Electromechanical coupling in the hyperpolarization-activated $K^+$ channel KAT1

Voltage-gated potassium ($K_v$) channels coordinate electrical signalling and control cell volume by gating in response to membrane depolarization or hyperpolarization. However, although voltage-sensing domains transduce transmembrane electric field changes by a common mechanism involving the outward or inward translocation of gating charges\(^1\)\(^-\)\(^3\), the general determinants of channel gating polarity remain poorly understood\(^4\). Here we suggest a molecular mechanism for electromechanical coupling and gating polarity in non-domain-swapped $K_v$ channels on the basis of the cryo-electron microscopy structure of KAT1, the hyperpolarization-activated $K_v$ channel from *Arabidopsis thaliana*. KAT1 displays a depolarized voltage sensor, which interacts with a closed pore domain directly via two interfaces and indirectly via an intercalated phospholipid. Functional evaluation of KAT1 structure-guided mutants at the sensor–pore interfaces suggests a mechanism in which direct interaction between the sensor and the C-linker hairpin in the adjacent pore subunit is the primary determinant of gating polarity. We suggest that an inward motion of the S4 sensor helix of approximately 5–7 Å can underlie a direct-coupling mechanism, driving a conformational reorientation of the C-linker and ultimately opening the activation gate formed by the S6 intracellular bundle. This direct-coupling mechanism contrasts with allosteric mechanisms proposed for hyperpolarization-activated cyclic nucleotide-gated channels\(^5\), and may represent an unexpected link between depolarization- and hyperpolarization-activated channels.
R1 (R171) and R2 (R174) are positioned above the gasket, whereas R3 (R176), R4 (R177) and R5 (R184) are located below the gasket (Fig. 2c, Extended Data Fig. 5a). This conformation of the KAT1 VSD corresponds to an ‘up’, or depolarized state, which—in the nominal absence of a field (0 mV)—is coupled to a closed pore domain. Limiting-slope analysis in KAT1 has suggested an effective $z_e$ of about 3 $e$ per channel (approximately 0.75 $e$ per sensor) consistent with R2, and possibly R1, serving as the main sensing charges. Accordingly, mutant channels R174Q and R171Q did not yield currents (data not shown). As described below, mutant cycle and metal-bridge data also indicate that the VSD structure corresponds to an up state and point to a number of residue–residue pairs with interactions that probably change on transitioning to the ‘down’ state during membrane hyperpolarization.

KAT1em voltage-sensing and pore domains interact through two major interfaces: the first near the intracellular face of the channel (Fig. 3a, b) with the participation of S4 and S5 overlaying the C-linker of the adjacent subunit, and the second near the extracellular side formed by the intercalation of S1 between S4 and S5 of the same subunit (Fig. 3c). At the first, intracellular interface, the extended length of the KAT1em S4 mediates interactions between S4, S5 and the C-linker. The intracellular ends of the S4 and S5 helices come to rest on top of the C-linker, forming a tightly packed interface. Notably, R310 from the

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**Fig. 1** Function and architecture of A. thaliana KAT1em. **a**, Macroscopic currents of full-length KAT1 and KAT1em, recorded in Xenopus oocytes using a family of hyperpolarizing pulses (top). Data are representative of 11 and 6 biologically independent cells for full-length KAT1 and KAT1em, respectively.

**b**, Sharpened cryo-EM density map of the channel octamer, side view.

**c**, Ribbon model of KAT1em, with domains labelled. Phospholipid is shown in red stick representation.

**d**, Sharpened cryo-EM density map of the channel tetramer, top view (view from extracellular side).

**Fig. 2** KAT1 pore and voltage-sensing domain structure and alanine scanning of pore inner gate region. **a**, View of the pore, with only two subunits shown for clarity. Sticks are shown for selectivity-filter residues, inner-gate-forming residue I292 and functionally important residues L287, T288 and V299. Residues are coloured by effect of alanine mutagenesis (see inset legend).

**b**, Deactivation energies ($\Delta G_{\text{closing}}$) of alanine mutants, calculated from conductance–voltage ($G(V)$) relations (Extended Data Fig. 4b). $\Delta G_{\text{closing}}$ refers to the difference in deactivation energy between a given mutant and wild type. The wild type (WT; $n = 11$) and L287A ($n = 19$), T288A ($n = 4$), L291A ($n = 10$), I292A ($n = 10$), T296A ($n = 10$), V299A ($n = 8$) and H301A ($n = 10$) mutants are shown; $n$ is the number of biologically independent cells. Six mutants did not yield currents: Y290A, N294A, M295A, N297A, L298A, and V300A (data not shown).

**c**, Rotated views of the KAT1em VSD. Stick side chains are shown for the hydrophobic gasket: F102 and V70, for key residues on S4: R165 (R0), R171 (R1), R174 (R2), R176 (R3), R177 (R4) and R184 (R5), and for counter-charges or dipoles: E63, D95, N99, D105 and D141.
The mutations at the intracellular sensor–pore interface might affect the energetics of the sensor, the pore or the coupling between sensor and pore. We consider it likely that at least some of these mutants alter coupling energetics (12 mutants in total, covering the entire S4–S5–C-linker interface) (Fig. 3a, b, Extended Data Fig. 6a, b). However, owing to the technical challenge of monitoring sensor function in KAT1 mutants (by gating currents or fluorescence) we cannot conclusively determine the contributions of each individual residue to sensor–pore coupling. As a partial and preliminary readout of sensor motion and function, we used limiting-slope analyses using macroscopic currents for the two VSD residues that are at the intracellular VSD–pore interface (K187A and D188A) as a way to estimate the amount of charge moved upon channel activation. Compared with the wild-type channel, the D188A mutant moves a similar amount of charge upon activation, despite its left-shifted ionic current activation; therefore, D188A may impair sensor–pore coupling (Extended Data Fig. 7c, d). Low expression levels of K187A prevented robust limiting-slope analyses (data not shown), and it is possible that K187A impairs VSD function rather than coupling. More generally, the result that the majority of KAT1 VSD-pore mutants generate channels with an energetic bias for the closed state over the open state is consistent with the hypothesis that the pore domain of KAT1 is closed by default and the VSD performs work to open the pore at negative potentials. Functional and structural experiments conducted in the isolated pore domain of KAT1 could be used to further test this hypothesis.

Within the plane of the membrane, the VSD and pore of KAT1 are separated by a hydrophobic window. This window is absent in the HCN1 structure, in which S4 and S5 form zipper-like interactions along their length. In contrast to what we observe at the intracellular sensor–pore interface, mutations designed to disrupt this charge to helix-dipole interaction—R310K/Q/N/E—did not yield any currents, despite wild-type-like expression of R310K (Extended Data Fig. 6h, i), supporting a critical role for R310 in channel gating. The rest of the S4–S5–C-linker interaction surface appears to be formed by van der Waals contacts and potential hydrogen bonds between Y193 (in S5) and T306 (in the C-linker) as well as between R197 (in S5) and T303 (in the C-linker).

We carried out extensive mutagenesis on most of the residues that make productive interactions at the intracellular interface (Fig. 3a, b, residues coloured by effect). All mutants that generated measurable currents (K187A, D188A, R190A, F191A, T303A, R307A and R314E) require more energy for channel opening; that is, the midpoints of activation shift towards more negative potentials (Fig. 3a, Extended Data Fig. 6a), with the exception of wild-type-like N192A and R314A. Many mutants (I189A, Y193F/A, F194V/A, R197K/Q/A, K200Q/A, T306A, F309A and R310K/Q/N/E) did not give currents (data not shown). However, when complementary RNAs (crRNAs) encoding various loss-of-function mutants (I189A, R197K, K200Q, T306A and R310K) were individually mixed and co-injected with a gain-of-function double-mutant (Q80A/R177K) crRNA, we observed currents with activation curves separated by a hydrophobic window. This bound phospholipid appears in a conformation that is not observed in other ion channel structures. The head group of this intercalated phospholipid is coordinated by charge—charge and hydrogen-bonding interactions between R197 and K200 on S5 and Y290 on S6 (Fig. 3e). All mutations introduced to the lipid-coordinating residues (R197K/Q/A, K200Q/A and Y290F/A) abrogated currents (data not shown), despite wild-type-like membrane expression of R197K and K200Q mutants (Extended Data Fig. 6h, i), suggesting a structural or functional role for the bound lipid. During a molecular dynamics simulation approximately 3.5 μs in duration, in which a lipidless KAT1 was initially placed in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer, lipid molecules from the bulk stably occupied binding conformations similar to that seen in the cryo-EM structure (Extended Data Fig. 6f, f). KAT1 and other plant plasma membrane K+ channels are strongly modulated by PtdIns(4,5)P2 through an unknown mechanism and the lipid in the hydrophobic window may indicate a binding site of PtdIns(4,5)P2 or some other modulatory lipid. Given the placement of this binding site at the functionally critical S4–S5–S6 interface, the bound lipid may constitute an integral component of the gating machinery. In addition, KAT1 is known to open very slowly: the time constants for gating and ionic currents are separated by approximately three orders of magnitude (time constants of gating current and ionic current activation are approximately 270 μs and 120 ms, respectively). A requirement for lipid binding (Fig. 3e) or reorientation upon gating is a speculative, but testable hypothesis to explain this kinetic disparity.

In contrast to what we observe at the intracellular interface, mutagenic perturbations at the extracellular interface formed by S1, S4 and S5 yielded mixed and nuanced effects on the channel energetics (Extended Data Fig. 6b–d). These mutations led to four distinct phenotypes: nonfunctional channels (F83A, L172A, F207A and C211A), wild-type-like channels (F83A/L, I166A and F215A), and channels that are energetically biased towards the closed state (V178A) or open state (M169A), compared with wild type. Mutations that abrogate ionic currents support the idea that the S1–S4–S5 interface might be important for gating and ionic currents are separated by approximately three orders of magnitude (time constants of gating current and ionic current activation are approximately 270 μs and 120 ms, respectively). A requirement for lipid binding (Fig. 3e) or reorientation upon gating is a speculative, but testable hypothesis to explain this kinetic disparity.

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for channel assembly and stability. However, its role as a major pathway of energy transfer from VSD to pore remains to be defined.

Given the structural and energetic relationship between VSD and pore, in particular the tight packing at the S4–S5–C-linker interface and the severe loss-of-function phenotypes of mutants at this interface, we investigated how KAT1 might open upon membrane hyperpolarization. As a first step towards answering this question, we sought to estimate the extent of the conformational change in the VSD associated with KAT1 opening. We used double-mutant cycle analysis (Fig. 4a–d, Extended Data Fig. 8) to investigate a subset of residue–residue interactions that might change upon hyperpolarization and construct hypothetical down-state models of the VSD, which correspond to the open channel at hyperpolarized potentials. Calculated \( \Delta G_{\text{nonadditive}} \) values greater than 1 kcal mol\(^{-1}\) (ref. 18) are interpreted as a state-dependent interaction between two residues, with negative values of \( \Delta G_{\text{nonadditive}} \) indicating stronger interaction in the down state, and positive values indicating stronger interaction in the up state. In brief, \( \Delta G_{\text{nonadditive}} \) is defined as \( \Delta G_{\text{nonadditive}} = \Delta G_{\text{mut1}} + \Delta G_{\text{mut2}} - \Delta G_{\text{mut1,2}} \), in which \( \Delta G_{\text{mut1}} \) is the difference in deactivation energy between single mutant 1 and wild-type, \( \Delta G_{\text{mut2}} \) is the difference in deactivation energy between single mutant 2 and wild-type, and \( \Delta G_{\text{mut1,2}} \) is the difference in deactivation energy between double mutant 1,2 and wild-type. On the basis of this mutant cycle analysis, we identified two residues on S4, R0 (R165) and V718, each of which exchanges different interaction partners upon VSD activation (Fig. 4a,b, Extended Data Fig. 8). Furthermore, metal bridging experiments point to a cadmium-dependent interaction between R165C (R0 on S4) and C77 (on S1) that promotes channel opening and thus represents an additional down-state interacting pair (Extended Data Fig. 9).

These down-state interacting pairs were then used to construct simplified, hypothetical ‘one-click’ and ‘two-click’ down-state VSD models, in which the S4 helix moves downward by one and two helical turns, respectively, in the context of the isolated KAT1 VSD (Fig. 4c, d). Molecular dynamics simulations were used to calculate the amount of gating charge displaced during these putative transitions, enabling comparison with electrophysiological limiting-slope estimates, which provide a lower bound of around 0.75 e per VSD in KAT1. We obtained 1.02 e for the one-click model and 1.57 e for the two-click model by molecular dynamics simulation (Extended Data Fig. 8c). Our hypothetical models, particularly the one-click model, are consistent with the limiting-state estimate in the literature, the double mutant cycle and metal-bridge constraints, and previous second-site suppressor studies (Extended Data Fig. 8e). Our proposed KAT1 VSD motion would encompass a displacement of approximately 5–7 Å, similar to that proposed for depolarization-activated channels and observations in other VSD structures. Thus, the main question becomes how a canonical downward VSD motion might lead to pore opening in a hyperpolarization-activated channel.

In our gating model, the downward, hyperpolarization-driven movement of S4 is directly coupled to a subsequent lateral reorientation of the C-linker of the neighbouring subunit, ultimately opening the S6 gate (Fig. 4e, f). Although it shares a similar architecture to KAT1, the structure of depolarization-activated EAG1 (also captured with an up-state voltage sensor and closed intracellular gate), shows S4 to be disengaged from the C-linker (Fig. 4g). According to our model, a downward movement of S4 of EAG1 would be unable to trigger channel opening upon membrane hyperpolarization (Fig. 4h), consistent with the depolarization-activated phenotype of EAG1. It is also worth noting that although KAT1 is nominally not domain-swapped, the tight interaction between S4 and the C-linker in its adjacent subunit at rest (0 mV) ultimately leads to a process of activation gating dominated by direct communication between subunits.

Our proposal for a direct coupling mechanism for KAT1 contrasts with the allosterically coupled nature of voltage-sensitive gating reported for HCN channels, where coupling might not be as strong as suggested for KAT1. Supporting this proposal, KAT1, unlike HCN, is not activated by cyclic nucleotides and the structural conformation of the KAT1em pseudo-CNBD is already compatible with an ‘activated’ ligand-binding domain conformation even in the absence of ligand (Extended Data Fig. 4c–g). Therefore, we suggest that KAT1 is perhaps mechanistically closer to a ‘reversed’ depolarization-activated, non-domain-swapped channel such as EAG1 or human ERG, even though KAT1 lacks the cytoplasmic Per-Arnt-Sim domain of EAG1 and human ERG. In view of these results, the present proposal is likely to have direct implications to the mechanism of gating and electromechanical coupling in non-domain-swapped channels such as EAG1 and human ERG, in which electric field transduction (and not nucleotide binding).
represents the sole driving force for channel gating. We anticipate that the KAT1 structure will serve as a framework for future functional and engineering studies of ion channels. Such efforts in plants might hold promise in improving carbon assimilation and optimal biomass production14.

Note added in proof: Since this paper was accepted, two contributions independently addressed electromechanical coupling in HCN channels13,14. These studies establish the structural and energetic underpinnings of the allosteric communication between the voltage sensors and the activation gate of HCN1. Further, they support the present conclusion that highlight the divergence between HCN channels and the direct coupling mechanism suggested here for KAT1.

Online content
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Methods

Molecular biology and biochemistry
A DNA construct encoding amino acids M1–S502 was codon optimized for sf9 expression and synthesized by Integrated DNA Technologies. This gene was subcloned into a modified pFastBac vector containing a C-terminal 3C protease site, eGFP, and His6, using restriction sites 5′ NotI and 3′ XbaI. Baculovirus was generated via the Bac-to-Bac method (Invitrogen). PO virus was amplified once to yield P1 baculovirus, which was used to infect sf9 cells (ATCC CRL-1711) at a 1:50 v/v ratio. Cells were not tested for mycoplasma nor further authenticated. Cells were collected 36–48 h post infection, washed in phosphate-buffered saline pH 7.4, dounce homogenized in hypotonic buffer A (20 mM HEPES, 20 mM KCl, 10 mM MgCl2) and ultracentrifuged. This hypotonic lysis cycle was repeated four times and was subsequently followed by one cycle in hypertonic buffer (buffer A plus 800 mM NaCl).

Membranes were resuspended in 50 mM HEPES pH7.4, 200 mM KCl with 40% glycerol and flash frozen. For purification all steps were performed at 4 °C. Membranes were thawed, diluted with glycerol-free buffer and detergent-extracted in 50 mM HEPES pH 7.4, 200 mM KCl, 1% n-dodecyl-β-d-maltopyranoside (DDM; Anatrace), 0.2% cholesteryl hemisuccinate Tris salt (CHS; Steraloids), asolectin (Sigma, crude) 0.05mg/ml for 90 min. Solubilized supernatant was isolated by ultracentrifugation and diluted with low-detergent buffer to drop DDM/CHS concentration to about 0.5% DDM, 0.1% CHS. Supernatant was batch bound to Cobalt IMAC Talon beads (clontech) for 2–3 h with 5 mM imidazole present. Beads were collected by low-speed centrifugation and washed in batch with 50 mM HEPES pH 7.4, 200 mM KCl, 0.05% DDM (Anatrace), 0.01% CHS (Anatrace), 0.05 mg ml−1 asolectin (Avanti) and 15 mM imidazole. Beads were transferred to plastic column and further washed exchanging stepwise to buffer containing diginton 0.05% (Millipore) and eluted in 50 mM HEPES pH 7.4, 200 mM KCl, 0.05% digitonin and 250 mM imidazole. Protein was cleaved by HRV 3C protease for 3 h, concentrated and subjected to size-exclusion chromatography (SEC) on a Superose 6 column (GE Healthcare) with running buffer: 50 mM HEPES pH 7.4, 200 mM KCl, 0.05% digitonin, 2 mM CaCl2. Peak fractions were collected and concentrated to 4–5 mg ml−1 (Millipore concentrator unit).

Cryo-EM analysis
Quantifoil 200-mesh 1.2/1.3 grids (Quantifoil) were plasma cleansed for 30 s in an air mixture in a Solarus Plasma Cleaner (Gatan). Grids were frozen in liquid nitrogen-cooled liquid ethane in a Vitrobot Mark IV (FEI) using the following parameters: 3.5 μl sample volume, 2.5 s blot time, blot force 3, 100% humidity, at a temperature of 22 °C and double filter papers on each side of the vitrobot.

Grids were screened on a 200 kV Talos side entry microscope (FEI) equipped with K2 summit direct detector (Gatan) using a Gatan 626 single-tilt holder. Replicate grids from the same preparation were shipped to the National Cryo-Electron Microscopy Facility at the National Cancer Institute. Grids were imaged on a Titan Krios with K2 detector (super-resolution mode) and GIF energy filter (set to 20 eV) at a nominal magnification of 130,000 corresponding to a super-resolution pixel size 0.32 Å per pixel. The dose rate was roughly 4.7 e− pixel−1 s−1 and the exposure time was 12 s, yielding a total post-GIF dose of 38–43 e− Å−2. A total of 1,502 movies were collected using Latitude Macroscopic currents were recorded 36–48 h post injection on a two-electrode voltage clamp setup, comprising a OC-720C (Warner Instruments), Digidata 1322A 16 bit digitizer (Axon Instruments) and a Windows XP PC running Clampex10.3. Oocytes were impaled with two 3M KCl-filled Ag/AgCl electrodes with resistances in the range 0.2–1.0 MΩ, in bath containing S0. For each mutant, more than four recordings were obtained, each from a different oocyte. Non-expression of a mutant was determined by absence of tail currents for more than 10 oocytes, and was confirmed in an independent injection session. KAT1 K+ currents were evoked by voltage steps of
was not experimentally observed, the last (most negative) voltage
Faraday’s number. The first derivatives of the raw data (\( \frac{dI}{dV} \))
iseochronal tail current
after the decay of the oocyte linear capacitive response. The conduct-
potential was set to +20 mV or +70 mV in order to measure the full
set at 0 mV except for extremely right-shifted mutants, the holding
1 s, going from 0 to −190 mV in 10-mV steps. The holding potential was
in modelling. The selection of the 1 kcal mol\(^{-1}\) threshold is based on
previous double-mutant-cycle work\(^{39}\).

Limiting-slope analysis of KAT1 channels
The ionic currents were recorded using the cut-open oocyte techni-
que. The extracellular solution contained (in mM) 120 K-MES,
2 CaCl\(_2\), 10 HEPES, pH 7.4. The intracellular solution contained (in mM)
120 K-MES, 2 EGTA, 10 HEPES, pH 7.4. The slow hyperpolarization was
elicited with a voltage ramp from 0 to −100 mV (1 mV s\(^{-1}\)). The inward
current was fitted using cubic spline interpolation, and linear leak-
leak correction was performed offline using a piecewise linear fitting
from the beginning of the curve to the first turning point, obtained from
the second derivative of the curve. Conductance-voltage relations
combinations by dividing the current by the driving force, and the
limiting slope (\( \chi \)) obtained by linear regression to the logarithm of
\( G(V) \) curve constrained by first and second turning points from
the current second derivative. Additional information is provided in
the Supplementary Methods.

Oocyte membrane expression test and confocal imaging
Oocytes for each construct (wildtype, R197K, K200Q, Y290F, and
R310K) were injected as described above. After 48 h, wild-type oocytes
were recorded and confirmed to give 1–2 μA of tail current. Then, 10
oocytes for each construct, as well as 10 uninjected oocytes, were
washed in SOS, mechanically lysed in hypotonic lysis buffer A via
pipette tip aspiration. Lysate was cleared of debris by centrifugation
(10 min, 1,000 g), and the supernatant was isolated and ultracentrifuged
(30 min, 100,000 g). The resulting membrane pellet was resuspended
in 40 μl extrusion buffer (50 mMHEPES pH 7.4, 200 mM KCl, 1.5% DDM,
0.3% CHS), rotated at 4 °C for 90 min and subsequently cleared by
centrifugation (30 min, 12,000 g). Supernatant was then subjected to
SDS–PAGE followed by in-gel GFP imaging using a ChemiDoc Imaging
System (BioRad).

For confocal imaging, oocytes were first injected and expression
confirmed by recording a subset as above. Oocytes submerged in SOS
were placed in a glass bottom dish (MatTek), and imaged in an Olympus
DSU spinning disk confocal microscope using a 10× objective. Regions of
the animal (dark) pole were imaged to avoid intrinsic autofluores-
cence of the vegetal (light) pole. Each sample received identical GFP
channel exposures (5 s) and DIC exposures (47 ns). Images were batch
normalized in SlideBook6 (3i) to allow for a fair comparison between
samples, and GFP images were false-coloured in ImageJ\(^{56}\).

System construction and molecular dynamics simulations
The deposited tetramer model was prepared for molecular dynamics
simulations by using Coot to manually build the missing S3–S4 loop,
and selecting rotamers for stubbed residues to avoid clashes. This
model was then embedded into a POPC lipid bilayer solvated with a
salt solution of 100 mM KCl. The symmetry axis of the protein was
aligned along the z-axis. Three K\(^+\) ions were placed at the selectivity
filter ion binding sites: S0, S2 and S4 of the selectivity filter, separated
by two additional water molecules occupying the binding sites S1 and
S3. The final system was in an electrically neutral state with orthonhomb-
periodic box dimensions of about 126 × 126 × 142 Å\(^3\), consisting of
about 227,000 atoms.

First, the all-atom system of the full channel was energy minimized for
5,000 steps, followed by a 100-ns equilibration simulation with gradu-
ally decreasing harmonic restraints being applied to the protein and
the K\(^+\) ions and the oxygen atoms of water in the selectivity filter. Then,
a further 400-ns simulation was carried out with all restraints being
removed. After this, the equilibrated system was simulated longer,
up to 3 μs, to study the spontaneous binding of lipids to the VSD–pore
interface using the special-purpose supercomputer ANTON2\(^{37}\).
An isolated VSD (residues 50–189) was used to estimate the gating charge, \( \Delta Q \), corresponding to the conformational change of the VSD between different states by calculating the average displacement charge, \( <Q_0> \), of each system. The one-click down and two-click down homology models of the KAT1 VSD were built using the program MOD-ELLER\(^{38}\), by shifting the S4 helix 3 and 6 residues downwards, respectively, from the up-state VSD in the cryo-EM structure, according to the click model of VSD movement proposed from the structural study of Ci-VSD\(^{39}\), which was consistent with the classic helical-screw or sliding helix model.

The up-state VSD was inserted into a pure POPC lipid bilayer and the z-coordinates of the Ca atoms of the two aromatic residues F111 and F155 were used to adjust the position of the VSD along the normal axis of the membrane, which was then solvated in a 100 mM KCl solution. The final neutralized system contained about 31,000 atoms. The one-click down and two-click down systems were constructed by only replacing the up-state VSD protein with the one-click down and the two-click down VSD proteins, respectively. Thus, the three VSD systems had exactly the same size and components, with different protein conformations.

Each VSD system was energy minimized for 5,000 steps and equilibrated for 20 ns with the restraints applied on the protein been gradually decreased from 5 to 0 kcal mol\(^{-1}\) at 0 mV. The equilibrated systems were then simulated at \(-300\) mV, \(-150\) mV, 0 mV, 150 mV, and 300 mV for 50 ns. Snapshots from the last 40-ns trajectories were used to calculate the average displacement charge of each system at different transmembrane voltages, using the partial charge and unwrapped z coordinate of all the atoms. The offset constant between the linearly fitted \(<Q_0>\) of the systems was the gating charge associated with the conformational change between different states.

All the systems were built using the program VMD\(^{40}\) and all the MD simulations other than the ANTON2 simulation were performed with the program NAMD\(^{41}\). The CHARMM36 force field\(^{62,63}\) was used for proteins, phospholipids and ions, and the TIP3P model\(^{64}\) for water molecules. Nose–Hoover thermostat and the semi-isotropic MTK barostat\(^{65,69}\). The temperature and pressure were constrained using the Langevin piston method\(^{103}\), and the van der Waals interaction was smoothly switched off at 10\(^{-12}\) Å. An electric field scaled by cell basis vectors was applied along the z-axis of the membrane, which was then solvated in a 100 mM KCl solution. The final neutralized system contained about 31,000 atoms.

All other data are available upon reasonable request to the corresponding author.
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Author contributions M.D.C. and E.P. conceived the project. M.D.C. performed structural, biochemical and most electrophysiological experiments. G.F.C. performed limiting-slope analyses and processed all electrophysiology data. R.S. constructed down-state models and ran simulations. All authors contributed to manuscript preparation.

Competing interests The authors declare no competing interests.

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Extended Data Fig. 1 | Structural and functional diversity of tetrameric ion channels. a, Two major classes of channels, domain-swapped and non-domain-swapped are distinguished by the relative positions of voltage-sensing and pore domains. b, Solved structures of non-domain-swapped ion channels, two subunits shown for clarity. c, $G(V)$ relations of each channel subclass. d, Gradient depiction of cyclic-nucleotide and voltage sensitivity for subclass members. Figure inspired by ref. 75.
**Extended Data Fig. 2 | KAT1em biochemistry and cryo-EM workflow.**

**a**, SEC of KAT1em purified in digitonin, run on a Superose 6 column. **b**, Stain-free SDS–PAGE of purified KAT1em. SEC and SDS–PAGE results correspond to the preparation used for imaging (d) and are representative of three independent purifications. **c**, SEC of KAT1em in 2N2 nanodiscs (yellow trace), showing putative octamer, tetramer and empty nanodisc. Fluorescence detection SEC of full-length KAT1–GFP (blue trace) showing putative octamer and tetramer. These two samples were not subjected to any cryo-EM experiments, and are included only for the purpose of comparison. **d**, KAT1em cryo-EM workflow. From 1,500 movies, 120,000 particles were picked and subjected to 2D classification, which then yielded 110,000 particles, which were classified in 3D without imposing symmetry (4 coloured classes). Particles from the best two classes (blue and green classes, 91,000 total) were subsequently refined, imposing C4 symmetry, and using successive masks to focus on one of the tetramers and finally on the transmembrane region of one of the tetramers. Additional details are given in Methods.
Extended Data Fig. 3 | Cryo-EM map and model validation. a, ResMap colouring of unfiltered half map of full molecule. b, Same ResMap colouring as a on sharpened full molecule map. c, d, Ninety-degrees-rotated angular-distribution plots for refined full molecule. e, FSC plot for map focused on the transmembrane region. FSC 0.143 criterion is used for resolution determination. f, FSC (map and model) plot from phenix.mtriage, indicating correspondence of tetramer atomic model to transmembrane domain-focused-refined density map. g, Details of sharpened cryo-EM density map are shown with fitted atomic model.
Extended Data Fig. 4 | KAT1em pore domain and pseudo cyclic nucleotide-binding domain. a, Side view of pore, with only two subunits shown for clarity. Permeation pathway is shown in blue, with inner gate radius calculated by MOLE74 (1.4 Å) or HOLE77 (1 Å), inner gate-forming I292 side chains shown as sticks. b, G(V) relations of pore alanine scan. Shaded error regions represent s.d., surrounding the symbols which represent the mean. Wild type (n = 11), L287A (n = 19), T288A (n = 4), L291A (n = 10), I292A (n = 10), T296A (n = 10), V299A (n = 8) and H301A (n = 10) are shown; n is the number of biologically independent cells. c, Overlay of KAT1em pseudo-CNBD (tan) and holoHCN1 CNBD (green, PDB ID: 5U6P). The ligand, HCN1-cAMP is shown as sticks in the cAMP-binding pocket. d, Overlay of KAT1em (tan) and EAG1 (blue, PDB ID: 5K7L). KAT1 lacks the ‘intrinsic ligand’ loop of EAG1. e, Top-down view of KAT1em (tan), holo HCN1 (green) and EAG1 (blue) overlay. Structures were aligned and superimposed on the basis of the transmembrane helices. Only C-linker hairpins are shown for clarity to compare relative rotation of the C-linker to the transmembrane domain for each structure. The relative rotation of the KAT1 C-linker matches that of EAG1 and not HCN1. f, g, Surface electrostatic potential of HCN1 (f) and KAT1 (g), respectively. Ligand-binding pockets are circled in black. KAT1 lacks a deep electropositive (blue) pocket as seen in HCN1.
**Extended Data Fig. 5** | The voltage-sensing domain of KAT1em in the up conformation. 

**a** | Diagram of key VSD features, showing hydrophobic gasket (F102 and V70, yellow) as well as all S4 charges (blue) and distributed countercharges (or counter dipoles) (red). Dashed lines indicate likely interactions in the up conformation.

**b, c** | Overlays of KAT1em (tan) with HCN (green, PDB ID: 5U6O) and Kv1.2/2.1 (pink, PDB ID: 2R9R), respectively, highlighting structural differences between S4 helices. Cα atoms of the positively charged residues of S4 are shown as spheres.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Structural and functional characterization of KAT1 VSD–pore interfaces. a, $G(V)$ relations of S4–S5–C-linker interfacial mutants. Wild type ($n = 11$) and K187A ($n = 8$), D188A ($n = 9$), R190A ($n = 6$), F191A ($n = 12$), N192A ($n = 9$), T303A ($n = 13$), R307A ($n = 14$), R314A ($n = 31$) and R314E ($n = 9$) mutants are shown; $n$ is the number of biologically independent cells. Shaded regions represent s.d. and symbols represent the mean. b, $G(V)$ relations of upper-interface mutants. Wild type ($n = 11$) and F81A ($n = 12$), F81L ($n = 19$), I166A ($n = 18$), M169A ($n = 5$), V178A ($n = 19$) and F215A ($n = 19$) mutants are shown; $n$ is the number of biologically independent cells. Shaded regions represent s.d. and symbols represent the mean. c, Deactivation energies of upper-interface mutants calculated from $G(V)$ relations in b (same sample sizes). d, Mapping of upper-interface functional data (shown in c). Displayed as sticks are key residues on S1: F80, F81, F83, key S4 residues: I166, M169, L172, V178, and key S5 residues: Y193, R197, K200, F207, C211, F215. e, f, Comparison of similar lipid-binding conformations observed in the structure (e) and after about 3.5 μs molecular dynamics simulation (f). g, Cryo-EM density map, with one bound lipid coloured green, contoured at the same contour level as the full map. h, SDS–PAGE and GFP in-gel imaging of Xenopus oocyte membrane fractions, extracted in gentle detergent (Methods). The experiment was performed once and each lane is derived from ten cells. i, Confocal imaging of Xenopus oocyte animal poles expressing various GFP-tagged constructs. Imaging was performed in a single session with normalized exposure times, and each image is representative of five independent oocytes.
Extended Data Fig. 7 See next page for caption.
Extended Data Fig. 7 | Detailed functional characterization of selected VSD–pore interface mutants. a, $G(V)$ relations for cRNA mixing-coinjection experiments. cRNA encoding loss-of-function mutants (I189A, R197K, K200Q, T306A and R310K), for which no currents were observed were selected. These loss-of-function mutant cRNAs were each individually mixed with cRNA encoding a gain-of-function double mutant (Q80A–177K). Data are mean ± s.e.m. Q80A–R177K ($n$ = 12), I189A + Q80A–R177K ($n$ = 7), K200Q + Q80A–R177K ($n$ = 8), R197K + Q80A–R177K ($n$ = 9), R310K + Q80A–R177K ($n$ = 8) and T306A + Q80A–R177K ($n$ = 9) are shown. b, Plot of activation midpoint ($V_m$) of $G(V)$ relations shown in a, c, d. Limiting-slope analyses for wild-type KAT1 (c) and D188A (d). Top, raw currents evoked by voltage ramp protocol. Middle, conductance–voltage relations, with conductance plotted on a log scale. Data points are black, fits are red. Blue vertical lines mark the first and second inflection points of the curve, the region between which was used to calculate limiting-slope ($z$) values (Methods). Wild type, $z = 2.83 ± 0.5$; D188A, $z = 3.28 ± 0.2$. Data are mean ± s.d. Bottom, data (black) and fits (red) on a linear scale. $n$ is the number of biologically independent cells.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | VSD movement during gating. a, Schematic of double-mutant cycle analysis. The difference between $\Delta \Delta G_x$ and the quantity $(\Delta \Delta G_x + \Delta \Delta G_y)$ determines the extent of differential interaction between residues $x$ and $y$ in the up and down states. b, $G(V)$ relations for single and double mutants, illustrating residue–residue pairs displaying additivity (gray) and non-additivity in different directions (green, up-state interaction; red, down-state interaction). Shaded regions represent s.d. and symbols represent the mean. Wild type ($n = 11$) and M64A ($n = 11$), V67A ($n = 33$), C77A ($n = 15$), Q80A ($n = 11$), D95A ($n = 12$), Q149A ($n = 21$), S168A ($n = 17$), V178A ($n = 19$), M64A/V178A ($n = 15$), V67A/Q80A ($n = 13$), V67A/S168A ($n = 16$), V67A/V178A ($n = 10$), C77A/S168A ($n = 14$), Q80A/R165A ($n = 6$), D95A/R165A ($n = 5$) and Q149A/R165A ($n = 14$) mutants are shown; $n$ is the number of biologically independent cells. c, Displacement of charge for the isolated VSD in the up, one-click down and two-click down conformations at different transmembrane potentials. Data are mean ± s.d. calculated using the last 40-ns snapshots ($n = 4,000$) of 50-ns trajectories. Each system was simulated once at each chosen potential. The gating charge was then calculated as the offset constant between the linear fits, resulting in a gating charge of $1.02 \, e$ and $0.55 \, e$ between the up and one-click down, and one-click down and two-click down states, respectively. d, Mapping of double-mutant cycle constraints onto up VSD structure. Thick red and green lines connect Cα carbons of interacting pairs. Thin grey lines connect negative-control pairs. e, Mapping of literature KAT1 down-state interacting pairs onto up structure. Thick red lines connect Cα carbons of interacting pairs.
Extended Data Fig. 9 | A cysteine–Cd$^{2+}$–cysteine bridge in the KAT1 VSD promotes channel opening. a, Raw current traces for all four combinations of C77(S) and R165(C). On washing with 100 μM CdCl$_2$, current increases only in the C77/R165C condition (red box, middle), and then decreases again upon EDTA wash. Representative data are shown from the same oocyte, and each experiment was repeated five independent times (five biologically independent oocytes) with similar results. b, Pulse protocol used during experiment. c, Mapping of C77 (on S1) and R165 (on S4) onto the up VSD structure of KAT1. Ca atoms are indicated by a red line.
Extended Data Table 1 | Cryo-EM data collection, refinement and validation statistics

|                         | KAT1em TMD (EMDB-21018) (PDB 6V1X) | KAT1em Full (EMDB-21019) (PDB 6V1Y) |
|-------------------------|-----------------------------------|------------------------------------|
| **Data collection and processing** |                                   |                                    |
| Magnification           | 130,000                           | 130,000                            |
| Voltage (kV)            | 300                               | 300                                |
| Electron exposure (e-/Å²) | 50                                | 50                                 |
| Defocus range (µm)      | -1 to -2.5                        | -1 to -2.5                         |
| Pixel size (Å)          | 0.532                             | 0.532                              |
| Symmetry imposed        | C4                                | C4                                 |
| Initial particle images (no.) | 124,211                          | 124,211                            |
| Final particle images (no.) | 91689                           | 91689                              |
| Map resolution (Å)      | 3.5                               | 3.8                                |
| FSC threshold           | 0.143                             | 0.143                              |
| Map resolution range (Å) | Not determined                    | ~3.5- 4.5 (ResMap)                |
| **Refinement**          |                                   |                                    |
| Initial model used (PDB code) | *de novo*                      | *de novo*                          |
| Model resolution (Å)    | 3.5                               | 3.71                               |
| FSC threshold           | 0.143                             | 0.143                              |
| Model resolution range (Å) | n/a                              | n/a                                |
| Map sharpening B factor (Å²) | -134                             | -137                               |
| Model composition       |                                   |                                    |
| Non-hydrogen atoms      | 13996                             | 27392                              |
| Protein residues        | 1784                              | 3568                               |
| Ligands                 | 8                                 | 16                                 |
| B factors (Å²)          |                                   |                                    |
| Protein                 | 49.0                              | 49.0                               |
| Ligand                  | 9.2                               | 9.2                                |
| R.m.s. deviations       |                                   |                                    |
| Bond lengths (Å)        | 0.0104                            | 0.0104                             |
| Bond angles (°)         | 1.35                              | 1.35                               |
| **Validation**          |                                   |                                    |
| MolProbity score        | 1.70                              | 1.70                               |
| Clashscore              | 3.64                              | 3.68                               |
| Poor rotamers (%)       | 0                                 | 0                                  |
| Ramachandran plot       |                                   |                                    |
| Favored (%)             | 90.27                             | 90.27                              |
| Allowed (%)             | 9.50                              | 9.50                               |
| Disallowed (%)          | 0.23                              | 0.23                               |
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on 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 statistical parameters 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

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- CryoEM data collection: Gatan Latitude
- Electrophysiological data collection: Clampex 10.3

Data analysis
- CryoEM data analysis: motioncor2, Cffind4, Relion2, ResMap
- Model building and analysis: Swiss-model web server, Chainsaw, Coot, Phenix, UCSF Chimera, Chimera X, Segger, MOLE, Hole
- Molecular Dynamics simulations: VMD, NAMD
- Electrophysiological data analysis: Clampfit 10.3, in-house python scripts

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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

Cryo-EM density maps of KAT1 have been deposited in the Electron Microscopy Data Bank under accession codes EMDB-XXXX (full molecule) and EMDB-YYYY (transmembrane-focused refinement). The atomic models of the KAT1 tetramer and octamer have been deposited in the Protein Data Bank under accession code ZZZZ and WWWW, respectively. All other data are available upon reasonable request to the corresponding author.
Life sciences study design

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

**Sample size**
No statistical methods were used to predetermine sample size. For cryoEM experiments, particle number was chosen to maximize map quality and resolution. For electrophysiology experiments, cell number was chosen based on convention in the field (at least 4). This was deemed to be sufficient to determine mean and standard error of the mean, allowing for comparison between different mutants.

**Data exclusions**
For cryoEM experiments, particles were excluded if they did not improve map quality. This is standard practice for cryoEM structure determination. For electrophysiological experiments, recordings were excluded from analysis if leak or endogenous currents prevented analysis. A record was determined to be an outlier, and thus excluded, if the V_h was more than 10 mV (approximately two standard deviations) outside the mean of the normalized ensemble, or if the z was more than two standard deviations outside the mean of the normalized ensemble. This is standard practice in electrophysiology.

**Replication**
Structure determination was completed once, as is standard. All electrophysiological results contain data from multiple cells, ensuring reproducibility. For mutants that failed to yield currents, at least 10 cells were measured, and the results were confirmed in a separate session of injection and recording. This is standard practice in electrophysiology.

**Randomization**
Randomization was not employed, as is standard for structural and electrophysiological work.

**Blinding**
Blinding was not employed, as is standard for structural and electrophysiological work.

**Reporting for specific materials, systems and methods**

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

#### Eukaryotic cell lines

Policy information about [cell lines](#)

| Cell line source(s) | Sf9 (ATCC CRL-1711) |
|---------------------|---------------------|
| Authentication      | none                |
| Mycoplasma contamination | not tested          |
| Commonly misidentified lines (See ICLAC register) | not used |

#### Animals and other organisms

Policy information about [studies involving animals](#), **ARRIVE guidelines** recommended for reporting animal research

| Laboratory animals | Xenopus oocytes |
|--------------------|----------------|
| **Wild animals**     | not used                         |
|---------------------|----------------------------------|
| **Field-collected samples** | not used                         |
| **Ethics oversight**    | University of Chicago Institutional Animal Care and Use Committee, animal usage protocol 71475 |

Note that full information on the approval of the study protocol must also be provided in the manuscript.