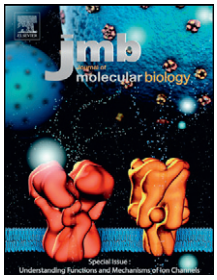

Hydrophobic Gating in Ion Channels

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http://dx.doi.org/10.1016/j.jmb.2014.07.030
Edited by D. L. Minor

Abstract

Biological ion channels are nanoscale transmembrane pores. When water and ions are enclosed within the narrow confines of a sub-nanometer hydrophobic pore, they exhibit behavior not evident from macroscopic descriptions. At this nanoscopic level, the unfavorable interaction between the lining of a hydrophobic pore and water may lead to stochastic liquid–vapor transitions. These transient vapor states are "dewetted", i.e. effectively devoid of water molecules within all or part of the pore, thus leading to an energetic barrier to ion conduction. This process, termed "hydrophobic gating", was first observed in molecular dynamics simulations of model nanopores, where the principles underlying hydrophobic gating (i.e., changes in diameter, polarity, or transmembrane voltage) have now been extensively validated. Computational, structural, and functional studies now indicate that biological ion channels may also exploit hydrophobic gating to regulate ion flow within their pores. Here we review the evidence for this process and propose that this unusual behavior of water represents an increasingly important element in understanding the relationship between ion channel structure and function.

Introduction

The unusual behavior of water in narrow hydrophobic pores, as opposed to bulk, macroscopic solution, can be described as an energetic balance between wetting and dewetting (i.e., drying). The first observations of these transitions were made from molecular dynamics (MD) simulations of explicit water in carbon nanotubes and simple model nanopores and led to the concept now referred to as "hydrophobic gating" [1–3]. At a simple level, the diameter of one water molecule is ~3 Å, yet within a hydrophobic pore of diameter less than ~14 Å, water molecules can begin to exhibit liquid–vapor transitions, switching stochastically between both wet and dry states. The most dynamic range for these transitions is between 9 and 12 Å, and below this range, the pore will be largely dewetted. Therefore, the hydrophobicity of the pore can result in a highly effective barrier to ion permeation (Fig. 1).

Ion channels are specialized membrane proteins that act as pores to enable ion movement across the cell membrane. In addition to their ability to be selective between different types of ions, they can also be switched or gated between an open state (i.e., ion conducting) and a closed state (non-conductive) by external signals such as changes in transmembrane voltage, binding of ligands, and mechanical stress. Interestingly, the pores of many ion channels also have internal dimensions within the range where hydrophobic gating is observed in model nanopores. It was therefore anticipated that some ion channels might also exhibit hydrophobic gating and that this property might be tunable by local changes in the diameter and/or hydrophilicity of the channel pore. Over the last decade, these ideas

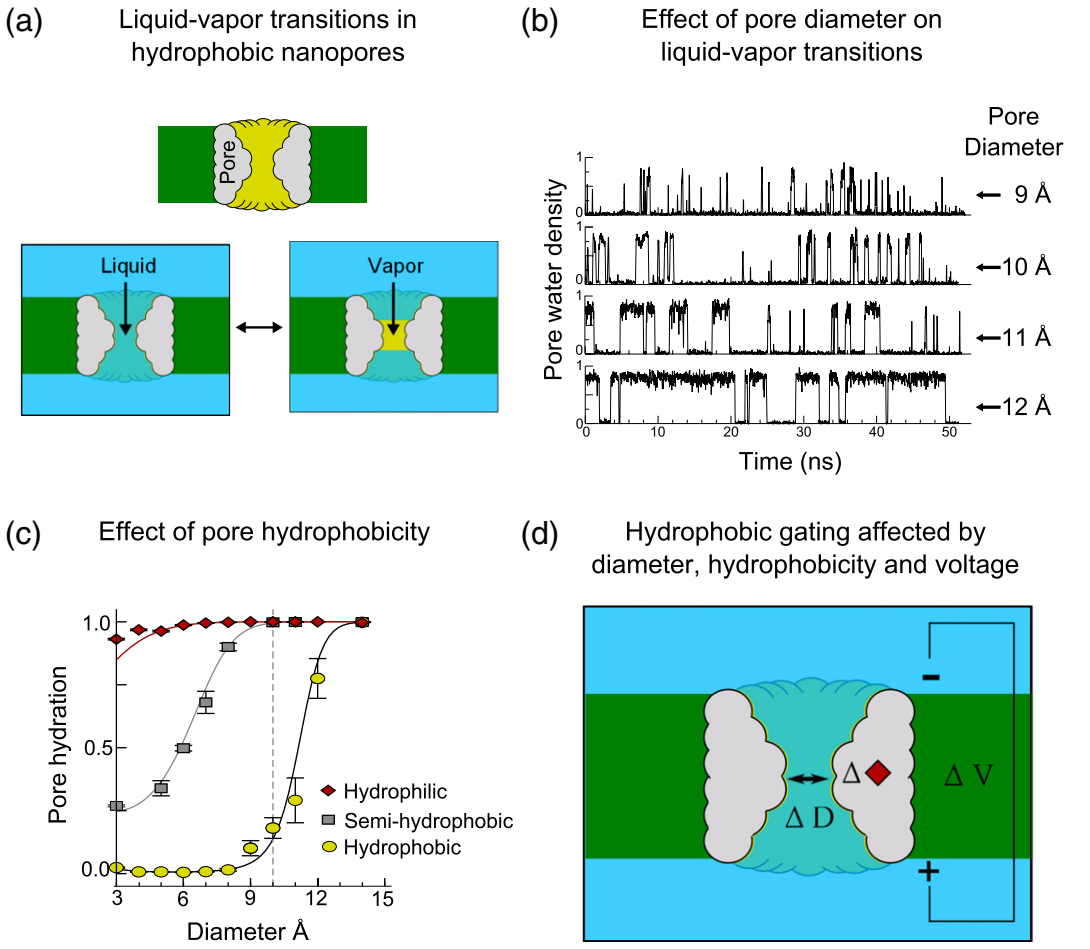

Fig. 1. Principles of hydrophobic gating. (a) Cartoon representation of a cross-section through a model hydrophobic nanopore. Hydrophobic surfaces are shown in yellow, and the membrane is shown in green. In solution, these nanopores can switch stochastically between both wet and dry states via liquid–vapor transitions within the pore. The dewetted vapor state presents an effective barrier to water and ion permeation. (b) These oscillations occur on the nanosecond timescale, and the stability of the wetted state is highly dependent upon pore diameter. (c) The probability of the pore being in the liquid or wetted state is dependent not only upon diameter but also on the hydrophobicity of atoms lining the pore. This was shown by progressively adding hydrophilic atoms to a model nanopore [4]. A fully hydrophilic pore remains fully occupied by water. However, a hydrophobic pore starts dewetting below 14 Å and becomes completely dewetted below ~8–10 Å. Semi-hydrophobic pores also exhibit similar dewetting below ~10 Å (vertical dotted line). (d) The process of hydrophobic gating has now been shown to be influenced by pore diameter, hydrophobicity, and also changes in transmembrane voltage. This figure is adapted from results within Refs. [1] and [4].

have gained momentum driven both by advances in computational techniques and by the increasing availability of crystal structures for many different classes of ion channels. In this review, we examine the evidence for hydrophobic gating in ion channels and highlight recent studies of both channels and model nanopores indicating that this unusual behavior of water may play a critical role in our understanding of ion channel permeation and gating.

Behavior of water in model hydrophobic pores

The concept of hydrophobic gating and its possible influence on the flow of ions through protein ion channels was first elaborated in a series of simulation studies of simple model nanopores with a hydrophobic central region. These narrow pores were not physically occluded but could be shown to form a hydrophobic gate due to liquid–vapor transitions of water within the pore [1,4,5]. In particular, it was shown that a functionally closed (i.e., dewetted; vapor state) pore could be opened, yielding a wetted liquid state either by increasing the diameter or by increasing the hydrophilicity in the narrowest region of the pore (e.g., via the introduction of molecular dipoles or polar groups) [4] (Fig. 1).

Subsequent simulation and theoretical studies confirmed that a narrow hydrophobic nanopore presents a

significant energetic barrier (i.e., a gate) not only to water but also to ions [6]. Recent experimental studies on (non-biological) nanopores have also provided further direct experimental evidence for hydrophobic gating. In particular, these studies have demonstrated experimentally that wetting of functionally closed hydrophobic nanopores can also be achieved by application of a voltage across the pore [7]. This idea, also known as "electro-wetting", is a key functional property of a hydrophobic gate and was originally predicted in simulation studies of simple model nanopores [8]. Other studies have even shown that an asymmetric flow of ions (i.e., rectification) can be introduced by simply altering the relative shape of the nanopore [9].

Hydrophobic gating in biological ion channels

These early descriptions of hydrophobic gating in model nanopores, combined with some of the first high-resolution channel structures quite naturally suggested that a similar mechanism may also exist in biological ion channels such as bacterial mechanosensitive channels, pentameric ligand-gated ion channels (pLGICs), and even members of the superfamily of tetrameric P-loop cation channels [10]. The concept of hydrophobic gating in ion channels has therefore attracted significant interest over the last decade, and there are now several examples where multiple layers of experimental evidence exist to support this mechanism.

Prokaryotic mechanosensitive channels

The bacterial mechanosensitive channels open in response to membrane tension to allow survival of bacteria under hypo-osmotic shock (for detailed review, see Ref. [11]). The first structure of the heptameric small conductance channel (MscS) was initially thought to be open because its central pore had a diameter of ~5 Å [12] (Fig. 2a). However, the pore is highly hydrophobic with branched hydrophobic side chains Leu109 and Leu105 pointing into the pore lumen. The first evidence for hydrophobic gating in these channels was reported in MD simulation studies where a vapor lock was observed within the pore [13,14]. Furthermore, a hydrophilic mutation of Leu109, which had been reported to have a gain-of-function phenotype [15], disrupted this hydrophobic gate *in silico*. This initial crystal structure was therefore considered to be in a closed, non-conductive state [13]. A later structure of an open form of MscS revealed an iris-like rotation of Leu105 and Leu109 away from the pore, causing a change in diameter of >8 Å and opening of its hydrophobic gate [16]. These studies therefore provided the first direct experimental evidence for hydrophobic gating in a biological ion channel. Further details of the MscS gating mechanism are reviewed extensively elsewhere [17,18].

Simulation studies have now extended this idea to other bacterial mechanosensitive channels (e.g., the pentameric MscL) [19] and are supported by a range of experimental observations such as the clustering of (hydrophilic) gain-of-function mutations onto the pore-lining face of the M1 helix [20,21], as well as a direct correlation between residue hydrophilicity and channel function at Gly22 in TM1 [22]. Furthermore, recent subunit titration experiments have demonstrated that dynamically altering the hydrophilicity of a single subunit (by sulfhydryl modification of G22C) is sufficient to open the channel to allow the passage of ions and small molecules (up to ~10 Å in diameter). This suggests that breaking open this hydrophobic gate represents the initial step in the opening process of MscL [23,24].

Pentameric ligand-gated ion channels

pLGICs mediate fast neurotransmission in the nervous system and were the subject of several groundbreaking structural studies that provided the first glimpse into the structure of a eukaryotic ion channel [25,26]. These structures suggested that branched aliphatic side chains within the pore formed a "hydrophobic girdle" with an internal diameter of ~6 Å. A detailed simulation study later demonstrated that this girdle created an energetic barrier to the movement of water and sodium ions through the pore [27].

Subsequent crystal structures of prokaryotic homologs of nAChR in different conformational states (GLIC and ELIC) have now significantly refined our understanding of gating in pLGIC channels (for detailed review, see Ref. [28]). Initially, the architecture of the pore-lining helix suggested that the ELIC channel represented a closed state, while the GLIC structure represented an open state [29–32]. Much like the nAChR, the GLIC channel contains a ring of branched hydrophobic residues within the inner pore, and MD simulations suggested a role for hydrophobic gating within this region (Ile9′–Ile16′) [33] (Fig. 2a). Later studies reported drying transitions during steered MD simulations of the GLIC transmembrane domain from a putative open-state conformation to a closed-state conformation [34] and also estimated the energetic cost of opening this hydrophobic gate [35]. This latter study found that the free-energy cost of hydrating the gate was ~11 kcal/mol, while the energy required for a solvated ion to subsequently move into this gate was only 4 kcal/mol greater. This suggested that the largest energy barrier to ion movement was due to hydration of the pore itself and that drying of this hydrophobic constriction therefore represented the major determinant of ion conductance. Interestingly, more recent structures of GLIC in an apparently

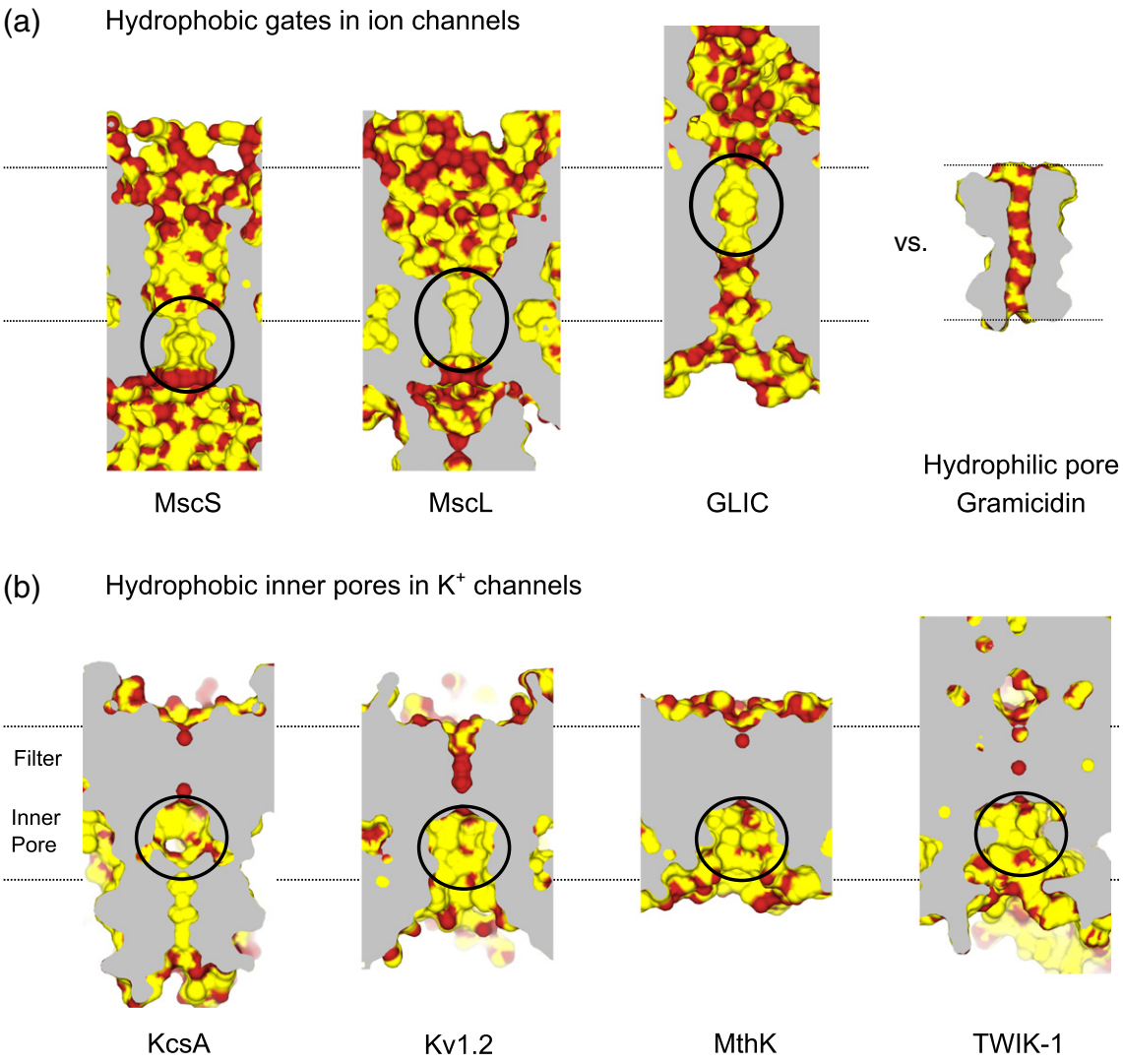

Fig. 2. Hydrophobic gates and pores in biological ion channels. (a) Longitudinal sections through the center of the pore lumen for several different ion channels. Carbon and sulfur atoms are colored yellow, and hydrophilic atoms are colored red. The approximate position of the channels within the membrane is marked by dotted lines. The channels shown are as follows: the closed pores of MscS (2OAU), MscL (2OAR), and GLIC (4NPQ). The positions of the hydrophobic gates are circled; in MscS, this gate contains Leu105 and Leu109; in MscL, Gly22 (Ala20 in 2OAR); and in GLIC, Ile-9′–Ile-16′. These pores are in marked contrast to gramicidin (1MAG), which is hydrophilic throughout the pore. (b) The inner pore of many K^+ channels is also hydrophobic. Shown are sections of KcsA (1K4C), Kv1.2 (2A79), MthK (3LDC), and TWIK-1/K2P1 (3UKM). The circled region of MthK contains Ala88 [58] while TWIK-1 contains Leu146 and Leu261 [71] (see also Fig. 3). Structures are colored and positioned as in (a).

closed (or resting) state [36] now appear to confirm the hydrophobic gating mechanism proposed by Zhu and Hummer [34,35].

The hydrophobic gate region within the nAChR and GLIC structures also appears to be conserved in a related eukaryotic glutamate-gated chloride channel [37]. Thus, although the precise details of the structural changes induced by ligand binding remain to be determined, the basic principle of hydrophobic gating within the pore may be more conserved than the more detailed mechanisms of ligand binding or ionic selectivity within the pLGIC superfamily.

Tetrameric cation channels

The superfamily of tetrameric “P-loop” cation channels includes various potassium, sodium, and calcium selective channels and the non-selective TRP and cyclic-nucleotide-gated channels. The

ability of these channels to select between different cations and to be gated by a diverse range of biochemical and biophysical stimuli enables them to play fundamental roles in the control of nearly all forms of cellular electrical activity. It is therefore not surprising that they have been the subject of intense investigation over the last 50 years [38].

Crystal structures of prokaryotic homologs have now provided us with detailed insights into the mechanisms of cation selectivity while comparison of their transmembrane pore architecture has led to the classical "helix-bundle-crossing" gating model in which the pore-lining helices intersect at the cytoplasmic entrance to seal the permeation pathway shut but then bend and splay outward to expose the inner cavity in the open state [39–43]. For many members of this superfamily, there is now such a wealth of supporting experimental evidence for this model of activation gating that it has found its way into many text books. Indeed, the intuitive simplicity of this mechanism and the way it has been adapted into the modular design of this superfamily is one of its major attractions.

However, despite the structural conservation within the transmembrane/pore modules of this superfamily, there now appear to be other structural and biophysical mechanisms that may also gate the pore. In particular, dynamic structural rearrangements within the selectivity filter are known to be important for gating and are extensively reviewed elsewhere [44,45]. Instead, we examine how hydrophobic gating may be important for the gating of K^+ channels, especially those that appear to lack a classical helix-bundle-crossing gate.

The hydrophobic inner pore of the K channel

Potassium channels are one of the best-characterized groups within this superfamily with functional studies stretching back over many decades; experiments from the 1960s in squid giant axons first indicated that the inner pore of the voltage-gated K^+ channel was relatively hydrophobic because of its relative affinity for quaternary ammonium (QA) blockers such as TEA and its longer-chain derivatives [46]. Furthermore, these QA ions were found to block the K^+ channel only after the channel had been opened, thus identifying a hydrophobic inner pore with an activation gate at its cytoplasmic mouth [47]. Other early studies also demonstrated that the open probability and conductance of these K^+ channels were sensitive to the osmolarity of the bulk surroundings and may involve depletion of water from the channel [48]. The availability of crystal structures for so many different types of K^+ channel now allows us to directly visualize these pores (Fig. 2b). These reveal that the region where the TM helices intersect at the bundle crossing is relatively hydrophobic, but perhaps more surprisingly, the lining of the whole inner pore in many K^+ channels is also hydrophobic. The relative hydrophobicity of the bundle-crossing gate is perhaps not unexpected because this permits tight packing of these helices in the closed state, but the hydrophobic nature of the rest of the inner cavity is of particular interest because ions clearly have to pass through this region to access the selectivity filter (Fig. 2b).

Kv channels

Although a number of open-state crystal structures now exist for voltage-gated (Kv) potassium channels [49,50], no such closed-state crystal structures are available; thus, the precise location of the "bundle-crossing" gate in these channels remains uncertain. However, several studies suggest that this gate may be located slightly higher up within the inner pore than initially predicted by comparison to the KcsA channel. Interestingly, the S6 pore-lining helix in many Kv channels contains a highly conserved Pro-Val-Pro motif thought to form a tight hydrophobic seal [51], and it was found that hydrophilic, but not hydrophobic, substitutions within this region could disrupt the closed state of the channel at resting voltages [52,53].

Advances in MD simulation methodologies have also now allowed extended timescale (microsecond-to-millisecond) simulations of the open-state transition to closed-state transition of the Kv channel pore. These simulations demonstrated that the hydrophobic nature of the inner pore appeared to promote dehydration of the cavity that then underwent a hydrophobic collapse leading to a tight constriction at the Pro-Val-Pro motif [54]. Further simulations with the voltage sensors intact also reported that, when the channel was open under depolarizing conditions, the inner pore remained fully hydrated, but when subjected to hyperpolarizing potentials, the channel exhibited a transient inward "tail" current followed by dewetting of the cavity, thereby halting ion conduction [55]. This dewetting step was concurrent with pore closure and occurred before the voltage sensor moved to the down position. Together, these results therefore suggest that hydrophobic gating mechanisms may even contribute to the gating of channels thought to possess a classical "bundle-crossing" gate.

Non-standard models of K^+ channel gating

Although comparison of the KcsA *versus* MthK structures has been extremely valuable in terms of understanding the classical K^+ channel "bundle-crossing" gating mechanism, there is now clear evidence that some channels within this superfamily do not utilize a bundle-crossing gate. In some cases, this may be explained by the presence of a filter gating mechanism, but in other channels, additional mechanisms have been proposed [56–61]. As a more general channel gating mechanism that also

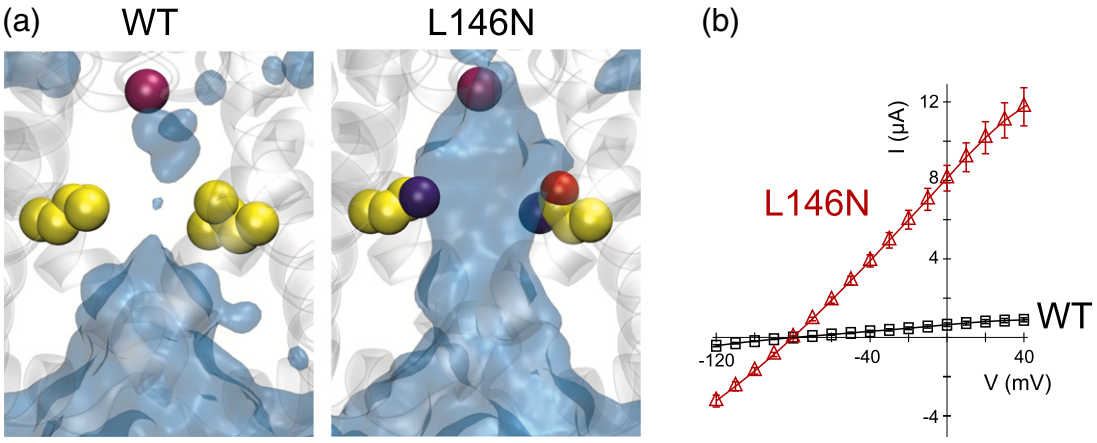

Fig. 3. Hydrophobic barrier in a K2P channel pore. (a) MD simulations of the TWIK-1 K2P potassium channel structure (3UKM) demonstrate that dewetting occurs deep within the inner pore thus creating an energetic barrier to ion permeation [71]. Shown are the average water densities within the inner pore during simulations of a wild type (WT) and the L146N mutant pore that disrupts this hydrophobic barrier. The cyan transparent surface is contoured at 0.50 of bulk water density, overlaid on a snapshot of the inner pore. The side chains at position 146 are highlighted with carbon atoms colored yellow. The K^+ ions at the S4 position are shown as purple spheres. (b) Averaged whole cell currents for WT TWIK-1* and L146N TWIK-1* mutant channels. Disruption of the hydrophobic barrier produces a large increase in channel activity. Hydrophobic gating may therefore contribute to the regulation of channels that do not possess a classical cytoplasmic bundle-crossing gate. This figure is adapted from results within Ref. [71].

obviates the requirement of a bundle-crossing gate, Roth *et al*. have suggested that liquid–vapor transitions within the pore may not only gate ion flow but also underlie the on–off transitions of single-channel currents [62]. Although this remains an appealing hypothesis consistent with the general principles of hydrophobic gating, it is technically challenging to relate such nanoscopic properties to experimentally observed single-channel gating events.

Both the small conductance (SK) and large conductance (BK) Ca^{2+}-activated channels appear to lack a bundle-crossing gate, and in addition to a filter gate, they are also thought to possess a gating mechanism involving hydrophobic residues deep within the inner cavity [56,58–60]. Unfortunately, there are no crystal structures available for these specific channels and thus our understanding of their precise inner pore structure is limited. However, several high-resolution structures are available for the homologous prokaryotic Ca^{2+}-activated K^+ channel, MthK [40].

MthK channel

MthK is considered to be the archetypal "open-state" structure and it was originally proposed that ligand-induced movement of the intracellular domains controlled opening and closing of a helix-bundle-crossing gate [40,63]. However, several studies now indicate that the selectivity filter, not bundle crossing, may play the dominant role in MthK channel gating [60,64]. The open MthK structure (Fig. 2) shows a hydrophobic inner pore with the narrowest constriction (~9 Å) defined made by Ala88 [58] (Fig. 2). Mutation of this alanine (Ala88) to valine or leucine results not only in a progressive decrease in channel conductance but also in a decrease in open probability [58]. By marked contrast, mutation to similarly sized branched hydrophilic side chains (Asn or Asp) causes both an increased conductance and an increased open probability. Analysis of high-resolution structures of MthK reveals a K^+ ion within the cavity near to the cytoplasmic mouth of the channel with Ala88 forming a hydrophobic gap in the middle of the inner cavity [65]. Such observations are therefore consistent with the existence of a hydrophobic barrier within the pore because the ability of water and ions to move through this constriction would be highly dependent upon the relative hydrophobicity of this region. However, further studies are clearly needed to determine the possible influence of this region on permeation and gating.

K2P channels

Another group of potassium channels also thought to lack a classical bundle-crossing gate is the subfamily of two-pore domain (K2P) channels [66]. The pore structure of these channels shares some similarity with classical tetrameric K^+ channels but is assembled as an asymmetrical "dimer of dimers". This pseudo-4-fold symmetry has recently been confirmed by crystal structures of the TWIK-1 and TRAAK channels [67,68]. However, the novel transmembrane architecture of K2P channels poses a number of important questions about how they gate. Studies that

examined the state-dependent access of QA ion blockers to the inner pore concluded that K2P channels do not utilize a lower bundle-crossing gate and suggested that gating occurs close to or within the selectivity filter [61,69]. External stimuli such as extracellular pH are thought to directly modulate this gate in a process similar to C-type inactivation, while internal stimuli are thought to induce subtle movements of the TM helices that can modulate channel activity without full constriction of a lower bundle-crossing gate [69,70].

TWIK-1 has a hydrophobic inner cavity

In an attempt to address how K2P channels gate, a recent MD simulation study examined the TWIK-1 crystal structure embedded in a phospholipid bilayer [71]. Interestingly, stochastic wetting and dewetting events were observed within inner pore. Examination of the residues lining the pore (Fig. 2b) revealed that the inner pore was highly hydrophobic, suggesting that the associated dewetting of this area might create an energetic barrier to ion permeation. In particular, two leucine residues (Leu146 on TM2 and Leu261 on TM4) line the narrowest point of the inner pore forming a "hydrophobic cuff" with a diameter of 8.5 Å. Mutagenesis of these two leucine residues to isosteric but polar side chains (asparagine) led to not only the retention of water *in silico* but also robust whole cell currents when expressed *in vivo* (Fig. 3) [71]. This suggested that a hydrophobic barrier within the inner pore might also contribute to the low levels of functional activity generally observed for TWIK-1.

This hypothesis was validated computationally with free-energy calculations that showed an energetic barrier to ion movement through the hydrophobic wild type, but not in the L146N mutant pore. Likewise, functional studies demonstrated that a series of hydrophilic, but not hydrophobic, substitutions within the cuff produced robust currents by disrupting this hydrophobic barrier. Furthermore, increased voltages were required to drive currents through the hydrophobic wild-type channel pore compared to the L146N mutant [71], possibly reflecting similar results obtained for the voltage-dependent hydration of nanopores [7].

Interestingly, both sequence and structural alignments suggest that the hydrophobic cuff in TWIK-1 is equivalent to the hydrophobic constriction formed by residue Ala88 in MthK (see above) [58]. In other K2P channels, the nature of the side chains at this position varies considerably, although THIK2 channels, which also exhibit low basal currents, have an isoleucine at this position on TM2 and changing this to a more polar side chain leads to a gain of function [72]. Furthermore, mutation of the equivalent position in TM2 of the *Drosophila* KCNKØ channel also suggests a correlation between channel activity and side-chain polarity [73]. However, the physiological and structural mechanisms that might modulate the hydrophobic cuff within TWIK-1 remain to be determined, as does the importance of equivalent hydrophobic barriers in other K2P channels.

Further experimental validation

In addition to the channels described above, hydrophobic pores have also recently been described in several other types of ion channels and transporters thereby adding further experimental systems in which these principles can now be tested and validated. For example, the behavior of water within the pores of the calcium-release-activated calcium channel [74] and the CorA family of Mg^{2+} transporters [75] have also recently been suggested to be important for their structural and functional properties.

Although the computational and theoretical studies that have highlighted the unusual behavior of water in model pores and ion channels are now being supported by a range of structural and functional data, more systematic methods are clearly required to assess the role of hydrophobic pore in channels and transporters. Computationally, improved water–water and water–protein interaction parameters are needed to describe the relative wettability of transmembrane pores (see discussion in Ref. [19]). Polarizable force fields and better descriptions of transmembrane voltage are also needed [76,77]. Furthermore, methods to define the relationship between dewetting on the nanosecond timescale with millisecond timescale single-channel biophysical properties are also clearly necessary.

Crystallographic studies of water in ion channel pores are challenging due to the resolution required, but indirect measurements of hydrophobicity can be achieved by examination of densities for non-polar gases, such as xenon, or lipids. Such density has been observed in the hydrophobic gate of ELIC [29] and GLIC [31], as well as TWIK-1 [67]. Furthermore, next-generation prediction and visualization software are also needed to combine and display both radius and hydrophobicity of the transmembrane pore when reporting new structures. The ability to functionally compare the effects of hydrophilic and hydrophobic pore mutations on channel pore properties also represents one of the more obvious experimental approaches. Indeed, this has been performed for several types of channels, but more extensive comparison of series of different substitutions or even unnatural amino acids and other forms of synthetic biology could be useful. Similarly, as shown for the MscL channels, dynamic alteration of the hydrophobic gate by reaction of hydrophilic MTS reagents to engineered cysteine mutations could also be considered [23]. Electric-field-induced wetting of ion channel pores might also be used as a test for hydrophobic gating. Finally, although direct changes

in hydrostatic pressure may be difficult to replicate experimentally, the role of water could also be tested by altering the relative osmolarity, and it may even be possible to modify other methods which detect water–protein interactions, such as X-ray radiolysis and electron paramagnetic resonance spectroscopy to monitor the dynamic accessibility of waters to channel pores in response to different gating signals [78,79].

Conclusions

In summary, the behavior of water in confined hydrophobic pores appears to contribute to the biophysical and functional properties of a range of different ion channels. However, a combination of structural, functional, and computational approaches will be required to address the role of hydrophobic gating in biological ion channels. For example, it remains intriguing that several K^+ channels that do not utilize a classical bundle-crossing gate all seem to possess a highly hydrophobic inner pore that can function as an effective barrier to ion permeation. In particular, it will be important to understand how physiological stimuli may affect these gates and whether this occurs through subtle structural changes to the relative hydrophobicity of the pore or through larger conformational changes in pore diameter. In reality, such effects may be inextricably linked and difficult to dissect. However, understanding how this unusual property of water in confined hydrophobic spaces influences ion permeation and gating clearly represents an emerging theme in ion channel biology, and the rapidly expanding number of high-resolution channel structures will undoubtedly help us to rise to this challenge.

Note added in proof: Since submission of the revised manuscript, a crystal structure of the mouse 5-HT3 receptor has been published, revealing a 4.6 Å diameter hydrophobic constriction of the pore which is discussed in the context of pLGIC gating [80].

Acknowledgements

We would like thank members of the Structural Bioinformatics and Computational Biochemistry group (Department of Biochemistry, Oxford) for their helpful discussions, in particular, Caroline Dahl and also Dr. Oliver Beckstein (Arizona State University). This work was supported by grants from the Wellcome Trust and the Biotechnology and Biological Sciences Research Council. P.A. is a Wellcome Trust OXION Training Fellow.

Conflict of Interest Statement: The authors declare no competing financial interests.

Received 5 June 2014;
Received in revised form 24 July 2014;
Accepted 28 July 2014
Available online 12 August 2014

Keywords:
hydrophobic gating;
nanopore;
ion channel;
potassium channel;
K2P channel

Abbreviations used:
MD, molecular dynamics; pLGIC, pentameric ligand-gated ion channel; QA, quaternary ammonium.

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