Energetics of divalent selectivity in a calcium channel: the ryanodine receptor case study

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26 June 2007

Abstract

A model of the ryanodine receptor (RyR) calcium channel is used to study the energetics of binding selectivity of Ca^{2+} vs. monovalent cations. RyR is a calcium-selective channel with a DDDD locus in the selectivity filter, similar to the EEEE locus of the L-type calcium channel. While the affinity of RyR for Ca^{2+} is in the millimolar range (as opposed to the micromolar range of the L-type channel), the ease of single-channel measurements compared to L-type and its similar selectivity filter make RyR an excellent candidate for studying calcium selectivity. Using a Poisson-Nernst-Planck/Density Functional Theory model of RyR to quantify the energies of selectivity, it is found that the Ca^{2+} vs. monovalent selectivity is driven by the charge/space competition mechanism that hypothesizes that selectivity arises from a balance of electrostatics and the exclude volume of ions in the crowded selectivity filter. While electrostatic terms dominate the energy of selectivity, it is found that the much smaller excluded-volume energies also play a substantial role. In the D4899N and D4938N mutations of RyR that are analyzed, substantial changes in the energy profiles far from the mutation site are found. These changes result in the loss of Ca^{2+} selectivity.

Introduction

Calcium-selective ion channels play an important role in many physiological functions including in the excitation-contraction (EC) coupling pathway that translates nerve signals into muscle contractions. EC coupling involves two kinds of calcium channels: depolarization of the transverse tubule activates the L-type calcium channel (a.k.a. the dihydropyridine receptor) that conducts Ca^{2+} to activate the ryanodine receptor (RyR) calcium channel. RyR, in turn, conducts Ca^{2+} out of the sarcoplasmic reticulum, a Ca^{2+}-storage organelle. It is the binding of this Ca^{2+} to the contraction proteins that initiates muscle contraction.

The L-type and RyR calcium channels have very different permeation and selectivity properties: the L-type channel has a small conductance (1) and micromolar Ca^{2+} affinity (2,3) while RyR has a large conductance and only millimolar Ca^{2+} affinity (4). On the other hand, both the L-type and RyR calcium channels have negatively-charged, carboxyl-rich selectivity filters, the EEEE locus of L-type (5,6) and the DDDD locus of RyR (with a neighboring EEEE locus) (7). Therefore, it is plausible that both channels share a mechanism for selectivity that is

determined by the EEEE/DDDD locus. In this paper, a model of RyR is used to understand how a EEEE/DDDD locus leads to a Ca^{2+}-selective channel. RyR is used because a model of permeation through it already exists (and is expanded on here) and because it is relatively easy to perform single-channel measurements, providing a very large data set to work with.

Besides barrier models, selectivity in calcium channels has been modeled before with general studies by Boda et al. (including the author) (8-12), specific studies of the L-type channel by Nonner et al. (13,14) and Corry et al. (15,16), and RyR by Chen et al. (17-19) and the author (20). From these studies two schools of thought have emerged with regard to why calcium channels prefer to bind/conduct Ca^{2+} over monovalent cations. Corry et al. (15,16) argue that the L-type channel must be a single-filing channel and that Ca^{2+} is preferred because calcium ions see a much larger electrostatic energy well in the selectivity filter from the four glutamates than monovalent ions (16). On the other hand, Nonner, Boda, the author, and co-workers argue that calcium channels have a small (but *not* single-filing) and crowded selectivity filter that prefers Ca^{2+} over monovalent cations because of the balance of electrostatic and excluded-volume forces (i.e., two ions cannot overlap) (8-12,14,20-22); two Ca^{2+} can balance the four negative glutamates in half the volume of four Na^{+}, a mechanism called charge/space competition (CSC).

Both sides argue that they qualitatively reproduce the important characteristics of the L-type channel (e.g., the anomalous mole fraction effect, AMFE, where micromolar concentrations of Ca^{2+} block Na^{+} current), but both sides have problems. For example, it is not practical for Corry et al. to simulate the low voltages and low Ca^{2+} concentrations where almost all experiments have been done. This requires them to extrapolate four orders of magnitude from simulation data at 18 mM Ca^{2+} to the required 1 μM Ca^{2+} of the AMFE (15). Moreover, they only simulate Ca^{2+} vs. Na^{+} selectivity and do not simulate other monovalent cations to see if their theory is consistent with experiments. They also do not simulate monovalent vs. monovalent selectivity (e.g., Na^{+} vs. K^{+}). This makes it difficult to determine by what mechanism their model channel distinguishes between monovalents, which they are known to do (1); *a priori*, electrostatics alone would not seem to be enough. Moreover, the mechanism involved in monovalent vs. monovalent selectivity must also be present in divalent vs. monovalent selectivity. Monovalent vs. monovalent selectivity might be one situation where the two models give qualitatively different results.

On the other hand, much of the work on the CSC mechanism has been done with equilibrium simulations that do not compute current, but only channel occupancy (8-12,14). When current was computed, it required data-fitting of excess chemical potentials (see below) (13,18,19)—rather than using a theory to compute them—which gave reasonable values for these potentials and reproduced the AMFE of the L-type channel (14). Much of the effort by the CSC school has been directed at studying a wide range of selectivity including Ca^{2+} vs. different monovalents (9,14,21) and monovalent vs. monovalent selectivity (9,12) to show that a crowded filter prefers small monovalents. In recent work they have also shown that reducing both the pore radius and the protein polarization can account for the very different Ca^{2+} affinities observed in L-type and RyR channels (11,12). These studies have shown mechanisms that work in principle.

To move beyond "in principle," a model of a real calcium channel that reproduces—*and predicts*—the experimental data over a wide range of ionic conditions and mutations is vital. In this paper, a model that *quantitatively* reproduces and *predicts* RyR experimental data in over 100 different ionic solutions is used to study the energetics of selectivity in RyR. The experimental verification of one of these predictions is also shown here. Specifically, the model predicted an AMFE between Ca^{2+} and monovalent cations (Na^{+} and Cs^{+}). In this AMFE, current is reduced by up to 65% which is significant, but not as dramatic as the >90% reduction found in the L-type calcium channel (2).

The Poisson-Nernst-Planck/Density Functional Theory (PNP/DFT) model used here has the advantage that it computes quickly (minutes for a whole current/voltage curve), computes the excess chemical potentials from thermodynamic formulas, and uses exactly 9 experimental data points to determine the ion diffusion coefficients of the 7 ion species in the model. Another advantage of the PNP/DFT model is that it naturally computes the components of the chemical potential of ion selectivity without additional work. That decomposition of energies is used here to dissect Ca^{2+} vs. monovalent selectivity in both native and mutant RyR. It is found that different energy terms become important under different circumstances and in mutations energy differences between mutant and native can extend 7.5 Å beyond the region where the mutation occurred. The results indicate the Ca^{2+} vs. monovalent cation selectivity in RyR is driven by the CSC mechanism.

Theory and methods

The Poisson-Nernst-Planck/Density Functional Theory model

The flux through the RyR pore is described by a constitutive relationship that is a generalization of the Poisson-Nernst-Planck (PNP) equations:

$$-\mathbf{J}_i(\mathbf{x}) = \frac{1}{kT} D_i(\mathbf{x}) \rho_i(\mathbf{x}) \nabla \mu_i(\mathbf{x}) \tag{1}$$

where $\mathbf{J}_i$, D_i, ρ_i, and μ_i are the local flux density, diffusion coefficient, density, and chemical potential, respectively, of ion species *i*. *k* is the Boltzmann constant and *T* is the temperature. The chemical potential is decomposed into different terms (14,22-27):

$$\mu_i(\mathbf{x}) = \overbrace{kT \cdot \ln\left(\frac{\rho_i(\mathbf{x})}{\Lambda_i^3}\right)}^{\text{ideal gas}} + \overbrace{z_i e \phi(\mathbf{x})}^{\text{mean electrostatic}} + \overbrace{\mu_i^{\text{SC}}(\mathbf{x})}^{\text{screening}} + \overbrace{\mu_i^{\text{HS}}(\mathbf{x})}^{\text{excluded volume}} \tag{2}$$

where *e* is the elementary charge and where Λ_i is the de Broglie wavelength and z_i is the valence of ion species *i*.

In this decomposition of the chemical potential, there are two electrostatic terms and an excluded-volume term in addition to the usual ideal gas term. The mean electrostatic potential ϕ is given by the average (i.e., long-time, many particle ensemble average) ion densities via the Poisson equation:

$$-\varepsilon_0 \nabla \cdot \left(\varepsilon(\mathbf{x}) \nabla \phi(\mathbf{x})\right) = e \sum_i z_i \rho_i(\mathbf{x}) \tag{3}$$

where ε_0 is the permittivity of free space and ε is the local dielectric coefficient. The sum on the right-hand side includes both the densities of the permeating ions and the protein charge densities. If the chemical potential is defined with only the ideal gas and mean electrostatic terms, then Eqs. (1) and (3) reduce to the normal PNP equations of charged, point ions.

Ion size is included in the screening and excluded-volume terms. The screening term μ_i^{SC} is the electrostatic energy *in addition* to the mean electrostatic potential that reflects an ion's ability to coordinate with other ions (i.e., to screen neighboring ions) (23,27). In this paper, ions are modeled as charged, hard spheres and therefore the excluded-volume term is purely due to hard-sphere (HS) repulsion. Both of these terms are computed using density functional theory (DFT) of classical fluids (*not* electron orbitals) as previously described (22,26). DFT is currently

the state-of-the-art theory in the study of physics of confined fluids (e.g., see the recent review by Wu (24)) and the specific DFT of charged, hard spheres used here has been tested against multiple Monte Carlo simulations to assess its accuracy (22,26,28).

The work shown in this paper is computed with a one-dimensional approximation of Eqs. (1)-(3) that was described previously (13,29) where the dielectric coefficient ε was constant at 78.4 throughout the system. How the model pore and input parameters were determined from experimental data is described in the Appendix. Also, in the Appendix comparisons between the model and experiments are shown.

The geometry of the model RyR pore is shown in Fig. 1.

Analysis of binding selectivity

The energetics of ion binding within the pore is most easily analyzed in equilibrium when no currents from any ion species are flowing (i.e., $\mathbf{J}_i = 0$ for all ion species). In equilibrium, the baths are identical in concentration and composition and the chemical potential is constant throughout the system (in the baths and the pore):

$$\begin{aligned} \mu_i^{\text{bath}} &= \mu_i^{\text{pore}}(x) \\ &= kT \cdot \ln\left(\frac{\rho_i(x)}{\Lambda_i^3}\right) + z_i e\phi(x) + \mu_i^{\text{SC}}(x) + \mu_i^{\text{HS}}(x). \end{aligned} \tag{4}$$

In this paper all the analysis of selectivity is done in equilibrium based on this decomposition of the chemical potential.

AMFE experiments

The experimental results shown in Fig. 2 were measured by the lab of Michael Fill (Rush University Medical Center) using standard, previously described methods (30). These experiments were performed on the cardiac isoform (RyR2) that still had regulatory proteins (e.g., the negatively-charged calsequestrin) attached to them. In this way, these channels are different from the "purified" RyR channels for which the theory was originally developed (7,20,31). Therefore larger discrepancies between theory and experiment than those described in the Appendix are to be expected. Still, the model is in very good agreement with experiment. The experiments were performed *after* the theoretical calculations and no parameters were changed in the model to better reproduce the experimental data.

Results

AMFE for Ca^{2+} and monovalent cations

Many mole fraction experiments have been performed in RyR, both for mixtures of monovalent cations with other monovalents and mixtures of divalents with other divalents (32,33). In these experiments, the relative concentrations of two ion species was changed while the total concentrations of both species was kept constant and the conductance was measured as a function of mole fraction. None of these experiments showed an AMFE where a minimum in the conductance vs. mole fraction curve is present until the RyR model of Gillespie et al. (20) predicted an AMFE for mixtures of Na^+ and Cs^+. This was experimental verified after the model calculations were done (Fig. 20A in the Appendix).

Here we present another AMFE prediction of the RyR model, this time with mixtures of Ca^{2+} and Cs^+ as well as Ca^{2+} and Na^+. Because increasing the bath Ca^{2+} concentration on the cytosolic side of RyR decreases open probability, it is not possible to perform true mole fraction experiments with symmetric bath conditions. Instead, we follow Almers, McCleskey, and Palade (2) who used symmetric, fixed concentrations of a monovalent cation and only increased lumenal [Ca^{2+}]. This protocol produced the classical AMFE in the L-type calcium channel that showed the block of Na^+ current by micromolar Ca^{2+} (2,3).

The calculations were completed—with all model parameters fixed—*before* the experiments were performed. The model predicted not only the presence of a minimum, but also that the minimum for Cs^+ would be deeper than for Na^+ (Fig. 2). Specifically, the theory predicted a reduction in current of 47% for Na^+ at 1 mM Ca^{2+} (compared to 1 μM Ca^{2+}) and a 59% reduction in current for Cs^+. The experimental results were 42% and 65%, respectively. Moreover, the theory predicted that 10 μM Ca^{2+} added to Na^+ would not substantially change the net current (Fig. 2, the two left-most squares) while 10 μM Ca^{2+} added to Cs^+ would decrease net current by about 20% (Fig. 2, the two left-most circles). These results and those shown in the Appendix (Fig. 22) indicate that the model can accurately reproduce Ca^{2+} vs. monovalent cation selectivity data over a very wide range of conditions (i.e., 0–50 mM Ca^{2+}; –150 to +150 mV applied voltage; Na^+, K^+, and Cs^+ as monovalents).

More study is planned to understand the cause of the AMFE in RyR. However, the computations shown here prove that the AMFE in RyR does *not* require the correlated motion of

multiple ions through a long, single-filing pore (34), because the model pore does not include a single-filing selectivity filter (it is 8 Å in diameter). Moreover, the model does not include the conservation of momentum necessary to model correlated ion motion; the Nernst-Planck equation used to describe ion flux (Eq. (1)) only includes conservation of mass (35). In general, the physical interpretations of the AMFE are highly model-dependent. Because classical barrier models of ion permeation do not include the physics known to exist in electrolytes, using these models to infer the occupancy—or any property—of a channel is problematic (14,20,36).

Dependence of Ca^{2+} vs. K^+ selectivity on bath Ca^{2+} concentration

To study binding selectivity (i.e., the amount of an ion species that accumulates at one location in the pore), it is easiest to analyze the equilibrium situation where the chemical potential of each ion species is constant in the baths and within the pore (Eq. (4)). In this case, the baths must be identical in ionic composition and concentration. Then, the *partitioning* of ion species *i* between the bath and location *x* in the pore can be written as

$$\ln\left(\frac{\rho_i(x)}{\rho_i^{\text{bath}}}\right) = -\frac{z_i e}{kT}\phi(x) - \frac{\Delta\mu_i^{\text{SC}}(x)}{kT} - \frac{\Delta\mu_i^{\text{HS}}(x)}{kT}. \tag{5}$$

where $\Delta\mu_i^{\text{SC}}(x)$ indicates the screening chemical potential at *x* minus the bath value (and similarly for the HS term). Each of these terms are shown in Figs. 3–6 for a bath containing 150 mM KCl and varying amounts of $CaCl_2$ ranging from 1 μM to 50 mM.

Fig. 3 shows the partitioning coefficient plotted logarithmically (the left-hand side of Eq. (5)) for K^+ and Ca^{2+} (panel B). For K^+ (panel A), the bath concentration is held constant and so the decrease in partitioning as [Ca^{2+}] increases is a direct result of K^+ being replaced by Ca^{2+} everywhere within the pore. For Ca^{2+} (panel B) on the other hand, partitioning reflects both an increase in $\rho_{\text{Ca}}(x)$ within the pore, but also an increase in bath [Ca^{2+}]. Even though more and more Ca^{2+} enters the pore as [Ca^{2+}] increases, the ratio of $\rho_{\text{Ca}}(x)$ to [Ca^{2+}]—the partitioning—*decreases* as more Ca^{2+} is added to the bath; the increase of Ca^{2+} concentration in the pore is proportionately smaller than the increase of Ca^{2+} concentration in the baths.

Fig. 4 shows the *mean electrostatic* component $z_i e\phi(x)$ of the partitioning in Eq. (5). Because Ca^{2+} has twice the charge of K^+ this component is twice as large for Ca^{2+} (panel B) as for K^+ (panel A). For both ion species, this term makes up much more than half of the

partitioning energy. Also, it is important to note that $\phi(x)$ changes from a deep energy well in the selectivity filter when [Ca^{2+}] is low to being very close to zero throughout the channel when [Ca^{2+}] is high; the mean electrostatic potential changes significantly as [Ca^{2+}] changes. This indicates that the *entire* pore is becoming more and more charge-neutral (on average over a long-time and many particles passing through the channel) as [Ca^{2+}] is increased. It must be the entire pore that is becoming electroneutral because any significant net charge in any region of the pore would create an electrostatic potential well or barrier in the electrostatic potential profile computed from the Poisson equation (Eq. (3)).

Fig. 5 shows the screening component of the partitioning $\Delta\mu_i^{SC}(x)$ in Eq. (5) that describes electrostatics beyond the mean electrostatic component (the component computed from Eq. (3)). The correlations due to electrostatics are most intuitively described as the ion's ability to screen the charge of another ion and therefore this component is called the *screening* (SC) component. In general, the smaller or higher-valence ion screens a charge more efficiently than the larger or lower-valence ion. This is reflected in Fig. 5 by the much more negative screening energy for Ca^{2+} (dashed lines) compared to the slightly negative screening energy for K^+ (solid lines). This component of the partitioning is always negative (favoring partitioning into the pore) and changes little as [Ca^{2+}] increases.

Fig. 6 shows the hard-sphere (HS) component $\Delta\mu_i^{HS}(x)$ in Eq. (5) that describes the contribution of the ions' excluded volume; it is the excess chemical potential of an uncharged, hard-sphere fluid with the same density profile $\rho_i(x)$ as the ionic fluid. This *excluded-volume* term is positive, indicating that it is more difficult to insert an ion-sized particle into the selectivity filter than into the bath. The size of the ions is hindering ion partitioning into the pore. This term is, however, small in the pore (<1 kT), and, in general, the smaller the ion, the smaller the excluded-volume term. Like the screening term, $\Delta\mu_i^{HS}(x)$ changes little as [Ca^{2+}] increases.

While these profiles are useful for understanding the partitioning of one ion species into the pore, by themselves they do not show why one ion species is favored over another. For this, the difference in the partitioning between the two ion species (K^+ and Ca^{2+} in this example) is necessary. Specifically, the relative concentrations in the pore are considered:

$$\overbrace{\ln\left(\frac{\rho_{\mathrm{Ca}}(x)}{\rho_{\mathrm{K}}(x)}\right)}^{\text{binding selectivity}} = \overbrace{\ln\left(\frac{[\mathrm{Ca}^{2+}]}{[K^{+}]}\right)}^{\text{number advantage}} + \overbrace{\left(z_{\mathrm{K}} - z_{\mathrm{Ca}}\right)\frac{e\phi(x)}{kT}}^{\text{mean electrostatic advantage}}$$
$$+ \overbrace{\frac{1}{kT}\left(\Delta\mu_{\mathrm{K}}^{\mathrm{SC}}(x) - \Delta\mu_{\mathrm{Ca}}^{\mathrm{SC}}(x)\right)}^{\text{screening advantage}} \qquad (6)$$
$$+ \overbrace{\frac{1}{kT}\left(\Delta\mu_{\mathrm{K}}^{\mathrm{HS}}(x) - \Delta\mu_{\mathrm{Ca}}^{\mathrm{HS}}(x)\right)}^{\text{excluded-volume advantage}}$$

Here, the *binding selectivity* is defined by the ratio of the ion concentrations in the pore and by Eqs. (2) and (5) is naturally decomposed into four energetic *advantages*, energy differences that each favor the binding of one ion species over the other. In this case, a positive term favors the binding of Ca^{2+} while a negative term favors K^{+}.

To describe the energetics of binding selectivity with a single number rather than an entire profile (like in Figs. 3–6), the relative concentrations of Ca^{2+} and K^{+} in Eq. (6) are only considered in the middle of the Asp-4899 region (i.e., at x = 20 Å). This location is not only where the ion densities are highest and is where ion selectivity occurs, but how the energy components change as $[Ca^{2+}]$ changes at that location is also similar to other locations in the pore.

All the terms of Eq. (6) are shown in Fig. 7 for $CaCl_2$ ranging from 1 μM to 50 mM. As $[Ca^{2+}]$ increases, the overall binding advantage of Ca^{2+} increases (solid line). This displacement of K^{+} by Ca^{2+} is determined by how each of the energy terms in Eq. (6) changes as $[Ca^{2+}]$ increases:

(1) *Number advantage (horizontal-hatched column).* The only term that favors K^{+} binding in the pore is its number advantage; there is more K^{+} in the baths than Ca^{2+} and therefore it is more probable that a K^{+} ion enters the channel. Even this advantage is overcome by the electrostatic and excluded-volume terms at just 0.1 mM $CaCl_2$ in the bath. It is important to note, however, that the number advantage is physiologically real; with $[Ca^{2+}]$ at 1 mM (approximately the free Ca^{2+} in the sarcoplasmic reticulum) the K^{+} number advantage is 5 kT, just 1 kT less than the combined electrostatic terms (the diagonal- and cross-hatched bars in Fig. 7). This term is especially important for the L-type Ca^{2+} channel where the Ca^{2+} affinity of the channel is 1 μM and the number advantage for K^{+} is 12 kT.

(2) *Mean electrostatic advantage (diagonal-hatched column).* The mean electrostatic potential inside the pore always favors Ca^{2+}, but it reduces to almost zero as $[Ca^{2+}]$ becomes comparable to $[K^+]$ (see also Fig. 4). The long-ranged average electrostatic potential only attracts Ca^{2+} to the pore when $[Ca^{2+}]$ is low. When $[Ca^{2+}]$ becomes comparable to $[K^+]$, this attraction is quite small and it is the decrease in the number advantage of K^+ that favors Ca^{2+} binding in this case (since the two other energy terms are virtually unchanged as $[Ca^{2+}]$ increases, as described below).

(3) *Screening advantage (cross-hatched column).* When $[Ca^{2+}]$ is higher than about 0.1 mM, then the largest energy term favoring Ca^{2+} binding in the pore is the screening advantage of Ca^{2+}. Fig. 5 showed that the screening term of Ca^{2+} in the selectivity filter was approximately -4.5 kT while for K^+ it was only about -1 kT; the large difference in these terms is the 3.5 kT screening advantage for Ca^{2+} shown in Fig. 7. Most importantly, this screening advantage is *unchanged* as $[Ca^{2+}]$ is increased to provide the largest continuous preference for Ca^{2+} over K^+ (and over other monovalent cations as described below). While the excluded-volume advantage of Ca^{2+} is also unchanged, that term is much smaller (see below) and while the mean electrostatic potential can be larger than the screening advantage, that term decreases to only 0.5 kT as $[Ca^{2+}]$ increases (see above).

(4) *Excluded-volume advantage (solid column).* This term is also unchanged as $[Ca^{2+}]$ is increased, but at approximately 0.5 kT it is generally the smallest term favoring Ca^{2+} binding in the pore. Because Ca^{2+} ions are smaller than K^+ ions (diameters of 2 Å vs. 2.76 Å, respectively), it easier to insert a Ca^{2+} ion into the crowded pore than a K^+ ion. While the excluded-volume advantage of Ca^{2+} is generally small, this term does have a significant effect on Ca^{2+} vs. monovalent selectivity as described below.

Combining these results, it is clear that the origin of the Ca^{2+} selectivity of RyR is electrostatics; the sum of the mean electrostatic and screening advantages is enough to overcome the large number advantage of K^+. However, it is not the average electrostatic attraction of the Ca^{2+} ions from any net charge in the selectivity filter that is solely responsible for the selectivity. While a Ca^{2+} ion always "feels" twice the electrostatic pull that a K^+ ion does because of its two positive charges, the net charge throughout the pore is close to zero in the pore as $[Ca^{2+}]$ increases above 10 mM (Fig. 7); that is, there is little electrostatic pull on the cations to move into the pore at high $[Ca^{2+}]$ (on average). When $[Ca^{2+}]$ becomes comparable to $[K^+]$ it is *only* the

superior ability of the Ca^{2+} to screen the protein charges that favors Ca^{2+} accumulation in the pore because all other terms are small in comparison. The number advantage is also an important term. Not only is it *physiologically real*, but when [Ca^{2+}] is less than about 3 mM Ca^{2+} (the physiological upper limit in the sarcoplasmic reticulum) it is larger than any other term. Moreover, when considering the L-type Ca^{2+} channel that has a Ca^{2+} affinity of 1 μM, the monovalent number advantage is a whopping 12 kT that must be overcome by the other energy terms.

Role of monovalent size in Ca^{2+} vs. monovalent cation selectivity

In vivo, RyR must select Ca^{2+} ions from a background of K^+ ions. An important check on any theory that reproduces this Ca^{2+} vs. K^+ selectivity is to use the same pore model to reproduce the *in vitro* selectivity data of different monovalent cations. The model of RyR described here does this. Besides the figures shown in the Appendix, Fig. 2 shows that the model can *predict* measurable differences in the selectivity of Ca^{2+} vs. Na^+ and Ca^{2+} vs. Cs^+. These two monovalents were chosen because they have similar conductances through RyR, and therefore any differences are mainly due to their size difference (Na^+ and Cs^+ diameters are 2 Å and 3.42 Å, respectively (37)).

The significant changes that occur when different monovalent cations compete with Ca^{2+} for the pore can be seen in Fig. 8. In this figure, the concentration profiles of the monovalents and Ca^{2+} in the pore are shown for [X^+] = 150 mM (X^+ = Li^+, Na^+, K^+, and Cs^+, listed from smallest to largest) and [Ca^{2+}] = 1 mM. As monovalent diameter is increased from 1.33 Å for Li^+ to 3.42 Å for Cs^+, the monovalent concentration throughout the pore decreases and the Ca^{2+} concentration increases. Within the selectivity filter itself, there is an >80% decrease in monovalent selectivity (Cs^+ vs. Li^+) and a 40% increase in Ca^{2+} concentration. To understand this substantial change, Fig. 9 shows the chemical potential decomposition of Eq. (6) for different monovalents:

(1) *Number advantage (horizontal-hatched column).* This term is constant since [Ca^{2+}] and [X^+] are constant.

(2) *Mean electrostatic advantage (diagonal-hatched column).* All of monovalents create the same mean electrostatic potential inside the selectivity filter and therefore this advantage for Ca^{2+} accumulation is constant.

(3) *Screening advantage (cross-hatched column).* Ca^{2+} still has a screening advantage because of its higher valence. The relative screening between two cations in the pore is not just a function of the valence, however. The relative size of the ions is also important. This can be seen from the analytic formulas of the mean spherical approximation for homogeneous electrolytes (14,23,27). Because of this, the screening advantage for Ca^{2+} is approximately 0.5 kT smaller when competing against Li^+ and than when competing against Cs^+.

(4) *Excluded-volume advantage (solid column).* This term favors the small ion. Since Li^+ is the only monovalent considered that is smaller than Ca^{2+}, it is the only one with an excluded-volume advantage (albeit very small at approximately 0.25 kT). Ca^{2+}, however, has a relatively large excluded-volume advantage over Cs^+ of approximately 1 kT.

Combining these results, it is the number and mean electrostatic terms that remain constant as monovalent size is changed; previously, when [Ca^{2+}] was changed, these terms changed substantially. Visa versa, the screening and excluded-volume terms that remained approximately constant as [Ca^{2+}] varied now change as monovalent size is varied. These two terms combined only change about 1.75 kT, but this is enough to change the relative concentrations of Ca^{2+} and monovalent in the selectivity filter from approximately 1:1 for Ca^{2+} vs. Li^+ to more than 7:1 Ca^{2+} vs. Cs^+. The excluded-volume term is the one that changes the most. Therefore, is the most significant factor in determining the amount of Ca^{2+} vs. monovalent selectivity, even though it is generally less than 1 kT in magnitude.

Effects of mutations on Ca^{2+} vs. K^+ selectivity

The model of RyR permeation and selectivity described here correctly reproduces and predicts the Ca^{2+} vs. monovalent cation selectivity. Without adjusting any parameters the model also reproduces the experimentally-measured decrease in conductance and selectivity when specific charged amino acids are mutated to neutral analogs. These include the mutations D4899N, E4900Q, and D4938N (see Appendix). In the model, these mutations are produced by changing the charge on these amino acids to zero; no other parameters (e.g., diffusion coefficients, pore radius) are changed.

Here, two of these mutations are considered in detail: D4899N and D4938N. Each results in a significant reduction of Ca^{2+} vs. K^+ selectivity; D4899N reduces the permeability ratio P_{Ca}/P_K from a WT value of 7.0 to 3.4 and D4839N reduces it to 3.3 (31,38). This loss of

selectivity is reflected in the cation profiles shown in Figs. 10 and 11. In both cases, there is a significant reduction in both Ca^{2+} and K^+ in the region where the mutation occurred (indicated by the vertical lines) and a neighboring region. In other parts of the pore, the profiles are virtually identical to the native (WT) profiles. The changes are very localized, but the resulting large changes in the current/voltage curves (see Appendix) show that these localized changes in the cation profiles have measurable effects.

To understand the differences in binding selectivity in these mutations compared to native RyR, the same energy decomposition of Eq. (6) can be used. In this case, however, it is more instructive to consider the entire profile through the pore rather than just a single location. Figs. 12 and 13 show the energetics for D4899N and D4938N (dashed lines), respectively, compared to native RyR (solid lines). In both cases, there is a significant (approximately 3 kT) loss of Ca^{2+} binding compared to K^+ in the mutated region (panel A). In the regions neighboring the mutation site—up to 7.5 Å away—there is also significant loss of Ca^{2+} binding; each mutation has far-reaching effects. Analyzing the energy components again gives insight into why this occurs:

(1) *Excluded-volume advantage (panel B).* This term does not change significantly in the mutant RyRs.

(2) *Mean electrostatic advantage (panel C).* Zeroing the charge in a region of the pore is expected to change the mean electrostatic potential in that region, as it does. But, in the two mutations the results are different. In D4899N (Fig. 12C), the region where the mean electrostatic potential differs from native profile by more than 1 kT is small compared to D4938N (Fig. 13C). For that mutant, the entire mutation site as well a neighboring region has a mean electrostatic potential difference (compared to native) of approximately 1.5 kT. In both mutations, the change in this potential is localized to the mutation site and approximately 2.5 Å on either side; in the rest of the pore the potential is the same as in native RyR.

(3) *Screening advantage (panel D).* The largest change is a reduction in the screening advantage of Ca^{2+} in and around the mutation site. This change—up to 2 kT—extends up to 7.5 Å away from the mutation sites.

Altogether, the charge-deletion mutations result in an environment with significantly smaller mean electrostatic and screening advantages of Ca^{2+} over K^+; Ca^{2+} retains some advantage, but in each case up to 2 kT less then in native RyR. Because electrostatic correlations

range over the local screening (Debye) length (22,26), changes in the mutation site produce changes in the ionic concentration a distance away. Both Ca^{2+} and K^+ concentrations are reduced and because of the loss of up to 4 kT between these two advantages, the K^+ concentration is now significantly higher than that of Ca^{2+}.

Discussion

In equilibrium, the energetics of Ca^{2+} vs. monovalent cation binding selectivity in the pore RyR can be decomposed into the four terms in Eq. (6): (1) the number advantage that describes which ion species has a larger concentration in the baths; (2) the mean electrostatic advantage that describes the average electrostatic well/barrier in the channel due to the average local net charge (through the Poisson equation); (3) the screening advantage that describes the ability of an ion to electrostatically coordinate with other ions within a screening (Debye) length; (4) the excluded-volume advantage that, in this paper, describes the energy needed to insert an uncharged hard sphere the size of an ion into a location. In this model of RyR, there is no explicit ion dehydration energy; all the experimental data—including the high conductance of Mg^{2+} through RyR—is reproduced with water as a uncharged, hard spheres in a continuum dielectric. This prediction of the model that the ion dehydration step is small must be tested and will be explored in future work.

Each of these four terms plays an important role in Ca^{2+} vs. monovalent cation selectivity, as detailed now.

Number advantage

In calcium-selective channels, the number advantage that monovalents generally have over divalents is the challenge that selectivity must overcome; all other energy terms must overcome the number advantage. For example, under physiological conditions in the sarcoplasmic reticulum, $[Ca^{2+}]$ is approximately 1 mM while $[K^+]$ is approximately 150 mM—a number advantage equivalent to 5 kT of chemical potential in favor of K^+. Also, in experiments (e.g., on the L-type calcium channel or in Fig. 2) $[Ca^{2+}]$ can be 1 μM (or less)—a number advantage of 12 kT (or more) in favor of the monovalent.

In RyR, when the number advantage for K^+ is removed by increasing $[Ca^{2+}]$, the mean electrostatic potential throughout the pore goes to zero as more Ca^{2+} enters (Fig. 4) and K^+ is

displaced (Fig. 3A). Recent work using grand canonical Monte Carlo simulations has shown that this displacement of K^+ is a nonlinear function of the environment in the pore and how important it is to do all calculations at the experimental [Ca^{2+}] (11,12,39). Currently, all MD and BD simulation require at least 10 mM Ca^{2+} in the bath. While simulation results from 18 mM Ca^{2+} have been extrapolated down to 1 μM Ca^{2+} (15), a theory is required to do this. Without further simulations, however, it is impossible to verify the theory or its assumptions. Rather, a theory like PNP/DFT that spans *all* concentration ranges can be applied. Since PNP/DFT directly computes the average thermodynamic quantities and does not simulate particle trajectories, bath concentrations are just input parameters for the theory.

Electrostatics

In general, the largest contributions come from the two electrostatic terms that always favor Ca^{2+} binding in the pore over monovalent cations. Also, it is only these terms that change substantially in mutations. These two terms, while both electrostatic in nature, are physically distinct: the mean electrostatic advantage reflects the long-time, many-particle *average* electrostatic potential in the pore produced by the *average* ion concentrations in the Poisson equation (Eq. (3)); on the other hand, the screening advantage reflects the ability to form long-time ion correlations (i.e., ion coordination or screening) produced by the ions' sizes and the *instantaneous* local electrostatic environment (on the atomic motion timescale) that is not included in the Poisson equation (22,26,27).

In total, the electrostatics of the system *are* the major driving force for Ca^{2+} vs. monovalent selectivity, in general agreement with Corry et al. (15,16). However, since the DFT approach naturally decomposes the electrostatics into these two physically different terms, the PNP/DFT approach can give a more thorough understanding of how the electrostatics contributes to selectivity. With 150 mM K^+ in the bath, the screening advantage of Ca^{2+} is always more than the mean electrostatic advantage if [Ca^{2+}] is more than 0.1 mM (Fig. 7). Moreover, the mean electrostatic advantage disappears as [Ca^{2+}] is increased while the screening advantage remains largely unchanged (Fig. 7). Therefore, it is the screening advantage of Ca^{2+} that is the dominant electrostatic term.

Ionic screening is a reflection of an ion's ability to coordinate with neighboring ions and thereby lower its energy. This coordination is a function of both the ion's charge and size (as

well as the other ions' charges and sizes) and is a balance of electrostatic and excluded-volume forces (14,23,26); a small monovalent ion can screen better than a large divalent (REF). An ion species' screening advantage then directly reflects the CSC mechanism of selectivity; the excluded-volume term reflects the purely entropic component. This is especially true for Ca^{2+} because its screening advantage of Ca^{2+} over monovalent cations is large (~4 kT), indicating that Ca^{2+} coordinates significantly better, especially in the crowded environment of the selectivity filter (Fig. 7). In other words, the large screening advantage of Ca^{2+} shows that Ca^{2+} can more efficiently balance the negative charges of the protein (e.g., Asp-4899 in the selectivity filter) than the monovalents.

Excluded volume

While the electrostatic terms are generally the largest, the excluded-volume (hard-sphere) term is generally the smallest—but still important in selectivity. If electrostatics were purely responsible for Ca^{2+} vs. monovalent selectivity, then there would be little difference in the concentration of Ca^{2+} and different monovalent cations in the selectivity filter. The calculations, however, show a large difference (Fig. 8); there is significantly less Ca^{2+} in the pore with the small Li^{+} (1.33 Å diameter) as the monovalent than with the large Cs^{+} (3.40 Å diameter). The chemical potential decomposition done in the DFT (Fig. 9) demonstrates that this difference is due to changes in both the screening (up to ~0.5 kT) and excluded-volume terms (up to ~1 kT). The larger the monovalent, the more both terms favor Ca^{2+} binding.

This trend reflects the CSC selectivity mechanism: it is the small ions (e.g., Li^{+} and Ca^{2+}) that can more efficiently balance the protein charges than the large ions (e.g., Cs^{+}) because they occupy less space in the crowded selectivity filter. Fig. 8 shows this in terms of ion concentrations in the pore. If Cs^{+} is replaced by Li^{+} as the monovalent, then Ca^{2+} concentration in the selectivity filter decreases approximately 30% while monovalent concentration increases approximately 500% (compare dotted and solid lines in Fig. 8). The small Li^{+} takes up only 6% the volume of the large Cs^{+} and therefore fits more easily into the selectivity filter. Ca^{2+} is displaced because more monovalents are in the filter to balance the negative Asp-4899 protein charges. The exact ratio of Ca^{2+} to monovalent concentration in the pore is a balance of the electrostatic and excluded-volume forces—charge/space competition.

It is important to note that, while changes in the excluded-volume advantage are relatively small at around 1 kT or less, the ion concentrations in the pore depend on all the energies *exponentially* (Eq. (4)); small changes in energies can have a large effect. It is because of this that any model must reproduce experimental data over a wide range of conditions. Only then can one have confidence that the energies in the model change correctly as conditions are changed. For this reason, all the data reproduced by the model—more than 100 different ionic solutions—are shown in this paper (main text and Appendix). Specifically, Figs. 2 and 22 show that the PNP/DFT model correctly reproduces RyR's Ca^{2+} vs. monovalent affinity as $[Ca^{2+}]$ is changed.

Flexible coordination in the selectivity filter

The balance of electrostatics and excluded volume in the selectivity filter that is the CSC mechanism of selectivity is consistent with a more general idea of selectivity that is emerging from the study other ion channels. In the potassium channel, Noskov and Roux (40) and Varma and Rempe (41) describe how the carbonyl oxygens in that selectivity filter form an environment that best coordinates K^+. In the sodium channel, Boda et al. (39) show how the amino acids of the DEKA locus arrange around the permeant ions, with Na^+ being coordinated best compared to K^+ and Ca^{2+}. In those channels and in the calcium channels studied previously with Monte Carlo simulations (8-12), the channel protein forms a flexible environment that coordinates the "correct" ion better than the other ions, leading to binding selectivity.

The same is true for RyR with the carboxyl groups of the DDDD locus (from Asp-4899) coordinating Ca^{2+} best among the permeant ions. This is quantified by the screening and excluded-volume advantages of Ca^{2+}. Both of these energy terms indicate how well an ion "fits into" the crowded environment of the selectivity filter, either by its ability to coordinate with (screen) neighboring ions and protein charges—the screening advantage—or by its ability to find space among the other atoms—the excluded-volume advantage.

Conclusion

A PNP/DFT model was used to analyze the energetics of equilibrium binding selectivity in RyR. The extension of a previous model (20) presented here uses 9 data points to determine model parameters that were then never changed. The model reproduces both native and mutant RyR permeation and selectivity data in over 100 different ionic solutions and predicted the

presence of different sized AMFEs when Ca^{2+} was added to Na^{+} and when Ca^{2+} was added to Cs^{+}. It had previously predicted an AMFE for mixtures of Na^{+} and Cs^{+} (20). While there are approximations in the model that need to be explored further (e.g., no dehydration penalty for ions moving from the bath into the pore), the PNP/DFT approach has advantages over other methods including fast computing time (minutes for an entire current/voltage curve) and arbitrarily small bath concentrations.

The model shows that Ca^{2+} vs. monovalent cation selectivity in RyR is determined by the CSC mechanism that balances the electrostatic attraction of the negative protein charges (especially Asp-4899) with the excluded volume of the ions and protein charges in the selectivity filter. This balance in favor of Ca^{2+} is achieved by having a selectivity filter that contains negatively-charged carboxyl groups on tethers so they are free to move in response to the permeant ions currently in the filter. In this sense the CSC mechanism is consistent with the selectivity by the flexible coordination provided by the channel protein seen in other channels (39-41). The PNP/DFT approach allows one to quantify the energies involved through the large screening advantage of Ca^{2+} over monovalent cations—a measure of the coordination of Ca^{2+} with the other charges in the selectivity filter—and in the excluded-volume advantage of Ca^{2+} over larger monovalent cations—a measure of much better the smaller ion fits into the crowded selectivity filter.

Acknowledgments

The author is extremely grateful to Prof. Michael Fill (Rush University Medical Center, Chicago) and his lab (especially Alma Nani) for performing the experiments on the anomalous mole fraction predictions and for performing them so quickly. Useful comments on the manuscript from Wolfgang Nonner, ???? are also gratefully acknowledged. The author was supported through NIH grant GM076013 (Bob Eisenberg, PI).

Appendix: Constructing the model

The model of ion permeation through the open RyR channel is a refinement of the model described in Ref. (20) that includes new mutation data that was not available when the first model was created (31). Specifically, two charge-neutralizing mutations of aspartates in the cytosolic (*cis*) vestibule of the pore (D4938N and D4945N) were shown to affect RyR conductance and selectivity: the conductances in 250 mM symmetric KCl were 65% and 92% of WT for D4938N and D4945N, respectively, and permeability ratios P_{Ca}/P_K were reduced from 7.0 to 3.3 and 6.5. Charge-neutralizing mutations (D or E to N or Q) of other charged amino acids in the cytosolic vestibule did not affect either K^+ conductance or Ca^{2+} vs. K^+ selectivity (31).

Previous experiments (7) showed that neutralizing the charge on two negatively-charged amino acids (Asp-4899 and Glu-4900) significantly reduced both conductance and selectivity: the conductances in 250 mM symmetric KCl were 20% and 56% of WT for D4899N and E4900Q, respectively, and permeability ratios P_{Ca}/P_K were reduced from 7.0 to 3.4 and 3.2. Except for the mutation E4902Q, charge-neutralizing mutations of other charged amino acids in the lumenal vestibule did not affect either K^+ conductance or Ca^{2+} vs. K^+ selectivity. While the conductance of E4902Q was found to be similar to WT, a small but statistically significant change from WT in Ca^{2+} selectivity was found (7) so E4902 was also included in this model.

Only Asp-4899 and Glu-4900 were explicitly included in the first model of RyR (20), although a region of negative charge in the cytosolic vestibule was required to reproduce the data. In hindsight, these were the then-unknown Asp-4938 and Asp-4945. In the model described here, all of the charged amino acids found in mutation experiments to affect RyR conductance and selectivity (while still producing functional and caffeine- and ryanodine-sensitive channels) were included: Asp-4899 in the selectivity filter, Asp-4938 and Asp-4945 in the cytosolic vestibule, and Glu-4900 and Glu-4902 in the lumenal vestibule (Fig. 1).

Since no high-resolution structures of the RyR are available, it was necessary to reverse-engineer the location of these amino acids. Several low-resolution electron microscopy structures of the entire RyR protein that were published after the initial model were used to guide the making of the model pore (42,43). Construction of the model pore was done in a way similar that described in Ref. (20), but the basic method is outlined here. Because of the homology

between RyR and the potassium channel (43), the pore was given a narrow selectivity filter with a wider cytosolic vestibule. The selectivity filter radius was chosen to be the same as in the previous model (4 Å) and 15 Å in length. The filter includes residues 4894 to 4899 (GGGIGD) as shown in low-resolution structures of the RyR pore (42). The cytosolic vestibule radius was chosen to be 7 Å in accordance with low-resolution RyR structures (M. Samsó, Harvard Medical School, personal communication, 2007), although the model cannot distinguish between different vestibule radii as it can between different selectivity filter radii (Fig. 15 of Ref. (20)).

As in the previous model, Glu-4900 was placed at the selectivity filter/lumenal vestibule junction and Glu-4902 was placed on the lumenal face of the channel. These are in accordance with other modeling of the RyR pore based on KcsA homology and mutation experiments (Fig. 2 of Ref. (7)). Asp-4938 was placed at the selectivity filter/cytosolic vestibule junction in accordance with low-resolution RyR structures (42) and 15 Å away from Asp-4899 (M. Samsó, Harvard Medical School, personal communication, 2007). Asp-4945 was placed 10 Å away from Asp-4938 toward the cytosolic end of the pore (42,43) because, as part of the same α-helix, they are approximately one helix turn apart. Because the tilt of this helix is not yet resolved (M. Samsó, Harvard Medical School, personal communication, 2007), the increase in pore radius near Asp-4945 was arbitrarily chosen to be 45°. The model is not sensitive enough to distinguish between different helix tilt angles.

Each of the aspartates and glutamates were assumed to be fully-charged and facing into the permeation pathway with the terminal carboxyl (COO^-) group on a flexible tether than can span a hemisphere of radius 5 Å for aspartates and 7 Å for glutamates (Fig. 1). In the one-dimensional Poisson-Nernst-Planck/Density Functional Theory (PNP/DFT) model (13,20), residues Asp-4938, Asp-4899, and Glu-4900 were modeled as two, independent, half-charged oxygen ions (2.8 Å diameter) confined to a region of the long axis of the pore spanned by each residue's hemisphere (8,11,12,14,20). For example, the centers of the oxygens for Asp-4899 were confined to 15 Å $< x <$ 25 Å in Fig. 1. The other residues in the model (Asp-4945 and Glu-4900) were modeled as regions of uniform regions of fixed charge (i.e., just a background charge and not as ions that take up space) because the pore radius where they were located was too wide for the residues to exert excluded-volume effects on the permeating ions; their presence was only felt electrostatically by the permeating ions.

Many important structural inferences were made from the first model (20) that were not changed in this model (e.g., selectivity filter radius of 4 Å and the location of Glu-4900 at the selectivity filter/lumenal vestibule interface and that its range of tethered movement overlapped with that of Asp-4899). Other structural parameters were constrained by known structural information (e.g., distance of Asp-4938 from Asp-4899 or distance of Asp-4945 from Asp-4938) or were chosen to have a reasonable value (e.g., range of tethered movement of side chains, location of Glu-4902, or pore radius in the cytosolic vestibule). The results were insensitive to the exact choice of these latter values. Given the constraints of the previous model and known structural information and the insensitivity of the other parameters, there were no adjustable parameters with respect to the structure in this model.

There were, however, some parameters for the ions that had be determined from the experimental data: the diffusion coefficients of the permeating ions and water are inputs to the PNP/DFT model. Because water does not contribute to the current and Cl^- does not permeate the channel, these were given diffusion coefficients of 1% of bulk within the pore. Previously it was shown that the results of the model did not change even when bulk diffusion coefficients were used (20). For the cations, three different diffusion coefficients were used within the pore, one in each of the following regions: in the cytosolic vestibule where Asp-4938 was confined (0 Å $< x <$ 10 Å), in the selectivity filter (10 Å $< x <$ 25 Å), and in the lumenal vestibule where Glu-4900 was confined (25 Å $< x <$ 32 Å). In all other regions, bulk (infinite dilution) diffusion coefficients were used. The resulting piecewise constant profile was smoothed as described (20).

For K^+ the three diffusion coefficients were determined by reproducing the experimental current in symmetric 250 mM KCl in native RyR (80 pA at +100 mV) and in the mutants E4900Q (10 pA at +20 mV) and D4839N (52 pA at +100 mV). The diffusion K^+ coefficients (from cytosolic to lumenal) were 122.1×10^{-11}, 6.91×10^{-11}, and 40.3×10^{-11} and m^2/s. For all non-K^+ cations (Li^+, Na^+, Rb^+, Cs^+, Mg^{2+}, and Ca^{2+}) only one diffusion coefficient was left undetermined by assuming that the ratio of bulk to cytosolic vestibule diffusion coefficients for K^+ was the same for all other cations and by assuming that the ratio of selectivity filter to lumenal vestibule diffusion coefficients for K^+ was the same for all other cations. The one open diffusion coefficient in the selectivity filter was determined for the monovalent cations by reproducing the current at +100 mV in 250 mM symmetric conditions: Li^+ (21.2 pA), Na^+ (48.1 pA), Rb^+ (71.5 pA), and Cs^+ (51.9 pA). For the divalent cations, selectivity filter diffusion

coefficient was determined by reproducing the current at –100 mV in 250 mM symmetric KCl and 10 mM lumenal divalent-chloride: Mg^{2+} (–31 pA) and Ca^{2+} (–33 pA). The selectivity filter diffusion coefficients were found to be: 1.29×10^{-11} for Li^+, 3.65×10^{-11} for Na^+, 5.92×10^{-11} for Rb^+, 4.18×10^{-11} for Cs^+, 0.42×10^{-11} for Mg^{2+}, and 0.41×10^{-11} m^2/s for Ca^{2+}.

While no molecular dynamics simulations to determine diffusion coefficients inside a highly-charged calcium channel have been performed, these values for the selectivity filter diffusion coefficients are consistent with those used in other models of RyR (17-19) and consistent with diffusion coefficients used in models of other highly-charged ion channels (13,44-47) and of other channels (48-52). These values are also consistent with the fact that diffusion coefficients are reduced below bulk values by a combination of both geometric confinement (53,54) and—to an even larger extent—by a highly-charged environment (55-57) like that found in the selectivity filter of RyR or other calcium channels.

After determining the three diffusion coefficients for K^+ and one diffusion coefficient for Li^+, Na^+, Rb^+, Cs^+, Mg^{2+}, and Ca^{2+} using exactly 9 experimental data points out of more than a thousand, the model reproduces all the permeation and selectivity data of RyR2 (the cardiac isoform of RyR) in over 100 different ionic solutions—some yet to be published—*without readjusting any parameters*. The comparison of the revised model and experimental data for two mole fraction curves and 55 current/voltage curves in pure monovalent-chloride, biionic, and monovalent/divalent mixtures in native and mutant RyR of Ref. (20) are shown here and in the main text. Comparisons of model results and previously unpublished experiment data will be published later.

The additional structural and mutation data have substantially improved the results of the model. The new model also reproduces the conductances of mutations not in the previous model without any adjustable parameters; in 250 mM symmetric KCl, the model conductance is 718 pS for D4945N (experimental 737±11 pS (31)) and 792 pS for E4902Q (experimental 782±4 pS (7)).

Details of the modeling not described here are discussed in Ref. (20).

Figs. 14-22 show the results of the model compared to experiments in 66 ionic solutions in both native and three mutants (D4899N, E4900Q, and D4938N). These experimental data have been published previously (7,20,31). Comparisons of the model to as-yet unpublished experimental data will be shown in future work.

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