

Perspectives on: Ion selectivity

Structural studies of ion selectivity in tetrameric cation channels

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Introduction

The transfer of ions across biological membranes is central to physiological processes like nerve excitation, muscle cell contraction, and hormone secretion. To this end, ion channels play a vital role by providing pathways, the ion conduction pores, within membranes to allow specific ions to traverse down their concentration gradients. This ability to select for specific ionic species is known as ion selectivity and is a fundamental property defining ion channel function. In tetrameric cation channels, the largest and best characterized superfamily of ion channels, selectivity arises from the unique structural and chemical environment within part of the ion channel pore known as the selectivity filter. Our understanding of the molecular details governing ion selectivity in this group of channels has come a long way with the advancement of genetic, biochemical, and electrophysiological analysis of ion channels and, more recently, the structural characterization of several of them. Here, we review the contributions made by ion channel structural biology, specifically the determination of high resolution crystal structures of various tetrameric cation channels, beginning with the groundbreaking work of the MacKinnon group on the first K⁺ channel structure and subsequent work on both K⁺-selective and nonselective cation channels. These studies offer a wealth of direct insight into the structural details underlying their selectivity properties (Doyle et al., 1998; Zhou et al., 2001; Shi et al., 2006; Valiyaveetil et al., 2006; Alam and Jiang, 2009b; Ye et al., 2010). With the focus on channel selectivity, several recent structural studies on K⁺ channel gating and inactivation are not included in this discussion.

KcsA: the first view of a K⁺ channel

In the absence of detailed structural information, mutagenesis and electrophysiological analysis identified a region of the K⁺ channel imparting high K⁺ over Na⁺ selectivity. This so-called selectivity filter is formed by residues of the highly conserved TVGYG K⁺ channel

signature sequence (Heginbotham et al., 1992, 1994). However, the molecular mechanisms by which the selectivity filter imparts ion specificity were revealed for the first time with the structure determination of KcsA, a prototypical bacterial K⁺ channel (Doyle et al., 1998; Zhou et al., 2001). The KcsA filter was seen to contain four contiguous and chemically similar ion-binding sites composed of backbone carbonyl oxygen atoms from filter residues together with the hydroxyl oxygen atoms from the conserved threonine groups. The filter is positioned at the extracellular portion of the ion conduction pathway, just above a water-filled central cavity where hydrated ions can reside (Fig. 1). The ability to have hydrated ions enter the aqueous cavity serves to overcome what would otherwise be the large energy barrier that a hydrated K⁺ ion would encounter moving to the low dielectric environment of the lipid bilayers. Moreover, the structure revealed a group of “pore” helices oriented so as to point their C termini toward the central cavity, further stabilizing the ions inside (Roux and MacKinnon, 1999). From the cavity, ions must enter the selectivity filter in a dehydrated form, the energetic cost of which is compensated by the stabilization of K⁺ ions by eight oxygen ligands (mainly backbone carbonyl oxygen atoms) arranged in a square antiprism geometry at each ion-binding site within the selectivity filter. This allows for rapid and energetically efficient ion dehydration, transfer, and rehydration during the transport cycle. In the case of Na⁺ ions, the chemical environment of the selectivity filter was thought to be unfavorable for efficient ion binding and transfer, and attempts to visualize Na⁺ ions in the selectivity filter proved difficult even in the presence of a large excess of Na⁺. Moreover, the selectivity filter in these conditions (high Na⁺/low K⁺) was seen to “collapse” into a nonconducting conformation. This, in turn, led to the proposal that K⁺ ions are required to stabilize the selectivity filter

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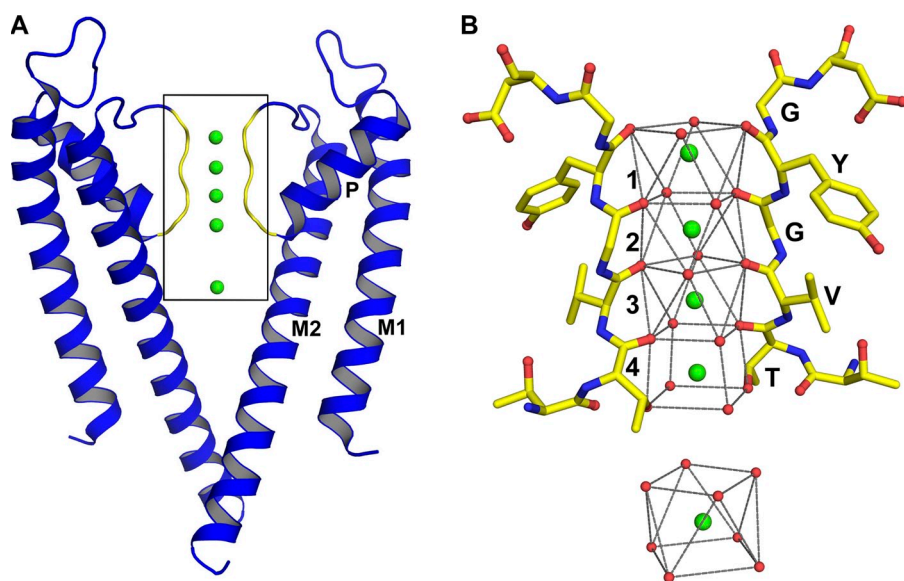


Figure 1. Structure of KcsA. (A) Overall structure of KcsA, with the front subunit removed for clarity. Selectivity filter (boxed) is colored yellow. Ions in the selectivity filter and cavity are shown as green spheres. M1, outer helix; M2, inner helix; P, pore helix. (B) Zoom-in view of the selectivity filter. The K^+ ion in the cavity is surrounded by eight water molecules (red spheres). The four ion-binding sites within the filter are labeled 1–4 from top to bottom.

of K^+ channels in a conductive conformation (Zhou et al., 2001; Valiyaveetil et al., 2006). This K^+ -dependent conformational change can be mitigated by replacing the first conserved glycine residue in the TVGYG signature sequence (underlined) with an unnatural D-alanine residue (KcsA^{D-Ala77}) (Valiyaveetil et al., 2006). Consequently, although still K^+ selective, the mutant channel is able to conduct Na^+ in the absence of K^+ . This points to the K^+ -dependent stabilization adding to, but not solely determining, the high K^+/Na^+ selectivity of KcsA, as further confirmed in a recent high resolution structural/functional study of the MthK K^+ channel, as discussed below.

Additional studies on ion occupancy in KcsA revealed the filter under high K^+ concentrations to be occupied simultaneously by two ions separated by water molecules, giving rise to the “hopping model” of 1,3 and 2,4 configurations within the selectivity filter (Moraes-Cabral et al., 2001; Zhou and MacKinnon, 2003). Collectively, the structural studies of KcsA seem to favor the classical snug-fit model (Mullins, 1960; Bezanilla and Armstrong, 1972) and explain high K^+ over Na^+ selectivity in the context of a fine energetic balance between hydration/dehydration energies and ion–ion repulsion to achieve selective ion conduction at near diffusion-limited rates. At the heart of this is the selectivity filter architecture of KcsA that is seemingly optimized for K^+ conduction and nonconductive for Na^+ .

In addition to the mentioned structural work, ongoing theoretical studies have suggested that a simple energetic picture of snug fitting is not adequate to explain selectivity, and kinetic factors also play important roles. Some computational studies invoke one or more of the following concepts in explaining K^+ channel selectivity: the coordination number of the ion, chemistry of carbonyl oxygen ligands, intrinsic dynamism of the

selectivity filter, the solvent exposure of the ion-binding sites, or the free energy landscapes of ion entrance and translocation in a multi-ion configuration (Noskov et al., 2004; Bostick and Brooks, 2007; Noskov and Roux, 2007; Thomas et al., 2007; Varma and Rempe, 2007; Varma et al., 2008; Dudev and Lim, 2009; Thompson et al., 2009; Egwolf and Roux, 2010). Although still under debate, the underlying mechanism of ion selectivity, as determined electrophysiologically, likely reflects a combination of both energetic and kinetic factors.

MthK: a Na^+ -permeable K^+ channel

Testament to the conservation of selectivity filter architectures in K^+ channels, the selectivity filter of the Ca^{2+} -regulated MthK K^+ channel adopts an identical

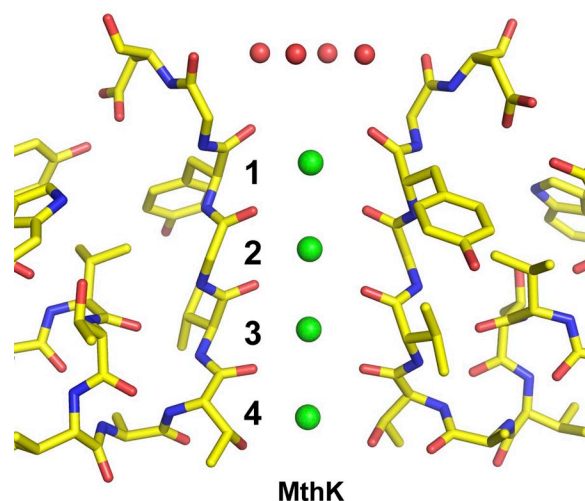


Figure 2. Selectivity filter of MthK. K^+ ions in the selectivity filter are shown as green spheres, and the four water molecules at the external entrance are shown as red spheres.

architecture to that of KcsA (Fig. 2) (Jiang et al., 2002). However, recent studies combining high resolution crystal structures of the MthK pore along with electrophysiological recordings have pointed to some structural/functional differences between the two (Ye et al., 2010). Unlike KcsA, the MthK filter in a low K^+ /high Na^+ environment is seen to retain a conductive conformation, suggesting that the requirement for K^+ ions to stabilize a conductive selectivity filter as observed in KcsA may only hold true for a subset of K^+ channels. Although still highly selective for K^+ , MthK is able to conduct Na^+ ions in a K^+ -free setting, reminiscent of the KcsA^{D-Ala77} mutant. Although at high K^+ concentrations two K^+ ions have equivalent occupancy at the four sites in the selectivity filter, low K^+ /high Na^+ concentrations lead to the replacement of one of the K^+ ions, whereas the other remains bound in the selectivity filter, preferably at site 1 or 3, upsetting the balance between the 1,3 and 2,4 K^+ ion configurations underlying efficient conduction. The binding of a single K^+ ion at low K^+ concentrations effectively blocks the permeation of Na^+ ions that would otherwise pass through the pore in the absence of K^+ . This gives rise to the so-called anomalous mole-fraction effect, a key property of multi-ion pores related to ratio-dependent permeability changes attributed to the presence of a second permeable ionic species observed in various cation channels such as MthK and other Na^+ -conducting K^+ channels, as well as Ca^{2+} channels (Almers and McCleskey, 1984; Hess and Tsien, 1984; Kiss et al., 1998). In context of the biological functioning of K^+ channels, this property could prevent Na^+ ions from “leaking” through under the low K^+ /high Na^+ concentrations of the extracellular environment.

The balance between the favorable 1,3 and 2,4 configurations for multiple K^+ ion binding and conduction is restored upon increasing K^+ concentrations.

NaK: a nonselective cation channel

The first picture of a nonselective cation channel pore came with the structure determination of the NaK channel from *Bacillus cereus*, which shares overall architecture and sequence similarities with its K^+ -selective counterparts (Fig. 3) (Shi et al., 2006; Alam et al., 2007; Alam and Jiang, 2009a,b). The channel's key distinguishing feature is the selectivity filter that, compared with those of K^+ channels, bears an altered ${}_{63}\text{TVGDG}_{67}$ signature sequence. The presence of the acidic D66 residue is reminiscent of selectivity filter sequences of the nonselective CNG channels (Yau and Baylor, 1989; Zagotta and Siegelbaum, 1996; Kaupp and Seifert, 2002; Matulef and Zagotta, 2003) known to contain an equivalent and highly conserved acidic residue, as seen in the common CNG signature sequence of TIGET. Although carrying only a single-amino acid substitution compared with K^+ channel filters, the structure of NaK reveals only two contiguous ion-binding sites in the selectivity filter equivalent in position and chemical environment to sites 3 and 4 of K^+ channels, whereas sites 1 and 2 are replaced by a wide vestibule (Fig. 3). The NaK filter is unique in its ability to use both protein oxygen atoms as well as water molecules for ion coordination (Fig. 3). This results in efficient binding of both Na^+ and K^+ ions, as well as divalent cations, albeit with different ligand chemistries as discussed below.

As seen in K^+ channels, K^+ ions tend to bind in the middle of an eight-liganded shell. In the absence of

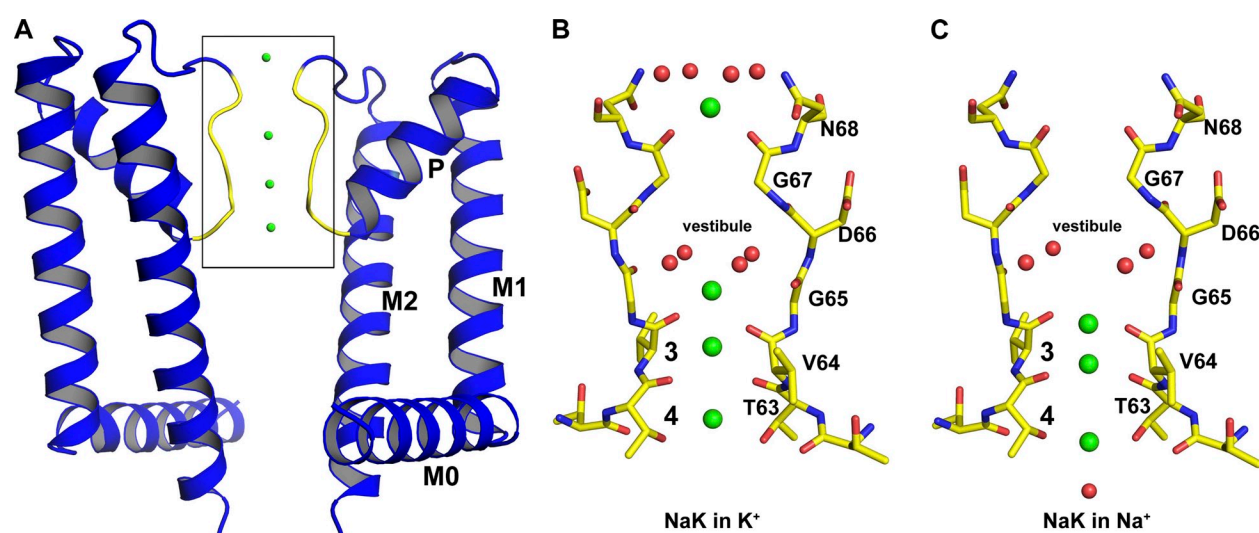


Figure 3. Structure of NaK. (A) Overall structure of NaK, with the front subunit removed for clarity. Selectivity filter is colored yellow. Ions in the selectivity filter are shown as green spheres. (B) Zoom-in view of the selectivity filter of NaK in K^+ (green spheres). The water molecules in the vestibule and at the external entrance are shown as red spheres. The two ion-binding sites within the filter are labeled 3 and 4. (C) Zoom-in view of the selectivity filter of NaK in Na^+ (green spheres).

sites 1 and 2, this requirement is fulfilled with the participation of four water molecules that coordinate K^+ in combination with the four backbone carbonyl oxygen atoms of residue V64 in the vestibule, effectively mimicking site 2 in K^+ channels, or G67 to create an ion-binding site at the external entrance. In contrast, Na^+ ions prefer to bind in plane with their ligands, as has also been suggested in the computational studies of ion selectivity and conduction in NaK (Noskov and Roux, 2007; Vora et al., 2008), allowing for shorter ligand-ion distances. Na^+ also binds in a pyramidal coordination scheme at site 4, with a water molecule from the cavity participating in Na^+ coordination. A similar Na^+ coordination scheme is also seen in the Na^+ complex of MthK (Ye et al., 2010), pointing to conserved Na^+ ion-binding modes in different members of the family. This unique ability to combine water molecules with carbonyl oxygen atoms has important consequences for ion binding, as the selectivity filter does not appear to undergo visible conformational changes to accommodate ions of different size.

As mentioned previously, the distinguishing feature of NaK is the presence in its selectivity filter of residue D66, equivalent to the highly conserved acidic residues in CNG channel selectivity filters, where they play a central role in Ca^{2+} permeation and blockage (Root and MacKinnon, 1993; Eismann et al., 1994; Seifert et al., 1999; Gavazzo et al., 2000). The NaK channel structure revealed two Ca^{2+} -binding sites, one each at site 3 and the external entrance. Ca^{2+} was also shown to block monovalent currents similar to CNG channels (Alam et al., 2007). Like CNG channels, residue D66 in NaK was also shown to play a central role in Ca^{2+} binding at the external entrance but, contrary to the predominantly accepted view, does not directly participate in ion coordination. Instead, it mediates Ca^{2+} binding at the external site via a through space electrostatic interaction between the negatively charged D66 side chain and the

Gly67-Asn68 peptide bond. In fact, both Ca^{2+} -binding sites exclusively use backbone carbonyl oxygen atoms as Ca^{2+} ligands (Alam et al., 2007). This carries far-reaching implications for Ca^{2+} -conducting channels, as it highlights the possibility that their ion conduction pores/selectivity filters could be lined exclusively with backbone carbonyl groups much like KcsA, with acidic residues imparting Ca^{2+} specificity through similar mechanisms as those seen in wild-type NaK.

Recent studies incorporating detailed mutagenesis and structural work on NaK variants with selectivity filters containing three and four contiguous ion-binding sites have been performed, allowing for an analysis of the mechanistic implications of having different numbers of inline sites, as well as offering the most accurate pictures to date of what CNG channel selectivity filters likely look like, as described below.

CNG-mimicking NaK mutants offer structural insight into Ca^{2+} blockage of CNG channels

Although previous studies on NaK have yielded novel insights into Ca^{2+} binding in cation channels (Alam et al., 2007), they fall short of explaining several key mechanistic differences between NaK and CNG channels. The selectivity filters of the two differ significantly after the conserved T(V/I)G residues in both amino acid composition and sequence length, with a majority of CNG channels containing an ETPP motif (Fig. 4 A). To reveal the structural mechanism of nonselective permeation and Ca^{2+} blockage in CNG channels, a set of NaK chimeras mimicking CNG channel pores were generated and reported in a recent study (Derebe et al., 2011b). In these chimeras, the filter sequence of TVGDGNFS in NaK was replaced by TVGXTPP, where X (corresponding to residue 66) is Glu (NaK2CNG-E), Asp (NaK2CNG-D), or Asn (NaK2CNG-N), simulating commonly seen CNG channel pores and their Glu-to-Asp and Glu-to-Asn mutants, respectively. The CNG mimics share

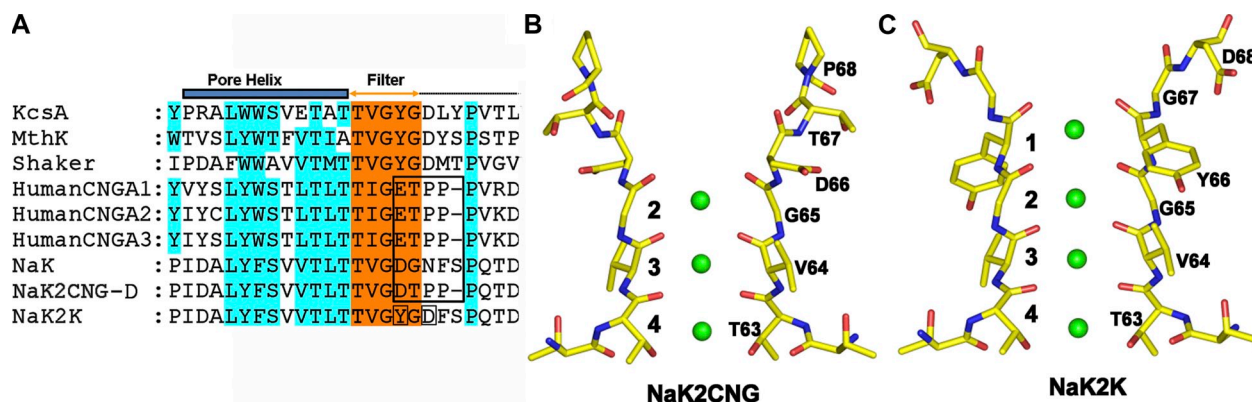


Figure 4. Selectivity filter structures of NaK mutants. (A) Partial sequence alignment between NaK, K^+ channels (MthK, KcsA, and Shaker), and human CNG channel α subunits (A1–A3). The sequences of NaK2CNG-D and NaK2K mutants are also shown in which the mutated residues are boxed. Residues forming the selectivity filter are shaded orange. (B) Structure of NaK2CNG chimera. The three ion-binding sites within the filter are labeled 2–4 from top to bottom. (C) Structure of the K^+ -selective NaK2K mutant.

several striking functional similarities in ion selectivity with their eukaryotic counterparts: they are nonselective and permeate Na^+ and K^+ equally well, and externally added Ca^{2+} serves as a permeating blocker, with the conserved acidic residue in the filter mediating Ca^{2+} binding. The Glu-to-Asn substitution in NaK2CNG-N is seen to abolish Ca^{2+} binding, whereas an equivalent Glu-to-Asp substitution in NaK2CNG-D yields a channel with roughly 10-fold higher Ca^{2+} affinity than NaK2CNG-E.

The high resolution structures of these chimeric channels reveal a hitherto unseen selectivity filter architecture comprising three contiguous ion-binding sites (equivalent to sites 2–4 of a K^+ channel) (Fig. 4 B). The introduction of an additional site in NaK2CNG chimeras results from a main chain conformational change at residue 66, leading to the elimination of the water-filled vestibule as was observed in wild-type NaK (compare Fig. 3 B with Fig. 4 B). Na^+ and K^+ are seen to bind in these chimeric selectivity filters using similar coordination schemes as seen in the wild-type channel, with K^+ binding in the center of each site and Na^+ preferring to bind at the upper and lower edges of the sites, with a water molecule participating in a pyramidal Na^+ coordination at site 4 (Alam and Jiang, 2009b; Derebe et al., 2011b). The structural analysis also provided an accurate picture of how Ca^{2+} binds inside the selectivity filter as well as the position of conserved acidic residues mediating it. Although NaK2CNG-E contains a single Ca^{2+} -binding site (site 3), Ca^{2+} ions can bind both sites 2 and 3 in NaK2CNG-D. Contrary to conventional models, and expanding on insight from wild-type NaK structures, the side chain of the conserved acidic residue in the filter does not participate in direct ion coordination, with Ca^{2+} ions instead coordinated exclusively by backbone carbonyl oxygen atoms. Furthermore, these structures also allowed for the pinpointing of equivalent interactions in wild-type CNG channel pores through structure-based mutagenesis that have hitherto not been predicted using NaK or K^+ channel models. Overall, these CNG-mimicking chimeras have provided the most realistic structural models to date of actual CNG channel pores and again highlight the possibility of Ca^{2+} specificity occurring without direct involvement of negatively charged groups, as seen in most Ca^{2+} -binding proteins (Katz et al., 1996; Pidcock and Moore, 2001).

The importance of number of inline K^+ -binding sites in determining K^+ selectivity

The structures of NaK and NaK2CNG chimeras showcase a set of tetrameric cation channels with two and three contiguous ion-binding sites, respectively, in the filter. In both cases, each of these sites maintains a geometry and ligand environment virtually identical to that of equivalent sites in K^+ channel selectivity filters,

yet both remain nonselective. It takes the addition of another contiguous site equivalent to site 1 of K^+ channel selectivity filters to make a channel K^+ selective, as demonstrated in a NaK mutant named NaK2K, which carries both a D66Y and N68D mutation (Fig. 4, A and C) (Derebe et al., 2011a). NaK2K has a selectivity filter sequence and structure identical to that of K^+ channels and exhibits high K^+/Na^+ selectivity. Moreover, protein packing surrounding the NaK2K selectivity filter is similar to that in MthK, and the channel can also conduct Na^+ in the absence of K^+ . In addition to high K^+/Na^+ selectivity, NaK2K also exhibits higher single-channel conductance for K^+ compared with wild-type NaK or the CNG channel mimics. In light of the NaK- and NaK2CNG-mutant selectivity filter structures, it therefore appears that a minimum of four contiguous ion-binding sites is central to conferring high K^+ selectivity in NaK2K, which likely holds true for canonical K^+ channels. This is further supported by the elimination of site 4 in the K^+ -selective NaK2K mutant or the MthK channel with an introduction of a T63A or T59A mutation, respectively. These mutants maintain three contiguous K^+ ion-binding sites in the filter (equivalent to sites 1–3 of a K^+ channel) but become virtually nonselective (Derebe et al., 2011a).

To summarize, the structural studies of NaK and its various mutants present an ensemble of tetrameric cation channel pores whose selectivity filters comprise two, three, or four contiguous ion-binding sites with similar chemical environments. Although the ligand geometry of each individual site in these channels is virtually identical to equivalent sites in the KcsA K^+ channel, only selectivity filters containing four ion-binding sites display marked K^+/Na^+ selectivity, suggesting that four equivalent and contiguous ion-binding sites in the filter are a prerequisite for selective and efficient K^+ conduction. The results of these studies clearly demonstrate that, in what likely is a complex interplay of various factors giving rise to a channel's selectivity properties, the number of inline sites in the filter is a key determinant, hitherto overlooked or otherwise unexplained by both the classical snug-fit model as well as by current theoretical models that, in some cases, rely on calculations based on single isolated K^+ -binding sites.

Concluding remarks

High resolution structures of ion channels have greatly aided our understanding of the molecular details underlying ion selectivity. Key features of ion channel architecture at the heart of unique selectivity mechanisms have emerged along with the binding modes that different ions display within the selectivity filters, laying out an overall framework for general mechanisms of selectivity that existing experimental and theoretical models are based on. However, it is pertinent to realize that the mechanisms of even K^+ selectivity are not yet fully

understood, with additional higher resolution structures of K⁺-selective and related channels serving to broaden our understanding in increasingly finer detail. In comparison, Na⁺ and Ca²⁺ channel selectivity are very poorly understood in terms of the underlying molecular details, and the tetrameric cation channel field is left yearning for the first atomic pictures of Na⁺- or Ca²⁺-selective cation channel pores.

This Perspectives series includes articles by Andersen, Nimigean and Allen, Roux et al., Dixit and Asthagiri, and Varma et al. (scheduled for the June 2011 issue).

We thank Mehabaw Derebe for figure preparation.

This work was supported in part by the Howard Hughes Medical Institute and by grant from the National Institutes of Health (GM079179 to Y. Jiang).

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