Pore Hydration States of KcsA Potassium Channels in Membranes*

Joseph R. Blasic†, David L. Worcester§**, Klaus Gawrisch††, Philip Gurnev***, and Mihaela Mihailescu‡

From the †Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, Maryland 20850, the §Department of Physiology and Biophysics, University of California, Irvine, California 92697, the ‡National Institute of Standards and Technology, Center for Neutron Research, Gaithersburg, Maryland 20899, the ¶Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland 20892, the **Physics Department, University of Massachusetts, Amherst, Massachusetts 01003, and the ***National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Background: Detecting functionally important wet spots in ion channels is an experimental challenge.

Results: The water distributions in two different channel proteins embedded in membranes were determined and quantified with neutron diffraction.

Conclusion: The water profiles report on the structural conformation and functional state of channels in membranes.

Significance: This study clearly shows the extent of hydration of membrane-embedded channels, important for channel gating.

Water-filled hydrophobic cavities in channel proteins serve as gateways for transfer of ions across membranes, but their properties are largely unknown. We determined water distributions along the conduction pores in two tetrameric channels embedded in lipid bilayers using neutron diffraction: potassium channel KcsA and the transmembrane domain of M2 protein of influenza A virus. For the KcsA channel in the closed state, the distribution of water is peaked in the middle of the membrane, showing water in the central cavity adjacent to the selectivity filter. This water is displaced by the channel blocker tetrabutylammonium. The amount of water associated with the channel was quantified, using neutron diffraction and solid state NMR. In contrast, the M2 proton channel shows a V-shaped water profile across the membrane, with a narrow constriction at the center, like the hourglass shape of its internal surface. These two types of water distribution are therefore very different in their connectivity to the bulk water. The water and protein profiles determined here provide important evidence concerning conformation and hydration of channels in membranes and the potential role of pore hydration in channel gating.

There is little experimental evidence or general consensus regarding the presence and dynamics of water in the nonpolar cavities found in channel proteins (1). These cavities serve as sites for uptake and transfer of both nonpolar and polar molecules, including ions, across membranes (2). In many ion channels, a central cavity is formed between membrane-spanning subunits in the pore domain that contains the ion conduction pathway and selectivity filter (3–5). Although the water in the cavity is thought to be essential for conduction, the presence of water is hard to reconcile with the hydrophobic surface of the cavity. Water in a hydrophobic cavity is like a small water droplet and by the Kelvin equation has increased vapor pressure caused by surface tension, making the droplet unstable to evaporation. Well known examples of this effect in biology are the gas vesicles of marine algae (6). These are formed by a protein wall with an inner hydrophobic surface and contain only gas of the surrounding medium. To overcome Kelvin equation instability, water in the hydrophobic cavities of ion channels must therefore depend on some external hydrogen bonding, the presence of an ion, proximal electric dipoles, and/or small enough size that surface tension is no longer an appropriate parameter.

Water in ion channel cavities is difficult to detect, and whether the channels are empty in the closed state is an open question. In vivo functional studies indicated that the gating of voltage-gated ion channels includes both a voltage-dependent component and a solvent-dependent component (7). Molecular dynamics simulations predicted that the solvent-dependent gating component, also referred to as “hydrophobic gating” (8), occurs via a dehydration (“dewetting”) transition that can drive the central cavity to be emptied and collapsed upon closing (9, 10). Experimentally, detecting hydrophobic gating transitions faces significant challenges: (i) only water molecules that are sufficiently ordered are, typically, visible in x-ray crystal structures, and (ii) structures for only a few voltage-gated channel conformations (usually, open) are available (5, 11–13). To date, the bacterial potassium channel KcsA3 from Streptomyces lividans is the best characterized model for pore domains of voltage-gated channels, in a closed conformation (3, 4, 14). How-

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‡To whom correspondence should be addressed: Inst. for Bioscience and Biotechnology Research, University of Maryland, 9600 Gudelsky Dr., Rockville MD 20850. Tel.: 240-314-6123; E-mail: emihailescu@ibbr.umd.edu. This is an Open Access article under the CC BY license.

The abbreviations used are: KcsA, potassium crystallographically sited activation channel from the soil bacteria S. lividans; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; DDM, n-dodecyl β-D-maltoside; C/L, channel to lipid; TBA, tetrabutyl-ammonium; SLD, scattering length density; MAS, magic angle spinning; DLPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; fwhm, full width at half maximum.
ever, its conformation, hydration state, and interactions with lipid membranes have remained largely unexplored experimentally, limiting our knowledge of the role of water in channel gating mechanisms. Neutron diffraction is the key technique that can address these problems for the following reasons: (i) isomorphous hydrogen-deuterium replacement does not compromise the original structure and is a highly sensitive probe, because of the large difference in scattering length between the two isotopes, and (ii) deuterium atoms in the bilayer can be detected with up to a tenth of an Angstrom accuracy, despite thermal disorder (15, 16). Neutron diffraction is used here, together with solid state NMR to detect and quantify water in KcsA channels reconstituted in lipid membranes with different conditions, including displacement of channel water by tetrabutyl-ammonium. Comparison is made of the water distributions of KcsA with that of the tetrameric M2 (matrix protein 2, integral membrane protein in the viral envelope of influenza A virus) proton channel and demonstrates the structural connection between channel morphologies and the corresponding water profiles in phospholipid membranes.

**Experimental Procedures**

*KcsA Expression and Purification*—KcsA was expressed and purified as described in Ref. 17 but substituting Terrific Broth (Thermo-Fisher) for Luria-Bertani. The KcsA C-terminal domain was removed by digestion with chymotrypsin at a ratio of 1:200 enzyme to KcsA, at 37 °C for 2 h. Concentrations of KcsA were determined by UV absorbance (ε = 34,950 M⁻¹ cm⁻¹ at 280 nm) after dialysis to remove imidazole. Deuterium-labeled KcsA was produced by growing *Escherichia coli* in 70% ²H₂O in M9 minimal media, whereas ¹⁵N-labeled KcsA was expressed using ¹⁵N ammonium chloride as nitrogen source. The degree of labeling was determined by MALDI-TOF mass spectrometry.

**Reconstitution in Lipid**—Equimolar mixtures of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (Avanti Polar Lipids) were used. Approximately 4 mg of lipid mixture was solubilized in 1 mM n-dodecyl β-D-maltoside (DDM) (Anatrace) and mixed with KcsA at several different channel to lipid (C/L) molar ratios (Table 1). After 30 min, samples were rapidly diluted 20 fold with water, and the proteoliposomes were pelleted by centrifugation at 100,000 x g for 90 min. The supernatant was removed, and the pellets dispersed in 10 ml of water and then recovered by centrifugation as before. This was repeated twice, and the final pellet was dispersed in 400 μl of water. Samples for neutron diffraction were spotted on clean glass microscope coverslips and allowed to dry at room temperature. Several separate preparations yielded roughly 25% recovery of lipid material at channel to lipid (C/L) ratios higher by 15–20% compared with the original mixtures (Table 1). Samples prepared at the same target C/L ratio yielded highly reproducible compositions, neutron diffraction repeat spacings, and water distributions (see “Results”).

**Formation of KcsA Tetrabutyl-Ammonium (TBA) Complex**—5 mM TBA was added to purified KcsA in 1 mM DDM. The pH was reduced to ~4 by the addition of 1 M HCl, and the acidified solution was incubated at room temperature with rocking for 10 min and then neutralized by the addition of an equal volume of 1 M NaOH. Samples were then mixed with lipid and washed extensively. POPC was used for the reconstitution of KcsA + TBA and the control sample (without TBA). Inclusion of POPG lipid was avoided to prevent issues of phase separation between POPC and POPG when samples were handled and low pH.

**Quantification of Protein to Lipid Ratio**—To quantify lipid recovery, the fluorescent lipid lissamine rhodamine B 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids) was added to the mixture of POPC/POPG at a ratio of 1:6,000. Prior to fluorescence measurements, an equal volume of 6% SDS (w/v) was added to disperse the lipid. Lipid recovery was assessed based on fluorescence compared with a standard curve. Recovered protein concentration was determined by UV absorbance, as above. The samples contained 2.5 mol% of DDM compared with lipid as measured by ¹H NMR. Deuterium contents in protein or lipid were determined by MALDI-TOF and electrospray mass spectrometry.

**Functional Assays**—Assays were performed in 50 mM Tris, pH 4, for channel activity and pH 7 as control. Prior to assay, the potassium benzoate analog (PFBI) (Life Technologies) was added to the buffer to a final concentration of 10 μM. Potassium-loaded vesicles were added, and fluorescence was measured (excitation, 340 nm; emission, 500 nm). Ion selectivity control experiments were performed in the same way using liposomes formed in 200 mM NaCl solution and the sodium benzoate analog indicator (SBFI) (Life Technologies).

**Single Channel Recordings**—Bilayer lipid membranes were formed from 1:1 equimolar mixture of POPC and POPG using the monolayer opposition technique (18) on a round hole in a film, separating two compartments of an experimental chamber (*cis* and *trans*). Hexadecane (Sigma) was used for partition pretreatment. The bilayer bathing solution contained 200 mM KCl buffered with MES at pH 4. KcsA was added to the *cis*-side in the nearest vicinity of the membrane with a sharp tip, and the membrane was reformed several times after KcsA addition. This method usually yielded stable reconstitution of several KcsA channels. Application of voltage and registration of transmembrane currents were achieved with a pair of Ag/AgCl electrodes with 2 M KCl/agarose bridges, connected to an Axopatch 200B patch clamp amplifier (Molecular Devices). The applied transmembrane voltage was defined as negative if potential was higher on the *cis*-side of the chamber. The amplifier signal was in-line filtered by a built-in 8-pole Bessel filter and saved into computer memory with a 5-KHz sampling fre-

### TABLE 1

| Target C/L (molar) | Measured average C/L (molar) | Change (%) |
|-------------------|------------------------------|------------|
| 1/150             | 1/123                        | 18         |
| 1/300             | 1/239                        | 20         |
| 1/300             | 1/231                        | 23         |
| 1/600             | 1/492                        | 18         |
| 1/600             | 1/460                        | 23         |
| 1/1200            | 1/1041                       | 13         |
| 1/1200            | 1/1050                       | 12         |

*Measured versus target C/L ratios in lamellar lipid samples.* The average values were determined by measuring a few aliquots extracted from each KcsA sample.
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Neutron Diffraction—After KcsA reconstitution in liposomes, lamellar samples containing roughly 1 milligram of lipid (1000–1500 bilayers) and a few hundred micrograms of protein were produced by fusing the proteoliposomes on the surface of thin glass coverslips and allowing the bulk water to slowly evaporate. Samples were then maintained at 93% relative humidity throughout the measurements, in the vapor of saturated KNO$_3$ salt solutions. Neutron diffraction measurements were performed using the instrument MAGIK at the National Institute of Standards and Technology (Gaithersburg, MD). Diffraction from aligned lamellar samples, probing the axis perpendicular to the bilayer plane (z axis) was used to determine one-dimensional scattering length density (SLD) profiles of the bilayer membranes (Equation 1).

Data Treatment—Structure factors $F(h)$ were obtained as the square root of the integrated peak intensities, corrected for background, Lorentz factors, absorption, and extinction effects, as described previously (19–21). Structure factors were converted, by Fourier synthesis, into a projection of the SLD of the bilayer onto the normal, according to the following equation.

$$
SLD(z) = A \sum \omega_i \rho_i + \frac{2}{d} \sum \varphi_i |f(h)| \cos \left( \frac{2\pi h z}{d} \right) 
$$

(Eq. 1)

The first term in Equation 1 is the average scattering length density of the unit cell: one lipid plus all the other components (water, protein) that contribute their scattering length density $\rho_i = b_i/(A'd/2)$ and their molar fraction ($\omega_i$) to the repeat unit of area per lipid (A) and height (d). The second term describes the fluctuations in SLD about the average, where $f(h) = k^* F(h)$ are the structure factors, calibrated by an amplitude factor ($k$), to reflect the composition of the bilayer (20). For a centrosymmetric system, the phase factors ($\varphi_i$) can be $+1$ or $-1$. They were determined here by H$_2$O/2H$_2$O contrast methods (19). The area per lipid (A) varies with composition, hydration, and temperature and was not determined explicitly in these experiments. For convenience, all profiles are determined here on an absolute relative scale (22) that leaves (A) undetermined.

Profile Amplitude Calibration—To determine a calibration (scale) factor ($k$) for each set of structure factors, we employed deuterium contrast in samples of known composition. Two homologous samples, containing either protonated (H-POPC) or chain deuterated, 1-palmitoyl-1,2-oleoyl-sn-glycero-phosphocholine (D31-POPC) lipid components were measured under constant conditions. The measured compositions of the two homologous samples were: POPC:POPG:DDM:KcsA = 0.486:0.486:0.024:0.004, and D31-POPC:POPG:DDM:KcsA = 0.236:0.250:0.486:0.024:0.004, at 1/235 channels to lipids, molar ratio. In addition, each of the two samples were measured in a series of H$_2$O/2H$_2$O water contrast conditions (100:0, 80:20, 50:50, and 0:100 H$_2$O:2H$_2$O), ensuring a sufficiently large data set for the analysis of the diffraction data (supplemental Tables S1 and S2). The differences in the resulting structure factors from (deuterated versus protonated) sample analogues ($\Delta f = k^{D31} F^{D31} - k^{H} F^{H}$) were converted into the deuterium difference profiles ($\Delta SLD$; Equation 2) to identify the distribution of the deuterated component in the bilayer (15), D31 in this case.

$$
\Delta SLD^{D31}(z) = \left[ A \sum \omega_i (\rho_i^{D31} - \rho_i^{H}) + \frac{2}{d} \sum k^{D31} F_i^{D31} - k^{H} F_i^{H} \right] \cos \left( \frac{2\pi h z}{d} \right)
$$

(Eq. 2)

The calibration factors ($k^{H}, k^{D31}$) were determined by requiring that the deuterium signal from the labeled hydrocarbon chains cancels in the aqueous phase ($\Delta SLD^{H/D31}(z \equiv d/2) = 0$) and performing a simultaneous analysis of the deuterium signal (D31 and $^2$H$_2$O) from the two isomorphous samples. The uncertainties in the calibration factors were determined from the standard deviations in the structure factors (supplemental Tables S1 and S2) and including the uncertainties in sample compositions ($\omega_i$; Equation 1), where the major contributors are the protein to lipid ratios (Table 1).

$^2$H$_2$O Profile Analysis and Modeling—We used the difference in the calibrated structure factors (supplemental Tables S1 and S2) to find a minimal set of Gaussians that would best fit the data. The Fourier inverted Gaussian model (Eq 3) was compared with the experimental structure factors (supplemental Tables S1 and S2) via a $\chi^2$ minimization procedure,

$$
\Delta F_{\text{water}} = \sum a_i e^{-\left( \frac{\pi z - \sigma_i}{\sigma_i} \right)^2} \cos \left( \frac{2\pi h z}{d} \right)
$$

(Eq. 3)

where $Z_i$ indicates the mean positions of the Gaussian distributions, $\sigma_i$ indicates their standard deviations, and $a_i$ indicates their amplitudes. The procedure was applied simultaneously for all data sets, for both the H and D31 samples.

NMR Measurements—Experiments were conducted on a AV800 NMR spectrometer (Bruker Biospin, Inc., Billerica, MA) equipped with a $^1$H/$^{13}$C/$^2$H$_2$O-gr-HR-MAS-800-SB-13.44 probe for determination of water content and a $^1$H/$^{13}$C/$^{15}$N-PH-MAS-800-SB-13.44 probe for determination of hydrogen to deuterium exchange. Samples were deposited as thin film on the inner wall of a ZrO$_2$ 4-mm rotor by rotation of the horizontally oriented rotor containing KcsA/lipid proteoliposomes in H$_2$O. A stream of air was applied to remove excess water. This resulted in the formation of stacked bilayers on the rotor wall with a thickness similar to neutron experiments. The samples were then equilibrated in H$_2$O or $^2$H$_2$O vapor of a saturated KNO$_3$ salt solution (93% relative humidity, 21.5 ± 0.5°C). After hydration, rotors were sealed with a Kel-F cap, and the water content was measured by $^1$H MAS NMR at a MAS frequency of 5 kHz. Integral intensity of the water resonance at 4.8 ppm was determined by spectral deconvolution. Water content is reported relative to intensity of the choline resonance at 3.25 ppm. Measurements on $^{15}$N-KcsA/POPC/POPG samples were conducted to determine the amount of hydrogen to deuterium exchange in KcsA. The $^{15}$N MAS NMR spectra were recorded at ambient temperature by $^1$H-$^{15}$N cross-polarization, a MAS frequency of 5 kHz, a 3.7-μs $^1$H 90° pulse, 1-ms $^1$H-$^{15}$N cross-polarization at...
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$\gamma / 2 \pi B_1 = 40$ kHz with a 100–80% linear ramp on the $^1$H channel, $(\gamma / 2 \pi)B_1 = 62$ kHz $^1$H decoupling during $^{15}$N detection using a spinal-64 sequence, 77,000 scans, 1024 data points at a dwell time of 5 $\mu$s, and 1-s delay time between scans.

**M2 Channel Sample Preparation**—Oriented lipid multilayers with and without M2 (residues 22–46) peptides were prepared by slow evaporation of solvent on thin glass microscope slide coverslips at temperatures of about 37 °C. Solvents were ethanol/water mixtures, usually 3/2 by volume. Thin samples of about 1000 layers or less gave distinctly better diffraction, especially with annealing at high humidity and 37 °C. Multilayers prepared this way are centrosymmetric, with channels oriented both ways across the membrane. Similar results were obtained with 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine or POPC, of which DLPC gave best diffraction, based on mosaic spread and resolution. Peptide/lipid ratios were high, 1/3 or 1/4 molar, so that channel features would be prominent in profiles.

**Results**

**Structural and Functional Characterization of KcsA in Lipid Samples**—To obtain structural information from KcsA/lipid samples amenable for neutron diffraction measurements, we produced oriented lamellar samples of lipid bilayers containing KcsA (Fig. 1) starting from proteoliposome solutions. The reconstituted channels were found to adopt a structurally stable tetrameric state (Fig. 2a). We tested the viability of the tetrameric channels in proteoliposomes using our bulk functional assay (“Experimental Procedures”) (Fig. 2, b–d). At neutral pH, KcsA was demonstrated to adopt a closed, stable conformation, with a very low open probability, even when the intracellular C-terminal amino acids were removed (23, 24). Our preparations of both C-terminal truncated KcsA (ctd-KcsA: amino-acids 126–160 removed) and full-length (fl-KcsA) in proteoliposomes resulted in a functional population of channels that are conductive at pH 4 and nonconductive at pH 7 (Fig. 2, a–d). The selectivity for conduction of $K^+$ versus $Na^+$ ions was also verified (data not shown). Lamellar samples (Fig. 1a) were obtained from proteoliposomes containing ctd-KcsA at neutral pH. For further characterization, we performed single channel recordings of KcsA extracted from the lamellar samples by resuspension in buffer solution. The data show that channels were viable, displaying characteristic conductance upon incorporation in planar black lipid membranes (Fig. 2, e–g). Measured single-channel current-voltage curves match well with KcsA currents obtained by LeMasurier et al. (25) (Fig. 2f). Neutron diffraction was then performed on fully characterized samples, under controlled hydration and temperature, maintaining the bilayers in the biologically relevant (Lα) phase. Although thermal disorder limits the amount of observable Bragg intensity to 4 or 5 orders of diffraction, this does not limit the amount of information that can be extracted by deuterium difference analysis, as will be described below. Unique sets of diffraction intensities can be clearly distinguished depending on deuteration (Fig. 3, a and b). Despite the high protein concentration, the quality of the lamellar samples was remarkably high, as demonstrated by examining both out of plane (Fig. 3, a and b) and in-plane diffraction data (Fig. 3c) and the mosaic spread of the oriented samples (Fig. 3d). The data show that the samples were homogeneous with no indications of in-plane crystallization of the protein or domain formation. The repeat spacing, $d$ (Fig. 1a), was identical (51.4 ± 0.1 Å) for KcsA samples of the same composition (Fig. 3, a and b), but 1.8 Å smaller compared with neat bilayers (not shown), indicating a reorganization of the lipid and water to accommodate the channel proteins.

**Water Distribution in Lipid Membranes with KcsA**—The one-dimensional SLD profile of the bilayer with KcsA was determined from corrected and calibrated structure factors for various $H_2O$/$^2H_2O$ contrasts (supplemental Table S1). The typical bilayer SLD profile in $H_2O$ shows peaks associated with the phospholipid headgroups and a trough across the hydrocarbon region (Fig. 4a). Gradual in situ exchange of $^2H_2O$ for $H_2O$ results in increasing SLD in those regions of the bilayer where water is present (Fig. 4a). Deuterium difference analysis singles out the water distribution and exchangeable hydrogens (Fig. 4b), all other contributions being cancelled. Both protein orientations are equally present in the lamellar samples, preserving the centrosymmetric symmetry of the membrane (Fig. 1a). Two major water peaks, symmetrically located at −25.7 and 25.7 Å, are due to water presence around the phospholipid headgroups and in the interbilayer space of the stack.
includes contribution from the hydrated segments of the protein that are exposed on both surfaces of the membrane. The deuterium peak in the middle of the hydrocarbon region is due to structural water and exchangeable protons on the protein surface in the internal cavity. When we examined the morphology of uniformly deuterated KcsA in bilayers (Fig. 5), we found that, in contrast to the $^2$H$_2$O profile, the density distribution of the protein is lower at the center of the bilayer, where the transmembrane helices stretch across the bilayer, and higher at the bilayer surfaces because of the additional density contributions from the pore helices and selectivity filter (Fig. 1b). Remarkably, the $^2$H$_2$O distribution in the hydrocarbon region does not follow closely the density profile of the protein, as would be expected if all $^2$H$_2$O signal came from exchangeable protons. The distinct profile of the water in the cavity region indicates that the channel cavities are filled with water.

Channel Blocker TBA Displaces Water from the Internal Cavity—Quaternary ammonium ions (TBA), as well as other substituted amine compounds were found to block the channel conductance by accessing the pore mainly from the intracellular side of the membrane when the channel is open (26, 27). Crystal structures of KcsA with bound TBA show that the blocker molecule binds in the cavity, close to the entrance to the selectivity filter (27). We added TBA to detergent-solubilized KcsA in acidified buffer (pH ~4) to open the KcsA gate and allow TBA access to the binding pocket (see “Experimental Procedures”). Our functional assays performed on liposomes prepared from POPC with KcsA/TBA show that potassium ion flux is essentially absent in the presence of TBA (Fig. 2d). Two samples prepared in parallel (KcsA/POPC and KcsA+TBA/POPC) were measured by neutron diffraction. The two samples displayed similar bilayer profiles, with the largest difference at the bilayer center: the trough is deeper for the sample without TBA, possibly because of the presence of water that contributes negatively to the SLD (Fig. 6a). When $^2$H$_2$O/$^2$H$_2$O contrast was applied, a deuterium signal in the center of the bilayer was found for KcsA/POPC, but not for KcsA/POPC/TBA (Fig. 6b). This indicates that TBA ions displace water from the central cavity.

Structural Perturbations of Bilayers with KcsA—To characterize the extent to which KcsA affects the bilayer structure, chain-deuterated lipid species (D31-POPC) and $^2$H$_2$O/$^2$H$_2$O contrast were used for samples with and without KcsA (Fig. 7,a and b). D31 profiles, determined by deuterium difference analysis (“Experimental Procedures”) were used to examine the distribution of the hydrocarbon chains in the bilayer. Notably, calibrated D31 profiles obtained at any of the measured $^2$H$_2$O/
$^2$H$_2$O contrast conditions overlap almost perfectly, indicating that data from different measurements are internally consistent. When we compared the chain profiles for bilayers with and without KcsA, we found that, in the presence of KcsA, the hydrocarbon region becomes thinner by 2.6 Å compared with the neat bilayer (measured as the full width at half maximum of the D31 distribution) (Fig. 7c), suggesting that the acyl chains rearrange to accommodate protein inclusion and an overall area expansion of the bilayer. At the same time, the phospholipid headgroup region and the water envelope associated with it expand toward the bilayer interior, indicating that the surface of the bilayer is significantly distorted by the presence of KcsA (Fig. 7c). In contrast to bilayers with KcsA, no $^2$H$_2$O peak could be detected in the middle of a neat bilayer (Fig. 7c), indicating that there is no significant water penetration into the hydrocarbon region in the absence of the channel protein. Taken together, the results indicate that lipid chains rearrange to fill density gaps in the hydrocarbon region where protein density is minimal (Fig. 5), whereas the phosphate groups and interfacial water redistribute to accommodate the bulkier polar region of the channel. Water redistributes to hydrate not only polar protein loop regions exposed to the membrane surface but also the internal cavity.

Quantifying KcsA Hydration in Bilayers by Neutron Diffraction—To quantify water in bilayers with KcsA from the neutron data, we used composition-based calibration of the deuterium signal found by H$_2$O/$^2$H$_2$O (“Experimental Procedures”). The water profile, calculated on a per lipid, absolute scale (22), was then modeled and analyzed in terms of a minimal set of Gaussian distributions. We could describe the overall $^2$H$_2$O profile across the repeat unit ($d/H_{11002}$ to $d/H_{11002}$) of samples with KcsA by a minimum set of six Gaussian distributions (three pairs, symmetrically positioned relative to the bilayer center). The central part, corresponding to the cavity, could be modeled by a pair of Gaussians positioned at $d/H_{11006}$ = 2.7 ± 0.08 Å from the bilayer center and characterized by a full width at half maximum (fwhm) of 7.4 ± 0.1 Å (Fig. 8a). We employed the Gaussian analysis to evaluate in detail the amount of $^2$H associated with either the cavity or the bilayer surfaces, upon H$_2$O/$^2$H$_2$O exchange. We determined that 214 ± 19 deuterium atoms were associated with a channel cavity in the bilayer. Furthermore, integration of the entire $^2$H$_2$O envelope across the repeat unit (−d/2 to d/2) of samples with KcsA by a minimum set of six Gaussian distributions (three pairs, symmetrically positioned relative to the bilayer center). The central part, corresponding to the cavity, could be modeled by a pair of Gaussians positioned at ± 2.7 ± 0.08 Å from the bilayer center and characterized by a full width at half maximum (fwhm) of 7.4 ± 0.1 Å (Fig. 8a). We employed the Gaussian analysis to evaluate in detail the amount of $^2$H associated with either the cavity or the bilayer surfaces, upon H$_2$O/$^2$H$_2$O exchange. We determined that 214 ± 19 deuterium atoms were associated with a channel cavity in the bilayer. Furthermore, integration of the entire $^2$H$_2$O envelope across the repeat unit (−d/2 to d/2) yielded 10.6 ± 0.2 water molecules per lipid headgroup for neat bilayers and 14.1 ± 1.9 for bilayers with KcsA (at C/L = 1/235) (Fig. 8, a and c). To a good approximation, we determined that nearly 1000 water molecules are associated with one channel protein in the bilayer, assuming that the hydration of each lipid headgroup does not change significantly when KcsA is present. The $^2$H$_2$O envelope corresponding to the membrane surfaces and filling the space between adjacent membranes in lamellar samples has a more complicated structure. Here it could be separated into a minimum of two pairs of Gaussians: a major contribution located at d/2 = 25.7 ± 0.1 Å with fwhm = 11.4 ±
0.4 Å and a minor contribution located at 18.7 ± 0.2 Å and fwhm = 7.8 ± 0.4 Å (Fig. 8b). Conceivably, the minor contribution may distinguish water bound to polar segments of the protein exposed to the membrane surface, from water hydrating the lipid headgroups. By comparison with a bilayer incorporating KcsA, the water distribution in the neat bilayer is concentrated only at the membrane surfaces and can be described with a good approximation by a single pair of Gaussians, at $d/2 = 26.6 ± 0.1$ Å and fwhm = 14.7 ± 0.1 Å (Fig. 8d).

Although the minimal Gaussian distribution model was used here as an analysis tool for quantification purposes, clearly it does not provide a sufficient set to qualitatively separate all individual contributions from bound or free water around protein segments and phospholipid headgroups. Complications arise from the fact that individual contributions overlap extensively because of the thermal disorder and the presence of exchangeable hydrogens. In the hydrocarbon region, no water can be detected in the absence of channels, implying that the water peak found there is due to the water-accessible KcsA cavity. However, the amide protons of all amino acids and a few -OH groups on serine and threonine residues present in KcsA that are directly exposed to the solvent accessible regions are labile for exchange to deuterium and contribute to the measured $^2$H$_2$O signal.

Quantifying the Amount of Water and H/$^2$H Exchange by Solid State NMR—We ask the question as to what extent protons in KcsA exchange when samples are hydrated from $^2$H$_2$O vapor for several hours, the time course of the diffraction measurements. To answer this question, we reconstituted $^{15}$N-labeled KcsA into POPC/POPG lipid and formed lipid multilayers on the inner wall of a 4-mm rotor (ZrO$_2$) for MAS NMR experiments while maintaining hydration conditions identical to those used for diffraction. By using $^1$H MAS NMR, we first determined the extent of water uptake of bilayer membranes in the presence of KcsA channels. We found that the total water content increased from 8.5 waters per lipid for membranes without KcsA to 17.5 waters for samples with $^{15}$N-KcsA (1/130, channels/lipids molar ratio) (Fig. 9, a and b). This substantial increase agrees with results from neutron diffraction and indicates that roughly 1000 water molecules per tetramer are associated with KcsA in the lipid membrane. We then carried out $^1$H-$^{15}$N cross-polarization measurements of the $^{15}$N-KcsA/lipid sample hydrated in H$_2$O and $^2$H$_2$O. The intensity of the band of amide resonances centered at 120 ppm for the sample hydrated in H$_2$O dropped by 64% after exposure to $^2$H$_2$O for 26 h (Fig. 9c). Intensity of amide resonances recorded by $^1$H-$^{15}$N cross-polarization is somewhat dependent on differences in motional properties of amides. However, it is safe to assume that for a relatively rigid molecule like KcsA, this effect is unlikely to be significant. Therefore, 64% of amide protons were exchanged to deuterons, assuming that all amide protons con-
tribute equally to intensity. This high degree of exchange suggests that all amide bonds and -OH groups exposed to water are likely to have exchanged and that includes surface-exposed polar regions and the cavity. To estimate their proportion, we examined the crystal structure of KcsA (Fig. 9d). Roughly 100 amide protons and 20 exchangeable -OH protons populate the cavity-apposed inner helix of KcsA tetramer, yielding an estimate of 120 exchangeable protons in the hydrophobic region. The remaining 94 deuterium atoms, of the 214 found by neutron diffraction, account for at least 47 structural water molecules (2H2O) associated with the cavity. This number of water molecules is higher than allowed by the cavity dimensions in the crystal structure, based only on strict spatial constraints. Although a very accurate determination of the number of water molecules in the cavity is difficult, our results indicate that both channel cavity and surface-exposed polar regions of the protein are highly hydrated. The shape of the water distribution in membranes with KcsA is unlike what we have observed for other membrane proteins (21), but it is strongly suggestive that the water profile follows the shape of the transmembrane cavity (Figs. 4b and 5).

Water Distribution in the M2 Proton Channel from Influenza A Virus—As a comparative example, we examined the water distribution for a different type of homotetrameric channel, formed by the transmembrane part (residues 22–46) of the M2 protein from H1N1 influenza A virus. The M2 protein and the M2 peptide (residues 22–46) form tetrameric proton channels in lipid bilayers, which are required for viral replication. Anti-viral drugs such as amantadine and rimantadine block this channel and thereby inhibit its function of acidifying the viral interior, an essential step in replication. The M2 (residues 22–46) peptide is strongly hydrophobic for the 19, central, membrane-spanning residues. Of these, only Ser-31, His-37, and Trp-41 have side chains with exchangeable protons (28, 29). The channel gate is formed by His-37 and Trp-41, and amantadine and rimantadine bind close to Ser-31 (28, 29). The neutron diffraction results reveal distinct changes in the water distribution across lipid membranes when the M2 peptides are present (Fig. 10). The neat lipid bilayer (without M2) shows a typical water distribution, flat across the hydrocarbon region, with no water penetration observed beyond the lipid headgroup region (±10 Å). The water distribution with M2 displays a
FIGURE 8. Gaussian models of the water distributions at the membrane surface. a, same experimental water profiles as in Fig. 4b for bilayers with KcsA at increasing $^2\text{H}_2\text{O}$ (from light to dark blue: 20%, 50 and 100% $^2\text{H}_2\text{O}$), shown on an expanded scale for better visibility of the uncertainty limits of the profiles (light blue bands). The uncertainties were calculated by a Monte Carlo sampling method (44) and include contributions from standard deviations in the structure factors and calibration factors caused by sample composition. The Gaussian model fitting (red) was applied simultaneously for all $^2\text{H}_2\text{O}$ contrasts. b, water profiles in the interbilayer space for samples with KcsA. Minimum set Gaussian distributions that can describe the experimental profiles (yellow) and their envelope (red), shown only for 100% $^2\text{H}_2\text{O}$, for clarity. c, experimental water profiles for neat bilayers shown on an expanded scale (light blue, 20%; dark blue, 50% $^2\text{H}_2\text{O}$), with uncertainty limits (light blue bands). d, water profiles in the interbilayer space for neat bilayers and Gaussian model fitting curves (red).

FIGURE 9. Water content and hydrogen to deuterium exchange determined by NMR. a and b, $^1\text{H}$-MAS NMR spectrum of POPC/POPG (1/1, mol/mol) bilayers (a) and of KcsA/POPC/POPG bilayers (b) hydrated at 93% relative humidity. Water content of samples was determined by spectral deconvolution of the water resonance at 4.8 ppm and lipid resonances. c, $^1\text{H}$-$^1\text{H}$ cross-polarization MAS NMR spectra of $^{15}\text{N}$-KcsA hydrated with $\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$. Visible are the band of amide resonances centered at 120 ppm and its spinning sidebands at $\pm 5$ kHz ($\pm 61.7$ ppm). d, crystal structure of KcsA (Protein Data Bank code 1K4C) (4). Only two opposing units of the tetramer are shown. The boundaries of the hydrocarbon region (yellow lines) were drawn approximately, based on the center of mass positions of two tryptophan (W) clusters, assumed to line up at the bilayer-water interfaces (45). Amide protons around the cavity within these boundaries (blue spheres) account for $\approx 25$ per unit. Additional exchangeable protons are present on -OH groups of Thr-74, Thr-75, Thr-101, Thr-107 (green), and Ser-102 (purple), on the inner transmembrane helix and base of the selectivity filter. A single hydrophilic threonine residue is present on the outer transmembrane segment (green), at the position of the cavity.
Hydration of Ion Channels in Membranes

![Graph showing water distribution in the M2 proton channel.](image)

**FIGURE 10. Water distribution in the M2 proton channel.** Water distribution determined by neutron diffraction for DLPC lipid bilayers with and without the M2 peptide. The DLPC/M2 peptide molar ratio is 1/4. Relative humidity is 86%. Near the bilayer center, the water distribution is flat for DLPC lipid (gray) but distinctly V-shaped with the channel-forming M2 peptides present (red). The d-spacing is 44.5 Å for the DLPC bilayer and 39.25 Å for the DLPC + M2 peptide bilayer.

striking V-shaped profile with significant water penetration toward the bilayer center. The profile resembles the shape of a truncated cone, as observed in the crystal structure of the proton conducting transmembrane domain of M2 (28, 29). A detailed comparison of structure, stability, and dynamics of KcsA and M2 channels, based mainly on NMR studies, has been made (30) and provides useful complimentary information to the neutron diffraction results presented here.

**Discussion**

It has been suggested that *dewetting* transitions in the central hydrophobic cavity play a key role in the function of some ion channels (8–10). However, there are no experimental data relating the structure of the cavity to dehydration transitions. For the mammalian channel Kv1.2, which has a similar cavity architecture and size as KcsA, predictions of molecular dynamics simulations probing gating transitions over hundreds of microseconds have suggested that, at reverse (hyperpolarizing) and even zero transmembrane voltages, a cavity collapse can be observed (9, 10). This hydrophobic collapse is accompanied by a complete *dewetting*, driving the number of water molecules in the cavity to 0. The prediction implies that voltage sensors may act to prevent the intrinsically more stable collapsed conformation. Contrary to this, none of the available crystal structures of Kv channel pores in closed conformation show a collapsed cavity. This includes the smaller pore channel, KvLm, crystallized in lipid cubic phases, lacking the K⁺ ion in the cavity (31). Because the “down” states of Kv channels have been structurally unattainable, most information about the closed conformation of Kv channel pore domains was provided by crystal structures of KcsA (3, 4, 14). Here we show that, in the absence of an applied field, the KcsA channels in bilayers are essentially filled with water in the closed state and therefore not collapsed. The high resolution structures of the closed KcsA crystalized in detergents reveal a 10 Å diameter central cavity (3, 4, 14), which allows enough space for up to 23 water molecules, but only water that is tightly bound to the polar segments of the protein or the 8 waters coordinating the single K⁺ ion in the central cavity are typically resolved (Fig. 1b). However, non-zero electron densities associated with channel cavities indicate that they are filled with water (3). We estimate that at least 47 water molecules are associated with the cavity, in the sense that they are at the position of the cavity across the membrane. This number is higher, by a factor of ~2, than what is consistent with the cavity dimensions from the crystal structure. Additional water in the cavity may be enabled by the fluid bilayer environment that allows membrane fluctuations and protein conformations not achievable in crystals. Alternatively, the additional water may be just outside the cavity, associated with hydrophilic side chains within the membrane via hydrogen bonds. Very few of these are along the transmembrane segments (Fig. 9c). Most of them are clustered at the water-membrane interfaces, at the exits of the cavity (3, 4, 12, 13). Such water clusters in the membrane could form hydrogen-bonded networks that help to stabilize water in the cavity.

Nonpolar or weakly polar pores play a prominent role in many ion channels, as well as other transport proteins (32–35). Computationally, the stability of water clusters in nonpolar confinements has been discussed in the simpler contexts of carbon nanotubes and fullerenes (36, 37), but it is difficult to assess in the case of proteins where small conformational changes can alter the number of polar residues exposed to the aqueous medium (38). Experimentally, NMR spectroscopy has provided evidence for the presence of disordered water in weakly polar cavities (39, 40). Our neutron diffraction studies show that the transmembrane cavities of KcsA and M2 channels have a high occupancy of water and that the water profile reports on the protein conformation in the membrane environment. It will be interesting to see how hydrophobic mutations in the cavity region (e.g. the semi-conserved Thr-107 of K⁺ channels (3) or Gly-34 of M2, found to inhibit channel conductance (29, 41)) would alter the hydration of these channels. How the cavity water is structurally organized and what the rates of exchange are between this water and the bulk during channel gating are critical questions that remain to be answered and may, potentially, be addressed in the future using neutron and NMR techniques (42, 43).

**Author Contributions**—M. M. designed the study. J. R. B., D. L. W., and M. M. planned the experiments, prepared samples, carried out neutron measurements, and analyzed data. K. G. performed NMR investigations. P. G. did single-channel conductance measurements. J. R. B., D. L. W., and M. M. wrote the manuscript with contributions from all co-authors.

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