a tetramer and thus contains four proton pores. Unlike classic HCN channels, HCNL1 is not gated by cyclic nucleotides. The channel is present in zebrafish sperm and carries a proton inward current that acidifies the cytosol. Our results suggest that protons rather than cyclic nucleotides serve as cellular messengers in zebrafish sperm. Through small modifications in two key functional domains, HCNL1 evolutionarily adapted to a low-Na\(^+\) freshwater environment to conserve sperm’s ability to depolarize.

HCNL1 channel | proton channel | voltage-sensing domain | HCN channel

Significance

We discovered a subfamily of voltage-gated ion channels, called HCN-like channels, consisting of two members, HCNL1 and HCNL2. In contrast to classic pacemaker HCN channels in the heart and brain, HCNL1 conducts protons rather than potassium or sodium ions. The pore domain, which exists in most voltage-gated channels, is nonconducting. Instead, protons permeate the channel via the voltage-sensing domain involving the S4 motif. Key to proton conduction is a methionine residue that interrupts the regular spaced series of arginine residues in S4. We show that fish sperm use this unusual ion pathway to create a hyperpolarization-activated proton influx that counterbalances an alkaline-activated K\(^+\) channel.

Author contributions: S.F., U.B.K., R.S., and T.K.B. designed research; L.W., T.W., S.F., W.B., H.G.K., J.F.J., C.T., R.S., and T.K.B. performed research; R.F. contributed new reagents/analytic tools; L.W., T.W., S.F., R.S., and T.K.B. analyzed data; and L.W., T.W., U.B.K., R.S., and T.K.B. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2001214117/-/DCSupplemental.


www.pnas.org/cgi/doi/10.1073/pnas.2001214117

PNAS | June 16, 2020 | vol. 117 | no. 24 | 13783–13791
at \(-65\) mV (Fig. 1B). Currents were half maximal at \(V_{1/2} = -98.8 \pm 6.8\) mV with a slope \(s = 5.8 \pm 1.5\) mV (mean \(\pm SD, n = 6\) experiments). However, although it carries a CNBD, HCN1, unlike any other HCN channel, was insensitive to cyclic nucleotides (cAMP: \(V_{1/2} = -96.6 \pm 3.6\) mV, \(s = 6.6 \pm 1.3\) mV, \(n = 5\); cGMP: \(V_{1/2} = -100.1 \pm 3.0\) mV, \(s = 5.8 \pm 1.2\) mV, \(n = 6\)).

The HCN1 Channel Is Selective for Protons. We determined the ion selectivity of HCN1 from the reversal potential (\(V_{rev}\)) of tail currents, which is diagnostic of the permeant ions. Surprisingly, tail currents behaved anomalously: upon prolonged stimulation, tail currents changed from inward to outward (SI Appendix, Fig. S2), suggesting that either the ion selectivity of HCN1 is changed or ions are redistributed at the membrane (accumulation or depletion). Because of their low concentration, protons are particularly prone to redistribution during proton channel activity (14). To test whether HCN1 conducts protons, we recorded currents from HCN1- and HCN2-expressing (control) CHO cells and simultaneously measured intracellular pH (pHi) using the fluorescent pH dye BCECF (2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein). Activation of HCN1, but not HCN2 (a classic HCN channel), acidified the cell cytoplasm and large organic cations instead of alkali metal ions (15).

Proton-selective channels (15) change or ions are redistributed at the membrane (accumulation or depletion), which minimizes proton redistribution, and large cations instead of alkali metal ions (15–17). Even under these conditions, large HCN1 currents were still observed. The \(V_{rev}\) at defined pH differences (\(\Delta pH = \text{extracellular pH} - \text{intracellular pH}\)) behaved as predicted from the Nernst equation (Fig. 1D and E), indicating that only protons pass HCN1. We also tested the selectivity for protons over other ions by comparing \(V_{rev}\) in the presence of the large, impermeant organic cation NMDG\(^+\) versus various alkali ions.

Exchanging NMDG\(^+\) with alkali ions (at \(pH_o = 6.5, pHi = 5.5\)) did not change \(V_{rev}\) significantly (Fig. 1F), demonstrating that the HCN1 channel is exquisitely selective for protons. \(Cl^-\) also did not change \(V_{rev}\) (Fig. 1F). To determine the relative permeability of \(H^+\) versus \(Na^+\) quantitatively, we reduced the \(H^+\) concentration (pHi = pHo = 7) and recorded reversal potentials in HCN1-expressing CHO cells. Here, a shift in \(V_{rev}\) could be observed: Replacing NMDG\(^+\) with \(Na^+\) resulted in a \(\Delta V_{rev}\) of 7.2 ± 2.6 mV. According to the Goldman–Hodgkin–Katz (GHK) equation (18), the relative permeability of protons to sodium is \(P_{Na}/P_{H} = 3 \times 10^6\).

HCN1 from the common carp Cyprinus carpio and the goldfish Carassius auratus also mediated proton currents (SI Appendix, Fig. S3), suggesting that HCN1 channels represent a family of hyperpolarization-activated proton channels.

HCN1 Conducts Protons via the VSD. We set out to identify the proton permeation pathway in HCN1. In voltage-gated ion channels, except the depolarization-activated proton channel H1, a stretch of conserved residues in the PD comprises the selectivity filter of the pore (19, 20). In classic HCN channels, a short conserved CIGYG sequence motif in the pore loop determines the ion selectivity (Fig. 2B, yellow). Mutations in this motif abolish ion conduction in HCN channels (21). This canonical CIGYG motif is absent in HCN1 channels, and the corresponding string of amino acid residues is not conserved among HCN1 channels from different species (WPFFLE in \(dHCHN1\), WISTK in caHCNL1 and ccHCNL1) (Fig. 2B and SI Appendix, Table S1). This comparison suggests that the PD in HCN1 channels is nonconducting, and protons might permeate via another pathway.

In \(Na^+\) and \(K^+\) channels, mutations that introduce gaps in the regular spacing of the S4 Arg residues in the VSD can induce pores (22–25). The resulting currents through the VSD have been termed “gating pore currents” or “omega currents.” A
number of pathologic conditions, termed “channelopathies,” are caused by channels with these gating pore currents (25). Probably by a related mechanism, H1 conducts protons through its VSD because it lacks a central PD (15, 16). Mutations of the S4 segment of H1 abolish proton permeation (17, 26, 27) or alter ion selectivity (28). Therefore, we compared the S4 sequences of HCN, HCN1/2, and H1 channels with emphasis on interruptions of the Arg residue pattern. The S4 segment of classic HCN and HCN1/2 channels is highly conserved: It carries a string of up to eight regularly spaced Arg residues that is interrupted in both channel types at the fifth position by a serine residue (Fig. 2B). Strikingly, in HCN1/2 but not classic HCN channels, the string of Arg residues is additionally interrupted at the third position by a methionine residue (M169 in zebrarfish HCN1) (Fig. 2B). The S4 segment of H1, compared to that of the HCN and HCN1/2 channels, contains a shorter string of positively charged residues, consisting of only three Arg residues.

Four experimental observations argue that the VSD represents the proton permeation pathway in HCN1. First, pharmacological experiments support the notion that protons permeate through the VSD rather than the PD (Fig. 3A). The open-channel blocker ZD7288 of classic HCN channels did not block HCN1 currents (Fig. 3B), although hydrophobic amino acid residues important for drug binding (29) are conserved in HCN1 (Fig. 3A). By contrast, an open-channel blocker of H1 channels, 2-guanidinobenzimidazole (2GBI) (30, 31), and its membrane-permeable congener 5-chloro-2-guanidinobenzimidazole (CGBI), both blocked HCN1 (but not classic HCN2) currents in a dose-dependent fashion (Fig. 3B and C). A key feature of VSDs is a conserved phenylalanine in the transmembrane segment S2 referred to as a gating charge transfer center (32). Mutation of the respective Phe to Ala in H1 leads to an increase of the efficacy of 2GBI (30). Mutating the homologous Phe in HCN1 to Ala (F96A) also changes the efficacy of 2GBI; however, in the HCN1 context, the affinity of the mutant was lower (Fig. 3C). In both channel types, the conserved Phe residue of the gating charge transfer center participates in the binding of GBI compounds. In summary, these results suggest that GBI compounds block HCN1 currents by occluding a proton permeation pathway in the VSD.

Second, we examined whether the PD of HCN1 channels contributes to ionic currents. Various constructs lacking the PD were nonfunctional (SI Appendix, Fig. S4). Therefore, we took a less invasive strategy: in HCN channels, replacing the canonical selectivity filter GYG by AAA produces nonconducting, dominant-negative channels (21). We constructed a congruous HCN1 mutant in which the respective amino acids (PLE; positions 271 to 273) (Fig. 2B) were replaced by AAA (HCN1-AAA). In this functional mutant, proton permeation and selectivity were not altered (Fig. 3D–F), suggesting that the proton permeation pathway of HCN1 does not involve the central PD.

Third, we investigated a channel mutant where M169 in the S4 segment was replaced by an Arg residue (M169R). Thereby, we generated a sequence of charges in S4 that is similar to that of classic HCN channels that do not transport ions via S4 (Fig. 2B). This mutant, in inside-out patches from Xenopus oocytes, carried only small transient currents, much smaller than the currents that we robustly obtained for wild-type channels (Fig. 4A). Low expression of the mutant was not responsible for the small current because patch clamp fluorometry of GFP-tagged variants of wild-type and M169R channels demonstrated similar expression levels, despite large differences in patch current (Fig. 4B and C). We studied the M169R mutant in more detail in CHO cells. Here, in whole-cell recordings, the transient currents were larger and robust (Fig. 4D). We reasoned that the transient currents could either originate from fast channel inactivation or might represent gating currents due to S4 movement across the electric field of the membrane. To discriminate between the two possibilities, we activated M169R channels and, when the peak current was reached, stepped to various voltages from −80 to +80 mV under symmetric ion concentrations (Fig. 4E, Left). Under these conditions, ionic currents, but not gating currents, are expected to reverse direction at Vrev = 0 mV. However, currents did not reverse (Fig. 4E, Right), a behavior incompatible with ionic currents. Currents also did not reverse under Na+ - and K+-based solutions (Fig. 4E, Right). We conclude that the residual currents produced by the
M169R mutant predominantly represent gating currents. The charge–voltage relationships, obtained by integrating on- and off-gating currents, saturated, and their voltage dependence was similar (Fig. 4F). On-gating charges of HCNL1 were much larger than off-gating charges (Fig. 4F). A similar behavior of H1 and Shaker K⁺ channels has been attributed to a phenomenon called “voltage sensor immobilization” (27, 33). During prolonged activation, the voltage sensor enters a more stable conformation from which it recovers only slowly. This immobilization results in an apparent loss of off- compared to on-gating charges. Voltage sensor immobilization is initially small and increases during long stimulation times. Therefore, we recorded on- and off-gating currents of HCNL1 for different pulse lengths. For short stimulation times, on- and off-gating charges were similar (Fig. 4G). With longer stimulation times, the ratio of off- to on-gating charges decayed exponentially (τoff,on = 50.6 ± 8.3 ms, n = 5, Fig. 4G), which is diagnostic for voltage sensor immobilization. In summary, the M169R mutant, proton currents are suppressed, and gating currents become apparent, suggesting that M169 lines the pore.

Fourth, we mutated M169 to cysteine, which can be chemically modified with 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET). Activation of the M169C mutant produced proton currents that were blocked by MTSET modification; the wild-type HCNL1 control was not affected by MTSET (Fig. 4H). Collectively, these results show that protons pass HCN1 via the VSD and M169 lines the proton permeation pathway; furthermore, the “classic” central pore region of HCN1 does not pass protons or alkali metal ions.

**HCNL1 Mediates Hyperpolarization-Activated Currents in Zebrafish Sperm.** Next, we studied the native HCN1 channel in zebrafish sperm by whole-cell patch clamping. In a Cs⁺-based solution, which eliminates K⁺ currents carried by the K⁺-selective cyclic-nucleotide-gated channel (CNGK) (12), we recorded hyperpolarization-activated currents that were similar to currents of heterologously expressed HCN1 (V1/2 = −105.3 ± 11.4 mV, s = 13.0 ± 2.1, n = 5) (Fig. 5A). These currents were also not cyclic nucleotide sensitive (cAMP: V1/2 = −100.6 ± 17.2 mV, s = 11.0 ± 2.6 mV, n = 4; cGMP: V1/2 = −110.0 ± 6.6 mV, s = 10.0 ± 2.6 mV, n = 3). Furthermore, during current activation, the fluorescence of the pH dye pHrodo Red increased (Fig. 5B, 5.3 × 10⁻³ ± 1.9 × 10⁻³ pA⁻¹ s⁻¹, n = 8), indicating acidification by proton flux into sperm. Finally, CIGBI (100 μM) blocked the inward current in sperm (Fig. 5C, 75 ± 9%, n = 4). These results show that HCN1 mediates hyperpolarization-activated currents in zebrafish sperm.

**HCNL1 Forms Tetramers and Is Expressed in the Head of Zebrafish Sperm.** We examined the presence of HCN1 protein in zebrafish sperm by two independent monoclonal antibodies directed against N- (anti-Nterm) and C-terminal (anti-Cterm) epitopes of HCN1. In Western blots of HCN1-AAA-expressing oocytes, anti-HCN1 and anti-HA antibodies labeled a polypeptide with an apparent molecular weight (Mr) of about 62 kDa, similar to the
Fig. 4. Position M169 in the S4 segment is key for proton permeation in HCNL1. (A) Excised inside-out patch clamp recordings from *X. laevis* oocytes expressing wild-type HCNL1 (black) or mutant HCNL1-M169R (orange). (B) Excised inside-out patch clamp recording from a *X. laevis* oocyte expressing HCNL1-eGFP (Left) or HCNL1-M169R-eGFP (Right). eGFP was excited by cyan light; the images of the patch pipettes were recorded under identical illumination and exposure time. The patch domes are encircled by a red trace. (Scale bars, 20 μm). (C) Maximal (negative) current (*I*<sub>max</sub>) recorded at −140 mV as a function of the patch dome fluorescence (black, HCNL1-eGFP; red, HCNL1-M169R-eGFP). Lines are linear fits (black, slope s = 5.9 × 10<sup>−4</sup> pA/F; red, s = 5.6 × 10<sup>−7</sup> pA/F). Data from patches shown in B are encircled in gray. (D) Whole-cell patch clamp recording of a CHO cell expressing HCNL1-M169R. (E) Excised whole-cell patch clamp recording from *X. laevis* oocytes expressing HCNL1-M169R before (black) and after application of 1 mM MTSET (red). (H, Right) Relative inhibition of HCNL1-M169C (79.2 ± 15.2%, n = 12) and wild-type HCNL1 (8.1 ± 13.4%, n = 5) by MTSET. Error bars denote SD.

Fig. 5. HCNL1 acidifies zebrafish sperm upon hyperpolarization. (A, Left) Whole-cell patch clamp recording of hyperpolarization-activated currents in zebrafish sperm in the presence of Cs<sup>+</sup> to block CNGK. (A, Right) GVs derived from tail currents with or without 100 μM cAMP or cGMP in the intracellular solution. Error bars denote SD. (B) Whole-sperm patch clamp fluorometry recording with the fluorescent pH indicator pHrodo Red. (C) Block of hyperpolarization-activated current in zebrafish sperm by 100 μM CIGB (73 ± 9%, n = 4). Error bars denote SD.

calculated M<sub>w</sub> of 60.4 kDa (Fig. 6A). In Western blots of control oocytes, no band was detected, demonstrating the specificity of these antibodies. The antibodies also detected a smear between 65 and 75 kDa. Upon treatment with PNGase, the smear collapsed into a single 62-kDa band, suggesting that HCNL1 proteins are glycosylated (Fig. 6B). The anti-C-term antibody detected
HCNL1 protein in tissue of zebrafish testis and in zebrafish sperm (Fig. 6B) but not in the ovary, eyes, and brain (SI Appendix, Fig. S5A); PNGase treatment also lowered the Mw of HCN1 in testis and sperm (Fig. 6B).

Classic HCN channels are composed of four subunits that form a single pore (34), whereas Hv1 forms a dimer (35, 36) with two proton pores (17). In Western blots of chemically cross-linked HCN1 protein, bands at 59.3, ∼120, ∼180, and ∼240 kDa were detected. The higher molecular species are multiples of the monomer (SI Appendix, Fig. S5B), suggesting a tetrameric organization. Thus, each HCN1 channel probably carries four proton pores (SI Appendix, Fig. S5C).

We studied the cellular distribution of HCN1 in sperm by immunocytochemistry. The anti-Nterm, anti-Cterm, and anti-HA antibodies stained HCN1-HA-transfected but not control CHO cells, demonstrating that these antibodies are suitable for immunocytochemistry (Fig. 6C and D). Both anti-HCN1 antibodies stained heads from zebrafish sperm, whereas the flagellum was only weakly recognized (Fig. 6E and F). Moreover, currents recorded from isolated sperm heads and from...
whole sperm were similar in amplitude. Thus, HCNL1 is predominantly localized to the sperm head. This finding is remarkable because CNGK, the only other ion channel identified in zebrafish sperm, is also localized to the head.

**HCNL1 Is Functionally Linked to the K⁺ Channel CNGK.** Finally, we addressed the physiological role of HCNL1. We previously identified in zebrafish sperm the K⁺-selective CNGK channel (12). CNGK is pH dependent: At alkaline pH, CNGK opens, and at acidic pH, CNGK closes. Current clamp recordings from zebrafish sperm show that intracellular alkalization hyperpolarizes the membrane potential, suggesting that CNGK is involved in setting the resting membrane potential (12). We confirm here the K⁺ dependence of the resting membrane potential in single sperm cells loaded with the fluorescent voltage-sensitive dye di-8-ANEPPS (4-(2-[[3-(diocetylamino)-2-naphthalenyl]ethenyl]-1-(3-sulpropyloxy)pyridinium): When external K⁺ was high, like in the seminal fluid of most freshwater fish species (37), the membrane potential was depolarized; when external K⁺ was low, like in fresh water, the membrane potential was more hyperpolarized (Fig. 6C). Next, we tested whether hyperpolarization by low extracellular K⁺ concentrations also activates HCNL1. Indeed, reducing the extracellular K⁺ concentration from 5.4 mM to 40 μM leads to intracellular acidification (Fig. 6H, 8.8 ± 5.1% ΔF/F, see SI Appendix, Materials and Methods for details, n = 22), suggesting that hyperpolarization by CNGK activates HCNL1, which leads to subsequent proton influx. In turn, proton influx through HCNL1 decreases pHᵢ and thereby should lower the open probability of the pH-sensitive CNGK. The reciprocal interaction of the two channels creates an intricate negative feedback loop (Fig. 6f). In conclusion, the HCNL1 function in zebrafish sperm is similar to that of classic HCN channels: It limits hyperpolarization and initiates recovery from hyperpolarization.

Upon spawning into fresh water, sperm are exposed to a hypoosmotic shock, which eventually activates motility. We emulated a hypoosmotic shock during spawning by rapid mixing in a stopped-flow device pHrodo Red-loaded sperm in isoosmotic solution with a hypoosmotic solution. Changes in pHᵢ were detected by changes in fluorescence of the pH-sensitive dye. Challenging sperm with such a hypoosmotic shock triggered a decrease of pHᵢ (Fig. 6f, 12.1 ± 4.5% ΔF/Fᵢ₀, n = 5). These experiments suggest a role of HCNL1 during sperm activation.

**Discussion**

HCNL1 is the founding member of a family of hyperpolarization-activated channels that are highly selective for protons; only three other proton channels have been identified: H₁ and Otopetrin in eukaryotes (15, 16, 38) and M2 in the influenza virus (39, 40).

Although HCNL1 channels and classical HCN channels are highly homologous, two specific modifications have completely changed their physiology. First, the PD of the channel carries mutations that “plug the pore.” The central pore, key to ion permeation in all other members of voltage-gated ion channels, is nonfunctional in HCNL1. Second, replacing a single arginine, permeation in all other members of voltage-gated ion channels, by hydrophobic residues in segments S1 to S3 and that separates the extra- from the intracellular side (32, 41–43). During activation, the voltage-sensing S4 segment travels through the HCS. While S4 moves, the constriction is always occupied by one of the positively charged residues of S4, and no ions can pass the HCS. However, mutations of Arg residues in S4 of Kᵢ, Naᵢ, and Caᵢ channels can cause voltage-dependent currents flowing through the VSD, so-called gating pore currents (22–25). Some of these mutants have been identified in human voltage-gated channels that give rise to nonselective currents through the VSD and cause channelopathies (25). A similar mechanism may give rise to proton currents in HCNL1, with the major difference that the currents through HCNL1 are exquisitely proton selective. We envision that hyperpolarization might relocate M169 to the VSD constriction site and thereby generate a proton-selective pore. Remarkably, introducing at position M169 an Arg residue that “fills the gap” in the string of regularly spaced Arg residues abolishes proton permeation, and gating currents become apparent. Similarly, in H₁, an Arg residue introduced at the fourth S4 position in register with the three other Arg residues blocks proton permeation (17) and reveals gating currents (26, 27). In this respect, HCNL1 and H₁ seem to share a common mechanism of proton permeation. For H₁, the mechanism of proton permeation is not completely understood and is debated in the community (44, 45). The discovery of a second channel possessing an analogous, yet hyperpolarization-activated, proton permeation pathway will give ample opportunities to gain insight into the requirements for proton pores in VSDs. Proton selectivity of HCNL1 is therefore within the same order of magnitude as that of the H₁ proton channel (46). Channels that conduct ions through a classic PD typically feature a much lower selectivity (P_K+P_Na+ = 1,000 to 10,000 for K⁺ channels and P_K+_P_Na+ = 100 to 500 for Na⁺ channels) (18). Given the extremely low concentration of protons in most physiological environments (10⁻³ to 10⁻⁸ M), this exquisite proton selectivity is essential for HCNL1 to actually function as a proton-conducting channel (21).

The pore sequence of HCNL2 is more conserved than that of HCNL1: HCNL2 channels carry AISYG, QISYG, or AISYG sequence motifs (SI Appendix, Table S1), which is similar to the canonical CIGYG motif of classic HCN channels. In K⁺ channels, however, the analogous Gly to Ser exchange at the first position of the GYG motif renders the Kᵢ pore nonconducting (47), and a CISYG sequence in human HCN4 results in nonconducting, dominant negative subunits (48), suggesting that the pore of HCNL2 might be functional as well. Nonfunctional HCNL2 channels carry the characteristic Met in S4 that is crucial for proton conduction in HCNL1. Therefore, we speculate that HCNL2 also conducts protons via the same VSD pathway. Functional expression of HCNL2 in heterologous systems was unsuccessful. HCNL2 transcripts are enriched in zebrafish hair cells (49).

Zebrafish sperm become activated by hypoosmotic shock during the release into fresh water. After activation, sperm have about 1 min to find the egg and the microopyle, a tiny hole in the chorion of the egg through which the sperm can reach the plasma membrane for fertilization. The molecular signaling pathways that control the sperm’s journey to the egg are not known.

Fresh water is extremely low in Na⁺ and K⁺ (0.1 to 0.7 mM). Not much is known about the ion channel inventory of zebrafish sperm, except for the alkaline-activated, K⁺-selective CNGK channel (12). CNGK is active under resting conditions and closes upon intracellular acidification (12). HCNL1 is the second channel type identified in zebrafish sperm. CNGK and HCNL1 are both present in the sperm head. Classic HCN channels in the heart and brain carry a Na⁺ inward current that depolarizes the cell. However, because of the low Na⁺ and K⁺ concentrations of fresh water, HCN channels instead would strongly hyperpolarize zebrafish sperm. The proton selectivity enables HCNL1 to depolarize cells in a freshwater environment. Thus, the hyperpolarization produced by the K⁺-selective CNGK channel upon release into fresh water activates HCNL1; the ensuing proton...
influx limits the hyperpolarization in two ways: 1) Proton influx directly depolarizes the membrane potential, and 2) the intracellular acidification will close CNGK channels. Although CNGK and HCN1L1 carry a CNBD, both channels are insensitive to cyclic nucleotides, suggesting that protons instead of cyclic nucleotides serve as cellular messengers in zebrafish sperm.

Across phyla, Ca\(^{2+}\) ions control sperm motility and navigation (50). The sperm-specific Ca\(^{2+}\) channel CatSper mediates Ca\(^{2+}\) influx in many but not all species from marine invertebrates to humans. Some testosel fish, including zebrafish, and birds and amphibians lack CatSper channels (51). The channel(s) and mechanisms that promote voltage-activated Ca\(^{2+}\) influx in CatSper-deficient sperm are not known. In marine invertebrates, the interplay between hyperpolarization, alkalinization, and, ultimately, depolarization activates CatSper channels (52, 53). The opening of K\(^{+}\)-selective, cyclic nucleotide-gated CNGK channels hyperpolarizes sperm; HCN channels counteract hyperpolarization. During recovery from hyperpolarization, CatSper channels open. We hypothesize that, in zebrafish sperm, a similar interplay between the pH-sensitive Ca\(^{2+}\)-channel and the proton-selective HCN1L1 may control a voltage- and/or pH-gated Ca\(^{2+}\) conductance. The swimming pattern of zebrafish sperm depends on the intracellular Ca\(^{2+}\) concentration (12), and Ca\(^{2+}\) signaling might be crucial for successful navigation to the micropyle. We speculate that activation of CNGK and HCN1L1 during spawning changes the intracellular Ca\(^{2+}\) concentration and thereby activates and modulates sperm swimming.

To further delineate the signaling pathway that controls zebrafish sperm motility and navigation, it will be necessary to identify additional molecular components that control the intracellular Ca\(^{2+}\) concentration.

Materials and Methods

Expression in CHO Cells and *X. laevis* Oocytes. For channel expression in *X. laevis* oocytes, oocytes were injected with mRNA obtained from in vitro transcription and incubated at 14 to 16 °C for 1 to 5 d. For channel expression in CHO cells, either a stable cell line was generated, or for transient expression, cells were transfected at least 24 h before use. Additional information on channel expression is provided in SI Appendix, Materials and Methods.

**Biochemistry.** Zebrafish sperm were solubilized using a hypotonic solubilization buffer and sonication. *X. laevis* oocytes were mechanically devitalized by hypotonic homogenization by trituration. Standard procedures were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and Western blot. Anti-hCN1L1 antibodies were generated from stable rat hybridoma clones. Cross-linking was performed with the amino-specific cross-linker disuccinimidyl suberate. Additional information on biochemical protocols is provided in SI Appendix, Materials and Methods.

Electrophysiology and Fluorometry. Electrophysiological recording of CHO cells and *X. laevis* oocytes was performed using standard experimental procedures. For pH fluorometry, cells were loaded with the pH indicator BCECF. To record gating currents, online leak subtraction was applied using the p4p4 protocol. In some cases, leak currents were subtracted off-line. Junction potentials were calculated with pClamp 10 and subtracted off-line. For whole-cell recordings of intact zebrafish sperm and isolated sperm heads, gigaseals were formed at the neck region of the sperm cell. For patch clamp fluorometry measurements of zebrafish sperm, sperm cells were loaded with the pH indicator pHrodo Red AM prior to recording. All electrophysiological measurements were performed at room temperature. Changes in pH, of single zebrafish sperm in response to changes in the extracellular K\(^{+}\) concentration (Fig. 5B) were measured by superfusing sperm isolated with HEPES solution containing different concentrations of K\(^{+}\). The fluorescence signal was determined from the spatial average of a region of interest covering the sperm head. Additional information on electrophysiological and fluorometric recording procedures is provided in SI Appendix, Materials and Methods.

Immunochemistry. A stable CHO cell line expressing HCN1-L1 was mixed with fresh sperm or CHO cells adhered onto glass cover slips, fixed with ice-cold methanol, and stained with anti-hCN1L1 antibodies, a monoclonal anti-HA antibody, and DAPI. For detection, fluorescently labeled secondary antibodies (Alexa488 or Cy3) were used. After mounting, cells were imaged under a confocal microscope. Zebrafish sperm were immobilized on adhesion microscope slides. Fixation, staining, and imaging were performed as for CHO cells. Additional information on immunochemical protocols is provided in SI Appendix, Materials and Methods.