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SYMPOSIUM REVIEW:

TITLE: Changing Perspectives on How the Permeation Pathway Through Potassium Channels is Regulated.

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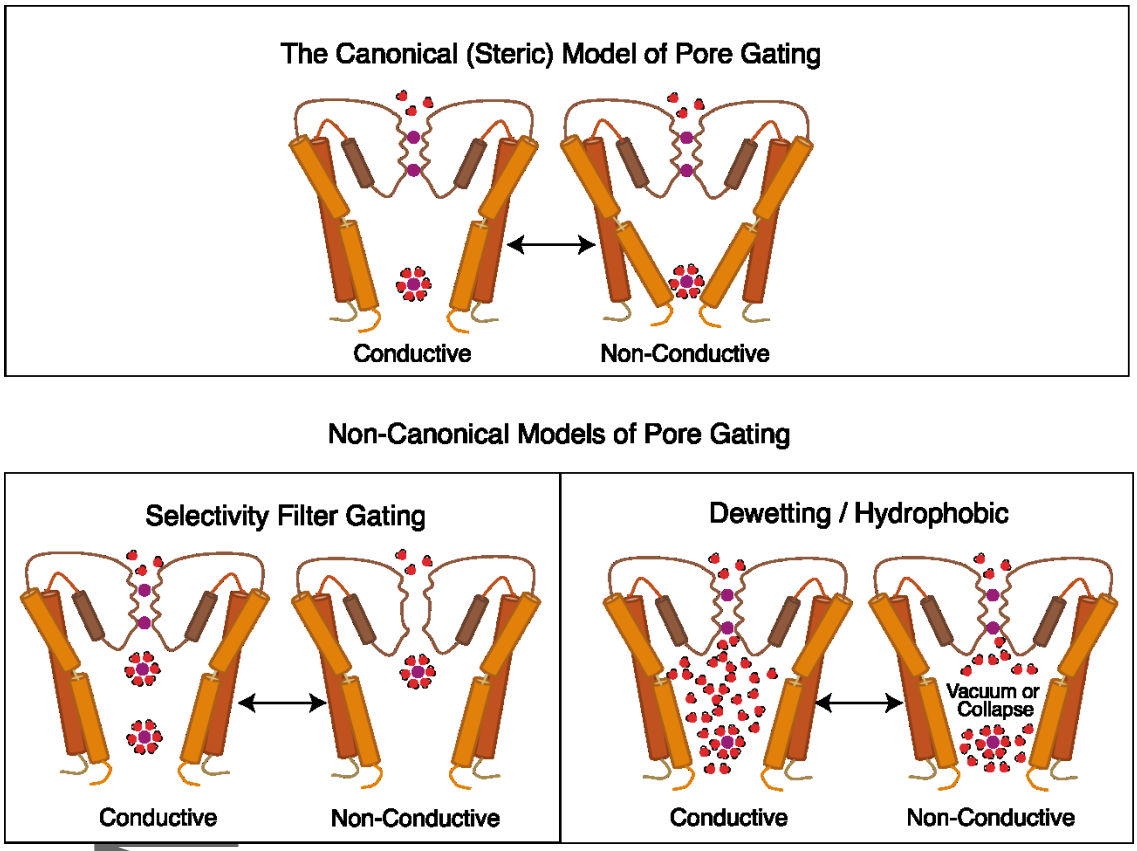
ABSTRACT:

The primary means by which ion permeation through potassium channels is controlled, and the key to selective intervention in a range of pathophysiological conditions, is the process by which channels switch between non-conducting and conducting states. Conventionally, this has been explained by a steric mechanism in which the pore alternates between two conformations: a 'closed' state in which the conduction pathway is occluded and an 'open' state where the pathway is sufficiently wide to accommodate fully hydrated ions. Recently, however, 'non-canonical' mechanisms have been proposed for some classes of K^+ channels. The purpose of this review is to illuminate structural and dynamic relationships underpinning permeation control in K^+ channels, indicating where additional data might resolve some of the remaining issues.

ABSTRACT FIGURE:



Hypothetical mechanisms of control over ion permeation through potassium channels. The upper panel portrays the canonical steric pore-gating mechanism, while the lower panels depict emerging models. Of these, the left-hand panel describes the situation where steric changes have been ruled out and gating has been ascribed to the selectivity filter, whereas the right-hand panel shows a 'dewetting' model, which is neither contingent upon, nor excludes, steric changes within the pore.

Katrina Black gained her PhD from the University of Melbourne for research into K^+ channel permeation and is currently a postdoctoral researcher at The Walter and Eliza Hall Institute of Medical Research. **Ruitao Jin** and **Sitong He** qualified with Bachelor of Science degrees at the University of Nanjing and are now PhD students at La Trobe University in Melbourne; they study the mechanism of ion channel conduction using advanced molecular modelling methods. **Jacqueline Gulbis** heads a structural biology laboratory at The Walter and Eliza Hall institute in Melbourne. She has a keen interest in the gating and regulation of inward rectifier K^+ channels.

Introduction

Orchestrated ion channel activity propagates the electrical signals that allow the human central nervous system and vital organs to function, underpinning neurological, cardiac, endocrine as well as essential developmental and homeostatic functions. While the interplay of ion channels in excitable cells has remained a source of fascination, their subtler roles in facilitation of cell division, apoptosis, and immune cell activation via the cell cycle and signal transduction pathways are less well appreciated but profoundly important for human health. Naturally occurring mutations impairing normal function and the anomalous expression of native K^+ channels have both been linked to

neurological and organ-related conditions. Aberrant expression of K^+ channels in cancer cells has been decisively linked to invasiveness and malignancy of brain, colon and breast cancers (Becchetti, 2011; Leanza et al., 2013; 2017; 2015; Peruzzo et al., 2016; Simon et al., 2015). The dearth of scientific tools appropriate to investigating channel biology and pathophysiology has hindered understanding of the pathophysiology and impeded discovery of K^+ channel-selective pharmaceuticals. Development of selective agents requires a rational approach based on mechanistic criteria. These are perhaps the most important reasons to re-evaluate the relationship of structure and dynamics to mechanism.

Single particle cryo-EM now rivals crystallography in providing high-resolution structural information and has provided access to new and interesting ion channel structures that reveal a wide range of structural diversity, with the advantage that the conformational heterogeneity within populations can also be sampled. Fresh insight into mechanism has been more limited than might be expected, however, and a significant proportion of emerging K^+ channel data is not explicable by the canonical mechanism of gating. Whether this is because all classes of K^+ channels operate on a slightly different basis or if it is simply that we have not yet discovered a common thread linking disparate findings; a universal principle that manifests according to the unique features of each type of K^+ channel.

A major challenge in equating ion channel structure and function lies in the interpretation of structures with respect to physiological status. In soluble proteins, the presence of potentiating agents, cofactors, or partner proteins has guided structure interpretation. In ion channels this has not been the case, and one might reasonably question why not. In large part this appears to be due to fixed preconceptions (*e.g.* specific expectations of conformation). Occasionally structures and their imprudent interpretation are the culprits, and there are instances where gating models have been substantiated by precise measurements on side chains not visible in the maps or, conversely, by overlooking features that are present. Thus, in drawing conclusions from structure, it is vital to critically view the electron density, especially that of moderately-resolved structures.

In this overview, we have tried to present the literature as objectively as possible. Some of the points of view expressed here are not those of the originating authors and we have tried to be clear when this is the case. We have mentioned caveats in instances where the experimental logic may have a bearing upon interpretation. The literature on K^+ channels is extensive, and we have restricted the review to studies of most relevance to pore gating, regrettably not finding the space for many issues of great interest, including permeation by direct coulombic repulsion, voltage-dependent activation gating and channel regulation by specific membrane lipids or fatty acids. We have focused on papers that have strongly influenced the way gating is perceived and others that have made salient findings that merit wider discussion. Figure 1 shows a timeline of discoveries.

Conservation of Sequence and Architecture within Potassium Channel Families

The human genome encodes approximately eighty distinct K^+ channel genes characterised on the basis of sequence homology. The K_V channels represent the largest of the four major classes of K^+ channel defined by the International Union of Basic and Clinical Pharmacology: voltage-dependent (K_V), calcium- or sodium-activated (K_{Ca}), inward rectifiers (K_{IR}; Kir), and the 'two-pore' channels (K_{2P}; K2P) (Fig. 2). These classical K^+ channels have a conserved subunit architecture where inner and

outer membrane-spanning helices position an angled pore helix. Four monomeric (or in K2P channels, two pseudo-dimeric) subunits fuse together to form each functional channel. The α-helical scaffold positions the ion selectivity filter, a signature element (T T V/I G Y/F G) responsible for conferring the exquisite K^+ selectivity that sets K^+ channels apart from other cation channels. Notably, the consensus sequence is the *only* part of the pore that enjoys high sequence identity *across* family boundaries. Within K^+ channel families and subfamilies (but not across the wider spectrum), transmembrane helices exhibit strong sequence identity and conservation of pore morphology.

Importantly, conformations observed in structures are largely unaffected by the truncation of regulatory domains or co-crystallisation with partnering subunits or ligands. The lattice packing in crystals rarely has a significant impact on pore conformation as intermolecular contacts are typically restricted to ancillary domains and/or extra- and intracellular faces of the pore, while the transmembrane regions are enveloped in a loose detergent micelle. Single particle cryo-EM structures are free of lattice constraints altogether but exhibit similar conformational conservation within families and subfamilies. There is thus an argument to be made that pore morphology is determined by the primary amino acid sequence conserved within each specific K^+ channel family and subfamily.

Initiation of Gating

To control ion permeation, K^+ channels switch between activated, resting (deactivated) and unresponsive (inactivated) states. In essence, the reversible transition referred to as 'activation gating' relates the presence of an activation stimulus to measurable conduction. The response is typically mediated by protein domains located N- or C-terminal to the pore, but knowledge of how these domains initiate or gradate the response remains limited – an exception being the tightly coupled voltage-sensor domains of K_V channels (Fig. 2). These respond to depolarisation by inducing a tiny outward current arising from the positive charges on conserved arginine and lysine residues of S4 (Bezanilla et al., 1991; Papazian et al., 1991). This 'gating current' precedes and triggers changes in the pore that enable K^+ currents, although the precise nature of these changes and how they are transduced from the voltage-sensor remain unclear. The process in other classes of K^+ channel is understood at a more elementary level; In some K_{Ca} channels, calcium binding to the intracellular domains initiates a gating response (Schreiber and Salkoff, 1997), whereas in Kir channels the switch has been linked to changes within a cytoplasmic assembly (Clarke et al., 2010). Pores leave the conducting state by either deactivation or inactivation. The latter may refer to fast intracellular block, a process coupled to activation of the pore (Zagotta et al., 1990), or to 'C-type' inactivation by structural changes in the selectivity filter or its immediate surrounds (Cuello et al., 2010a; Hoshi et al., 1991; López-Barneo et al., 1993). It was recently proposed that a 'C-type gate' may be responsible for both activation and inactivation in K2P channels (Lolicato et al., 2017). This review analyses the changes within the pore that follow these triggered events.

Origins of the Canonical Gating Model

In 1952, seminal reports by Alan Lloyd Hodgkin and Andrew Fielding Huxley described findings gained from measuring ion permeability in giant nerve fibres of the squid *Loligo pealii* under voltage clamp, leading to the first clear description of the normal waxing and waning of action potentials

(Hodgkin and Huxley, 1952a; 1952b). The origins of the canonical ion channel gating model are, however, to be found in the landmark experiments of Clay Armstrong, in which he studied the effect of intracellular application of the tetraethylammonium ion TEA^+ and other quaternary ammonium (QA) cations on action potentials in giant squid axons and at amphibian Nodes of Ranvier. Demonstrating that ion channels are gated pores (Armstrong, 1971), he probed the underlying processes by making use of the fact that QA ions interfere with normal deactivation, assigning the "activation gate" to an 'axoplasmic' position at or near the intracellular face (Armstrong and Hille, 1972).

Initial experiments aimed at pinpointing the nature of the gate, and its precise location in the conduction pathway, focused largely on *Shaker*, the archetypal voltage-dependent K^+ (K_V) channel from *Drosophila melanogaster* (Tempel et al., 1987). To ascertain whether the transition between resting and activated states involved significant changes in pore conformation, investigators sought to identify residues that exhibited a change in reactivity at activating voltages relative to resting potentials. Two studies critical in shaping opinion showed increased susceptibility to pore block at voltages favouring K^+ conduction, indicating differences in internal accessibility in active and resting channels (Holmgren et al., 1997; Liu et al., 1997). In deference to Armstrong's findings, these state-dependent differences in accessibility were attributed to conformational changes at the intracellular mouth.

In 1998, the first crystal structure of a K^+ channel, C-terminally truncated KcsA from *Streptomyces lividans* (Doyle et al., 1998), implicated the inner helix bundle crossing as the probable site of a permeation gate. In the absence of a K_V channel structure it provided a framework for interpretation of further studies on *Shaker* (del Camino and Yellen, 2001; Hackos et al., 2002; Holmgren et al., 1998). In retrospect this had some serious shortcomings, incorrectly defining the locations of many point mutations. Structural comparison of KcsA with a Ca^{2+}-gated K^+ channel, MthK, (Jiang et al., 2002a) was pivotal in gaining general consensus for the model of a universal gate located at the inner helix bundle operating by reversible steric occlusion of the ion conduction pathway. Relative to KcsA, the inner helices of MthK splay apart, with its mouth 12 Å wide, the polypeptide backbones diverging at a 'hinge point' at a glycine located midway along the inner helix. This led to the further suggestion that the inner helices of K^+ channels bend at a conserved glycine 'hinge' to mediate gating (Jiang et al., 2002b). Not all findings concurred with the specifics of the model, and a study that utilised Cd^{2+} bridges to 'lock' *Shaker* in an open state argued for a loosely constricted pore mouth in both conducting and non-conducting states (Webster et al., 2004). A *Shaker* gating mutant with increased modification rate kinetics was isolated (Del Camino et al., 2005); the findings were consistent with the canonical gating model in that the state-dependency of block infers conformational change concomitant with gating, but equivocal regarding the role of the inner helices. Reanalysis of early *Shaker* mutational data in the appropriate context of a K_V channel structure would resolve some inconsistencies. However, to pinpoint the reasons for state-dependency of block in *Shaker* would require structures of a native K_V1 channel assembly in complementary conformations unequivocally corresponding to conducting and non-conducting states.

Cryo-EM structures of distantly related members of the K_V clan, the human ether-a-go-go K^+ channel (hEAG; $K_V10.1$) and human ERG-related gene (hERG; $K_V11.1$) have been discussed as the canonical

open and closed states of a generic K_V pore (Wang and Mackinnon, 2017), but the comparison is not entirely satisfactory. Only a single conformation of each was published; the structure of hEAG in the presence of inhibitory calmodulin (5K7L) was assigned as a 'closed' channel (Zubcevic et al., 2014) based on a narrow opening thought to measure less than 1 Å (van der Waals'; vdW) between side chains of a glutamine residue (Glu476). A caveat is that inspection of the map indicates that its resolution and quality are insufficient to determine the position of the labile glutamine side chain past Cβ; the effective vdW spacing thus approximates 5 Å. In contrast, hERG (5VA1) was assigned to an 'open' state because of its significantly more dilated opening (Wang and Mackinnon, 2017). At 27%, the sequence identity between hERG1 and hEAG1 is moderate, about half that of the percentage identity within either subfamily, indicating that although the relationship is close one might expect to observe significant differences in the proteins. Complementary 'open' and 'closed' structures of one or both of these individual channels would resolve these anomalies and pinpoint the molecular changes that are most critical for gating and permeation.

Importantly, state-dependent reagent accessibility of the type observed in *Shaker* has *not* been demonstrated in the K2P channel TREK-1 (Piechotta et al., 2011) (Rapedius et al., 2012) or the K_{Ca} channels BK, SK4 and MthK; these are blocked by, cysteine-reactive MTS reagents or metal ions under both activating and deactivating conditions (Bruening-Wright et al., 2007; Klein et al., 2007; Wilkens and Aldrich, 2006) (Posson et al., 2013; 2015). Evidence for state-dependent accessibility in K_{IR} channels is also ambiguous, with conflicted reports of permissive or restrictive accessibility in Kir6.1 (as part of the K_{ATP} complex) (Proks et al., 2003) and Kir2.1 (Xiao et al., 2003). A key implication is that the canonical mechanism may not apply in these classes.

The Effect of Changing Pore Width on the Integrity of the Selectivity Filter

It has been widely supposed that the canonical gating model is relevant to all selective ion channels. In the ryanodine receptor Ca^{2+}-channel, for instance, conformational classes observed by cryoEM reveal the vdW-delimited pore diameter increases from from ~2 to 9 Å (Georges et al., 2016) when potentiating ligands are bound, supporting the canonical gating model. Similarly, in the non-selective cation channel NaK, a substantial conformational change of the pore relieves a steric occlusion (Alam and Jiang, 2009). Complementary structures confirm each inner helix 'bends' at a conserved glycine, revealing the anticipated disruption of the inner helix hydrogen bonding that allows it to do so. In contrast, only incremental changes in width have been reported for any native K^+ channel. To consider why this might be, we first turn our attention to the key factor distinguishing classical K^+ channels from Na^+ and Ca^{2+} selective channels: its unique selectivity filter. As the selectivity filter forms at the common interface of the four subunits, the inclination of each subunit to the molecular 4-fold axis is crucial to its integrity, and hence to selectivity and conduction.

We questioned whether bending at glycine residues present in the inner helices (Jiang et al., 2002b), might be important in cushioning the selectivity filter from the effects of motions of the inner helices associated with pore gating. However, observations based on a multiple mutant of KcsA (KcsA-OM) indicate that only limited movement of the inner helices is tolerated before the selectivity filter distorts. In a bid to force the inner helices apart, multiple point mutations were introduced at the helix bundle crossing of C-terminally truncated KcsA (Cuello et al., 2010a). Whereas structures of KcsA invariably exhibit a narrow pore, five crystal structures of KcsA-OM revealed conformations of the pore ranging from narrow to very wide (Cuello et al., 2010a).

To evaluate the capacity of glycines in the inner helices to preserve selectivity filter geometry during large conformational changes (Gly99 and Gly104), we superimposed 57 residues of each KcsA-OM structure onto a well-resolved structure of KcsA (1K4C), from residue 33 in the middle of the outer helix to residue 90 at the top of the inner helix. This superimposed all structural elements in the outer bilayer leaflet, with the exception that it did not compel the inner helices to superimpose. Unexpectedly, we found the inner helices of KcsA-OM diverged from native KcsA at different points in the structures (Fig. 3A-E). In the three structures with narrower pores (14, 16 and 17 Å) divergence occurred in the vicinity of the glycine residues. However, disruption of hydrogen bonding ('helix-breaking') in the inner helix was not evident near either glycine, suggesting neither was playing a specific 'bending' role. However, in the two widest pore structures 3F7V and 3F5W, with distances between the Cα of opposing Thr112 of 23 and 32 Å respectively, the inner helix backbones diverge very close to the external face of the pore, above the selectivity filter. While the data verify that the inner helices can move relative to the outer and pore helices, they do not support the idea that Gly99, Gly104 or any other specific residue acts as a hinge for substantial inner helix motion.

The reason for glycine conservation is thus unclear; it may aid packing of the pore helices against the inner helices (Shang and Tucker, 2008) or the lability of the α-hydrogen-bonds at a glycine may facilitate transitory alignment of the peptide carbonyl dipoles with K^+ ions. The break may also stabilise water molecules, allowing hydrogen-bonding to the polypeptide backbone in an otherwise hydrophobic environment.

One would predict that divergence from native KcsA conformation in the direct vicinity of the selectivity filter would have the greatest effect on selectivity filter integrity. This is precisely what is observed. Although there is evidence of mild selectivity filter distortion in the narrowest of the KcsA-OM mutant structures, in very widest pores, 3F7V and 3F5W, the inner helices diverge at the outer face and the selectivity filter effectively collapses, compromising K^+ coordination (Fig. 3F). This has been attributed to C-type inactivation, aided by steric interactions between a cavity side chain (F103) and the base of the selectivity filter (Cuello et al., 2010b). MD simulations suggest that C-type inactivation is caused by rapid, spontaneous transitioning of the selectivity filter to a constricted state, stabilised by changes in the water network behind the selectivity filter, but only when the pore is wide (Li et al., 2018).

The data indicate that forcing a naturally narrow pore conformation to widen ultimately collapses the K^+ selectivity filter, in part because extensive hydrophobic interfaces between the inner, outer and pore helices preclude truly independent movement of the inner helices. For comparison, K^+-channels naturally adopting a wide pore (*e.g.* MthK) exhibit intact selectivity filters and it would be informative to test whether the converse is true; *i.e*. that forcing the inner helix backbone of a wide (*e.g.* BK) pore to adopt a narrower conformation similarly compromises the selectivity filter. While this has not yet been put to the test it is an important step toward clarifying whether the pore conformation is indicative of gating state, as has been thought, or is a family characteristic determined by the primary amino acid sequence.

The Relationship Between Pore Conformation and Open Probability

Before discussing more recent models of gating, we look to studies investigating the correlation between pore conformation and open probability (P_O), as this is the basis of the canonical steric

model. KcsA has been extensively studied as a canonical K^+ channel pore. A series of illuminating studies have sought to define its conformational plasticity at the inner helix bundle, many exploiting the known pH-dependency of KcsA activity (Cordero-Morales et al., 2006) for *in vitro* experimentation. Conformational transitions at the bundle crossing have been inferred from a variety of biophysical methods, including electron paramagnetic resonance (EPR) (Perozo et al., 1999) nuclear magnetic resonance (NMR) (Baker et al., 2007) (Chill et al., 2006; Takeuchi et al., 2007) (Imai et al., 2010) and proximity-induced quenching of fluorescent tags (Blunck et al., 2006).

The activation of KcsA at low pH has been correlated to increased interhelix distances near the C-terminus of the inner helix. Estimates of changes in pore diameter during gating were gained by introducing a single cysteine into tandem dimers of KcsA, followed by single site-directed spin labelling and electron paramagnetic resonance (EPR) on the derivatised mutants (Liu et al., 2001). Dipolar broadening, weighted by P_O, was the parameter used to indicate the change in distance between inner helices at the Cα of Thr112 near the bundle crossing. The data were interpreted as an increase in width of approximately 3 Å in acid conditions. A caveat on the results is that labelling residues on all sides of the inner helix near the bundle crossing may have interfered with helix-helix interactions in some mutants.

In 2006, fluorescence lifetime spectroscopy was utilised to report on conformation at the inner helix bundle of labelled KcsA by proximity-induced quenching. Two populations distinguished by 1 ns and 3 ns time constants were assigned to wide and narrow bundle crossings respectively. The spectroscopic distribution did not reflect the experimentally determined open probability of native KcsA however, and no significant differences were found in the distribution of fluorescence lifetimes between the wild type pore (low open probability) and two KcsA mutants known to have high open probability under the same pH conditions (E71A and A73E). Concluding that the conformation at the bundle crossing of KcsA was not directly correlated with channel activity, these unexpected findings were rationalised in terms of an additional gate in the selectivity filter that opens and closes independently of an intracellular gate (Blunck et al., 2006).

A recent study took this a step further (Cuello et al., 2017). A series of cysteine-pair mutants were designed using the KcsA-OM 3F5W structure as a model in an effort to capture and stabilise the elusive 'open' conformation. One such cysteine pair mutant, A28C-E118C, did just this, spontaneously crosslinking into a wide pore of similar conformation to 3F5W. KcsA-activating (E71A) or 'deep C-type inactivating' (Y82A) mutations evaluated on this background are revealing. Both triple mutants exhibit wide pores that superimpose closely onto 3F5W. The structures differ significantly only in that the A28C-E118C/E71A mutant (5VKH) has a selectivity filter indistinguishable from native KcsA, while in A28C-E118C/Y82A (5VKE) it has collapsed, resembling the distorted filter of 3F5W. The normal, conducting, selectivity filter of the triple mutant A28C-E118C/E71A thus differs markedly from the collapsed form observed in other wide KcsA structures, KcsA-OM-3F5W, KcsA-OM-3F7V and A28C-E118C/Y82A (Fig. 4).

To consider why this might be, we note that in native KcsA, Glu71 makes direct and water-mediated hydrogen bonds, both to the polypeptide backbone of the selectivity filter and Asp80 in a network behind the selectivity filter that also includes Trp68. Truncation of the Glu71 sidechain naturally disengages the outer selectivity filter from the pore scaffold, preventing its collapse as the pore widens. Both triple mutants have similar open probability to their corresponding single point mutant

counterparts, E71A and Y82A, the former constitutively active, with P_O near 100%, and the latter rapidly inactivating, yet two structures of E71A (normal and 'flipped at Asp80') (Cordero-Morales et al., 2006) and a conductive double Y82A mutant Y82A-F103A (no single Y82A structure is available) exhibit narrow pores and selectivity filters barely distinguishable from native KcsA structures. Findings that E71A can conduct when the conformation is locked-wide, yet Y82A cannot, were interpreted in the context of a conventional gating cycle. This does not definitively address the key issue of whether pore widening is required for conduction to occur; for this one would need to carry out experiments that lock the pore in a narrow state prior to evaluating function. The experimental outcomes of Cuello *et al* demonstrate that an intact selectivity filter is as crucial for K^+ conduction as it is for ion selectivity, and that the network of polar interactions behind the selectivity filter determine its integrity. The authors have qualified that, in view of differences in the full-length KcsA structure (3EFF), the locked-open structures may not reflect dynamic physiological changes.

Conformational transitions in KcsA have also been studied by solution dynamics and functional analysis using strategies of cysteine-pair (L24C-R117C and H20R-H124R) mutant crosslinking (Kim et al., 2016). To select and interchange between specific permeation states of KcsA, the lipid composition of bicelles were varied, amongst other tactics. For instance, disulfide-linking the L24C-R117C subunits together with copper phenanthroline as a catalyst rendered KcsA non-functional, while adding the reducing agent tris-2-carboxyethylphosphine (TCEP) rescued activity *in situ*. The NMR sampling of liposome-reconstituted KcsA mutants revealed considerable conformational heterogeneity; the study elegantly demonstrating that both conducting and non-conducting states are characterised by ensembles of slowly interconverting conformations (Kim et al., 2016). Stopping short of pinpointing a relationship between conformation at the bundle crossing and gating state, the study highlights a persistent issue besetting the ion channel field – that relating structure to function is invariably indirect, as experimental and structural data must be obtained under different conditions. It also demonstrates the nightmare complexities of interpretation. For example, the non-functional disulfide-linked L24C-R117C mutant is crosslinked into a pinwheel, inner helix-to-outer helix of adjacent subunits, rather than a tight collar, at a position below the membrane surface. While this would not appear to be a particularly firm constraint on pore width, it does limit the conformational dynamics.

The Hydration State of Potassium Ions Underpins the Canonical Gating Model.

The dehydration energy of K^+ is the rationale behind the expectation that it must be fully hydrated to pass between the selectivity filter and intracellular opening. The larger ionic radius of K^+ than other biological cations translates to a weaker charge distribution than for divalent ions or the monovalent Na^+ and Li^+, such that exchanging its oxygen ligands incurs a lower energetic cost. It is precisely these qualities that allow K^+ to serially exchange between stacked coordination shells as it passes through the selectivity filter. In contrast, Ba^{2+}, Ca^{2+} and Mg^{2+} can bind in the selectivity filter at the S4 position but do not transfer between sites. Using alchemical free energy (FEP) calculations to determine sequential dehydration energies of K^+ when it leaves bulk solution (Song and Corry, 2009), Corry demonstrated that the energy required to remove a water from a fully hydrated ion (n = 7 or 8) is relatively low but increases significantly for each water removed as n approaches zero. The immediate environment also affects the energetics and, as computing power increases, quantum

mechanical evaluation of the dehydration energy within the proteinaceous environment of specific channels is feasible and would provide a more complete picture.

Importantly, while dehydration energy is the main contributor to the energetic barrier to permeation, the loss of aligned water dipoles can be offset by interactions with the protein, and K^+ ions can travel several Angstroms through very narrow pores if the positive charge is shielded by dipoles. In proteins, these include dipoles in the polar environment and cation-pi interactions. Roux showed that in gramicidin A, K^+ ions with reduced hydration shells pass through a narrow conduction pathway; one-dimensional potential of mean force (PMF) simulations indicate K^+ passes through with only two coordinating waters, charge-stabilised by the alignment of water dipoles and polar groups of the protein (Allen et al., 2004; 2006). Nanofluidic data shows that some divalent ions pass through spacings smaller than their large (inner and outer) hydration shells, but at rates markedly slower than bulk diffusion (Esfandiar et al., 2017), suggesting their outer solvation layer is labile. These are factors to be considered in viewing emergent gating models and raise the interesting question of whether some K^+ channels have evolved away from Na^+ and Ca^{2+} selective channels by offsetting the costs of partial ion dehydration.

Present Perspectives on Gating

Current opinion on the importance of switching between wide and narrow-mouthed conformations during pore gating is changing, depending on the class of K^+ channel. The ion selectivity filter has been the focus of several investigations.

1. Ca^{2+}-dependent K^+ channels:

The K_{Ca} channels are a relatively disparate grouping classified on the basis of their dependence on Ca^{2+} for gating. Here we discuss two types of K_{Ca} channels for which conformational states have been sampled by cryo-EM.

BK channels: A growing body of evidence suggests that the large conductance BK channels *Slo1* and *Slo2* and their prokaryotic counterpart MthK (Wilkens and Aldrich, 2006) (Posson et al., 2013) (Posson et al., 2015) (Jia et al., 2018) do not undergo large-scale conformational change of the pore. Like K_V channels, the BK channels have six transmembrane helices. A large intracellular assembly in BK channels is referred to as the 'gating ring', and the Ca^{2+} binding sites (Schreiber and Salkoff, 1997) are within the individual RCK domains that comprise it (Jiang et al., 2001). Single particle cryo-EM structures of *Slo2.2* carried out in the presence of a potentiator, Na^+, distinguished two classes of conformers co-existing on the grids, which were designated 'open' (5U70) and 'closed' (5U76) (Hite and Mackinnon, 2017). The comparison between the two is illuminating. While only subtle differences in positioning of the sensor domains relative to the pore occur between the two structures, there are marked changes in the orientation and positioning of the domains comprising the intracellular 'gating ring' relative to the pore.

The *Slo2.2* structures superimpose closely in the region of the selectivity filter over 57 residues (260 to 317). Near residue Leu323, however, the inner and outer helices of each subunit in the 'open' conformer diverge as a unit from those of the 'closed' conformer. The interdependent motion is a consequence of extensive helix packing interactions between the inner and outer helices of each subunit. Despite this, the nominally 'open' and 'closed' pores are both easily wide enough to

accommodate fully hydrated K^+ ions along the entire length of the conduction pathway, from the selectivity filter to the distal face of the intracellular 'gating ring' (side chains of the putative gating residue Met333 in the ion conduction pathway are not visible in cryo-EM maps of either structure). The structural data concurs with findings of several groups (above) that BK channels are not controlled by a steric gate within the pore. State-dependent accessibility experiments on *Slo1* have indicated that the upper pore is accessible to relatively large quaternary ammonium cations under both activating and deactivating conditions (Wilkens and Aldrich, 2006); similarly, methanethiosulfonate (MTS) reagents successfully modify introduced cysteines just below the selectivity filter under deactivating conditions (Zhou et al., 2011).

The selectivity filter has thus been implicated as the conduction gate, essentially by default. It has been variously proposed that BK, SK (Bruening-Wright et al., 2007) and MthK (Posson et al., 2013; Thomson and Rothberg, 2010) channels gate at the selectivity filter. However, as there are no discernible differences between the selectivity filters in structures of *Slo1* (Hite et al., 2017; Tao et al., 2017; Wang and Sigworth, 2009), *Slo2* (Hite and Mackinnon, 2017), MthK (Ye et al., 2010) and SK4 (Lee and Mackinnon, 2018) the precise basis of mechanism has proved difficult to pin down.

KCa3.1: The small-conductance channel calcium-activated K^+ (SK) channel, KCa3.1 (SK4), co-assembles with calmodulin which confers its sensitivity to calcium. Calcium binding is concomitant with activation and two structures of KCa3.1 show that in the absence of Ca^{2+}, the N-terminal lobe of CaM is disordered, but that the interaction of CaM with Ca^{2+} causes the lobe to fold and interact with the intracellular region of the channel (Lee and Mackinnon, 2018). In the absence of Ca^{2+}, the KCa3.1 conduction pathway tapers to an hourglass-like constriction at Val282, with vdW spacing less than the ionic diameter of K^+, and thus no conduction is possible. When Ca^{2+} is bound, the radius at the constriction point increases by a mere 0.7 Å, yielding a vdW spacing just larger than the diameter of ionic K^+. Is this incremental increase sufficient to allow K^+ to pass through, or is it simply one facet of a more complex pore-gating mechanism? There remains the possibility that by partial depletion of its hydration shell, replenished on either side of a shallow opening, potassium may be able to pass through a much smaller opening than has been surmised.

2. K2P channels

The K2P channels are unusual in that they assemble as dimers with pseudo-4-fold molecular symmetry. A study focussing on the state-dependence of block by quaternary amines concluded that the intracellular mouth is permanently wide (Piechotta et al., 2011), consistent with structures of two members: TREK-1 (Lolicato et al., 2017) and TRAAK (Brohawn et al., 2014). It has been posited that a non-canonical gate is located at the selectivity filter in K2P channels, but its mechanism has not yet been resolved. For example, one group suggest gating is driven by ion-flux (Schewe et al., 2016). Another provides compelling evidence for a 'C-type' gate based on molecular changes in the region surrounding the selectivity filter of TREK-1 (Lolicato et al., 2017). In the latter example, the authors posit a 'cryptic' binding pocket behind the selectivity filter formed by the pore and M4 helices, where activators can bind and stabilise the active 'conformation'. Their findings lend insight into how a C-type gate at the selectivity filter of K2P channels might operate; likening it to C-type inactivation, except in the opposing context of activation (Lolicato et al., 2017).

The asymmetry of the K2P pore appears to have functional relevance and it has been proposed that the fourth transmembrane helix, M4, may function as a gating control (Dong et al., 2015). The importance of M4 in K2P channel gating is supported by the outcomes of mutagenesis-driven electrophysiology for many K2P subfamilies (Brohawn et al., 2014) including TREK, TWIK, TALK and TASK, using a variety of activating and inhibitory gating stimuli. Structurally, both TREK-2 (Dong et al., 2015) and TRAAK (Brohawn et al., 2014) have been crystallised in alternate conformations distinguished by the position of M4 relative to the pore. The conformations have been described as 'up' or 'down', and in the 'up' state M4 appears to block lateral fenestrations to the pore cavity near the centre of the bilayer. All except the TREK-2 'down' state have ions in all four sites in the selectivity filter, suggesting the channels are conduction-enabled. Molecular dynamics simulations on TREK-2 lend support by indicating that the 'down' state is less likely to be conductive than the 'up' state (Brennecke and de Groot, 2018). It has also been suggested that in the 'down' state acyl lipid tails can project into the fenestrations, potentially blocking conduction (Brohawn et al., 2014). In other work, inhibitors or activators identified from screens appear to act in an allosteric manner, binding at an extracellular 'cap' domain typifying K2P channels, or in fenestrations below the selectivity filter. It has been proposed that the modulatory agents act on the selectivity filter (Schewe et al., 2019).

3. Kir channels

At the other end of the conformational spectrum are the inward rectifier (Kir) channels. The first structure of a Kir channel, the prokaryotic channel KirBac1.1 (Kuo et al., 2003), exhibited an hourglass-like pore tapering to a very narrow intracellular opening, leading the authors to suggest a closed conduction gate. However, all subsequent structures of Kir channels revealed similarly narrow intracellular openings, whether or not activating ligands were bound. Crystal structures of Kir2.2 with the activating ligand phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) bound (Hansen et al., 2011), revealed a pore of comparable dimensions to the apo-structure (Huang et al., 1998). Kir3.2 (GIRK2, the KCNJ6 gene product) channel, co-crystallised with an activating $G\beta\gamma$ protein complex (Krapivinsky et al., 1998; Wickman et al., 1994), has a cytoplasmic opening only incrementally wider than other Kir channels (Whorton and Mackinnon, 2013). The ATP and ADP-bound structures of K_{ATP} (a Kir6.2/sulfonylurea-receptor complex) determined by single particle cryo-EM reveal equally narrow pore conformations (Lee et al., 2017). It is important to address the apparent anomaly of two serine-to-arginine mutants of KirBac3.1 that have gained traction as 'open' Kir channels. In both single S129R (Bavro et al., 2012) and double S129R/S205L (Zubcevic et al., 2014) mutants, the intracellular opening is as narrow as in wild type structures. Four arginine residues introduced into the conduction pathway were overlooked in both depictions and discussion of the structures and are only evident upon inspection of structures and maps. Compensatory adjustment to the inner helix polypeptide backbone causes it to deviate relative to the wild type channel, accommodating the arginine side chains within the conduction pathway. The cytoplasmic opening to the conduction pathway, delineated by the interatomic distance between the closest nitrogen atoms of the arginine guanidinium moieties, remains less than 6 Å in both mutants and thus too narrow to permit passage of fully hydrated K^+ ions.

Molecular Dynamics Provide Novel Insights into Permeation Gating

Molecular dynamics used to simulate events during permeation have provided insight into the dynamic events during conduction and gating. A model now enjoying a renaissance is based on the water density and dynamics within the pore and is variously termed 'hydrophobic gating', 'dewetting', or 'vapour plugging'. The first suggestion of dewetting transitions within a biological ion channel arose 15 years ago from MD on a non-selective mechanosensitive ion channel, MscS (Anishkin and Sukharev, 2004). In the crystal structure of homoheptameric MscS, the pathway is constricted at two leucines one turn of a helix apart (Leu105 and Leu109) and thus deemed 'closed'. In the simplified MD model, a cut down MscS was embedded in a thick octane layer and soft harmonic restraints used to tie the protein backbone atoms to the coordinates of the original crystal structure. Prior to MD, the pore cavity was loaded with water and equilibrated for 100 ps. During subsequent MD, the water moved away from the constriction in the first 100 ps, leaving a vacuum. It was proposed that the lack of water around this relatively large constricted area was in effect a hydrophobic gate. In a related study using a longer equilibration time of 500 ps, simulations on the full-length MscS channel structure (1MXM; 2OAU) embedded in a POPC bilayer (Sotomayor and Schulten, 2004) showed similarly intermittent water permeation through the pore while harmonic restraints were applied. Interestingly, the Leu105/Leu109 constriction proved unstable in the absence of restraints on backbone atoms and, as the harmonics were gradually released, spontaneous closure of the pore occurred. The collapse was surprising, as 1MXM was presumed to represent a 'closed' state prior to any simulations (minimum vdW spacing 5.7 Å). A later MscS structure (2VV5) (Wang et al., 2008) appears 'open' by comparison to 1MXM, highlighting the difficulty inherent in interpretation of ion channel structures. While the simulations were of high quality, computational power was comparatively limited in 2004 and simulating a large heptamer in a lipid bilayer at the time would have been pushing the boundaries; therefore a caveat is that release of the positional restraints before sufficient equilibration of the structure in the membrane might have contributed to the collapse observed. Another caveat is that the water model used was parametrised on water-water, rather than water-hydrophobic (*e.g.* Leu side chain), interactions.

The first all-atom MD of ion permeation and gating on a K^+ channel simulated the transmembrane pore of $K_V1.2$ at both depolarising (activating) and hyperpolarising (deactivating) potentials (Jensen et al., 2010). At negative voltages, K^+ ions were depleted from the cavity and it was suggested that, in the absence of hydrated ions, a transition from liquid water to vapour eventually results in complete dewetting of the cavity, physically closing the pore. The dewetting phenomenon was also observed when the voltage-sensing domains of $K_V1.2$ were included in the simulations (Jensen et al., 2012), revealing a series of conformational changes of the inner (S5) and outer (S6) helices, the S4-S5 linker, and hydrophobic residues within the cavity. Although dewetting was observed, the key event controlling conduction was attributed to conformational changes that resulted in tight packing of the PVP motif (a motif implicated in gating K_V channels), closed off the pore and prevented K^+ flux.

In 2014, simulations utilised the crystal structure of the K2P potassium channel TWIK-1 (3UKM) as a starting model (Aryal et al., 2014). In the crystal structure, the vdW spacing between opposite leucine CD2 atoms is sufficient for hydrated K^+ to pass, at ~8 Å. In TWIK-1, the number of waters around a hydrophobic constriction within the pore (Leu146 and Leu261) dropped to zero only a few nanoseconds after the start of all-atom MD. Lateral fenestrations between M2 and M4 (see above) were maintained during preparatory (500 ns) coarse-grained simulations and at the start of the subsequent atomic simulations but ablated by the end of only 100 ns of atomic simulation. Both

coarse-grain and full atomic simulations were performed in Na^+, with only three K^+ ions present in the simulation (one in the cavity and two in the selectivity filter). The K2P 'vacuum' states dominated simulation time (over 50%) and one needs more information to evaluate this outcome. For instance, the relationship of conformational changes of the pore to 'dewetting' transitions was not elaborated, *i.e.* whether water loss is manifest as a vacuum where cavity shape is retained or is due to cavity collapse. Critically, the structures serving as input to all-atom simulations (output of coarse grain MD simulations) were not reported and may have illuminated alternative reasons for why a vacuum state arose in nanoseconds.

A recently published hypothesis is that selectivity filter dilation corresponds to activation, but that conformational changes at the bundle crossing are essential to facilitate this. Modified (and mutated) versions of the wide-pore KcsA-OM structures 3F7V and 3F5W were utilised to represent 'open' KcsA states in MD simulations. A mutation that constitutively activates KcsA (E71A) (Cordero-Morales et al., 2006) was incorporated into some starting models, whereas the nominally 'closed' state of KcsA was provided by 1K4C. The results inferred that high affinity binding of K^+ ions within the selectivity filter results in non-conductive channels and that dilation of the filter is required to reduce binding affinity and enable K^+ conduction (Heer et al., 2017). The authors concluded that selectivity filter dilation is the basis of activation gating, accommodated by positional adjustments of the pore, inner and outer helices. Building on the argument, they posited that the selectivity filter is the universal gate in K^+ channels, but that conformational changes at the bundle crossing are required to allow allosteric gating at the filter, challenging the canonical model of steric activation while accepting the premise of conformational change. The study provided new insight into how a selectivity filter gate might conceivably work, mechanistically. However, there are caveats. The starting model is always critical to MD and in simulations of the wide pore channels 'conductive' selectivity filter conformations were incorporated into the model – with ion positions maintained by harmonic restraints. In simulations of the constitutively active KscA mutant (E71A), the intracellular gate is maintained in an 'open' conformation by stringent harmonic constraints. This enforces a combination of selectivity filter and pore conformation that is naturally incompatible in native KcsA. Secondly, relatively coarse sampling, at 0.5 Å steps along the reaction coordinate, and simulation windows of only 600 ps may have been insufficient to properly evaluate the selectivity filter dynamics and any propensity to collapse.

Conclusions

Although this is not a comprehensive overview, our intent is to illustrate the ideas on permeation control taking hold in the K^+ channel field and provide some insight into their robustness. Discriminating between the potential models is not likely to prove straightforward, and these are early steps. While at first glance there is no apparent connection between models advanced for BK and K2P channels, both focus on the selectivity filter as the control point and the experimental or simulation methods employed in each case are sufficiently different that it is difficult to compare and thus a shared basic mechanism cannot yet be excluded.

A logical next step would be to identify unifying principles that connect new to past findings. An important question is why K^+ channels appear to operate in a distinct manner from other cation channels, where there is structural evidence for a steric gate. It may be because K^+ channels carry out a greater diversity of functions, from propagation of action potentials through to mediating the

leak currents that set the resting membrane potential, or perhaps because the relatively higher chemical propensity of potassium to exchange oxygen ligands, and lower free energy of dehydration, de-emphasises a key criterion of the canonical model – that the size of the intracellular mouth must accommodate fully hydrated ions. Another question is of the gate location and whether the choice is limited to the selectivity filter and intracellular mouth. The inner helices have conformational plasticity, but it is not clear that its purpose is to allow wide pores to narrow or narrow pores to widen. A critical factor is that large changes in pore morphology can impact on the selectivity filter. This prompts us to outline criteria for an effective gate. First and foremost, its role is to raise or lower the free energy barrier to permeation sufficiently to control ion flux. It must also be responsive to cellular or extracellular signals, both before and during activity. Lastly, but of no less importance, it must not compromise the integrity of the selectivity filter, at least during conduction. To find a mechanism(s) meeting these criteria may require a broader perspective, considering the pore in the context of the macromolecular assembly and membrane within which it exists, the specific coordination requirements of K^+ and the mode of action of known regulatory agents.

ADDITIONAL INFORMATION

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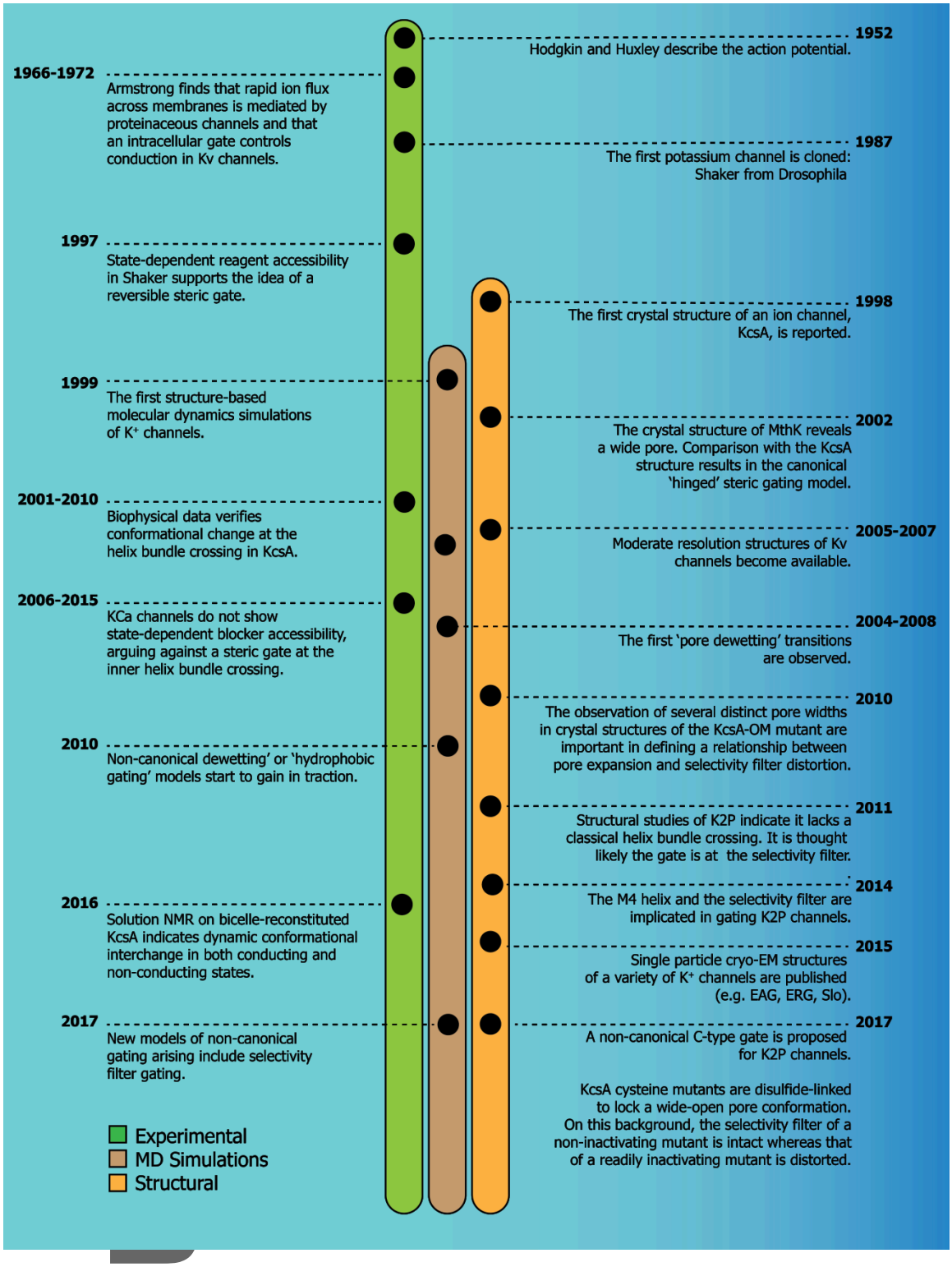

Figure 1. Timeline of major advances.

Figure 2.

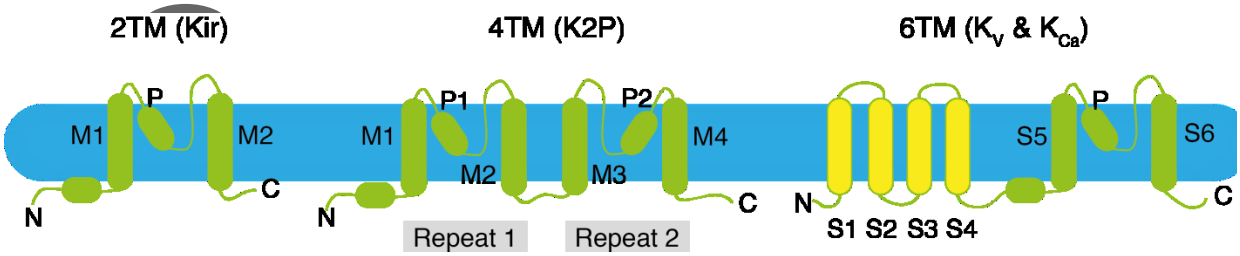

Figure 2. Four major classes of K^+ channel have been distinguished. Schematic showing the secondary structure elements, showing the nomenclature used in each case (TM=transmembrane helix). The 2TM class represent the canonical pore of K^+ channels, as in the prokaryotic channel KcsA from *Streptomyces lividans* and the Kir channels present in eukaryotes. The K2P channels have an internal sequence repeat and form a functional dimer. The 6TM channels have an additional transmembrane domain (the voltage sensor domain) comprising four helices (S1 to S4). In K_V channels, S4 is known as the voltage sensor due to several positive charges on arginine and lysine residues that move in response to a change in the membrane electric field. Note: In addition, the family members of each class have characteristic intracellular or extracellular domains that form a larger assembly.

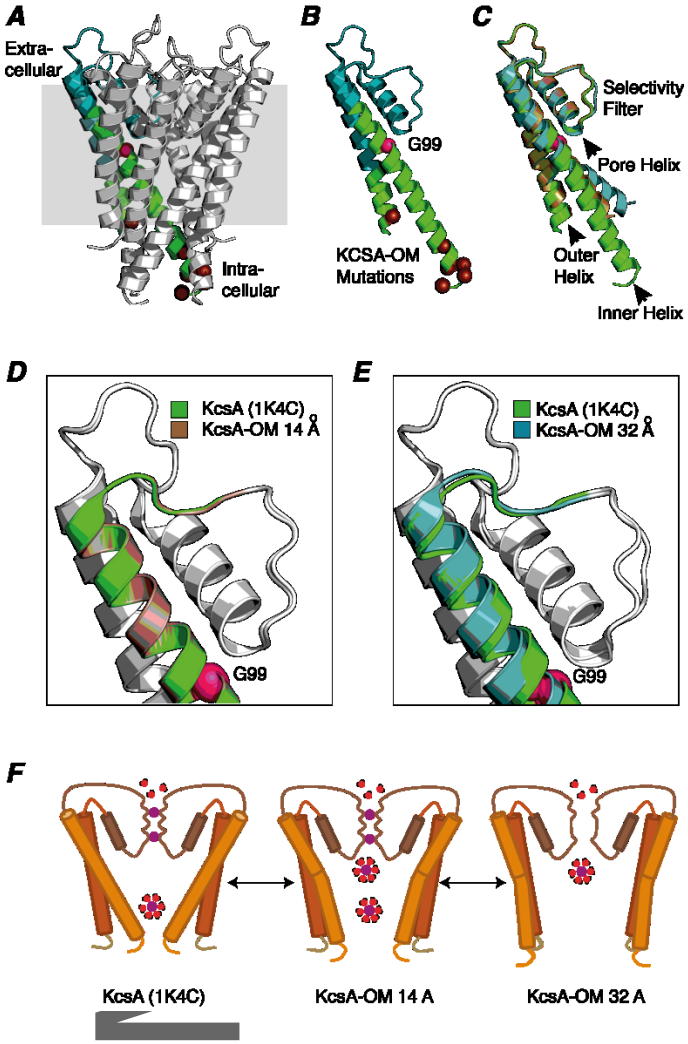

Figure 3. Substantial pore widening in KcsA does not entail bending at a specific site. (*A*) a well-resolved structure of KcsA (1K4C), shown as a ribbon diagram with one subunit picked out in colour. (*B*) The subunit is depicted in the same orientation, with inner helix closest to the viewer. The teal segment delineates the 57 residues over which pairwise superpositions of KcsA-OM onto C-truncated KcsA backbone were carried out. Gly99 is indicated by a magenta sphere at Cα, while the sites (Cα) of seven point-mutations defining KcsA-OM are depicted as brown spheres. (*C*) The result of pairwise superpositions of two KcsA-OM structures (14 and 32 Å) onto C-truncated KcsA. (*D*) Close up of the superposition of the 14 Å pore onto 1K4C within the outer leaflet of the membrane shows tight correspondence of all elements; divergence between the structures occurs close to Gly99. Only the inner helix is coloured, with outer and pore helices in grey. (*E*) in contrast, the widest of the KcsA-OM structures (32 Å) reveals that the inner helix diverges along its entire length, starting near the extracellular surface. Note that differences are evident in the selectivity filter in this superposition. (*F*) The KcsA-OM structures show that when the pore widens substantially, the inner helices bend a little but still move relative to the pore helix, distorting the selectivity filter.

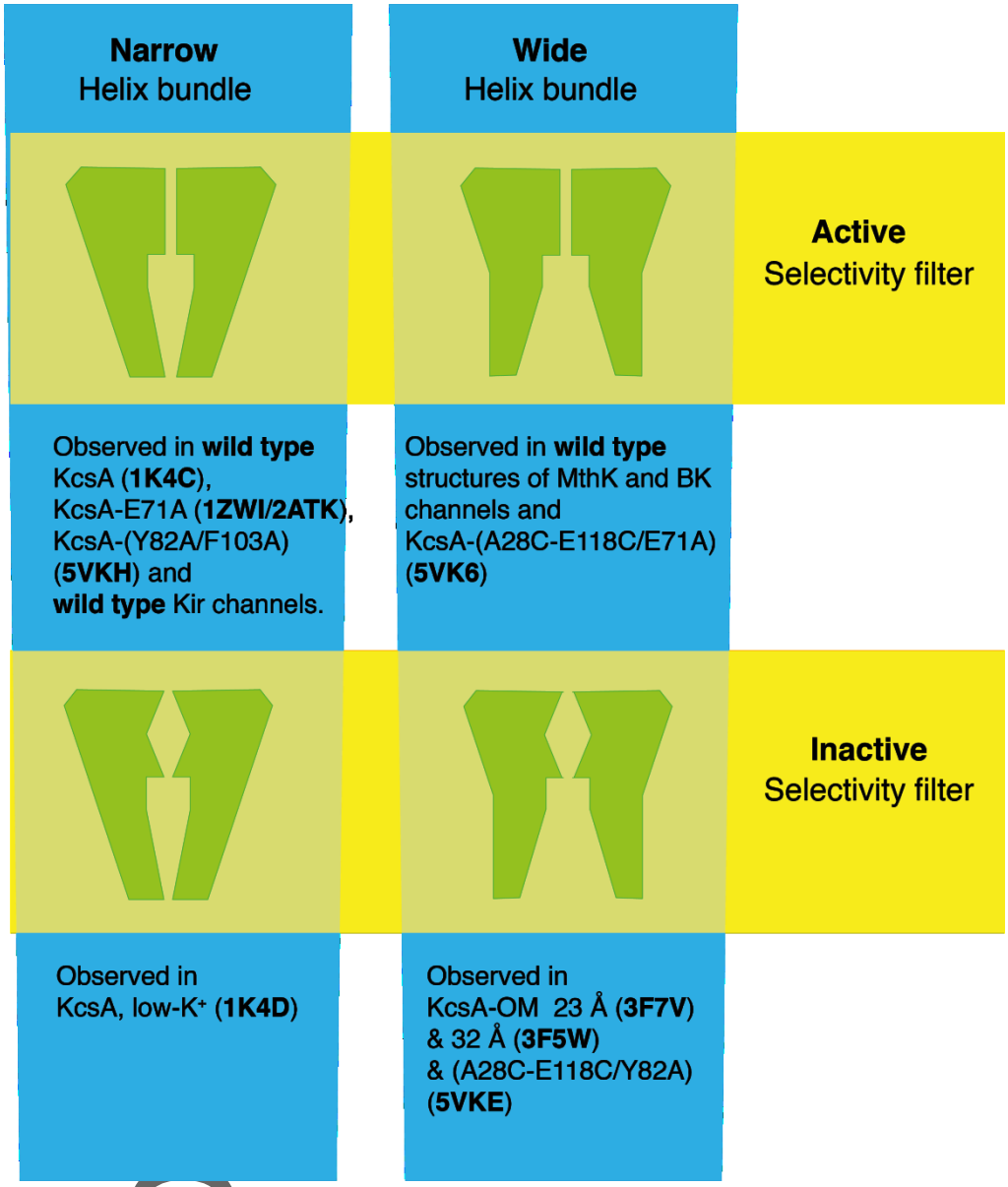

Figure 4. The conformation of the selectivity filter is contingent upon the pore architecture. Crystal structures show different conformations of K^+ pores at an intracellular helix bundle crossing. Widening a narrow pore by multiple mutagenesis near the intracellular face invokes changes at the selectivity filter. However, in KcsA mutants (at E71 and F103) where the selectivity filter 'disengages' from the scaffold, the selectivity filter remains normal. There is as yet no structure explicating the effect of forcing a channel with a wide-pore architecture (*e.g.* MthK) to assume a narrow conformation; *i.e.* whether or not the selectivity filter distorts (bottom left). It is important to note that these are crystal structures and NMR sampling indicates that both active and inactive states of KcsA mutants exhibit conformational heterogeneity in the vicinity of the helix bundle.

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