

Inherent Leakage of Hydrophobic Gating in BK Channels

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The big potassium (BK) channels remain open with a small limiting probability of $P_o \sim 10^{-7}$ at minimal Ca^{2+} and negative voltages below -100 mV. The molecular origin of such an “intrinsic opening” is not understood. Here, free-energy analysis of K^+ permeation shows that the intrinsic opening of BK channels is likely an inherent property of the vapor barrier, generated by hydrophobic dewetting of the inner pore in the Ca^{2+} -free state. The vapor barrier only gives rise to a finite free-energy barrier, of ~ 8 kcal/mol, and it cannot completely shut down K^+ flow even when the voltage sensor domains are fully deactivated. The resulting “leaking” currents can be measured at negative voltages as intrinsic opening. The shallow limiting slope of P_o at negative voltages arises primarily from the electric field effects on the permeating ion through the vapor barrier. We further demonstrate that the vapor barrier can be perturbed by truncation of the cytosolic domains, inner pore mutations, and ligand binding to the pore, leading to predictable changes in limiting slope measurements highly consistent with existing experimental data. Therefore, the intrinsic opening paves the way for direct experimental study of hydrophobic gating in BK and many other ion channels.

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I. INTRODUCTION

Large conductance Ca^{2+} -activated K^+ (BK) channels, also known as KCa1.1 or SLO1 channels, exhibit the highest single-channel conductance of 100–300 pS among all K^+ channels [1–5]. Formed by four Slo1 subunits, functional BK channels can be activated independently and synergistically in response to membrane depolarization, through the voltage sensing domains (VSDs), and binding of intracellular Ca^{2+} and Mg^{2+} , through the C-terminal domains (CTDs) [Fig. 1(a)] [6–9]. By linking intracellular Ca^{2+} signal to cell excitability, BK channels play a central role in many important physiological processes [10,11], and they are implicated in many cardiovascular and neurological diseases [12]. BK channels are considered promising therapeutic targets for multiple diseases including epilepsy [13], lysosomal storage diseases [14], temporal lobe seizures [15], hypertension [16], and dyskinesia [17]. There is a great need to further understand the gating and regulatory mechanisms of BK channels at the molecular level.

BK channels differ significantly from canonical voltage-gated K^+ (Kv) channels in both the tetrameric topology and pore conformations. The voltage sensor domains (VSDs) are domain-swapped with respect to the pore-gate domain (PGD) in Kv channels [18–21]. In contrast, cryo-EM structures of the whole-length BK channels reveal that VSDs and PGDs are not domain-swapped [22–27], which is a key feature that likely underlies distinct voltage gating characteristics

and mechanisms [28]. Another important difference is that a classical Kv channel is closed by pinching the domain-swapped pore lining helices at the intracellular entrance, creating a bundle crossing gate that physically obstructs the K^+ entrance from the cytoplasmic side [3,29–31]. However, bundle crossing is absent in all available cryo-EM structures of BK channels [22–27], and the pore of BK channels remains physically open with a diameter of ~ 10 Å even in the Ca^{2+} -free, deactivated state. Molecular dynamics (MD) simulations suggested that BK channels likely followed the hydrophobic gating mechanism [32–34]. Here, changes in the shape and surface hydrophobicity of the inner pore in the Ca^{2+} -free state allow it to undergo spontaneous hydrophobic dewetting, giving rise to a (water) vapor barrier to block the ion flow. The dry pore remains physically open and accessible to quaternary ammonium channel blockers [32], which has been observed experimentally but not understood previously [35,36]. The hydrophobic gating mechanism also readily explains methanethiosulfonate reagent accessibility experiments [37], as well as systematic scanning mutagenesis studies and free-energy calculations showing that modulation of pore hydrophobicity is strongly correlated with BK channel activation [34,38].

Hydrophobic gating has now been recognized for dozens of channels, which are activated by voltage, mechanical force, and/or ligand binding and include both domain-swapping and non-domain-swapping channels [39,40]. It has been proposed that hydrophobic gating could work synergistically with bundle crossing in channels where both features coexist [41]. Yet, the experimental support for hydrophobic gating in ion channels has been indirect and largely based on structural, mutagenesis, and computational analysis. No effective experimental assay has been developed to directly validate and further explore this noncanonical gating mechanism. The key difficulty is the lack of effective assays to functionally study

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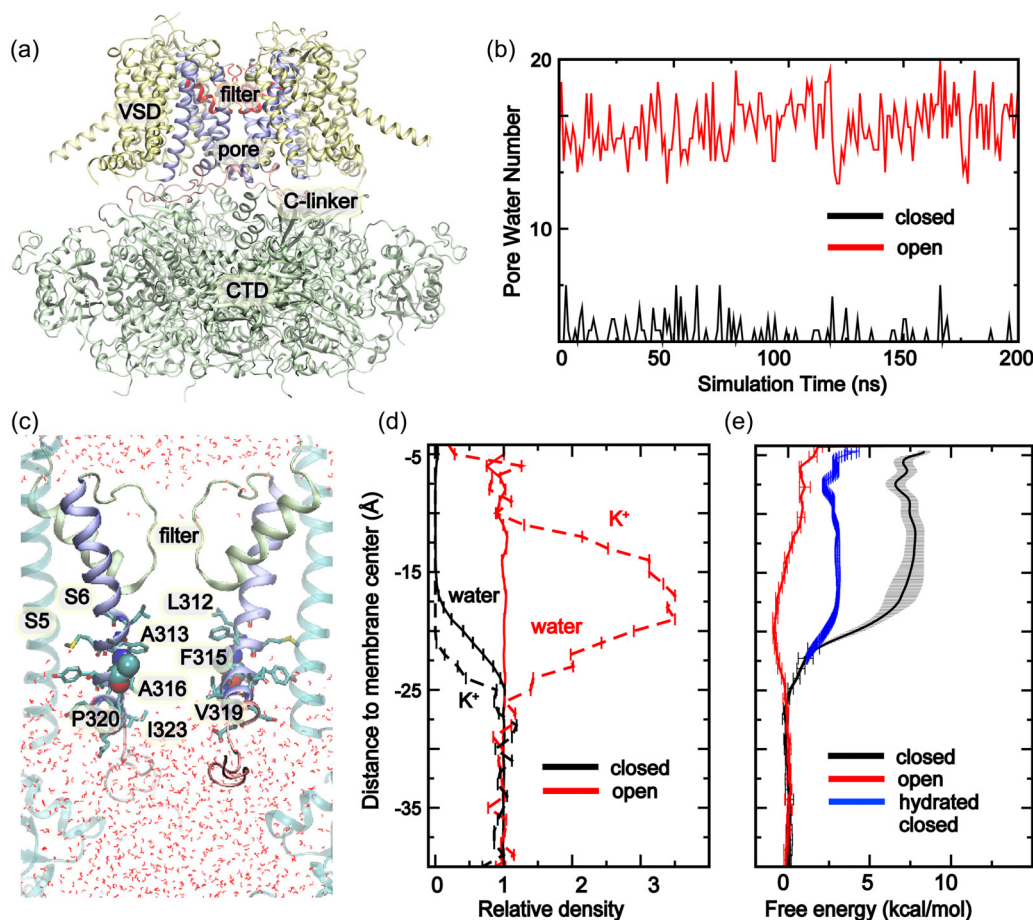


FIG. 1. Structure, hydration, and ion permeation free-energy of hBK channels. (a) The overall structure of Ca^{2+} -free hBK channel (PDB: 6v3g), the C-terminal domain (CTD), C-linker, VSD, pore, and filter are colored in green, pink, yellow, blue, and red, respectively. (b) Representative traces showing the number of pore water molecules as a function of time for the Ca^{2+} -free (black) and bound (red) states. (c) A representative snapshot of the dewetted pore (taken at 100 ns of sim 1, Table S1). Water molecules are represented as wires, the pore-lining residues (L312, A313, F315, V319, and I323) are presented in sticks, and A316 is highlighted with van der Waals spheres. (d) Average water and K^+ density profiles in the open (red) and closed (black) states, derived directly from the last 150 ns of equilibrium simulations (Table S1, *sim 1* and 2). (e) PMFs of K^+ permeation calculated from umbrella sampling for the closed state (black trace, Table S2, *wt0v*), the average K^+ density profile of the open state (red trace, Table S1, *sim 2*), and umbrella sampling for the closed channel with a hydrated pore (blue trace, Table S2, *wt-hydrated*). The error bars show the standard deviations from three replicas.

the gate in the closed state, when dewetting has happened and the vapor barrier is formed. Curiously, it is known that BK channels have a small but measurable “intrinsic opening” at very negative voltage ($\leftarrow 100$ mV) and minimal intracellular Ca^{2+} [6,42–44]. The open probability (P_o) at these negative voltages is weakly voltage-dependent, distinct from canonical Kv channels where P_o continues to decrease exponentially as a function of voltage [45,46]. We observe that a vapor barrier only gives rise to a finite free-energy barrier to ion permeation [32] and thus cannot completely shut down ionic current even if the channel is completely driven into the deactivated conformational state (such as under 0 mM Ca^{2+} and large negative voltages). That is, unlike physical blockage formed by bundle crossing, hydrophobic gating channels are inherently leaky, which could give rise to the intrinsic opening of BK channels observed.

In this work, we exploit atomistic simulations and available electrophysiology data to test the hypothesis that intrinsic

opening of BK channels derives from inherent leakage in hydrophobic gating. We show that the free-energy profiles of K^+ permeation through the vapor barrier quantitatively predict intrinsic opening probabilities. Free-energy analysis further reveals that the shallow voltage dependence of P_o is primarily due to the electric field effects on the permeating ion through the vapor barrier. In addition, we explored three orthogonal approaches to perturb the vapor barrier, namely, truncation of the entire cytosolic gating ring, binding of a ligand to the pore, and mutations of pore-lining residues. We showed that these perturbations of the vapor barrier led to predictable changes in intrinsic opening, with the predictions on the first two perturbations confirmed by published limiting slope measurements. Taken together, our analysis strongly supports the notion that intrinsic opening is an inherent property of hydrophobic gating. Therefore, measurement of intrinsic opening could provide an experimental strategy to directly study hydrophobic gating, such as using established

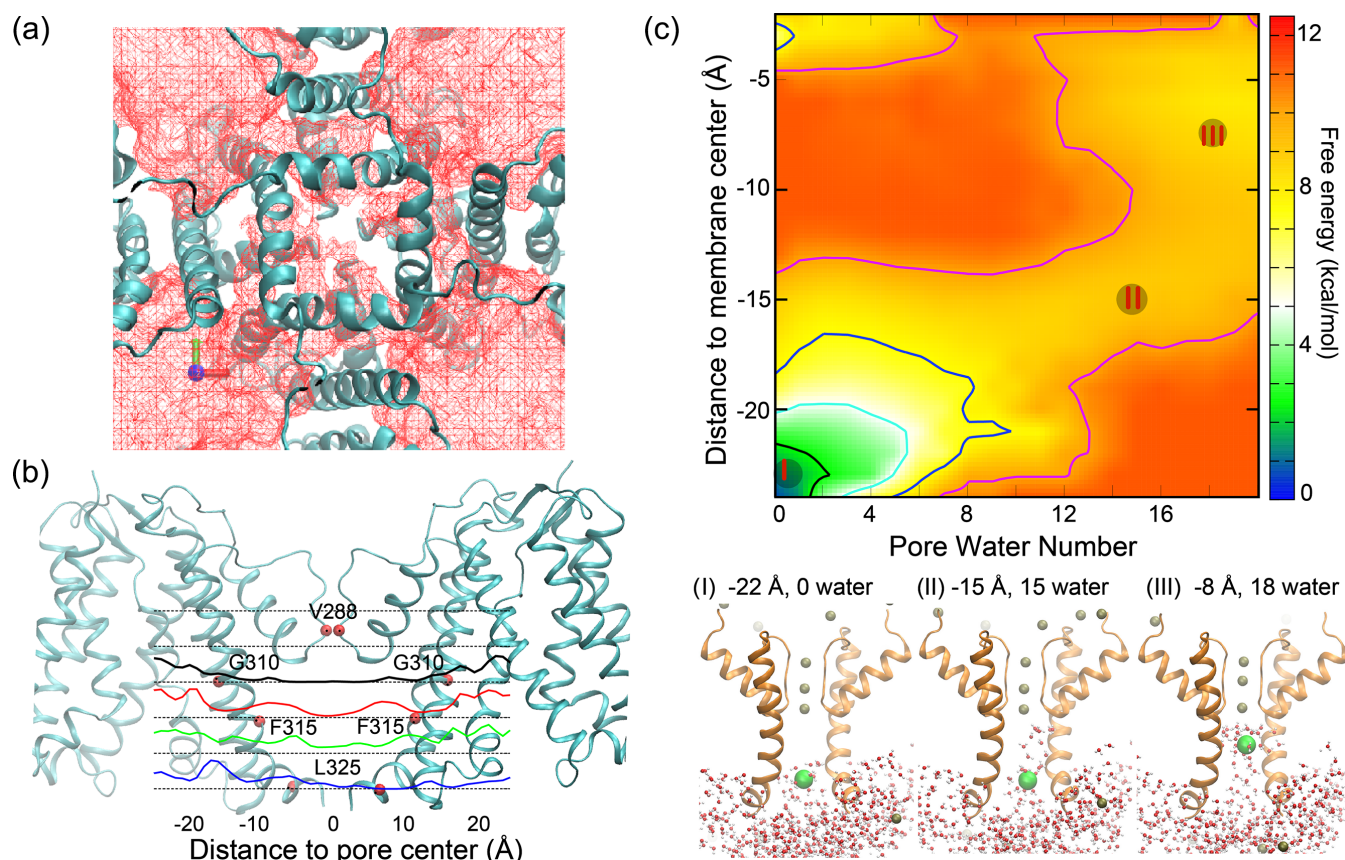


FIG. 2. Pore fenestration of lipid tails and K^+ -driven pore hydration. (a) Density of lipid tails near the pore of wt hBK in the Ca^{2+} -free state, calculated using the last 150 ns of equilibrium simulations (Table S1, *sim 1*). The density is plotted as red mesh with a cutoff of $0.03 \text{ atom}/\text{\AA}^3$. (b) Distribution of lipid tails at various z -positions along the central axis of the pore. The mean lifetime of lipid tails in the pore is $<10 \text{ ns}$ (see Fig. S3). (c) Free-energy surface as a function of pore water number and distance to the membrane center, calculated from WHAM analysis of the umbrella sampling simulations for the closed state (Table S2, *wt0v*). Contours are drawn every 2.5 kcal/mol . Snapshots are shown for three representative locations (I–III) along the pathway, with the biased K^+ ion highlighted as the green sphere and other K^+ ions near the pore shown in silver spheres. Only two pore lining S6 helices and filter loops/helices are shown for clarity.

limiting slope experiments to measure the leaking current when the vapor barrier is formed.

II. RESULTS

A. Leakage of vapor barrier explains both the magnitude and kinetics of intrinsic opening

Starting from the relaxed cryo-EM structure [Fig. 1(a), PDB 6v3g, see Methods], the pore of human BK (hBK) in the Ca^{2+} -free, closed state readily underwent hydrophobic dewetting transitions during equilibrium atomistic simulations [e.g., see Fig. 1(b)], similar to what was observed previously using different structures [32–34]. The closed pore remained stably dewetted throughout subsequent production simulations, and the number of pore waters fluctuated between 0 and up to 5, consistent with a dry pore occupied by water vapor phase [Fig. 1(c)]. The resulting relative density profiles show that there is depletion of water, and consequently K^+ , densities from around $-15 \text{ \AA}$ (near P320) to the filter in the closed state pore [Fig. 1(d)]. This dry inner pore region is lined by a series of hydrophobic residues including L312, A313, F315, A316, V319, and P320 [Fig. 1(c)]. The structure

of the dry pore is stable during all simulations (see Fig. S1 of the Supplemental Material [47]) with a diameter of $\sim 7 \text{ \AA}$ (Fig. S2), physically open and accessible to hydrated ions, channel blockers, and various side chain modifying reagents. In contrast, the pore in the Ca^{2+} -bound open channel remained fully solvated throughout the simulations [Fig. 1(b)], and there is no water or K^+ depletion in the pore [Fig. 1(d)]. Note that the K^+ density peaks at about three times that of the bulk in the open channel, due to the presence of eight conserved acidic residues near the pore entrance (E321/E324). It has been shown that deleting these charges leads to an $\sim 50\%$ drop in the single channel conductance [48].

Lipid tails can enter the dewetted pore through the fenestration gap between the pore lining S6 helices [Figs. 2(a) and 2(b)]. This has been observed consistently in previous simulations and likely reflected in densities within the pore in multiple cryo-EM maps [24,27,32–34]. It is possible that the presence of the lipid tails in the pore helps stabilize the dewetted state and drives hydrophobic gating [49]. During the current equilibrium simulations, lipid tails that enter the dry pore are highly dynamic with an average residence time of $<10 \text{ ns}$ (Fig. S3), which suggests that lipid tails do not show a strong preference of nonspecific interactions with the pore

wall compared to the hydrophobic membrane interior. The implication is that the presence of a lipid tail in the pore is likely a consequence, rather than a driver, of pore dewetting. Previous simulations revealed the coupling of lipid fenestration with the side chain rotamer state of F315 [32]. When the pore is dewetted, the F315 sidechain can sample both the pore and helix-helix interface facing states, the former of which facilitates lipid fenestration into the dry pore. Two independent free-energy analyses using either the classical force field or a polarizable one have further confirmed that lipid presence in the pore did not appear required for dewetting of the BK pore [34,50].

We then calculated the potential of mean force (PMF) of K^+ permeation through the pore in both open and closed states. The results show that, while there is no free-energy barrier for the open (and hydrated) pore, the vapor barrier in the closed (and dry) pore imposes a free-energy barrier of $\Delta W \sim 8$ kcal/mol for K^+ to reach the filter from the cytosolic entrance [Fig. 1(e), red versus black trace]. Permeation of K^+ through the pore in the closed state is strongly coupled to pore rehydration [Fig. 2(c)]. K^+ essentially retains its full first solvation shell upon entering the pore, significantly increasing the number of pore water to ~ 18 when approaching the selectivity filter. Note that strong coupling of the pore water number and distance to the membrane center allows the 1D projections [such as 1D PMFs shown in Fig. 1(e)] to accurately capture the “true” free-energy barrier (~ 8 kcal/mol).

A major implication of the finite nature of the free-energy barrier imposed by the vapor region is that, even if the channel is driven to 100% in the closed conformational state (e.g., under large negative voltages and no intracellular Ca^{2+}), there would be a nondiminishing probability of $P_o \sim e^{-\Delta W/RT} \sim 10^{-6}$ for K^+ to cross the barrier and leak through at room temperature. Strikingly, this estimate is in quantitative agreement with the intrinsic opening probabilities of BK channels reported in the literature [6,17,51,52]. The intrinsic opening probability has been attributed to some unknown spontaneous activation of the pore while all VSDs are in the resting state [51,53]. Curiously, limiting slope experiments show such spontaneous opening transitions to be extremely fast, no slower than 20 μs for the wild type (wt) full-length hBK channel. The fast transition kinetics is inconsistent with a large-scale pore conformational change, such as those involved in voltage-driven activation of BK channels on ms timescales. Instead, the fast transient opening is reminiscent of sporadic rehydration dynamics of hydrophobic nanopores, which readily occurs at tens of ns timescales for carbon nanotubes [54,55] to $\sim \mu s$ for protein pores with complex and roughed surface [34].

It has also been observed that the transiently open pore in limiting slope experiments possesses similar single channel conductance to that of the open state driven by membrane voltage and/or Ca^{2+} binding [6,17,51,52]. To examine if a transiently hydrated pore in the closed conformational state would support conductance similar to the conformationally open pore, we calculated the PMF of K^+ permeation of the closed but (pre)hydrated pore. Here, the hydration level of the pore was maintained by imposing a restraint potential on the number of pore water (N_{water}) to 30 (see Methods) [34,56].

The resulting free-energy barrier of K^+ entering the pore is reduced dramatically to below 3 kcal/mol [Fig. 1(e), blue trace], which is much lower than the estimated ~ 5 –6 kcal/mol barrier imposed by the selectivity filter itself [57,58] and should thus be no longer rate-limiting to K^+ conductance. That is, a transiently hydrated pore in the closed conformational state is expected to have conductance similar to that of the conformationally open pore. Indeed, direct conductance calculations by Gu and de Groot using atomistic simulations have shown that the single channel conductance of the BK channel is mostly determined by the hydration level of the central cavity regardless of whether the pore is in the conformationally open or close state [33]. Taken together, inherent leakage of the vapor barrier can readily explain both the magnitude and kinetics of intrinsic opening of hBK channels derived from existing limiting slope experiments.

B. Shallow voltage dependence of intrinsic opening arises mainly from the electric field effects on the permeating K^+

The shallow voltage dependence of intrinsic opening is distinct from the steep dependence driven by VSD activation under depolarizing voltages [51,53]. Its molecular origin is unknown, even though it presumably arises from the voltage dependence of the putative pore conformational equilibrium with resting VSDs. To examine if vapor barrier leakage could give rise to the observed voltage dependence, we calculated additional free-energy profiles of K^+ permeation at -40 , -80 , -120 , and -160 mV with the backbone heavy atoms of the pore restrained to be the equilibrated conformationally closed state (Table S2; see Methods). The results, summarized in Fig. 2(a), show that the free-energy barrier of K^+ permeation slightly increases at more negative voltages, from ~ 8 kcal/mol at 0 mV to ~ 9.5 kcal/mol at -160 mV. Strikingly, the leakage probabilities estimated from the barrier height are in quantitative agreement with published experimental measurements, in terms of both the magnitude and slope of voltage dependence [Fig. 2(c), filled black circles versus black empty triangles, circles, and diamonds]. Note that the intrinsic opening reported from the Rothberg lab [17] is ~ 10 -fold higher compared to results reported from other labs [6,52,59], likely reflecting differences in experimental conditions. The slope estimated from calculated $\log(P_o)$ values from -40 to -160 mV is 0.003 mV^{-1} [Fig. 3(c), green line], compared to about 0.005 mV^{-1} from analysis of the experimental measurements. This is an important observation that a large-scale conformational response of the pore is not required for recapitulating the limiting slope of BK channels.

To determine the origin of the voltage dependence of the K^+ permeation free-energy barrier, we evaluated the contribution of a local electric field. The electric potential along the pore axis was calculated from equilibrium simulations at different voltages with the backbone of the pore restrained (Table S1, *sim 6–10*) (see Methods and Fig. S4). The work to move a K^+ from bulk solvent to the inner pore region against the electric field can then be estimated as $\Delta\Delta W_{(v)} = q \Delta E_{(v)}$, where $q = +1$ for K^+ , and $\Delta E_{(v)}$ is the relative electric potential with respect to the bulk region. The electric field contribution can then be added to the 0 mV

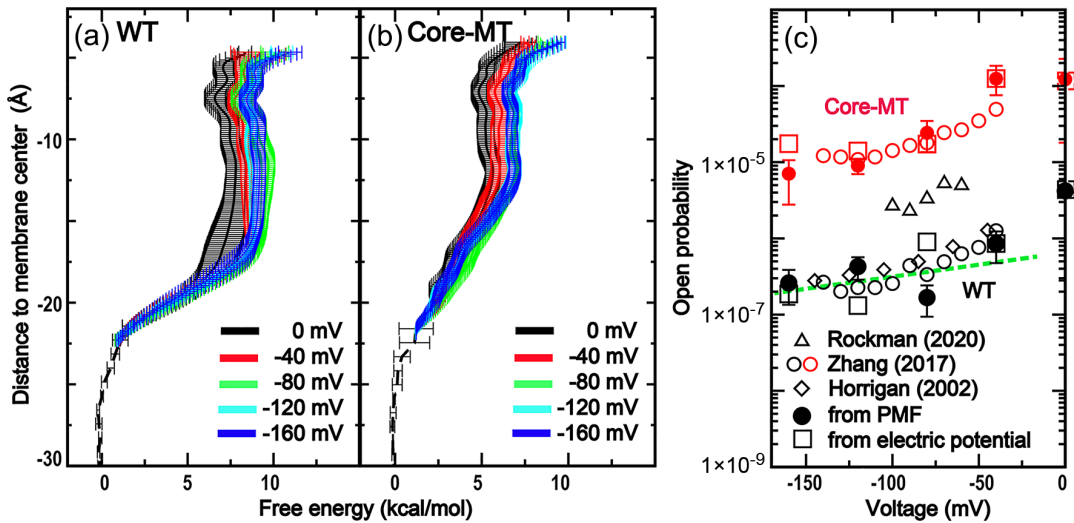


FIG. 3. Intrinsic opening of full-length and core-MT hBK channels. (a), (b) PMFs of K⁺ permeation through the closed pore of the full-length and core-MT hBK channels, calculated using umbrella sampling at different voltages (Table S2, sim *wt0mv* to *core-160mv*). (c) Intrinsic opening probabilities estimated from PMFs in panels (a) and (b) (solid circles) and from 0 mV PMFs and electric potential profiles (empty squares). The error bars show the standard deviations from three replicas. Three representative experimental measurements for the full-length channel extracted from the Rothberg lab [17], the Horrigan/Aldrich labs [6], and the Cui lab [52] (black empty triangles, circles, and diamonds) are shown for comparison. Only one set of experimental results is available for core-MT hBK channels from the Cui lab [52] (red empty circles). The green dashed line shows the linear fit to opening probabilities calculated from PMFs at -40 to -160 mV.

PMF [Fig. 3(a), black trace] to directly estimate the voltage dependence of K⁺ permeation barrier as $\Delta W_{(v)}^* \sim \Delta W_{(0)} + \Delta \Delta W_{(v)}$, and then the leakage as $P_{o,(V)} \sim e^{-\Delta W_{(v)}^*/RT}$, where $\Delta W_{(0)}$ is the free-energy barrier at 0 mV. Remarkably, the resulting leakage probability estimates seem to well reproduce the values derived from the full PMF calculations [Fig. 3(c), empty squares versus filled circles]. Therefore, the shallow voltage dependence of the intrinsic opening of BK channels arises mainly from the effects of the electric field on the permeating K⁺ as it enters the inner pore and crosses the vapor barrier.

C. Intrinsic opening reports vapor barrier perturbations: CTD truncation, drug binding, and pore mutations

To further examine if the vapor barrier leakage is indeed the origin of intrinsic opening, we explore three distinct approaches to perturb the inner pore properties and test if they can lead to predictable changes in intrinsic opening that can be measured experimentally. The first approach involves the Core-MT construct of BK channels where the whole CTD is removed and replaced by an 11-residue minitail from K_v channels [52,60]. Core-MT BK channels are devoid of Ca²⁺ and Mg²⁺ activation but retain voltage-dependent gating. It has been shown that, while the voltage sensor movement (Q-V relationship) of core-MT only exhibits a small change compared to the full length channel (shifted to the left about 20 mV), the intrinsic opening probability increases by more than two orders of magnitude [52]. The equilibrated conformations of the pore are similar in core-MD and full-length hBK channels (Fig. S5). However, the absence of the CTD increases the flexibility of the C-linkers [Fig. 1(a)], potentially leading to dynamic destabilization of the vapor lock in the closed state. Indeed, free-energy calculations reveal that the

barrier of K⁺ entering the pore is reduced to ~ 5 kcal/mol at 0 mV [Fig. 3(b), black trace], which translates into an ~ 150 -fold increase in leakage probability, in excellent agreement with published experimental results [52]. The voltage dependence of the intrinsic opening of core-MT predicted from the PMFs [Fig. 3(b)] is also in quantitative agreement with the measured results [Fig. 3(c), solid versus empty red circles]. Following the same procedure as described above, we further show that the experimentally determined limiting slope can be fully explained by the electric field effects and does not require pore conformational response to different membrane voltages [Fig. 3(c), red empty squares versus circles].

In the second approach, we examine the effects of mutating the pore-lining residues on the vapor barrier and predict the effects on limiting slope measurements. A previous systematic scanning mutagenesis study revealed a strong correlation between the overall hydrophobicity of the pore with gating voltage of BK channels [38]. Here we focus on two mutations of A316, located at the center of the vapor barrier. A316I installs a larger hydrophobic sidechain and increases the half activation voltage ($V_{1/2}$) from 163 to 353 mV in 0 Ca²⁺; A316S installs a hydrophilic sidechain and reduces $V_{1/2}$ to 40 mV [38]. Equilibrium simulations suggest that these mutations do not lead to significant changes of the pore conformation itself (Fig. S2). However, alterations of the inner pore hydrophobicity seem to have significant impacts on the vapor barrier. The barrier to K⁺ entering the pore is increased to ~ 9 kcal/mol for A316I and decreased to ~ 3 kcal/mol for A316S at 0 mV [Figs. 4(a) and 4(b)]. Interestingly, a previous free-energy analysis of BK inner pore hydration free-energy also showed that A316S destabilizes the dry vapor state by ~ 4 kcal/mol while A316I stabilizes the vapor state by ~ 2 kcal/mol [34], suggesting that pore rehydration is the major source of free-energy

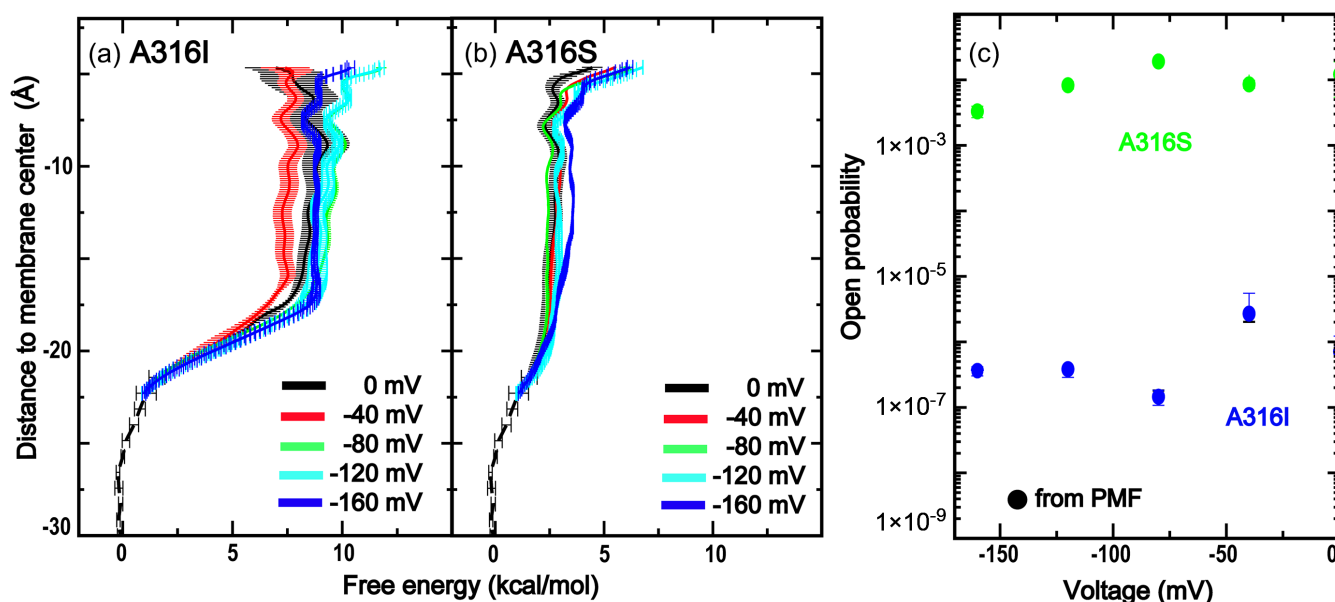


FIG. 4. Effects of pore mutations on the intrinsic opening of full-length hBK channels. (a), (b) PMFs of K⁺ permeation through the closed pore of the full-length A316I and A316S mutant hBK channels, calculated using umbrella sampling at different voltages (Table S2, sim 316/0mv to 316S - 160mv). (c) Intrinsic opening probabilities estimated from PMFs. The error bars show the standard deviations from three replicas.

barrier for K⁺ (leakage) permeation in the closed state. Consequently, the calculation predicts that A316S should have a much larger intrinsic opening probability ($\sim 10^{-2}$), while A316I should have a smaller intrinsic opening probability ($\sim 10^{-7}$). Experimental limiting slope measurements are currently not available in the literature, and the predicted effects of A316I and A316S on intrinsic opening remain to be validated.

Lastly, we examine the action of small molecule NS11021, a negatively charged activator (NCA) that has been shown to activate several potassium channels including BK. Structural, biochemical, and electrophysiological data have previously demonstrated that NS11021 binds to the inner pore of BK channels [17,61]. Atomistic simulations further showed that NS11021 binding to the deactivated BK pore was dynamic, stabilizing by nonspecific interaction of the nonpolar trifluoromethyl-phenyl (FB) group with the hydrophobic inner pore surface [62]. Critically, the presence of the negatively charged tetrazol-phenyl group of NS11021 significantly increases the hydration level of the pore and promotes the activated state, which explains the ability of NCAs to act as a broad-spectrum activator. The most striking effect of NS11021, though, is that destabilization of the vapor barrier by NS11021 can be expected to increase inherent leakage and thus the intrinsic opening of BK channels. This is exactly what has been observed computationally and experimentally. Free-energy analysis showed that the barrier to K⁺ permeation is reduced by ~ 3 kcal/mol [62], which translates into an ~ 150 -fold increase in inherent leakage. This is in good agreement with the limiting slope measurement showing an ~ 60 -fold higher intrinsic opening in NS11021-bound state of hBK channels [17], further supporting the notion that intrinsic opening directly reports inherent leakage and thus the strength of the hydrophobic gate.

III. DISCUSSION

Hydrophobic gating has emerged as a major noncanonical mechanism in ion channels. Specifically for BK channels, they possess a large inner pore with a minimal diameter of $\sim$ nm in the activation state to support the large conductance. The ion permeation can be efficiently shut off without complete collapse of the pore to form a physical constriction point. Instead, relatively small conformational changes are required to change the shape and hydrophobicity of the pore such that it can spontaneously dewet to create a vapor barrier during deactivation. In this work, we combine atomistic simulation and existing electrophysiology data to demonstrate that the well-known intrinsic opening of BK channels derives from the leakage vapor barrier, due to a finite free-energy barrier it provides for K⁺ permeation. We show that vapor barrier leakage quantitatively recapitulates both the magnitude and kinetics of hBK intrinsic opening observed experimentally. Our study reveals that the shallow voltage dependence of intrinsic opening at negative voltages does not require conformational response of the pore when VSDs are fully deactivated; instead, it can be readily explained by the effects of local electric field on the permeating K⁺ during leakage.

We demonstrate that various mutations and drug binding can affect the dynamics and surface properties and/or hydration level of the pore to modulate the stability of vapor barrier in deactivated BK channels. These perturbations lead to predictable changes in the inherent leakage probability and can be validated directly by measuring intrinsic opening. Therefore, limiting slope experiments provide an experimental essay to study hydrophobic gating. This essay will enable one to systematically study how hydrophobic gating can be control by channel protein properties and binding of various ligands in complex cellular environments. As the intrinsic equilib-

rium for channel opening is predominantly determined by liquid-vapor equilibrium in the inner pore region, hydrophobic gating can be controlled and regulated solution conditions including the presence of certain metabolites, salt concentration, and external electric field. Many distal mutations as well as Ca^{2+} binding can dramatically modulate the intrinsic open probability of BK channels [6,59,63]. Understanding their impacts on the vapor barrier itself in the closed state may provide a fundamental basis for further understanding the allosteric mechanism of BK activation and regulation.

To the best of our knowledge, BK channels are the only channel where the intrinsic opening has been experimentally detected. This may be attributed to several unique properties of BK channels that make them particularly amenable to limiting slope measurements. First, BK has the largest open channel conductance (up to 300 pS) among all channels. Second, it can be independently controlled by Ca^{2+} and voltage, allowing accurate quantification of the number of channels in limiting slope experiments. Third, it has a large pore opening in the closed state, such that the vapor barrier imposes a modest barrier (~ 8 kcal/mol) and gives rise to a larger and more detectable leakage current ($\sim 10^{-6}$). Nonetheless, the current study firmly supports that the vapor barrier of hydrophobic gating is inherently leaky. Thus, the intrinsic opening is a fundamental property of hydrophobic gating channels that likely has significant implications on the physical mechanism of gating and regulation of these channels.

IV. METHODS

A. Atomistic simulations: Initial structures, equilibration, and production runs

The cryo-EM structures of hBK channel in the Ca^{2+} -bound (PDB: 6v38) or Ca^{2+} -free (PDB: 6v3g) states [24] were used in all simulations reported in this work. Several short loops missing in one structure were rebuilt using the other structure as template with Swiss-PDB server (e.g., as A567-E576 and N585-E591 is missing in 6v38 but present in 6v3g), as described previously [34]. The missing N- and C-terminal segments as well as several long loops (H55-G92, A614-N682, S835-I870) are presumably dynamic and thus not included in the current simulations. Residues before and after the missing segments are capped with either an acetyl group (for N-terminus) or an N-methyl amide (for C-terminus). Standard protonation states under neutral pH were assigned for all titratable residues. We note that PDB 6v3g contains a spurious π -helix turn in residues 317-32 of helix S6, which has not been observed in any other structures of BK channels thus far [22–26,64] and is unstable in simulations [34]. All simulations of the Ca^{2+} -free state reported in this work were thus initiated from a carefully relaxed 6v3g structure from a previous work [34] (see Fig. S1a). The relaxed pore structure was stable during all simulations (e.g., see Fig. S1b).

All initial structures in various states were first inserted in model POPC lipid bilayers and then solvated in TIP3P water using the CHARMM-GUI web server [65]. The systems were neutralized and 150 mM KCl added. For the full-length hBK channel system, the final simulation boxes contain about

~ 900 lipid molecules (POPC) and $\sim 130\,000$ water molecules and other solutes, with a total of $\sim 520\,000$ atoms and dimensions of $\sim 180 \times 180 \times 160 \text{ \AA}^3$. For the core-MT system (with CTD removed), the final simulation boxes contain about ~ 600 lipid molecules (POPC) and $\sim 55\,000$ water molecules and other solutes, with a total of $\sim 260\,000$ atoms and dimensions of $\sim 160 \times 160 \times 100 \text{ \AA}^3$. The CHARMM36m all-atom force field [66] was used with the CHARMM36 lipid force field [67]. All simulations were performed using CUDA-enabled versions of Gromacs 2019 [68,69]. Electrostatic interactions were described by using the Particle Mesh Ewald (PME) algorithm [70] with a cutoff of 12 $\text{\AA}$. van der Waals interactions were cut off at 12 $\text{\AA}$ with a smooth switching function starting at 10 $\text{\AA}$. Covalent bonds to hydrogen atoms were constrained by the SHAKE algorithm [71], and the MD time step was set at 2 fs. The temperature was maintained at 298 K using the Nose-Hoover thermostat [72,73]. The pressure was maintained semi-isotropically at 1 bar at membrane lateral directions using the Parrinello-Rahman barostat algorithm [74].

All systems were first minimized for 5000 steps using the steepest descent algorithm, followed by a series of equilibration steps where the positions of heavy atoms of the protein and lipid were harmonically restrained [75]. Specifically, six equilibration step (25 ps for steps 1–3, 100 ps for steps 4–5, and 10 ns for step 6) were performed, where the restrained force constants for proteins were set to 10, 5, 2.5, 1.0, 0.5, and 0.1 kcal mol $^{-1} \text{ \AA}^{-2}$, respectively. For lipids, the phosphorus is restrained with force constants of 2.5, 2.5, 1.0, and 0.5, 0.1 and 0.0 kcal mol $^{-1} \text{ \AA}^{-2}$, respectively. In the last equilibration step, only protein heavy atoms were harmonically restrained, and the system was equilibrated 10 ns under NPT (constant particle number, pressure, and temperature) conditions.

As summarized in Table S1, three replicas of 200 ns atomistic simulations were first performed for the Ca^{2+} -free and -bound states of WT hBK channel (*sim 1* and 2). In addition, three replicas of simulations were performed for the core-MT hBK channel in the closed state, generated by removing the CTD region (residue after R342) from the equilibrated full length hBK channel structure (*sim 3*). Two additional equilibrium simulations were performed for the A316I and A316S mutant full-length hBK channels in Ca^{2+} -free state (*sim 4* and 5). The remaining 20 sets of simulations (*sim 6* to 25) were initiated from various equilibrated structures as noted for various constructs under different membrane voltages to characterize the electric potentials and/or generate relaxed initial structures for subsequent free-energy calculations (see below). The external electric field was imposed as described in previous studies [76].

B. Calculation of K^+ permeation free-energy profiles

Umbrella sampling [77] was used to calculate the ion permeation free-energy profiles in the closed states under different membrane voltages. To generate initial structures for umbrella sampling, an additional 200 ns simulations with all backbone atoms restrained were performed (-160 to 0 mV) for each system (*sim 6–25*, Table S1), to relax the ion, water, lipid, and protein configurations at the volt-

ages imposed. A snapshot was then selected at ~ 100 ns (Table S2), where a single K^+ ion was located near the cytosolic pore entrance (~ 25 Å below the membrane center and ~ 10 Å to E321/E324). The initial conformations for umbrella sampling were then generated by 5-ns steered MD simulations, during which a moving harmonic pulling potential was imposed on the selected K^+ ion with a force constant of $2.0 \text{ kcal mol}^{-1} \text{ Å}^{-2}$ (Fig. S1c). Umbrella sampling windows were placed at 2 Å intervals covering $z = -6$ to -22 Å for the selected K^+ . For each window, 3×20 ns simulations were performed (Table S2). A spring force constants of $2.0 \text{ kcal mol}^{-1} \text{ Å}^{-2}$ was used to restrain the distance along the membrane normal (z) between the ion and membrane center.

Final 1D and 2D PMFs were calculated using the weighted histogram analysis method [78]. The error bars of the PMF were estimated as standard errors among the three independent sets of free-energy profiles. We note that there is a high probability of multiple K^+ occupancy in the hydrated metal-bound pore or ~ -22 Å below the membrane center. The single-ion PMF calculated from umbrella sampling is thus only valid for the dewetted pore region from -6 to -22 Å. The overall PMFs of K^+ permeation for the closed state of channel from -6 to -30 Å were constructed from umbrella sampling and equilibrium MD using linear interpolation at the -22 to -24 Å region as done previously [32,34]. The height of the free-energy barrier is then estimated by the difference between the bulk region (30 – 40 Å below membrane center) and the plateau region in free-energy barrier (-15 to -10 Å for full length channel and -12 to -10 Å for core-MT).

To obtain the PMF of K^+ permeation with the hydrated pore (Table S2, *wt-hydrated*), we harmonically restrained the pore water count (N_{water}), defined using the PLUMED coordination collective variable as previously described [34,56]. N_{water} was restrained to 30 using a force constant of $1.0 \text{ kcal/mol } N_{\text{water}}^2$. This water count was selected to ensure that the pore is fully hydrated [34].

C. Simulation analysis

Unless stated otherwise, snapshots were extracted every 50 ps after 50 ns of all equilibrium MD trajectories for calculation of statistical distributions. Molecular illustrations were prepared using VMD [79]. The ion and water distribution is calculated by MDAnalysis [80]. Pore water molecules were identified as those occupying the internal cavity below the selectivity filter, roughly from L312 to the plane defined by the center of mass of P320. Pore profiles are calculated using program HOLE [81]. Electric potential maps were calculated using the VMD's PMEPot and VolMap plugins from *sim 6* to *sim 26* (Table S1) [82]. The bin size is set to 1 Å during these calculations. To calculate the electric potential profile along the pore axis, a cylinder region is selected, the center of which is located at the COM filter. The radius is set to 5 Å, which allows the cylinder to cover the internal cavity of the pore without involving protein atoms. The cylinder is then sliced along the z axis with 1 Å intervals, and the potential of each slice is then estimated as the averages of the potential of all bins in this slice.

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J.C. and Z.J. conceived and initiated the studies. Z.J. performed molecular dynamics simulations and analysis. Z.J. and J.C. wrote the manuscript.

The authors declare no competing interests.

DATA AVAILABILITY

The data that support the findings of this article are openly available [83].

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