The HCN channel voltage sensor undergoes a large downward motion during hyperpolarization

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Voltage-gated ion channels (VGICs) contain positively charged residues within the S4 helix of the voltage-sensing domain (VSD) that are displaced in response to changes in transmembrane voltage, promoting conformational changes that open the pore. Pacemaker hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are unique among VGICs because their open probability is increased by membrane hyperpolarization rather than depolarization. Here we measured the precise movement of the S4 helix of a sea urchin HCN channel using transition metal ion fluorescence resonance energy transfer (tmFRET). We show that the S4 undergoes a substantial (~10 Å) downward movement in response to membrane hyperpolarization. Furthermore, by applying distance constraints determined from tmFRET experiments to Rosetta modeling, we reveal that the carboxy-terminal part of the S4 helix exhibits an unexpected tilting motion during hyperpolarization activation. These data provide a long-sought glimpse of the hyperpolarized state of a functioning VSD and also a framework for understanding the dynamics of reverse gating in HCN channels.
cysteine accessibility mutagenesis studies have suggested that the conformational change of the VSD during channel activation is conserved between spHCN and mouse HCN1 channels.

To measure the structure and dynamics of the VSD during channel activation, we used tmFRET on intact spHCN channels in the plasma membrane. The tmFRET measures the distance between a donor fluorophore and an acceptor nonfluorescent transition metal ion, such as Ni²⁺, Co²⁺, and Cu²⁺. Minimal transition metal ion binding sites were introduced by site-directed mutagenesis. Efficient and specific fluorophore labeling was achieved using amber stop codon suppression to introduce the fluorescent, noncanonical amino acid L-Anap. Anap is a small, environmentally sensitive fluorophore with a short linker to the protein backbone, and it is well suited as a tmFRET donor for distance measurements. We also employed PCF to allow simultaneous measurement of the structure (with tmFRET) and function (with current).
electrophysiology) of intact spHCN channels, while controlling membrane voltage and rapidly applying intracellular ligands (for example, cAMP and transition metals) (Fig. 1d). By combining these methods, we established a powerful way to measure the conformational dynamics associated with the gating of ion channels in a membrane environment.

PCF measurements of spHCN channels during activation by hyperpolarization. We introduced amber stop-codons individually at five positions along the S4 transmembrane helix of an spHCN channel with a C-terminal yellow fluorescent protein (YFP) fusion (Fig. 1c,f). YFP was used to confirm the expression of the full-length channel, correlate with Anap incorporation and calculate the relative quantum yield of l-Anap for tmFRET (Supplementary Fig. 1). The five different spHCN constructs were expressed in Xenopus oocytes, which were also injected with l-Anap and an l-Anap-specific aminoacyl tRNA synthetase/tRNA plasmid (pANAP)39. Inside-out patches were excised from the oocyte plasma membrane, and fluorescence and current were recorded simultaneously using PCF (Fig. 1d).

Substantial Anap fluorescence, YFP fluorescence and K+ current were observed from all five engineered spHCN channels. Anap fluorescence was directly proportional to ionic current and YFP fluorescence (Fig. 1d and Supplementary Fig. 2). In addition, little or no Anap fluorescence was observed in the absence of pANAP or in the absence of the introduced amber stop codon (Fig. 1g). These results indicate that the fluorescent labeling was efficient and specific for each of the five l-Anap sites introduced into the channels.

The functional properties of the engineered channels were measured using a family of hyperpolarizing voltage pulses from 0 mV to −110 mV in the presence of 1 mM cAMP. All channels exhibited appreciable hyperpolarization activation (Supplementary Fig. 1). The conductance–voltage (G–V) relationships were generally similar to that of wild-type spHCN channels in four of the channels (Fig. 1b, Supplementary Fig. 1b and Supplementary Table 1). However, the F359Anap site at the end of S4 helix resulted in activation kinetics that were substantially slower, and a G–V curve that was shifted by more than 30 mV in the hyperpolarizing direction, in comparison to wild-type spHCN (Fig. 1b, Supplementary Fig. 1b and Supplementary Table 1). These results suggest that, for all of our sites except F359Anap, introduction of l-Anap into the S4 helix did not substantially perturb the hyperpolarization-dependent gating of these channels.

The environmental sensitivity of Anap fluorescence allowed us to test for any changes to the environment of the S4 residues during voltage-dependent activation of spHCN channels. For spHCN-S346Anap, there was a substantial increase in fluorescence during hyperpolarizing voltage pulses to −100 mV (61.2 ± 3.3%, n = 6 patches) (Fig. 2a and Supplementary Video 1). This increase was accompanied by a shift in the peak emission spectrum to longer wavelengths (8.3 ± 1.5 nm, n = 3 patches) (Fig. 2a and Supplementary Fig. 3a,f), suggesting that S346Anap enters a more hydrophilic environment at −100 mV. An increase in fluorescence was also observed for L348Anap, S353Anap and F359Anap, and a decrease was observed for W355Anap (Supplementary Video 2), with or without a shift in the peak emission (Fig. 2b,c and Supplementary Fig. 3). For S346Anap, the time course and voltage dependence of these fluorescence increases matched the time course and voltage dependence of the ionic currents (Fig. 2d,e). The fluorescence change–voltage (F–V) relationship for S346Anap is shifted to negative voltages compared to the G–V (Fig. 2e), similar to the way the gating charge–voltage (Q–V) relationship of related HCN channels is shifted compared to the G–V, suggesting some charge movement after opening48. In contrast, an Anap site in the S1 transmembrane segment (W218Anap) exhibited little or no fluorescence change when hyperpolarized to −100 mV (Supplementary Fig. 4b and Supplementary Fig. 3e). These results suggest that the fluorescence changes at the S4 Anap sites are reporting, at least in part, the rearrangement of the VSD during channel activation.

Hyperpolarization-induced movement of S4 measured by tmFRET. To measure the S4 movement directly, we performed tmFRET experiments. Transition metals, such as Ni2+, Co2+ and Cu2+, have absorption spectra that overlap with the emission spectrum of l-Anap (Fig. 3a)37 and thus can serve as non-fluorescent FRET acceptors that quench the donor’s fluorescence in a highly distance-dependent manner. Because the absorption of most transition metals is low, with multiple transition dipoles, tmFRET can measure short distances (10–25 Å) with little or no orientation dependence37,38. Various metals can be reversibly attached to engineered metal-binding sites on the protein, extending the useful distance range for tmFRET37. Finally, by measuring the degree of metal
L182H, L186H channels was much greater at hyperpolarized voltages. The quenching for each state of the channel, the FRET efficiency can be accurately determined for each state, even if there is an environmental change in fluorescence.

A transition metal ion binding site was introduced into the spHCN-S346Anap channel as a dihistidine (HH) motif (L182H, L186H). On application of 1 mM Co²⁺, there was substantial quenching of Anap fluorescence, which was reversible by the addition of the metal chelator EDTA (Fig. 3c). The quenching at −100 mV was substantially more in spHCN-S346Anap, L182H, L186H channels (35.3 ± 1%) than in spHCN-S346Anap channels lacking the HH site (14.5 ± 1%) (n = 3 patches). These results indicate that the quenching arose primarily from FRET between S346Anap and Co²⁺ bound to the HH site in the HCN domain, although some solution quenching by 1 mM Co²⁺ was also present.

Remarkably, Co²⁺ quenching of Anap in spHCN-S346Anap, L182H, L186H channels was much greater at hyperpolarized voltages than at depolarized voltages (Fig. 3c). We quantified the apparent FRET efficiency by calculating the fractional decrease in Anap fluorescence in 1 mM Co²⁺ and correcting for the solution quenching in spHCN-S346Anap lacking the HH site (see Methods). The FRET efficiency increased by tenfold, from 0.03 ± 0.01 at 0 mV to 0.30 ± 0.01 at −120 mV (Fig. 3d) with a voltage dependence that is similar to that of previously measured gating currents from spHCN [36–39]. These results suggest that the S346 position in the S4 helix moves downward—closer to the HCN domain—during hyperpolarization activation of HCN channels.

To measure the movement of the S4 helix more precisely we switched to a new method for tmFRET, called ACCuRET (Anap Cyclen-Cu²⁺ resonance energy transfer) [39]. Instead of a HH motif, ACCuRET introduces a higher affinity transition metal ion binding site using TETAC (1-(2-pyridin-2-ylsulfanyl)ethyl)-1,4,7,10-tetrazacyclododecane) (Fig. 4a) [39]. TETAC is a cysteine-reactive compound with a short linker to a cyclen ring that binds transition metal ions with subnanomolar affinity [31]. Single cysteines introduced into the protein react with TETAC to create a high-affinity metal-binding site for tmFRET. This binding site has two major advantages over HH: (1) it avoids using high (millimolar) concentrations of transition metals in solution, which cause solution quenching and could bind to other endogenous sites or have unwanted effects on the protein; (2) TETAC increases and shifts the absorbance of Cu²⁺ to create greater overlap with the emission of Anap, extending the range of distances that can be measured with tmFRET (Fig. 3a). These advantages recently enabled the accurate measurement of absolute distances and distance changes in soluble and membrane proteins [32–34].

We employed ACCuRET to measure the size of the movement of the S4 helix during hyperpolarization activation of spHCN. In combination with the Anap sites, we introduced a single cysteine (L186C) into the second helix of the HCN domain of cysteine-depleted spHCN constructs. Neither the cysteine mutations nor Cu²⁺-TETAC labeling of L186C had an appreciable effect on hyperpolarization-dependent gating (Supplementary Fig. 5). Similar to the effect of Co²⁺ on the HH motif, the modification by 10 µM Cu²⁺-TETAC caused a significant reduction in Anap fluorescence for spHCN-S346Anap, L186C, particularly at −100 mV (Fig. 4b,c). This Cu²⁺-TETAC quenching had no effect on the shape of the Anap emission spectrum and was negligible in the absence of the introduced cysteine, indicating that it arose from FRET between S346Anap and Cu²⁺-TETAC bound to L186C on the HCN domain (Fig. 4b,c). Like the effect of Co²⁺ on the HH motif, the quenching was much greater at −100 mV (62.5 ± 3.6 %) than at 0 mV (16.7 ± 0.8 %) (n = 5 patches) (Fig. 4c).

We performed similar ACCuRET experiments for the three other Anap positions closer to the C-terminal end of the S4 (L348, S353 and W355). For each position, there was substantial quenching by Cu²⁺-TETAC modification of L186C (Fig. 4c). At 0 mV the quenching increased steadily as the Anap position moved towards the C-terminal end of the S4 helix, as expected from the decreasing FRET distance in the cryo-EM structure of hHCN1 (ref. [39]). Unexpectedly, however, the increase in quenching at −100 mV compared to that at 0 mV progressively diminished as Anap sites became closer to the C-terminal end of the S4 helix (Fig. 4c). These data imply that S4 is not simply translating as a rigid body directly towards the HCN domain during hyperpolarization activation.

**Distance changes between the Anap sites and HCN domain by hyperpolarization.** We used our tmFRET data to calculate the distances between each Anap site and L186 at 0 mV and −100 mV. FRET efficiencies were calculated from the fractional decrease in Anap fluorescence after application of Cu²⁺-TETAC and corrected for the small Cu²⁺-TETAC-dependent quenching in Anap constructs lacking L186C (Supplementary Fig. 6 and see Methods). We also determined the R₀ values (the distance predicted to produce 50% FRET efficiency) for each site at both 0 mV and −100 mV, using emission spectra and fluorescence intensity data (Supplementary Figs. 2 and 3 and see Methods). The measured FRET efficiencies for each site at 0 mV were plotted against the β-carbon distances between each FRET pair (in distance/R₀ units) determined from an spHCN homology model built from the cryo-EM structure of hHCN1 (also at 0 mV) (Fig. 4d). The FRET efficiencies decreased...
with distance, and this relationship provides an empirical measure of the distance dependence of tmFRET that can be used to measure the distances at −100 mV.

The distance dependence measured for tmFRET at 0 mV had the expected $R_0$ value, but was shallower than predicted by the Förster equation (Fig. 4d). Previously this shallower distance dependence has been attributed, in part, to heterogeneity of the interatomic distances in proteins, and could be accounted for by convolving the Förster equation with a Gaussian distribution of distances\(^40\). We found a Gaussian distribution of distances with a standard deviation $\sigma$ of 7.5 Å (inset). The distances of these five pairs at −100 mV estimated using the FCG equation are also shown with square markers. 

**Fig. 4** | The distance change measured by ACCuRET decreases as Anap is positioned closer to the C-terminal end of S4. 

- **a**, Cartoon showing ACCuRET between S346Anap and Cu\(^{2+}\)−TETAC attached to L186C in the HCN domain. Also shown is the reaction of Cu\(^{2+}\)−TETAC with a cysteine in a protein.
- **b**, Representative spectra of the Anap emission at 0 mV and −100 mV, before and after quenching by Cu\(^{2+}\)−TETAC, for spHCN-S346Anap, L186C channels.
- **c**, Summary of the fraction of Anap fluorescence quenched by Cu\(^{2+}\)−TETAC for the four Anap sites in S4, without (left, n = 4 for S346, n = 3 for L348, S353 and W355) and with (right, n = 5 for S346, n = 4 for L348 and S353, n = 7 for W355) the introduced cysteine L186C. Data shown are mean ± s.e.m., n is number of patches. *P < 0.05 by two-tailed Student’s t-test.
- **d**, Distance dependence of the measured FRET efficiency at 0 mV for four \(l\)-Anap sites (circle markers) in S4 and the W218Anap site in S1 versus the $\beta$-carbon distances between each site and L186C (based on the spHCN homology model). Also shown are the predicted distance dependencies of the Förster equation (black) and the FCG equation (green), using a Gaussian distribution with a standard deviation $\sigma$ of 7.5 Å (inset). The distances of these five pairs at −100 mV estimated using the FCG equation are also shown with square markers.
- **e**, Summary of the distances of each FRET pair at 0 mV and −100 mV, calculated from the FCG equation in panel d, using the FRET efficiencies in Supplementary Fig. 5a.
- **f**, Helical-wheel display of the C-terminal $\alpha$-helical part of S4 (S346 to F359) viewed from the extracellular side, highlighting the magnitude of the hyperpolarization-induced distance changes for the four Anap sites in the S4.
deviation of 7.5 Å could explain the distance dependence of the FRET efficiency that we observed at 0 mV (Fig. 4d). Such wide distance distributions have been directly observed using double-electron-electron resonance spectroscopy in other proteins and arise from rotameric and conformational heterogeneity. The wide distribution observed in the distances between sites in the S4 and L186 in the HCN domain may be partially due to flexibility of the HCN domain. Note, however, that our mean distances estimated from the average tmFRET measurements of many channels are more accurate than the standard deviation of the individual distances. The distances calculated from the FRET efficiencies at 0 mV using the Förster convolved Gaussian (FCG) fit closely matched those in our homology model based on the known hHCN1 structure (r.m.s. deviation = 1 Å) (Fig. 4d). This is similar to our previous estimates of the accuracy of tmFRET using FCG theory, which have ranged from 1.5 to 2.9 Å. Therefore, FCG theory provides an effective way to accurately determine the atomic distances based on the FRET efficiencies.

Using our calibrated distance dependence at 0 mV and our measured FRET efficiencies and Rv values determined at −100 mV (see Methods), we calculated the distances for each FRET pair at −100 mV. While this estimate assumes the same Gaussian distribution of distances at 0 mV and −100 mV, this assumption had only a small effect on the estimated distances at −100 mV because they were all very near Rv (Fig. 4d,e). The distances at −100 mV deviated from the distances at 0 mV. The change in distance between 0 and −100 mV decreased steadily along the S4 helix, from 11.6 Å at S346 to only 0.8 Å at W355. These results support our proposal that the movement of S4 is not simply a rigid body movement directly towards L186 in the HCN domain. Furthermore, since our sites on the same side of the helix (S346 and S353, as well as L348 and W355) reported very different distance changes, and residues on different sides of the helix (L348 and S353) reported more similar distance changes (Fig. 4f), the majority of the distance changes cannot result from a rotation. Instead, our results suggest that the S4 helix moves downward and tilts during hyperpolarization activation.

The interpretation above assumes that L186 in the HCN domain does not move substantially during activation. To test for movement of the HCN domain, we measured tmFRET between L186C and W218Anap on the S1 helix, and calculated distances at 0 mV and −100 mV as above (Supplementary Fig. 4d,e). Interestingly, W218Anap moved a little closer to the HCN domain at −100 mV (distance change, ΔD = 2.7 Å). This could reflect a small upward movement of the HCN domain, or a subtle downward movement of S1 or a combination of the two. In any case, this small movement was included in our structural modeling below, and did not appreciably affect our measurements of the much larger movements of some sites on the S4 segment.

**Structural model for HCN S4 movement by Rosetta modeling.** To estimate the actual movement of the S4 helix, we used our distances as constraints in Rosetta-based structural modeling of the conformational change of the VSD (Fig. 5a and Supplementary Video 3). The models at 0 mV and −100 mV accurately reproduced the distances and distance changes measured from our ACCuRET experiments (Fig. 5b and Supplementary Video 4). At 0 mV, the model was very similar to the spHCN homology model based on the HCN1 cryo-EM structure at 0 mV (ref. 19) (Fig. 5a). However, with the −100 mV constraints, the model revealed a large downward movement of S4 of about two turns of the helix (Fig. 5a,c and Supplementary Video 3). In addition, the C-terminal end of the S4 helix tilted away from the HCN domain, producing a kink in the helix near position R344. Little or no rotation of S4 was observed in the model (indeed, rotation caused a poorer fit to our measured distances), and the HCN domain underwent only a small upward movement. Applying different constraining weights for the experimentally determined distances at −100 mV generated models with very similar movement, reflecting the robustness of our modeling at the hyperpolarized voltage (Supplementary Fig. 7).

While our tmFRET method does not directly measure the positions of side chains, extensive salt bridges between S4 arginines and S1–S3 aspartates were present for the models at both voltages (Fig. 5d). In addition, the S1–S3 form a negatively charged electrostatic surface that interfaces with S4 positive charges (Fig. 5c and Supplementary Video 3). At 0 mV, both R3 (R341) and R4 (R344) were above the ‘phenylalanine cap’ F260 in the hydrophobic constriction site (HCS) and interacting with the extracellular negative cluster (ENC: D308, D257). Both R5 (R347) and R6 (R350) formed salt bridges with the intracellular negative cluster (INC: D263, D300). At −100 mV, R3 and R4 moved down below the HCS and were stabilized by interacting with the INC while the K1 (K335) formed a salt bridge with D308 of the ENC. The 3w-helical nature of S4 in the gating charge transfer center (indicated in Fig. 1e) positions the voltage-sensing arginines, R3 and R4, in line on the same side of S4, at both voltages. Therefore, this region moves almost vertically to exchange their ion-pair partners without much rotation (Fig. 5d and Supplementary Video 3). Together, our experiments provide an explicit model of the conformational dynamics of the spHCN VSD during hyperpolarization activation, based on experimentally determined distance measurements under physiological conditions.

**Discussion**

The model for the movement of the S4 helix presented here is consistent with the gating characteristics of HCN channels. A downward movement corresponding to two turns of the S4 helix could produce an equivalent charge movement of up to two electronic charges per subunit (eight charges per channel) during voltage-dependent activation of spHCN. This is more than enough charge movement to reproduce the steepness of the G–V, Q–V and F–V curves for these channels15,46. Furthermore, the large downward translation of S4 strongly supports a previous model based on scanning cysteine accessibility mutagenesis experiments in mouse HCN1 channels11. In addition, the similar results from the cysteine accessibility studies for spHCN and mouse HCN1 channels confirm that the spHCN channel shares very similar VSD movement with its mammalian orthologs21,24. The tilting motion of the S4 that we have observed has not been previously described in any other VGIC, but is compatible with the short S4–S5 linker seen in these non-domain-swapped channels.

The mechanism for coupling this S4 movement to pore opening in HCN channels remains a mystery. Unlike the previously proposed mechanisms in domain-swapped channels, the coupling in non-domain-swapped HCN channels does not require an intact S4–S5 linker25. The S4–S5 linker is also not required for gating in the related depolarization-activated EAG and hERG channels, which are also not domain-swapped46,47. Considering the tight packing of S4 and S5 helices in HCN channels5, the downward and tilting movement of the S4 helix could bypass the short S4–S5 linker to influence the S5 helix directly, thus altering channel opening47,48. Potentially, the tilting motion of the C-terminal end of the S4 could move the N-terminal end of the S5 helix to allow the S6 bundle-crossing gate to open when the S4 is in the ‘down’ position at hyperpolarized voltages. Our model of the S4 movement provides a framework to decipher this coupling and the reverse gating mechanism of HCN channels.

Despite decades of research on the VSD of VGICs, it remains controversial how the S4 voltage sensor moves during voltage-dependent activation. Using a combination of tmFRET, PCF and Rosetta modeling, we have provided a glimpse into the movement of the S4 at hyperpolarized voltages under physiological conditions that does not rely on gating modifiers to trap a potentially down state of the VSD. Our model is incompatible with other models
proposing that the S4 helix has relatively little ‘up/down’ movement, which are based on cryo-EM, scanning cysteine mutagenesis and FRET. On the other hand, our model is similar to a recently proposed movement of the S4 in VSD2 of the depolarization-activated two-pore channel 1 (TPC1) inferred from structures of mouse TPC1 (‘up’ state) and Ba²⁺-inhibited Arabidopsis TPC1 (‘down’ state) at 0 mV and −100 mV (Supplementary Video 5). Furthermore, a similar movement of S4 was recently proposed for voltage-gated sodium channels based on the ‘down’ state trapped by spider toxins, as well as that of VSD4 trapped by α-scorpion toxins (Supplementary Video 5). The similarity between our model, obtained from tmFRET experiments and using a functioning VSD at hyperpolarized voltages, and the models based on cryo-EM structures with cation- or toxin-trapped states provides further support for the assumption that these high-resolution ‘down’ states of the VSD may represent physiological conformations of the S4 at negative voltages. Our methods further provide an avenue to measure not only a snapshot of the ‘down’ state structure, but also the

Fig. 5 | Rosetta model of S4 movement in HCN channels based on experimentally determined distance constraints. a, Model structures of the VSD of spHCN at 0 mV and −100 mV. Side view parallel to the membrane (top). View from the intracellular side (bottom). b, Comparison of the measured distance changes using tmFRET from Fig. 4 and the distance changes in the Rosetta models. c, A vacuum electrostatic surface illustration of the S1-S3 helices and the HCN domain, showing a negative charged surface (red) facing the S4 helix. d, Structural diagrams showing the ion-pair partners between arginines or lysine (blue) within S4 and aspartates (red) within S1–S3 at 0 mV and −100 mV in the Rosetta models. The ‘phenylalanine cap’ F260 in the HCS of the charge transfer center is highlighted in magenta. The α-carbon distance changes between K1, R3–R6 residues at 0 mV and −100 mV are illustrated.
conformational dynamics of the VSDs as they move to promote channel opening.

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**Author contributions**

G.D., T.K.A. and W.N.Z. conceived and designed experiments and G.D. performed experiments. T.K.A. performed pilot experiments. F.D. designed and performed Rosetta-based computational modeling. G.D., T.K.A., F.D. and W.N.Z. analyzed data. W.N.Z. and G.D. wrote the manuscript. G.D., T.K.A., F.D. and W.N.Z. edited the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods
Molecular biology. The full-length spHCN cDNA (a gift from U.B. Kaupp; GenBank: Y16880) was subcloned into a modified pcDNA3.1 vector (Invitrogen) that contained a C-terminal eYFP; a T7 promoter and 5’ and 3’ untranslated regions of a Xenopus β-globin gene. For the ACCuRET experiments, an oocyte depleting spHCN construct was used with the following mutations: C211A, C224A, C69A, C373A. Point mutations were made using QuickChange II XL Site-Directed Mutagenesis kit (Agilent Technologies). The sequences of the DNA constructs were confirmed by fluorescence-based DNA sequencing (Genewiz). The messenger RNA was synthesized using the HcToBe T7 ARCA mRNA Kit (New England Biolabs) or the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (ThermoFisher) from the linearized plasmid.

Oocyte expression and electrophysiology. The animal-use protocols were consistent with the recommendations of the American Veterinary Medical Association and were approved by the Institutional Animal Care and Committee of the University of Washington. Xenopus oocytes were prepared as previously described40. t-Anap (free-acid form, AsisChem) was made as a 1 mM stock in water at a high pH by adding NaOH and was stored at −20 °C. The pANAP plasmid (purchased from Addgene) contained the orthogonal tRNA/aminocyl tRNA synthetase specific to t- Anap. The pANAP plasmid (40–50 nl of 200 ng μl−1) was injected into the Xenopus oocyte nucleus. Then, t- Anap (~50 nl of 1 mM) and channel mRNA were injected separately into the oocyte cytosol.

Channel currents were recorded from the oocytes 2 to 4 d after injection, using the inside-out configuration of the patch-clamp technique with an EPC-10 (HEKA Elektronik) or Axopatch 200B (Axion Instruments) patch-clamp amplifier and PATCHMASTER software (HEKA Elektronik). Inside-out patch-clamp recordings were made approximately 5 min after patch excision, when the rundown of HCN channels due to phosphorylation and 4.5-bisphosphate (PI(4,5) P) depletion was almost complete47. Borosilicate patch electrodes were made using a P97 micropipette puller (Sutter Instrument). The initial pipette resistances were 0.5–0.7 MQ. Recordings were made at 22 °C to 24 °C. A µFlow microvolume perfusion system (ALA Scientific Instruments) was used to change solutions during the experiment. All recordings were made in the presence of 1 mM cAMP on the cytosolic side of the patch. For oocyte patch-clamp recording and Co2+-HH tmFRET experiments, the standard bath and pipette saline solutions contained 130 mM KCl, 10 mM HEPES, 0.2 mM EDTA, pH 7.2. Co2SO (1 mM) was added to the perfusion solution with EDTA eliminated. For ACCuRET experiments, stabilization buffer (SBT) (130 mM KCl, 30 mM Tris; 0.2 mM EDTA, pH 7.4) was used for the bath and the pipette solution. The Cu2+-TETAC was prepared as previously described40. TETAC (Toronto Research Chemicals) was used as a 100 mM stock in DMSO and stored at −20 °C. On the day of the experiment, 1 μl each of 100 mM TETAC stock and 110 mM CuSO4 stock were mixed together and incubated for 1 min until the solution turned a deeper shade of blue, indicating binding of Cu2+ to the TETAC. To this mixture, 98 μl of SBT was added, giving a solution of 1.1 mM Cu2+ and 1 mM TETAC. The 10% overabundance of Cu2+ ensured that all the TETAC was bound with Cu2+.

This solution was then diluted 1:100 in SBT buffer, giving a final concentration of 10 μM Cu2+-TETAC, and perfused onto the patch for 1 min before fluorescence measurements.

Fluorescence measurements. PCF simultaneously records fluorescence and currents signals in inside-out patches. Our PCF experiments were performed using a Nikon Eclipse TE2000-E inverted microscope with a ×60, 1.2 numerical aperture water immersion objective. Epifluorescence recording of t- Anap was performed with wide-field excitation using a Lambda LS Xenon Arc lamp (Sutter Instruments) and a filter cube containing a 576/30 nm excitation filter and a 460/50 nm emission filter. A 425-nm, long-pass emission filter was used for the spectral measurement of t- Anap. YFP was measured with a filter cube containing a 490/10 nm excitation filter and a 535/30 nm emission filter. Images were collected with a 200-ms exposure using an Evolve 512 EMCCD camera (Photometrics) and MetaMorph software (Molecular Devices). For the ACCuRET experiments, the steady-state Anap fluorescence at −100 mV was captured a few seconds after switching the voltage from 0 mV to −100 mV. For comparison of the Anap and YFP fluorescence intensities, the settings of the EMCCD camera were kept the same.

For spectral measurements, images were collected by a spectrograph (Model: 2150i, 300 g mm−1 grating, blaze = 500 nm; Acton Research) mounted between the output port of the microscope and the Evolve 512 EMCCD camera. The membrane patch was positioned in the entrance slit of the spectrograph with the pipette parallel to the slit. The slit width was made slightly smaller than the width of the patch to block light from regions of the patch attached to the glass. The slit width used had little or no effect on the shape of the emission spectrum (Supplementary Fig. 8b). The spectrograph produced an image on the camera where the vertical dimension was positioned along the axis of the pipette, and the horizontal dimension was the wavelength. The wavelength scale was calibrated with known filters and laser lines. Anap spectra were recorded by measuring line scans through the patch area, background subtracted using line scans through the nonfluorescent region in the pipette outside of the patch and corrected for the filter characteristics of the spectrograph.

Fluorescence experiments on free t- Anap in solvents were performed using a Jobin Yvon Horiba FluoroMax-3 spectrophluorometer (Edison). Absorption spectra were recorded with a DU 800 spectrophotometer (Beckman Coulter). Submicrometer fluorimeter cuvettes (100 μl; Starna) were used for both the fluorescence and spectrophotometry. For emission spectra of free t- Anap-ME (AsisChem), an excitation wavelength of 350 nm and 1-nm slits for excitation and emission were used.

Rosetta modeling. A homology model of the spHCN channel based on the human HCN1 structure55 (PDB 3U60) was created using RosettaCM without any experimental constraints55. The spHCN homology model and HCN1 structure were very similar (r.m.s. deviation of α-carbons for the HCN domain and VSD was 0.8 Å). To obtain models of the spHCN channel at 0 mM and −100 mV, the structures were refined using RosettaCM with three types of constraints applied to the homology model: (1) coordinate constraint (fitting the Cartesian coordinates of an atom) were applied on every nonhydrogen atom except for those in the S4 helix. These were ‘top-out’ constraints, which are roughly harmonic up to 1 Å from the minimum, and flat beyond; (2) harmonic pairwise distance restraints were applied on all backbone hydrogen bonding groups; and (3) experimentally derived distances at 0 mV (state 1) and −100 mV (state 2) based on our tmFRET measurements were applied as ‘flat-bottom’ harmonic constraints, where the constraint energies were zero in a 1-Å window centered on the measured distance, and harmonic beyond this. This constraint roughly corresponds to the estimated resolution of our tmFRET measurements. For setting the constraint weight for a satisfactory model, we performed a grid search of the weights on each of the three constraint types. The weights that overall weights that satisfied the experimental constraints in both states were used: a weight of 15 kcal mol−1 Å−2 on the experimental data, and a weight of 2 kcal mol−1 Å−2 on the hydrogen-bond and coordinate constraints. To better establish the robustness of our modeling procedure, we varied the weight on the experimental data at −100 mV in 11 increments, ranging from 7.5 to 30 kcal mol−1 Å−2 and showed that the final model is relatively insensitive to the experimental weights in this range (see Supplementary Fig. 7). All modeling was carried out starting with the complete spHCN tetramer homology model with C4 symmetry imposed. Since only FRET constraints between the HCN domain and the S4 helix were determined experimentally, we only expect to accurately measure the rearrangements in those regions. Therefore, we imposed coordinate constraints outside the S4 helix to resist movements and, hence, did not observe appreciable movements in other regions. This yielded a voltage-sensor-activated but closed-pore conformation, which is compatible with the weak coupling observed in these channels55. No manual adjustment or refinement was used. The model at 0 mV (state 1) was highly similar to the homology model, while the model at −100 mV (state 2) showed a large downward and tilting rearrangement of the S4 helix (Fig. 5). Rosetta scripts are available in Supplementary Dataset 1. The coordinates for the models were deposited at PDB-dev with accession code: PDBDEV_00000032. A structural morph was created between the state 1 and state 2 models (Supplementary Videos 3, 4, and 5), highlighting the hyperpolarization-dependent movement of the S4 helix. Structural representations and morphs were created using the PyMOL software (https://pymol.org).

Data analysis. Data were analyzed using IgorPro (Wavemetrics) and ImageJ (NIH). The G/V relationships were measured from the instantaneous tail currents at +40 mV following voltage pulses from 0 mV to between 0 and −110 mV. The leak tail currents following pulses to +40 mV were subtracted and the currents were normalized to the maximum tail current following voltage pulses to −110 mV (G/Gmax). The relative conductances were plotted as a function of the voltage of the main pulse and fitted with a Boltzmann equation:

$$G / G_{\text{max}} = \frac{1}{1 + \exp \left( \frac{V - V_{1/2}}{V_{s}} \right)}$$

where V is the membrane potential, V1/2 is the potential for half-maximal activation, and Vs is the slope factor.

Fluorescence images from the membrane patches were imported into ImageJ (ref. 55) for analysis. Regions of interest were drawn by hand around the dome of the patch, excluding any regions that appeared attached to the glass (see Fig. 1d). For each patch, a background region was selected in the pipette outside the area of the patch. The mean gray value of the background region of interest was subtracted from the mean gray value of the region of interest of the patch to give the mean fluorescence intensity (f).

The FRET efficiency was calculated using the following equation (1) as previously described56:

$$F_{\text{RET}}^\text{eff} = \frac{E_{\text{RET}} \times F_{\text{HF}}} {E_{\text{RET}} \times F_{\text{HF}} + E_{\text{MF}} \times F_{\text{HF}} + F_{\text{HF}}}$$

or

$$F_{\text{RET}}^\text{eff} = \frac{E_{\text{RET}} \times C_{\text{HF}}} {E_{\text{RET}} \times C_{\text{HF}} + E_{\text{MF}} \times C_{\text{HF}} + C_{\text{HF}}}$$

(1)
Where $F_{\text{HH}}$ and $F_{\text{noHH}}$ are the fractions of fluorescence that are unquenched by Co$^{2+}$ in channels with and without HH sites, respectively, for example $F_{\text{HH}} = F_{\text{HH, metal}} - F_{\text{HH, no metal}}$. Similarly, $F_{\text{Cys}}$ and $F_{\text{noCys}}$ are the fractions of fluorescence that are unquenched by Cu$^{2+}$-TETAC in channels with and without cysteines, respectively. These equations for calculating FRET efficiency assume that the decrease in $F_{\text{HH}}$ is due to nonspecific energy transfer, such as solution quenching or FRET to a metal ion bound to an endogenous metal binding site.

Alternatively, the FRET efficiency was calculated using the following equation (2), which only account for nonspecific decreases in fluorescence (for example, photobleaching) but not energy transfer.

$$F_{\text{R}} = 1 - \frac{F_{\text{HH}}}{R_{\text{HH}}} \quad \text{or} \quad F_{\text{R}} = 1 - \frac{F_{\text{Cys}}}{R_{\text{Cys}}}$$

The FRET efficiencies produced by equations (1) and (2) were similar because of the low degree of background quenching (Supplementary Fig. 6). For this paper we primarily used equation (1) to calculate the FRET efficiencies. To calculate the mean, and standard error of the mean, for the FRET efficiency, we used the mean and standard error of the mean for our fractional quenching measurements (that is, $F_{\text{HH}}$, $F_{\text{noHH}}$, $F_{\text{Cys}}$, and $F_{\text{noCys}}$) in Monte Carlo resampling (1 x 10$^6$ cycles; NIST Uncertainty Machine v.1.3.4; https://uncertainty.nist.gov).

The $R_g$ values, the distances that predict 10% energy transfer, were calculated using the established equation as previously published.

$$R_g = C \sqrt{J/Q} \frac{\eta}{\kappa}$$

Where $C$ is the scaling factor, $J$ is the overlap integral of the donor emission spectrum and the acceptor absorption spectrum, $Q$ is the quantum yield of the donor, $\eta$ is the index of refraction and $\kappa$ is the orientation factor. $\eta$ was assumed to be 1.33 and $\kappa$ was assumed to be 2/3, a reasonable assumption for an isotropic metal ion environment. Assuming $\kappa = 2/3$ in the case of an isotropic acceptor limits the worst-case error in $R_g$ to $-11\%$ to $+12\%$ (ref. 71). The $J$ values for each $l$-Anap site at 0 mV and $-100$ mV were calculated using the emission spectrum of each site at each voltage (Supplementary Fig. 3) and the absorption spectrum of Cu$^{2+}$-TETAC in solution. Since the shape of the Anap emission spectrum does not change appreciably in different solvents (Supplementary Fig. 8a, inset), we shifted the free $l$-Anap spectrum measured in the cuvette to match the peak of the emission spectra measured on the microscope for each Anap site at 0 mV and $-100$ mV (Supplementary Fig. 3). This strategy removes the contamination of the Anap emission spectrum by the YFP emission peak at around 530 nm. The YFP emission is most likely due to the direct excitation of YFP by the light used to excite Anap, although the possibility of a small amount of FRET between Anap and YFP cannot be completely excluded. Finally, we estimated the quantum yield of $l$-Anap at each site $\pm 100$ mV using the slopes of the plots of Anap intensity versus YFP intensity at 0 mV (Supplementary Fig. 2) and the relative brightness at $-100$ mV versus 0 mV ($Q_{\text{HH, metal}}/Q_{\text{HH, no metal}}$) (Fig. 2c). For each site, $Q_{\text{HH}} = 0.3 \times$ slope (Anap versus YFP) (Supplementary Fig. 2a–e) and $Q_{\text{HH, metal}} = Q_{\text{HH}} \times (\beta_{\text{HH, metal}}/\beta_{\text{HH, no metal}})$. A scaling factor of 0.3 was used for converting the slopes to quantum yields to make the quantum yields fall in the range of the quantum yields for Anap in different solvents (0.2–0.5) (ref. 70). This estimate of quantum yield assumes that the change in brightness of Anap is primarily due to a change in quantum yield and not extinction coefficient, as appears to be true for the solvents that we tested (Supplementary Fig. 8c). $R_g$ values calculated using these estimated spectral overlaps and quantum yields were as follows: 13.1 Å (0 mV) and 14.9 Å (−100 mV) for S346Anap; 12.6 Å (0 mV) and 13.5 Å (−100 mV) for L348Anap; 13.8 Å (0 mV) and 14.1 Å (−100 mV) for S353Anap; 13.3 Å (0 mV) and 13.0 Å (−100 mV) for W355Anap; 13.9 Å (0 mV) and 13.9 Å (−100 mV) for W218Anap.

**Statistics and reproducibility.** Data parameters were expressed as mean ± s.e.m. of n independent patches. Statistical significance (*P < 0.05) was determined by using a two-tailed Student’s t-test.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The coordinate files of the State 1 and State 2 models were deposited in PDB-dev (https://pdb-dev.wwpdb.org/) with accession code PDBDEV_00000032. Source data for Figs. 3 and 4 and Supplementary Fig. 6 are available online. Other data are available from the corresponding author upon reasonable request.

**Code availability**

Rosetta scripts used for the modeling are in Supplementary Dataset 1.

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- PatchMaster 2x73.2 (HEKA Elektronik), MetaMorph 7.8 (Molecular Devices), FluorEssence 3.5 (Horiba Scientific), DU800 3.0 (Beckman Coulter)

Data analysis:
- RosettaCM 2019.19, Igor 6, ImageJ 1.49 (NIH), PyMol 7

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