Insights into the mechanisms of K+ permeation in K+-channels from computer simulations

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ABSTRACT

Ion permeation, selectivity, and the behaviour of the K^+ channel selectivity filter has been studied intensively in the previous two decades. The agreement among multiple approaches used to study ion flux in K^+-channels suggests a consensus mechanism of ion permeation across the selectivity that has been put to test in recent years with the proposal of an alternative way by which ions can cross the selectivity filter of K^+-channels via direct Coulomb repulsion between contacting cations. Some past experimental work by Zhou and MacKinnon showed that mutation of site S4 reduces the total occupancy of the selectivity filter to less than two ions on average by lowering the occupancy of the S2-S4 configuration without changing the S1-S3 configuration much, and this reduction of occupancy means that ion configurations others from the ones involved in the canonical mechanism are likely to be involved. At that time, calculations using complicated kinetic networks to relate occupancy to conduction did not provide deeper insight into the conduction mechanism. Here, to help solve this enigma, umbrella sampling simulations have been performed to evaluate the potential of mean force of two KcsA mutant channels, where the S4 site is substituted. Our new results provide insights into the significance of threonine in this position, revealing the effect of substitution on the alternate mechanisms of conduction proposed, involving either water or vacant sites.

INTRODUCTION

The first high-resolution crystal structure of a prokaryotic K^+ channel, KcsA from *Streptomyces lividans*, revealed the atomic configuration of the K^+ channel pore domain.[1] This assembly, which is generally conserved in eukaryotic K^+ channels, is formed from the symmetrical arrangement of four subunits, each containing two transmembrane α-helices connected by a re-entrant pore loop. The pore loop contains a pore helix and the highly conserved selectivity filter structure. The selectivity filter is a critical determinant of ion permeation, selectivity (discrimination between ionic species) and inactivation (blockage of ionic current following channel opening) processes in K^+ channels.

The canonical sequence of the selectivity filter, TVGYG, from each subunit, forms four contiguous binding sites (S1-S4) at the extracellular side of the channel (Figure 1A-B).[1-2] Sites S1, S2 and S3 are enclosed by the backbone carbonyl groups of the selectivity filter sequence, which can accommodate single dehydrated K^+ ions. The S4 site is unique in that it is forged from carbonyl (upper) and hydroxyl (lower) groups from the initial threonine residue, yet exhibits analogous occupancy. In addition, sites have been determined above and below the selectivity filter, at the extracellular mouth (S0 and Sext) and in the central cavity (S_{CAV}), respectively.[3]

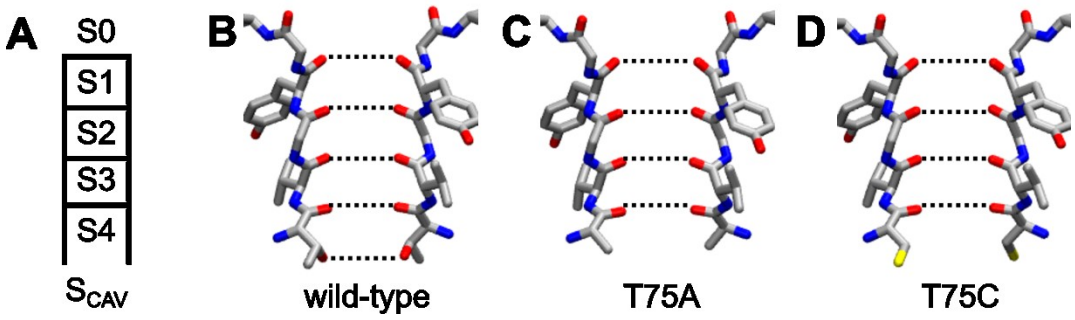

Figure 1. (A) Definition of selectivity binding sites. Structure of the selectivity filter in the (B) wild-type, (C) T75A and (D) T75C KcsA channels. Carbon, oxygen and nitrogen atoms are shown in grey, red and blue, respectively.

Various electrophysiological studies of KcsA mutants have been performed to discern the importance of individual selectivity filter sites with regards to the rate of conduction, inactivation and recovery from inactivation.[4-7] Ion occupancy of the S1-S3 sites can be tuned by synthetic substitution of individual amide bonds to ester linkages, translating to functional effects in the inactivation process.[5-6] Using this

protocol, the inactivation rate and rate of recovery from inactivation were diminished as a consequence of mutations of the S2 and S3 sites respectively, whereas negligible effects for both phenomenon were observed for S1. Side-chain substitutions have also been examined to perturb the S4 site. The T75G KcsA channel exhibited a minor reduction in the inactivation rate, but a major decline in the rate of recovery from inactivation.[6] On the contrary, the T75A KcsA channel completely abolished channel inactivation, as a result of an inversion of the allosteric coupling between the upper and lower gates.[7]

Thus far, the conduction properties have been characterised for two KcsA mutant channels, where the threonine side-chain forming the S4 site has been substituted. The conductance of the T75C/A108S double mutant (at 200 mV) was reported to decrease by a factor of ~2-4, relative to the wild-type (at 180 mV), in a concentration-dependent manner.[4] Moreover, the recorded conductance of the non-inactivating T75A mutant channel was reduced by a factor of ~17, relative to the wild-type, at a potential of +100 mV.[7] Although macroscopic currents have also been determined for the T75G mutant channel, detailed information regarding this mutant is not included in the relevant publication.[6] Consequently, we sought to understand the rationale behind the drastic reduction in conduction rates on mutation of the S4 site. To this end, high-resolution crystal structures of T75C (PDB ID 1S5H; closed state) [4] and T75A (PDB ID 6BY3; open state)[7] were utilized to perform umbrella sampling calculations, in order to simulate conduction through the altered selectivity filter and evaluate the energy of this process. The structure of the selectivity filter in the mutant channels under investigation is displayed in Figure 1C and D.

The microscopic mechanism of conduction has been under intense scrutiny since the publication of the initial KcsA structure.[8] Initial reports from crystallographic information and computational experiments supported a process involving both K^+ ions and water molecules.[8-11] In this mechanism, referred to as KWK or 'soft' knock-on mechanism, K^+ ions and water molecules occupy alternative sites in the selectivity filter, with ions occupying either S2/S4 or S1/S3 configurations. Following this, ion transport occurs by coordinated movements between these sites, prompted by an incoming intracellular ion. This is supported by various independent computational studies which have calculated the maximum energetic barrier of process in the 2-3 kcal/mol range,[9-10, 12] and the associated transport of water

molecules in K^+ channels reported experimentally.[13-14] An alternative mechanism of conduction involving vacant sites has also been proposed by Furini and Domene, exhibiting similar energetics.[12] In this mechanism, known as the KK or 'hard' knock-on mechanism, the selectivity filter can accommodate two or three K^+ ions simultaneously, in adjacent sites or separated by empty sites. In this way, direct electrostatic repulsion drives permeation. Evidence supporting this rationale has gained traction in recent years, as a result of extensive unbiased MD simulations in voltage conditions, and re-examination of the initial electron density profiles within the same study.[15] In the wild-type channel, the electron density profiles suggest the same occupancy probability for binding sites S1 to S4, which is compatible both with the KWK and KK conduction mechanisms. However, in the T75A and T75C mutant channels, the only configuration left for the KWK mechanism is S1S3, while for the KK mechanism several occupancy states of the filter are still possible (S2S3, S1S3, S1S2). Here, the energy maps for both conduction schemes were calculated, and the results are compared with experimental data on ion conductances and density profiles with the aim to help resolve an old quiz. As stated in the original publication by Ming Zhou and Roderick MacKinnon, the mutation actually reduces the total occupancy of the filter to less than two ions on average by lowering the occupancy of the S2-S4 configuration, without changing the S1-S3 configuration much, and this reduction of occupancy means that ion configurations others from the ones involved in the canonical mechanism are necessary.[16] Although these authors carried out calculations using more complicated kinetic networks to relate occupancy to conduction, they reported that these calculations did not provide deeper insight into the conduction mechanism.[16]

MATERIALS AND METHODS

Model Setup

High-resolution crystal structures of T75C (PDB ID 1S5H; closed state)[4] and T75A (PDB ID 6BY3; open state)[7] were used as the atomic coordinates of the mutant channels. Residues 26-114 and 26-121 were included in the models of T75C and T75A, respectively. N- and C-termini were acetylated and methylated respectively. The amino acid E71 of KcsA was modelled in the protonated state to form a diacid hydrogen bond with D80.[17] Default ionisation states were used for the remaining amino acids. Four water molecules were placed at the back of the selectivity filter, in

agreement with crystallographic data and previous molecular dynamics (MD) simulations. SOLVATE 1.0 was used to solvate internal cavities of the protein. The structures were aligned perpendicular to the bilayer and inserted into a neutral membrane containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) molecules. The VMD solvate plugin was then used to create a cubic water box around the membrane-protein system.[18] The overlapping water and lipid molecules around the ion channel structure were removed with a cut-off distance of 1.2 Å. Potassium and chloride ions were added using Autoionize Plugin of VMD to neutralise the systems and obtain a concentration of 150 mM.[18] The final system size was approximately 90,000 atoms.

Molecular Dynamics Simulations

MD simulations were performed with NAMD 2.12.[19] CHARMM36 parameters were used for the protein and lipids,[20] the TIP3P model was used for water,[21] and the CHARMM NBFIX parameters for ions as in a similar previous study of the wild type channel.[12] The particle mesh Ewald method was used for the treatment of periodic electrostatic interactions, with an upper threshold of 1 Å for grid spacing.[22] Electrostatic and van der Waals forces were calculated every time step. A cut-off distance of 12 Å was used for van der Waals forces. A switching distance of 10 Å was chosen to smoothly truncate the non-bonded interactions. Only atoms in a Verlet pair list with a cut-off distance of 13.5 Å (reassigned every 20 steps) were considered. The SETTLE algorithm was used to constrain all bonds involving hydrogen atoms, to allow the use of a 2 fs time step throughout the simulation.[23] MD simulations were performed in the NPT ensemble. The Nose-Hoover-Langevin piston was employed to control the pressure with a 200 fs period, 50 fs damping constant, and the desired value of 1 atmosphere.[24-25] The system was coupled to a Langevin thermostat to sustain a temperature of 300 K throughout. In the equilibration process, the same protocol was used for all of the systems. The systems were subjected to 10,000 steps of minimization, with harmonic constraints (force constant 20 kcal $mol^{-1}Å^{-2}$) on protein atoms, lipid headgroups and crystallographic water and ions. Harmonic restraints were gradually reduced to a force constant of 2 kcal $mol^{-1}Å^{-2}$ and removed in consecutive steps from the lipid headgroups, protein side-chains and protein backbone over the course of a 3.5 ns trajectory. Unbiased MD simulations were then performed for 20 ns to equilibrate the

system. Coordinates and velocities from this point were used as the starting point for subsequent biased simulations.

Umbrella Sampling Simulations

Umbrella sampling has been used to calculate the Potential of Mean Force (PMF) of ion translocation through the mutated KcsA selectivity filter. Ion permeation involving three ions was examined, simulating the events connecting the S1/S3/S_{CAV} and S_{EC}/S1/S3 configurations, with either a water molecule (KWK mechanism) or a gap (KK mechanism) in the S2 site. The initial and final configurations were considered to be equivalent, and thus the energetics obtained is representative of the permeation of a single ion. The three ions are denoted K1 (exterior ion), K2 (central ion) and K3 (interior ion). Two biasing potentials were initialized acting on the K3 ion and the center of mass of the K1 and K2 ions. Individual simulations were predominantly spaced 0.5 Å apart, adopting a force constant of 20 kcal $mol^{-1}Å^{-2}$ for the harmonic potential. In the extremities of K1 (extracellular) and K3 (intracellular), windows were spaced 1 Å apart, adopting a force constant of 10 kcal $mol^{-1}Å^{-2}$ for the harmonic potential. In a single scenario (T75C-KK), the chosen collective variables resulted in degenerate states, *i.e.* mixing of different ion configurations in simulations at fixed harmonic potentials. Thus, simulations with three biasing potentials acting on individual ions were performed. Ions were moved to their starting configurations during an initial 10 ps trajectory, with weak restraints applied to the backbone atoms of residues E71 to D80. Simulations of 500 ps were then performed for each configuration. The positions of water molecules in the selectivity filter were monitored throughout, and simulations exhibiting water molecules with unconventional behaviour were removed from subsequent analysis. The initial 100 ps of the trajectories were considered as equilibration and also removed. The weighted histogram analysis method was used to unbias the data and obtain the PMF in two (T75C-KWK, T75A-KWK, T75A-KK) or three-dimensions (T75C-KK).[26] Error estimates were obtained by calculating PMF profiles for 100 ps portions of the trajectory and combining them.

It should be noted here, that differences in the activation gate are neglected, on the basis of structural studies of voltage-gated K^+ channels which exhibit comparable ion occupancies in the crystal structures of representative open-conductive and closed-conductive states.[27-28] Furthermore, substitution of T75 position has dramatic

consequences on the coupling of the activation-inactivation gates.[7] Therefore, further mutations in this position in either of structures used would likely destabilize the channel structure.

RESULTS

KWK Mechanism

Firstly, the long-established KWK mechanism in the T75A and T75C mutant channels is investigated, denoted T75A-KWK and T75C-KWK, respectively. In the following discussion, it can be assumed that all selectivity filter sites are filled with K^+ ions or water molecules. The 2D PMF of the T75A mutant channel (Figure 2A) exhibits three minima corresponding to S1/S3, S0/S2, S0/S2/$S3_B$ and S2/$S3_B$ configurations, where B represents the lower boundary of the site. The S1/S3 site is the most thermodynamically stable and progression of ions from S1/S3 to S0/S2 poses the largest barrier to conduction (~5 kcal/mol), where the behaviour of the third ion is omitted (Table 1). This mechanism is inconsistent with the traditional KWK mechanism, which involves S1/S3/$S4_B$ and S0/S2/S4 configurations. It is therefore apparent that elimination of the S4 site abolishes the knock-on effect of the intracellular ion and increases the maximum barrier to conduction. Following evolution of the S0/S2 configuration, the remainder of the permeation event can be completed with low energetic barriers (~2 kcal/mol) on approach of the third ion.

Table 1. Maximum barrier to the conduction process in KcsA mutant channels

Simulation	Energy Barrier (kcal/mol ± SD)
T75A-KWK	5.7 ± 1.1
T75A-KK	5.6 ± 0.7
T75C-KWK	7.2 ± 0.7
T75C-KK	5.3 ± 0.2

In contrast to the above, the wild-type and T75C mutant channel differ primarily by the electrostatics of the S4 site, as threonine and cysteine are similar sizes. Configurations S1/S3/S_{CAV}, S0/S2/S_{CAV}, S0/S2/S4 and S2/S3 represent minima in the conduction mechanism in the 2D PMF of the T75C channel (Figure 2B). As expected, the S4 site is restored, though the $S4_B$ site remains unviable. S0/S2/S4 constitutes the lowest energy state, followed by S1/S3/S_{CAV}, the energy of which fluctuates in a ~2 kcal/mol range dependent on the position of the ion in the central

cavity. Similar to the T75A mutant channel, conduction is initiated by the interchange between S1/S3 to S0/S2 configurations. In this case, the barrier to this process is at a minimum (~3-4 kcal/mol), when the cavity ion is approximately 6 Å below the S3 site. Movement of this ion towards the S4 site, requires an additional 3 kcal/mol. Overall, transition from S0/S2/S4 and S2/S3 limits conduction to the greatest extent, with a calculated barrier in excess of 6 kcal/mol.

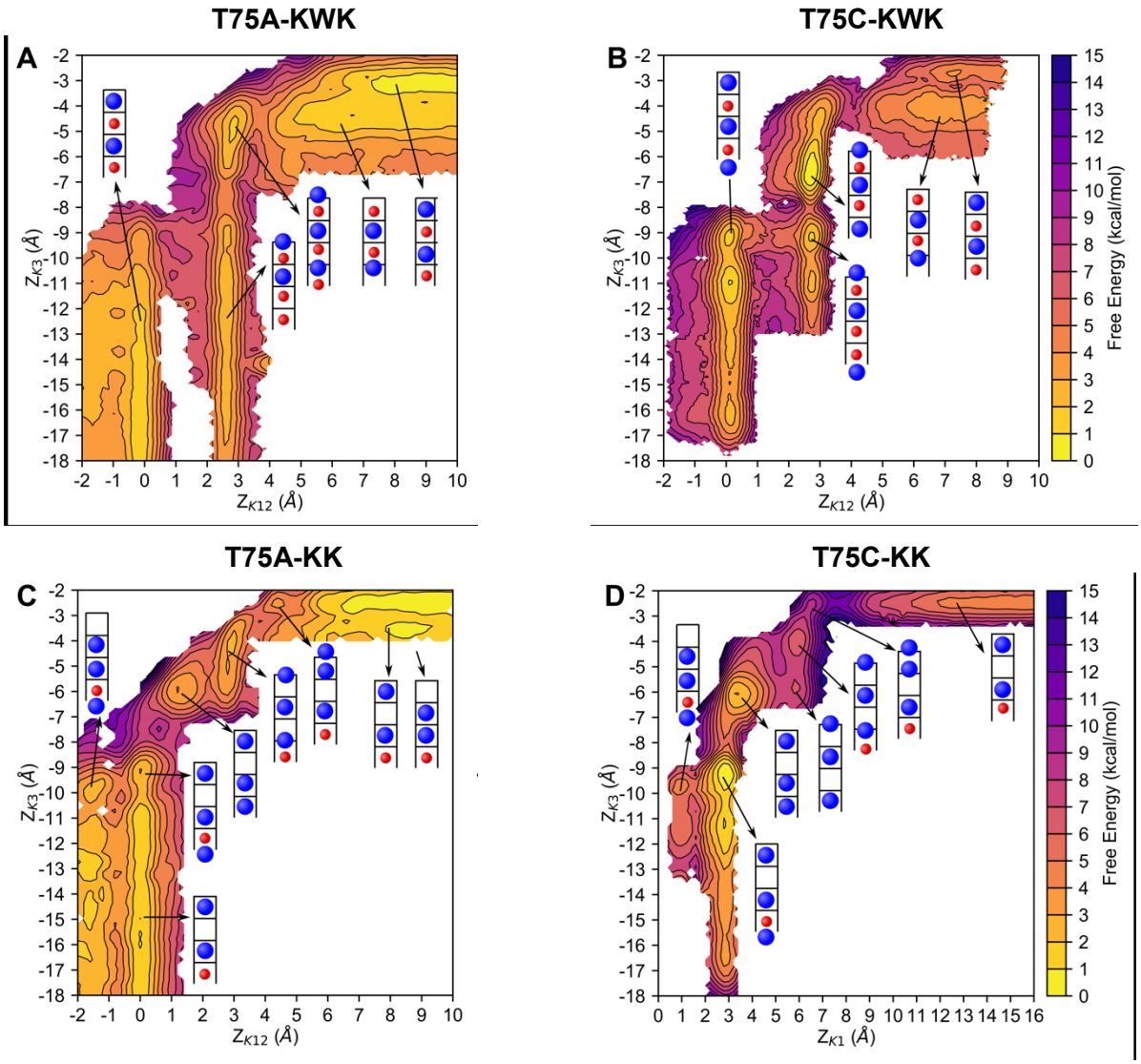

Figure 2. PMF profiles of (A) T75A-KWK, (B) T75C-KWK, (C) T75A-KK and (D) T75C-KK simulations, obtained using umbrella sampling. Figure 2D is a projection onto two collective variables, derived from the 3D-PMF calculated directly from the simulation. Ion configurations are represented using a simplified representation of the selectivity filter, defined in Figure 1A, with K^+ ions and water molecules shown as blue and red spheres, respectively.

KK Mechanism

Next, the energetics of the KK mechanism in the T75A and T75C mutant channels is evaluated, denoted T75A-KK and T75-KK, respectively. Here, sites not occupied by K^+ ions are empty unless specifically mentioned. In the T75A mutant channel, four minima can be identified on the permeation pathway in the 2D PMF corresponding to configurations: S1/S3/S_{CAV}, S1/S2/S4, S0/S2/S3 and S0/S1/S3 (Figure 2C). Minima representing S2/S3/S_{CAV} configurations are also found. S1/S3 and S2/S3 configurations are similar in energy and separated by low energy barriers (~2 kcal/mol); thus, it is likely that S2/S3 can unwittingly evolve from S1/S3 but does not necessarily play a direct role in conduction. The energy of certain selectivity filter configurations varies dependent on the position of the ion in the central cavity. The maximum barrier to conduction (5 kcal/mol) arises from the progression of one ion from the cavity to S4, and the accompanying movement of a second ion from S3 to S2. The remaining energetic barriers are of the order of 1-3 kcal/mol. However, intermediary steps with direct ion-ion interactions (S1/S2/S4, S0/S2/S3 and S0/S1/S3) are visibly higher in energy than the initial S1/S3 state.

In the 3D PMF of the T75C channel mutant (Figure 1D), an additional minimum is observed representing a S0/S2/S4 configuration, which is similar in energy to S0/S2/S3. In this case, however, S0/S2/S4, S0/S2/S3 and S0/S1/S3, form part of the elevated energy region of the PMF, although S1/S2/S4 is excluded. As a result, high energy barriers (between 5-6 kcal/mol) are realized in three separate regions of the PMF: i) entrance of ion to S4 from the cavity, when other ions occupy S1 and S3, ii) movement of ion to S0 from S1, when other ions occupy S2 and S4, and iii) a concerted movement of ions from S2 and S0, to S1 and S_{EC}, respectively, to return to the S1/S3 starting conformation. Moreover, the energy barrier of the backward transition of movement iii) is in excess of 7 kcal/mol. It should be noted that the marked stability of the cavity site is a result of occupation of the S4 site by a water molecule, intersecting the cavity and S3 ions.

DISCUSSION

By using umbrella sampling simulations, the PMF for both KK and KWK mechanisms in the T75A and T75C mutant channels has been evaluated. From these, the maximum barrier to conduction has been calculated for all scenarios to be in the

range 5-7 kcal/mol. This is a significant increase from values calculated for the wild-type KcsA channel, which is frequently cited as 2-3 kcal/mol[9-10, 12] justifying the diminished conductances recorded experimentally.[4, 7]

The increased barriers can be attributed to differences in the observed mechanisms of conduction, as a result of tuning the properties of the S4 site. For example, the threonine-cysteine substitution maintains a comparable side-chain volume yet transforms the electrostatic properties, rendering the $S4_B$ site inhospitable. On the other hand, both side-chain volume and polarity are reduced on substitution of threonine to alanine, which merges the S4 site with the central cavity, effectively removing both S4 and $S4_B$ regions. In the KWK mechanism, the difference is stark. In the absence of the $S4_B$ site, in both T75A and T75C, the approach of an intracellular K^+ ion, to promote the concerted movement of ions from S1/S3 to S0/S2 in the KWK mechanism, is unworkable. Thus, removal of threonine in this scenario eliminates the long-established “knock-on” aspect of conduction. In the KK mechanism, the sequence of atomic movements is unchanged. However, two-ion configurations are evidently more favorable than those with three ions. This observation is in agreement with experimental data which documents a reduction in the total ion occupancy of the T75C filter to less than two (~1.7).[4]

Subsequently, it is of interest to examine if the calculated PMF profiles can rationalize the ion occupancies estimated from the crystal structure of the T75C channel.[4] In the mutated filter, the ion occupancies of S2 and S4 are diminished, whilst those in S1 and S3 are basically sustained. In the accompanying publication, Zhou and Mackinnon contemplate this in terms of the KWK mechanism, suggesting that the S1/S3 configuration may be more energetically favorable than the S2/S4 configuration in the mutant channel. However, in the PMF profile of this process (T75C-KWK), configurations involving S2 are energetically equivalent to those that involve S1 and S3, and in fact, the S0/S2/S4 configuration is found to be the most thermodynamically stable, advocating that this is not the case. What is more, the free energy of the S1/S3/$S_{CAV(-9.0)}$ configuration in the PMF profile of the KK mechanism is at least 2-6 kcal/mol lower than other configurations involving the S2 site, with energetic barriers of ~5 kcal/mol to evolve from this. Considering this model, it can be suggested that the S1/S3 configuration, where the S2 site is vacant, is primarily responsible for the elevated electron densities in S1/S3. It then follows that the S2

density may arise from configurations where S2 is occupied, but S4 remains empty, such as S0/S2/S_{CAV} (KWK), S2/$S3_B$ (KWK), S2/S3 (KK) and S0/S2/$S3_B$ (KK).

Finally, we remark on the significance of the ion in the central cavity of the T75C channel. Our PMF profiles reveal several minima in the central cavity which overlap well with the electron density presented in the crystal structure and stabilize certain selectivity filter configurations. It is worth noting that the S1/S3/$S_{CAV(-9.0)}$ configuration, in the KK mechanism, includes a water molecule in the region considered as S4, inferring that a potential hybrid pathway, involving both vacant and hydrated sites may exist.

CONCLUSIONS

The agreement among multiple approaches used to study ion flux in K^+-channels suggests a consensus mechanism of ion permeation across the selectivity that has been put to test in recent years with the proposal of an alternative way by which ions can cross the selectivity filter of K^+-channels via direct Coulomb repulsion between contacting cation.[29] Some past experimental work showed that mutation of S4 reduces the total occupancy of the filter to less than two ions on average by lowering the occupancy of the S2-S4 configuration, without changing the S1-S3 configuration much. Although this resulting reduction of occupancy means that ion configurations others from the ones involved in the canonical mechanism are likely to be involved, calculations using complicated kinetic networks to relate occupancy to conduction did not provide deeper insight into the conduction mechanism.[30] Here, to help solve this enigma and provide further insight into conduction in K^+-channels, umbrella sampling simulations have been performed to evaluate the potential of mean force of KcsA mutant channels, where the S4 site is substituted. Substitution of the threonine residue to hydrophobic residues of differing sizes (alanine and cysteine) notably increases the maximum barrier to ion permeation, in line with the reduced conductance rates obtained from single-channel recordings in prior experimental studies. Moreover, insights are provided into the deviant ion occupancies reported for the T75C mutant channel, indicating the dominant mechanism of conduction in this channel involves vacant sites in the selectivity filter. Overall, the barriers for KWK or KK conduction in the T75A channel are similar. Therefore, there is no preferred mechanism for ions to travel along the pore which is in line with the data we reported for the wild type KcsA and KirBac channels previously.[12] The movement

of ions can be accompanied at times by water molecules as exemplified in streaming potential experiments but this is not necessarily always the case. We propose that the permeation mechanism by which multiple ions in the selectivity filter of a K^+-channel are hit by an incoming ion applies equally to the mutant structures, and the energy patterns suggest a picture in which the ions can travel either alone or accompanied by water molecules. The fractional contribution of each mechanism will then be reflected in the electron density reported from crystallographic experiments. This then explains why the S1/S3 configuration, where the S2 site is vacant, is primarily responsible for the elevated electron densities in S1/S3 in the mutant structures; S2 is the only site where a vacant can be realistically materialised as both S1 and S3 are easily accessible by water molecules (or ions) from the intracellular and extracellular media. This model further supports the view that in K^+-channels with the threonine residue at S4 in the selectivity filter mutated, there is not a preference for ions to travel through the selectivity filter either in isolation without intervening water molecules or accompanied by water molecules.

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ABBREVIATIONS

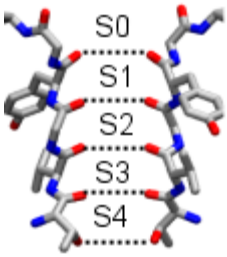
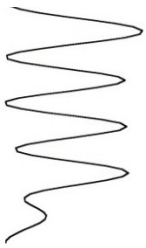
MD: Molecular dynamics; PMF: potential of mean force

REFERENCES

1. Doyle, D. A.; Cabral, J. M.; Pfuetzner, R. A.; Kuo, A.; Gulbis, J. M.; Cohen, S. L.; Cahit, B. T.; MacKinnon, R., The structure of the potassium channel: Molecular basis of k^+ conduction and selectivity. *Science* **1998,** *280*, 69-77.
2. Kuo, A.; Gulbis, J. M.; Antcliff, J. F.; Rahman, T.; Lowe, E. D.; Zimmer, J.; Cuthbertson, J.; Ashcroft, F. M.; Ezaki, T.; Doyle, D. A., Crystal structure of the potassium channel kirbac1.1 in the closed state. *Science* **2003,** *300* (5627), 1922-6.
3. Zhou, Y. F.; Morais-Cabral, J. H.; Kaufman, A.; MacKinnon, R., Chemistry of ion coordination and hydration revealed by a k+ channel-fab complex at 2.0 angstrom resolution. *Nature* **2001,** *414* (6859), 43-48.
4. Zhou, M.; MacKinnon, R., A mutant kcsa k+ channel with altered conduction properties and selectivity filter ion distribution. *Journal of Molecular Biology* **2004,** *338* (4), 839-846.
5. Devaraneni, P. K.; Komarov, A. G.; Costantino, C. A.; Devereaux, J. J.; Matulef, K.; Valiyaveetil, F. I., Semisynthetic k+ channels show that the constricted conformation of the selectivity filter is not the c-type inactivated state. *Proc. Natl. Acad. Sci. U. S. A.* **2013,** *110* (39), 15698-15703.
6. Matulef, K.; Annen, A. W.; Nix, J. C.; Valiyaveetil, F. I., Individual ion binding sites in the k+ channel play distinct roles in c-type inactivation and in recovery from inactivation. *Structure* **2016,** *24* (5), 750-761.
7. Labro, A. J.; Cortes, D. M.; Tilegenova, C.; Cuello, L. G., Inverted allosteric coupling between activation and inactivation gates in k+ channels. *Proc. Natl. Acad. Sci. U. S. A.* **2018,** *115* (21), 5426-5431.
8. Doyle, D. A.; Cabral, J. M.; Pfuetzner, R. A.; Kuo, A. L.; Gulbis, J. M.; Cohen, S. L.; Chait, B. T.; MacKinnon, R., The structure of the potassium channel: Molecular basis of k+ conduction and selectivity. *Science* **1998,** *280* (5360), 69-77.
9. Aqvist, J.; Luzhkov, V., Ion permeation mechanism of the potassium channel. *Nature* **2000,** *404* (6780), 881-4.
10. Berneche, S.; Roux, B., Energetics of ion conduction through the k+ channel. *Nature* **2001,** *414* (6859), 73-77.
11. Morais-Cabral, J. H.; Zhou, Y. F.; MacKinnon, R., Energetic optimization of ion conduction rate by the k+ selectivity filter. *Nature* **2001,** *414* (6859), 37-42.
12. Furini, S.; Domene, C., Atypical mechanism of conduction in potassium channels. *Proc. Natl. Acad. Sci. U. S. A.* **2009,** *106* (38), 16074-16077.
13. Miller, C., Coupling of water and ion fluxes in a k+-selective channel of sarcoplasmic-reticulum. *Biophysical Journal* **1982,** *38* (3), 227-230.
14. Alcayaga, C.; Cecchi, X.; Alvarez, O.; Latorre, R., Streaming potential measurements in ca-2+-activated k+ channels from skeletal and smooth-muscle - coupling of ion and water fluxes. *Biophys J* **1989,** *55* (2), 367-371.
15. Kopfer, D. A.; Song, C.; Gruene, T.; Sheldrick, G. M.; Zachariae, U.; de Groot, B. L., Ion permeation in k+ channels occurs by direct coulomb knock-on. *Science* **2014,** *346* (6207), 352-355.
16. Zhou, M.; MacKinnon, R., A mutant kcsa k+ channel with altered conduction properties and selectivity filter ion distribution. *J Mol Biol* **2004,** *338* (4), 839-46.
17. Berneche, S.; Roux, B., The ionization state and the conformation of glu-71 in the kcsa k+ channel. *Biophysical Journal* **2002,** *82* (2), 772-780.
18. Humphrey, W.; Dalke, A.; Schulten, K., Vmd: Visual molecular dynamics. *J Mol Graphics* **1996,** *14* (1), 33-38.
19. Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K., Scalable molecular dynamics with namd. *J Comput Chem* **2005,** *26* (16), 1781-802.
20. Klauda, J. B.; Venable, R. M.; Freites, J. A.; O'Connor, J. W.; Tobias, D. J.; Mondragon-Ramirez, C.; Vorobyov, I.; MacKerell Jr, A. D.; Pastor, R. W., Update of the charmm all-atom additive force field for lipids: Validation on six lipid types. *J Phys Chem B* **2010,** *114* (23), 7830-7843.

21. Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L., Comparison of simple potential functions for simulating liquid water. *J Chem Phys* **1983,** *79* (2), 926-935.
22. Darden, T.; York, D.; Pedersen, L., Particle mesh ewald: An n·log(n) method for ewald sums in large systems. *J Chem Phys* **1993,** *98* (12), 10089-10092.
23. Miyamoto, S.; Kollman, P. A., Settle: An analytical version of the shake and rattle algorithm for rigid water models. *J. Comput. Chem.* **1992,** *13* (8), 952-962.
24. Feller, S. E.; Zhang, Y.; Pastor, R. W.; Brooks, B. R., Constant pressure molecular dynamics simulation: The langevin piston method. *J Chem Phys* **1995,** *103* (11), 4613-4621.
25. Martyna, G. J.; Tobias, D. J.; Klein, M. L., Constant pressure molecular dynamics algorithms. *J Chem Phys* **1994,** *101* (5), 4177-4189.
26. Kumar, S.; Bouzida, D.; Swendsen, R. H.; Kollman, P. A.; Rosenberg, J. M., The weighted histogram analysis method for free-energy calculations on biomolecules .1. The method. *J Comput Chem* **1992,** *13* (8), 1011-1021.
27. Jiang, Y. X.; Lee, A.; Chen, J. Y.; Ruta, V.; Cadene, M.; Chait, B. T.; MacKinnon, R., X-ray structure of a voltage-dependent k+ channel. *Nature* **2003,** *423* (6935), 33-41.
28. Long, S. B.; Tao, X.; Campbell, E. B.; MacKinnon, R., Atomic structure of a voltage-dependent k+ channel in a lipid membrane-like environment. *Nature* **2007,** *450* (7168), 376-82.
29. Furini; Domene, C., Atypical mechanism of conduction in potassium channels. *Proc Natl Acad Sci U S A* **2009,** *106* (38), 16074-7.
30. Zhou, Y.; MacKinnon, R., The occupancy of ions in the k+ selectivity filter: Charge balance and coupling of ion binding to a protein conformational change underlie high conduction rates. *J Mol Biol* **2003,** *333* (5), 965-75.

Graphical Abstract

	Selectivity Filter	Electron density	Permeation Mechanism
Wild Type			?
Mutant