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VIEWPOINT

When is a hydrophobic gate not a hydrophobic gate?

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The flux of ions through a channel is most commonly regulated by changes that result in steric occlusion of its pore. However, ion permeation can also be prevented by formation of a desolvation barrier created by hydrophobic residues that line the pore. As a result of relatively minor structural changes, confined hydrophobic regions in channels may undergo transitions between wet and dry states to gate the pore closed without physical constriction of the permeation pathway. This concept is referred to as hydrophobic gating, and many examples of this process have been demonstrated. However, the term is also now being used in a much broader context that often deviates from its original meaning. In this Viewpoint, we explore the formal definition of a hydrophobic gate, discuss examples of this process compared with other gating mechanisms that simply exploit hydrophobic residues and/or lipids in steric closure of the pore, and describe the best practice for identification of a hydrophobic gate.

Introduction

At the turn of the millennium, Mark Sansom and Nigel Unwin sat next to each other on the long bus journey from the airport to a conference somewhere in deepest New England, discussing the wide, but very hydrophobic, nature of the central pore within the *Torpedo* nicotinic acetylcholine receptor structure that Nigel had just solved. Recent studies on the thermodynamics of liquid-to-vapor transitions in water confined between two hydrophobic plates (Lum et al., 1999), along with a chemical physics simulation study of confined water (Brovchenko et al., 2000), hinted that the hydrophobic nature of the pore in this new channel structure could act as a potential barrier to permeation and that it might not be conductive even though it appeared wide enough to accommodate a hydrated or partially hydrated ion. This chance conversation developed and ultimately led to the concept of hydrophobic gating, which is now shown to occur in several different classes of ion channels (Fig. 1).

The phenomenon of hydrophobic gating was initially demonstrated using molecular dynamics simulations in model nanopores (Beckstein et al., 2001; Allen et al., 2003; Beckstein and Sansom, 2004) and in carbon nanotubes designed to mimic ion channels (Hummer et al., 2001; Sansom and Biggin, 2001). It was shown that, if hydrophobic enough, narrow pores wider than a hydrated ion could form a hydrophobic gate that prevents permeation due to liquid–vapor transitions of water within the pore. A functionally closed, dewetted pore could then be opened in two ways: firstly, by increasing the diameter of the narrowest region of the pore; or secondly, by increasing the hydrophilicity of that region allowing it to become wetted and thus permeable (Beckstein and Sansom, 2004; Fig. 2 A). This phenomenon is now referred to as "hydrophobic gating" (Beckstein et al., 2001; Beckstein and Sansom, 2003), thereby reflecting the behavior of water within these confined spaces, but has also sometimes been called a vapor lock (Anishkin and Sukharev, 2004) or bubble gating (Roth et al., 2008).

We are generally familiar with the anomalous behavior of water at the macroscale, for example, where surface tension promotes the formation of droplets on a hydrophobic surface. However, these properties can also manifest when water is confined within hydrophobic nanoscale environments, such as those found in some ion channel pores, where it promotes alternating evaporation and condensation events. These spontaneous dewetting transitions present a significant energetic barrier to a hydrated or partially hydrated ion without the usual requirement for steric occlusion of the pathway. By contrast, water confined by polar surfaces in similar size pores exists exclusively in the condensed phase, and these pores are therefore permeable. Other factors such as temperature (Beckstein and Sansom 2004) and transmembrane voltage have also been shown to affect this process (Dzubiella and Hansen, 2005; Li et al, 2007; Trick et al, 2017; Klesse et al, 2020b), but the primary determinants of such hydrophobic gates appear to be the radius of the pore and its local hydrophobicity.

Over the last two decades, advances in structural biology along with recent artificial intelligence–driven structure prediction algorithms, such as AlphaFold (Jumper et al., 2021), have

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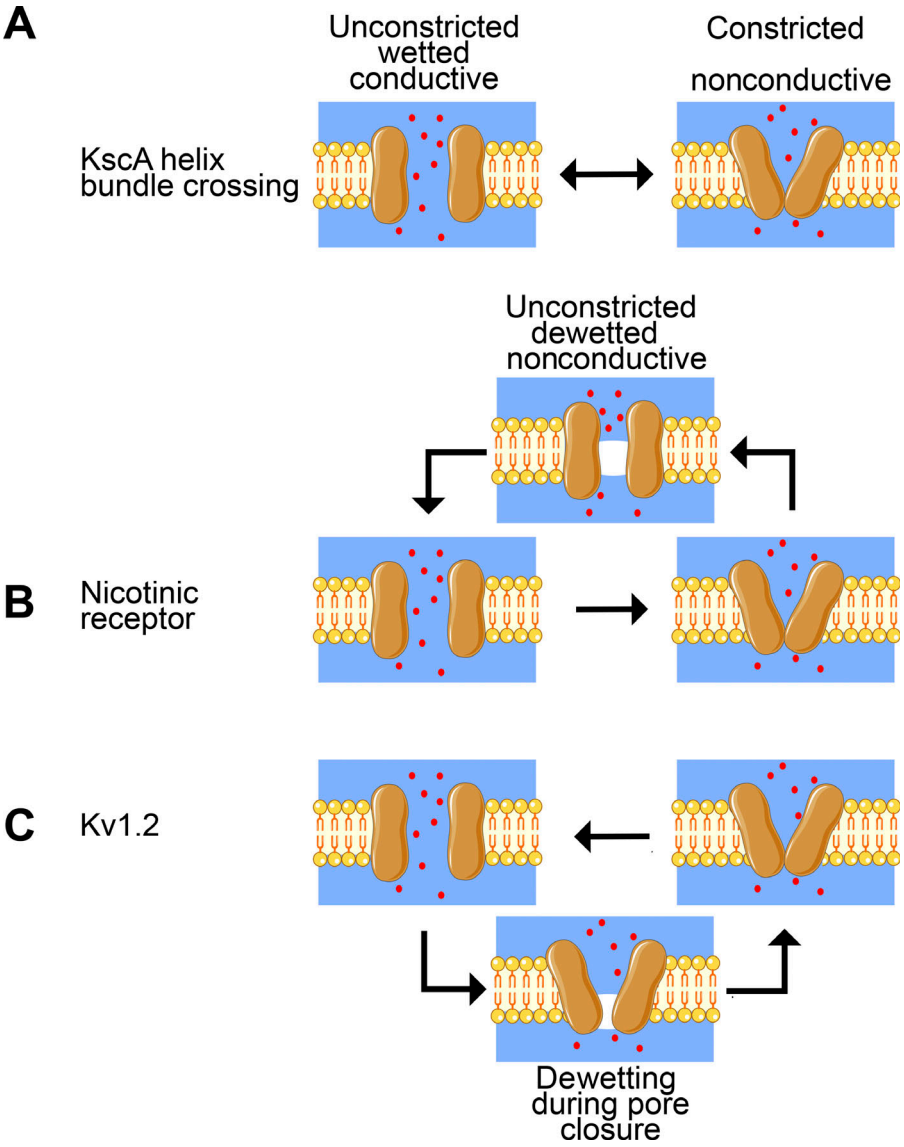

Figure 1. **Different gating mechanisms.** Water (blue), ions (red), ion channel (brown), and lipid bilayer (yellow). **(A)** Gating via helix bundle crossing, for instance in the KscA channel. Ion movement is prevented by steric occlusion of the helix bundle crossing in the nonconductive state. **(B)** Ion movement is prevented by a hydrophobic gate in the resting state of the nicotinic receptor where the pore pathway is not sterically occluded (see middle top panel). Further changes then open the hydrophobic gate, and the channel becomes conductive. **(C)** Similar to KcsA, the closure of the bundle crossing gate in Kv1.2 produces a physically occluded closed state. The hydrophobicity of the bundle-crossing gate permits tight packaging of the helices in the closed state and also produces dewetting during closure. The formal definition of hydrophobic gating would exclude such cases where pore dewetting immediately precedes steric closure of the pore.

revealed a plethora of 3-D ion channel and nanopore structures in different conformational states. However, in nearly every case, these structures still require functional annotation (Klesse et al., 2019). In some cases, it is relatively easy to define whether a given structure corresponds to an open or a closed state by determining the physical dimensions of the pore with applications such as HOLE (Smart et al., 1996) or CHAP (Klesse et al., 2019). Likewise, sterically occluded pores can quickly be annotated as functionally closed. However, due to the process of hydrophobic gating, a simple determination of pore radius is often not enough because hydrophobicity also plays a major role in defining pore permeability.

There is considerable experimental evidence to support the anomalous behavior of water at the nanoscale (Eriksson et al., 2019; Brandner et al., 2011). However, it is not currently possible to directly observe water dynamics within a channel pore, and evidence for hydrophobic gating relies mainly on molecular simulation studies supported by mutagenesis studies (Yazdani et al., 2020). Molecular dynamics simulations therefore help bridge the gap between structural and functional observations, and more detailed computational approaches such as free energy profiles for ion permeation can also be determined, as well as using the behavior of water in a channel pore as a proxy for ionic permeability (Trick et al., 2016). Along with other groups, we have used many of these tools to demonstrate how molecular simulations can aid the functional annotation of channel pore structures with the freely available CHAP software, specifically developed for this purpose (Klesse et al., 2019). As part of these studies, it appears that the essential parameters of pore radius and hydrophobicity are so influential that a simple heuristic could also be developed that is capable of predicting the permeability of any given pore structure (Fig. 2 B; Rao et al., 2019). However, when simulating water in confined environments, the water models and force fields used must also be chosen carefully (Lynch et al., 2021), and in some cases, the use of polarizable force fields may be required to capture effects where the electronic structure of an atom is altered by other charges in its immediate environment (Klesse et al, 2020a; Yue et al, 2022).

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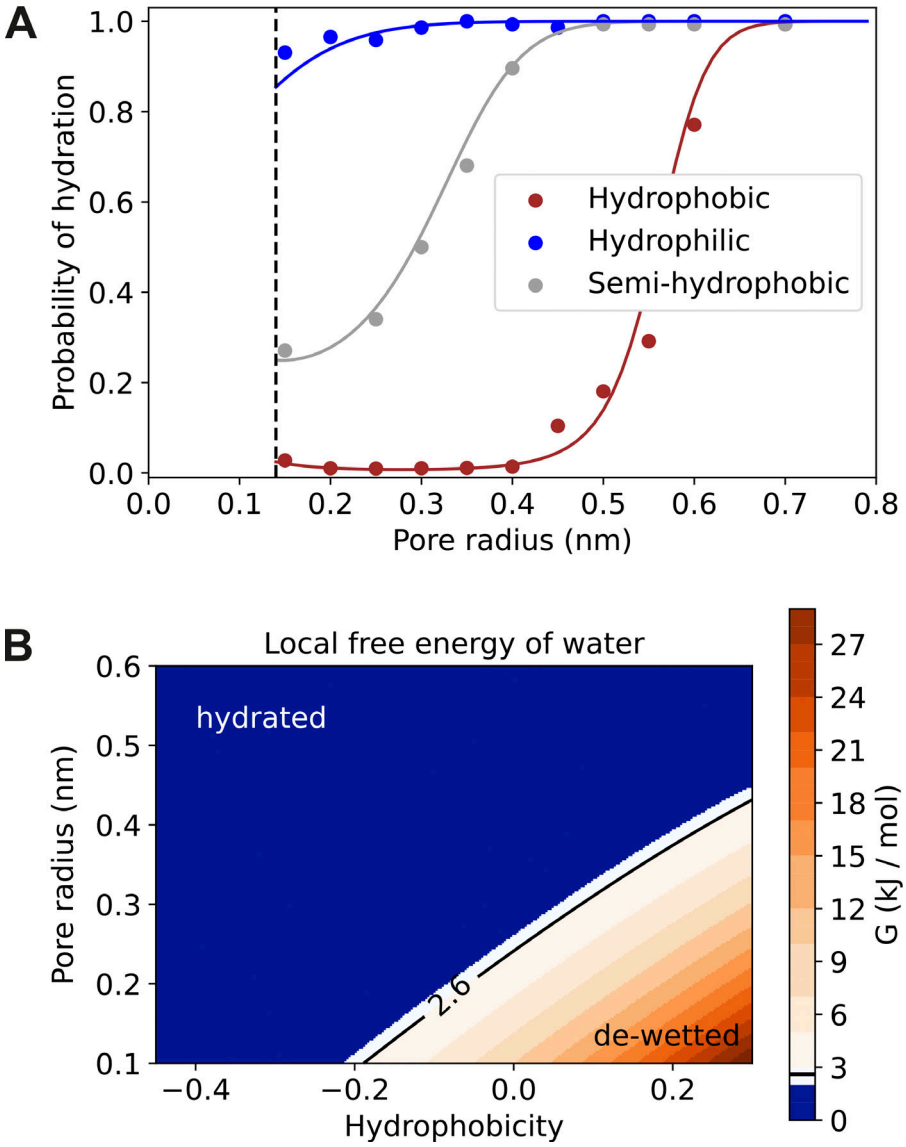

Figure 2. **The role of pore radius and hydrophobicity. (A)** Pore hydration probability depends on its radius and hydrophobicity. Based on studies of a model nanopore, a hydrophilic pore remains hydrated if its radius is larger than that of a water molecule (0.13 nm, indicated by the dotted line). By contrast, a hydrophobic pore dewets at radii below 0.6 nm (original data from Beckstein and Sansom [2004]). **(B)** Using a simple heuristic based on analyzing the hydration of many different pore structures (Rao et al., 2019), the radius and hydrophobicity of a pore can be used to predict its hydration state. The hydrophobicity employs the Wimley-White scale, with more positive values indicating greater hydrophobicity. The water-free energy is based on mean measurements from pore radius, hydrophobicity, and water-free energy profiles obtained from number density calculations.

The concept of hydrophobic gating in channels and nanopores has therefore gained considerable traction over the past few years with many different examples now being observed. However, the increased visibility of this phenomenon has also resulted in the term hydrophobic gate being used in a much broader context that often deviates from its original definition. For instance, nonpolar residues have been proposed to form a hydrophobic gate in several cases where wet–dry transitions have not been observed (Chen et al., 2021; Pant et al., 2022), whilst in other cases, the direct occlusion of pores by lipids has been referred to as hydrophobic gating (Anderson and Thompson, 2022).

There are several structural mechanisms that can regulate a hydrophobic gate (Fig. 1). Typically, these involve a change in radius (without steric closure) that promotes dewetting or smaller scale changes such as rotation of side chains that can change both radius and hydrophobicity or sometimes a combination of both. However, based upon its original meaning, the expression hydrophobic gating should only be used to describe the mechanism by which movement of a hydrated ion through the dewetted sections of a channel pore is prevented by the free energy barrier created by liquid–vapor transitions within the pore, rather than by van der Waals clashes with tightly packed pore residues, even if these residues are hydrophobic. It is important to note that this definition of hydrophobic gating also formally excludes cases where dewetting immediately precedes the subsequent steric closure of a pore, e.g., the dewetting events observed prior to closure of the helix-bundle crossing gate in simulations of the Kv1.2 pore domain (Jensen et al., 2010).

Examples of hydrophobic gates in ion channels

According to this original definition of a hydrophobic gate, genuine examples have been demonstrated in several distinct classes of ion channels, in particular, within the family of pentameric ligand-gated ion channels (pLGICs or Cys-loop receptors; Gharpure et al., 2020). As Mark Sansom and Nigel Unwin suspected during their long bus journey, hydrophobic residues in the pore-lining M2 helix of the then recently solved

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Torpedo nicotinic acetylcholine receptor (nAChR) do indeed act as a hydrophobic gate that creates a large desolvation barrier for permeating ions (Beckstein and Sansom, 2006; Plazas et al., 2005), an effect also confirmed in more recent *Torpedo* structures (Zarkadas et al., 2022). Likewise, in GLIC, a bacterial homolog of nAChR, tilting of the pore-lining helices induces cooperative drying and closing of the pore by dewetting and not by constriction (Zhu and Hummer, 2010). Similarly, in the serotonin receptor channel (5-HT3R), strong dewetting within the central hydrophobic gate region can be observed (Trick et al, 2016; Yuan et al, 2016; Klesse et al, 2020a), whilst the glycine receptor (GlyR) also appears to show the same features (Cerdan et al., 2018; Dämgen and Biggin, 2020; Kumar et al., 2020). That being said, recent structures of Cys-loop receptors, where both resting and desensitized structures have been elucidated, have shown that the minimum radius in both states can be very similar; this, therefore, implies a potential role for steric occlusion in the resting state gate, even if simulations show substantial dewetting in this region.

The TWIK-1 K2P potassium channel has also been shown to possess a hydrophobic barrier deep within its inner pore, and stochastic dewetting of this hydrophobic constriction acts as a major barrier to K^+ permeation. The hydrophobic nature of the TWIK-1 pore restricts full hydration of the inner cavity, and polar substitutions at this hydrophobic cuff increase channel activity by directly disrupting this hydrophobic barrier to water and hydrated ions (Aryal et al., 2014). However, like many other aspects of TWIK-1 function, the mechanisms that regulate this barrier in the wild-type channel remain unclear.

Hydrophobic gates have also been found in the small conductance (SK) and large conductance (BK) Ca^{2+}-activated channels (Aryal et al, 2015b; Jia et al, 2018). In the functional closed state of the BK channel, the pore undergoes hydrophobic dewetting transitions and is not permeable to ions even though its pore remains physically open and accessible to relatively large molecules (Jia et al., 2018). Simulations suggest that upon Ca^{2+}-binding, the pore-lining helices bend and twist to move hydrophobic residues away from the pore and expose two glutamic acid residues to the pore, thus making it more hydrophilic (Jia et al., 2018; Yazdani et al., 2020).

The heptameric small conductance mechanosensitive channel (MscS) has a hydrophobic pore with branched hydrophobic leucine side chains pointing into the pore. Anishkin and Sukharev (2004) demonstrated dewetting in MscS, which allowed for reinterpretation of the first MscS structure that had previously been considered open (Bass et al., 2002). Mutation of this leucine ring to polar residues caused a gain of function phenotype (Miller et al., 2003), whilst later work showed that the gate could also be opened by a rotation of these same leucine residues away from the pore, thus increasing its diameter at that point (Wang et al., 2008). Simulation studies have also suggested a similar hydrophobic gate in the related large-conductance mechanosensitive channel (MscL; Anishkin et al., 2010), though it may be the precise dynamics of the dewetting process itself that are more important for gating (Najem et al., 2018).

A hydrophobic gate has also been proposed as the major determinant of inactivation in the mechanosensitive Piezo channels (Zheng et al., 2019). Members of the transient receptor potential (TRP) channel family have also been shown to exhibit hydrophobic gates that involve the motion of polar side chains to stabilize either the dry or wet state of the channel (Kasimova et al., 2018; Zheng et al., 2018a, 2018b).

Other examples include hydrophobic gates in the Ca^{2+} release-activated Ca^{2+} (CRAC) channel formed by two rings of pore-lining valine and phenylalanine residues that are rotated out of the pore upon channel opening (Yamashita et al., 2017), whilst in simulations of the magnesium channel CorA, spontaneous and reversible hydration events concurrent with small-amplitude fluctuations in pore diameter can be observed (Neale et al., 2015). Likewise, in the Bestrophin chloride channel (BEST1), dewetting events that act as a hydrophobic gate have been observed in the narrow neck region of the pore that is lined by three rings of hydrophobic amino acids (Rao et al., 2017).

Other mechanisms of gating involving nonpolar residues

Although nonpolar pore-lining residues are clearly important in the formation of hydrophobic gates, not every hydrophobic residue along the pore of a channel constitutes such a gate. As described above, the underlying principle of the hydrophobic gating mechanism is the creation of a free-energy barrier to permeation that is associated with stripping ions of their hydration shell. A genuine hydrophobic gate is not sterically occluded and must remain wide enough to allow a hydrated ion to pass if the pore was otherwise hydrophilic in that region. However, in some cases, hydrophobic pore segments and/or constrictions are sometimes referred to as hydrophobic gates in the sense they contain patches of hydrophobic residues that play an important role in gating and/or selectivity, but they do not meet the formal criteria originally described for this process.

A particularly interesting case is the early study of the Kv1.2 pore domain where a dewetting transition was observed immediately prior to steric occlusion of the pore by the closure of the bundle crossing gate (Jensen et al., 2010). Such bundle-crossing gates are clearly important in the gating of many members of this superfamily of tetrameric cation channels where the pore-lining helices intersect at the cytoplasmic entrance to seal the permeation pathway shut (see cartoon in Fig. 1, A and C). In such cases, it is quite common to find patches of small hydrophobic residues at the point where these helices intersect as this permits tight packing of the helices in the closed state (Yonkunas and Kurnikova, 2015; Yelshanskaya et al., 2022). Consequently, as these hydrophobic helices move toward the sterically occluded closed state, it is therefore likely that at some point in this transition a dewetting event will occur, and in some cases this is thought to assist the closure of the gate (Yonkunas and Kurnikova, 2015). However, such dewetting events immediately preceding such hydrophobic collapse and steric occlusion of a gate would not normally be classified as a classical hydrophobic gating mechanism.

In another, more recent case, two pore regions lined by hydrophobic residues were identified as hydrophobic gates within the transient Cl^- conductive channel-like state of the glutamate transporter (Glt_{Ph}; Chen et al., 2021). These hydrophobic residues at the extracellular and intracellular openings clearly play

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an important role in the selectivity of the putative Cl^- permeation pathway in Glt_{Ph}, and a similar role has also been proposed in its human homolog (EAAT1; Pant et al., 2022). However, no dewetting transitions were demonstrated, and computational studies suggest uninterrupted aqueous pathways in both cases. Thus, despite their location and obvious functional importance, these hydrophobic residues may not meet the criteria required of a genuine hydrophobic gate.

Another area of possible confusion arises from the fact that bound lipids and/or detergents are now being identified in many experimentally determined ion-channel structures (Bocquet et al, 2009; Miller and Long, 2012; Brohawn et al, 2014; Aryal et al, 2015a; Reddy et al, 2019; Jin et al, 2022; Turney et al, 2022). Such lipids can influence channel function in various ways ranging from lipid-induced conformational changes (Jin et al., 2022) to direct blocking of a pore. For example, molecular dynamics simulations suggest that lipids can favorably assemble into a bilayer within the large hydrophobic pore of the calcium homeostasis modulator protein, CALHM2, but not within the smaller, more hydrophilic pore of CALHM1 (Syrjanen et al., 2020). There is also evidence that CALHM4 and CALHM6 may be gated by a lipid block in a similar manner to CALHM2 (Drożdżyk et al., 2020). Hydrophobic residues within these pores thus clearly play a role in this "lipid plugging" process, but its mechanism and time scale are fundamentally different from that of hydrophobic gating. Dewetting occurs on a nanosecond timescale, whereas lipid plugging functionally closes the channel on a much longer time scale. Steric block by lipids may therefore be a possible mechanism for regulating large pores, but such plugging events are unlikely to be used for the dynamic regulation of a typical ion channel gate.

Direct pore occlusion by lipids has also been suggested in mechanosensitive channels such as TRAAK (Brohawn et al., 2014) and MscS (Reddy et al., 2019). Many K2P K^+ channels such as TRAAK and the related TWIK-1 have large side portals or fenestrations at the interface between their transmembrane helices that potentially expose the ion conduction pathway to the lipid core of the bilayer (Brohawn et al, 2014; Aryal et al, 2015a), though to what extent such lipid intrusion contributes to gating remains to be seen. In the TWIK-1 channel, simulations demonstrated that lipid tails could enter the fenestrations, but not far enough to sterically occlude the pore (Aryal et al, 2015a). Interestingly, it has recently been argued that lipids seen within the pore of the mechanosensitive Piezo channel may result from a computational artifact because absolute binding free energy calculations indicate that the presence of these lipids within the pore is thermodynamically unfavorable and most likely results from being kinetically trapped in the pore during simulations (Jiang et al., 2022). Three pore-wetting mutations targeting the S6 helix of the human Na_X channel have also recently been shown to unlock a voltage-insensitive leak conductance (Noland et al., 2022) and are referred to as a possible hydrophobic gate. However, further work is required to investigate whether this S6 gate is indeed a genuine hydrophobic gate or whether lipids identified in the structure occlude the pore.

Dynamic conformational changes in the amino terminus of the human Pannexin channel (PANX1) may also be associated with lipid movement in and out of its pore (Kuzuya et al., 2022), and similar mechanisms have also been proposed for some of the other large-pore channels such as innexin (Burendei et al., 2020) and connexin (Lee et al., 2020). Such lipids blocking the permeation pathway in these channels have also been referred to as a hydrophobic gate (Anderson and Thompson, 2022), but these lipids sterically occlude the pore without evidence of a vapor lock being formed. Such references have therefore also added further confusion to the original definition of a hydrophobic gate, which is based on a fundamentally different principle of gating.

Functional annotation of a hydrophobic gate

To help avoid such confusion, we summarize a series of best practices as nearly every newly solved ion channel structure requires some form of functional annotation to address whether it is open i.e., conductive or not. As an initial analysis step, the radius profile of the permeation pathway can be calculated with tools such as HOLE (Smart et al., 1996) or CHAP (Klesse et al., 2019). The radius and hydrophobicity profile can be directly determined with CHAP from the PDB structure with no need for MD simulations. If the narrowest constriction exceeds the radius of the respective hydrated ion and is not hydrophobic, then the structure is considered to be open, and ion permeation can be further studied using more demanding approaches such as computational electrophysiology (Kutzner et al., 2011).

However, if the narrowest constriction suggests a hydrated ion might pass, but appears to be hydrophobic, then further analysis is needed. Based on radius and hydrophobicity, a simple heuristic can also be used to predict the energetic barrier for water (see Fig. 2 B; Rao et al., 2019), and if this score indicates a significant barrier, then the channel structure should be embedded in a lipid bilayer and simulated with an atomistic force field. Appropriate backbone or Cα atom restraints should also be used to ensure that the functional annotation refers to the input structure and not some subsequent conformational change. If dewetting of the pore or a discontinuous water pathway is seen that creates an associated free energy barrier (Klesse et al., 2019), then the structure can be considered to be closed and nonconductive due to a hydrophobic gate, but only if there is no evidence that this dewetted region subsequently constricts to sterically block the permeation pathway.

Conclusion

In summary, attempts to functionally annotate the ever-increasing number of ion channels and pore structures have led to confusion about the formal definition of a hydrophobic gate. Not every hydrophobic cluster within a channel pore necessarily generates pore dewetting, and a genuine hydrophobic gate only functions as a gate if that region of the pore would otherwise remain permeable if it were more hydrophilic. Furthermore, dewetted regions within a pore do not function as hydrophobic gates if that region subsequently becomes sterically occluded. The term hydrophobic gating should therefore only be used to describe the mechanism by which dewetting events generate a free energy barrier for hydrated ions to prevent permeation through an otherwise wide enough pathway. Clarity

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in these matters will therefore assist in the functional annotation of channel pore structures.

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