Supplementary information:

Materials and Methods.

Protein expression and purification. KcsA-WT, G77A and G77C cloned in the expression vector pQE70 were transformed in the *E. coli*-C41 strain (Lucigen) and grown overnight in Luria-Bertani (LB) supplemented with 1% glucose and 0.4 mg/ml at 37 °C. The overnight culture was diluted a 100-fold into 1 L of LB media supplemented with 0.2% glucose, 0.4 mg/ml ampicillin, 0.5 % glycerol and 5 mM $MgCl_2$ (1) and growth at 37 °C until it reached an optical density at 600 nm of ~ 0.6. At this point, the cell cultures were cooled down to 29 °C under continuous agitation for 1 hour. Then, protein expression was initiated by the addition of 0.1 mM isopropyl thiogalactoside and the media was supplemented with 10 mM $BaCl_2$ (a very effective K^+ channel blocker) and 0.4 mg/ml ampicillin to reinforce the plasmid selection. The cell cultures were incubated at 29 °C under constant agitation for 24 hours. Next day, the cells expressing KcsA or the mutant channels were harvested by centrifugation at 4600 rpm at 4 °C. Protein expression levels were routinely monitored by western blot analysis. The harvested cells were resuspended in a buffer (Buffer TKS: 50 mM Tris-HCl: 150 mM KCl, pH 8.0) supplemented with + 170 ug/ml phenylmethylsulfonyl fluoride and treated with 1.0 mg/mL of egg lysozyme and 5 mM EDTA and rotated at room temperature for 1 h. The cell suspension then was frozen at -80 °C and next day they were thawed and supplemented with 5 mM $MgCl_2$ and 50 ug/ml of DNase for 1 hour. The cell suspension was broken by passing it through a microfluidizer and then spun down at 100,000 g for 1 h to pellet the cell membranes. The cell membranes were resuspended with Buffer TKS supplemented with protease inhibitors, and aliquots were stored at -80 °C until further use. KcsA was solubilized from the *E. coli* membrane by extracting it with Buffer TKS + 20 mM Dodecyl Maltoside (DDM) + protease inhibitors for 1 h at room temperature. The solubilized KcsA-WT or mutant channels was separated from the insoluble material by centrifugation at 100,000 g. Then, the supernatant was applied to a Talon resin column (Clontech), which was washed with Buffer TKS supplemented with 15 mM imidazole and 2 mM DDM. The protein was eluted by applying TKS buffer supplemented with 400 mM imidazole and 2 mM DDM. The pure protein was concentrated, incubated with chymotrypsin (1:50) for 2 hours at room temperature and applied to a size-exclusion chromatography column Enrich SEC 650 10x300 column (Bio-Rad) for final purification and to determine if the purified channel was properly folded.

Macromolecular crystallography: C-terminal truncated KcsA (wilt-type, G77A and G77C) was mixed with an antibody fragment needed for the crystallization process. The channel-Fab complex was purified by passing it through a size exclusion chromatography column ENrich SEC 650 10x300 column (Bio-Rad) pre-loaded with Buffer TKS + 0.5 mM Dodecyl Maltoside (the G77A or G77C channels did not crystallize in the commonly used decyl maltoside detergent, instead a systematic search was conducted to determine the optimal conditions for crystallization). For the G77A, the Fab-channel complex was passed through a SEC column pre-equilibrated in a buffer containing 150 mM NaCl. Crystal trials were performed by the sitting-drop method in 22-26 % PEG400 (v/v), 50 mM magnesium acetate, 50 mM sodium acetate (pH 4.8.-5.4) at 20 °C. Crystals appeared within a week period and they were as soon as possible cryo-protected by rising the concentration of

PEG-400 to 40% by increasing it 5% per day. A data set were acquired from a single crystal for the G77A or the G77A mutants at the beamline 14-1, at the Stanford Synchrotron Radiation Laboratory (SSRL). Image processing and data reduction were performed with HKL2000(2).

Crystal structure determination. The crystal structures of the closed state in high-K^+ concentration of the KcsA-G77A (PDB=**6NFU**), high-Na^+ concentration of the KcsA-G77A (PDB=**6PA0**) and the KcsA-G77C (PDB=**6NFV**) were solved by molecular replacement using the atomic model of the antibody fragment from a Fab-KcsA complex structure at 2 Å resolution (PDB-1K4C)(3) as the search model. The structural models for the these mutant channels were built using coot(4) and refined with Phenix(5). The protocol for refining these structures included 2-5 cycles of rigid body, energy minimization, simulated annealing and individual B-factor refinements. Table 1 contains the statistics of the crystallographic data analysis. The crystal structural figures used on this paper were made using Pymol (https://pymol.org).

Electrophysiological recordings: KcsA-G77A mutant or KcsA wild-type channels were reconstituted in Asolectin liposomes as described before(6). In Brief, KcsA-WT and G77A mutant were reconstituted at a 1 to 100 protein-to-lipid ratio (weight/weight), which is a protein to lipid ratio that allows reliable measurements of macroscopic currents. Asolectin liposomes (10 mg of dried lipids) were made in the presence of 1 mL of the following buffer: 200 mM KCl and 5 mM MOPS-buffer at pH 7.0 (reconstitution buffer). The mixture of KcsA and liposomes were incubated overnight under constant agitation at 4°C with bio-beads SM-2 from Bio-Rad. Following 24 hours incubation time, proteoliposomes suspension was spun down by centrifugation at 100,000 g for 2 hours. The pellet of channel-containing liposomes was resuspended in 60 μL of reconstitution buffer. A drop of the proteoliposomes suspension containing KcsA-WT or G77A mutant was placed on a microscope slide and subject to overnight dehydration in a desiccation chamber with Drierite desiccant. 24 hours later, the drop was rehydrated with 20 μl of reconstitution buffer at 4°C overnight. Usually, all the samples were ready to patch after a 24 hours rehydration period, since it yielded giant liposomes suitable for patch-clamp measurements. Electrophysiology measurements were conducted in symmetrical 5 mM MOPS at the desired pH in the presence of 200 mM KCl. Macroscopic currents or single channel recording were recorded from several independent experiments (n=3-5 repeats) with a patch-clamp amplifier Axopatch 200 B, and currents were sampled at 40 kHz with an analogue filter set to 10 kHz. Patch pipettes, after fire polishing, displayed a resistance of 2.0 MΩ (they were filled with 200 mM KCl and 5 mM MOPS-buffer at pH 4.0).

Isothermal Titration Calorimetry (ITC). C-terminal truncated KcsA-WT or G77A mutant were applied to a size exclusion chromatography column ENrich SEC 650 10x300 column (Bio-Rad) pre-equilibrated with a buffer containing 50 mM Tris-Cl pH 8.0 + 5 mM Dodecyl Maltoside +150 mM NaCl (Sample buffer). The samples were concentrated to ~380 μM and they were dialyzed extensively against a buffer with the following buffer composition: 50 mM Tris-Cl pH 8.0, 150 mM NaCl and 5 mM DDM (dialysis buffer). ITC determinations were conducted in a Nano Isothermal Titration Calorimeter (TA Instruments) at 25 °C. The protein sample (~380 uM KcsA WT or G77A) was placed in a sample cell that contains a reaction volume of ~170 μL. The KCl solution used to titrate the dialyzed KcsA sample were prepared in the dialysis buffer used during the last dialysis step and they

were degassed for 30 minutes previous starting the titration experiments using a TA Instrument degassing instrument (model 63256). 2 µL injections of the KCl solution were applied into the sample cell (containing the KcsA sample) under constant stirring every 300s. Controls experiments were performed by applying 2 µL injections of the KCl solution every 300s into the reaction chamber containing only the dialysis buffer. The data handling and analysis was performed applying a one site independent binding model contained in the NanoAnalyze software from TA instrument. The total heat changes were corrected by subtracting the heat signature associated with the dilution of the last injections. The corrected and normalized heat change was plotted against the molar ratio of the titrant, as it has been done before(7). The apparent K_d for K^+ binding to the KcsA or the G77A selectivity filters was calculated by fitting the data to a model that considers one-independent binding site. The binding stoichiometry was set to one (N=1) since according to the canonical model for ion permeation, upon K^+ depletion, an alternating ion vacancy at either the S2 or the S3 sites has been proposed based on X-ray crystallographic analysis(3, 8).

Liposomes flux assay to assess KcsA K^+ selectivity. To assess the ion selectivity of KcsA-WT and the G77A mutant a fluorescent liposome flux assay that has been used before to evaluate the function of ion channels was adapted for this purpose (9). C-terminal truncated KcsA-containing proteoliposomes, 1:100 mass to mass ratio, were preloaded with 200 mM KCl and then diluted into a buffer containing non-permeant ions such as Na^+ or NMG^+. In these conditions, the large K^+ concentration difference between the inside and the outside of the proteoliposomes promote the selective exit of K^+ ions through KcsA if the channel is open (the C-terminal truncated version of KcsA displays a higher channel activity at neutral pH), which in turn produces a membrane electrical potential (negative inside the proteoliposomes). Upon addition of 1 µM of the membrane permeable pH-sensitive ACMA dye and 1 µM of the protonophore CCCP, a clear time dependent reduction of fluorescence intensity emitted at 480 nm is recorded, as a consequence of ACMA quenching by protons (i.e., protons will enter via CCCP only if KcsA is active and K^+ selective). When the KcsA-containing liposomes (WT or G77A) are diluted in a buffer containing permeant ions, no membrane potential is generated and consequently no quenching of the ACMA fluorescence is detected. The assay is finished by the addition of 1 µM valinomycin, which empty the remaining vesicles and produce the maximum quenching of the fluorescent signal.

Table S1.

Statistic	G77A ** (PDB=6NFU)	G77A in Sodium	G77C ** (PDB=6NFV)
Space Group	I 4		I 4
Cell Dimension			
a=b, c **(Å)**	155.08, 75.79	155.01, 76.10	156.15, 76.11
α=β=Y **(°)**	90	90	90
Resolution (Å)	31.18-2.09 (2.16-2.09)	34.66-2.05 (2.12-2.05)	34.9-2.13 (2.20-2.13)
R_{merge}	0.075 (0.49)	0.099 (0.85)	0.1 (0.7)
I/σI	30.38 (5.0)	15.76 (0.88) [C1/2=0.54)	33.74 (1.6)
Completeness (%)	99.89 (99.89)	97.55 (83.94)	99.29 (99.54)
Redundancy	7.9 (8.2)	5.2 (3.5)	3.3 (2.8)
Refinement			
No. reflections	53355 (5319)	55579 (4870)	51020 (4913)
R_{work}	0.21 (0.25)	0.1981 (0.33)	0.205 (0.288)
R_{free}	0.22 (0.30)	0.2350 (0.38)	0.242 (0.315)
No. atoms	4356	4277	4165
Protein	4006	4006	4016
Ligand/ion	4	1	4
Waters	305	229	104
Other ligands	45	42	45
Protein residues	534	534	534
Bond lengths (Å)	0.004	0.008	0.009
Bond angles (°)	0.99	1.18	1.25
Wilson B-factor	35.46	42.76	50.54
Average B-Factor, $Å^2$	43.40	49.87	60.29
Protein	42.87	49.55	60.26
Ligands	56.15	61.6	71.49
Water	48.52	53.28	56.68
Ramachandran Favored (%)	97.35	97.92	97.54
Ramachandran outliers (%)	0.00	0.00	0.00

*Highest resolution shell is show in parenthesis.

** Data sets were collected from a single crystal.

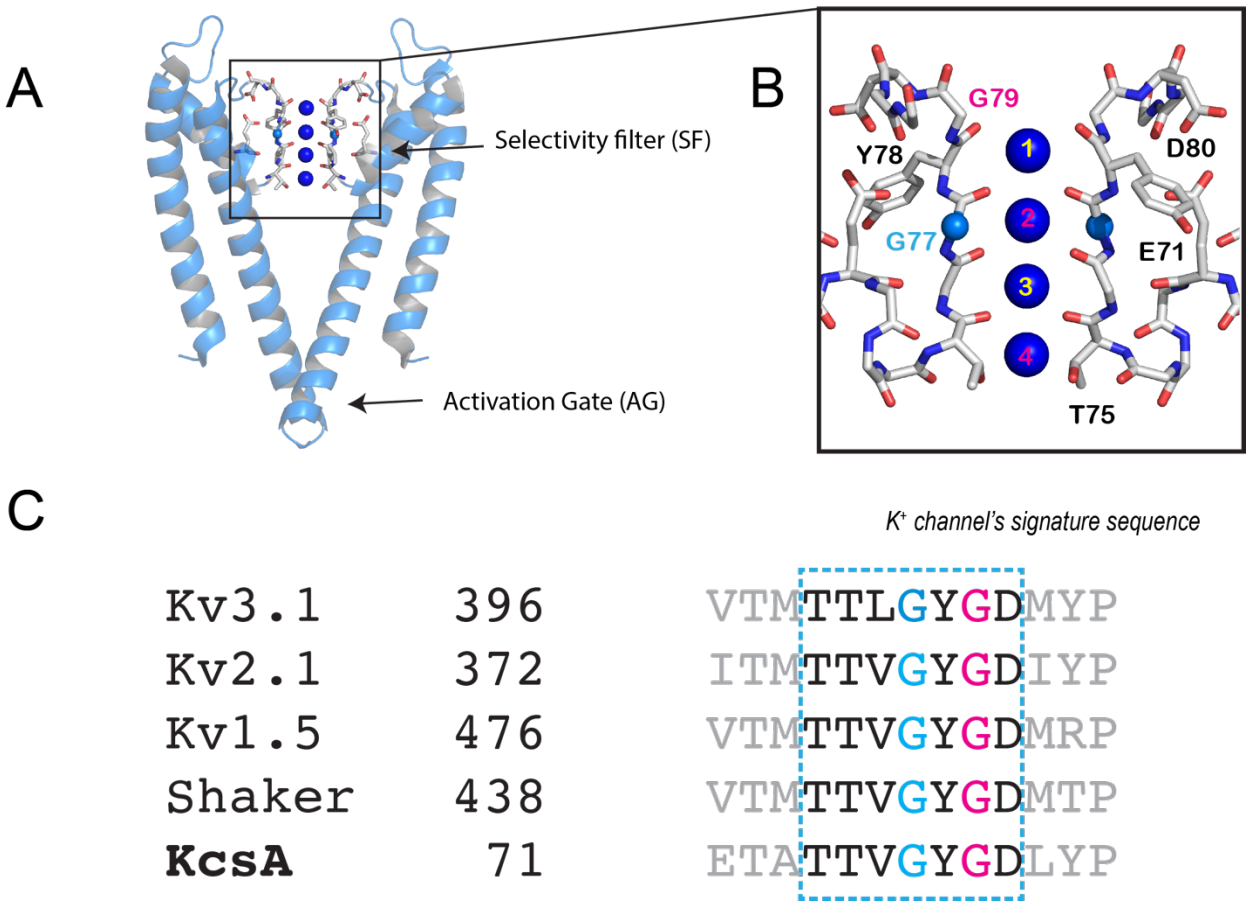

Fig. S1. The signature sequence of a K^+ channel. (A) A cartoon representation of KcsA in blue, only two subunits are shown for clarity, highlighting the position of the channel's selectivity filter coordinating four (4) K^+ ions. (B) An inset showing a magnification of the channel's selectivity filter, underlining the position of the backbone carbonyl groups from two subunits relative to the four coordinated K^+ ions. Amino acid residues E71, G79, G77 and T75 are determinant key players for the function of the channel. (C) A sequence alignment of 5 representative members of the K^+ channels super family indicating the position of the so called "*K^+ channel's signature sequence*". In blue and red are underscored the two highly conserved glycine residues (G77 and G79) within this stretch of amino acid residues. A perturbation analysis by mutagenesis of the Glycine 77 is the subject of this study.

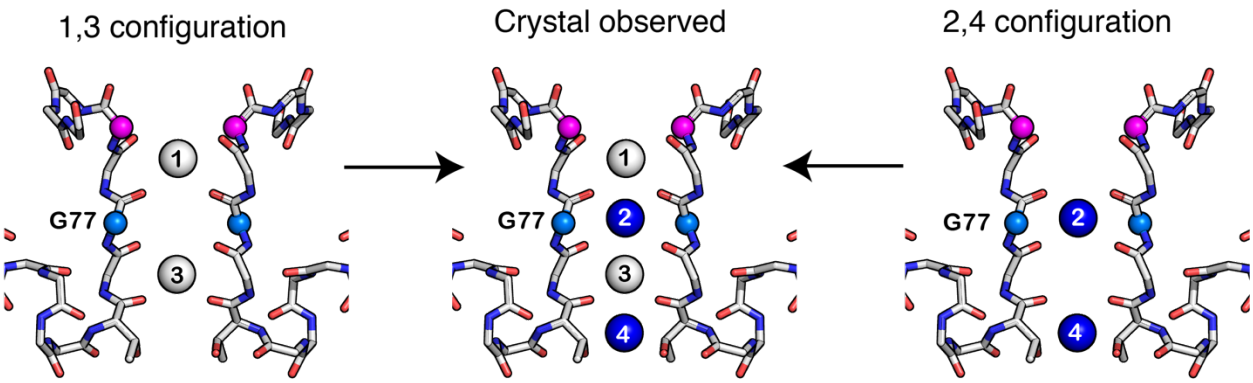

Fig. S2. Two-alternating K^+ ion bound configurations coexist in equilibrium within a K^+ channel selectivity filter. The 4 K^+ ions seen in the selectivity filter of all the crystal structures of K^+ channels solved to date (crystal observed, central panel) presumably corresponds to the superposition of two iso-energetic K^+ ion bound configurations. The 1,3 (left panel) and 2,4 (right panel) ion bound configurations are likely to have the same probability to exist in solution, hence within the crystal lattice, the superposition of these two alternating K^+ ions-water molecules configurations yields a structure that displays four bound K^+ ions.

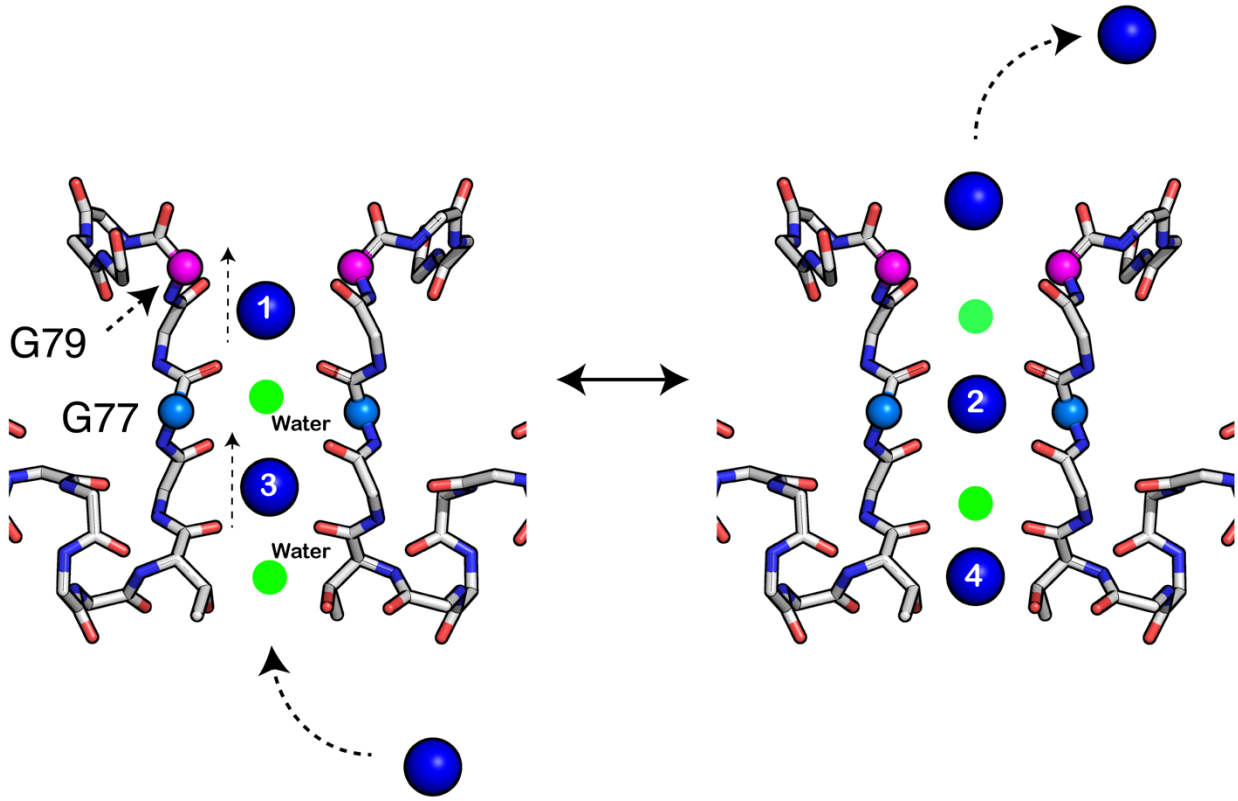

Fig. S3. Model for ion permeation according to the "canonical model". The panel on the left displays a K^+ channel selectivity filter with two K^+ ions, which are bound to the S1 and the S3 sites. In between these two bound K^+ ions, this model proposes the existence of a water molecule, which has never been seen in crystal structures. When a K^+ ion moves from the intracellular milieu and occupies the S4 site, strong electrostatic repulsion drives the ion bound at the S3 site to diffuse or to permeate to the S2 site. During every cycle of transport one water molecule is transported per each K^+ ion.

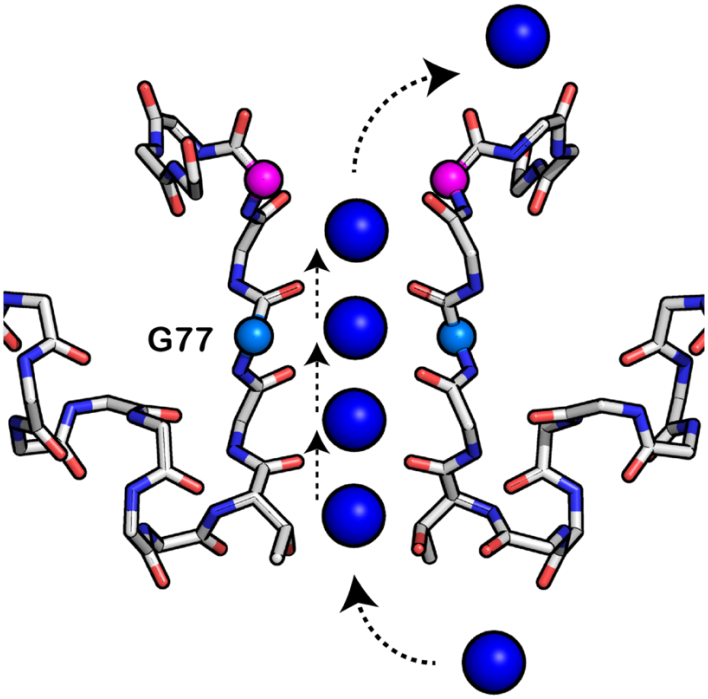

Fig. S4. An alternative model for ion permeation in K^+ channels, known as the hard-knock-on mechanism or direct Coulomb knock-on. The hard-knock-on mechanism assumes the maximum occupancy, at steady state conditions, of the K^+ ions bound to a K^+ channel selectivity filter. This means that at any given moment there are 4 K^+ ions coordinated in the filter and that not water molecules move per transport cycle. The K^+ ions interact directly exerting a very strong electrostatic repulsion, which drives the high-permeation rates characteristic of K^+ channels.

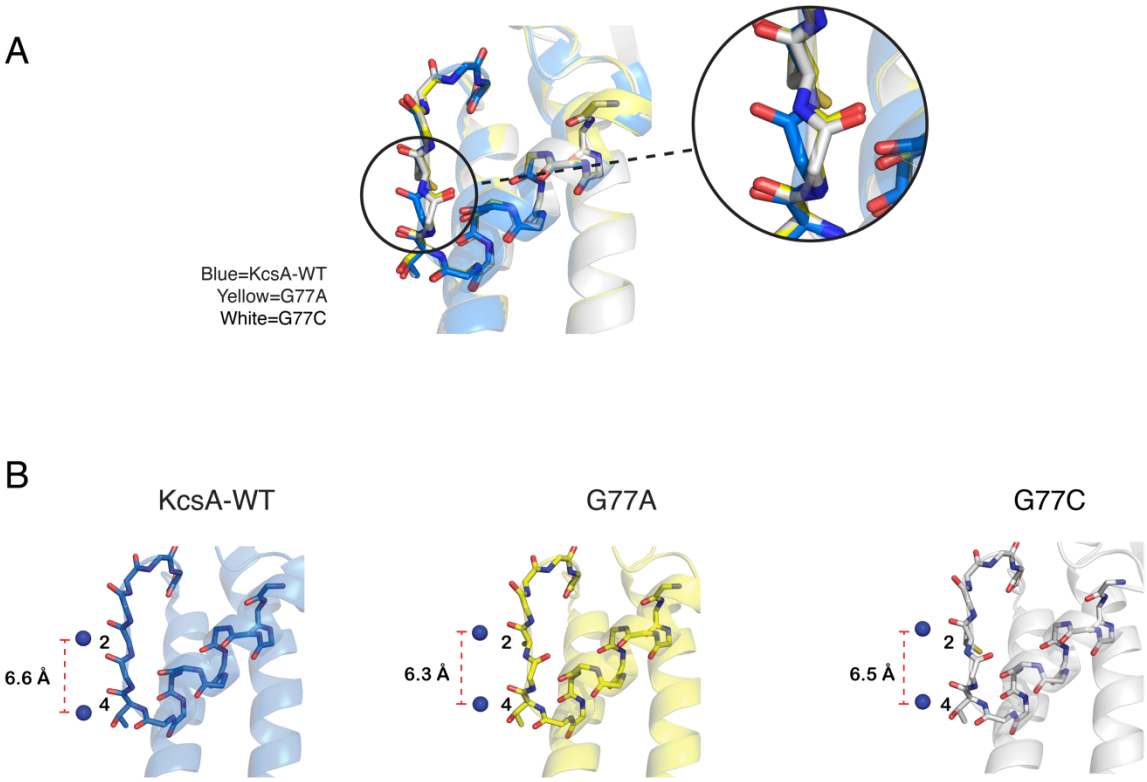

Fig. S5. **A structural alignment of the selectivity filters of the KcsA-WT and the G77A and G77C mutants.** (**A**) A structural alignment of the KcsA-WT (light-blue) and G77A (yellow) or G77C (white) selectivity filter mutants indicates that they have a very similar structure, r.m.s.d=0.20 and 0.32 Å for the selectivity filter atoms, respectively. The inset zoom into the main structural difference between the three selectivity filter structures, the back-flipping of the Val76 carbonyl group, which no longer coordinate a K^+ ion at the S3 site. (**B**) The panel highlights the interatomic distance differences between the K^+ ions bound to the S2 and the S4 sites of the selectivity filter of the KcsA-WT (blue), G77A (Yellow) and G77C (white).

References:

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