A constricted opening in Kir channels does not impede potassium conduction

Katrina A. Black1,2,9, Sitong He3,9, Ruitao Jin3,9, David M. Miller1,2,9, Jani R. Bolla4, Oliver B. Clarke5, Paul Johnson6, Monique Windley7, Christopher J. Burns8,2, Adam P. Hill7, Derek Laver6, Carol V. Robinson4, Brian J. Smith3 & Jacqueline M. Gulbis1,2

The canonical mechanistic model explaining potassium channel gating is of a conformational change that alternately dilates and constricts a collar-like intracellular entrance to the pore. It is based on the premise that K$^+$ ions maintain a complete hydration shell while passing between the transmembrane cavity and cytosol, which must be accommodated. To put the canonical model to the test, we locked the conformation of a Kir K$^+$ channel to prevent widening of the narrow collar. Unexpectedly, conduction was unimpaired in the locked channels. In parallel, we employed all-atom molecular dynamics to simulate K$^+$ ions moving along the conduction pathway between the lower cavity and cytosol. During simulations, the constriction did not significantly widen. Instead, transient loss of some water molecules facilitated K$^+$ permeation through the collar. The low free energy barrier to partial dehydration in the absence of conformational change indicates Kir channels are not gated by the canonical mechanism.
Potassium channels propagate electrical signals, maintain membrane potentials and facilitate numerous downstream cellular processes. To control ion permeation, K⁺ channels switch between activated, resting and unresponsive (inactivated) states. The reversible transition distinguishing the conducting and non-conducting states of the pore has been explained by a conformational change causing an intracellular opening to the conduction pathway to expand sufficiently to accommodate fully hydrated K⁺ ions. The inward rectifier K⁺ (Kir) channels are important in brain and cardiac function and are notable for their ability to limit K⁺ efflux from cells. Upon membrane depolarization, linear polyamines (e.g. spermine) released from a shallow cation-binding site in the cytoplasmic assembly of the channel penetrate deeply into the pore and stem, or rectify, outward K⁺ flow in a process known as open-channel block. Structures of Kir potassium channels superimpose over the entire transmembrane pore, whether prokaryotic17,18, eukaryotic19,20 or chimeric21. All feature an hourglass-like constriction at the intracellular opening, leading to the widespread conclusion that the canonical permeation gate at the helix bundle crossing is particularly narrow, it rapidly tapers at either end, delineated by crystallography or single-particle cryo-EM. The opening is not wide enough to accept a fully hydrated K⁺ ion, but is sufficient to accept an extended linear polyamine. The pore conformation is not significantly influenced by crystal packing forces; in almost all Kir structures, the lattice contacts between adjacent proteins are mediated by the intracellular domains, while the transmembrane domains are solubilized in detergent micelles and make minimal interactions with neighboring molecules.

Structures of Kir channels in association with potentiating ligands do not exhibit the increase in the width of the conduction pathway in a conducting channel predicted by the canonical model. In crystal structures of Kir2.2 with the activating ligand phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) bound, the pore remains as narrow as in the apo-structure. The Kir3.2 (GIRK2, the KCNJ6 gene product) channel co-crystallized with an activating Gβγ protein complex has an intracellular opening only incrementally wider than other Kir channels. ATP and ADP-bound structures of K ATP (a Kir6.2/sulfonylurea–receptor complex) determined by single-particle cryo-EM reveal equally narrow pore conformations. Moreover, although single S129R and double S129R/S205L mutants of KirBac3.1 show subtle divergence of the inner helix backbone, the intracellular opening, delineated by a 6 Å distance between nitrogen atoms of opposing arginine guanidiniums within the conduction pathway, is no wider than wild type.

The lack of certainty circumscribing gating mechanism in Kir channels prompted us to investigate the requirement for, and degree of, conformational change accompanying gating and conduction. We carried out molecular dynamics to evaluate the free-energy cost incurred by ions passing from the pore cavity through a constriction to the intracellular solution. In parallel, we tested whether KirBac3.1 channels, with their inner helices constrained together by covalent linkages encircling the intracellular mouth, retain the ability to function. Inter-subunit disulfide bonds and chemical cysteine-crosslinkers were employed to afford some latitude in the tightness of crosslinking. We rationalized that loss of function would only result if conduction was contingent on pore widening, and that the use of different crosslinkers or pore blockers might be useful to gauge the approximate opening size required for K⁺ to pass. In parallel, we employed all-atom molecular dynamics to determine the free-energy barrier that would be faced by K⁺ passing through a tight constriction. Crosslinking the pores did not prevent K⁺ conduction but did prevent entry of a narrow blocker of Kir channels, spermine, which has a cross-sectional width between that of the ionic and hydrated diameters of K⁺. Thus, data presented here indicate that inward rectifiers (Kir) are able to conduct without requiring the substantial conformational changes predicted by the canonical gating mechanism. Instead, each K⁺ transiently loses water from its hydration shell as it passes through. On the basis of these findings, we conclude that the fundamental mechanism of controlling currents in Kir channels, in some cases triggered by partner proteins, is presently unknown.

Results

A 2.0 Å crystal structure delineates the conduction pathway. The molecular architecture of Kir channels has been described previously. A near atomic resolution crystal structure (2.0 Å) of wild-type KirBac3.1 (Supplementary Table 1 and Supplementary Fig. 1) was determined, providing an accurate starting model for simulations. KirBac3.1 adopts the canonical fold of a K⁺ channel, with inner and outer membrane-spanning helices supporting an inclined, shorter, pore helix that supports the signature selectivity filter (Fig. 1a). Although the internal cavity is not particularly narrow, it rapidly tapers at either end, delineated by Tyr132 and Thr96 (Fig. 1b, c).

MD simulations address free-energy barriers to permeation. To determine the energetic barrier that would be encountered by K⁺ ions passing through the constriction located at Tyr132 in the inner helix bundle crossing, we adapted a molecular dynamics (MD) method previously employed to determine the free-energy landscape for gramicidin A. Steered molecular dynamics simulations, in which K⁺ ions are moved from the transmembrane cavity to the cytosol, were carried out on the pore of KirBac3.1 (residues 33–138 of the 2.0 Å structure) embedded in a POPC membrane separating saline reservoirs. Initially, K⁺ ions were included in the selectivity filter and internal cavity (upper and lower sites), along with a surfeit of water molecules in the cavity. In addition, a homology model was prepared in which cysteine substitutions at residues 129 and 135 formed cystine bridges between adjacent inner helices, encircling the conduction path at Tyr132; after minimization, this model was subject to simulation protocols, as for wild type. Umbrella sampling simulations (Supplementary Fig. 2 and Supplementary Table 2) were performed on structures extracted from a 1-dimensional pulling trajectory along the molecular axis, and a weighted histogram analysis method (WHAM) used to calculate the potential of mean force (PMF) experienced by each ion. Detailed methods are provided in the Supplementary Information.

Over the duration of the simulations the narrowing of the shallow intracellular opening did not prevent K⁺ passing through. The simulations report free-energy barriers at the Tyr132 collar of 6 kJ mol⁻¹ for wild type and 1 kJ mol⁻¹ for the disulfide-linked mutant. These values fall within the range expected for thermal fluctuations and are too low to impair K⁺ flux (Fig. 2a, b).

The hydration shell of K⁺ ions is transiently depleted. Physical factors that determine the size of aperture through which an ion can pass are its coordination number, which defines the molecular volume, and its propensity to release and exchange ligands. In the selectivity filter, each K⁺ rapidly exchanges between four eight-coordinate binding sites, whereas in solution a predicted coordination number of six to seven is consistent with observation. Notably, theoretical ab initio quantum mechanical calculations concur with the findings of diffraction and spectroscopic studies in coming to the conclusion that in solution K⁺ is coordinated by two subpopulations of water: four tightly and two loosely bound water molecules.
To evaluate the hydration state of $\text{K}^+$ during conduction, coordination numbers were determined from sampling data as a function of linear distance from the selectivity filter along the conduction pathway by enumerating the number of oxygen atoms within 3.0 Å of each $\text{K}^+$. Within the pore cavity, $\text{K}^+$ diffuses with a coordination number of approximately six. A fleeting reduction in the number of solvating water molecules to three or four occurs as the ion passes through the collar of tyrosine side chains but is almost immediately replenished. Transient interactions between $\text{K}^+$ and the Tyr132-hydroxyl groups occur after the ions pass the low energetic barrier near the center of the tyrosine aromatic ring. The total coordination number at each point along the $z$ axis, including the Tyr-OH interactions, is presented (Fig. 2c, d and Supplementary Fig. 3). Potassium ions exhibit a slightly higher coordination number in wild type than in the disulfide-linked S129C-A135C mutant, but the mutant shows a small relative gain in hydroxyl coordination; this may be due to geometric constraints imposed on the Tyr132 side chain by the disulfide links. The retention of a kernel of water molecules means the free-energy barrier to permeation at the constriction is not prohibitive (Fig. 2e–g).

To detect any relationship between $\text{K}^+$ penetration and the size and shape of the aperture at the Tyr132 collar, small fluctuations in the internuclear distances between diagonally opposed Tyr132-hydroxyl oxygens were statistically analyzed (Supplementary Fig. 4). All 11 structures extracted at the precise instant individual
K\(^+\) ions pass through the Tyr132 collar were compared to the entirety of the data (Supplementary Fig. 4). In all cases, the ions permeated mildly asymmetric Tyr132 collars with openings inadequate to accommodate fully hydrated ions (Supplementary Fig. 5). The mean values for the shorter and longer Tyr132-OH diagonals of the 11 structures correspond to van der Waals (vdW) spacings of 4.0 and 6.0 Å (Supplementary Fig. 6), smaller than the time-averaged hydration diameter of K\(^+\) of 6.6 Å\(^3\). The conformation of the pore is constrained by crosslinking. To covalently constrain the dimensions of the hourglass-like constriction, paired cysteine point mutants were introduced into KirBac3.1 upon a cysteine-less background, in which each of the three native cysteine residues were substituted for serine or valine (C262S, C71V, C119V). This allowed us to covalently link the four inner helices into a tetramer without obstructing the conduction pathway. Two mutants (A133C-T136C and S129C-F135C) were selected for study.

**Fig. 2** Molecular dynamics simulations indicate that K\(^+\) passes through the Tyr132 collar in a partially hydrated state. Wild-type panels are shown in a, c, and e, and a disulfide-linked mutant in b, d, and f. a The potential of mean force along the axis of the wild-type structure is shown as a function of the distance between a K\(^+\) cavity ion ‘pulled’ along the conduction pathway (\(z\) axis) and the center of mass of the four Thr96 side chains (the global reaction coordinate). SEM are depicted as vertical gray bars and represent \(n=200\) bootstraps. The Tyr132 side chain position from \(C{\beta}\) (~20 Å) to OH (~25 Å) is indicated by dotted lines. b A comparable plot for disulfide-linked S129C-F135C. c Normalized histograms enumerate the number of oxygen atoms coordinating K\(^+\) as a function of distance from Thr96. Data are accumulated from umbrella sampling simulations of wild-type KirBac3.1. Each bin represents the number of oxygen atoms within 3.0 Å of K\(^+\), expressed as a percentage. d Histograms for disulfide-linked S129C-F135C. e Potential of mean force (PMF) for the wild-type channel is depicted as a function of the distance along the molecular axis from Thr96 and the number of coordinating ligands per ion. f PMF for disulfide-linked S129C-F135C. Energy units are kJ mol\(^{-1}\). g Selected structures from simulations revealing K\(^+\) ions in a partially dehydrated state as they pass Tyr132. Potassium ions are represented as green spheres and water depicted in red and white.
F135C) were constructed and utilized for structure determination and functional experiments (Fig. 3a).

Following purification of A133C-T136C and S129C-F135C, covalent linkages between adjacent subunits were generated, either by direct disulfide formation using Cu²⁺ as the oxidant or by the crosslinking reagents 1,1-methanediyl bismethanethiosulfonate (MTS-1-MTS), which forms compact linkages (C=S–S–CH₂–CH₂–S–S⁻) with slightly more tolerance than direct disulfides (C=S–S⁻), and dibromobimane, a rigid bicyclic molecule. In all, six unique mutant-crosslinker combinations were constructed.

Crosslink formation was validated by denaturing polyacrylamide gel electrophoresis, native mass spectrometry and X-ray crystallography. A change in migration on SDS-PAGE signified formation of covalent links (Fig. 3b, c). Native mass spectrometry demonstrated that disulfide-bonded and crosslinked tetramers fail to dissociate in the gas phase under conditions that release wild-type subunits (Fig. 3d). Recorded masses were consistent with tetrameric species with four crosslinks in each (Supplementary Table 3). Mass peaks were not observed for monomeric, dimeric or trimeric species, while incomplete crosslinking was excluded by the difference between calculated and observed masses. Native mass spectrometry verified the presence of covalent tetramers in all six variants.

To visually inspect the channels for intact crosslinks or unanticipated effects on structure, crystal structures of two of the KirBac3.1 adducts, S129C-F135C-MTS-1-MTS and A133C-T136C-bimane (Supplementary Table 1) were determined. In both mutants, four crosslinks encircle the conduction pathway at the Tyr132 as anticipated (Fig. 3e). Both revealed electron density bridging between adjacent inner helices, consistent with the predicted covalent linkages (Fig. 3f). An overlay of both crosslinked structures onto the native pore is illustrated (Fig. 3g).

Crosslinked Kir mutants conduct K⁺ currents. KirBac3.1 and fully crosslinked mutants were reconstituted into liposomes and a well-established fluorimetric liposomal flux assay was used to measure activity. ACMA-based assays are essentially qualitative, but are adaptable to different ions and have been used to verify measure activity. ACMA-based assays are essentially qualitative, but are adaptable to different ions and have been used to verify measure activity. ACMA-based assays are essentially qualitative, but are adaptable to different ions and have been used to verify measure activity. ACMA-based assays are essentially qualitative, but are adaptable to different ions and have been used to verify measure activity. ACMA-based assays are essentially qualitative, but are adaptable to different ions and have been used to verify measure activity. ACMA-based assays are essentially qualitative, but are adaptable to different ions and have been used to verify measure activity.

K⁺/Na⁺ to Na⁺/N-methyl-D-glucamine (NMDG⁺) (i = inside buffer; o = outside buffer) and a less specific ionophore, monensin, substituted for valinomycin. Baseline fluorescence (i.e. the same as empty liposomes) was observed in the Na⁺/NMDG⁺/o experiment on wild-type KirBac3.1, consistent with a K⁺ selective pore. To validate that the K⁺ conductance arises specifically from Kir K⁺ channels, a classical Kir channel blocker (spermine) was added to inner and outer solutions of proteoliposomes reconstituted with wild-type KirBac3.1; this abolished signal, indicating the K⁺ flux occurred via KirBac3.1.

The cysteine-pair mutants were assayed under identical experimental conditions to wild type. All eight variants of the S129C-F135C or A133C-T136C mutants, either with reduced cysteine sulfhydryl moieties or as covalently-linked adducts with inner helices tethered at Tyr132, mediated K⁺ movement out of liposomes, with no significant difference in signal from wild type in any of the crosslinked populations (Fig. 4c), indicating that crosslinking did not prevent K⁺ flux through the Kir channels. The differences in rate (and overall signal) between crosslinked and reduced forms of the pore are not significant, which indicates the enforced constriction is not rate limiting. The rates (s⁻¹) and experimental time constants (tau) have been included as Supplementary Table 5.

Although ACMA assays do not replicate the fast physiological rates of ion channel function, published titration studies demonstrate their internal consistency. The rates calculated from our experimental curves of KirBac3.1 fall in the mid-range of rates reported for Kir3.2 titrations under very similar conditions, indicating that in the context of the ACMA assay, the rates measured for KirBac3.1 fall into a physiologically relevant range.

Disulfide-locked pores are too narrow for spermine entry. To gauge the degree of width restriction afforded by disulfide linkages, and hence the relative plasticity at the Tyr132 collar, we utilized an open-channel blocker specific to Kir channels (spermine) and compared its binding in native and disulfide-linked pores using two methods, MD and functional assay. Spermine reversibly blocks activated Kir channels in a voltage-dependent manner, entering from the cytosol and occluding the conduction pathway. The response to membrane depolarization is conceptually similar to the outward motion of conserved arginine side chains in the voltage sensors of Kᵥ channels. A crystal structure of KirBac3.1 with spermine bound shows the extended polyanion in the cavity with its tail end protruding into the cytoplasm through a snug opening at the Tyr132 collar. In the presence of spermine, the opening is ~1 Å wider than in wild type, the incremental expansion being induced by spermine entry.

To determine whether spermine was able to bind and block conduction under the assay conditions, the polyanion was applied to proteoliposomes containing disulfide-linked A133C-T136C. In contrast to the inhibitory effect of spermine on wild-type KirBac3.1, spermine failed to inhibit K⁺ conduction through the disulfide-linked pore, indicating that it struggled to penetrate the Tyr132 collar to reach its blocking site within the conduction pathway (Fig. 5a). This suggests the narrowest (transverse) dimension of spermine is wider than the disulfide-linked cytoplasmic opening. It also indicates that the permeating K⁺ species is only partially hydrated, as fully hydrated K⁺ is bulkier than spermine (Fig. 5b).

Steered MD and umbrella sampling simulations were employed to estimate the free-energy required to move intracellular spermine into the pore cavity. Figure 5c plots the position of a central methylene carbon, C8, of spermine, as it moves from the cytosol into the cavity, against the PMF. The
Fig. 3 Crosslinking around Tyr132 forms covalent tetramers and limits inner helix movement. a Sites of the paired cysteine mutations relative to Tyr132, with Cα shown as spheres. Sites of 129 and 135 are colored orange, and 133 and 136 shown in green. For clarity, only two subunits are depicted. b In the presence of crosslinker, the cysteine-pair mutant S129C-F135C MTS-1-MTS migrates on SDS-PAGE as a tetramer, whereas reduced S129C-F135C migrates as a monomer. Both samples are of reconstituted proteoliposomes and are representative of at least \( n = 20 \) gels. c All six mutant-crosslinker combinations migrate as tetramers and are representative of at least \( n = 10 \) gels. d Native mass spectrometry using collision-induced dissociation in the gas phase results in loss of signal for wild type and reduced cysteine-pair mutants, but not for covalently crosslinked mutants. Spectra for wild-type (upper) and dibromobimane-linked A133C-T136C (lower). e Comparative cross-sectional slices through crystal structures at the Tyr132 collar. Only the inner helices are shown. Crosslinkers are depicted as yellow sticks and K\(^+\) ions as deep cyan spheres. f Electron density for the crosslinkers is present at all subunit interfaces of S129C-F135C (MTS-1-MTS) and A133C-T136C (dibromobimane). Left panel shows a 3.1 Å composite omit map (\( 2|F_o| - |F_c| \)) contoured at 1.5 \( \sigma \) and showing strong density for the bridging S-CH\(_2\)-S moiety. Right panel shows a simple 4.1 Å \( 2|F_o| - |F_c| \) omit map contoured at 1 \( \sigma \) (brown mesh), overlaid with an unsharpened \( |F_o| - |F_c| \) omit map contoured at 3 \( \sigma \) (purple mesh) revealing the bimane moiety. g Superposition of Cα traces of crosslinked structures S129C-F135C MTS-1-MTS (cyan) and A133C-T136C DBB (purple) onto the native pore structure (black). Two subunits have been omitted for clarity in the left-hand view.
resultant PMF values indicate a significantly greater energetic barrier to spermine at the Tyr132 collar in disulfide-linked A133C-T136C than in wild type (unconstrained), with respective maxima at site 2 of 15 kJ mol\(^{-1}\) for wild type and 25 kJ mol\(^{-1}\) for the disulfide-linked mutant. The 10 kJ mol\(^{-1}\) difference between them infers ~50-fold lower probability of the spermine passing the disulfide-linked constriction of the cysteine-pair mutant relative to the unconstrained channel, whereas 15 kJ mol\(^{-1}\) difference at site 3 approximates a 400-fold lower probability of it reaching that site. The approximate differences in PMF at positions 1 to 4 are 5, 10, 15 and 45 kJ mol\(^{-1}\), respectively, with the disulfide-linked mutant always the higher of the two.

In wild-type channels at zero field, the energetic barrier of 15 kJ mol\(^{-1}\) faced by spermine as its leading amine N1 passes the Tyr132 collar (Fig. 5c; 1->2) is the same as that experienced by N2 passes the tyrosine collar (3->4). At sites 1 and 3, and prior to spermine engaging the channel, the energy is approximately equal. In contrast, in the disulfide-locked pore, there is a significant barrier at site 4 above the PMF at site 3, 40–45 kJ mol\(^{-1}\), preventing spermine from penetrating much beyond the barrier at Tyr132. The MD-predicted differences in free-energy of spermine binding at serial sites between wild-type and disulfide-linked A133C-T136C KirBac3.1 accord with the experimental assay results that spermine blocks wild type but not disulfide-linked channels.

Probability density profiles corresponding to the position of spermine in the transmembrane cavity at zero field, and 25 and 50 mV nm\(^{-1}\) (Fig. 5d) illustrate the impact of the 15 kJ mol\(^{-1}\) barrier to spermine penetration in wild-type channels at different field strengths. At zero field, there is only a small probability that spermine traverses the tyrosine collar and reaches site 3, whereas at 25 and 50 mV nm\(^{-1}\) progressively more spermine penetrates past the constriction at Tyr132. The calculations indicate that the probability of spermine penetrating further into the pore increases with the field strength applied, in accord with the known voltage-dependence of polyamine block\(^{16}\). Sites 1 and 3 exhibit near equivalence in energy under these simulation conditions (noting that ions are absent from the upper cavity).

**Discussion**

Although the conventional view that gating entails switching from a narrow to a wide conformation has proved challenging to verify, studies on BK\(^{44,45}\) and K2P\(^{46,47}\) channels have indicated that conformational change of K\(^+\) channel pores during gating is not universal. Underpinning the canonical gating model is the idea that the intracellular opening to the pore must be sufficiently wide to accommodate a flow of hydrated K\(^+\) ions during conduction. Data presented here suggest that the natural limiting width for permeation is closer to the ionic diameter of K\(^+\) and that the requirement for substantial conformational change...
within the pore is obviated by the propensity of K\(^{+}\) for ligand exchange, analogous to carbonyl exchange in the selectivity filter. The MD simulations on KirBac3.1 indicate that the coordination shell of K\(^{+}\) is fleetingly depleted as it passes through an intra-cellular opening that, whereas smaller than the diameter of a hydrated K\(^{+}\) ion, is sufficient to accommodate partially hydrated ions. We verified this in functional assays where the width of the opening was covalently constrained, finding that this did not prevent K\(^{+}\) passing through and demonstrating that the constriction is ineffective at stemming K\(^{+}\) flux. To verify the covalent bonds linking subunits together were effective at preventing pore dilation at the collar, we carried out spermine block experiments on the disulphide-linked (most tightly constricted) mutant, A133C-T136C. The inability of spermine to block the crosslinked, but not the reduced, mutant verifies the covalent disulphide linkages are effective at preventing relative movement of the inner helices.

**Fig. 5 Spermine enters wild type but not disulphide-linked pores.**

- **a** Summary of potassium flux experiments investigating spermine block in wild-type and disulphide-linked A133C-T136C channels. Data are shown as mean ± SEM (n = 4 (channel-free, wild type)) or 3 (A133C-T136C disulphide, A133C-T136C reduced experiments); details of replicates are in Supplementary Table 4. Each black or white circle represents the mean of an individual experiment.
- **b** Comparative dimensions of permeant cations (K\(^{+}\) is depicted in deep cyan, oxygen in magenta, carbon in yellow and nitrogen in blue). The PMF represents the energetic barrier to spermine block. Positions 1 to 4 refer to the positioning of a middle carbon (C8) of spermine relative to the pore—shown in the schematic below. **c** The voltage-dependency of spermine block is illustrated by the trend in probability density of spermine entering the pore at field strengths of 0, 25 and 50 mV nm\(^{-1}\). The shaded region of each panel corresponds to 80% of structures in the MD simulations, shown relative to positions 1-4. The distance of C8 relative to the center of mass of Thr96 is annotated for major peaks (red). In the region 18.0-27.5 Å, the occupancy sequentially changes from ~25 to 40-50% with increasing field (0-25 to 50 mV nm\(^{-1}\)). The field-free distribution of spermine in the cavity predicts only a small probability of spermine occupancy at position 4.
Potassium conduction through K+-selective channels requires multiple transfers between coordination shells. As K+ enters the selectivity filter its hydration shell is exchanged for peptide carboxyl oxygen atoms; these are serially exchanged as it transfers between the four binding sites and to then be replaced by water molecules as K+ exits the selectivity filter. Although ion dehydration represents a significant contribution to the free-energy barriers governing ion permeation, it cannot be considered in isolation; dipole moments, environmental permittivity (dielectric) and the energetics of hydration within a protein cavity of limited size will differ from the energetics in the selectivity filter or the hydrogen-bonded network of bulk water. The large ionic radius of K+ relative to other biological cations means its hydration shell is less strongly bound and its relative dehydration energy lower. The energetic costs incurred by transient partial dehydration may be offset by favorable interactions with the protein, including dipoles in the polar environment. Work on gramicidin A indicates that K+ can travel several Angstroms through very narrow pores. The gramicidin A pore accommodates water in single file across the lipid bilayer, and one-dimensional PMF simulations indicate K+ can pass through with only two coordinating waters, the positive charge stabilized by the alignment of dipoles from water and polar groups of the protein. In contrast, the distance through the hourglass-like constriction at Tyr132 of KirBac3.1 is very short, as in human Kir2 channels, affording ions almost simultaneous access to water molecules on either side of the opening (Fig. 6 and Supplementary Movie 1) to replenish their hydration shell as they pass through. A recent study demonstrating that K+ favors cation–π over cation–water interactions suggests that in KirBac3.1 (and in all human Kir3 and Kir6 subfamily members) a weakly bound hydration shell and the aromatic rings at the constriction are particularly favorable for partial dehydration.

Our data demonstrate that, in Kir channels, control over ion flux is not explained by the canonical pore-gating model of conformational change and that, although some K+ channels conduct through very wide pores, the width is not essential; the limiting aperture for conduction is effectively set by the ionic diameter of K+ (<3 Å). Interestingly, a recent study noted spontaneous transition of K+ from bulk to cavity through a narrow aperture, which is also inconsistent with the constriction acting as a gate. There are several possible alternatives to the canonical model. In BK45 and K2P47 channels, the idea that K+ can pass through with only two coordinating waters, the positive charge stabilized by the alignment of dipoles from water and polar groups of the protein. The distance through the hourglass-like constriction at Tyr132 of KirBac3.1 is very short, as in human Kir2 channels, affording ions almost simultaneous access to water molecules on either side of the opening (Fig. 6 and Supplementary Movie 1) to replenish their hydration shell as they pass through. A recent study demonstrating that K+ favors cation–π over cation–water interactions suggests that in KirBac3.1 (and in all human Kir3 and Kir6 subfamily members) a weakly bound hydration shell and the aromatic rings at the constriction are particularly favorable for partial dehydration.

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**Methods**

**Experimental objectives and study design.** The objectives of the study were to investigate (and cross validate) the conformational requirements of gating and conduction in inward rectifier potassium channels. Molecular dynamics were employed to evaluate the conformational changes and free-energy barriers to ions passing along the conduction pathway of KirBac3.1. Structure analysis and native mass spectrometry were used to validate recombiant purified mutant channels in which the dimensions of the pore were constrained by covalent linkages. The function of crosslinked mutants reconstituted into liposomes was evaluated by an established fluorimetric assay.

**Materials.** Detergents were purchased from Anatrace, lipids from Avanti Polar Lipids and all other reagents, unless otherwise stated, from Sigma Aldrich. Kir-bac3.1 mutant cDNA was purchased from DNA2.0. Chromatography columns and resins are from GE Healthcare.

**Protein preparation.** Kirbac3.1 was expressed in C41(DE3) (Lucigen, Cat# 60442) or LEM021(DE3) (New England Biolabs, Cat# C2528E) E. coli at 18°C for 18 hr, or at 16°C for 72 hr, respectively. Cells were harvested and resuspended in 20 mM TRIS pH 8.0 and 130 mM KCl containing EDTA-free Complete Protease Inhibitor (Roche) followed by lysis by two passages through a Stansted homogenizer (Stansted Power) at 100 MPA. Membranes were harvested by centrifugation at 265,000×g for 40 min at 4°C and the protein solubilized in 100 ml of 20 mM TRIS pH 8.0, 150 mM KCl, 1 mM PMSF and 1% (w/v) 3,12-Anzergent for 45 min. Insoluble material was removed by centrifugation. Imidazole to 20 mM was added to the supernatant, which was loaded on a 5 ml IMAC HiTrap column (GE) charged with Co2+. Protein was eluted with a linear gradient of imidazole, up to 500 mM, in buffer containing 20 mM TRIS pH 8.0, 500 mM KCl, 0.1 mM PMSF and 0.05% (w/v) dodecylmaltoside (DDM). Fractions containing KirBac3.1 were concentrated in a 100 kDa molecular weight cutoff centrifugal concentrator (Millipore) and applied to a Superdex 200 10/30 column (GE) equilibrated with 20 mM TRIS pH 8.0, 150 mM KCl, 0.02% (w/v) DDM and 0.5 mM TCEP. Fractions corresponding to the KirBac3.1 tetramer were concentrated with a 100 kDa centrifugal concentrator (Millipore) to between 10 and 40 mg ml−1, snap-frozen in liquid N2 and stored at −80°C as single use aliquots.

**Crosslinking.** KirBac3.1 mutants were diluted to 0.03–4 mg ml−1 (depending on the crosslinker) in crosslinking buffer (20 mM TRIS pH 8.0, 150 mM KCl and 0.02% (w/v) DDM) and crosslinked by addition of MTS-1,MTS, dibromobimane or CuSO4 to 0.5 mM, 2.5 mM and 1 mM, respectively, followed by incubation at room temperature for 30 min. EDTA was added to 5 mM to the CuSO4 crosslinking reaction and MMTS to 100 mM to the MTS-1,MTS and dibromobimane reactions. Excess crosslinking reagents were removed with Micro Bio-Spin 6 columns (BioRad) equilibrated in crosslinking buffer. The crosslinked tetramer was isolated from higher molecular weight species by size exclusion chromatography with a Superdex 200 10/30 column (GE) equilibrated with crosslinking buffer. Fractions corresponding the crosslinked KirBac3.1 tetramer were recovered and concentrated with a 100 kDa centrifugal concentrator (Millipore) to between 10 and 20 mg ml−1, snap-frozen in liquid N2 and stored at −80°C as single use aliquots, until required.

**Cryocrstallization and data collection.** KirBac3.1 and crosslinked KirBac3.1 mutants at a concentration of 2.3 to 4 mg ml−1 were crystallized in sitting drops by vapor diffusion at the Bio21 C3 crystallization facility in Parkville against 3.5 M ammonium acetate, 0.1 M pH 6.5 TRIS and 100 mM KCl, followed by crystallization at 10% PEG 300, 30% PEG 400, 20% PEG 600, 15% PEG 300, 15% PEG 600, 0% PEG 1000, 5% PEG 1000, 10% PEG 2000, 1% PEG 1000 and 0% PEG 1000 (w/v) DDM and 0.5 mM TCEP. Fractions corresponding the crosslinked KirBac3.1 tetramer were concentrated with a 100 kDa centrifugal concentrator (Millipore) to between 10 and 40 mg ml−1, snap-frozen in liquid N2 and stored at −80°C as single use aliquots, until required.

**Crystallographic and data collection.** KirBac3.1 and crosslinked KirBac3.1 mutants at a concentration of 2.3 to 4 mg ml−1 were crystallized in sitting drops by vapor diffusion at the Bio21 C3 crystallization facility in Parkville against 2.5% PEG 4k, 0% PEG 8k–17% PEG 400:90 mM KCl, 100 mM PEG 8k, 0.1 M pH 7.5 TRIS, 50 mM EDTA or 10 mM CaCl2, for reduced KirBac3.1, 33% (v/v) PEG 400: 0.1 M MES, pH 6.5; 4% (v/v) ethylene glycol; 0.1 M NaCl for dibromobimane crosslinked KirBac3.1 A133C-TRIS6C and 13% (v/w) PEG MME 2000: 0.1 M TRIS-HCl; pH 7.1; 0.1 M CaCl2 and 1% (w/v) LDAO for MTS-1-MTS crosslinked KirBac3.1 S129C-F135C. crystals were cryoprotected prior to data collection by cryoprotecting at 265 μl MTAO pH 8.0 to 2 M. All crystallographic data were collected at beamline MX2 at the Australian Synchrotron using Blu-Ice.

**Structure determination and refinement.** Crystallographic data were processed and scaled using HKL2000 and processed and scaled using XDS and reduced using the CCP4 suite. Electron density in all structures was phased by molecular replacement using Phaser as implemented in the CCP4i suite. A molecular replacement model for the wild-type structure was derived from previous protein data bank entries from our group and the 2.0 Å refined model (wild type) reported.
**Fig. 6 Schematic representation of the findings.**

a. A hydrated K\(^+\) (cyan sphere) within the wild-type Kir pore has a hydration shell of 6–7 water molecules (red and yellow sticks). b. As the pore narrows to a bottleneck, the ion partially dehydrates by shedding 3–4 water molecules. c. The hydration shell of K\(^+\) is replenished from the other side of the opening. d. A spermine molecule penetrates into the cavity of the wild-type channel, preventing outward ion conduction. e. When the Tyr132 collar is disulfide-linked, f. K\(^+\) permeates but spermine cannot enter the conduction pathway.

**Structural comparison.** Superposition of the S129C-F135C-MTS-1-MTS structure onto a 2.0 Å native KirBac3.1 structure shows a close fit of the polypeptide backbone of the entire assembly; the RMS deviation calculated for coordinate displacement of ~1136 Ca positions (residues 12–295) is 1.1 Å, whereas for the pore alone (residues 33–136) it is 0.6 Å. The fit of the A133C-T136C-bimane structure, in which a rigid pyrazole-based linkage connects residue 133 on one subunit to residue 136 on an adjacent, to the 2.0 Å native shows minimal distortion of either pore or cytoplasmic assembly (which were superimposed separately because of a relative interdomain ‘twist’ rotation\(^{38}\)). The RMS deviation calculated for coordinate displacement over the transmembrane pore (residues 33–136) is 0.6 Å and for the cytoplasmic assembly (residues 137–295) is 1.1 Å.

**Molecular dynamics.** The 2.0 Å crystal structure of native KirBac3.1 (PDB 6O9U) was used as an initial model for simulations. The disulfide-bonded model was derived from this template by introducing cysteine substitutions at residues 129 and 135 or 133 and 136 of each subunit via homology modeling using MODELLER v9.14 software\(^{65}\). The whole simulation system, including the formation of disulfide bonds between adjacent inner helices, was prepared using the CHARMM-GUI Membrane Builder server\(^{66}\), with N- and C-terminal residues patched as standard termini. Transmembrane pores (residues 33–138) of native and disulfide-linked channels were separately embedded within a standard POPC bilayer, each containing 162 lipids, using the replacement method. In addition, each starting model contained four potassium ions: three sites within the selectivity filter (S1, S3, S4) and one in the internal cavity (near the selectivity filter). One additional potassium ion or spermine molecule was placed in the lower cavity for potassium or spermine calculations respectively. Spermine was placed into channel cavity at the same position observed in structure 2WLK. Twenty-nine water molecules were also placed in the cavity for potassium calculations and 35 for spermine calculations. The simulation cells comprised ~60,000 atoms, of which approximately 30,000 were water molecules, in a box of dimensions 8.2 × 8.2 × 8.8 nm\(^3\). TIP3P water parameters were used in order to solvate all systems and all ionizable residues were assumed to be in their dominant protonation states at pH 7. Sufficient K\(^+\) and Cl\(^-\) ions were introduced by replacement of water molecules to bring the systems to an electrically neutral state at an ionic strength of 0.15 M. The principal axis of the pore was aligned along the z axis.

All simulations were performed using the GPU-accelerated GROMACS software package (version 2018.3)\(^{65}\) and CHARMM36m force field\(^{66}\). CMAP corrections were applied for all components; all atoms including hydrogen atoms were included. To obtain the equilibrated channel-ion-solvent-membrane complex, an elongated CHARMM-GUI Membrane Builder preparation protocol was followed. Steepest descent energy minimization was followed by six sequential steps of equilibration with a gradual decrease in the restraining force applied to different...
components. After 101.5 ns NVT and NPT equilibrations, the resulting structures were utilized in steered molecular dynamics (SMD). The LINCS algorithm was applied. 

KirBac liposome reconstitution. Lipids (50% 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine:POPC, 5% 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-i-serrine:POPS, 45% total) were dissolved in chloroform:methanol (65:35), dried to a thin film under \( N_2 \); and residual solvent removed by lyophilization overnight. Water was added to a final lipid concentration of 25 mg ml\(^{-1}\) followed by incubation at 37 °C for 1 h and 5 freeze/thaw cycles. An equal volume of 2x inside buffer (IB) was added to give a final buffer composition during 20 mM Tris-HCl pH 8.5 and 150 mM KCl following by extrusion to 400 nm (two rounds of 11 passages through the filter) in a mini-extruder (Avanti Polar Lipids) preheated to 65 °C. In some experiments, NaCl was substituted for KCl.

Liposomal fluorometric assay. Fluorimetric assays were performed with 24 h of Kirbac reconstitution. Each 5 μl aliquot of prepared liposomes was diluted to a final volume of 110 μl in inside buffer (OB: 20 mM Tris-HCl pH 8.5 and 150 mM NaCl), resulting in a final buffer composition of 20 mM Tris-HCl pH 8.5, 143.2 mM NaCl and 6.8 mM KCl. In some experiments, the outer buffer (OB) contained 150 mM NMDG\(^+\) instead of NaCl. The pH sensitive dye 9-amino- chloro-2-methoxyacridine (ACMA) was added to a final concentration of 1 μM from a freshly prepared 200 μM stock in 100% (v/v) ethanol. Fluorescence emission at 483 nm (excitation at 419 nm) was monitored over time with path lengths of 2 and 10 mm for excitation and emission, respectively. Baseline fluorescence was monitored for 3 min before the proton specific ionophore carbonyl cyanide m-chlorophenylhydrazine (CCC (5 mM), (t > 35) the K\(^{+}\) specific ionophore was added to 20 mM from a freshly prepared 2 μM stock made up in 100% (v/v) ethanol. The assay was allowed to reach a final electrochemical equilibrium over a further 20 minutes. In some experiments, the K\(^{+}\) channel blocker spermine was added to a final concentration of 500/1000 μM in IB/OB before the establishment of the fluorescence baseline. Control liposomes were prepared identically to proteoliposomes with the exception of the addition of an equal volume of crossing buffer in place of protein. All fluorescence experiments were normalized to the fluorescence measured at \( t = 0 \), which represents maximal fluorescence (100%); and the fluorescence measured after the addition of valinomycin, which represents the minimal fluorescence where all liposomes have equilibrated (0%). A first-order exponential decay was fitted from \( t = 0 \) to \( t = 35 \) to determine the change in fluorescence due to K\(^{+}\) influx through reconstituted Kirbac channels.

Mass spectrometry. Protein samples were buffer exchanged into 200 mM ammonium acetate pH 8.0, 0.5% C8E4 using Biospin-6 (BioRad) columns prior to mass spectrometry analyses and were directly introduced into the mass spectrometer using gold-coated capillary needles prepared in-house. Data were collected on both a modified quadrupole-time-of-flight (Q-ToF) mass spectrometer (Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer 72) (Thermo Fisher Scientific Inc., Bremen, Germany) optimized for analysis of high mass complexes. The instrument parameters used for data collection on Q-ToF were: capillary voltage 1.55 kV, cone voltage 200 V, extractor 5 V, collision voltage 400 V, back pressure 5.6 mbar and argon collision gas pressure 0.2 MPa, and 1500 Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer 72). Data were collected in negative mode, using the Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer 72 (Thermo Fisher Scientific Inc., Bremen, Germany) optimized for analysis of high mass complexes. The instrument parameters used for data collection on Q-ToF were: capillary voltage 1.55 kV, cone voltage 200 V, extractor 5 V, collision voltage 400 V, back pressure 5.6 mbar and 2 μm argon collision gas pressure 0.2 MPa, and 1500 Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer 72). Data were collected in negative mode, using the Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer 72).
Liposomal flux assay. Each liposomal flux experiment was performed across at least three independent reconstitution experiments. Each independent measurement was the result of averaging between two and six technical replicates. To assess significance, a two-sided Dunnett’s multiple comparisons test was carried out to compare each experimental condition to either channel-free liposomes or wild-type KirBac1.1 proteoliposomes. Significance was determined by a family-wide threshold of 0.05, with the P-value for each comparison adjusted for multiplicity. For each experimental condition, the number of independent (and constitutive technical) replicates and P-values as determined by the Dunnett’s multiple comparisons are summarized in Supplementary Table 3.

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

Data availability. Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file. Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession codes PDB 6O9V, PDB 6O9U, PDB 60WV. Source data underlying Figs. 4c and 5a are provided as a Source Data file.

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References

1. Sun, C. et al. Association study between inwardly rectifying potassium channels 2.1 and 4.1 and autism spectrum disorders. Life Sci. 213, 183–189 (2018).

2. Sicca, F. et al. Gain-of-function defects of astrocytic Kir4.1 channels in children with autism spectrum disorders and epilepsy. Sci. Rep. 6, 34325 (2016).

3. Sicca, F. et al. Autism with seizures and intellectual disability: possible causative role of gain-of-function of the inwardly-rectifying K+ channel Kir1.1. Neurobiol. Dis. 43, 239–247 (2011).

4. Haj-Yasein, N. N. et al. Evidence that compromised K+ channel expression is associated with autism spectrum disorders in children with autism spectrum disorders and epilepsy. (2016).

5. Katz, B. Les constantes electriques de la membrane du muscle. Arch. Sci. Physiol. 3, 285–299 (1949).

6. Ashcroft, F. M., Heiny, J. A. & Vergara, J. Inward rectification in the transverse tubular system of frog skeletal muscle studied with potentiometric dyes. J. Physiol. 359, 269–291 (1985).

7. Ficker, E., Taglialeta, M., Wible, B. A., Henley, C. M. & Brown, A. M. Spermine and spermidine as gating molecules for inward rectifier K+ channels. Science 266, 1068–1072 (1994).

8. Lopatin, A. N., Makhina, E. N. & Nichols, C. G. Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. Nature 372, 366–369 (1994).

9. Kuo, A. A. & Gregory, K. J. Crystal structure of the potassium channel KirBac1.1 in the closed state. Science 300, 1922–1926 (2003).

10. Clarke, O. B. et al. Domain reorientation and rotation of an intracellular assembly regulate conduction in Kir potassium channels. Cell 141, 1018–1029 (2010).

11. Wang, M. R. & Mackinnon, R. Crystal structure of the mammalian GIRK2 K+ channel and gating regulation by G proteins, IP3, and calcium. Cell 147, 209–228 (2011).

12. Hansen, S. B., Yao, X. & Mackinnon, R. Structural basis of IP32 activation of the classical inward rectifier K+ channel Kir2.2. Nature 477, 495–498 (2011).

13. Nishida, M., Cadene, M., Chait, B. T. & Mackinnon, R. Crystal structure of a K+ channel prokaryotic Kir channel chimera. EMBO J. 26, 4005–4015 (2007).

14. Huang, C. L., Feng, S. & Hilgemann, D. W. Direct activation of inward rectifier potassium channels by IP3 and its stabilization by Gbg. Nature 391, 803–806 (1998).

15. Wickman, K. D. et al. Recombinant G protein-beta gamma subunits activate the muscarinic-gated atrial potassium channel. Nature 368, 255–257 (1994).

16. Krapivinskaia, G. et al. G protein binding to GIRK4 subunit is critical for G protein-gated K+ channel activation. J. Biol. Chem. 273, 16946–16952 (1998).

17. Whorton, M. R. & Mackinnon, R. X-ray structure of the mammalian GIRK2-βG protein complex. Nature 498, 190–197 (2013).

18. Lee, K. P. K., Chen, J. & Mackinnon, R. Molecular structure of human KATP in complex with ATP and ADP. Elife 6, e23481 (2017).

19. Bavo, V. N. et al. Structure of KirBac potassium channel with an open bundle crossing indicates a mechanism of channel gating. Nat. Struct. Mol. Biol. 19, 158–163 (2012).

20. Zübeck, E. et al. Control of KirBac1.1 potassium channel gating at the interface between cytoplasmic domains. J. Biol. Chem. 289, 143–151 (2014).

21. Allen, T. W., Andersen, O. S. & Roux, B. Energetics of ion conduction through the gramicidin channel. Proc. Natl Acad. Sci. USA 101, 117–122 (2004).

22. Hub, J. S., de Groot, B. L. & van der Spoel, D. g-wam-A free weighted histogram analysis implementation including robust error and autocorrelation estimates. J. Chem. Theory Comput. 6, 3713–3720 (2010).

23. Bostick, D. L. & Brooks, C. L. Selectivity in K+ channels is due to topological control of the permeant ion’s coordinate state. Proc. Natl Acad. Sci. USA 104, 9260–9265 (2007).

24. Im, W. & Roux, B. Ions and countermers in a biological channel: a molecular dynamics simulation of OmpF porin from Escherichia coli in an explicit membrane with 1 M KCl aqueous salt solution. J. Mol. Biol. 319, 1177–1197 (2002).

25. Varma, S. & Rempe, S. B. Coordination numbers of alkali metal ions in aqueous solutions. Biophys. Chem. 124, 192–199 (2006).

26. Conway, B. Ionic Hydration in Chemistry and Biophysics (Elsevier, 1981).

27. Lee, S.-Y., Letts, J. A. & Mackinnon, R. Functional reconstitution of purified human Hv1 H+ channels. J. Mol. Biol. 387, 1055–1060 (2009).

28. Lacin, E. et al. Dynamic role of the tether helix in IP32-dependent gating of a G protein-gated potassium channel. J. Gen. Physiol. 149, 799–811 (2017).

29. Brohawn, S. G., del Mármol, J. & Mackinnon, R. Crystal structure of the human K2P TRAAK, a lipid- and mehano-sensitive K+ ion channel. Science 335, 436–441 (2012).

30. Roux, X., Pedi, L., Diver, M. M. & Long, S. B. Crystal structure of the calcium release-activated calcium channel Orai. Science 338, 1308–1313 (2012).

31. Yang, H. et al. Pore architecture of TRIC channels and insights into their gating mechanism. Nature 538, 537–541 (2016).

32. Park, E., Campbell, E. B. & Mackinnon, R. Structure of a CLC chloride ion channel by cryo-electron microscopy. Nature 541, 500–505 (2017).

33. Claessens, I. W. & Sleijnger, P. A. Dual activation of neuronal G protein-gated inwardly rectifying potassium (GIRK) channels by cholesterol and alcohol. Sci. Rep. 7, 4592 (2017).

34. Su, Z., Brown, E. C., Wang, W. & Mackinnon, R. Novel cell-free high-throughput screening method for pharmacological tools targeting K+ channels. Proc. Natl Acad. Sci. USA 113, 5748–5753 (2016).

35. Papazian, D. M., Timpe, L. C., Jan, Y. N. & Jan, L. Y. Alteration of voltage-dependence of Shaker potassium channel by mutations in the S4 sequence. Nature 349, 305–310 (1991).

36. Wilkins, C. M. & Aldrich, R. W. State-independent block of BK channels by an intracellular quaternary amnonium. J. Gen. Physiol. 128, 347–364 (2006).

37. Poisson, D. J., McCoy, J. G. & Nümieg, C. M. The voltage-dependent gate in MhK potassium channels is located at the selectivity filter. Nat. Struct. Mol. Biol. 20, 159–166 (2013).

38. Piechotta, P. L. et al. The pore structure and gating mechanism of K2P channels. EMBO J. 30, 3607–3619 (2011).

39. Lolicato, M. et al. K2P2.1 (TREK-1) crystal structure of an inwardly rectifying K+ channel from human. Nature 471, 338–348 (2011).

40. Allen, T. W., Andersen, O. S. & Roux, B. Ion permeation through a narrow channel: using gramicidin to ascertain all-atom molecular dynamics potential of mean force methodology and biomolecular force fields. Biophys. J. 90, 3447–3468 (2006).

41. Allen, T. W., Andersen, O. S. & Roux, B. Molecular dynamics—potential of mean force calculations as a tool for understanding ion permeation and selectivity in narrow channels. Biophys. Chem. 124, 251–267 (2006).
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Author contributions

K.A.B. and D.M.M. planned and carried out protein biochemistry and assays and crystallized the protein. B.J.S., R.J. and S.H. designed and performed all molecular simulations. J.R.B. and C.V.R. devised and performed mass spectrometry experiments. D.L., P.J., M.W. and A.P.H. contributed to experimental design and interpretation. J.M.G., O.B.C., D.M.M. and K.A.B. carried out crystallography. C.J.B. was responsible for reagent synthesis. J.M.G. conceived the project, guided the experimental work and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to B.J.S. or J.M.G.

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50. Pašalić, H. et al. Cation-ion interactions in competition with cation microhydration: a theoretical study of alkali metal cation-pyrene complexes. J. Mol. Model 23, 131 (2017).

51. Bernsteiner, H., Zangerl-Plessl, E.-M., Chen, X. & Stary-Weinzinger, A. Conduction through a narrow inward-rectifier K+ channel pore. J. Gen. Physiol. 151, 1231–1246 (2019).

52. Hilgemann, D. W. & Ball, R. Regulation of cardiac Na\(^+\),Ca\(^{2+}\) exchange and KATP potassium channels by PIP2. Science 273, 956–959 (1996).

53. Fan, Z. & Makielski, J. C. Anionic phospholipids activate ATP-sensitive potassium channels. J. Biol. Chem. 272, 5388–5395 (1997).

54. Rapedius, M. et al. Long chain CoA esters as competitive antagonists of phosphatidylinositol 4,5-bisphosphate activation in Kir channels. J. Biol. Chem. 280, 30760–30767 (2005).

55. McPhillis, T. M. et al. Blu-Ice and the Distributed Control System: software for data acquisition and instrument control at macromolecular crystallography beamlines. J. Synchrotron Radiat. 9, 401–406 (2002).

56. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).

57. Kabsch, W. XDS. Acta Crystallogr. D. Biol. Crystallogr 66, 125–132 (2010).

58. Collaborative Computational Project, Number 4. The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D. Biol. Crystallogr 50, 760–763 (1994).

59. McCoy, A. J. et al. Phaser crystallographic software. J. Appl Crystallogr 40, 658–674 (2007).

60. Brunger, A. T., DelaBarre, B., Davies, J. M. & Weis, W. I. IUCr. X-ray structure determination at low resolution. Acta Crystallogr. D. Biol. Crystallogr 65, 128–133 (2009).

61. Emsley, P. & Cowtan, K. IUCr. Coot: model-building tools for molecular graphics. Acta Crystallogr. D. Biol. Crystallogr 60, 2126–2132 (2004).

62. Adams, P. D. et al. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D. Biol. Crystallogr 58, 1948–1955 (2002).

63. Smart, O. S. et al. Exploiting structure similarity in refinement: automated NCS and target-structure restraints in BUSTER. Acta Crystallogr. D. Biol. Crystallogr 68, 368–380 (2012).

64. Bricogne, G. & Irwin, J. J. Maximum-likelihood refinement of incomplete models with BUSTER+. T宁T. In IUCr Conference Proceeding (1996).

65. Webb, R. & Sali, A. Comparative Protein Structure Modeling Using MODELLER. Curr. Protoc. Bioinformatics 54, S.6.1–S.6.37 (2016).

66. Wu, E. L. et al. CHARMM-GUI Membrane Builder toward realistic biological membrane simulations. J. Comput. Chem. 35, 1997–2004 (2014).

67. Abraham, M. J. et al. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX 1–2, 19–25 (2015).

68. Huang, J. et al. CHARMM36m: an improved force field for folded and intrinsically disordered proteins. Nat. Methods 14, 71–73 (2017).

69. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. J. Mol. Graph. 14, 27–28 (1996).

70. Hernández, H. & Robinson, C. V. Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry. Nat. Protoc. 2, 715–726 (2007).

71. Sobott, F., Hernández, H., McCammon, M. G., Tito, M. A. & Robinson, C. V. A tandem mass spectrometer for improved transmission and analysis of large macromolecular assemblies. Anal. Chem. 74, 1402–1407 (2002).

72. Gaul, J. et al. High-resolution mass spectrometry of small molecules bound to membrane proteins. Nat. Methods 13, 333–336 (2016).
Author/s: 
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