The conserved potassium channel filter can have distinct ion binding profiles: Structural analysis of rubidium, cesium, and barium binding in NaK2K

Yee Ling Lam, Weizhong Zeng, David Bryant Sauer, and Youxing Jiang

Potassium channels are highly selective for K⁺ over the smaller Na⁺. Intriguingly, they are permeable to larger monovalent cations such as Rb⁺ and Cs⁺ but are specifically blocked by the similarly sized Ba²⁺. In this study, we used structural analysis to determine the binding profiles for these permeant and blocking ions in the selectivity filter of the potassium-selective NaK channel mutant NaK2K and also performed permeation experiments using single-channel recordings. Our data revealed that some ion binding properties of NaK2K are distinct from those of the canonical K⁺ channels KcsA and MthK. Rb⁺ bound at sites 1, 3, and 4 in NaK2K, as it does in KcsA. Cs⁺, however, bound predominantly at sites 1 and 3 in NaK2K, whereas it binds at sites 1, 3, and 4 in KcsA. Moreover, Ba²⁺ binding in NaK2K was distinct from that which has been observed in KcsA and MthK, even though all of these channels show similar Ba²⁺ block. In the presence of K⁺, Ba²⁺ bound to the NaK2K channel at site 3 in conjunction with a K⁺ at site 1; this led to a prolonged block of the channel (the external K⁺-dependent Ba²⁺ lock-in state). In the absence of K⁺, however, Ba²⁺ acts as a permeating blocker. We found that, under these conditions, Ba²⁺ bound at sites 1 or 0 as well as site 3, allowing it to enter the filter from the intracellular side and exit from the extracellular side. The difference in the Ba²⁺ binding profile in the presence and absence of K⁺ thus provides a structural explanation for the short and prolonged Ba²⁺ block observed in NaK2K.

INTRODUCTION

Rapid and selective conduction of potassium ions across cell membranes is central to many biological processes, including nerve excitation, muscle cell contraction, signal transduction, and hormone secretion (Hille, 2001). These are two seemingly mutually exclusive properties, as high selectivity is normally achieved by high affinity ion binding, whereas the opposite is required for a high flux rate. With an ingenious architecture in the channel pore, tetrameric potassium channels can specifically allow K⁺ ions to permeate and traverse down their electrochemical gradients at a rate close to the diffusion limit. The pore of K⁺ channels has a unique structure known as the selectivity filter, which is formed by the conserved signature sequence TVGYGD (Heginbotham et al., 1994). Within the selectivity filter, the backbone carbonyl oxygen atoms from the TVGY residues and the hydroxyl oxygen from the threonine side chain point toward the center and form four contiguous ion-binding sites (numbered 1–4 from the extracellular side) for dehydrated K⁺ ions (Fig. 1), mimicking the hydration shell of a K⁺ ion (Doyle et al., 1998; Zhou et al., 2001). Having four contiguous ion-binding sites in the filter has been shown to be a prerequisite for selective K⁺ conduction, and structural deviation from the four-site filter architecture will lead to the loss of selectivity (Derebe et al., 2011a; Sauer et al., 2011). During permeation, two K⁺ ions occupy the four-site filter with equal probability, most likely hopping between 1, 3, and 2, 4 configurations. This equal distribution of two conducting ions within the filter is necessary for efficient ion permeation (Morais-Cabral et al., 2001).

As Na⁺ and K⁺ are the two most abundant cations in life, K⁺ channels appear to have evolved to achieve high selectivity primarily by the exclusion of smaller sodium ions. Indeed, K⁺ channels select poorly among ions that are of a similar size as or slightly larger than K⁺, such as Rb⁺, Cs⁺, and Tl⁺. Some K⁺ channels are actually more selective for Rb⁺ or Cs⁺ than K⁺ (Eisenman et al., 1986; Heginbotham and MacKinnon, 1993; LeMasurier et al., 2001). However, the conduction of these larger ions in the K⁺ channel is not as efficient as that of K⁺, likely because of the imbalanced ion distribution in the filter. The Ba²⁺ ion, which has the same size as K⁺ but twice the...
that Cs\(^+\) and Ba\(^{2+}\) binding in NaK2K is different from that observed in KcsA, despite the fact that both channels have a virtually identical selectivity filter structure and similar ion permeation and blockage properties. In addition, Ba\(^{2+}\) has a different binding profile within the NaK2K filter in the presence and absence of K\(^+\), and this K\(^+\)-dependent difference in Ba\(^{2+}\) occupancy provides structural insight into the Ba\(^{2+}\) lock-in effect in K\(^+\) channels.

### MATERIALS AND METHODS

#### Protein expression, purification, and crystallization

K+-selective NaK double mutant NaK2K, containing the mutations D66Y and N68D, was encoded in the pQE60 vector, expressed in the Escherichia coli strain SG13009, and purified as previously described (Derebe et al., 2011a). In brief, expression was induced with 0.4 mM IPTG (isopropyl \(\beta\)-d-thiogalactopyranoside) in the presence of 5 mM BaCl\(_2\) for 18 h at 25°C. Cells were lysed in 100 mM KCl and 50 mM Tris-HCl, pH 8.0, and the expressed channel proteins were solubilized with 40 mM \(\alpha\)-decyl-\(\beta\)-maltoside (DM) and purified on a column (Talon IMAC; Takara Bio Inc.). Eluted proteins were digested overnight with thrombin, 1 U thrombin/2 mg protein, and further purified on a (10/30) size exclusion column (Superdex 200; GE Healthcare) in 100 mM KCl, 20 mM Tris-HCl, pH 8.0, and 4 mM DM. The NaK2K channel used in functional studies contains an additional mutation, F92A, which increases flux through the channel.

Purified NaK2K channels were concentrated to \(~20\) mg ml\(^{-1}\) and crystallized in the same conditions previously described using sitting drop vapor diffusion at 20°C (Alam and Jiang, 2009a). All crystals were initially grown in complex with K\(^+\) by mixing equal volumes of protein solution (which contained 100 mM KCl) with a well solution of 62.5–70% (±) 2-methyl-2,4-pentanediol (MPD) and 100 mM buffer at various pH (Mes for pH 6.0–6.5, HEPES for pH 7.0–7.5). In all soaking experiments, the crystal was soaked...
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Data collection and structure determination

X-ray diffraction data were collected at the Advanced Light Source beamlines 8.2.1 and 8.2.2 and the Advanced Photon Source beamlines 19ID and 23ID. Data were processed and scaled in HKL-2000 (HKL Research, Inc.; Otwinowski and Minor, 1997). As all crystals for the soaking experiment were obtained from the same space group, with unit cell dimensions around \( a = b = 68 \text{ Å}, c = 89 \text{ Å} \), and contained two molecules (subunits A and B) in the asymmetric unit. The fourfold axis of channel tetramer coincided with the crystallographic tetrad. Only the tetramer generated from subunit A was used for structural analysis in this study, as the filter residues of subunit C have much lower and more stable B factors.

For occupancy estimation, the total area of the four peaks above the baseline in the one-dimensional electron density profile was set to represent electrons from two \( K^+ \) ions and two water molecules in the NaK2K–K⁺ complex (total of 56 e⁻). The area of individual peak in the one-dimensional profile of various soaked crystals was then normalized against the peaks of the NaK2K–K⁺ complex to calculate the number of electrons each peak represents; ion occupancy at each site was then calculated using the following equation, assuming a total occupancy of 1 for water (10 e⁻) and ion (N e⁻) at each site. \( N \times (1 - Y) = \text{number of electrons calculated from the peak area (1), where } N \text{ is the number of electrons for each filter ion (18 for } K^+, 36 \text{ for } Rb^+, \text{ and } 54 \text{ for } Cs^+ \text{ and } Ba^{2+} \text{) and } Y \text{ is the occupancy for the respective ion.}

Electrophysiology

All single-channel recordings were performed using giant liposome patches as described previously (Derebe et al., 2011a,b). A protein/lipid (3:1 POPE/POPG) ratio of 0.05–0.1 µg/mg was used to reconstitute proteoliposomes. Giant liposomes were obtained by air drying 2–3 µl liposome sample on a clean coverslip overnight at 4°C followed by rehydration in bath solution at room temperature. Patch pipettes were pulled from borosilicate glass (Harvard Apparatus) to a resistance of 8–12 MΩ filled with pipette solution containing 150 mM XCl, 1 mM EGTA, and 10 mM HEPES, pH 7.4, buffered with NaOH, where X is the monovalent cation (or mixed cations) used in each experiment, as discussed in the Results section, with a total concentration of 150 mM. The initial bath solution contained 150 mM KCl, 10 mM HEPES, and 1 mM EGTA, pH 7.4, buffered with Tris. A gigaseal (>10 GΩ) was obtained by gentle suction when the patch pipette attached to the giant liposome. To get a single layer of membrane in the patch, the pipette was pulled away from the giant liposome, and the tip was exposed to air for 1–2 s. Membrane voltage was controlled and current recorded using an amplifier (Axopatch 200B; Molecular Devices)

### Table 1

Data collection and refinement statistics for NaK2K in complex with \( Rb^+ \), \( Cs^+ \), or \( Ba^{2+} \)

<table>
<thead>
<tr>
<th>Statistic</th>
<th>NaK2K–Rb⁺</th>
<th>NaK2K–Cs⁺</th>
<th>NaK2K–Ba⁺ (in KCl)</th>
<th>NaK2K–Ba⁺ (in NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>( I )</td>
<td>( I )</td>
<td>( I )</td>
<td>( I )</td>
</tr>
<tr>
<td>Cell dimensions ( a = b, c (\text{Å}) )</td>
<td>68.011, 89.346</td>
<td>68.106, 89.593</td>
<td>68.065, 89.614</td>
<td>68.042, 89.627</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0000</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50–1.58</td>
<td>50–1.70</td>
<td>50–1.82</td>
<td>50–1.85</td>
</tr>
<tr>
<td>( R_{	ext{sym}} ) (%)</td>
<td>4.9 (78.6)</td>
<td>4.3 (77.0)</td>
<td>5.4 (99.8)</td>
<td>6.3 (93.1)</td>
</tr>
<tr>
<td>I/σ</td>
<td>48.3 (2.1)</td>
<td>49.8 (1.7)</td>
<td>37.0 (1.8)</td>
<td>39.2 (2.3)</td>
</tr>
<tr>
<td>Number of total reflections (unique)</td>
<td>205,718 (27,782)</td>
<td>165,756 (22,437)</td>
<td>128,478 (18,212)</td>
<td>150,140 (17,542)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.86 (99.82)</td>
<td>99.86 (99.64)</td>
<td>99.53 (100.00)</td>
<td>99.24 (99.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.4 (7.3)</td>
<td>7.4 (6.8)</td>
<td>7.1 (7.2)</td>
<td>7.4 (7.0)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.58</td>
<td>1.70</td>
<td>1.82</td>
<td>1.85</td>
</tr>
<tr>
<td>( R_{	ext{free}}/R_{	ext{work}} )</td>
<td>0.2043/0.2335</td>
<td>0.2070/0.2358</td>
<td>0.1972/0.2324</td>
<td>0.1998/0.2266</td>
</tr>
<tr>
<td>Number of atoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>1,485</td>
<td>1,485</td>
<td>1,485</td>
<td>1,485</td>
</tr>
<tr>
<td>MPD/ion</td>
<td>3/6</td>
<td>3/6</td>
<td>4/9</td>
<td>4/8</td>
</tr>
<tr>
<td>Water</td>
<td>79</td>
<td>49</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td><strong>RMSD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond angles (Å)</td>
<td>0.007</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>1.07</td>
<td>1.08</td>
<td>1.20</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest resolution shell. 5% of the data was used in the \( R_{	ext{free}} \) calculation.

Electrophysiology

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channels, their binding profiles in NaK2K were analyzed. The NaK2K channel was first crystallized in the presence of KCl as previously described (Derebe et al., 2011a). Crystals were then soaked into stabilization solutions in which KCl was replaced by 100 mM RbCl or CsCl, and their structures were determined to 1.58 Å and 1.7 Å, respectively (Protein Data Bank accession nos. 4PDM and 4PDL; Table 1; see Materials and methods). In both cases, the binding profiles are almost identical to that of the NaK2K–K complex, with a main chain root mean square deviation (RMSD) of <0.2 Å.

The binding profile of Rb+ in the NaK2K filter is similar to that of KcsA (Zhou and MacKinnon, 2003). The Fo–Fc ion omit map of the NaK2K–Rb+ complex reveals three discrete density peaks with similar intensity at sites 1, 3, and 4, indicating equivalent Rb+ binding at these three sites (Fig. 2 A). This equivalent Rb+ occupancy can be further confirmed by the equivalent Rb+ anomalous scattering as shown in the anomalous difference map (Fig. 2 B). To estimate the relative occupancy of Rb+, we compared the one-dimensional electron density profiles along the central axis of the filter between

**RESULTS**

Rubidium and cesium binding in the filter of NaK2K

Because larger group I monovalent cations such as Rb+ and Cs+ are known to be permeable ions for K+-selective channels, their binding profiles in NaK2K were analyzed. The NaK2K channel was first crystallized in the presence of KCl as previously described (Derebe et al., 2011a). Crystals were then soaked into stabilization solutions in which KCl was replaced by 100 mM RbCl or CsCl, and their structures were determined to 1.58 Å and 1.7 Å, respectively (Protein Data Bank accession nos. 4PDM and 4PDL; Table 1; see Materials and methods). In both cases, the binding profiles are almost identical to that of the NaK2K–K complex, with a main chain root mean square deviation (RMSD) of <0.2 Å.

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with a digitizer (Digidata 1322A; Axon Instruments). Currents were low-pass filtered at 1 kHz and sampled at 20 kHz. Except the Cs+ current measurement, only patches containing a single channel were used for further experiments. Upon the observation of single-channel activity, the bath solution with 150 mM KCl was replaced by another salt as indicated in each experiment. All chemicals used for solution preparation were purchased from Sigma-Aldrich.

In the study of intracellular Ba2+ blocking, the free Ba2+ concentration was controlled by mixing 5 mM EGTA with an appropriate amount of BaCl2 calculated using the software MAXCHELATOR. Owing to the two possible orientations of reconstituted NaK2K in the liposomes, 30 µM tetracentyl ammonium, an intracellular pore blocker, was added in the bath solution to ensure that the channel in the recording had its intracellular side facing the bath solution for internal Ba2+ blocking assay and its extracellular side facing the bath solution for external Ba2+ blocking assay. Tetracentyl ammonium was then removed through bath solution exchange before Ba2+ block measurements.

Figure 2. Rb+ and Cs+ binding in NaK2K. (A) F–F, ion omit map (6σ, blue mesh) of the NaK2K–Rb+ complex and the one-dimensional electron density profile (from a 1.85-Å ion omit map) along the central axis of the filter. The electron density distribution of K+ ions in the NaK2K–K complex is also plotted (in gray) for comparison (and for all other one-dimensional profiles). The Rb+ ions are modeled as purple spheres. (B) Anomalous difference Fourier map (7σ, red mesh) of the NaK2K–Rb+ complex and the one-dimensional anomalous scattering profile at the selectivity filter region. (C) F–F, ion omit map (6σ) of the NaK2K–Cs+ complex and the one-dimensional electron density profile along the filter. The Cs+ ions are modeled as cyan spheres. The density at site 4 is modeled as a Na+ ion (orange sphere). (D) Anomalous difference Fourier map (7σ) of the NaK2K–Cs+ complex and the one-dimensional anomalous scattering profile at the selectivity filter region.
the Rb⁺ and K⁺ complexes (see Materials and methods; Fig. 2 A and Table 2). The occupancy calculation was performed under the assumptions that the filter of the K⁺ complex is occupied by two water molecules and two K⁺ ions on average and that the area under the four peaks of its one-dimensional electron density profile reflects the total number of electrons in the filter (56 e⁻ for 2 K⁺ and 2 H₂O; Zhou and MacKinnon, 2003). Accordingly, upon comparison of their one-dimensional electron density profiles, we estimated the number of electrons each Rb⁺ peak represents and calculated the Rb⁺ occupancy at sites 1, 3, and 4 to be 0.69, 0.68, and 0.58, respectively.

Distinct from KcsA, Cs⁺ ions predominantly bind at sites 1 and 3 in NaK2K (Fig. 2, C and D). Although the Fo–Fc ion omit map of the NaK2K–Cs⁺ complex shows three electron density peaks within the filter, the density at site 4 is clearly not coming from Cs⁺, as no anomalous scattering is observed at this site in the anomalous difference map. The site 4 density is likely from a mixture of Cs⁺ and water molecules, with Cs⁺ having a quarter of the occupancy. The equivalent density in most K⁺ channel structures is NaK2K, so that the external entrance is unexpectedly strong and is partially contributed by Cs⁺, which is indicated by the anomalous scattering signals. The equivalent density in most K⁺ channel structures was generally weak and assigned as four water molecules for site 0 hydration shell. In light of the fourfold symmetry-related density peaks at the external entrance is unexpectedly strong and is partially contributed by Cs⁺, which is indicated by the anomalous scattering signals. The equivalent density in most K⁺ channel structures was generally weak and assigned as four water molecules for site 0 hydration shell. In light of the fourfold symmetry, the close proximity of these peaks, and the weaker anomalous scattering than the filter Cs⁺, we believe that each peak represents a mixture of Cs⁺ and water molecules, with Cs⁺ having a quarter of the occupancy. Neither Rb⁺ nor K⁺ seems to bind at the equivalent external site, as no obvious anomalous signal is observed in Rb⁺-soaked crystals; and the electron density at the equivalent position is much weaker in the Rb⁺ or K⁺ complex and is likely from a water molecule only. It is unclear whether this off-center external Cs⁺ binding is NaK2K specific or not.

Rb⁺ and Cs⁺ permeation in NaK2K

To correlate the structurally defined ion-binding profile of Rb⁺ and Cs⁺ in the NaK2K filter with their permeation properties, we performed single-channel recording of NaK2K reconstituted into liposomes in the presence of Rb⁺ or Cs⁺ (Fig. 3). As equal distribution of two K⁺ ions between 1,3 and 2,4 configurations appears to be essential for efficient ion permeation in K⁺ channels (Morais-Cabral et al., 2001), the imbalanced binding of Rb⁺ and Cs⁺ would imply a lower permeation rate. Indeed, the single-channel conductance of NaK2K in 150 mM of symmetrical RbCl is ~20 pS (Fig. 3 A), much lower than that of 120 pS in KCl (Derebe et al., 2011a). With respect to selectivity, NaK2K is also slightly more selective for K⁺ with a permeability ratio (Pₖ/P₀) of ~1.5 (Fig. 3 B).

Cs⁺ binds almost exclusively at sites 1 and 3 within the NaK2K filter, representing an extreme case of imbalanced ion distribution between 1,3 and 2,4 configurations. This implies a high energy difference between the two configurations and would suggest an extremely low efficiency of ion permeation. As expected, the Cs⁺ current, if permeable, is too small to measure (Heginbotham and MacKinnon, 1993). With K⁺ as the permeating ion, Cs⁺ can reduce the K⁺ current and serve as a permeating blocker similar to Ba²⁺, as discussed in the section below (Fig. 3 C).

External K⁺-dependent barium block of the NaK2K channel Ba²⁺, which has an identical crystal radius as K⁺, is known to bind K⁺ channel filters and block the K⁺ current. It has been shown that the kinetics of internal Ba²⁺ blocking (mean blocking dwell time) depends on the presence of external K⁺ (Neyton and Miller, 1988a,b; Piasta et al., 2011). To test if this also occurs in NaK2K, we performed a similar blocking assay on NaK2K using single-channel recording. In this assay, the extracellular solution (in pipette) contained 150 mM NaCl with or without K⁺, and the intracellular solution (bath) contained 150 mM KCl with and without BaCl₂. Shown in Fig. 4 A are sample traces of outward NaK2K currents without Ba²⁺ (control in bi-ionic condition), with 10 µM internal Ba²⁺ but no external K⁺, and with 10 µM internal Ba²⁺ and 5 mM external K⁺. In the absence of Ba²⁺ (Fig. 4 A, top trace), NaK2K exhibits two closing states, as demonstrated by

<table>
<thead>
<tr>
<th>Site/ion</th>
<th>Rb⁺</th>
<th>Cs⁺</th>
<th>Ba²⁺ in K⁺</th>
<th>Ba²⁺ in Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.44</td>
</tr>
<tr>
<td>S1</td>
<td>0.69</td>
<td>0.68</td>
<td>0.95 (as K⁺ ion)</td>
<td>0.24</td>
</tr>
<tr>
<td>S2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S3</td>
<td>0.68</td>
<td>0.75</td>
<td>0.74 (as Ba⁺⁺ ion)</td>
<td>1.00</td>
</tr>
<tr>
<td>S4</td>
<td>0.58</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable.

*H₂O was not taken into account in the occupancy calculation for site 0 Ba²⁺, as water has never been observed at this site.

\begin{table}
<table>
<thead>
<tr>
<th>Site/ion</th>
<th>Rb⁺</th>
<th>Cs⁺</th>
<th>Ba²⁺ in K⁺</th>
<th>Ba²⁺ in Na⁺</th>
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<tbody>
<tr>
<td>S0</td>
<td>NA</td>
<td>NA</td>
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<td>0.44</td>
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<td>S2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S3</td>
<td>0.68</td>
<td>0.75</td>
<td>0.74 (as Ba⁺⁺ ion)</td>
<td>1.00</td>
</tr>
<tr>
<td>S4</td>
<td>0.58</td>
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</tbody>
</table>
\end{table}
the nonconducting dwell time histogram: brief closings with a time constant of \(\tau_1\) and longer closings with a time constant of \(\tau_2\). The rapid closings are an intrinsic property of the pore and were also observed in other K⁺ channels with a submillisecond time constant (Li et al., 2007; Piasta et al., 2011). These spontaneous closings are likely a result of the fast gating at the filter region. The longer closings can be attributed to gating at the intracellular bundle crossing. The addition of 10 µM Ba²⁺ at the intracellular side (Fig. 4 A, middle trace) does not change the kinetics of brief closings. However, it increases the time constant for the longer closings \(\tau_2\) to \(\sim 18\) ms. This increase of closing time can be attributed to Ba²⁺ block, which lasts longer than the kinetics of the intracellular gating. When 5 mM K⁺ is included in the extracellular solution (5 mM KCl/145 mM NaCl), a third extremely long closing state with a time constant in the order of seconds \(\tau_3\), \(\sim 3,200\) ms in this measurement) is observed. The two Ba²⁺-induced closing states \(\tau_2\) and \(\tau_3\); Fig. 4 A, bottom trace) in the presence of external K⁺ are reminiscent of the two Ba²⁺ block states with fast and slow time constants (termed \(\tau_f\) and \(\tau_s\)) observed in KcsA (Piasta et al., 2011). Similar to KcsA, the time constant of fast Ba²⁺ block \(\tau_f\) in NaK2K appears to be independent of external K⁺, as it remains almost unchanged in the presence or absence of external K⁺. The slow Ba²⁺ block \(\tau_s\), however, depends on external K⁺. It represents an external K⁺-induced Ba²⁺ lock-in state in which external K⁺ binding prevents the Ba²⁺ ion in the filter from escaping to the extracellular side and prolongs Ba²⁺ block to a timescale of seconds.

We also measured the effect of external Ba²⁺ block in NaK2K (Fig. 4 B). In this assay, the channel in the patch has its extracellular side facing the bath solution containing 145 mM NaCl and 5 mM KCl, with or without Ba²⁺, and the intracellular solution (in pipette) contained 150 mM KCl. Similar to other K⁺ channels, the Ba²⁺ block in NaK2K is also much weaker from the external side, as shown in the sample trace of outward currents with 1 mM Ba²⁺ (Fig. 4 B). However, the external Ba²⁺...
of bound ions within the filter is unevenly distributed, with the strongest density at site 3 and much weaker density at sites 2 and 4 (Fig. 5 A). As confirmed by the anomalous difference map (Fig. 5 B), Ba\(^{2+}\) binds almost exclusively at site 3, a finding that is very different from Ba\(^{2+}\) binding in KcsA, which occurs mainly at site 4 with weaker binding at site 2 when K\(^+\) is absent (Jiang and MacKinnon, 2000; Lockless et al., 2007). Analysis of site 3 intensity in the one-dimensional electron density profile yielded a Ba\(^{2+}\) occupancy of 0.74 (Fig. 5 A and Table 2). The presence of site 3 Ba\(^{2+}\) excludes the K\(^+\) binding at neighboring sites, resulting in much weaker density at sites 2 and 4.

still induces two block states with fast and slow time constants in the presence of external K\(^+\), as seen in KcsA.

Barium binding in the NaK2K filter in the presence of potassium
To examine the structural basis of the K\(^+\)-dependent Ba\(^{2+}\) block in the NaK2K channel, we soaked NaK2K crystals in a stabilization solution containing 20 mM Ba\(^{2+}\) in addition to 100 mM K\(^+\) and determined the structure to 1.82 Å (Protein Data Bank accession no. 4PDV). Although the filter structure is virtually the same as that of the NaK2K–K\(^+\) complex (RMSD of 0.1 Å), the electron density of bound ions within the filter is unevenly distributed, with the strongest density at site 3 and much weaker density at sites 2 and 4 (Fig. 5 A). As confirmed by the anomalous difference map (Fig. 5 B), Ba\(^{2+}\) binds almost exclusively at site 3, a finding that is very different from Ba\(^{2+}\) binding in KcsA, which occurs mainly at site 4 with weaker binding at site 2 when K\(^+\) is absent (Jiang and MacKinnon, 2000; Lockless et al., 2007). Analysis of site 3 intensity in the one-dimensional electron density profile yielded a Ba\(^{2+}\) occupancy of 0.74 (Fig. 5 A and Table 2). The presence of site 3 Ba\(^{2+}\) excludes the K\(^+\) binding at neighboring sites, resulting in much weaker density at sites 2 and 4.

Figure 4. Ba\(^{2+}\) block in NaK2K. (A) Internal Ba\(^{2+}\) block in the presence and absence of external K\(^+\). The nonconducting dwell time histograms with exponential fits for each recording are shown beside each trace. Only the patch that contains a channel with its intracellular side facing the bath solution was used in the recording. (B) External Ba\(^{2+}\) block in the presence of 5 mM external K\(^+\). Only the patch that contains a channel with its extracellular side facing the bath solution was used in the recording.
observations indicate that Ba\(^{2+}\) and Na\(^+\) are also partially occupying site 4 in the crystal in addition to K\(^+\), resulting in a stronger site 4 density than that of site 2. One observation is that the site 4 ion has some weak anomalous scattering, indicating the presence of Ba\(^{2+}\) at low occupancy. The other observation is that the site 4 density is diffusive and extended toward the bottom of site 4 where Na\(^+\) typically binds (Alam and Jiang, 2009b), and the soaking solution does contain Na\(^+\) from NaOH used for Mes buffering. In the structure of the NaK2K–Ba\(^{2+}\) complex in NaCl (discussed in the next section), we did demonstrate that Na\(^+\) can co-reside at the bottom of site 4 with Ba\(^{2+}\) occupying site 3.

Interestingly, even with Ba\(^{2+}\) occupying site 3, K\(^+\) remains bound at site 1, as indicated by the strong electron density without obvious anomalous signal, eliminating the possibility of this density coming from Ba\(^{2+}\). The exclusion of K\(^+\) at sites 2 and 4 upon Ba\(^{2+}\) binding actually enhances the occupancy of site 1 K\(^+\) to ~0.95 as estimated based on the peak height in the one-dimensional electron density profile. The high occupancy of site 1 K\(^+\) (close to unity) and site 3 Ba\(^{2+}\) indicates that their binding at these two sites can take place simultaneously. This co-residence of Ba\(^{2+}\) at site 3 and K\(^+\) at site 1 is a snapshot of the lock-in state, which explains how K\(^+\) prevents Ba\(^{2+}\) from exiting to the extracellular side and prolongs Ba\(^{2+}\) blockage observed in our electrophysiology data. Two
An additional anomalous density peak, although weak, was observed on the extracellular face of the channel just above site 0, indicating a second weak Ba\(^{2+}\) site. The presence of external Ba\(^{2+}\) can also be confirmed by the strong electron density that was not observed in the NaK2K–K\(^+\) complex. Unlike the site 0 Ba\(^{2+}\) seen in the NaK2K–Ba\(^{2+}\) complex in NaCl (discussed in the next section) or the asymmetrical external Cs\(^+\), this bound Ba\(^{2+}\) is further outside on the central axis and fully hydrated (Fig. 5A, inset). Similar weak binding of a fully hydrated divalent cation at the external entrance has also been observed in other NaK mutants, such as a hydrated Ca\(^{2+}\) in the NaK2CNG mutant (Derebe et al., 2011b).

**Barium binding in the NaK2K filter in the absence of potassium**

To reveal the structural basis of fast Ba\(^{2+}\) blockage in the absence of external K\(^+\), we performed a Ba\(^{2+}\) soaking experiment in the presence of 100 mM NaCl in stabilization solution instead of KCl and determined the structure of the Ba\(^{2+}\)/Na\(^+\)-soaked NaK2K crystal to 1.85 Å (Protein Data Bank accession no. 4PDR). In the absence of K\(^+\), Ba\(^{2+}\) binds at three positions, as demonstrated in the anomalous difference map (Fig. 6). Even though site 3 is still the major Ba\(^{2+}\) site with full occupancy (~1.0), Ba\(^{2+}\) also binds at site 1 and site 0. Because of the close proximity of these two sites, Ba\(^{2+}\) is unlikely to simultaneously occupy both of them. As indicated by the stronger anomalous signal, site 0 Ba\(^{2+}\) has a higher occupancy (~0.44) than that of site 1 (~0.24). Despite multiple binding sites, the likelihood of having two double charged Ba\(^{2+}\) ions within the NaK2K filter is low because of the strong electrostatic repulsion. Thus, the low probability of Ba\(^{2+}\) binding at site 1 could be the result of high Ba\(^{2+}\) occupancy at site 3. Nevertheless, without K\(^+\) competition, Ba\(^{2+}\) binding at site 3 as well as site 1 or 0 reveals the steps along the path of Ba\(^{2+}\) release to the extracellular side. Although fully occupied site 3 Ba\(^{2+}\) almost completely excludes the binding of any other ion at site 2, Na\(^+\) can still reside at the bottom edge of site 4 with the coordination of Thr63 hydroxyl groups and a water molecule in the cavity as commonly seen in multiple NaK mutant structures (Fig. 6A, inset). As sites 0 and 1 become the main secondary sites for Ba\(^{2+}\) in the absence of K\(^+\) competition, Ba\(^{2+}\) binding is no longer observed at the external entrance above site 0.

**DISCUSSION**

By using high resolution structures along with anomalous signals, we can accurately define how various permeating and blocking cations bind in K\(^+\)-selective NaK2K channel. Combined with single-channel electrophysiology, this approach provides structural insights into ion permeation and blocking in the K\(^+\) channel. Despite the seemingly identical filter structure to canonical K\(^+\) channels, the ion-binding profile of NaK2K shows some distinct features. Although Rb\(^+\) binding in NaK2K is similar to other K\(^+\) channels, Cs\(^+\) in NaK2K binds mainly at sites 1 and 3 and serves as a permeating blocker. With respect to selectivity, NaK2K is slightly more selective for K\(^+\) than Rb\(^+\) and Cs\(^+\) based on the reversal potential in bi-ionic conditions with the sequence of K\(^+\) > Rb\(^+\) > Cs\(^+\) > Na\(^+\), which is distinct from other K\(^+\) channels (Heginbotham and MacKinnon, 1993; Hille, 2001; LeMasurier et al., 2001).

The most notable difference is the Ba\(^{2+}\)-blocking site. In KcsA, site 4 is the primary site for Ba\(^{2+}\), with site 2 being the secondary site in the absence of K\(^+\). In NaK2K, Ba\(^{2+}\) blocks primarily at site 3 with K\(^+\), if present, residing at site 1. The presence of both Ba\(^{2+}\) and K\(^+\) in the filter represents the so-called lock-in state of Ba\(^{2+}\) block, where extracellular K\(^+\) binding at site 1 traps the Ba\(^{2+}\) at site 3 and prolongs the duration of Ba\(^{2+}\) block. The removal of external K\(^+\) would allow Ba\(^{2+}\) exit to the external side via binding at sites 1 and 0. It is also worth noting the difference in Ba\(^{2+}\) binding between NaK2K and MthK (see Guo et al. in this issue). In MthK, Ba\(^{2+}\) binds primarily at site 2, with secondary binding at sites 3 and 4 in the absence of K\(^+\) (in a Na\(^+\)-only condition). The presence of high K\(^+\) can outcompete Ba\(^{2+}\) in the filter, resulting in low occupancy Ba\(^{2+}\) binding mainly at site 4.

Despite the difference in Ba\(^{2+}\) binding between NaK2K and KcsA (or MthK), the principle of K\(^+\)-dependent Ba\(^{2+}\) lock-in is likely the same (Fig. 7). In the absence of external K\(^+\), the outward electrochemical driving force can quickly dislocate Ba\(^{2+}\) from its primary blocking site (site 3 or 4) and push the ion through the filter via its secondary site (site 1 or 2). As site 1 has been shown, both structurally and functionally, to be the common K\(^+\)-selective site in multiple channels (Neyton and Miller, 1988a; Spassova and Lu, 1999; Ye et al., 2010; Piasta et al., 2011; Sauer et al., 2013), the presence of external K\(^+\) results in K\(^+\) occupation at site 1, which prevents Ba\(^{2+}\) from accessing its secondary site and thereby blocks its passage to the external side. In NaK2K, the external K\(^+\) and the permeating Ba\(^{2+}\) are both competing for site 1, whereas in KcsA, the K\(^+\) occupation at site 1 prevents Ba\(^{2+}\) from accessing the adjacent site (site 2) as a result of the electrostatic repulsion. With K\(^+\) occupying site 1, the unblock of the filter Ba\(^{2+}\) can occur via two pathways: either by exiting to the inside, which would be against the electrochemical gradient under most experimental conditions, or by outcompeting with site 1 K\(^+\), which depends on the K\(^+\) affinity and external concentration. Either process will lead to a prolonged Ba\(^{2+}\) block. The high K\(^+\) selectivity of site 1 may also explain the weak Ba\(^{2+}\)-blocking effect from the external side, as K\(^+\) occupation at site 1 prevents external Ba\(^{2+}\) from accessing its primary binding site (Fig. 4).

The observed Ba\(^{2+}\)/K\(^+\) binding profile in NaK2K is surprisingly similar to what was deduced from the Ba\(^{2+}\) block study in BK channels by Neyton and Miller (1988a),
which admirably was performed long before any K' channel structure was available. As recapitulated in Fig. 8, two K'-selective sites external to Ba\textsuperscript{2+}, the lock-in and enhancement sites in BK, were proposed in their study. The highly K'-selective lock-in site is equivalent to site 1 and has high K' affinity even in the presence of Ba\textsuperscript{2+}, consistent with the co-residence of site 1 K' and site 3 Ba\textsuperscript{2+} observed in the NaK2K filter. The enhancement site, equivalent to site 2, is adjacent to Ba\textsuperscript{2+} and can only start to be occupied by K' at high concentration, consistent with low K' occupancy in our structure. Their study also revealed another low selective lock-in site internal to Ba\textsuperscript{2+}, which is equivalent to site 4. Indeed, our previous selectivity study of NaK2K did demonstrate that sites 1 and 2 are the two most K' preferred sites, whereas site 4 is the least K' selective one, which can also be occupied by Na' even in the presence of Ba\textsuperscript{2+} at site 3, as we show here (Sauer et al., 2013).

Given the high conservation of filter architecture in all K' channels, it remains a mystery why these seemingly identical K' channel filters exhibit quite different ion binding profiles, most notably in K' and Ba\textsuperscript{2+}. As demonstrated in our previous study, when competing against Na', K' clearly prefers sites 1 and 2 in NaK2K (Sauer et al., 2013), whereas sites 1 and 3 are preferred in MthK (Ye et al., 2010). In this study, we demonstrated that the primary and secondary binding sites for Ba\textsuperscript{2+} in NaK2K are sites 3 and 1 (or 0), respectively, whereas in KcsA they are sites 4 and 2, respectively (Lockless et al., 2007). Even within the same channel filter, K' appears to prefer two...
particular sites at low concentrations, even though all four sites within the filter have very similar structures and chemical environments. The origin of these differences remains elusive even with the available high resolution structures and warrant further investigation.

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