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Review

Mechanisms of valence selectivity in biological ion channels

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Abstract. Transmembrane ion channels play a crucial role in the existence of all living organisms. They partition the exterior from the interior of the cell, maintain the proper ionic gradient across the cell membrane and facilitate signaling between cells. To perform these functions, ion channels must be highly selective, allowing some types of ions to pass while blocking the passage of others. Here we review a number of studies that have helped to elucidate the mechanisms by which ion channels discriminate between ions of differing charge, focusing on four channel families as examples: gramicidin, ClC chloride, voltage-gated calcium and potassium channels. The recent availability of high-resolution structural data has meant that the specific inter-atomic interactions responsible for valence selectivity can be pinpointed. Not surprisingly, electrostatic considerations have been shown to play an important role in ion specificity, although many details of the origins of this discrimination remain to be determined.

Key words. Ion channel; selectivity; permeation; Brownian dynamics; molecular dynamics; gramicidin; calcium channel; ClC; potassium channel.

Introduction

Ion channels provide pathways for the movement of charged particles across cellular membranes and mediate the interaction between a cell and its environment. The transport properties of ion channels are central to the physiology of living organisms, underlying phenomena such as the conversion of sensory input into electrical signals, the conduction of electrical signals such as in nerve conduction and the conversion of electrical stimuli into actions [1]. They are now known to be narrow water-filled pores through which ions can pass shielded from the non-polar interior of the membrane.

Not surprisingly, there are many different types of ion channels to carry out the vast variety of functions required of them. These channels differ in their localisation within the organism, when they open and close to allow or prevent the flow of ions, the types of ions that they will pass and the rate at which they do so. Some channels allow many types of ions to pass, whereas others are much more selective, allowing, for example, just one of the predominant physiological ion species, Na^+, K^+, Ca^{2+} and Cl^- passage. Also, the opening of channels can be initiated in many different ways. There are ligand-gated channels that open when one or two chemical molecules, known as neurotransmitters, secreted from nerve terminals bind to the channel. Some channels, the so-called voltage-gated channels, open in response to step changes in the electric field, while others, mechanosensitive channels, respond to pressure on the membrane. The importance of a fourth class of ion channels, the second-messenger-mediated channels, is now beginning to be realized. In these the activation of one channel triggers a cascade of biochemical events inside the cell which in turn activates a cluster of the second-messenger-mediated ion channels. Here we briefly summarize our understanding of how ion channels

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discriminate between ions of different valence. How does a calcium channel, for instance, select just the calcium ions out of a mixture containing many more Na^+, K^+ and Cl^- ions?

That many ion channels are selectively permeable to specific ionic species has been known for many decades. Hodgkin and Huxley managed to measure the flux of ions across the plasma membrane of a giant squid axon [2, 3]. They found that Na^+ and K^+ appeared to move along separate pathways with distinguishable kinetics and responses to changes in membrane potential and electrolyte composition. The electrostatic wave, or 'action potential', passing along the axon could be described by the separable flow of Na^+ ions with both a rapid activation and deactivation, and K^+ ions with slower kinetics [4]. Separate pathways for the remaining physiological ions Ca^{2+}, Cl^-, H^+ and NO^- were also postulated. However, it was not known at that time how these pores were physically formed. Subsequently, the origins of the selective currents were found to lie in different ion channel proteins [5]. Once the currents passing through single ion channels could be determined using the patch clamp technique [6], it was unequivocally shown that different ion channel proteins are responsible for the selective passage of different ion types.

Explaining how particular channel proteins discriminate between the different ion types is an ongoing project. Early discussions of this issue focused on the kinetics of transport using rate models. Recently, however, structural information has become available through site-directed mutagenesis, nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction. This has allowed molecular models to be constructed and the specific atomic interactions responsible for ion selectivity in a number of channels to be illuminated.

Here we examine four examples of valence selective ion channels, namely, gramicidin, ClC chloride channels, voltage-gated calcium channels and potassium channels. Gramicidin is selective for monovalent cations, ClC for anions, calcium channels for divalent cations and potassium channels for some monovalent cations. By comparing the mechanisms of selectivity amongst this diverse group of channels, we hope that common elements can be found to reinforce our understanding of their operation.

Gramicidin channel

The gramicidin A pore is formed by a linear polypeptide consisting of 15 hydrophobic amino acids in an alternating L-D sequence. It was one of the first antibiotics discovered by Hotchkiss and Dubois [7]. To form a cation-selective pore, two gramicidin peptides briefly link head-to-head by hydrogen bonds between their formyl end groups, creating a cylindrical passage across the membrane with length 25 Å and radius ~2 Å, as illustrated in figure 1. The opening of transmembrane pores can be recorded as stepwise increases in conductance across the membrane, each channel-opening event lasting between 30 ms and 60 s (for reviews see [8–11]). This peptide has become one of the most widely studied and understood transmembrane channels due primarily to its small size that enabled atomic resolution structures to be determined using solution and solid-state nuclear magnetic resonance (NMR) long before such information was available for any other transmembrane channel [12–17]. The protein also has characteristics reminiscent of much larger channels such as the family of potassium channels. Most notably, as illustrated in figure 1C, all the polar regions of gramicidin, including a number of carbonyl oxygens, line the pore where they can interact with permeating ions.

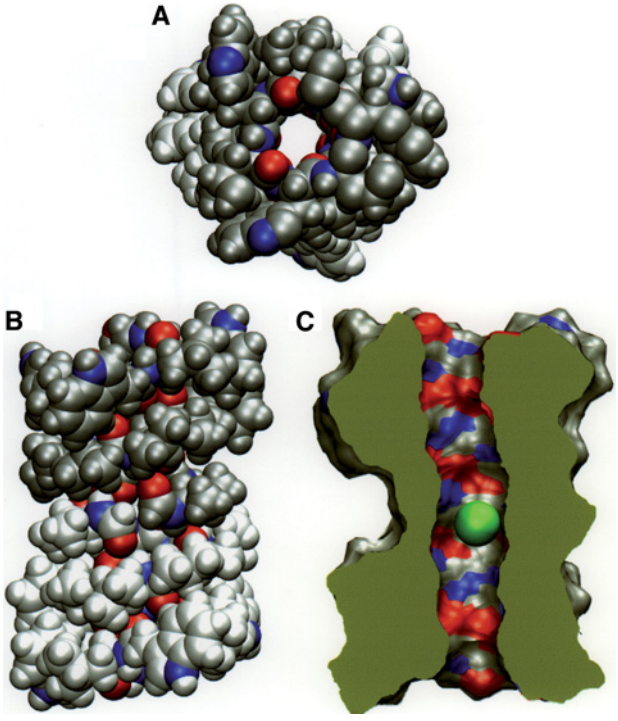

Figure 1. Gramicidin channel structure. Top (*A*) and side (*B*) views of the channel are shown as well as the surface of the pore (*C*) with a permeating potassium ion in green shown for scale. Oxygens are shown in red and nitrogens in blue.

Gramicidin channels have been extremely well characterized using single channel current recording techniques. Studies in a variety of bilayers [18–22] show that monovalent cations pass through the channel at a conductance in the order of 20 pS at 500 mM concentration. Myers and Haydon [23] observed that anions neither permeate nor block the channel, a fact that has been confirmed numerous times [24]. Divalent cations also do not permeate through the pore, but rather are observed to block the channel in single channel and whole cell studies.

It was noticed early on that the gramicidin peptide has a net neutral charge and furthermore that the carbonyl and amide dipoles all lay roughly parallel to the channel axis and anti-parallel to one another [25–27]. Thus, there was no obvious mechanism for charge discrimination. Urry [28] first postulated that the carbonyl oxygens lining the pore could more effectively coordinate cations than the amide groups could anions. This was a result of libration of the carbonyl dipole being sufficient to allow a slight net distortion in their position upon cation entry that could create a binding site favourable to accommodate the cation. The anion, on the other hand, is repelled by the negative charge on these groups [29]. This hypothesis gains some strength when examining recent NMR structures [15] that show the carbonyl oxygens slightly closer to the central axis than the amide hydrogens. Sung and Jordan [27] posed an alternative mechanism of cation-anion selectivity. They calculated the solvation energy profiles of Cs^+ and Cl^- and found a large barrier to Cl^- permeation at the mouth of the channel which prevents permeation or blockage. This was created by the local charge distribution originating from outward pointing carbonyl oxygens. They found, however, that if anions could overcome this energy barrier they would be stable in the center of the channel due to the quadrupolar nature of the charge distribution. That valence selectivity was a consequence of electrostatic interactions between the permeating ions and partial charges in the protein or membrane was supported by a number of studies by Lauger and colleagues [30, 31]. They were able to increase the cation transport rate by introducing charged residues into the protein or by altering the charge composition of the surrounding membrane.

In contrast to these studies, Dorman et al. [32] argued that the charge specificity of the gramicidin channel arises from the solvation characteristics of the ions rather than the specific charge distribution in the protein. Using a semimicroscopic approach containing atomistic and continuum elements they found that an anion binds water more strongly than a cation of the same size. Thus, the anion was less likely to dehydrate and enter the channel. The understanding of valence selectivity was further complicated when Roux [33] used a free-energy perturbation approach in molecular dynamics to reassert that the specific interaction of the ions with the carbonyl oxygens was important. In this study, the carbonyl oxygens were found to provide an energetically favourable interaction with cations, whereas the amide groups were less effective at stabilizing anions. This result is consistent with Urry's first postulations, but the importance of interactions with the hydrating water was also stressed in the later study.

At this time the debate over the origins of valence selectivity centered over whether it was a thermodynamically or kinetically controlled property. In the model of Sung and Jordan [27] anions were energetically favoured in the center of the channel, but the large barrier to entry resulted in very slow kinetics. Urry [28], Dorman et al. [32] and Roux [33], however, all suggested that cations were thermodynamically favoured in the channel, whereas anions preferred to remain out of the channel surrounded by water.

More recent studies seem to have re-emphasized a simpler version of the thermodynamic explanation, although the importance of solvation properties cannot be discounted. A number of continuum calculations using either Poisson's equation [34], Poisson-Nernst-Planck theory [35, 36] or a dynamic lattice Monte-Carlo theory [37] to determine the energy profile for ions inside the channel all indicate that the charge distribution in the structure alone is enough to account for the selectivity for monovalent cations. In these models a static structure taken from one of the NMR studies is assumed, and the energy on the ion is described by a dielectric-continuum model. In all cases a net negative potential that will attract cations while repelling anions is found along the length of the channel. These studies suggest that there is a slight asymmetry in the charge distribution in the protein, such that the regions close to the channel axis are negative and the regions further away slightly positive. Although most of this charge asymmetry presumably arises from the backbone carbonyl groups [15], a specific distortion away from their mean position by a permeating ion does not appear to be required to explain cation specificity, although such a distortion may still take place [32, 38–42]. Continuum electrostatic calculations allowing for protein flexibility have also been carried out [41, 42] which find a slightly greater cation-protein attraction that the models with static structures.

Recent detailed molecular dynamics (MD) studies of ion permeation in gramicidin support the notion of an asymmetric charge distribution underlying valence selectivity [40]. A decomposition of the potential of mean force for a permeating K^+ ion shown in figure 2A indicates that the electrostatic interaction of the ion with the protein creates an attractive energy well of almost 40 kcal/mol (68 kT). Furthermore, this attractive component creates two binding sites near each end of the channel where the interaction with the carbonyl oxygens is greatest. The single file of water also attracts ions into the channel, but these attractive interactions are largely canceled by the dehydration barrier encountered by an ion entering the pore. No similar MD studies for anions are reported in the literature, but the electrostatic interaction can be expected to be less favourable for anions than for cations.

A slight charge asymmetry may be all that is required for the gramicidin channel to select cations over anions. But a question still remains as to why these channels are blocked by divalent ions. Both the continuum calculations [34] and molecular dynamics simulations [40] indicate that there is a large repulsive Born energy component

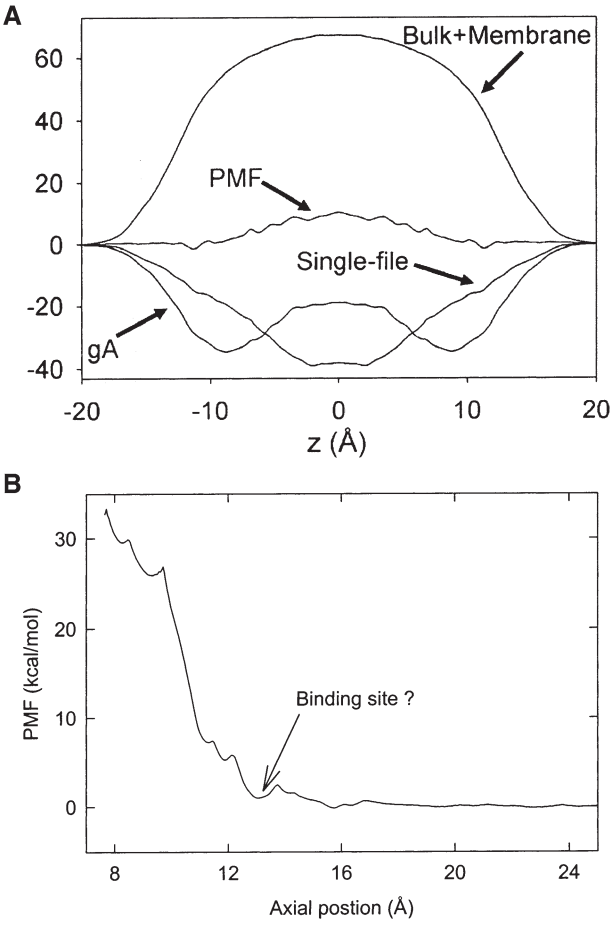

Figure 2. Potentials of mean force (PMF) in gramicidin found from molecular dynamics simulations. (*A*) The total PMF for K^+ is decomposed into an attraction between the ion and the protein (gA), an attraction between the ion and the single-file water in the pore (single-file) and a dehydration barrier (Bulk+membrane). Reproduced from [40]. (*B*) The PMF calculated for a Ca^{2+} ion in gramicidin calculated using umbrella sampling and 0.8 ns of simulation for each point in the profile.

to the total energy of the ion in the channel as a result of removing the ion from the bulk water and placing it into the lower dielectric region of the membrane. Although a divalent ion will be attracted more strongly to the negative charge on the protein than a monovalent ion, this scales as z, the valence of the ion. The Born energy, on the other hand, scales as z^2, so this term dominates and a large energy barrier is found for divalent ions in the center of the channel. In microscopic terms, this just means that the divalent ion is bound to its hydrating water more strongly than monovalent ions.

A number of modeling [43], X-ray scattering [44] and NMR [24] studies suggest that divalent cations bind further from the center of the channel (~13 Å) than monovalent cations (~9–10 Å), consistent with them encountering a large energetic barrier beyond the mouth of the channel. In this position the ion can still be surrounded by the majority of its hydrating water, which would have to be removed if it were to permeate further. Molecular dynamics studies show very weak binding of Ca^{2+} at z=13 Å as shown in figure 2B and reported elsewhere [45]. However, the degree of binding seen in these simulations is unlikely to reproduce the degree of blockage reported experimentally.

In summary, these studies suggest that most probable cause of valence selectivity in gramicidin is a slight asymmetry in the charge distribution that leads to an attractive energy well to cations. The greater flexibility of the carbonyl over amide groups may also play a role but does not appear to be essential to achieve cation specificity. Although divalent ions are also attracted to the negative potential in the channel, the greater energy required to dehydrate them upon entering the channel may be what prevents them from passing.

Anion channels

The main role of anion channels is to selectively pass Cl^- ions while excluding all else [46]. One of the most well studied families of anion channels are the ClC chloride channels, ubiquitous amongst cellular organisms but first cloned from Torpedo [46, 47–50]. We also focus our attention on the ClC channels, as we now have some detailed structural information to aid our understanding. Until recently all the ClC proteins studied were believed to be anion-selective pores [48] permeable to a range of small inorganic anions [51]. However, recently it was determined that a ClC protein from bacteria actually acted as a Cl^--H^+ exchange transporter [52]. Following this, two mammalian ClC proteins (ClC-4 and ClC-5) have also been suggested to be transporters [53, 54], although many of the more familiar ClC proteins (e.g., ClC-0 and ClC-1) are still believed to act as passive channels rather than transporters.

Single channel and mutagenesis studies on ClC channels have been limited, most likely due to the very small currents they pass and the technical difficulties this implies. Most information about the origin of ion selectivity, therefore, has come since atomic structures of two bacterial ClC proteins (now believed to be transporters) were determined from X-ray crystallography [55, 56]. These studies show the protein to be a dimer, with two ion conductive pathways whose entrances are noted by the arrows in figure 3A. The structure also revealed three ion binding sites midway through the membrane surrounded by a number of residues that are highly conserved among the ClC family. In the central position, the Cl^- ion can form hydrogen bonds with two backbone amide groups and two side chain hydroxyl groups that help stabilize it in the center of the protein, as illustrated in figure 3B. It was noted that the Cl^- ion did not interact directly with

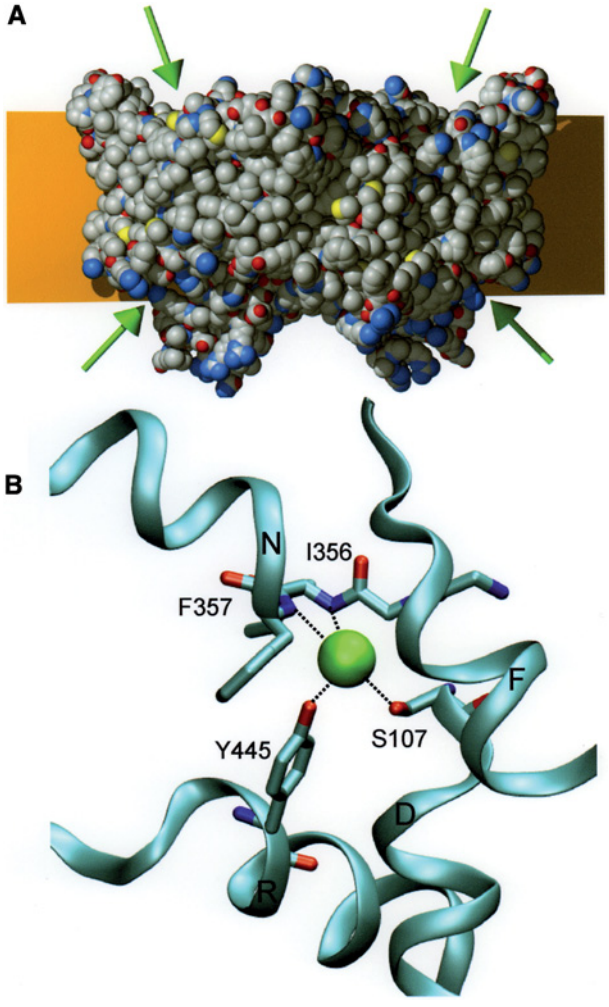

Figure 3. The structure of ClC proteins. The protein forms a dimer (*A*) with the entrances to the transmembrane ion-conducting pathway noted by the arrows. (*B*) In the central binding site, Cl^- is stabilized by interactions with backbone amides and polar side chains.

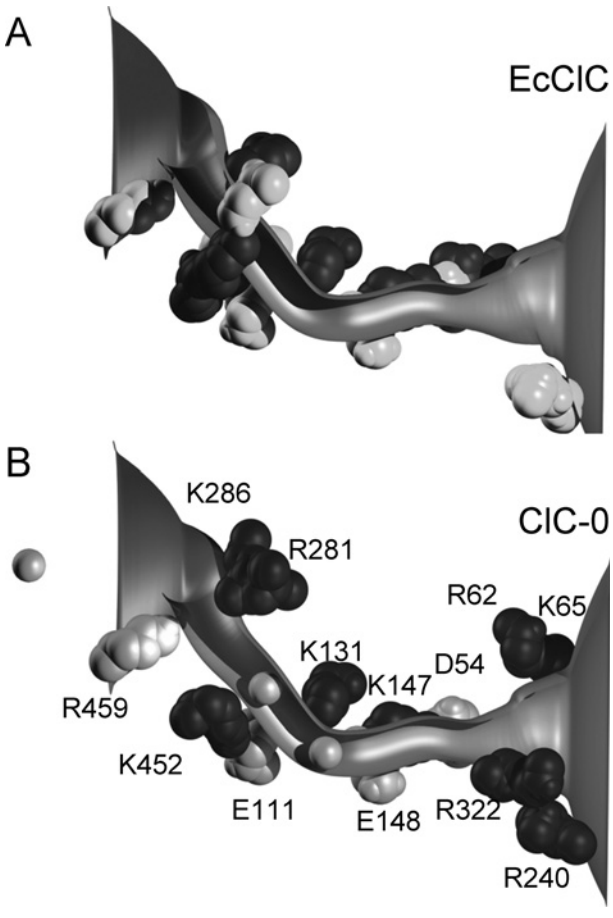

Figure 4. Charged residues surrounding the ClC pore. The acidic (light) and basic (dark) residues surrounding a hypothesized ion-conducting pathway (surface) are illustrated for a ClC protein from *E. coli* (*A*) and for the homology model of the protein ClC-0 from torpedo (*B*). There are considerably more basic residues near the pore in ClC-0 than in the bacterial protein. Adapted from [59].

any positively charged lysine or arginine residues and suggested that such a direct interaction may bind the ion too tightly to allow for rapid diffusion [55, 56]. The electrostatic attraction for anions was posited to lie in the weaker interactions, with the partial charges surrounding the ion binding sites and two macroscopic helix dipoles that focus on the center of the channel.

A number of computational studies soon followed the structural information and shed further light on the basis of anion selectivity in this family of channels. Miloshevsky and Jordan [57] determined the potential energy profile along a likely ion trajectory through the protein. As well as the hydrogen bonds surrounding the central ion binding site, this study highlighted the role of a number of other charged groups (e.g., E111, R147, E148) in electrostatically blocking or attracting anions into the protein, the importance of some of which had already been determined from mutagenesis [58]. Corry et al. [59, 60] also determined electrostatic energy profiles, but this time on homology models of the ClC-0 and ClC-1 channels in a hypothesized open state. They noted that although the bacterial protein did not contain excess positive charge along the probable ion conducting pathway, both the ClC-0 and ClC-1 homology models contained more lysine and arginine residues near the pore than glutamate and aspartate, as depicted in figure 4. Although the ion need not interact directly with these residues, their presence creates a large energy well that would attract anions and repel cations, and this attraction was greater for ClC-0 and ClC-1 than for the bacterial protein. Indeed the charge on these groups is sufficient to hold multiple ions in the channel and make conduction a multi-ion process.

A molecular dynamics study [61] also highlighted the role of backbone amide groups and polar side chains in attracting anions. A calculated PMF found a number of anion binding sites comparable to those seen in the crys-

tallographic data. In the binding sites interactions with the pore-lining residues were found to be most significant in stabilizing the ion, with the majority of the interaction coming from backbone amide groups rather than polar side chains. The remainder of the protein also contributed an attractive electrostatic background.

Together, the structural and computational studies yield a good indication of the origins of anion selectivity in ClC proteins. The interactions of the ion with the protein backbone, polar side chains, helix dipoles and charged residues all contribute to create an electrostatic attraction for anions into the central region of the protein. In the bacterial proteins the majority of this arises from interactions with the backbone amide groups; however, in the ClC-0 and ClC-1 proteins the attraction of charged residues may dominate. Until the mechanism of proton transport in the bacterial protons is determined, some care should be taken in inferring specific details of ClC channel function from transporter structures. But one can be reasonably confident in positing anion specificity to the specific charge distribution within the protein.

Calcium channels

The family of voltage-gated calcium channels provide an important example of divalent cation selectivity. Their role is to allow Ca^{2+} into the interior of a cell when the membrane depolarizes, creating a chemical signal that can be used to initiate responses in the cell, including muscle contraction and the secretion of neurotransmitters [62]. A central problem in understanding the function of these channels is to determine how they can be both highly selective while still passing millions of ions per second. Voltage-gated calcium channels are extremely discriminating, selecting calcium over sodium at a ratio of over 1000:1 [63]; yet the picoampere currents they carry require over 1 million ions to pass every second [64]. The degree of specificity is important for these channels, as Na^+ ions are more than 100-fold more numerous than Ca^{2+}. Furthermore, Na^+ and Ca^{2+} have very similar sizes, and much larger ions are known to permeate the channel [65]. Thus, these proteins must distinguish between ions using more than just size. As atomic resolution structures are not yet available for any member of the calcium channel family, the discussion of selectivity has revolved around a variety of other data. Here we focus on the L-type voltage-gated calcium channel, as it is the most widely studied, although it is hoped that many of the properties of this channel will apply to a variety of proteins in this family.

Monovalent ions conduct through the channel at much higher rates than any divalent ions [63, 66–69], but are blocked when the calcium concentration reaches only 1 μM [66, 70]. Indeed, the ability of some ion species to block currents carried by others gave the first clues to the origin of ion selectivity in these channels. In addition to Ca^{2+} blocking small metal monovalent cations, Sr^{2+} was also blocked by Ca^{2+} [71], while Cd^{2+}, Co^{2+} and La^{3+} block currents carried by Ca^{2+} even though all of these species were known to pass through the channel at some rate [72–74]. Together these data suggested a high affinity binding site, with an order of affinity estimated from bi-ionic reversal potentials to be $Ca^{2+} > Ba^{2+} > Li^+ > Na^+ > K^+ > Cs^+$ [63, 75–77]. Single channel conductance measurements, on the other hand, have the exact reverse order, with the conductance values given by $Ca^{2+} < Ba^{2+} < Li^+ < Na^+ < K^+ < Cs^+$ [63, 69, 78]. These properties were first explained with the so-called 'sticky-pore' hypothesis, in which ions that are bound with higher affinity pass through the channel more slowly and so have a lower conductance [79].

The sticky-pore model also suggested that ion conduction must be a multi-ion process, as otherwise there should not be a difference in conduction rates between ion types [79, 62]. Other strands of evidence also suggested a multi-ion pore. First, in many cases when two permeating species are mixed, the current is lower than for either species on its own [67, 80]. This so-called anomalous mole fraction effect is most easily explained supposing the pore contains either multiple binding sites or a single site holding more than one ion. Multiple ions were also suggested by the fact that calcium block of Na^+ currents is dependent on membrane voltage [68, 73, 81] and on the direction of ion movement [69, 78].

A multi-ion pore provides a simple explanation of the permeation and selectivity properties of the channel as depicted schematically in figure 5A and B. As soon as one Ca^{2+} ion occupies the channel, it binds tightly to the pore and thereby blocks the passage of Na^+. However, if

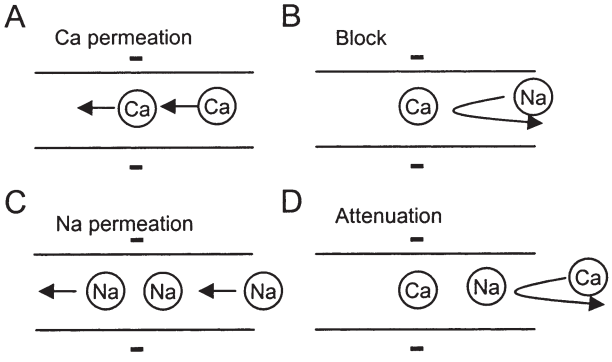

Figure 5. 'Electrostatic' model of permeation and selectivity in calcium channels. (*A*) When one Ca^{2+} ion is electrostatically bound to the negative charge of the EEEE locus, it can only be removed with the aid of Coulomb repulsion from another Ca^{2+} ion. (*B*) The lesser repulsion from Na^+ is unable to displace a resident Ca^{2+} ion, thus Ca^{2+} blocks Na^+ currents. (*C*) Na^+ is able to pass through the channel in the absence of Ca^{2+} in a 'knock-on' mechanism involving three ions. (*D*) Na^+ can attenuate Ca^{2+} currents by slowing the entry of the second Ca^{2+} ion required for conduction.

the second site in the pore is occupied by a calcium, then the strong electrostatic repulsion between the two ions allows one to overcome its interaction with the protein and exit the channel at a relatively fast rate [67, 80].

Using site-directed mutagenesis it was determined that four glutamate residues on the P-loop of the channel (often called the EEEE locus) were responsible for the high-affinity calcium binding site, as a mutation of one or more of these had a significant effect on channel selectivity [82–86]. Removing these glutamate residues was found to remove the specificity of the channel for Ca^{2+} [84], and no evidence of other high-affinity binding sites was apparent.

The determination of the atomic structure of a bacterial potassium channel by X-ray crystallography [87] also aided the understanding of calcium channel structures. The organization of the membrane spanning domains of voltage-gated calcium, potassium and sodium channels are all quite similar, and the discovery that the P-loop formed the selectivity filter of potassium channels gave credence to the view that this region would also be important in calcium channels.

An important difference is evident, however, in the structures of the potassium and calcium channel selectivity filters. Whereas ions are stabilized by the backbone carbonyl groups in voltage-gated potassium channels, most researchers believe that Ca^{2+} ions interact with the side chains of the glutamate residues in calcium channels. This belief is supported by a number of tests made to determine whether the Ca^{2+} bound to the carboxylate side chains of the glutamate residues, or whether binding was to the backbone carbonyl groups. Protons were found to be able to block Ca^{2+} currents [88], and point mutations within the EEEE locus suggested that multiple glutamates interacted with a single proton [89–91], suggesting both that these residues were in close proximity and that their charged side chains were accessible to the interior of the pore. Also, when individual residues in the EEEE locus were replaced with cysteine, the side chains of the cysteine residues appeared to be accessible to the solvent, as they could become ionized to react with methanethiosulfonate [92, 93]. The bulky methanethiosulfonate groups also blocked the pore upon reaction, supporting the conclusion that the EEEE locus lay inside the pore.

As the understanding of calcium channel permeation and selectivity was progressing through innovative experimental techniques, a number of theoretical models were also proposed to help elucidate the mechanisms underlying these properties. For a long time, especially when detailed structural information was lacking, the main approach was from rate theory. In this approach, the channel is represented as a series of energy wells and barriers, with each well representing an ion binding site and its depth being proportional to the binding affinity. Ion conduction is described as the hopping of ions between the wells, with the rate of flux over each barrier related to the exponential of its height. When the multi-ion nature of permeation was determined, the first models depicted this with two separate energy wells representing two binding sites, surrounded by two larger barriers. A single ion in the pore could move between these sites, but not over the barrier to exit the channel. When a second ion entered and occupied the second site, however, it was proposed that the Coulomb repulsion between them alters the energy landscape and reduces the barrier for dissociation [67, 80].

When it was discovered that there was only one binding region in the channel created by the EEEE locus, the idea of two similar binding sites had to be modified. A new model emerged in which there was only a single high-affinity binding site, but this was flanked by low-affinity sites that helped ions to step out of the large well [69, 94] and the role of ion-ion interaction was relegated.

The rate theory models are surprisingly simple, yet they capture a number of the salient features of permeation and selectivity. However, these models generally suffer an important flaw when determining the origins of specificity: the energy landscape is usually derived from measurements of binding affinities and are not directly related to the structure of the pore; a fact that has resulted in much discussion of the applicability of these models [62, 95, 96, 97]. Furthermore, the influence of multiple ions on the energies is hard to determine in this approach.

More recently four different approaches have been taken to model the permeation and selectivity of calcium channels by incorporating structural information: continuum theories, Brownian dynamics theories, Monte-Carlo and molecular dynamics.

In the first of these Nonner and Eisenberg [98] modeled the channel as a simple cylinder with conical vestibules and included the charge of the EEEE locus in the channel walls. By adjusting the diffusion coefficients and an *ad hoc* excess chemical potential parameter that helps ions to bind in the pore, they are able to use the drift-diffusion equations (or Poisson-Nernst-Planck theory as it is often known) to reproduce the conductance and selectivity of the channel. They find that Ca^{2+} ions bind to a broad region in the interior of the pore due to the Coulomb potential of the glutamate residues as well as the excess chemical potential and that the multi-ion repulsion may be important in speeding flux. However, questions raised about the validity of the continuum description of ions in narrow pores [99–101], the difficulty in treating ion-ion interactions in this model and the arbitrary nature of the adjustable parameters limit how much can be read into these conclusions.

Two later studies using different calculation schemes (Monte-Carlo simulations and a Mean Spherical Approximation method) presented a different explanation of the origins of ion selectivity [102, 103]. In these models eight

partially charged oxygens comprizing the glutamate side chains protrude into a cylindrical pore and can freely diffuse within a limited region of the channel. Cation binding is achieved due to the electrostatic attraction of the negatively charged oxygens. Selectivity of Ca^{2+} ions is proposed to be a result of ions competing to achieve charge neutrality in a selectivity filter having finite space. Ca^{2+} ions are preferred to Na^{+} in the model as they have the same charge-neutralizing effect as two Na^{+} ions while occupying less of the limited volume of the filter. Essentially, not enough Na^{+} ions can be squeezed in to the filter to achieve charge neutrality.

Another approach has been to actually simulate the trajectories of ions as they permeate the channel and observe how permeating ions interact with the protein and each other. This direction has been followed by Corry et al. [104–106] who carried out Brownian dynamics simulations in which the ions pass through the channel in a random walk subject to electrical forces. In these studies a rigid model of the channel is derived from analogy to other channels and a variety of experimental data. The most important aspect of the channel shape, however, is that it includes a relatively narrow region in which ions cannot pass each other, surrounded by the four glutamate residues as shown in figure 6A. Unlike the previous models, the glutamate charges do not compete for space within the pore, but the concentration of negative charge does attract cations within this region. Indeed, the electrostatic attraction of the protein is all that is required to account for ion permeation and selectivity in this model. The charge of the glutamate residues creates a deep energy well that strongly attracts multiple ions. In a process akin to that suggested by earlier rate models, repulsion between two resident Ca^{2+} ions is found to speed their exit, as illustrated in fig. 5A. Because the divalent Ca^{2+} ions are more strongly attracted by the channel, they can displace Na^{+} to occupy this region. Once there, the Ca^{2+} can only be moved by the repulsion from another divalent ion, and not by the lesser repulsion from Na^{+} (fig. 5B). As well as providing a simple mechanism for ion selectivity, depicted schematically in figure 5, this model was also used to calculate the conductance of the channel in a variety of situations and replicate and explain the observed I-V curves, current concentration curves, anomalous mole fraction effect (fig. 6B) and attenuation of Ca^{2+} currents by Na^{+}.

All the models described thus far assume simplified pore geometries, but one goal of ion channel modeling is to include all the atomistic detail of the pore in molecular simulations. Lipkind and Fozzard [107] created an atomic resolution homology model of a calcium channel and determined the forces on Ca^{2+} ions within it. They found that the glutamate residues form an electrostatic trap that firmly holds a single calcium ion, and that it can only be moved by the entry of two further calcium ions. Barreiro

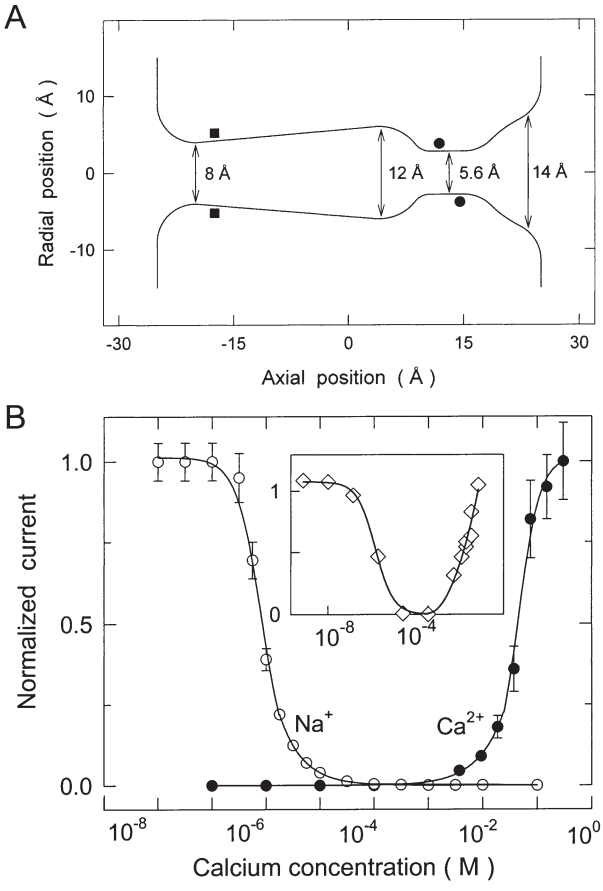

Figure 6. Brownian dynamics simulations of calcium channels. (*A*) The channel shape contains a narrow portion of the pore surrounded by the charge from the four glutamate residues (circles). (*B*) Results from Brownian dynamics simulations in mixed Na^{+} and Ca^{2+} solution reproduce the 'anomalous mole fraction' behaviour seen in the experimental data (inset [70]). Figures from [105].

et al. [108] carry out molecular dynamics simulations on a homology model pore containing either two Ca^{2+} or one Ca^{2+} and one Na^{+}. In a 100 ps simulation they find that the two Ca^{2+} move apart but the Ca^{2+} and Na^{+} do not. It is hypothesized that Na^{+} cannot displace Ca^{2+} but that another Ca^{2+} can, similar to that seen in the Brownian dynamics model, although the simulations are too short to draw serious conclusions.

The charge-space competition model was analysed further by Yang et al. [109] who conducted non-equilibrium molecular dynamics simulations of a model pore containing half-charged oxygens in an atomistic cylinder. In these simulations a second Ca^{2+} ion is required to release a Ca^{2+} already bound in the channel, but cation binding was found to be non-selective. Ramakrishnan et al. [110] also carry out non-equilibrium molecular dynamics simulations of a model pore containing glutamate residues in a β-barrel scaffold. Apparent off rates when the pore was preloaded with three Na^{+} ions are much higher than when the channel contained one Ca^{2+} and two Na^{+}, consistent

with Ca^{2+} blockage. As in the other models, the presence of multiple Ca^{2+} was able to release this block.

Together these models present two similar but distinct physical mechanisms underlying ion selectivity. In both, the electrostatic attraction of the negatively charged glutamate residues is essential for attracting multiple ions into the channel, and the size of the pore is such that ions cannot pass each other. The difference lies in whether this is of itself enough to create selectivity or whether a specific volume and flexibility of the pore is also necessary. In the simpler 'electrostatic' model, Ca^{2+} is bound more strongly by the channel due to its greater charge and can only be removed by the repulsion of a similarly charged ion, as represented in figure 5. In the 'charge-volume' model, the volume of the pore must be such that competition for space as well as electrostatic attraction is important. Given the limited structural information available, it is difficult to say exactly which, if either, of these mechanisms is correct. An advantage of the simpler model is that less is required, and it does not set limits on the size nor flexibility of the pore, only on the charge surrounding it. Also, it stresses a common theme that has arisen out of rate theory, continuum and simulation models, some of which are able to elegantly reproduce and explain a wealth of physiological data for a variety of different channel types. Thus, one wonders if the extra complication of setting limits on the channel volume and flexibility are necessary.

Potassium channels

Potassium channels selectivity transport K^+ across the membrane to hyperpolarize cells and are responsible for setting membrane potentials and controlling the duration of action potentials amongst many other functions [111, 112]. There are a vast variety of potassium channels, with measured single channel conductances ranging from 2 to 240 pS [112]. Potassium channels pass some monovalent cations (but notably do not pass Na^+), are blocked by divalent cations while anions are not believed to enter the pore.

Our understanding of this family of channels has taken a great leap forward since 1998, as the structures of a variety of potassium channels have been determined using X-ray crystallography. The first structure determined was of a pH-dependent K^+ channel from bacteria [87]. Since then additional structures of voltage-gated and calcium-gated potassium channels from bacteria [113–115], and more recently a mammalian voltage-gated potassium channel, Kv1.2 [116], have been determined. All these channels have a very similar architecture. They comprise four identical subunits arranged symmetrically about the ion-conducting pore. Each subunit itself consists of six transmembrane domains, the last two of which (S5 and S6) line the pore in conjunction with the 'P-loop' that joins the two as illustrated in figure 7.

The structures each contain three distinct regions: a narrow region formed by the P-loops believed to be selectivity filter, a larger central water-filled cavity and a hydrophobic region of the pore on the intracellular side. The selectivity filter is around 12 Å long, only just wide enough to fit a potassium ion and lined by a series of carbonyl oxygens of a highly conserved TVGYG amino acid sequence as noted in figure 7B. High-resolution images of the KcsA channel indicate the locations of ion binding in the pore [117, 118]. As indicated in figure 7B, each binding position is surrounded by eight carbonyl oxygens. Furthermore, from the relative electron density of the sites it was determined that each site was occupied by K^+ about one-half of the time-supportive of the belief that at any one time, only two of the four binding sites are occupied and that the ions are separated by a water molecule

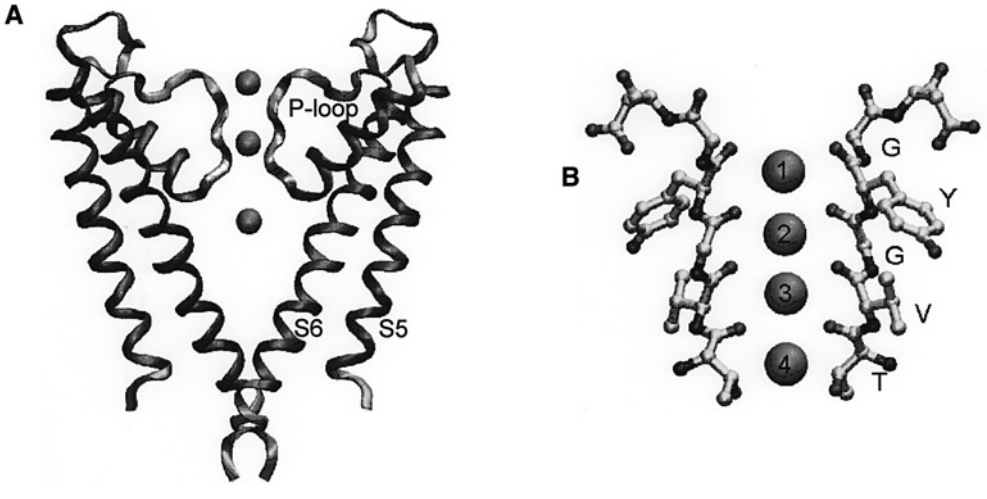

Figure 7. The structure of voltage-gated potassium channels. (*A*) the pore is formed by the S5 and S6 helices as well as the joining 'P loop'. (*B*) The four ion binding sites in the selectivity filter are each surrounded by eight carbonyl oxygens. Only two of the four subunits are shown.

in the intermediate site [119]. Thus two ions permanently occupy either the sites 1 and 3 or 2 and 4 with a water molecule sandwiched between them, and ions can probably oscillate between these two configurations without a substantial free-energy barrier.

The crystallographic studies also suggested the mechanism of K^+ permeation: a 'knock-on' mechanism that had been suggested previously. Additional ions coordinated by eight water molecules were located in X-ray data at the extracellular mouth of the channels and in the central cavity [118]. When one of these ions enters either end of the filter, the equilibrium of the two resident ions is disrupted, and the column moves along until one of the ions is ejected and the new ion takes up residence in the filter.

Since the first atomic resolution potassium channel structures were published, there have been many theoretical studies of ion permeation and selectivity. Many groups have conducted MD studies, varying most notably with respect to their treatment of the lipid bilayer environment due to the expense of simulating the membrane in its entirety. Bernèche and Roux embedded the protein in a DPPC bilayer [120, 121], Shrivastava and Sansom in a POPC bilayer [122], while others have used octanes [123, 124], non-polar atoms [125–127] or harmonic constraints [128–131] instead of a lipid bilayer. All these studies agree with the knock-on conduction mechanism described previously and have helped to determine the energy landscape experienced by the multiple ions in the channel. Such studies have also highlighted the favourable electrostatic interaction between the partial charges in the protein and the permeating ion. As illustrated in figure 8, a deep potential energy well is created for potassium ions by the carbonyl oxygens in the selectivity filter as well as by the helix dipoles. Brownian dynamics simulations of conduction through the channel have also been carried out in both simplified [132–135] and atomic detail [136] channel shapes. These studies also found that three ions were required for conduction and the conductance properties of the channel were be determined theoretically for comparison with experimental data. The variety of potassium channel conductances was also suggested to lie in changes in the radius of the intracellular pore region- as this altered the rate-limiting energy barrier to ion conduction [134, 136].

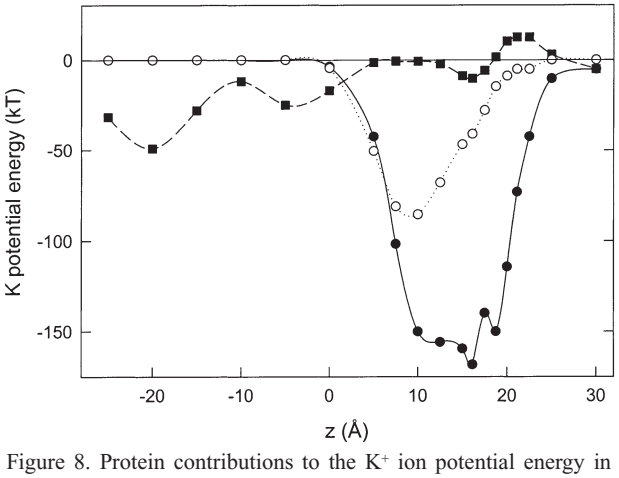

Figure 8. Protein contributions to the K^+ ion potential energy in potassium channels. Contributions from the selectivity filter (filled circles), pore helix (open circles) and inner helix (squares) are shown. Figure from [129].

Discussions of selectivity in the theoretical studies of the potassium channel have generally focused on the discrimination between K^+ and Na^+ ions, as this has been the most difficult aspect to explain. The rejection of anions can be explained quite simply using the structural information. The carbonyl oxygens that line the selectivity filter create a negative electrostatic potential that attracts cations and repels anions. Using electrostatic calculations, it has been found that an energy well of about 67 kT is presented to potassium ions, while a barrier of similar magnitude is presented to monovalent anions [136]. MD simulations also find a deep cation attractive potential in the selectivity filter that results in permanent occupation by K^+ ions (fig. 8).

The behaviour with divalent ions is more complex. Ba^{2+} is known to be able to enter the channel from either side, although it does so more quickly from the internal side [137–143]. Once there, Ba^{2+} blocks all current through the channel. The specific inhibitory properties of Ba^{2+} have been well studied [137–142], and it has been found that the mean blocking time is dependent on the surrounding K^+ concentration. Multiple binding sites for K^+ and Ba^{2+} in the channel were posited, including internal and external K^+ 'lock-in' sites that slowed Ba^{2+} exit. The specific location of Ba^{2+} binding in the pore was determined using X-ray crystallography [144], which showed that Ba^{2+} bound at the internal end of the selectivity filter and that there were K^+ sites both internal and external to this. Thus, it is apparent that Ba^{2+} binds in the channel due primarily to the attraction and coordination of the carbonyl oxygens that line to pore. But a question remains as to how Ba^{2+} blocks K^+ currents so effectively and why it cannot be expelled by either a K^+ ion or another Ba^+ as would be the case in the calcium channel.

One answer to these questions has been provided by a Brownian dynamics study of monovalent and divalent cations in the channel [106]. It was found that Ba^{2+} binds more strongly in the channel due to the stronger electrostatic interaction created by its larger charge. Indeed, the binding of Ba^{2+} in the potassium channel was found to be much stronger than that seen for Ca^{2+} in the calcium channels. Because of this, the repulsion created by either a nearby K^+ or Ba^{2+} was not found to be enough to move it from the channel. The reason that divalent ions block the potassium channel rather than conducting, as in the calcium channel, is simply because they are held in a stronger electrostatic trap. This study was also able to re-

produce the approximate locations of the Ba^{2+} and K^+ binding sites seen in the X-ray data.

It is worth making a comparison between potassium and sodium channels, both of which are selective for a subset of monovalent cations. Divalent ions are known to block sodium channels, but with a much lower affinity and duration than in potassium channels [145, 146]. Unlike the potassium channel, it is believed that the side chains of the P-loop residues line the pore rather than the backbone carbonyl oxygens, a situation more analogous to the hypothesized structure of calcium channels. In particular, two rings of charged residues, EEDD and DEKA, are believed to line the pore, as mutations of these residues have a large effect on the conductance and selectivity properties of the channel [147]. Indeed, appropriate mutations of these residues can confer divalent ion selectivity upon the channel [148]. Electrostatic calculations on a model sodium channel [106, 149] find that divalent binding is much weaker than in either the calcium or potassium channels. In this case other ions are not attracted into the channel to help push the divalent ion through and create a divalent ion current. But also being less strongly bound, the divalent ion dwell time is much shorter than for potassium channels, and so divalent block is less effective.

Conclusions

Many ion channels discriminate between different ion types, allowing some to pass while blocking others. To do this, ion channels must utilize the physical properties of the ions, finding something that is different between the desired ion type and the remainder. When discriminating among ions of different valence, the obvious difference is their charge, and not surprisingly, most studies highlight the electrostatic interaction between ions and the protein when explaining this aspect of selectivity. The recent availability of X-ray structures has meant that the protein atoms responsible for this electrostatic interaction can be pinpointed.

The simplest aspect of valence selectivity is the discrimination between anions and cations. The cation channels described here all appear to contain a net negative charge in the pore-lining region of the protein. In calcium channels this is due to the charged side chains of the four glutamate residues comprizing the EEEE locus. In both the potassium and gramicidin channels, on the other hand, it is the partially charged carbonyl oxygens that are used to attract cations and repel anions. Similarly, in the ClC chloride channels a combination of favourable interactions with the partial charges on the backbone and side chains of the pore-lining residues, possibly aided by a number of basic residues, attracts anions while repelling cations.

The discrimination between monovalent and divalent cations cannot just rely on presence of a net negative charge, as this acts to attract both kinds of ion. Rather, a number of studies suggest that it relies on the exact strength of the attraction to the permeating ions [104–108], but there are still some differing opinions on the exact mechanisms taking place. A divalent ion has a stronger electrostatic interaction with any charge on the protein than a monovalent ion due to its larger charge. This fact on its own appears to be enough to explain how potassium channels are blocked by Ba^{2+}. When it enters the selectivity filter it is electrostatically bound such that it is unlikely to leave, even with the aid of repulsion from nearby cations. Electrostatic calculations in model calcium channels suggest that the binding of divalent ions is not as strong as in the potassium channel [104–106]. One simple explanation for valence selectivity in this channel therefore is that once a divalent ion enters, it can only be forced out with the aid of the Coulomb repulsion from a second divalent ion, but not with the weaker repulsion from a monovalent ion [106]. Thus, once it enters only divalent ions will conduct. Another explanation involves competition for limited space within the channel [102, 103]. One hopes that when detailed structural information about these channels becomes available, we will be able to shed further light on the basis of divalent ion selectivity in calcium channels.

Despite its small size, gramicidin continues to be a difficult target for theoretical studies, as most computational models designed for bulk systems tend to run into problems in the narrow confines of this channel [34, 39]. For this reason the exact basis of divalent block in this channel remains slightly elusive, but experimental studies make it apparent that the divalent ions must be electrostatically bound near the mouth of the channel.

The recent high-resolution structural data from NMR and X-ray crystallography have proved invaluable for elucidating the origins of valence selectivity. Furthermore, many new techniques, such as resonance energy transfer, are also beginning to provide important structural information [150–152]. Although it was always suspected that the specific electrostatic interactions between ions and the protein were important for this discrimination, the atoms involved can now be pinpointed. Thus, as more structural information is generated, we can only expect our understanding of the mechanisms of valence selectivity in biological ion channels to be further enhanced.

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