Structural basis for gating pore current in periodic paralysis

Daohua Jiang1,6, Tamer M. Gamal El-Din1,6, Christopher Ing2,3, Peilong Lu1,4, Régis Pomès2,3, Ning Zheng1,5* & William A. Catterall1*

Potassium-sensitive hypokalaemic and normokalaemic periodic paralysis are inherited skeletal muscle diseases characterized by episodes of flaccid muscle weakness1,2. They are caused by single mutations in positively charged residues (‘gating charges’) in the S4 transmembrane segment of the voltage sensor of the voltage-gated sodium channel Na1.4 or the calcium channel Ca1.1.3,4. Mutations of the outermost gating charges (R1 and R2) cause hypokalaemic periodic paralysis1,2 by creating a pathogenic gating pore in the voltage sensor through which cations leak in the resting state1,4. Mutations of the third gating charge (R3) cause normokalaemic periodic paralysis5 owing to cation leak in both activated and inactivated states6. Here we present high-resolution structures of the model bacterial sodium channel NaAb with the analogous gating-charge mutations7,8, which have similar functional effects as in the human channels. The R2G and R3G mutations have no effect on the backbone structures of the voltage sensor, but they create an aqueous cavity near the hydrophobic constriction site that controls gating charge movement through the voltage sensor. The R3G mutation extends the extracellular aqueous cleft through the entire length of the activated voltage sensor, creating an aqueous path through the membrane only in the resting state. Crystal structures of NaAb(R2G) in complex with guanidinium define a potential drug target site. Molecular dynamics simulations illustrate the mechanism of Na+ permeation through the mutant gating pore in concert with conformational fluctuations of the gating charge R4. Our results reveal pathogenic mechanisms of periodic paralysis at the atomic level and suggest designs of drugs that may prevent ionic leak and provide symptomatic relief from hypokalaemic and normokalaemic periodic paralysis.

Na1.4 channels generate action potentials that initiate muscle contraction9. They are complexes of a pore-forming α-subunit and auxiliary β1 subunits.9–11 The α-subunit contains four homologous domains (I–IV), each with six transmembrane segments (S1–S6). Segments S1–S4 form the voltage sensor, and every third residue in S4 is positively charged. Upon depolarization, S4 moves outward through a narrow gating pore formed by S1–S3, catalysed by interactions with negative or polar residues in S2 and S3.12 The voltage sensor has an hourglass shape, with a narrow hydrophobic constriction site (HCS) that separates extracellular and intracellular compartments.6,11 Water-filled crevices on either side of the HCS focus the membrane electric field, assuring efficient coupling of voltage to conformational changes that open the central pore.12,13 Mutations in the arginine gating charges that occupy the HCS cause state-dependent cation leak through the voltage sensor, which we term ‘gating pore current’.14,15

Missense mutations of arginine gating charges in S4 of Na1.4 cause hypokalaemic periodic paralysis and normokalaemic periodic paralysis1,2,16,17. Mutations of R1 in domains I or III to H or Q, or mutation of R2 in domains I, II and III to W, G, Q or S cause hypokalaemic periodic paralysis1,2,16,17. Mutations of R3 in domain II to G, or Q, or of R3 in domain III to H or C cause normokalaemic periodic paralysis1,2. All these mutations result in non-selective gating pore current through the voltage sensor.2,16–19 Increased inward leak leads to Na+ overload, sustained depolarization and action potential failure, which paralyze skeletal muscles1,16–19. These pathophysiological effects suggest that mutations that cause hypokalaemic periodic paralysis result in an open aqueous pathway for ion movement in the resting state of the voltage sensor, but not in the activated state, and mutations that cause normokalaemic periodic paralysis result in an open aqueous pathway in the activated state, but not in the resting state. Molecular models and mutagenesis studies support this hypothesis20–22. To provide direct structural evidence for this pathophysiological mechanism, we introduced mutations known to cause periodic paralysis into NaAb, a voltage-gated Na+ channel from Arcobacter butzleri, the structure of which has been solved at high resolution.23 We characterized the resulting gating pore currents, solved the structures of mutant gating pores without and with a bound permeant ion, and investigated molecular dynamics20 of ion movement through the gating pores.

To reconstitute pathogenic hypokalaemic periodic paralysis gating pore currents in NaAb, we mutated R2 to S (R2S, analogous to Na1.4(R672S)) and expressed the mutant in Trichopolus ni insect cells. Transfected cells were voltage-clamped to −200 mV and depolarized in 10-mV steps to record Na+ currents. Half-maximal activation of central pore currents was observed at V1/2 = −105 ± 0.6 mV (Fig. 1a). To measure gating pore currents, cells were held at −100 mV, at which NaAb is in the slow-inactivated state and exhibits no central pore current. Gating pore current was examined by applying pulses from +100 to −200 mV in −10 mV steps. A nonlinear leak current component was observed in the resting state, beginning at −110 mV and increasing to −200 mV (Fig. 1b, c).

Mutations of the gating charge R3 that cause normokalaemic periodic paralysis (Na1.4(R675G/Q/W)) induce outward gating pore current in activated but not in resting states.6 In NaAb(R3G), central pore current was activated between −50 mV and 0 mV (Fig. 1d; V1/2 = −24.8 ± 1.1 mV). Steady-state inactivation was observed from −90 mV to −10 mV with half maximal inactivation at Vh = −47.7 ± 0.4 mV (Fig. 1d). NaAb(R3G) conducted outward gating pore current in both activated and inactivated states at potentials more positive than −60 mV (Fig. 1e, f). These physiological studies demonstrate that NaAb provides an accurate model of Na1.4, because gating pore current is observed only in the resting state for NaAb(R2S) and only in the activated and inactivated states for NaAb(R3G).

The pathogenic effects of gating pore mutations depend on inward leak of Na+. The R2S mutant gating pore was not significantly selective among Cs+, K+ or Na+ (Fig. 1g, P > 0.7). As is the case for Na1.4,24 the gating pore of NaAb(R2S) was exceptionally permeant to guanidinium (about 28-fold greater than Na+), but it was less permeant to methylguanidinium and ethylguanidinium (Fig. 1g). The outward gating pore currents conducted by NaAb(R3G) were higher for Cs+ than for K+ or Na+, which were similar to each other (Fig. 1h). However, NaAb(R3G)

---

1Department of Pharmacology, University of Washington, Seattle, WA, USA. 2Molecular Medicine, Hospital for Sick Children Toronto, Toronto, Ontario, Canada. 3Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada. 4Institute for Protein Design, University of Washington, Seattle, WA, USA. 5Howard Hughes Medical Institute, University of Washington, Seattle, WA, USA. 6These authors contributed equally: Daohua Jiang, Tamer M. Gamal El-Din. *e-mail: wcatt@uw.edu; nzheng@uw.edu © 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
was less permeant to guanidinium than to Na\(^+\) (Fig. 1h), and it was more than 16-fold less permeant to guanidinium than Na\(v\) Ab(R2S). The weak selectivity of R2S and R3G mutants for different inorganic cations and the high guanidinium permeability through the R2S mutant are characteristic of the corresponding mutations in Na\(v\)1.4\(^2\), further supporting the validity of Na\(v\) Ab as a model for structural studies of gating pore mutations.

To elucidate the structure of a pathogenic gating pore in its conductive conformation in an activated voltage sensor, we solved the structure of a Na\(v\) Ab analogue of a normokalaemic periodic paralysis-causing mutation, Na\(v\) Ab(R3G), at 2.7 Å resolution (Fig. 2). Voltage-gated sodium channels have a central pore module surrounded by four symmetrically located voltage sensors (Fig. 2a). The voltage sensors of Na\(v\) Ab and Na\(v\)1.4 are very similar in amino acid sequence by four symmetrically located voltage sensors (Fig. 2a). The voltage for half-maximal inactivation).

For Na\(v\) Ab(R3G) (\(V_h = -47.7 \pm 0.4\) mV, \(k = 7.5 \pm 0.3\) (n = 4); \(V_h\), the voltage for half-maximal inactivation), e, f, Gating pore Na\(^+\) currents and \(I-V\) curves for Na\(v\) Ab(R3G) (red) or Na\(v\) Ab(WT) (black) for voltage steps from 0 mV to the indicated potentials. n = 11. g, Gating pore current through Na\(v\) Ab(R2S) for Ca\(^+\) (n = 5), K\(^+\) (n = 7), Na\(^+\) (n = 5), N-methyl-D-glucamine (NMDG, n = 5), guanidinium (G, n = 7), methylguanidinium (MG, n = 5) and ethylguanidinium (EG, n = 5) at -200 mV. ***P = 0.00029. h, Gating pore current through Na\(v\) Ab(R3C) for Cs\(^+\) (n = 4), Na\(^+\) (n = 6), K\(^+\) (n = 6), guanidinium (n = 4) and NMDG (n = 4) at +100 mV. **P = 0.0011. Student’s t-test, two-sided.

Fig. 2 | Structures of the voltage sensor of Na\(v\) Ab(WT) and Na\(v\) Ab(R3G). a, Structure of Na\(v\) Ab(R3G) in top view. b, Comparison of the conformations of Na\(v\) Ab(WT) (grey) and Na\(v\) Ab(R3G) (rainbow) voltage sensor in side view. c–e, Structures of Na\(v\) Ab(WT) voltage sensor. c, Side view highlighting gating charges in sticks. d, Top view in space-filling format. e, MOLEC2 analysis of water-accessible space in magenta. f–h, Structures of Na\(v\) Ab(R3G) voltage sensor. f, Side view highlighting gating charges. g, Top view in space-filling format. h, MOLEC2 analysis of water-filled space in magenta. Green spheres in f and h indicate the positions of the missing side chain of R3. In d and g, the dotted red line circles the position where the gating pore would be in the activated state and the solid red line circles the open gating pore, respectively. See Extended Data Table 1 for details.

Fig. 1 | Functional properties of Na\(v\) Ab(WT), Na\(v\) Ab(R2S) and Na\(v\) Ab(R3G). a, Central pore Na\(^+\) currents (inset) and conductance–voltage (\(G-V\)) curve for Na\(v\) Ab(R2S) during 200-ms depolarizations from -200 mV to the indicated potentials. \(V_h\) (the voltage for half-maximal activation) = -105 ± 0.6 mV; slope factor, \(k = 10.0 \pm 0.9\). n = 4. b, c, Gating pore Na\(^+\) currents (\(I_p\)) and current–voltage (\(I-V\)) curves for Na\(v\) Ab(R2S) (blue) or Na\(v\) Ab(WT) (black) during test pulses from -100 mV to the indicated potentials (filled circles; \(V_h = -24.8 \pm 1.1\) mV, \(k = 9 \pm 1\); n = 4). Voltage dependence of steady-state inactivation (open circles) for Na\(v\) Ab(R3G) (\(V_h = -47.7 \pm 0.4\) mV, \(k = 7.5 \pm 0.3\) (n = 4); \(V_h\), the voltage for half-maximal inactivation)). e, f, Gating pore Na\(^+\) currents and \(I-V\) curves for Na\(v\) Ab(R3G) (red) or Na\(v\) Ab(WT) (black) for voltage steps from 0 mV to the indicated potentials. n = 11. g, Gating pore current through Na\(v\) Ab(R2S) for Ca\(^+\) (n = 5), K\(^+\) (n = 7), Na\(^+\) (n = 5), N-methyl-D-glucamine (NMDG, n = 5), guanidinium (G, n = 7), methylguanidinium (MG, n = 5) and ethylguanidinium (EG, n = 5) at -200 mV. ***P = 0.00029. h, Gating pore current through Na\(v\) Ab(R3C) for Cs\(^+\) (n = 4), Na\(^+\) (n = 6), K\(^+\) (n = 6), guanidinium (n = 4) and NMDG (n = 4) at +100 mV. **P = 0.0011. Student’s t-test, two-sided.
and structure (Extended Data Figs. 1 and 2). The voltage sensors of wild-type (WT) Na<sub>Ab</sub> and Na<sub>Ab(R3G)</sub> crystallize in the same conformation, with a root mean square deviation (r.m.s.d.) of 0.39 Å (Fig. 2b, Extended Data Fig. 3). These results indicate that the R3G mutation does not perturb the overall structure of the voltage sensor and, therefore, that its pathogenic effects are caused by the loss of the R3 side chain. These channels crystallize with an activated voltage sensor<sup>6,7</sup> (Fig. 2c), as would be expected at 0 mV. In Na<sub>Ab(WT)</sub>, R1, R2 and R3 are located extracellularly relative to the HCS, and their side chains point outward, toward the extracellular milieu (Fig. 2c). By contrast, R4 is located intracellularly relative to the HCS and its side chain points inward towards the cytosol (Fig. 2c). When viewed from the extracellular side, there is no water-accessible path into the cell through the wild-type voltage sensor (Fig. 2d); however, we observed a deep solvent-accessible cleft extending down to the R4 side chain in Na<sub>Ab(R3G)</sub> (Fig. 2g).

Analysis of the structure of chain B of Na<sub>Ab(WT)</sub> using the MOLE2 algorithm revealed an incomplete water-accessible path extending part of the way through the voltage sensor from both extracellular and intracellular sides, which is interrupted at the HCS by R3 (Fig. 2e). Strikingly, in Na<sub>Ab(R3G)</sub>, the water-accessible path continues all the way through the voltage sensor, and has a diameter of 2 Å at its narrowest point, similar to the size of Na<sup>+</sup> (Fig. 2h). By contrast, in chain A, R4 was captured in a rotamer conformation in which the arginine side chain partially blocks the inner end of the gating pore in Na<sub>Ab(R3G)</sub> (Extended Data Fig. 4a). Previously reported structures of Na<sub>Ab</sub> in the slow-inactivated state show that R4 adopts four slightly different rotamer conformations, with the most open having a diameter of 3 Å<sup>25</sup> (Extended Data Fig. 4b). These results elucidate the molecular mechanism by which mutations in S4 cause pathogenic gating pore currents and suggest that ion permeation through the gating pore is controlled dynamically by the state of the voltage sensor and by rotamer conformations of R4.

In contrast to voltage-gated sodium-channel mutations that cause normokalaemic periodic paralysis, those that cause hypokalaemic periodic paralysis result in a channel that conducts gating pore current in the resting state but is closed in the activated state (Fig. 1). Therefore, we hypothesized that Na<sub>Ab(R2G)</sub> would not have a continuous water-accessible path through its gating pore in the activated state. Analysis of the 2.9 Å structure of Na<sub>Ab(R2G)</sub> revealed a gap with additional solvent-accessible area in the extracellular aqueous cleft in comparison to the wild-type channel, but no change in the backbone conformation (Fig. 3a, Extended Data Fig. 3). Although the increased opening of the aqueous cleft in the voltage sensor is evident in space-filling models (Fig. 3b), the R3 and R4 side chains seal the voltage sensor in this activated state, interrupting the transmembrane path and preventing ion conductance. The solvent-accessible area penetrates about 21 Å into the membrane from the extracellular side (Fig. 3c), more than 7 Å deeper than in Na<sub>Ab(WT)</sub> (Fig. 2e), but it does not reach the cytosolic side. This structure illustrates why Na<sub>Ab(R2G)</sub> does not conduct gating pore current in the activated state (Fig. 1).

There are no crystal structures of the voltage sensor of a voltage-gated sodium channel in the resting state, because the resting state is only accessible at negative membrane potentials. However, we developed models of three resting states using disulfide locking of substituted cysteine residues and structure prediction with the Rosetta algorithm<sup>26</sup>; these are now considered consensus models of the actual resting states<sup>27,28</sup>. To model an open gating pore with the voltage sensor in the activated state, interrupting the transmembrane path and preventing ion conductance, solvent-accessible volume modelsled with MOLE2. Top view of Na<sub>Ab(R2G)</sub> with one guanidinium bound to each voltage sensor. h, mF<sub{o} </sub>– DF<sub>c</sub> electron density map (blue mesh) of residues around the guanidinium binding site at 1σ. i, Interaction network between guanidinium and amino acids in the voltage sensor of Na<sub>Ab(R2G)</sub>. Grey dashed lines show interatomic distances shorter than 4 Å. See Extended Data Table 1 for details.

blocks the gating pore (Fig. 3f). These structural models illustrate how R2 charge mutations that cause hypokalaemic periodic paralysis result in gating pore current in the resting state.

The Na<sub>Ab(R2S)</sub> mutant channel is much more permeant than Na<sub>Ab(R3G)</sub> to guanidinium ions<sup>24</sup> (Fig. 1). Guanidinium ions are chemically similar to the distal moiety of the arginine side chain, and guanidine compounds with hydrophobic substituents can block mutant gating pores<sup>24</sup>. We probed our gating pore structures for guanidinium-binding sites by soaking crystals of Na<sub>Ab(R2G)</sub> and Na<sub>Ab(R3G)</sub> with guanidinium and methylguanidinium to determine whether they would bind in place of the missing side chain of R2 or R3. The crystal structures did not show guanidinium binding to Na<sub>Ab(R3G)</sub>. However, crystals of Na<sub>Ab(R2G)</sub> soaked with guanidinium or methylguanidinium diffracted to 2.7 Å and 2.5 Å resolution, respectively, and unambiguous electron density was observed in place of each R2 side chain (Fig. 3g–i, Extended Data Fig. 5a, b). Bound guanidinium is clearly seen in 2F<sub{o}</sub> – F<sub>c</sub> maps (Fig. 3h). E32 and M29 from S1, N49 from S2, R1 and R3 from S4, and Q150 from an adjacent subunit form the binding site for guanidinium (Fig. 3i). M29 and R3 each bind guanidinium through hydrogen bonds (Fig. 3h, i). The carbonyl group of E32 and the carbonyl oxygen of R1 further lock guanidinium in place (Fig. 3h, i). The binding site is flanked by hydrogen bonds from N49 and Q150 that stabilize guanidinium from opposite sides (Fig. 3h, i). The binding site for methylguanidinium is almost identical (Extended
Data Fig. 5c, d). These structures capture guanidinium bound at a specific site in the closed R2G gating pore. The amino acid residues responsible for guanidinium binding are highly conserved in Na\textsubscript{Ab}, Na\textsubscript{1.4} and Ca\textsubscript{1.1} (Extended Data Fig. 1). Substituted guanidinium ions can block gating pore current without major effects on Na\textsubscript{1.4} function\textsuperscript{24}, suggesting that guanidinium-containing compounds specific for this binding site could provide a basis for structure-based drug design and be used therapeutically to relieve the symptoms of hypokalaemic periodic paralysis.

To examine relationships among structural fluctuations of the gating pore, ionic hydration and Na\textsuperscript{+} leakage, we performed molecular dynamics simulations of wild-type and R3G mutant voltage sensors in a hydrated lipid bilayer (Fig. 4). Multiple unbiased simulation repeats, with a total duration of 30 μs, show that the overall structures are conserved. Analysis of axial distributions of water molecules revealed a narrow region (−5 Å < z < 5 Å) that is more hydrated in the R3G mutant than in the wild-type voltage sensor, owing to the larger size of the lumen in the mutant (Fig. 4a–c, yellow; P < 0.002, see Extended Data Table 2). The average count of water molecules within the HCS was 3.9 ± 0.8 and 5.3 ± 0.4 for the wild type and R3G mutant, respectively (Fig. 4e). We performed umbrella-sampling simulations to compute the free energy of Na\textsuperscript{+} permeation along the principal axis of the voltage sensor. When Na\textsuperscript{+} was within the HCS, the number of water molecules in the HCS increased to 8.4 ± 0.3 in the wild type and 9.0 ± 0.3 in the R3G mutant, respectively. The free-energy profile for Na\textsuperscript{+} translocation forms a broad barrier spanning the HCS, centred at Co\textsubscript{R} of R3. The R3G mutation significantly decreases the height of this barrier from 18 ± 0.8 to 11 ± 1.4 kcal mol\textsuperscript{−1} (Fig. 4e). These values are consistent with the undetectable gating-pore conductance in the wild type and an upper limit of around 0.1 pS in the R3G mutant\textsuperscript{29}. Analysis of ionic coordination shows that, at the extracellular edge of the barrier, the first solvation shell of Na\textsuperscript{+} is almost exclusively composed of water, consistent with the hydrophobic nature of the bottleneck in the voltage sensor (Fig. 4f). The total coordination number of 5.81 ± 0.02 in bulk water drops to 4.88 ± 0.04 at the peak of the free-energy barrier, suggesting a large desolvation penalty for Na\textsuperscript{+} that is partly alleviated by the cavity created in the absence of the R3 side chain. Charge–charge repulsion is also likely to contribute substantially to the higher energy barrier to Na\textsuperscript{+} permeation in the wild type, impeding the gating pore leakage observed in the R3G mutant.

The location of R4 coincides with a secondary shoulder in the free-energy profiles (Fig. 4d, R108), indicating that movement of Na\textsuperscript{+} past R4 is not rate-limiting for permeation, even though transit of Na\textsuperscript{+} past R4 causes the largest displacement of water by protein ligands (Fig. 4e). Spontaneous disruption of the R4–E59 salt bridge in 3 ± 1% of simulation frames for the wild type and R3G mutant opens the inner end of the gating pore with sufficient frequency to support gating pore current (Fig. 4g–i). Na\textsuperscript{+} often makes direct contacts with the anionic side chains of D80 and E59 (Fig. 4f, g), and its movement is coupled to dynamic rearrangements of the R4 salt-bridge network.

Overall, our results provide an unprecedented high-resolution view of functional effects of ion channel mutations that cause periodic paralysis and define the structural basis for pathogenesis of this ion channelopathy. R2G and R3G mutations do not perturb the backbone structure of the voltage sensor, suggesting that the aberrant gating pore currents are not caused by conformational changes in transmembrane alpha helices. Instead, the absence of the positively charged R2 and R3 side chains opens an aqueous gating pore that allows diffusion of Na\textsuperscript{+} into the cell, depending on the functional state of the voltage sensor. Our structural studies show how this pathogenic gating pore current is gated in resting and activated states by transmembrane movements of the S4 segment. Although our studies of R2G and R3G mutants suggest a straightforward explanation for the pathogenic gating pore current, mutations that cause hypokalaemic periodic paralysis and normokalaemic periodic paralysis that substitute large side chains such as tryptophan also cause gating pore currents\textsuperscript{4,16,17}, perhaps by perturbing...
the local structure of the voltage sensor and thereby opening a pore across the membrane.

Our structures reveal the binding pose of a highly permeant ion, guanidinium, in the closed gating pore of the activated voltage sensor of Na\textsubscript{Ab}(R2G). Substituted guanidinium derivatives can block gating pore current without impairing voltage sensor function in Na\textsubscript{1,4}\textsuperscript{24}. Therefore, our high-resolution structural models may provide molecular templates for design and development of drugs that would mimic guanidinium, block gating pore current and provide symptomatic relief of periodic paralysis.

**Online content**

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0120-4.

Received: 18 December 2017; Accepted: 9 April 2018;
Published online 16 May 2018.

1. Venance, S. L. et al. The primary periodic paralyses: diagnosis, pathogenesis and treatment. *Brain* **129**, 8–17 (2006).
2. Cannon, S. C. Handbook of Experimental Pharmacology (Springer, Berlin, 2017).
3. Sokolov, S., Scheuer, T. & Catterall, W. A. Gating pore current in an inherited ion channelopathy. *Nature* **446**, 76–78 (2007).
4. Struyk, A. F. & Cannon, S. C. A Na\textsuperscript{+} channel mutation linked to hypokalemic periodic paralysis exposes a proton-selective gating pore. *J. Gen. Physiol.*** 130, 11–20 (2007).
5. Vicart, S. et al. New mutations of SCN4A cause a potassium-sensitive normokalemic periodic paralysis. *Neurology* **63**, 2120–2127 (2004).
6. Sokolov, S., Scheuer, T. & Catterall, W. A. Depolarization-activated gating pore current conducted by mutant sodium channels in potassium-sensitive normokalemic periodic paralysis. *Proc. Natl Acad. Sci. USA* **105**, 19980–19985 (2008).
7. Payandeh, J., Scheuer, T., Zheng, N. & Catterall, W. A. Crystal structure of a voltage-gated sodium channel. *Nature* **475**, 353–358 (2011).
8. Catterall, W. A. & Zheng, N. Deciphering voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels by studying prokaryotic ancestors. *Trends Biochem. Sci.* **40**, 526–534 (2015).
9. Catterall, W. A., Goldin, A. L. & Waxman, S. G. International Union of Pharmacology, XLVIII. Nomenclature and structure–function relationships of voltage-gated sodium channels. *Pharmacol. Rev.* **57**, 397–409 (2005).
10. Yan, Z. et al. Structure of the Na\textsubscript{1,4}-I complex from electric eel. *Cell* **170**, 470–482 (2017).
11. Shen, H. et al. Structure of a eukaryotic voltage-gated sodium channel at near-atomic resolution. *Science* **355**, eaal4326 (2017).
12. Catterall, W. A. Ion channel voltage sensors: structure, function, and pathophysiology. *Neuron* **67**, 915–928 (2010).
13. Starace, D. M. & Bezanilla, F. A proton pore in a potassium channel voltage sensor reveals a focused electric field. *Nature* **427**, 548–553 (2004).
14. Sokolov, S., Scheuer, T. & Catterall, W. A. Ion permeation through a voltage-sensitive gating pore in brain sodium channels having voltage sensor mutations. *Neuron* **47**, 183–189 (2005).
15. Gamal El-Din, T. M., Scheuer, T. & Catterall, W. A. Tracking S4 movement by gating pore currents in the bacterial sodium channel NaChBac. *J. Gen. Physiol.* **144**, 147–157 (2014).
16. Jurkat-Rott, K., Groome, J. & Lehmann-Horn, F. Pathophysiological role of omega pore current in channelopathies. *Front. Pharmacol.* **3**, 112 (2012).
17. Moreau, A., Gosselin-Badarouidine, P. & Chahine, M. Biophysics, pathophysiology, and pharmacology of ion channel gating pores. *Front. Pharmacol.* **5**, 53 (2014).
18. Wu, F. et al. A sodium channel knockin mutant (Na\textsubscript{1,4}-R266H) mouse model of hypokalemic periodic paralysis. *J. Clin. Invest.* **121**, 4082–4094 (2011).
19. Wu, F. et al. A calcium channel mutant mouse model of hypokalemic periodic paralysis. *J. Clin. Invest.* **122**, 4580–4591 (2012).
20. Gosselin-Badarouidine, P., Delemotte, L., Moreau, A., Klein, M. L. & Chahine, M. Gating pore currents and the resting state of Na\textsubscript{1,4} voltage sensor domains. *Proc. Natl Acad. Sci. USA* **109**, 19250–19255 (2012).
21. Moreau, A., Gosselin-Badarouidine, P., Boutjdir, M. & Chahine, M. Mutations in the voltage sensors of domains I and II of Na\textsubscript{1,5} that are associated with arrhythmias and dilated cardiomyopathy generate gating pore currents. *Front. Pharmacol.* **6**, 301 (2015).
22. Monteleone, S. et al. Mechanisms responsible for ω-pore currents in Ca\textsubscript{2+} channel voltage-sensing domains. *Biophys. J.* **113**, 1485–1495 (2017).
23. Chakrabarti, N. et al. Catalysis of Na\textsuperscript{+} permeation in the bacterial sodium channel Na\textsubscript{Ab}. *Proc. Natl Acad. Sci. USA* **110**, 11331–11336 (2013).
24. Sokolov, S., Scheuer, T. & Catterall, W. A. Ion permeation and block of the gating pore in the voltage sensor of Na\textsubscript{1,4} channels with hypokalemic periodic paralysis mutations. *J. Gen. Physiol.* **136**, 225–236 (2010).
25. Payandeh, J., Gamal El-Din, T. M., Scheuer, T.; Zheng, N. & Catterall, W. A. Crystal structure of a voltage-gated sodium channel in two potentially inactivated states. *Nature* **486**, 135–139 (2012).
26. Vargas, E. et al. Structural basis for gating charge movement in the voltage sensor of a sodium channel. *Proc. Natl Acad. Sci. USA* **109**, E93–E102 (2012).
27. Vargas, E. et al. An emerging consensus on voltage-dependent gating from computational modeling and molecular dynamics simulations. *J. Gen. Physiol.* **140**, 587–594 (2012).
28. Catterall, W. A., Wisedchaisri, G. & Zheng, N. The chemical basis for electrical signaling. *Nat. Chem. Biol.* **13**, 455–463 (2017).
29. Cooper, K. E., Gates, P. Y. & Eisenberg, R. S. Diffusion theory and discrete rate constants in ion permeation. *J. Membr. Biol.* **106**, 95–105 (1988).

**Acknowledgements** We thank the beamline staff at the Advanced Light Source (BL8.2.1 and BL8.2.2) for assistance during data collection and J. Li for technical and administrative assistance. This research was supported by National Institutes of Health research grants R01 NS015751 (W.A.C.) and R01 HL112808 (W.A.C. and N.Z.), by the Howard Hughes Medical Institute (N.Z.), and by Canadian Institutes of Health Research grant MOP 130461 (R.P.).

**Author contributions** D.J., T.M.G.E.-D., C.I., P.L., R.P., N.Z. and W.A.C. designed the experiments. T.M.G.E.-D., C.I. and P.L. performed the experiments. D.J., T.M.G.E.-D., C.I., P.L., R.P., N.Z. and W.A.C. wrote the paper with input from all co-authors.

**Competing interests** The authors declare no competing interests.

**Additional information** Extended data is available for this paper at https://doi.org/10.1038/s41586-018-0120-4.

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41586-018-0120-4.

**Reprints and permissions information** is available at http://www.nature.com/reprints.

Correspondence and requests for materials should be addressed to N.Z. or W.A.C.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
METHODS

Electrophysiology. All experiments were performed using T. ni insect cells (High Five Cells, Thermofisher). Molecular biology and patch-clamp measurements were performed as described previously\textsuperscript{15,30}. All constructs showed high level expression that enabled us to measure ionic current and gating pore currents 48 h after infection. Whole-cell sodium currents were recorded using an amplifier (Axopatch 200; Molecular Devices) with glass micropipettes (2–4 MΩ). The intracellular pipette solution contained (mM): 35 NaCl, 105 CsF, 10 EGTA and 10 HEPES, pH 7.4 (adjusted with CsOH). The extracellular solution contained (mM): 140 NaCl, 2 CaCl\textsubscript{2}, 1.8 MgCl\textsubscript{2} and 10 HEPES, pH 7.4 (adjusted with NaOH).

For Na\textsubscript{Ab}(R2S), the standard clamp protocol for measuring central pore currents consisted of steps from a holding potential of −200 mV to voltages ranging from −180 to 0 mV in 10 mV steps. For Na\textsubscript{Ab}(R3G), cells were held at −160 mV and 10 mV voltage steps ranging from −140 mV to +50 mV were applied. A P/−10 or P/−4 leak-subtraction protocol was used to subtract linear leak and capacitive currents from holding potentials of −200 or −160 mV, respectively.

To measure gating pore currents in Na\textsubscript{Ab}(R2S), cells were held at −200 mV for ~1 min to allow recovery from slow inactivation. Then, the cells were held at −100 mV for gating pore current measurements, which inactivates the central pore current. Depolarizing pulses in 10-mV steps were applied from −200 mV up to +50 mV. The intracellular pipette solution contained (mM): 140 CsF, 10 EGTA, and 10 HEPES, pH 7.4 (adjusted with CsOH). The extracellular solution contained (mM): 140 NaCl, 2 CaCl\textsubscript{2}, 1.8 MgCl\textsubscript{2} and 10 HEPES, pH 7.4 (adjusted with NaOH). To test gating pore selectivity for different cations, NaCl was replaced by an equimolar concentration of KCl, CsCl, LiCl, NMDG or 40 mM guanidinium sulphate, 100 mM Na-citrate pH 4.8–5.2. Crystals grew to full size in a week. Crystals were concentrated up to 17 mg ml\textsuperscript{−1} from holding potentials of +200 mV and 0 mV for gating pore current measurements, which inactivates 10 mV voltage steps ranging from −100 mV to 100 mV for gating pore current measurements. Because the channel and voltage sensor were initially devoid of water molecules and ions, a protein-restrained equilibration period of 30 ns was used to reduce the systematic sampling bias induced by the initial conditions (10 ns with protein heavy-atom restraints, 10 ns with backbone restraints, and 10 ns with C\textalpha{} restraints, all with a force constant of 2.39 kcal mol\textsuperscript{−1} A\textsuperscript{−2}). Unbiased production simulations of 15 replicates of ‘WT’ and ‘R3G’ systems were conducted for 1,000 ns each, resulting in aggregate sampling of 15 μs for each tetramer (× 4 × 15 μs = 60 μs for WT and R3G voltage sensors).

Simulation snapshots beyond t = 100 ns were extracted from unbiased simulations and used as initial conditions for biased simulations, using the entire tetramer. Umbrella sampling\textsuperscript{61,62} was used to compute the free energy or potential of mean force (PMF) profile for the movement of Na\textsuperscript{+} through voltage sensing domain. The range of the reaction coordinate, −2.0 to 2.0 nm with respect to the centre of the hydrophobic constriction, was discretized into ~130 unevenly spaced windows. For each window, biased simulations were initiated with a water molecule exchanged for Na\textsuperscript{+} in all four voltage sensors. Production simulations were performed for 70–100 ns per window with a harmonic restraining potential force constant of 2.39 kcal mol\textsuperscript{−1} A\textsuperscript{−2} and a flat-bottom cylindrical position restraint for all four Na\textsuperscript{+} ions simultaneously. The axial position of the permeating Na\textsuperscript{+} ion, z, was stored every 10 fs and the data from each of the four voltage sensors were used separately to generate four independent PMF profiles using g_wham\textsuperscript{51}, enforcing cyclic periodicity of the PMF in the bulk (at z = −2.5 nm). The initial 10 ns were excluded from each umbrella sampling run. We report the mean PMF over the four voltage sensors with error bars computed using the standard error of mean over all four PMFs. The total simulation time for each of the two systems (WT and R3G) was ~11 μs, yielding a total of ~45 μs of voltage sensor data.

Water occupancy of the voltage sensor was computed by counting the number of water oxygen atoms within a cylinder of radius 8.0 Å. We define the hydrophobic constriction centre as the geometric centre of C\textalpha{} atoms of residues 22, 57, 84 and 105. The range of the HCS is defined as −5 Å to 5 Å along the axial coordinate of the voltage sensor. Coordination of Na\textsuperscript{+} to channel ligands, water, ions and lipids was performed by computing the number of protein, water and lipid O atoms, as well as Cl\textsuperscript{−} ions, within the first solvation shell of Na\textsuperscript{+} (<3.0 Å). The average coordination number at a given axial position was computed over all simulation frames regardless of the subunit, but the total coordination number in bulk water and at the hydrophobic constriction reported in the text was based on the mean and standard error of mean over the four voltage sensors. Analysis of the trajectories was performed using MDTraj\textsuperscript{39} and molecular renderings were generated using Visual Molecular Dynamics\textsuperscript{55}.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability. Coordinates and structure factors have been deposited in the Protein Data Bank with the following accession numbers: Na\textsubscript{Ab}(R3G), 6C1E; Na\textsubscript{Ab}(R2G)–guanidinium, 6CIK; Na\textsubscript{Ab}(R2G)–methyleneamidinium, 6C1M; Na\textsubscript{Ab}(R2G), 6CI.P.

35 NAC 35 NAC, 12–21 (2010).

60 30. Gamal El-Din, T. M., Martinez, G. Q., Payandeh, J., Scheuer, T. & Catterall, W. A. A letter to Nature 60, 123–221 (2010).

61 31. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystalogr. D Biol. Crystalogr. 66, 213–221 (2010).

62 32. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystalogr. D Biol. Crystalogr. 66, 213–221 (2010).

63 33. Chen, V. B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystalogr. D Biol. Crystalogr. 66, 12–21 (2010).

64 34. Petek, M., Kosinov, P., Kovač, J. & Oyejika, M. MÖLE: a Voronoi diagram-based explorer of molecular channels, pores, and tunnels. Structure 15, 1357–1363 (2007).

© 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
35. Jefferys, E., Sands, Z. A., Shi, J., Sansom, M. S. P. & Fowler, P. W. Alchembed: a computational method for incorporating multiple proteins into complex lipid geometries. J. Chem. Theory Comput. 11, 2743–2754 (2015).
36. MacKerell, A. D. et al. All-atom empirical potential for molecular modeling and dynamics studies of proteins. J. Phys. Chem. B 102, 3586–3616 (1998).
37. Klauda, J. B. et al. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. J. Phys. Chem. B 114, 7830–7843 (2010).
38. Best, R. B. et al. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone $\phi$, $\psi$ and side-chain $\chi_1$ and $\chi_2$ dihedral angles. J. Chem. Theory Comput. 8, 3257–3273 (2012).
39. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. L. Comparison of simple potential functions for simulating liquid water. J. Chem. Phys. 79, 926–935 (1983).
40. Noskov, S. Y. & Roux, B. Control of ion selectivity in LeuT: two Na$^+$ binding sites with two different mechanisms. J. Mol. Biol. 377, 804–818 (2008).
41. Venable, R. M., Luo, Y., Gawrisch, K., Roux, B. & Pastor, R. W. Simulations of anionic lipid membranes: development of interaction-specific ion parameters and validation using NMR data. J. Phys. Chem. B 117, 10183–10192 (2013).
42. Abraham, M. J. et al. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX 1–2, 19–25 (2015).
43. Darden, T., York, D. & Pedersen, L. Particle mesh Ewald: an $N\cdot \log(N)$ method for Ewald sums in large systems. J. Chem. Phys. 98, 10089–10092 (1993).
44. Essmann, U. et al. A smooth particle mesh Ewald method. J. Chem. Phys. 103, 8577–8593 (1995).
45. Verlet, L. Computer ‘experiments’ on classical fluids. I. thermodynamical properties of Lennard–Jones molecules. Phys. Rev. 159, 98–103 (1967).
46. Hoover, W. G. Canonical dynamics: equilibrium phase-space distributions. Phys. Rev. A 31, 1695–1697 (1985).
47. Nosé, S. A molecular dynamics method for simulations in the canonical ensemble. Mol. Phys. 52, 255–268 (1984).
48. Parrinello, M. & Rahman, A. Crystal structure and pair potentials: A molecular-dynamics study. Phys. Rev. Lett. 45, 1196–1199 (1980).
49. Nosé, S. & Klein, M. L. Constant pressure molecular dynamics for molecular systems. Mol. Phys. 50, 1055–1076 (1983).
50. Hess, B. P-LINCS: a parallel linear constraint solver for molecular simulation. J. Chem. Theory Comput. 4, 116–122 (2008).
51. Torrie, G. M. & Valleau, J. P. Nonphysical sampling distributions in Monte Carlo free-energy estimation: umbrella sampling. J. Comput. Phys. 23, 187–199 (1977).
52. Roux, B. The calculation of the potential of mean force using computer simulations. Comput. Phys. Commun. 91, 275–282 (1995).
53. Hub, J. S., de Groot, B. L. & van der Spoel, D. g_wham—a free weighted histogram analysis implementation including robust error and autocorrelation estimates. J. Chem. Theory Comput. 6, 3713–3720 (2010).
54. McGibbon, R. T. et al. MDTraj: a modern open library for the analysis of molecular dynamics trajectories. Biophys. J. 109, 1528–1532 (2015).
55. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. J. Mol. Graph. 14, 33–38 (1996). 27–28.
Extended Data Fig. 1 | Sequence alignment of the voltage sensor of Na,Ab with those of human Na, 1.4 homologous domain (D)II, Na,1.4 DIV, Ca, 1.1 DII and Ca, 1.1 DIV. Coloured rectangles represent transmembrane helices. Black arrows indicate residues that form the guanidinium binding site, blue arrows indicate the hydrophobic constriction site and red arrows indicate the conserved intracellular negative cluster.
Extended Data Fig. 2 | Superposition of the Na$_\text{Ab}$(WT) voltage sensor and the *Electrophorus electricus* (electric eel) Na$_{\text{1.4}}$ DIV voltage sensor. 

a–b, Comparison of the conformations of Na$_\text{Ab}$(WT) voltage sensor (orange) and EcNa$_{\text{1.4}}$ voltage sensor DIV (PDB code: 5XSY) (grey) in side view and top view, respectively. Arginine sensors and hydrophobic residues in the HCS are labelled and shown with side chains in sticks.
Extended Data Fig. 3 | Superposition of the voltage sensors of Na$_v$Ab(WT) and mutant channels. a–b, Voltage sensor structure alignment between Na$_v$Ab(WT) (grey) and Na$_v$Ab(R3G) (green) in side view and top view, respectively. c–d, Voltage sensor structure alignment between Na$_v$Ab(WT) (grey) and Na$_v$Ab(R2G) (cyan) in side view and top view, respectively. Arginine sensors and hydrophobic residues in the HCS are labelled and shown with side chains in sticks.
Extended Data Fig. 4 | R4 side chain conformational changes.
a, Different conformations of the R4 rotamer in Na$_x$Ab(R3G) chain A (green) and chain B (orange). b, Different conformations of the R4 rotamer in the four subunits of Na$_x$Ab in the slow-inactivated state (PDB code: 4EKW).
Extended Data Fig. 5 | Electron density maps for bound guanidinium and methylguanidinium ions. a, 2mFo−DFc electron density map (blue mesh) of residues around the methylguanidinium binding site at 1σ. b, Overlay of guanidinium binding site (green) and methylguanidinium binding site (orange). c–d, Simulated annealing map (Fo−Fc) contoured at 3σ for methylguanidinium and guanidinium, respectively.
Extended Data Fig. 6 | Purification of Na\textsubscript{a}Ab(R3G). a, Representative gel-filtration chromatography of Na\textsubscript{a}Ab(R3G); highlighted peak fractions were concentrated for crystallization. b, Concentrated sample was visualized on SDS–PAGE by Coomassie blue staining.
## Extended Data Table 1  |  Data collection and refinement statistics

|                              | Na<sub>a</sub>Ab/R3G | Na<sub>a</sub>Ab/R2G Guanidinium | Na<sub>a</sub>Ab/R2G Methyl Guanidinium | Na<sub>a</sub>Ab/R2G Apo |
|------------------------------|----------------------|-----------------------------------|----------------------------------------|-------------------------|
| **Data collection**          |                      |                                   |                                        |                         |
| Space group                  | I222                 | I222                              | I222                                   | P2₁₂₂₁                   |
| **Cell dimensions**          |                      |                                   |                                        |                         |
| \(a, b, c\) (Å)              | 126.8, 127.0, 192.3  | 126.6, 126.6, 191.8               | 126.3, 126.2, 191.6                   | 125.5, 125.6, 192.0      |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 90           | 90, 90, 90                        | 90, 90, 90                             | 90, 90, 90               |
| Wavelength (Å)               | 0.99994              | 0.99994                           | 0.99994                                | 0.99994                  |
| Resolution (Å)              | 50.2-90 (3.00-2.90)  | 50.2-70 (2.80-2.70)               | 50.2-5.5 (2.64-2.55)                   | 50.2-80 (2.90-2.80)      |
| Rpim                         | 4.6 (62.6)           | 4.0 (62.0)                        | 3.9 (64.0)                             | 5.3 (58.1)               |
| \(I/\sigma I\)              | 16.6 (1.5)           | 18.5 (1.2)                        | 18.5 (1.0)                             | 14.5 (0.8)               |
| Completeness (%)             | 100 (99.9)           | 99.6 (96.5)                       | 99.4 (95.0)                            | 98.0 (81.6)              |
| Redundancy                   | 7.3 (7.2)            | 7.1 (5.4)                         | 5.3 (3.8)                              | 5.1 (3.2)                |
| **Refinement**               |                      |                                   |                                        |                         |
| Resolution (Å)              | 42.50-2.86           | 42.31-2.70                        | 42.31-2.52                             | 48.46-2.90               |
| No. reflections              | 35059                | 41173                             | 51039                                  | 67766                    |
| \(R_{work}/ R_{free}\)      | 21.25/23.99          | 20.98/24.59                       | 20.31/22.66                            | 23.35/26.03              |
| No. atoms                    |                      |                                   |                                        |                         |
| Protein                      | 3606                 | 3605                              | 3673                                   | 7160                     |
| Ligand/ion                   | 512                  | 449                               | 660                                    | 415                      |
| Water                        | 0                    | 5                                 | 35                                     | 0                        |
| **B-factors**                |                      |                                   |                                        |                         |
| Protein                      | 108.7                | 97.8                              | 103.1                                  | 112.89                   |
| Ligand/ion                   | 128.2                | 107.5                             | 130.8                                  | 115.8                    |
| Water                        | 54.5                 | 75.5                              |                                        |                          |
| R.m.s deviations            |                      |                                   |                                        |                         |
| Bond lengths (Å)             | 0.010                | 0.010                             | 0.009                                  | 0.012                    |
| Bond angles (°)              | 1.311                | 1.215                             | 1.253                                  | 1.703                    |
| Ramachandran plots           |                      |                                   |                                        |                         |
| Favorable                    | 93.2%                | 92.5%                             | 94.0%                                  | 92.1%                    |
| Allowed                      | 6.8%                 | 7.3%                              | 5.4%                                   | 7.4%                     |
| Outliers                     | 0.0%                 | 0.2%                              | 0.6%                                   | 0.5%                     |
## Extended Data Table 2 | Statistical analysis of voltage sensor water occupancy from molecular simulations

| Degrees of freedom | Axial Interval (Å, Å) | t-statistic | q value |
|--------------------|-----------------------|-------------|---------|
| (-20, -19)         | 1.411                 | 2.291E-01  |
| (-19, -18)         | 2.687                 | 1.004E-02  |
| (-18, -17)         | 4.389                 | 7.674E-05  |
| (-17, -16)         | 4.802                 | 1.896E-05  |
| (-16, -15)         | 4.545                 | 4.465E-05  |
| (-15, -14)         | 4.249                 | 1.148E-04  |
| (-14, -13)         | 2.181                 | 5.422E-02  |
| (-13, -12)         | 0.740                 | 5.420E-01  |
| (-12, -11)         | 0.217                 | 8.721E-01  |
| (-11, -10)         | 2.760                 | 1.276E-02  |
| (-10, -9)          | 4.283                 | 1.078E-04  |
| (-9, -8)           | -0.110                | 9.364E-01  |
| (-8, -7)           | 1.914                 | 9.279E-02  |
| (-7, -6)           | -5.668                | 4.626E-07  |
| (-6, -5)           | -5.839                | 2.674E-07  |
| (-5, -4)           | -9.376                | 6.032E-15  |
| (-4, -3)           | -12.075               | 9.500E-21  |
| (-3, -2)           | -11.945               | 9.674E-21  |
| (-2, -1)           | -10.018               | 2.422E-16  |
| (-1, 0)            | -5.812                | 2.674E-07  |
| (0, 1)             | -7.910                | 1.027E-11  |
| (1, 2)             | 1.488                 | 1.993E-01  |
| (2, 3)             | 1.813                 | 1.073E-01  |
| (3, 4)             | 2.797                 | 1.205E-02  |
| (4, 5)             | 5.497                 | 9.078E-07  |
| (5, 6)             | 2.476                 | 2.672E-02  |
| (6, 7)             | -3.688                | 8.593E-04  |
| (7, 8)             | -8.074                | 5.198E-12  |
| (8, 9)             | -3.257                | 3.462E-03  |
| (9, 10)            | -1.302                | 2.522E-01  |
| (10, 11)           | 1.220                 | 2.809E-01  |
| (11, 12)           | 0.482                 | 7.211E-01  |
| (12, 13)           | -2.870                | 1.024E-02  |
| (13, 14)           | 1.919                 | 9.279E-02  |
| (14, 15)           | -0.003                | 9.978E-01  |
| (15, 16)           | 0.887                 | 4.569E-01  |
| (16, 17)           | 1.843                 | 1.044E-01  |
| (17, 18)           | 1.319                 | 2.522E-01  |
| (18, 19)           | 0.263                 | 8.686E-01  |
| (19, 20)           | 0.249                 | 8.686E-01  |

Results of two-way t-tests on differences in average water count in 1 Å segments of the voltage sensor axial coordinate comparing wild-type and R3G mutant simulations. In each segment, we compare the mean of 60 values (n = 60, obtained from pooling the mean water counts of the four voltage sensor proteins from each of 15 simulation repeats). The HCS region (-5 to 1 Å, bold) has the largest effect size, indicating a region of biological significance.
Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a Confirmed
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
  State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection  |  N/A
Data analysis    |  N/A

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data Availability. Coordinates and structure factors have been deposited in the Protein Data Bank: NavAb/R3G, 6C1E; NavAb/R2G Guanidinium, 6C1K; NavAb/R2G Methyl Guanidinium, 6C1M; NavAb/R2G ap, 6C1P. Correspondence and requests for materials or electrophysiology data should be addressed to N.Z. (nzheng@uw.edu) or W.A.C. (wcatt@uw.edu).
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size       | Not applicable |
|-------------------|----------------|
| Data exclusions   | None           |
| Replication       | n values given in Figure Legends |
| Randomization     | Not applicable |
| Blinding          | Not applicable |

Materials & experimental systems

Policy information about availability of materials

n/a | Involved in the study
- Unique materials
- Antibodies
- Eukaryotic cell lines
- Research animals
- Human research participants

Method-specific reporting

n/a | Involved in the study
- ChIP-seq
- Flow cytometry
- Magnetic resonance imaging