The conduction pathway of potassium channels is water free under physiological conditions

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Ion conduction through potassium channels is a fundamental process of life. On the basis of crystallographic data, it was originally proposed that potassium ions and water molecules are transported through the selectivity filter in an alternating arrangement, suggesting a “water-mediated” knock-on mechanism. Later on, this view was challenged by results from molecular dynamics simulations that revealed a “direct” knock-on mechanism where ions are in direct contact. Using solid-state nuclear magnetic resonance techniques tailored to characterize the interaction between water molecules and the ion channel, we show here that the selectivity filter of a potassium channel is free of water under physiological conditions. Our results are fully consistent with the direct knock-on mechanism of ion conduction but contradict the previously proposed water-mediated knock-on mechanism.

INTRODUCTION
Potassium (K⁺)-selective channels allow for the selective and efficient conduction of K⁺ ions across the cell membrane. They are involved in numerous physiological and pathophysiological processes (1). K⁺ channels have been characterized in great detail by numerous studies using electrophysiology, x-ray crystallography, molecular dynamics (MD) simulations, and spectroscopic approaches. The first atomic resolution structure of a K⁺ channel was determined around 20 years ago for the channel KcsA, explaining many phenomena observed experimentally before (2, 3). KcsA has become a textbook example of how a protein structure can explain protein function. A selectivity filter (SF), comprising four K⁺ binding sites, is formed in the homotetrameric arrangement by segments of the four subunits with the signature sequence TVGYG (Fig. 1A). This narrow pore allows for high selectivity and near diffusion–limited conduction rates. Despite these achievements, some elementary properties of the K⁺ channel remain under debate. For example, it was proposed early on that water molecules are cotransported with K⁺ ions and that the conduction through the SF occurs via alternating water molecules and ions in a mechanism that we refer to in the following as “water-mediated” knock-on (4). This mechanism involves a cycle of [W, K, W, K], where W refers to water and K is K⁺, and [K, W, K, W] states occupying the SF binding sites S1 to S4 (Fig. 1, A and B).

The water-mediated knock-on mechanism had been the accepted model in the field until 2014 when a study suggesting that ions are in direct contact with each other and that no water molecules are involved in the conduction process appeared (5). The proposed “direct” knock-on mechanism (Fig. 1C) (5) was first observed in computational electrophysiology simulations of KcsA and furthermore supported by reanalysis of x-ray crystallographic data, indicating that, also under crystallographic conditions, adjacent binding sites are simultaneously occupied by K⁺ ions. More recently, the latter result was confirmed by anomalous x-ray diffraction studies of the K⁺ channel NaK2K (6). NaK2K is a K⁺-selective double mutant (D66Y and N68D) of the nonselective channel NaK and a well-established model system for K⁺-selective channels (7, 8). In contrast, a recent investigation using two-dimensional (2D) infrared (IR) spectroscopy appeared to favor the water-mediated rather than the direct knock-on mechanism (9).

![Fig. 1. Comparison between water-mediated and direct knock-on ion permeation mechanisms.](https://advances.sciencemag.org/tb-media/10.1126/sciadv.aaw6756/figures/fig1.png)
However, more recently, it could be shown that the 2D IR data are also in accordance with the direct knock-on mechanism (10). Notably, all of these previous investigations were either based on x-ray analysis of detergent-solubilized proteins or MD simulations using the so-obtained structures.

At this stage, it would thus be highly interesting to directly investigate the role of water molecules in the SF experimentally under conditions which are close to the native state. This means, in the context of membrane proteins, to study them embedded in a lipid bilayer at room temperature and under physiological buffer conditions. Solid-state nuclear magnetic resonance (NMR) is a structural biology technique that has emerged over the past two decades and that is quite suitable for this task. Solid-state NMR has already been used successfully to investigate the structure and dynamics of several membrane proteins (11, 12), including the ion channels KcsA-Kv1.3 (13), wild-type KcsA (14, 15), VDAC (voltage-dependent anion channel) (16), M2 (17, 18), and NaK and NaK2K (19). Furthermore, it has been demonstrated that solid-state NMR can probe the interaction of the protein of interest
with water molecules, for instance by hydrogen/deuterium (H/D) exchange experiments, which are sensitive to processes occurring on long time scales, i.e., seconds to hours or even days (20). In addition, $^1$H spin diffusion experiments allow for the detection of bound water molecules, which interact with the protein on a shorter time scale of 1 to 100 ms, via magnetization exchange (21, 22). Here, we have investigated the K$^+$ channel NaK2K (23) by $^1$H-detected solid-state NMR (24–26) and MD simulations to investigate whether water molecules are present in its SF under equilibrium conditions, i.e., without an applied voltage.
RESULTS

For this purpose, we prepared a deuterated detergent–solubilized sample with a $^1$H, $^{13}$C, $^{15}$N-labeling scheme and subsequently exposed it to water. Deuterons of water-exposed residues can then exchange to protons in a process known as H/D exchange, similar to what has been observed before for KcsA (27), with a broader feature in the background. The sharp signals could be readily assigned with the help of a set of 3D experiments: (H)COCA(N)H, (H)CACO(N)H, (H)CANH, and (H)CONH (fig. S1 and table S1). We found that the assignable signals correspond to three regions of the protein (Fig. 2, C and D). The strongest and sharpest signals are from the extracellular part (G47-D52 and H)CONH (fig. S1 and table S1). We observe clear diagonal peaks for those residues in the 2D NH spectrum, we do observe diagonal peaks for those residues in the 2D NH spectrum, which dominates the relaxation behavior. In contrast, the amine protons of residues G65 to F69 build up relatively fast and then decay with a relaxation time that corresponds to rigid protons in the protein (see figs. S8 and S9 for all spin diffusion buildup plots before and after the washing procedure, respectively). Those residues are all in contact with two structural water molecules at the back of the SF and not by additional water molecules. Although T63 and V64 are barely visible in the 2D NH spectrum, we observe diagonal peaks for those residues in the spin diffusion experiments. No magnetization transfer from water is observed for either T63 or V64, which is consistent with the magnetization transfer from water to the SF residues only coming from the bound water behind the SF. Our results agree well with previous observations for KcsA, where G79 and L81 (corresponding to G67 and F69 in NaK2K) are in close proximity to the structural water molecules (table S3), i.e., G67 and D68 build up the fastest, followed by F69, Y66, and lastly G65. This order can only be explained if all the water magnetization comes from the two structural water molecules at the back of the upper SF and not by additional water molecules in the SF. If water molecules were present in the SF as well, as predicted by the water-mediated knock-on mechanism, then a different buildup behavior would be expected. For example, in this case, G65 and Y66 should have shown a buildup comparable to G67 and D68, which are in close proximity to the structural water molecules. Although T63 and V64 are barely visible in the 2D NH spectra, we observe clear diagonal peaks for those residues in the spin diffusion experiments. No magnetization transfer from water is observed for either T63 or V64, which is consistent with the magnetization transfer from water to the SF residues only coming from the bound water behind the SF. Our results agree well with previous observations for KcsA, where G79 and L81 (corresponding to G67 and F69 in NaK2K) are in close proximity to a bound water molecule (27, 30).

DISCUSSION

In summary, both the H/D exchange results and the water-to-protein magnetization transfer experiments show that there is no water in the central SF binding sites as long as K$^+$ ions are present at a physiological concentration, preventing water from entering the SF. We note that H/D exchange in the lower part of the SF (T63 and V64) would probably require both the presence of water in the SF and...
carbonyl flipping (29, 31) of the SF residues, as this would expose the SF amides to the pore. Another potential explanation could be that the H/D exchange of T63 and V64 comes from the water behind the SF because of structural rearrangements. However, such structural rearrangements would have been detected in crystal structures of NaK2K in the presence and absence of K+ ions (7, 8). Further, a recent solid-state NMR study on KcsA showed that mutations removing hydrogen bonds and increasing the water accessibility behind the SF did not result in H/D exchange in the lower part of the SF (32). In the same study, increased dynamics was shown to be related to carbonyl flipping in the SF. It would thus be interesting to investigate the proximity of water molecules to the SF residues in these mutants. Our data are consistent with increased dynamics during the washing procedure, which would allow water to enter the SF when no ions are present, resulting in H/D exchange in the lower part of the SF. Previous and current results from MD simulations show that both aspects, carbonyl flipping and water in the SF, are linked: If water is present in binding sites S1 to S4, then the corresponding SF residues are prone to frequent flips (figs. S10 and S11) (33). Therefore, we can interpret our results such that the K+ ions prevent H/D exchange in two ways: They stabilize the SF in a conductive, nonflipped conformation and at the same time prevent water binding at the K+ binding sites. As the upper part of the SF exhibits some degree of H/D exchange, it is likely that binding site S1 is partially occupied by water. In conclusion, our data strongly support the direct knock-on mechanism of ion conduction.

MATERIALS AND METHODS

Sample preparation

The NaK2K construct (NaK D66Y and N68D double mutant; plasmid provided by Y. Jiang) is missing the first 19 amino acids and has a C-terminal hexahistidine tag. The NaK2K construct was cloned from the original pQE60 vector into a pET28a expression vector using the NCO I and Hind III cleavage sites on both plasmids (protein sequence: MADKEFQVLFVLTILTLISGFYSTVEGLRPIDALYFSV-VTTTVGDGNFSPQTDFGKIFTIYIFGIGLVFGEHKLAVNY-VQLPSLSNLVRPGSKRSHHHHHH).

The protein was expressed in E. coli OverExpress C43(DE3) (Lucigen) using 2 liters of deuterated M9 medium. The cells were adapted to D2O conditions as described previously (24). Deuterated M9 medium (D2O 99.9% D; D214, Eurisotop) was supplemented with α-ketobutyric acid (60 mg/liter; CDLM-7318-PK, Cambridge Isotope Laboratories), d-glucose 13C (4 g/liter; 552151, Sigma-Aldrich), and lyophilized deuteriated 15N ammonium chloride (1 g/liter; 299251, Sigma-Aldrich). At an OD600 (optical density at 600 nm) of 0.8, the cultures were induced with 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside) and grown for 24 hours at 25°C. Additional labeled deuterated glucose (1 g/liter) and deuteriated 15NHCl (0.25 g/liter) were added to the cultures 6 hours after induction. Cells were harvested and resuspended with 10 mL of 100 mM M9 tris (pH 7.5), 100 mM NaCl, 1 mM MgCl, and protease inhibitor cocktail (11836170001, Sigma-Aldrich) per gram of cell pellet. The sample was lyzed using a microfluidizer (LM10, Microfluidics) with five cycles of working pressure of 15,000 psi. The membrane fraction was isolated by ultracentrifugation at 150,000 relative centrifugal force (rcf) for 2 hours and solubilized in 80 mL of solubilization buffer [50 mM tris (pH 7.5), 100 mM NaCl, and 40 mM DM (n-Decyl-β-maltoside; GLYCON Biochemicals)] at room temperature for 3 hours. The protein was batch-purified by immobilized metal affinity chromatography using 5 mL of bed volume of TALON Superflow beads (GE Healthcare Life Sciences). Protein concentrations were determined using Bradford reagent supplemented with α-cyclodextrin (5 mg/mL; AppliChem), and 5.4 mg of protein was mixed with E. coli total lipid extract (100500, Avanti Polar Lipids) in a ratio of 2:1 (w/w). H/D back exchange and proteoliposome formation were performed by dialysis for 8 days at 100-fold dilution against 500 mL of 100% H2O with 20 mM tris (pH 8.0) and 50 mM KCl, followed by ultracentrifugation at 300,000 rcf for 4 hours at 4°C. Half of the pellet was removed for NMR experiments (sample A, K+). The second half of the sample was resuspended in 1 mL of 20 mM tris (pH 8.0) and kept rotating at 4°C. The buffer was refreshed twice over the course of 14 days, each time after ultracentrifugation at 150,000 rcf for 30 min at 4°C.

From this, half of the sample was removed for NMR experiments (sample B, no ions). The other half of the sample was resuspended in 1 mL of 20 mM tris (pH 8.0) with 50 mM KCl. The sample was kept rotating at 4°C for 8 days, during which time the buffer was refreshed once and used for NMR experiments (sample C, washed to K+).

Solid-state NMR

Sample A (K+) was packed into a 19-mm solid-state NMR rotor (Bruker BioSpin), and 1 μL of D2O was added to enable deuterium locking. To prevent leakage, a spacer was added between the bottom cap and the sample, and the drive cap was glued using a silicon-based glue. The washed samples [sample B (no ions) and sample C (washed to K+)] were packed into 13-mm rotors (Bruker BioSpin), and the drive caps were glued as well to prevent leakage. Experiments on sample A were recorded at 40-kHz magic angle spinning (MAS) using a four-channel (1H, 13C, 15N, and 2H) 1.9-mm probe (Bruker BioSpin) on a spectrometer with an external magnetic field strength corresponding to 900 MHz (Bruker BioSpin). Four CP-based 3D experiments [(H)CANH, (H)CONH, (H)CACO(N)H, and (H)COCA(N)H] were recorded for backbone chemical shift assignments of sample A, closely following earlier work by Fricke et al. (24). The assigned chemical shifts confirm protein folding into a single dominant conformation. Spin diffusion [H(H)NH] experiments with mixing times of 5, 12, 20, 35, and 100 ms were recorded using a 3D version of the standard (H)NH experiment, with the addition of a 1H acquisition period followed by a spin diffusion sequence on the 1H channel (90° pulse, spin diffusion mixing time, 90° pulse) before the CP-based transfer to 15N. A recycle delay of 1 s and 90° pulses of 83.3, 50, and 35.7 kHz for 1H, 13C, and 15N, respectively, were used for all experiments acquired on sample A. Experiments on samples B and C were recorded at 58- to 60-kHz MAS using a three-channel (1H, 13C, and 15N) 1.3-mm probe (Bruker BioSpin) on a spectrometer with an external magnetic field strength corresponding to 600 MHz (Bruker BioSpin). For sample B, a 3D (H)CANH spectrum was recorded, and for sample C, a 3D (H)CANH and (H)CONH spectra were recorded to identify new peaks compared to sample A. Spin diffusion (H(H)NH) experiments with mixing times of 20, 40, and 60 ms were recorded on sample C. A recycle delay of 1 s and 90° pulses of 100, 50, and 35.7 kHz for 1H, 13C, and 15N, respectively, were used for all experiments acquired on samples B and C. The chemical shifts were referenced to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) using an external reference. The sample temperature was kept at 25°C, as measured from the 1H chemical shift of water. Proton detection with a 30-ms acquisition time was used for all experiments.
Further experimental parameters for the 3D experiments are listed in tables S4 to S7.

Data analysis

All NMR data were processed using TopSpin 4 and analyzed using NMRFAM (National Magnetic Resonance Facility at Madison)–Sparky (34) and CcpNmr Analysis (35). High-resolution figures based on 3D structures were produced using PyMOL and UCSF (University of California, San Francisco) Chimera (36).

MD simulations

For calculations of the solvent accessible surface area (SASA) and backbone NH hydrogen protection, as well as for the time traces showing the correlation between the water presence in binding site S3 and carbonyl flipping of V64, MD trajectories of NaK2K, as reported in a recent publication (10), were used. In short, the high-resolution structure of the open-state NaK2K (PDB ID: 3ouf), with the F92A mutation, was inserted in a POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) membrane, surrounded with explicit water and K+ and Cl– ions, and simulated for 6.5 and 10.5 μs with the Amber99sb and CHARMM36m force fields (37–40) [labeled as AMBER (Assisted Model Building with Energy Refinement) and CHARMM (Chemistry at Harvard Macromolecular Mechanics) in the current publication], respectively, under an applied voltage of 300 mV. All the simulation details can be found in the previous publication (10). SASA patterns were calculated using the gmx sasa program, with a probe radius of 0.14 nm, and averaged over all trajectories. The backbone NH hydrogen bond protection patterns report the fraction of frames in which a given NH participated in hydrogen bonding with another protein residue and were calculated using the gmx hbond program over all trajectories.

To calculate the average occupancy of each site at a voltage of 0 mV, 50 frames per force field were randomly selected from the applied voltage simulations and further simulated for additional 200 ns per frame without any voltage, resulting in 10 μs of sampling per force field. All remaining simulation parameters and details were identical to those used for simulations with an applied voltage. The occupancy of each site was calculated with a custom Fortran code.

For simulations and analyses, the GROMACS package was used (41–47), together with GromacsWrapper (48). Visualizations and movies were made with visual molecular dynamics (VMD) (49).

Fig. S10. The relationship between carbonyl flipping and water presence at S3 in MD simulations under an applied voltage performed with the AMBER force field.

Fig. S11. The relationship between carbonyl flipping and water presence at S3 in MD simulations under an applied voltage performed with the CHARMM force field.

Table S1. Chemical shift assignments of deuterated NaK2K, in the presence of 50 mM K+, based on 100% H2O back-exchanged uniformly (2H, 13C, and 15N)-labeled NaK2K.

Table S2. Chemical shift assignments of NaK2K SF residues, in the presence of 50 mM K+, based on 100% H2O back-exchanged uniformly (2H, 13C, and 15N)-labeled NaK2K.

Table S3. Distances (Å) to amide protons of the SF in NaK2K (PDB ID: 3ouf) from the closest water molecule and binding sites (S1 to S4).

Table S4. Experimental parameters for 3D (H)CACO(N)H experiments acquired at 25°C on 100% H2O back-exchanged uniformly (2H, 13C, and 15N)-labeled NaK2K.

Table S5. Experimental parameters for 3D (H)COCA(N)H experiments acquired at 25°C on 100% H2O back-exchanged uniformly (2H, 13C, and 15N)-labeled NaK2K.

Table S6. Experimental parameters for 3D (H)CACO(N)H and (H)COCA(N)H experiments acquired at 25°C on 100% H2O back-exchanged uniformly (2H, 13C, and 15N)-labeled NaK2K.

Table S7. Experimental parameters for 3D H(H)NH spin diffusion experiments acquired at 25°C on 100% H2O back-exchanged uniformly (2H, 13C, and 15N)-labeled NaK2K.

Movie S1. Typical K+ ion permeation process through NaK2K, observed in a 20 ns trajectory segment of MD simulations under an applied voltage.

REFERENCES AND NOTES


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Acknowledgments: The plasmid of the NaK2K construct (NaK66Y and N680 double mutant) was provided by Y. Jiang. Funding: This work was supported by the Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), the Max Planck Society, the Deutsche Forschungsgemeinschaft (DFG FOR 2518 ‘Dynion’ Project P3 to H.S. and Project P5 to W.K. and B.L.d.G.), and the European Research Council (ERC Starting Grant [grant agreement no. 337490] to A.L.). Author contributions: K.H., C.S., D.M., and S.L. produced the protein samples. C.O., V.C., and K.H. recorded the solid-state NMR spectra. W.K. and B.L.d.G. performed the MD simulations. C.O., K.H., W.K., A.L., H.S., and B.L.d.G. analyzed the data. A.L. conceived this study. C.O., K.H., and A.L. wrote the manuscript. All authors commented on the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The NaK2K chemical shifts have been deposited in the Biological Magnetic Resonance Data Bank (BMRR) under accession code 27746. Raw data may be requested from the authors.

Submitted 15 January 2019
Accepted 21 June 2019
Published 31 July 2019
10.1126/sciadv.aaw6756

Citation: Öster, C., Hendriks, W., Kopec, V., Chevelkov, C., Shi, D., Michl, S., Lange, H., Sun, B. L. de Groot, A. Lange, The conduction pathway of potassium channels is water free under physiological conditions. Sci. Adv. 5, eaaw6756 (2019).
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Sci Adv 5 (7), eaaw6756.
DOI: 10.1126/sciadv.aaw6756