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ORIGINAL PAPER

Sieving experiments and pore diameter: it's not a simple relationship

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Abstract The classic sieving experiment for estimating an ion channel's diameter with successively larger ions is re-examined. Using a very reduced model of a calcium channel, it is shown that sieving experiments measure a combination of three mechanisms: the cross-sectional area available to the sieving ions (the classic interpretation), the exclusion of the sieving ions from a pore crowded with amino acid side chains that protrude into the permeation pathway, and competitive selectivity of the sieving ions with other ions in the bath (even if those are present only at trace concentrations). The latter two can be called sieving-by-crowding because they stem from the excluded volume of the amino acids in the permeation pathway. The model shows that—to a first-order approximation—sieving experiments measure the available volume inside a selectivity filter, rather than the available cross-sectional area. The two are only the same if the narrow part of the pore does not have flexible amino acid side chains interacting directly with the permeant ions; this may be true of potassium channels, but not calcium, sodium, and other channels with "crowded" selectivity filters.

Keywords Ion channel · Sieving experiment · Calcium channel · Modeling

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Introduction

Ion channels facilitate vital cellular functions such as initiating and propagating neuronal action potentials and initiating muscle contraction (Hille 2001). This conduction of ions is passive (i.e., it does not require ATP) down the ions' electrochemical potential gradients. But channels are not simply holes in the cell membrane that allow anything and everything to flow through. Many channels are highly selective, statistically allowing only one particular kind of physiological ion through.

The diversity in ion selectivity (e.g., potassium, sodium, and calcium channels) necessarily requires a diversity in channel structure, particularly in the selectivity filter. For example, the selectivity filter of potassium channels is lined with backbone carbonyl oxygens because the side chains' terminal groups face away from the permeation pathway (Doyle et al. 1998). On the other hand, the selectivity filter of calcium channels has the terminal groups of their aspartates and glutamates in the permeation pathway, directly interacting with the permeating ions (Koch et al. 2000; Wu et al. 2000).

These two channel types are then two fundamentally different "holes in the wall." The selectivity filter of K^+-selective pores (both potassium channels and valinomycin) has a relatively empty permeation pathway lined with carbonyl oxygens, while that of calcium channels has a permeation pathway filled with flexible side chains. In this paper we argue that this fundamental difference between "empty" and "crowded" pores makes the interpretation of sieving experiments, at least in calcium channels, difficult. The distinction between empty and crowded is, of course, mostly conceptual since potassium channels shrink in size in the absence of K^+ [although valinomycin does not (Varma et al. 2008)].

Sieving experiments attempt to estimate the diameter of the narrowest part of the channel to be the diameter of the largest particle to pass through the channel. There is a natural inclination to assume that the pore diameter is static [e.g., Dwyer et al. (1980) and its 12+ citations over the past decade]. Sieving experiments on calcium channels (the focus of this article) have used this idea to define a lower bound for the "minimum pore radius" (Tinker and Williams 1993) at the "narrowest point" of the pore (McCleskey and Almers 1985). However, pores are proteins and therefore probably flexible enough to adapt their size to the permeating ions. Sieving experiments would then give a lower bound for the dynamic diameter.

In this paper we examine the possibility that even the concept of the dynamic radius may not be appropriate for all channels. Specifically, we show that large sieving ions can be excluded from a model calcium channel selectivity filter *with no confinement at all* (radial or otherwise) due to crowding from the amino acids in the permeation pathway. There is no pore diameter in this model, but the results show a maximum ion radius (which depends on the concentration of the amino acids), similar to those of sieving experiments. In this model, the only steric constraint on the sieving ions is the space available between the amino acids, suggesting that this is measured in sieving experiments of crowded pores.

We demonstrate this with a "toy" model of a calcium channel selectivity filter where the content of the selectivity filter is a bulk (homogeneous and infinite in all directions) fluid so that there are no geometric constraints of pore size. Instead, the model selectivity filter contains the amino acid side chains that interact with the ions in the permeation pathway. Defining the concentration of these charged amino acids is the only way that the geometry of the pore contributes in the model we use. While this model of a selectivity filter is obviously oversimplified, it does capture the essential selectivity properties of calcium channels (Nonner et al. 2000) because, to a first-order approximation, it is the concentration of amino acids—not the shape of the selectivity filter—that determines the binding selectivity properties of such a pore (Malasics et al. 2009).

This model is useful for reconsidering sieving experiments because it strips away any radial constraints on the sieving ions. Instead, this model reduces the problem to partitioning of the sieving and other ions into the selectivity filter. This kind of competition between ions has been studied in the L-type calcium channel (with high Ca^{2+} affinity), the ryanodine receptor (RyR) calcium channel (with relatively low Ca^{2+} affinity), and other calcium-selective pores (Boda et al. 2000, 2001, 2002b, 2006, 2007b, 2008, 2009; Gillespie 2008; Gillespie and Boda 2008; Gillespie et al. 2005, 2008, 2009; Gillespie and Fill 2008; Malasics et al. 2009; Miedema et al. 2004, 2006; Nonner et al. 2000, 2001; Nonner and Eisenberg 1998; Rodriguez-Contreras et al. 2002). These studies have shown that competition between ion species for the selectivity filter of a calcium channel is determined (to first order) by a balance of cations entering the filter to screen the negative amino acids and a lack of space in the crowded filter. The ions that bind in the selectivity filter because of this charge/space competition are those that screen the amino acids while occupying the least volume (i.e., small, divalent cations such as Ca^{2+}). This selectivity mechanism has also been proposed for sodium channels (Boda et al. 2002a, 2007a).

Here, we find that this balance of electrostatics and entropy (i.e., ion size) determines how large a sieving ion can fit into the selectivity filter. Our results show that the concentration of amino acids within the selectivity filter plays a critical role in determining the size of the ions that can fit between the amino acid side chains (and therefore that are conducted). The larger the sieving ion, the more free energy it takes to fit it between the amino acid side chains. Even though the pore is highly charged to attract cations, it becomes energetically favorable to exclude large cations. Instead, other, smaller cations (e.g., Ca^{2+}) enter the pore—even when those are only present in trace (micromolar or less) concentrations.

This ion size dependence on amino acid concentration is oversimplified in our model, but the general conclusions remain valid: the same charge/space competition that defines the other selectivity properties of calcium channels also defines the rejection of large ions because they cannot fit into the selectivity filter crowded with amino acid side chains. Therefore this "sieving-by-crowding" mechanism approximately measures the volume (not area) available in the smallest part of the pore. In real calcium and sodium channels, the situation will be more complicated. There, sieving measurements are probably a combination of the traditional radial size limitations of the pore and sieving-by-crowding.

Theory and methods

Model of the selectivity filter

The model of the selectivity filter we use was introduced by Nonner et al. (2000). It simplifies an ion channel in equilibrium to a system of two macroscopic fluids in equilibrium and separated by a semi-permeable membrane. One fluid represents the bath far away from the membrane and the other represents the electrolyte inside the selectivity filter. Here, the dielectric constants of the two fluids are taken to be the same (78.4) because we are not attempting to model a real channel, merely to demonstrate principles. This assumption ignores the effect of the dehydration of

ions as they enter the filter, but this does not affect any of the general conclusions of this paper.

The filter is defined by a fluid of ions that model the amino acids of the channel protein, producing its selectivity properties. In calcium channels, the selectivity properties are conveyed by a combination of glutamates and aspartates, both in highly selective surface membrane channels (e.g., L- and T-type calcium channels) and intracellular channels (e.g., RyR and inositol trisphosphate receptors). Each of the glutamates and aspartates has a terminal COO^- group that Nonner et al. and we model as two half-charged, independent oxygen atoms ($O^{1/2-}$) that cannot leave the selectivity filter. Although a very reduced model of amino acids, this model has successfully modeled calcium selectivity in the pores described in the "Introduction" section. It has also reproduced and predicted experimental data in many cases (Boda et al. 2009; Gillespie 2008; Gillespie and Boda 2008; Gillespie and Fill 2008; Gillespie et al. 2009, 2005; Miedema et al. 2004, 2006; Nonner et al. 2000; Rodriguez-Contreras et al. 2002). Since we are interested in qualitative results, we ignore the effects of the exact mobility and tethering of the carboxyl oxygens, which are likely to affect selectivity.

The channel geometry is defined by the concentration of oxygens ρ_{Ox}. This concentration makes no distinction between n oxygens in a selectivity filter volume v or αn oxygens in a proportionately larger volume αv. Because the fluid representing the selectivity filter is infinite in all directions, the only steric constraint for sieving and other ions is the space between the oxygens.

As described below, ρ_{Ox} defines the selectivity properties of the model channel. On the face of it, it may seem that the shape of the selectivity filter should have first-order effects on the binding selectivity of the filter. However, a recent study has shown that this is, in fact, not the case; the concentration of oxygens within a confining cylinder is the major determinant of how many ions accumulate in that cylinder, as opposed to whether the pore is long and narrow or short and wide (Malasics et al. 2009).

Figure 1 shows that this model produces calcium selectivity. Specifically, in the figure and in the rest of this paper we consider three filters with different levels of calcium selectivity: the weakly calcium selective with $\rho_{Ox} = 10$ M, moderately calcium selective with $\rho_{Ox} = 25$ M, and strongly calcium selective with $\rho_{Ox} = 40$ M. For each, Fig. 1 shows the normalized concentration of Na^+ and Ca^{2+} in the filter when $[Na^+]$ in the bath is 100 mM and $[Ca^{2+}]$ varies.

The affinity of the pore Ca^{2+} can be quantified by the bath $[Ca^{2+}]$ at which these two lines cross. The lower the concentration of the oxygens, the lower the affinity of the filter is for Ca^{2+}. Specifically, the "cross-over" concentration of Ca^{2+} is ~500, 50, and 5 μM for the weakly, moderately, and strongly selective filters, respectively. This Ca^{2+} affinity is greater than similar models of calcium channels that include the pore outside of the selectivity filter and do not strictly enforce charge neutrality (see below). In those models, the same ρ_{Ox} will produce lower Ca^{2+} affinity because ions accumulate in the regions just outside the selectivity filter, leaving some space charge in the filter. For example, in RyR, only ~3 monovalent cations will accumulate in the selectivity filter (which has ~4 negative charges) (Gillespie 2008; Gillespie et al. 2005). This is even more pronounced in narrower channels (Boda et al. 2002b, 2006, 2007b, 2009). However, when the permeation pathway is surrounded by a low-dielectric protein—something that energetically favors (promotes) charge neutrality in the filter—the Ca^{2+} affinity is similar to that calculated here (Boda et al. 2007b). Therefore, the results of the Nonner et al. (2000) model should only be interpreted as qualitatively correct as it generally includes the same principles of the more complex models.

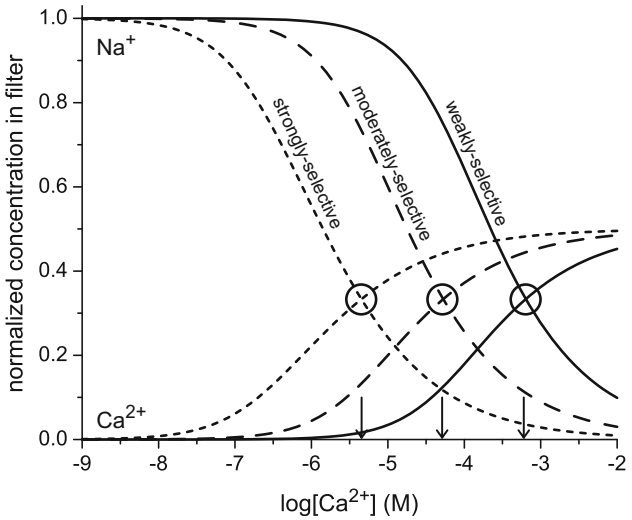

Fig. 1 The reduced model selects Ca^{2+} over Na^+. The filter accumulates Ca^{2+} as $[Ca^{2+}]$ increases and displaces Na^+ for all three filter types: weakly selective with $\rho_{Ox} = 10$ M (*solid lines*), moderately selective with $\rho_{Ox} = 25$ M (*dashed lines*), and strongly selective with $\rho_{Ox} = 40$ M (*dotted lines*). The concentrations are scaled so that the Na^+ concentration in the filter is 1 when $[Ca^{2+}]$ is 0. In the bath, $[Na^+] = 100$ mM. The Ca^{2+} affinity of each filter can be quantified by the $[Ca^{2+}]$ for which the Ca^{2+} and Na^+ concentration lines intersect. The intersection is *circled* and the Ca^{2+} affinity for that filter is indicated with an *arrow*

The mean spherical approximation

The model system is divided into a "bath" and a "filter." The permeant ions (subscript i) are at a given concentration ρ_i^{bath} in the bath and a given concentration of oxygens ρ_{Ox} is in the filter. The output of the model is the concentration ρ_i^{filter} of the permeant ions in the filter. In equilibrium,

$$\rho_i^{\text{filter}} = \rho_i^{\text{bath}} \exp\left(\mu_i^{\text{ex}}\left(\{\rho_k^{\text{bath}}\}\right) - \mu_i^{\text{ex}}\left(\rho_{\text{Ox}}, \{\rho_k^{\text{filter}}\}\right)\right) \quad (1)$$

where μ_i^{ex} is the excess chemical potential of species i. Specifically,

$$\begin{aligned}\mu_i^{\text{ex}} &= \mu_i^{\text{ES}} + \mu_i^{\text{HS}} \\ &= z_i e_0 V + \mu_i^{\text{SC}} + \mu_i^{\text{HS}}\end{aligned} \quad (2)$$

where, for the hard-sphere ions we use, the hard-sphere (HS) term describes the entropic component due to ions not being able to overlap and the electrostatic (ES) terms include a mean electrostatic potential V and a screening (SC) term that describes electrostatic correlations. Below we describe how each term is computed.

For the HS term we use the formulation of Salacuse and Stell (1982):

$$\begin{aligned}\frac{\mu_k^{\text{HS}}}{kT} = &\frac{3\xi_2\sigma_k + 3\xi_1\sigma_k^2}{\Delta} + \frac{9\xi_2^2\sigma_k^2}{2\Delta^2} \\ &+ \xi_0\sigma_k^3\left(1 + \frac{\xi_3}{\Delta} + \frac{3\xi_1\xi_2}{\xi_0\Delta^2} + \frac{3\xi_2^3}{\xi_0\Delta^3}\right) - \ln(\Delta)\end{aligned} \quad (3)$$

where the σ_k is the diameter of ion species k,

$$\xi_n = \frac{\pi}{6}\sum_j \rho_j \sigma_j^n, \quad (4)$$

and

$$\Delta = 1 - \xi_3. \quad (5)$$

For the SC term we use the mean spherical approximation (MSA) of Blum (1975, 1980) with the formulas taken from Nonner et al. (2000) where several typos were corrected:

$$\frac{\mu_k^{\text{SC}}}{kT} = -\frac{e_0^2}{4\pi\varepsilon\varepsilon_0 kT}\left[\frac{z_k^2\Gamma}{1+\Gamma\sigma_k} + \eta\sigma_i\left(\frac{2z_k - \eta\sigma_k^2}{1+\Gamma\sigma_k} + \frac{\eta\sigma_k^2}{3}\right)\right] \quad (6)$$

where the z_k is the valence of ion species k, ε the dielectric constant, ε_0 the permittivity of free space, and kT the thermal energy. The screening parameter (similar to the Debye-Hückel screening parameter) is given implicitly by

$$4\Gamma^2 = \frac{e_0^2}{\varepsilon\varepsilon_0 kT}\sum_k \rho_k\left(\frac{z_k - \eta\sigma_k^2}{1+\Gamma\sigma_k}\right)^2 \quad (7)$$

with

$$\eta = \frac{\pi}{2}\frac{1}{\Omega\Delta}\sum_k \frac{z_k\rho_k\sigma_k}{1+\Gamma\sigma_k} \quad (8)$$

and

$$\Omega = 1 + \frac{\pi}{2\Delta}\sum_k \frac{\rho_k\sigma_k^3}{1+\Gamma\sigma_k}. \quad (9)$$

In the bath, the concentrations of the permeant ions are given and $V = 0$. This completely defines the excess chemical potential (Eq. 2) in the bath. In the filter, however, ρ_{Ox} is given and the concentrations of the permeant ions and V (the Donnan potential, denoted V_D) must be to determined. If there are N permeant ion species, this gives $N + 1$ unknowns, but only N equations (Eq. 1). Another equation is needed. For this we choose charge neutrality of the electrolyte in the filter because it is a homogeneous fluid. Specifically, we require

$$0 = z_{\text{Ox}}\rho_{\text{Ox}} + \sum_k z_k \rho_k^{\text{filter}}. \quad (10)$$

Equations 1 and 10 (in conjunction with Eq. 7) are solved numerically with Newton's method.

Results

The model of the selectivity filter that we use assumes that the filter is charge neutral (Eq. 10) so that the Donnan potential, V_D, in the filter can be determined (Eq. 2). Therefore, if we only consider sieving cations and their associated anions in the bath, then (assuming for simplicity that the pore is perfectly cation selective) the sieving cations will always accumulate in the filter at the same concentration, namely the one that neutralizes the concentration of the oxygens. However, a larger and larger Donnan potential develops and soon becomes unphysically large. This continues until the sieving cations cannot physically fit between the oxygens (discussed below). Therefore, we will include another cation species (Ca^{2+}) against which the sieving cations must compete.

On the face of it, this appears to be different from how sieving experiments are done where only the sieving cation and its associated anion are in the bath [e.g., McCleskey and Almers (1985) and Tinker and Williams (1993)]. Below we will show that even trace concentrations (<1 nM) of Ca^{2+} affect the accumulation of sieving cations in the selectivity filter. Most importantly, when the sieving cations become large enough, they cannot fit between the oxygens and even a trace amount of Ca^{2+} will accumulate in the filter in significant amounts. This produces results that look similar to those of actual sieving experiments—but without any radial geometric constraints of the channel protein (because our channel is homogeneous in all directions). These results are then significant because de-ionized water contains $\sim$1 μM Ca^{2+}, and even with calcium buffers in the system it is possible to have nanomolar Ca^{2+} concentration.

To demonstrate this effect of trace amounts of Ca^{2+}, we consider three kinds of calcium channels: weakly calcium selective with $\rho_{\text{Ox}} = 10$ M, moderately calcium selective with $\rho_{\text{Ox}} = 25$ M, and strongly calcium selective with $\rho_{\text{Ox}} = 40$ M. These concentrations of oxygens correspond

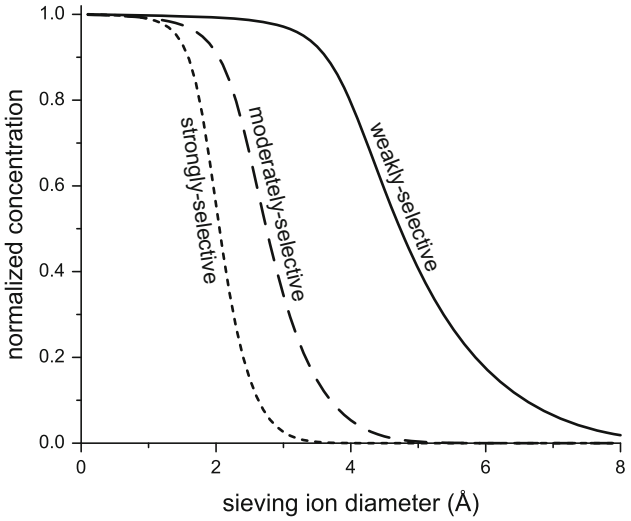

Fig. 2 Normalized concentration in the filter of sieving cations of different diameters in the presence of 1 μM Ca^{2+}. The sieving cations have a concentration of 100 mM in the bath

to a wide range of space-filled fractions, that is the percentage of the available volume taken up by the oxygen atoms:

$$\rho_{Ox}\frac{4\pi}{3}\left(\frac{\sigma_{Ox}}{2}\right)^3 \tag{11}$$

where $\sigma_{Ox}/2$ is the radius of each oxygen. At 10 M the oxygens occupy 6.9% of the available space—a gas-like density—while at 40 M they occupy a liquid-like 27.7%. The 25 M filter has an intermediate value of 17.3%.

We first analyze the competition of 100 mM sieving cations of various sizes with 1 μM Ca^{2+} in these three channels. The results are shown in Fig. 2. For all the channels, when the sieving cations are small enough, the size of these ions does not affect their concentration in the filter. This is what would be expected from the traditional view of sieving. However, at larger sizes, the concentration of the sieving ions goes down and eventually reaches 0. For the weakly, moderately, and strongly selective channels this occurs when the sieving cations have a diameter of ~8, 5, and 4 Å. Moreover, the more selective the pore is for Ca^{2+}, the sharper the transition to sieving particle exclusion.

As the sieving cation size increases, it is Ca^{2+} that is accumulating in the selectivity filter and excluding the sieving ions (data not shown). This kind of competitive selectivity depends on the concentrations of all the ions. In Fig. 3, the effect of [Ca^{2+}] is shown for all three calcium channels types: (a) weakly selective, (b) moderately selective, and (c) strongly selective. In all cases, the higher the Ca^{2+} concentration, the smaller the sieving particle that is totally excluded. Moreover, even nanomolar [Ca^{2+}] affects all the pores to some extent. The arrow indicates the

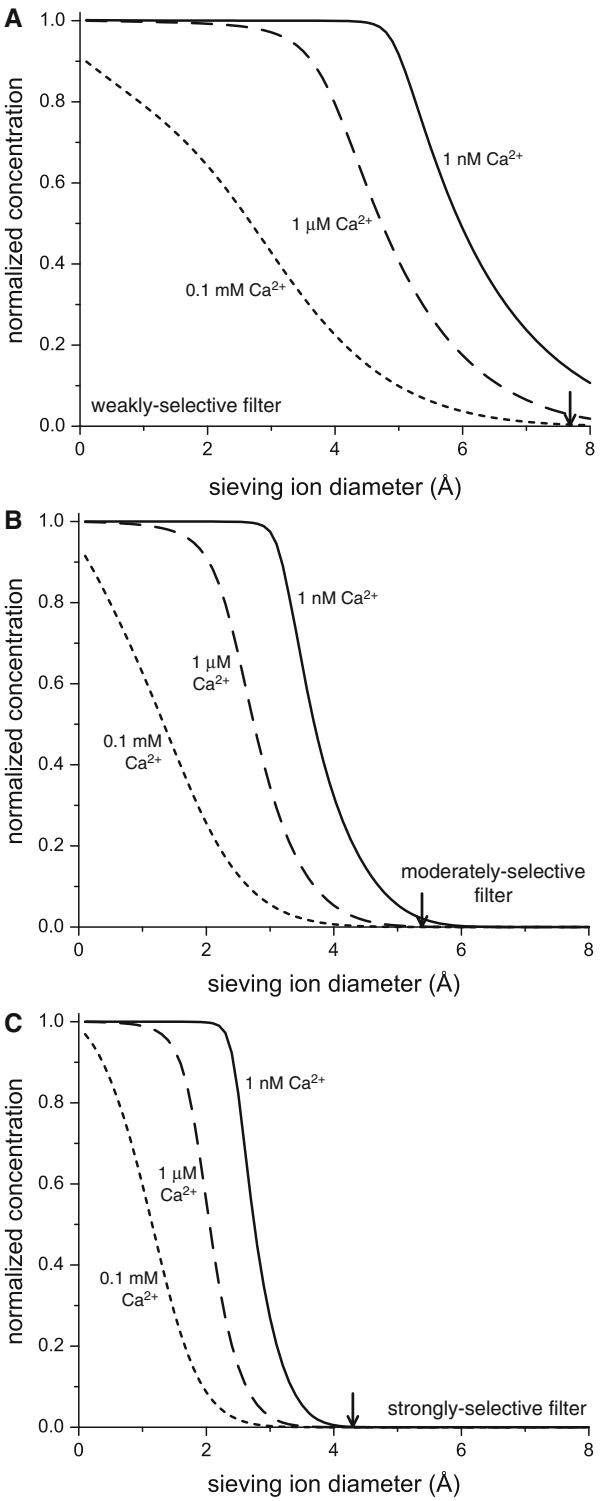

Fig. 3 Normalized concentration in the filter of sieving cations of different diameters in the presence of 1 nM (*solid lines*), 1 μM (*dashed lines*), and 0.1 mM Ca^{2+} (*dotted lines*). The sieving cations have a concentration of 100 mM in the bath. Three kinds of model calcium channels with different Ca^{2+} affinity are considered: **a** weakly calcium selective with $\rho_{Ox} = 10$ M, **b** moderately calcium selective with $\rho_{Ox} = 25$ M, and **c** strongly calcium selective with $\rho_{Ox} = 40$ M. The *arrow* indicates the largest diameter for which the model filter admitted sieving ions in the absence of Ca^{2+}

largest sieving ion that would fit into the filter when $[Ca^{2+}] = 0$.

The competition between ions also depends on the valence of the sieving ions; a more highly charged ion will compete more effectively with Ca^{2+} for the pore. This is shown in Fig. 4 for the moderately selective filter with the valence of the sieving cation +1, +2, and +3. However, for all three kinds of selectivity filters, even the high-valance sieving cations are eventually displaced from the filter completely (data not shown).

Discussion

The results shown in Figs. 1–4 show that large ions can be excluded from a calcium-selective homogeneous fluid by trace amounts of Ca^{2+}. This is because the larger the sieving ions, the more difficult it is to fit them in between the oxygens of the filter fluid at the densities required by charge neutrality. To see this, consider the percentage of the total volume taken up by the oxygens (subscripted Ox) and the sieving cations (subscripted S) (i.e., their packing fraction):

$$\eta = \frac{4\pi}{3}\left[\left(\frac{\sigma_{Ox}}{2}\right)^3 \rho_{Ox} + \left(\frac{\sigma_S}{2}\right)^3 \rho_S\right] = \frac{\pi}{6}\rho_{Ox}\left(\sigma_{Ox}^3 + \frac{|z_{Ox}|}{z_S}\sigma_S^3\right). \quad (12)$$

A graph of the total space-filled fraction from Eq. 12 is shown in Fig. 5. Note how rapidly η increases because of its dependence on the cube of the sieving cation diameter σ_S.

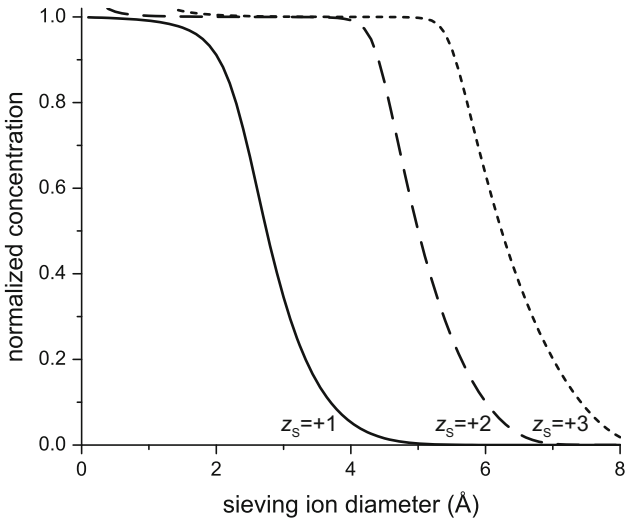

Fig. 4 The effect of sieving cation valence on the exclusion of the sieving ion from the moderately selective filter for $z_S = +1$ (*solid line*), +2 (*dashed line*), and +3 (*dotted line*). The Ca^{2+} concentration in the bath is 1 μM and the sieving ion concentration is 100 mM

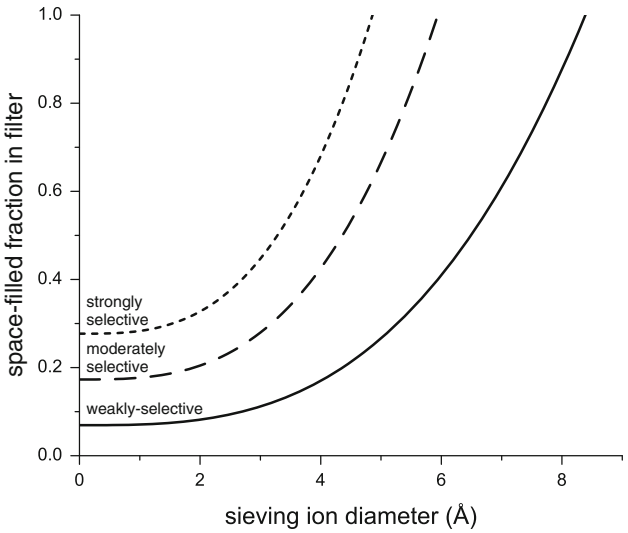

Fig. 5 The total space-filled fraction from Eq. 12 for different sieving cation diameters

For spheres of equal size the recently proven Kepler conjecture gives that $\eta < 74.05\%$. For unequally sized spheres the exact answer is not known, but certainly η must be less than 1. This latter estimate indicates that the diameter of the sieving ions cannot be greater than ~8.4, 5.9, and 4.9 Å for the weakly selective, moderately selective, and strongly selective filters, respectively. These are similar to the sizes when Ca^{2+} was present, but, as shown in Fig. 2, Ca^{2+} competes with the sieving cations and accelerates their exclusion for the filter.

Energetics of sieving ion exclusion

In the simplified model of the selectivity filter, even well below these sizes of sieving ions, the crowding of large ions and oxygens requires a large energy to bring in the sieving ions. Large ions are repelled by the filter because it is difficult to fit them in; the hard-sphere term of the electrochemical potential (μ_S^{HS} in Eq. 3) becomes large and positive. However, charge neutrality of the filter is required in our model so cations must go into the filter. Consequently, one of two things occurs: other cations such as Ca^{2+} go into the filter or, in the absence of other, smaller cations, the electrostatic potential in the filter (the Donnan potential V_D) becomes more negative to overcome the positive (repulsive) potential of fitting in more large ions. When both the oxygen concentration and the sieving ions are large enough, even trace amounts of other cations (Ca^{2+} in our case) enter the filter because this becomes more energetically favorable than packing more large ions into an already crowded environment.

This can be seen quantitatively by rearranging the terms of Eqs. 1 and 2 (Gillespie 2008). For an ion species i to

partition out of the bath and into the filter so that $\rho_i^{\text{filter}} > \rho_i^{\text{bath}}$, the difference between the filter and bath excess chemical potentials ($\Delta\mu_i^{\text{ex}}$) must be negative because

$$\ln\left(\frac{\rho_i^{\text{filter}}}{\rho_i^{\text{bath}}}\right) = -\Delta\mu_i^{\text{ex}} = -\left(\mu_i^{\text{ex,filter}} - \mu_i^{\text{ex,bath}}\right) = -\frac{\Delta\mu_i^{\text{ES}}}{kT} - \frac{\Delta\mu_i^{\text{HS}}}{kT}. \quad (13)$$

This can be subtracted for the two species competing for the pore, namely the sieving ions (S) and Ca^{2+} (Gillespie 2008):

$$\overbrace{\ln\left(\frac{\rho_{\text{Ca}}^{\text{filter}}}{\rho_{\text{S}}^{\text{filter}}}\right)}^{\text{binding selectivity}} = \overbrace{\ln\left(\frac{\rho_{\text{Ca}}^{\text{bath}}}{\rho_{\text{S}}^{\text{bath}}}\right)}^{\text{number advantage}} + \overbrace{\frac{1}{kT}\left(\Delta\mu_{\text{S}}^{\text{ES}} - \Delta\mu_{\text{Ca}}^{\text{ES}}\right)}^{\text{total electrostatic advantage}} + \overbrace{\frac{1}{kT}\left(\Delta\mu_{\text{S}}^{\text{HS}} - \Delta\mu_{\text{Ca}}^{\text{HS}}\right)}^{\text{excluded volume advantage}}. \quad (14)$$

This equation then decomposes the binding selectivity (the logarithm of the partitioning) into three energetic components (advantages). In this case, a positive advantage indicates the term favors Ca^{2+} accumulation in the pore and a negative advantage sieving ion accumulation.

Figure 6 shows the results of this energetics decomposition for the moderately selective filter with sieving cations at 100 mM and Ca^{2+} at 1 μM in the bath. The only term that favors sieving ions accumulating in the filter is the large number advantage they have over Ca^{2+}. The Ca^{2+} is energetically favored in the filter (relative to the sieving cations) because of its smaller size and larger charge; its +2 charge attracts it into the filter via the negative Donnan potential and its size makes it more easily coordinated by the oxygens and allows it to more easily fit into the crowded filter environment (Gillespie 2008). On the contrary, the larger the sieving cations, the larger their excluded-volume term in the filter becomes, soon tipping the energetics balance in favor of Ca^{2+} accumulating in the filter. This balance of energy (electrostatics) and entropy (excluded volume) is the charge/space competition mechanism of selectivity, and the exclusion of large ions is an extension of this principle. As shown in Fig. 3, this exclusion of large ions is sensitive to the amount of Ca^{2+} because that reduces the number advantage the sieving ions have (horizontally hatched bar in Fig. 6).

A similar result was found for the RyR calcium channel where micromolar Ca^{2+} significantly affected the current of the large cation Cs^+ (3.40 Å diameter), but 10 times more Ca^{2+} was needed for a similar effect on the smaller Na^+ (2.00 Å diameter) (Gillespie 2008). In the RyR model, the differences in monovalent cation versus Ca^{2+} selectivity were predominantly due to a ~1 kT change in the excluded-volume advantage when Ca^{2+} was competing with the small Na^+ as compared to competing with the large Cs^+. The predictions of this model were later confirmed by experiments, providing evidence that the energetics calculated by the RyR model are relatively accurate (Gillespie 2008; Gillespie and Fill 2008; Gillespie et al. 2009, 2005).

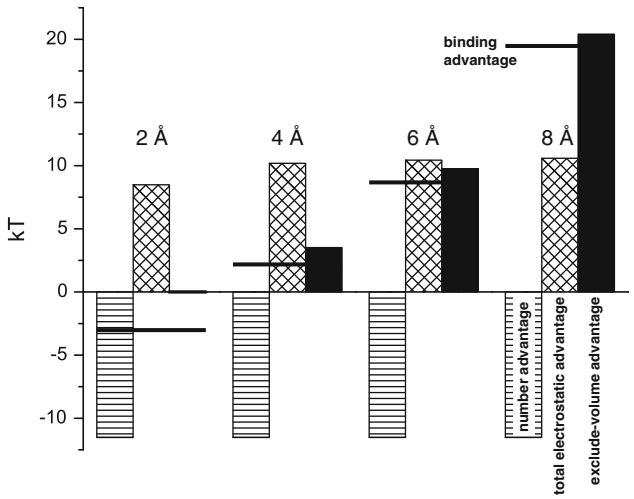

Fig. 6 Energetics of sieving ions competing with Ca^{2+} for the moderately selective filter. The Ca^{2+} concentration in the bath is 1 μM and the sieving ion concentration is 100 mM. Each of the terms in Eq. 14 is shown for the indicated monovalent cation diameter: number advantage (*horizontal hatch*), total electrostatic advantage (*cross hatch*), excluded-volume advantage (*solid*), and their sum, the binding advantage (*line*)

Connecting the simple model to reality

While the RyR model also produces selectivity by the charge/space competition mechanism like the simplified model we use here, a major difference in the models is that here we assume the electrolyte in the filter to be a homogeneous, charge-neutral fluid. In real channels that is clearly not true. As discussed earlier, the charge neutrality condition overemphasizes the Ca^{2+} selectivity compared to models where the selectivity filter is a narrow part of a pore that directly connects two baths. In those models, the selectivity filter is generally not charge neutral because it is more energetically efficient to have the preferentially selected ions (e.g., Ca^{2+}) in the filter while the less preferred ions (e.g., large monovalents) accumulate at the mouths of the pore (Boda et al. 2002b, 2006, 2007a, 2007b, 2009; Gillespie 2008). In fact, the ions in those accumulation sites can have concentrations well above bath values even though they are rarely, if ever, in the selectivity filter itself.

However, the models that enforce charge neutrality in the filter and those that do not both produce the same qualitative result: large ions are excluded because they do

not fit in between the oxygen ions of the filter—even if they can fit into an empty pore—and other, smaller cations are in the selectivity filter. This is because, to be conducted, an ion must pass through the selectivity filter and compete for space with the amino acids in the permeation pathway. The space available between the amino acids is primarily determined by their concentration and not the confining protein (Malasics et al. 2009). Therefore, even though charge neutrality is enforced in this paper, the qualitative results are identical to those of less restrictive models.

Those other models, in fact, reproduce experimental results well and are consistent with the interpretations of this paper (Boda et al. 2009; Gillespie and Boda 2008). The sieving cations do not produce a current because they cannot pass through the selectivity filter. Only the other, smaller cations will produce a current, but these are, by experimental design, at extremely low bath concentration so their current will be immeasurably small (Gillespie et al. 2009).

This "sieving-by-crowding" then gives the same experimental measurements, but the interpretation of the experiment is a generalization of the traditional view. The results of this study show that large ions can be entropically rejected from a homogeneous fluid containing a concentration of particles. Therefore, the mechanism must also apply in the more complicated case of a selectivity filter that is crowded with amino acid side chains (in addition to any other ion selection/rejection effects). This process is increased when the sieving ions compete with more preferred ions.

In our study, the only steric effect for sieving ion rejection is through the volume accessible between the oxygens (or amino acids in general). Sieving ions are excluded because the space-filled fraction of the amino acids and ions gets large. In the nanocosm of a channel, if amino acids form a fluid in the permeation pathway, then similarly a sieving experiment is a measure of how much *volume* is accessible in the selectivity filter, rather than how much *cross-sectional area* is accessible. When the pore does not contain amino acids (the empty pipe channel), then the two are the same; the ion can fit into the pore if and only if the pore is wide enough. However, when amino acids do interact with the ions in the permeation pathway, the ions must fit in between them. This depends (to first order) on the volume of the ion.

Conclusion

In a simplistic system without radial confinement (and therefore without the concept of a dynamical pore radius), we have demonstrated that there is a maximum radius of ions that enter a selectivity filter crowded with amino acids. We do not suggest that there is no dynamic radius of a channel, but our example does point out that this may be a fuzzy concept. We do, however, suggest that the physical mechanism of large ion rejection in our model is significant in interpreting sieving experiments. Certainly many things affect ion exclusion (e.g., Na^+ is excluded from potassium channels) and therefore there are many things that influence the outcome of a sieving experiment. However, fitting large ions between crowded amino acids is probably a dominant effect because of the fundamental physical limit of having a $\sim$75% space-filled fraction and because of the large amounts of free energy required to fit a large particle into a dense system (even if it is infinite and homogeneous in all directions).

Therefore, sieving-by-crowding is likely to be a mechanism present in any ion channel where amino acid side chains protrude substantially into the permeation pathway. As shown with our weakly selective example, charged side chains at a low packing fraction attract counterions to high enough packing fractions to exclude them from the selectivity filter. This is because the packing fraction scales with the cube of the ion's radius. As a result, it is possible for the selectivity filter to be rather wide and still exclude sieving ions that would easily fit into an empty cylinder of the same width. We therefore propose that sieving experiments be interpreted to approximately indicate the available *volume* within a selectivity filter instead of the available *area* unless there is independent evidence that the pore is an "empty pipe."

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References

Blum L (1975) Mean spherical model for asymmetric electrolytes I: method of solution. Mol Phys 30:1529–1535

Blum L (1980) Solution of the Ornstein-Zernike equation for a mixture of hard ions and Yukawa closure. J Stat Phys 22:661–672

Boda D, Busath DD, Henderson D, Sokołowski S (2000) Monte Carlo simulations of the mechanism of channel selectivity: the competition between volume exclusion and charge neutrality. J Phys Chem B 104:8903–8910

Boda D, Henderson D, Busath DD (2001) Monte Carlo study of the effect of ion and channel size on the selectivity of a model calcium channel. J Phys Chem B 105:11574–11577

Boda D, Busath D, Eisenberg B, Henderson D, Nonner W (2002a) Monte Carlo simulations of ion selectivity in a biological Na^+ channel: charge-space competition. Phys Chem Chem Phys 4:5154–5160

Boda D, Henderson D, Busath DD (2002b) Monte Carlo study of the selectivity of calcium channels: improved geometry. Mol Phys 100:2361–2368

Boda D, Valiskó M, Eisenberg B, Nonner W, Henderson D, Gillespie D (2006) The effect of protein dielectric coefficient on the ionic selectivity of a calcium channel. J Chem Phys 125:034901

Boda D, Nonner W, Valiskó M, Henderson D, Eisenberg B, Gillespie D (2007a) Steric selectivity in Na channels arising from protein polarization and mobile side chains. Biophys J 93:1960–1980

Boda D, Valiskó M, Eisenberg B, Nonner W, Henderson D, Gillespie D (2007b) Combined effect of pore radius and protein dielectric coefficient on the selectivity of a calcium channel. Phys Rev Lett 98:168102

Boda D, Nonner W, Henderson D, Eisenberg B, Gillespie D (2008) Volume exclusion in calcium selective channels. Biophys J 94:3486–3496

Boda D, Valiskó M, Henderson D, Eisenberg B, Gillespie D, Nonner W (2009) Ionic selectivity in L-type calcium channels by electrostatics and hard-core repulsion. J Gen Physiol 133:497–509

Doyle DA, Morais Cabral J, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science 280:69–77

Dwyer TM, Adams DJ, Hille B (1980) The permeability of the endplate channel to organic cations in frog muscle. J General Physiol 75:469–492

Gillespie D (2008) Energetics of divalent selectivity in a calcium channel: the ryanodine receptor case study. Biophys J 94:1169–1184

Gillespie D, Boda D (2008) The anomalous mole fraction effect in calcium channels: a measure of preferential selectivity. Biophys J 95:2658–2672

Gillespie D, Fill M (2008) Intracellular calcium release channels mediate their own countercurrent: the ryanodine receptor case study. Biophys J 95:3706–3714

Gillespie D, Xu L, Wang Y, Meissner G (2005) (De)constructing the ryanodine receptor: modeling ion permeation and selectivity of the calcium release channel. J Phys Chem B 109:15598–15610

Gillespie D, Boda D, He Y, Apel P, Siwy ZS (2008) Synthetic nanopores as a test case for ion channel theories: the anomalous mole fraction effect without single filing. Biophys J 95:609–619

Gillespie D, Giri J, Fill M (2009) Reinterpreting the anomalous mole fraction effect: the ryanodine receptor case study. Biophys J 97:2212–2221

Hille B (2001) Ion channels of excitable membranes, 3rd edn. Sinauer Associates, Sunderland

Koch SE, Bodi I, Schwartz A, Varadi G (2000) Architecture of Ca^{2+} channel pore-lining segments revealed by covalent modification of substituted cysteines. J Biol Chem 275:34493–34500

Malasics A, Gillespie D, Nonner W, Henderson D, Eisenberg B, Boda D (2009) Protein structure and ionic selectivity in calcium channels: selectivity filter size, not shape, matters. Biochim Biophys Acta Biomembr 1788:2471–2480

McCleskey EW, Almers W (1985) The Ca channel in skeletal muscle is a large pore. Proc Natl Acad Sci USA 82:7149–7153

Miedema H, Meter-Arkema A, Wierenga J, Tang J, Eisenberg B, Nonner W, Hektor H, Gillespie D, Meijberg W (2004) Permeation properties of an engineered bacterial OmpF porin containing the EEEE-Locus of Ca^{2+} channels. Biophys J 87:3137–3147

Miedema H, Vrouenraets M, Wierenga J, Gillespie D, Eisenberg B, Meijberg W, Nonner W (2006) Ca^{2+} selectivity of a chemically modified OmpF with reduced pore volume. Biophys J 91:4392–4400

Nonner W, Eisenberg B (1998) Ion permeation and glutamate residues linked by Poisson-Nernst-Planck theory in L-type calcium channels. Biophys J 75:1287–1305

Nonner W, Catacuzzeno L, Eisenberg B (2000) Binding and selectivity in L-type calcium channels: a mean spherical approximation. Biophys J 79:1976–1992

Nonner W, Gillespie D, Henderson D, Eisenberg B (2001) Ion accumulation in a biological calcium channel: effects of solvent and confining pressure. J Phys Chem B 105:6427–6436

Rodriguez-Contreras A, Nonner W, Yamoah EN (2002) Ca^{2+} transport properties and determinants of anomalous mole fraction effects of single voltage-gated Ca^{2+} channels in hair cells from bullfrog saccule. J Physiol 538:729–745

Salacuse JJ, Stell G (1982) Polydisperse systems: statistical thermodynamics, with applications to several models including hard and permeable spheres. J Chem Phys 77:3714–3725

Tinker A, Williams AJ (1993) Probing the structure of the conduction pathway of the sheep cardiac sarcoplasmic reticulum calcium-release channel with permeant and impermeant organic cations. J Gen Physiol 102:1107–1129

Varma S, Sabo D, Rempe SB (2008) K^+/Na^+ selectivity in K channels and valinomycin: over-coordination versus cavity-size constraints. J Mol Biol 376:13–22

Wu X-S, Edwards HD, Sather WA (2000) Side chain orientation in the selectivity filter of a voltage-gated Ca^{2+} channel. J Biol Chem 275:31778–31785