DIVALENT EFFECTS ON PERMEATION AND GATING OF T-TYPE CALCIUM CHANNELS

By

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Cells carefully regulate their intracellular ion composition and their membrane potential. Voltage gated calcium channels change both of these properties by allowing $\text{Ca}^{2+}$ to enter the cell, causing membrane depolarization and the entry of $\text{Ca}^{2+}$ which acts as important signaling molecule. Much research over the last few decades has gone into discovering the different types of voltage gated calcium channels and their permeation and gating properties. Many models have been proposed to explain how ions permeate through calcium channels. The second chapter of this thesis uses a 2 binding site 3 barrier model of permeation to fit a wide range of permeation data previously collected from $\alpha$1G T-type calcium channels. Using this model of permeation we were able to fit permeation and block data well over a wide range of concentrations and voltages for four different ions ($\text{Ca}^{2+}$, $\text{Ba}^{2+}$, $\text{Mg}^{2+}$, $\text{Na}^{+}$).

T-type calcium channels are expressed in a wide range of cells in a diverse set of tissue types. In the third chapter of this thesis, electrophysiological measurements were made of the effects of block and permeation of $\text{Fe}^{2+}$ on $\alpha$1G channels and the model of permeation was extended to this physiological ion to examine the role that calcium channels can play in iron overload condition and in cadmium toxicity. $\text{Fe}^{2+}$ entry through calcium channels has potentially deleterious consequences as this entry
is unregulated and in iron overload condition where cells have an impaired iron efflux mechanism, calcium channels may play a role in increasing the labile iron pool inside a cell. This labile iron pool is unbound iron that is redox active and can cause oxidative damage to proteins and lipids. We observed currents through α1G calcium channels carried by Fe²⁺ showing that α1G calcium channels can allow ferrous iron (Fe²⁺) to enter into cells. Rates of Fe²⁺ entry in physiological concentrations of Fe²⁺ using the extended model of permeation showed that α1G calcium channels can be a mechanism for non transferrin bound iron entry.

The fourth chapter of this thesis focuses on the permeation and blocking properties of Cd²⁺ on α1G channels. Cd²⁺ is known to have cytotoxic effects and while the exact mechanism of Cd²⁺ toxicity is still being elucidated, cadmium is increasingly contaminating the environment leading to increased exposure to humans. Electrophysiology experiments show that Cd²⁺ permeates extremely well through the T-type calcium channel and only incompletely block the channel. The model of permeation was extended again for α1G channels to include Cd²⁺, and it was able to fit the data collected in Cd²⁺ and to predict the transport rate of Cd²⁺ through the channels in Cd²⁺ concentrations seen in populations exposed to environmental containment. The calculated rates of Cd²⁺ entry showed that in Cd²⁺ exposure, α1G calcium channels can be a mechanism for Cd²⁺ entry into cells.
CHAPTER 1

Introduction
1.1: INTRODUCTION

For a living system to function it must carry out a series of chemical and physical processes in a well orchestrated manner. A series of cellular structures, enzymes and signaling molecules are used to control the course of these processes, crucially the plasma membrane composed of an insulating lipid bilayer (Fricke, 1925; Gorter and Grendel, 1925). The plasma membrane allows the cell to control its intracellular composition by the regulated use of transporters and channels to store energy in the form of a concentration gradient. Transporters allow for the conversion of energy e.g. a gradient to chemical energy (ATP synthase), chemical energy to a gradient (Na\(^+\)/K\(^+\) ATPase), and one chemical gradient into a different chemical gradient (co-transporters).

An important aspect of understanding ion channels is to understand why cells and organisms express them. Ion channels allow ions to diffuse down their concentration gradients, an entropically favorable mechanism. This can be seen by the fact that current flow through the channels is spontaneous, therefore it must have a negative Gibbs free energy (Clausius, 1867; Gibbs, 1876). This increase in entropy must serve a useful purpose for evolution to have selected ion channels to be expressed ubiquitously in advanced organisms. For potassium channels the movement of ions down their concentration gradient (from inside the cell to outside) is used to create an electromotive force (emf) giving the inside of a cell a negative potential compared to the extracellular side. Boltzmann’s student Walter Nernst as first formulated the emf necessary to balance the rates of diffusion and drift.
\[ z \cdot q \cdot V = kT \cdot \ln \frac{[K^+]_o}{[K^+]_i} \]

Where the left side of the equation is the energy of a charged particle moving in an electric field, \( z \) is the valance of the charged particle, \( q \) is the elemental charge, and \( V \) is the voltage. The right side of the equation is the free energy of a concentration gradient where \( k \) is Boltmann’s constant, \( T \) is temperature, \( \ln \) is the natural log, \([K^+]_o\) is the concentration of potassium on the extracellular side and \([K^+]_i\) is the concentration of potassium on the intracellular side. Dividing both sides by \( z \cdot q \) gives the Nernst potential (\( E_K \)).

If potassium channels create an electrostatic potential from a chemical potential, it still leaves the question of why other channels are needed. Observing that the most critical roles sodium and calcium channels play are in synaptic signaling, action potential propagation, and calcium signaling it can be seen that sodium channels and calcium channels are used by an organism to signal and to process signals to help that organism survive. (Signaling by ion channels can be seen in action potentials where a signal is transmitted over a distance without loss of information and signal processing can be seen in synaptic signaling where a membrane potential change leads to an increase in intracellular \( \text{Ca}^{2+} \) from calcium channels leading to synaptic release of neurotransmitters). By coupling to an existing gradient, channels can perform fast signaling as they are limited only by ionic diffusion. With the exception of the channel thermogenin in brown fat, the purpose of which is to produce heat to maintain an organism’s body temperature; the energy associated with the opening of sodium and calcium ion channels is lost from a form that can be used for other means (i.e. a
concentration and an electrostatic gradient that can be used for the uptake of nutrients) to a form of energy unusable to the cell, and a dissipated concentration and electrostatic gradient that must be restored using ATPases. The role of sodium and calcium channels in an organism is to transmit and process information to control and coordinate different chemical and physical processes.

**1.2: BASIC STRUCTURE OF ION CHANNELS**

There are two main types of ion channels. Ligand-gated ion channels are regulated by chemical messengers and voltage gated ion channels are regulated by the membrane potential. Voltage gated ion channels have four domains that are independent proteins for potassium channels and one protein for calcium and sodium channels. Each domain is composed of 6 transmembrane alpha helixes. The forth helix (S4) contains basic amino acid residues that are positive in physiological solutions and respond to the voltage of the cell. When the cell is hyperpolarized, the positive charges are attracted to the negative charge inside the cell and the S4 is more thermodynamically stable in the ‘down’ state. As the cell is depolarized, the S4 is less attracted to the inside of the cell and transitions to the ‘up’ state, where it moves through the electric field and the positive charges are located towards the extracellular side. The pore domain is composed of the S5-S6 transmembrane helixes and the extracellular P loop between them. The two main properties of ion channels are how ions permeate through the channel and how the channel gates.

**1.3: VOLTAGE GATED CALCIUM CHANNELS**
Calcium channels allow for the rapid influx of Ca\(^{2+}\) into a cell. Because cells keep the intracellular Ca\(^{2+}\) concentration low this acts to not only depolarize the cells but allows Ca\(^{2+}\) to act as a rapid signaling molecule. Classic examples of this are at the synapse of neurons and muscle contraction in myocytes where calcium channels convert an action potential signal into an increase in intracellular Ca\(^{2+}\) that coordinates vesicular release or the contraction cycle.

### 1.4: T-TYPE CALCIUM CHANNELS

Early experiments with calcium channels showed that there were two distinct types of calcium channels based on their electrophysiological characteristics (Hagiwara et al., 1975; Llinas and Yarom, 1981). Some calcium channels were found to activate at low voltages (~ -70 mV), have small single channel conductances, and inactivate relatively quickly and completely. They were called low voltage activated (LVA) or T-type (for transient or tiny) calcium channels, in comparison to high voltage actived (HVA) calcium channels which are actived at higher voltages (~ -20 mV). Three T-type calcium channels have been cloned; α1G (Perez-Reyes et al., 1998), α1H (Cribbs et al., 1998), and α1I (Lee et al., 1999). The three channels show very similar properties with the exception that α1I has activation and inactivation kinetics that are ~ 10 times slower than the other two (Frazier et al., 2001), and α1H has an extracellular site that binds a diverse set of metal ions to inhibit the channel (Kang et al., 2006).

Using Northern blots T-type calcium channels were found to be strongly expressed in human brain, ovary, and placenta tissue and weakly in testis, small intestine, colon and
the heart (Perez-Reyes, 2003). Analysis of human fetal tissue shows increased α1G expression in the heart, spleen, lung and kidney (Monteil et al., 2000) compared to adult tissue. In addition to changes during development, T-type calcium channels also show expression changes during the cell cycle (Day et al., 1998) and during cell proliferation (Rodman et al., 2005b). Certain disease states have also shown an increase in T-type expression including in hypertrophied hearts (Nuss and Houser, 1993) and in vascular smooth muscle cells during hypertension (Self et al., 1994).

1.5: PERMEATION

Permeation is how ions pass through an ion channel down their concentration gradient. The concentration gradient provides the driving force for ion movement and the ion channel provides the regulated pathway for the ions to transverse through the cell membrane. One of the main characteristic of an ion channel is its ion selectivity. Ion channels are able to be highly selective in the ion they allow to permeate while still allowing ions to pass through near the diffusion limit. Different ion channels use different mechanisms to achieve this depending on what ion they are selective for and how selective they are for a particular ion. There are three main differences between ions, their size, charge, and coordination chemistry (how the electron orbital shell interacts with ligands).

1.6: POTASSIUM CHANNEL PERMEATION

The most work on permeation has been on the selectivity of K⁺ over Na⁺ in potassium channels. K⁺ has the same charge as Na⁺ and is only 0.4 Å larger but potassium channels are able to select the larger ion over the smaller one while passing
K⁺ ions through the pore near the diffusion limit. The ion channel must be in contact with the ions to be so highly selective, therefore the ions must dehydrate at least partially before they enter the pore and the pore must be almost as narrow as the potassium ions (Hille, 2001). The difference in size has been postulated to account for the selectivity difference but this is hard to account for the large difference in selectivity seen for such a small size difference especially give that the pore is not a rigid pore but is a protein that in physiological conditions is undergoing thermal fluctuations. Using molecular dynamic simulations of the potassium channels it was shown that the fluctuations of the selectivity filter in potassium channels underwent movements larger than the difference in the radius between Na⁺ and K⁺ (Berneche and Roux, 2001). This is in agreement with the B-factor of the potassium channel KcsA which indicates the pore can fluctuate on the order of 0.75 to 1 Å (Zhou et al., 2001). This makes it unlikely that the selectivity filter would be able to select based on size alone at the speed the ions diffuse through the pore. The mechanism of selectivity now gaining acceptance is that the potassium channel selects for K⁺ over Na⁺ based on the difference between their optimal coordination number (Varma et al., 2008). K⁺ thermodynamically ‘prefers’ (has a lower free energy) when binding with 8 weak ligands (4 crowned on top and the bottom of the ion in a square planar formation) while Na⁺ prefers 6 ligands (4 in a square planar configuration around the center of the ion and 1 on either end) (Varma and Rempe, 2008).

This is supported by the crystal structure that shows the ionic pore is lined with carbonyl backbones of amino acids that point into the lumen of the conduction pathway (Zhou et al., 2001). This gives a series of steps that are energetically
unfavorable for Na\(^+\) compared to solution, as the ion channel cannot replace in the proper coordination the water molecules of its hydration shell with the carbonyl groups. K\(^+\) on the other hand coordinates water in the same configuration that the carbonyl backbones form so there is very little energetic difference between K\(^+\) in the aqueous solution and the protein, allowing it to flow at the diffusion rate through the pore (Aqvist and Luzhkov, 2000). This gives the first strong evidence that the selectivity filter of an ion channel uses coordination chemistry to select between very similar ions.

It is important to keep in mind when using thermodynamics that it is the free energy of the entire system that is to be minimized. For ion channel selectivity then it is not the simple explanation that the free energy of K\(^+\) in solution is the same as the free energy of K\(^+\) in the selectivity filter of a potassium channel, and that Na\(^+\) has a lower energy in solution than in the selectivity filter. This neglects the free energy change associated with the protein when different ions bind in the selectivity filter (Bostick and Brooks, 2009). Even though the crystal structure of multiple potassium channels have been deciphered and numerous techniques and theories have been used to discover how potassium channels can discriminate Na\(^+\) from K\(^+\) there is still a lot of unanswered questions.

1.7: CALCIUM CHANNEL PERMEATION

One of the key features observed for calcium channel permeation is that they can pass a large amount of monovalent cation current in the absence of any divalents. As Ca\(^{2+}\) is increased into the µM, range the current through calcium channels
decreases. As the concentration of Ca\(^{2+}\) is increased into the millimolar range, the current through the calcium channels increases again see Figure 2.5 (Almers and McCleskey, 1984). These two effects can be explained by the selectivity filter of the calcium channel allowing relatively large monovalent currents to permeate in the absence of divalents, binding one Ca\(^{2+}\) ion with high affinity to block the large monovalent currents, and two Ca\(^{2+}\) ions with lower affinity that allows Ca\(^{2+}\) to pass through the channel at a fast speed. Assuming Ca\(^{2+}\) enters the pore at the diffusion limit, with a K\(_D\) of 1 µM for Na\(^+\) block, a \(k_{off}\)

\[
k_{off} = k_{on}(at[Ca^{2+}]=1\mu M)\approx10^{-6} M \times 3 \times 10^8 M^{-1} s^{-1} = 300 s^{-1}
\]

can be calculated where Ca\(^{2+}\) will exit the pore at approximately 300 ions per second. This is around 1000 times slower than the current seen through calcium channels in physiological solutions. This would suggest that \(k_{off}\) is 1000 times faster when the pore of the ion channel will be occupied by two Ca\(^{2+}\) ions, i.e. K\(_D\) ~ 1 mM for two Ca\(^{2+}\) ions.

1.8: STRUCTURAL DETERMINANTS OF CALCIUM CHANNELS

The key motif of the permeation pathway for calcium channels was discovered to be four acidic residues in the selectivity filter in the extracellular loop between S5 and S6 that forms the pathway ions transverse through the membrane (Schlief et al., 1996). This structure explains the permeation property observed that one Ca\(^{2+}\) binds strongly in the pore and two Ca\(^{2+}\) ions with less affinity. With four acidic residues in the pore, the high density of negative charge will strongly interact with any counter cations. For one Ca\(^{2+}\) ion in the pore the strong negative charge would make it
energetically favorable for the Ca\(^{2+}\) ion to stay in the pore due to the enthalpic energy from the electrostatic forces. For two Ca\(^{2+}\) ions in the pore, the enthalpic costs for one Ca\(^{2+}\) leaving will be much less, hence the lower affinity and faster \(k_{off}\). The structure of the permeation pathway of the calcium channels is dynamic and dependent on the availability of Ca\(^{2+}\). In the presence of calcium the channels appear to have a pore of 3.4 Å in diameter, but in the absence of Ca\(^{2+}\) the large (~6 Å in diameter) cation tetramethylammonium can pass through the pore (McCleskey and Almers, 1985). This is most likely due to the channel pore being very electrostatically charged from the 4 acidic residues in the pore. The negative charges of the carboxyl groups would repel each other, widening the pore of the channel when there is low concentration of counter divalent cations to screen the negative charges from each other.

1.9: SELECTIVITY AMONG DIVALENT

Being able to distinguish between monovalents and divalents is a critical component in understanding permeation in calcium channels, but calcium channels are also able to distinguish among different divalents. It was discovered early on that some of the other group II elements from the periodic table (Sr\(^{2+}\) and Ba\(^{2+}\)) could permeate about as well as Ca\(^{2+}\) through calcium channels (Hagiwara et al., 1974). Interestingly, while calcium channels are able to pass large currents of Ca\(^{2+}\) or Ba\(^{2+}\) when they act as the sole charge carrier, when a solution of the two ions are mixed but the total concentration of \([\text{Ca}^{2+}] + [\text{Ba}^{2+}]\) is held constant the current decreases for certain mixtures. This anomalous mole fraction effect (AMFE) has been shown on the single channel level of L-type calcium channels (Friel and Tsien, 1989). The explanation for the AMFE on the single channel conductance is that the ions permeate
through a single file pore with multiple binding sites and the decrease is due to ion-ion interactions in the pore (Eisenman et al., 1986).

Currents through calcium channels with Ba\(^{2+}\) and Sr\(^{2+}\) were not surprising given their very similar properties including their ionic radius and coordination chemistry from being in the same column of the periodic table. It was found that Mg\(^{2+}\) did not permeate well and blocks the pore from conducting current unlike the other group II elements (Hagiwara and Takahashi, 1967). Mg\(^{2+}\) differs from Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) in three main ways, first Mg\(^{2+}\) has a much smaller effective ionic radius (72 pm) than Ca\(^{2+}\), Ba\(^{2+}\), or Sr\(^{2+}\) (100, 135, and 118 pm respectively), Mg\(^{2+}\) has a much larger dehydration energy because of its smaller size, and Mg\(^{2+}\) is in the 3\(^{rd}\) period of the periodic table so that its outer electron shell can not hybridize with a d orbital shell (Shannon, 1976). The smaller size leads to a much stronger electrostatic field in Mg\(^{2+}\) because the charge is spread over a smaller volume. Also the difference in the ability to hybridize with the d orbital will changes the coordination chemistry of Mg\(^{2+}\) compared to Ca\(^{2+}\), Ba\(^{2+}\), or Sr\(^{2+}\). While Mg\(^{2+}\) can hybridize its s and p orbitals the permeate ions are able to hybridize the s, p and d orbitals which may form a radically different outer electron shell configuration (Glendening et al., 2012).

Other divalents were also show to block currents through calcium channels including Zn\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\), and Ni\(^{2+}\) (Hagiwara and Takahashi, 1967) in early studies that were done on a diverse set of tissues and conversely a diverse set of calcium channels (Akaike et al., 1978; van Breemen et al., 1973). It was shown that Mn\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\) and Be\(^{2+}\) could permeate through the calcium channels in the muscle fibers of a beetle, while Co\(^{2+}\), Ni\(^{2+}\), and Mg\(^{2+}\) could not (Fukuda and Kawa, 1977).
While some divalents were classified as blockers it was found that strong hyperpolarizing potentials could relieve block of calcium channels by certain divalents (Swandulla and Armstrong, 1989) suggesting that the blockers were also permeate but they permeated at a much slower rate than Ca\(^{2+}\) or Ba\(^{2+}\). Diverse response of divalents to calcium channels to different divalent cations is well known, but there is no unifying theory to explain the effects of different divalents on calcium channels.

It was found that how ions bound in the pore and ion – ion competition in the pore could change how divalent blockers could affect the current. In an early study on calcium channels in barnacle muscle fibers it was found that while the currents were larger with Ba\(^{2+}\) as the charge carrier compared to Ca\(^{2+}\), the Ba\(^{2+}\) currents were blocked more strongly by Co\(^{2+}\) (Hagiwara et al., 1974). This can be explained by the pore binding Ca\(^{2+}\) more strongly than Ba\(^{2+}\), which would cause the exit rate of Ca\(^{2+}\) from the pore to be slower causing a smaller current but in the presence of Co\(^{2+}\), Ca\(^{2+}\) could bind more strongly and out compete Co\(^{2+}\) for binding sites in the pore leading to less block. A similar phenomenon occurs in T-type calcium channels where macroscopic currents carried by Ca\(^{2+}\) and Ba\(^{2+}\) look similar but Mg\(^{2+}\) is able to block currents carried by Ba\(^{2+}\) more strongly than it could block Ca\(^{2+}\) currents (Serrano et al., 2000).

1.10: MODELS OF PERMEATION

There have been many theories and models of how ions permeate through ion channels. The earliest models of transport across lipid membranes treated the membrane as a semipermeable barrier and used Fick’s first law to calculate fluxes
(Orbach and Finkelstein, 1980; Overton, 1902). Observing that cells had a negative resting potential due to the concentration gradient of potassium ions it was discovered in 1902 that the lipid membrane could have different permabilities for different ions (Bernstein, 1902). Later it was discovered that the permeability of the membrane could change with time (Cole and Curtis, 1939; Hodgkin and Katz, 1949). This gave rise to a model where the membrane had a time dependent conductance for different types of ions that would allow the ions to be transported down their concentration gradient (Hodgkin and Huxley, 1952b). The Goldman-Hodgkin-Katz equation was the first comprehensive equation based on physical parameters to explain ion flux across a membrane (Goldman, 1943; Hodgkin and Katz, 1949).

Later when it was discovered that fluxes across membranes are mediated by proteins in the form of ion channels and transporters, the study of permeation changed to include modeling of how these proteins worked. A different set of approaches have been used to model permeation through ion channels with various levels of approximations and assumptions. The most common methods used for ion channels are reaction rate theory, continuous electrodiffusion theory, Brownian dynamics, and molecular dynamics (reviewed in the next two sections). Developing an accurate model of permeation has the potential to help many fields of study. Using electrophysiological data the currents can be measured but only the net charge movement is measured not the specific ions that are carrying the charge. A model of permeation would allow the details of what ions are being transported to be elucidated, leading to a more comprehensive understanding of how all physiologically relevant ions are distributed in the human body.
In addition to calcium other divalents are physiologically important including magnesium, iron, copper, and zinc (Mertz, 1981). Reports have shown that misregulation of how trace metal ions are transported and where they accumulate are associated with neurological diseases such as amyotrophic lateral sclerosis, Alzheimer’s, Parkinson’s, Huntington’s, and prion diseases (Berg and Hochstrasser, 2006; Bourassa and Miller, 2012; Kell, 2010), glomerular diseases and chronic kidney failure (Marumo and Li, 1996; Shah, 2010), numerous cardiac diseases (Cavill, 2003; Iseri et al., 1975; Rubart and Zipes, 2005), liver diseases (Fujita and Takei, 2011; Suzuki et al., 1996), and in diabetes (Chen et al., 2009; Joshi and Shrestha, 2010) to name a few. Having a comprehensive understanding of how ions are transported into and out of cells would help to understand how changes in trace metal ion transport affect human health.

Ion channels are also important in numerous tissues where they control the membrane potential including in muscle, neurons, and pancreatic cells where they can cause excitation and couple that excitation to contraction or secretion by allowing Ca\(^{2+}\) entry. Having a model of permeation would allow more accurate physiological models of these cells. Transport through ion channel and artificial narrow channels in solution is also being researched for industrial and biomedical applications in biosensing to detect different solutes including drugs, proteins, and viruses (Krishnamurthy and Cornell, 2012; Moradi-Monfared et al., 2012; Oh et al., 2008); for high throughput DNA sequencing (Luan et al., 2012; Reiner et al., 2012; Reisner et al., 2012); and for solution control and desalination (Gong et al., 2010; Richards et al.,
A comprehensive model of permeation would help to fully develop channels as a tool for these applications.

1.11: EYRING RATE THEORY MODEL

An Eyring rate theory model assumes that ion channels can be modeled as ions transitioning into, out of and between different sites in an ion channel. The first model to use Eyring rate theory for calcium channels was presented in Almers and McCleskey 1984, see Figure 2.1. This model was able to reproduce the observation that one Ca\textsuperscript{2+} ion binds strongly to the pore while 2 bind less strongly. The way Almers and McCleskey’s model did this was intuitive, the first ion in the channel was bound in an energy well and when the second ion entered the channel an ion repulsion factor (Q) was used to model the electrostatic repulsion between the two ions. The ion repulsion factor used in the original paper was 11.89 that was scaled the same as the electrostatic force, i.e. for two divalents q\textsubscript{1} = q\textsubscript{2} = 2 the ion repulsion factor is raised to the power of q\textsubscript{1} \cdot q\textsubscript{2} = 4 which increased the exit rate of Ca\textsuperscript{2+} when are two ions compared to one Ca\textsuperscript{2+} in the pore (Tsien et al., 1987).

In a 2-site 3-barrier model of permeation most of the rates for ion movements have to be estimated from fitting the model to data, there is no way \textit{a priori} to calculate the parameters of the model given how little is known about the structure of the pore or a calcium channel. This gives the model many free parameters to fit, a drawback to the model but one that can be countered by fitting the model to a large data set to constrain the parameters of the model. Because the model uses average rates calculated by fitting to data, processes that may be important for ion permeation
such as thermal fluctuations and coordination chemistry can be taken into account. The main disadvantage of the 2S3B model is that there are not 2 distinct binding sites within a fixed distance of the ion channel. It was shown by mutational studies that the four acidic residues form a single binding site for blocking monovalent currents (Ellinor et al., 1995). This can be overcome by a more complicated Erying rate theory model but at the expense of a much larger number of free parameters (see conclusions).

A larger 3 Binding Site 4 Barrier Erying rate theory model (3S4B) has also been proposed to fit calcium channel permeation data (Dang and McCleskey, 1998). This model has one high affinity site in the center and two low affinity sites on either side. The 3S4B model has a much larger number of binding sites that increase the number of different states and transition rates in the permeation model that makes the computing and confinement of the parameters extremely difficult. An attempt was made to use this model to fit a large permeation data set from α1G calcium channels but the large number of states made matrix methods produce large numerical errors and the large number of parameters made other optimization techniques not feasible due to the large amount of time needed to calculate all parameters. In addition, the lack of repulsion factor made the Ca$^{2+}$ entry and exit rates too slow to fit single channel data in low Ca$^{2+}$ where the entry and exit rates can be measured.

1.12: OTHER PERMEATION MODELS

Molecular dynamics has also been used to study the permeation through ion channels on an atomically detailed level. Molecular dynamics of ion channels poses a
problem because of the large number of atoms needed, the long times needed to simulate to observe ions passing through the pore, and the need for the crystal structure (or having to use a homology model of a crystal of a similar protein). To overcome these problems approximations in the simulations have been used. In real ion channels, the electromotive force is caused by an imbalance of charged ions, but given the large area need to simulate the solutions properly an external electromotive force is often applied in the simulations (Jensen et al., 2010; Roux, 2008). In addition, the boundary conditions of molecular dynamic simulations are often set so that transport out of one side of the simulation is modeled as transport into the other side. This gives a uniform solutions on the boundaries but poses a problem for ion channels that need two different solutions to function properly (Kutzner et al., 2011; Roux, 2011). Recently a new method for performing simulations was developed that overcame these two assumptions and used two different bulk solutions and modeled over 100 ns for the bacterial channel PorB (Kutzner et al., 2011). While the timescale used was relatively short (100 ns) this level of modeling is useful to validate and understand some aspects of permeation.

Brownian dynamics been used to study ion flux through channels at longer times scales (Roux et al., 2004). This method of simulation achieves longer timescales by making two main assumptions to lower the computation costs for each time step. The first is that Brownian dynamics does not model the water as explicit molecules but models the water as a continuum and the interactions between water and the ions is modeled as frictional and random forces (Roux et al., 2004). Brownian dynamics also fixes the atoms of the protein in place and does not model their movements as
molecular dynamics does. It is assumed that the ions moving through the pore move fast compared to the protein fluctuations (Gordon et al., 2012).

For the Poisson-Nernst-Planck model, the channel is constructed as a rigid solid of a fixed permittivity. The model calculates the equilibrium concentrations of ions in solution and in the constructed channel pore as a continuum to calculate the current through a channel. It also must model the channel as a rigid solid with a fixed shape and a permittivity coefficient (\( \varepsilon_c \)) that must be estimated for there is no empirical way for it to be calculated (Corry et al., 1999). The biggest criticisms of Poisson-Nernst-Planck theory models is that by modeling the ions as a continuum it ignores the effects of induced charges on the surface of the ion channel caused by ions approaching a medium of low permittivity from a medium of high permittivity (Krishnamurthy and Cornell, 2012). This error becomes large as the ion channel modeled becomes narrower and the induced surface charges become large. The Poisson-Nernst-Planck, molecular dynamics and Brownian dynamics models all treat ions as perfectly spherical, and can not account for the coordination chemistry of different ions.

1.13: GATING

The permeation pathway allows for the flow of ions down its concentration gradient into the cell. This process is highly regulated by the gating mechanism of the channel. Voltage gated ion channels are gated by four voltage sensor domains that senses the voltage across the cell membrane. There are three main states that voltage gated channels can take, deactivated, activated and inactivated. The ‘deactivated’ state
is often called ‘closed’, but both deactivated and inactivated states are usually assumed impermeable to ions.

The deactivated state is observed at hyperpolarized potentials and represents a state where the S4 helix is in the down state; the channel is not passing current but can be activated by depolarizing the potential across the membrane. A channel activated by depolarization will have its S4 move to the up position, which couples to the activation mechanism, and the channel will be able to conduct current. The activation mechanism has been located on the intracellular side of potassium channels from experiments and the crystal structure shows it is composed of the S5-S6 ends of each domain (Jiang et al., 2003; Miller, 1987). The activation gate has also been found to be on the intracellular side of T-type calcium channels by electrophysiological experiments (Obejero-Paz et al., 2004). Activated channels can undergo the process of inactivation where the channels stop conducting current even at depolarizing potentials where the S4 is in the up position. The structural mechanism for inactivation of calcium channels has not been elucidated to date. Both Carlos Obejero-Paz and I attempted experiments to discern topologically where it is in relationship to the selectivity filter unsuccessfully (unpublished data), though some have proposed the inactivation gate is on the intracellular side and is formed by the domain I-II loop (Perez-Reyes, 2010). For a channel to conduct current again it must recover from inactivation by being held at hyperpolarizing voltages for a period of time.

1.14: IRON IN PHYSIOLOGY
Iron is an essential metal for biological life playing a role in enzymatic structure and function along with cell signaling and oxygen transport. Iron is regulated on two levels, the organism, and the cellular level. In humans iron is absorbed through the placenta as a fetus and a newborn has ~270 mg of iron (Rios et al., 1975). This level is reduced in pre-term infants because iron transfers from the maternal stores occurs primarily during the third trimester (Leong and Lönnerdal, 2012) and early clamping of the umbilical after birth prevents ~100 mL of blood returning from the placenta within the first 3 minutes of birth, causing ~33% reduction in total body iron of the newborn (Chaparro et al., 2006). Total iron levels of newborns is also reduced in anemic mothers (Singla et al., 1996) but not iron deficient mothers (Harthoorn-Lasthuizen et al., 2001).

The average adult human has ~3-5 grams of total body iron. Beyond the iron stores a human is born with, the rest must be absorbed through the intestinal wall from dietary sources (Leong and Lönnerdal, 2012). Iron absorption must be highly regulated as the body has no regulatory mechanism for iron excretion during excessive iron uptake (Kontoghiorghes and Kolnagou, 2005) and excess iron can cause oxidative damage. Iron is absorbed in the upper part of the intestine through the epithelial cells (enterocytes) by two main mechanisms, heme bound and non-heme bound iron uptake. Heme bound iron is found abundantly in meat products as hemoglobin and myoglobin and is absorbed more efficiently by the body than non-heme bound iron (Bezwoda et al., 1983). Heme is thought to be taken up from the lumen by receptor mediated endocytosis or by a transporter, though the exact transporter responsible is still debated (Fuqua et al., 2012; Shayeghi et al., 2005). Heme oxygenase breaks down heme in the
enterocytes releasing its iron into the intracellular iron pool. Iron is also absorbed into enterocytes in its free form by the divalent metal ion transporter 1 (DMT1) protein expressed on the luminal side of enterocytes (Gunshin et al., 2005). Iron is reduced to its ferrous form (Fe$^{2+}$) on the brush border by duodenal cytochrome B and possibly other reductases to facilitate its transporter by DMT1 (McKie, 2008). Intracellular iron in the enterocytes is then stored bound to the protein ferritin or exported into the blood by the transporter ferroportin (FPN1) (Ward and Kaplan, 2012). Because FPN1 exports ferrous iron but the main iron binding protein in the blood, transferrin binds iron in the ferric form (Fe$^{3+}$) enterocytes express a multicopper ferroxidase hephaestin on their apical side to convert Fe$^{2+}$ to Fe$^{3+}$ (Vulpe et al., 1999).

Reticuloendothelial macrophages are also a major source of iron into the plasma as they recycle iron from senescent red blood cells (Knutson et al., 2005). To control non-hemoglobin iron levels in the plasma the liver produces a 25 amino acid peptide hormone, hepcidin (Nemeth and Ganz, 2009). Hepcidin circulates in the blood and binds to the extracellular domain of FPN1 causing its internalization and degradation, reducing iron export into the plasma (Nemeth et al., 2004). Hepcinin is released in response to iron loading in the liver, to prevent iron uptake from the enterocytes and also in response to bacterial infection, to reduce the plasma iron levels, depriving pathogens of a vital co-factor (Johnson and Wessling-Resnick, 2012). “As there is no active mechanism to excrete iron from the body”, the regulation of iron influx has to be carefully balanced with iron lost from the body (Kohgo et al., 2008). Iron loss for men is approximately 1 mg per day with the major pathways of iron loss being hemoglobin passing through the bowel (~0.4 mg), ‘mucosal’ iron loss passing
through the bowels (~0.15 mg), shedding epidermal cells (~0.1 mg), excreted in urine (~0.1 mg) and sweat (~0.1 mg) (Green et al., 1968). The only pathway for excretion that was increased during high saturation levels of transferrin was uptake and loss by skin cells, which increased iron loss from 0.1 to 0.7 mg of iron a day (Green et al., 1968). Adult women lose on average 0.5 mg more iron a day than men due to the menstrual cycle and the associated blood loss (Leong and Lönnerdal, 2012). During pregnancy women also lose an additional 500-600 mg of iron due to fetal uptake (~245 mg), placental and cord iron formation (~75 mg), and blood loss during delivery (150-250 mg). Iron can also be lost due to gastrointestinal bleeding from lesions or the use of non-steroidal anti-inflammatory drugs (Baccini et al., 2006). Occult blood lose in the gastrointestinal tract is especially problematic for infants who have allergic reactions to the proteins in cow milk (Breastfeeding, 2005) and contributes to the epidemic of anemia in developing countries where it is present in 42% of preschool-aged children (Iannotti et al., 2006).

Iron circulates in the plasma bound to transferrin, a soluble protein with two high affinity iron binding domains \( (K_D \sim 10^{23} \text{ M for } Fe^{3+}) \). Transferrin bound to \( Fe^{3+} \) is then able to bind to the transferrin receptor expressed on the surface of most cells. The transferrin - transferrin receptor complex is then taken up by most cells through receptor mediated endocytosis (Bartnikas, 2012). The acidity of endosome lowers the affinity of transferrin for iron causing its release where DMT1 transporters on the vesicle membrane can pump it into the cytosol (Gkouvatsos et al., 2012). Cells regulate their iron uptake by regulating the amount of transferrin receptors expressed on their surface. Most iron in a cell not being used as enzyme cofactors is stored
bound to ferritin. The excess non-protein bound iron in the cell is called the labile iron pool, a dynamic concentration that is the result of iron entry, exit and storage mechanisms (Salgado et al., 2010). While the labile iron pool is normally held at very low levels in a healthy individual, it is redox active and can cause hydroxyl free radicals through the Fenton reaction.

Other mechanisms of non-transferring mediated iron entry have also been discovered including, calcium channels (Gaasch et al., 2007a), TRP channels (Mwanjewe and Grover, 2004), and NMDA receptors (Pelizzoni et al., 2011). This mechanism of iron entry is very low for most cells because the free iron concentration of iron in the plasma is extremely low except in the case of iron overload (see following section).

1.15: IRON IN THE BRAIN

One area of current interest in iron uptake is in the brain (Mills et al., 2010). Unlike peripheral cells, neurons are exposed to the cerebral spinal fluid (CSF), not to the blood because of the blood-brain barrier. The blood-brain barrier transports iron into the CSF and while there is controversy over the exact mechanism of how the epithelial cells of the blood vessels in the brain transport iron there is strong evidence that iron is released into the CSF in its free form (Bradbury, 1997). In contrast to the plasma, the CSF has more iron than what transferrin can bind (Moos and Morgan, 1998). Iron in also more likely to be in its Fe\(^{2+}\) oxidative state due to the presence of high levels of ascorbic acid which reduces Fe\(^{3+}\) to Fe\(^{2+}\), and low levels of ceruloplasmin which oxidizes Fe\(^{2+}\) to Fe\(^{3+}\) (Qian and Shen, 2001). The internal
composition of the CSF suggests there is a substantial pool of Fe$^{2+}$ only loosely bound to ligands such as ascorbic acid and ATP (Bradbury, 1997). Measurements of total iron in the CSF range from 0.2 to 2.5 µM for different subjects (Bleijenberg et al., 1971; Felgenhauer, 1974) with measurements of the ‘loosely bound iron’ being measured as 0.55 ± 0.27 µM (Gutteridge, 1992). The question arises: does this loosely bound iron play a role in iron homeostasis of the cells in the brain? There is growing evidence it does. Firstly, almost completely depleting transferrin from the CSF of rat by knocking out functional oligodendrocytes leaves most regions of the brain able to take up iron at a similar rate compared to wild type rates (Connor et al., 1987; Gocht et al., 1993). Secondly, astrocytes, oligodendrocytes, and microglial cells show almost not transferrin receptor expression in the adult mouse (Moos, 1996). Therefore, there must be another mechanism of iron uptake in the brain other than the transferrin receptor mediated iron entry commonplace in the peripheral tissue.

Understanding iron fluxes in the brain has taken on considerable significant as it has been found that iron accumulation is associated with the development and progression of Alzheimer’s disease (Cornett et al., 1998; House et al., 2007; Loeffler et al., 1995), Parkinson’s disease (Berg and Hochstrasser, 2006; Jenner and Olanow, 1996; Youdim et al., 1993), multiple sclerosis (Craelius et al., 1982; Ge et al., 2007), and allergic encephalomyelitis (Forge et al., 1998; Xu et al., 1998). Whether the increase in iron is a cause or an effect of the various diseases has not been established but independent of a diagnosed disease, the human brain is found to accumulate iron as it ages and that an increase in brain iron is correlated with poor performance on motor and cognitive tasks (Pujol et al., 1992).
There is very little known about the physiology of how iron is distributed in the brain. Various mechanisms of iron uptake have been reported including direct uptake of Fe$^{2+}$ by DMT1 into neurons (Cheah et al., 2006) though it was shown that this may have been an artifact and that DMT1 in neurons is only expressed on early endosomes (Pelizzoni et al., 2012). Additionally it has been shown in two studies that voltage gated calcium channels could allow iron uptake into cultured neurons (Gaasch et al., 2007b; Pelizzoni et al., 2011). In both studies, they show an L-type channel blocker could reduce the uptake of iron into neurons compared to controls using fluorescent and radioisotope measurements. This introduces an interesting facet of iron handling in neurons, if calcium channels opening allow Fe$^{2+}$ from the CSF to enter the cell in an activity dependent manner then neurons would have to regulate the efflux of Fe$^{2+}$ out of the cell to balance iron uptake with iron efflux.

This is very different from how peripheral cells maintain iron homeostasis, where they regulate the uptake of iron on the cellular level and the rate of efflux of the cell is maintained by the organism’s hepcidin levels. In neurons, it could be that iron entry through calcium channels is governed by the opening of the channels in the neurons and not by any specific iron regulated pathway. This would put great dependence on the rate of iron efflux being regulated properly and could explain the increasing iron concentrations in disease states and the aging brain.

One consequence of this mechanism of iron handling is that neurons would not want to express iron-sequestering proteins such as ferritin. Ferritin binds free iron and prevents it from causing redox damage. Bound iron will also be unavailable for export from neurons by ferroportin. Normally as iron levels increase in a cell ferritin will be
upregulated by iron-responsive proteins (IRPs). IRPs are cytosolic proteins that bind to mRNAs of proteins involved in iron handling at their iron-responsive elements (IREs). Cells deficient in iron will have IRPs bound to the 5’ end of ferritin and ferroportin mRNAs, suppressing the expression of these proteins while cells with high iron levels will not bind to the IREs and allow the expression of ferritin and ferroportin (Anderson et al., 2012). Normally IRPs will also bind to the transferrin receptors mRNAs causing its down regulation when cellular iron is high. If this mechanism was used in cells that had unregulated iron entry that could not be down regulated by IRPs then iron would increase in a continuous manner leading to excessive amounts of iron in the cell.

In agreement with a mechanism of unregulated iron entry through calcium channels, most neurons do not express cytosolic ferritin, to prevent excessive iron build up in the cells (Cheepsunthorn et al., 1998). The ferritin that was found to be expressed in neurons was localized to the nucleus where it has been shown to prevent oxidative damage caused by damage and may not be related to cytosolic iron storage (Cheepsunthorn et al., 1998). The lack of ferritin would allow iron entering the cell to be transported back out efficiently by ferroportin but would leave the cell unable to sequester intracellular free iron where it can cause redox damage. This mechanism would also suggest they that neurons have no way of storing iron but it was found that neurons do express the mitochondrial form of ferritin(Snyder et al., 2010) but this form or ferritin lacks any IREs and is regulated independently from cytosolic free iron levels (Levi et al., 2001). The iron taken in by calcium channels would have to be effluxed at the same rate to prevent iron build up. Ferroportin was found to be
expressed widely in neurons and that it was even found to be expressed on presynaptic vesicles (Wu et al., 2004), where it could theoretically be able to directly couple calcium channel activity leading to iron influx to iron efflux through vesicle release.

**1.16: IRON OVERLOAD**

Iron is absorbed from the diet through the small intestine. This step is highly regulated to balance iron intake with iron loss (Fleming and Ponka, 2012). High iron levels normally cause the liver to release hepcidin, which binds to the iron transporter ferroportin (Fpn) and causes its internalization and degradation. When this occurs on the enterocytes of the small intestine it prevents the uptake of dietary iron (McLaren et al., 1991). Hemochromatosis is a disorder where excess iron is absorbed into the body. This can be caused by mutations in numerous proteins on the hepcidin-FPN degradation pathway that leads to increased iron absorption in the gastrointestinal tract even with high body iron levels. This misregulation causes the total body iron in the human to increase from the normal 5 grams seen in healthy individuals to over 25 grams in severe iron overload cases (Flaten et al., 2012). The excess iron deposits first in the liver and then in various other organs including the heart, pancreas and joints where it builds up and can cause organ failure. Clinical symptoms usually do not occur until after 30-60 years of life when total body iron content exceeds 15 grams (Aaseth et al., 2007). Symptoms of iron overload include inflammation of the joints, especially in the fingers, ankle and foot, enlargement of the liver, diabetes mellitus, decreased secretion from the pituitary and the gonads, impotence, heart failure, liver failure and liver cancer (Niederau et al., 1996).
Hemochromatosis is probably the most common genetic disorder in northern European populations (Flaten et al., 2012). The primary treatment for hemochromatosis is phlebotomy to remove iron bound in the hemoglobin of red blood cells to deplete the iron in the body to normal levels (Aruoma et al., 1988). The treatment involves removing ~500 mL of blood once a week until serum ferritin levels fall to ~50 µg/L. After serum ferritin levels have fallen, one blood draw every three months is usually sufficient to prevent iron overload symptoms (Barton et al., 1998). The maximum amount of iron that can be removed with this method in one year is 10-15 grams. For patients with body stores of over 20 grams of iron, the use of erythropoietin has been shown to increase the rate of iron mobilization from the tissue to the red blood cells where it can be cleared from the body by venesection (De Gobbi et al., 2000). The proper treatment of hemochromatosis greatly improves the survival rate and prevents most symptoms of the disease (Niederau et al., 1996).

A more complicated issue of iron overload occurs in blood disorders (thalassemia) where patients need chronic blood transfusions. Each pint of blood transfused into a patient contains 250 milligrams of iron. This constant input of iron into the body becomes problematic because “there are no regulatory mechanisms for iron excretion” (Flaten et al., 2012). Phlebotomy can not be used to treat these patients because they already have an anemic condition. The current treatment for iron overload due to thalassemia is chelation treatment with deferoxamine; an iron chelator that is administered by subcutaneous injections, binds iron and allows it to be passed through the kidney and excreted into the urine.

1.17: CADMIUM EXPOSURE
Cadmium is associated with zinc ore on earth leading to high environmental contamination around zinc mining operations. Other sources of cadmium release into the environment include industrial uses of cadmium (nickel-Cd batteries) and use of cadmium tainted phosphate used in fertilizer. Cadmium is not degradable so exposure is becoming a greater risk for the general public as levels continually build up in the environment. Cadmium is taken up by humans in contaminated water, in food that has absorbed cadmium and in cigarette smoke.

Cadmium is not an essential biological metal. To date no enzyme has been found to use cadmium ions (Cd\(^{2+}\)) as a cofactor other than carbonic anhydrase in some diatoms which can substitute Cd\(^{2+}\) for the Zn\(^{2+}\) normally used in zinc-deficient environments (Lane and Morel, 2000). Cadmium has been found to be toxic to humans though no direct mechanism of adverse effects has been found, Cd\(^{2+}\) seems to disrupt multiple signaling pathways leading to disruption of proper cell function (Thevenod, 2009). Cd\(^{2+}\) is not redox active and can not cause free radical damage like iron. It is speculated that Cd\(^{2+}\) can replace other divalents as co-factors in enzymes that disrupts the enzymes’ proper structure and function. The toxicity of Cd\(^{2+}\) is compounded by the fact that the half-life of Cd\(^{2+}\) in the body is 20-30 years (Moulis and Thevenod, 2010). This means even small amounts of Cd\(^{2+}\) intake over a long enough time can lead to high concentrations of Cd\(^{2+}\) in the body over time. Because Cd\(^{2+}\) is not a biological ion there is no dedicated transporter for Cd\(^{2+}\), instead it is transported into cells by mimicking other divalents (‘ionic mimicry’) (Bridges and Zalups, 2005). The long half-life of Cd\(^{2+}\) in the body suggest it can easily get transported into cells but not out of cells into the plasma where it could be filtered.
passed through the kidneys and excreted. Cells that can uptake $\text{Cd}^{2+}$ well but not efflux it will show a buildup of $\text{Cd}^{2+}$ over time.
1.17: FIGURE LEGEND

**Figure 1.1**: Structure of Ca2+ and Na+ channels. Redrawn from (Zakon, 2012).

**Figure 1.2**: Concentration dependence of Ca2+ block and permeation. Redrawn from (Almers and McCleskey, 1984).

**Figure 1.3**: Erying Rate Theory Models of Permeation. Left: 2 Binding Site 3 Barrier model of permeation. Right: 3 Site 4 Barrier model of permeation. Redrawn from (Dang and McCleskey, 1998)
FIGURE 1.1

P-loop

Domain 1  Domain 2  Domain 3  Domain 4
FIGURE 1.2

Fraction of current vs. log [Ca²⁺] vs. single channel current

9  8  7  6  5  4  3  2  1
log [Ca²⁺]
CHAPTER 2

Evaluation of a 2-Site 3-Barrier Model For Permeation in Ca,3.1 (α1g) T-Type Calcium Channels: Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, and Na$^{+}$

Kyle V. Lopin, Carlos A. Obejero-Paz, and Stephen W. Jones


Whole cell data was taken from the paper “Permeation and gating in CaV3.1 (alpha1G) T-type calcium channels effects of Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, and Na$^{+}$” by Khan, Gray, Obejero-Paz, and Jones in Journal General Physiology (2008); single channel experiments and simulations were performed by Carlos Obejero-Paz; all modeling fits used were performed by Kyle V. Lopin
2.1: ABSTRACT

We explored the ability of a 2-site 3-barrier (2S3B) Eyring model to describe recently reported data on current flow through open Cav3.1 T-type calcium channels, varying Ca\(^{2+}\) and Ba\(^{2+}\) over a wide range (100 nM – 110 mM) while recording whole-cell currents over a wide voltage range (-150 mV to +100 mV) from channels stably expressed in HEK 293 cells. Effects on permeation were isolated using instantaneous current-voltage relationships (IIV) following strong, brief depolarizations to activate channels with minimal inactivation. Most experimental results were reproduced by a 2S3B model. The model described the IIV relationships, apparent affinities for permeation and block for Ca\(^{2+}\) and Ba\(^{2+}\), and shifts in reversal potential between Ca\(^{2+}\) and Ba\(^{2+}\). The fit to block by 1 mM Mg\(^{2+}\)\(_i\) was reasonable, but block by Mg\(^{2+}\)\(_o\) was described less well. Surprisingly, fits were comparable with strong ion-ion repulsion, with no repulsion, or with intermediate values. With weak repulsion, there was a single high affinity site, with a low affinity site near the cytoplasmic side of the pore. With strong repulsion, the net charge of ions in the pore was near +2 over a relatively wide range of concentration and voltage, suggesting a ‘knock-off’ mechanism. With strong repulsion, Ba\(^{2+}\) preferred the inner site, while Ca\(^{2+}\) preferred the outer site, potentially explaining faster entry of Ni\(^{2+}\) and other pore blockers when Ba\(^{2+}\) is the charge carrier.

2.2: INTRODUCTION

Paradoxically, understanding ion selectivity in calcium channels began with the recognition that a calcium channel is fundamentally a nonselective cation channel (Almers and McCleskey, 1984; Almers et al., 1984; Hess and Tsien, 1984; McCleskey and Almers, 1985). In the absence of Ca\(^{2+}\)\(_o\), calcium channels have a high conductance to monovalent cations. Initial models proposed 2 distinct binding sites,
each of which could bind a single Ca\(^{2+}\) with high affinity (Almers and McCleskey, 1984; Hess and Tsien, 1984). That explained why monovalent ions carry current in the absence of Ca\(^{2+}\)\(_o\), and why such currents are blocked by micromolar concentrations of Ca\(^{2+}\). To explain permeation, the models assumed that electrostatic repulsion increases the rates of Ca\(^{2+}\) exit from the pore, when both sites are occupied by Ca\(^{2+}\). Those pioneering models for calcium channel selectivity were based on Eyring rate theory, and included 2 binding sites and 3 barriers (2S3B), where one barrier is the transition state between the binding sites, and the others are for ion entry/exit.

Several theoretical and practical objections have been raised to the use of Eyring models for permeation in calcium channels. The ‘preexponential’ factors have been criticized (Nonner et al., 1999), but this does not affect the actual calculation of rate constants (McCleskey, 1999) (see also Materials and Methods). Previous calcium channel models predict significant changes in the net charge of all ion bound within the pore (as a function of voltage and concentration), while Poisson-Nernst-Planck calculations suggest a nearly electroneutral pore (Nonner and Eisenberg, 1998). Eyring models often overpredict the nonlinearity of current-voltage (IV) relationships (Levitt, 1986; Nonner et al., 1999). And mutational studies on L-type calcium channels demonstrate that high-affinity binding can be disrupted by single mutations within the pore, arguing against two physically separate ion binding sites (Yang et al., 1993).

One approach is to determine whether Eyring models, when strongly constrained by experimental data, provide physically realistic parameters: “Let the data decide” (Bergling, 1999). We examine here whether a 2S3B model can describe permeation through a T-type calcium channel, under a wide range of ionic conditions (varying Ca\(^{2+}\) and Ba\(^{2+}\) from 10\(^{-7}\) to 0.11 M; varying extracellular and intracellular Mg\(^{2+}\)), and a wide voltage range (usually -150 mV to +100 mV) (Khan et al., 2008).
The data set was based primarily on whole-cell recordings, but the use of instantaneous IV relationships (IIV) (Hodgkin and Huxley, 1952a) isolated effects on permeation from effects of voltage or ion concentration on channel gating. The data exhibited well-known features of calcium channel permeation, notably high conductance to Na$^+$ in the absence of divalent ions, block by Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$, and permeation at millimolar concentrations of Ca$^{2+}$ and Ba$^{2+}$. Less standard features of our data set include complex, nonlinear IIVs, complex Ca$^{2+}$ vs. Ba$^{2+}$ selectivity (similar currents over a wide range of concentration and voltage, yet Ca$^{2+}$ selectivity by the criteria of reversal potentials and sensitivity to Mg$^{2+}$ block), and nearly voltage-independent apparent dissociation constants ($K_D$) for Ca$^{2+}$ or Ba$^{2+}$ permeation despite strongly voltage-dependent $K_D$ values for block by those ions (Khan et al., 2008).

This extensive data set is a stringent test for any model of calcium channel permeation.

We find that most, but not all, features of the data were described well by a 2S3B model. The primary discrepancies relate to block by Mg$^{2+}$. Our simulations demonstrate that ion-ion repulsion is not necessary to produce Ca$^{2+}$ block (at µM concentrations) and Ca$^{2+}$ permeation (at mM concentrations), although for our particular dataset a model without repulsion did not accurately describe the timecourse of Ca$^{2+}$ block. Furthermore, for models with weak ion-ion repulsion, only one of the two sites has to bind Ca$^{2+}$ with high affinity.

2.3: MATERIALS AND METHODS

The 2S3B model was based on Almers & McCleskey (1984). Rate constants were calculated as

$$k = k_\alpha e^{(z\delta VF + \Delta G)/RT}$$
where \( z \) is the charge on the ion, \( V \) is the membrane potential, \( \Delta G \) is the difference in energy levels between a well and a barrier, and \( \delta \) is the fraction of the electrical distance across the membrane between the well and barrier (outside = 0, inside = 1). When both sites were occupied, electrical repulsion between bound ions enhanced exit from the pore by a factor \( Q^{z_1+z_2} \) (where \( Q \) is a constant, and \( z_1 \) and \( z_2 \) are the charges on the two ions in the pore). Ion entry into an open site was not affected by occupancy of the other site (Almers and McCleskey, 1984).

For comparison to most previous Eyring models, we use a preexponential factor \( k_o = 6.1 \times 10^{12} \text{ s}^{-1} \), or for entry rates \( k_o = C \times 6.1 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1} \), where \( C \) is the ion concentration in molar. Taken literally, this assumes that the fastest possible rate of ion entry at 1 M concentration equals the fastest possible rate of ion movement from site to site within the pore. That is physically unrealistic (Nonner et al., 1999), but if a different preexponential factor were chosen, the same rate constant can be produced by simply changing the ‘energy barrier’ for all ion entry steps by a constant factor (McCleskey, 1999). The appropriate factor depends on the maximal rate of ion entry via diffusion, which depends (e.g.) on the diameter of the pore. One study proposed that \( k_o \) should be multiplied by 0.00673, which is is equivalent to lowering all of the energy levels in the model by 5.00 RT units (Yue and Marban, 1990). Thus, the ‘energies’ for ion entry should not be interpreted literally, since they exaggerate the barrier heights, but the rate constants are unaffected. That is sufficient for our purpose here, which is to calculate the rate of ion flow through the pore.

The experimental data were from Khan et al. (2008). Briefly, whole-cell recordings were made from HEK 293 cells stably transfected with rat Ca\(_V\)3.1 (\( \alpha \)1G). Currents were evoked from a holding potential of -100 mV, or -120 mV in
experiments with < 0.5 mM Ca\(^{2+}\) or Ba\(^{2+}\), to prevent resting inactivation. Channels were maximally activated (with minimal inactivation) by 2 ms depolarizations to +60 mV (Serrano et al., 2000), followed by voltage steps from +100 to -150 mV in 10 mV intervals (+100 to -120 mV in some conditions where currents were extremely large). ‘Instantaneous’ current-voltage relationships (IIVs) were measured by fitting the initial portion of current decay (from peak, typically 0.2 – 0.5 ms, to 20 ms) to a single exponential. Voltages were corrected for junction potentials when > 1 mV. Series resistance was compensated, usually by 80% or more. The average residual series resistance error following compensation was estimated to be 0.75 MΩ (producing 0.75 mV of voltage error per nA of current). No correction was made for this effect. IIV relations calculated in this manner isolate effects of ions and voltage on permeation from effects on gating. Effectively, the IIV relationships should be directly proportional to the single channel i-V relationship, simply multiplied by the average number of channels per cell. We estimated the average number of channels per cell to be 8000, a value that gave a reasonable scaling factor between our whole-cell IIV relationships and available single-channel current measurements (see Discussion).

24 data sets were included, with IIVs in 10 mV increments from -150 or -120 mV to +90 or +100 mV (Khan et al., 2008). The data are freely available in Excel spreadsheet form, as online supplementary material for Khan et al. (2008). For the dose-response relationships for Ca\(^{2+}\)\(_o\) and Ba\(^{2+}\)\(_o\), [Ca\(^{2+}\)\(_o\) or [Ba\(^{2+}\)\(_o\) = 0.0001, 0.01, 0.1, 0.5, 2, 8, and 110 mM, with standard Mg\(^{2+}\)\(_i\) (1 mM calculated free Mg\(^{2+}\)\(_i\)) and 0 Mg\(^{2+}\)\(_o\). For Mg\(^{2+}\)\(_o\) block, [Mg\(^{2+}\)\(_o\) = 0.3, 1, 3, and 10 mM (with 2 mM Ba\(^{2+}\)\(_o\) and Mg\(^{2+}\)\(_o\) = 1, 3, 10, and 30 mM (with 2 mM Ca\(^{2+}\)\(_o\)), all with standard Mg\(^{2+}\)\(_i\). Two data sets were included with pipet solutions containing no Mg\(^{2+}\)\(_i\) or ATP (100 nM Ca\(^{2+}\)\(_o\) and 2 mM Ca\(^{2+}\)\(_o\)). IIV relations were similar with a pipet solution containing MgATP but with Mg\(^{2+}\)\(_i\) buffered to 0.2 mM, suggesting that the effect depends on Mg\(^{2+}\)\(_i\) not MgATP\(_i\).
Both extracellular and intracellular solutions contained 145 mM Na\(^+\), reduced where extracellular divalent concentrations were 30 mM or higher (Khan et al., 2008).

Final parameter estimation was done using MatLab with the function ‘nlinfit’ using a Levenberg-Marquardt algorithm for nonlinear regression, modified to minimize the sum of absolute errors between the IIV data and the model calculations. Minimizing the sum of squared errors unduly emphasized the largest currents (which have the largest experimental error), which occasionally led to substantial errors in the estimated reversal potentials. State occupancies were calculated using matrix methods, and the current was calculated as the rate of net charge movement over the central barrier. 95% confidence intervals were found using the MatLab function ‘nlparci’ using the Jacobian and residuals calculated by the Levenberg-Marquardt algorithm. There were 26 free parameters: 5 barrier or well energy levels × 4 ions, 5 electrical distances, and an ion-ion repulsion factor (Table 1). The parameter sets presented here are with the ion-ion repulsion factor fixed, to a range of different values. The barriers for ion entry were constrained to be > 6 RT units, except for Ca\(^{2+}\) entry from the extracellular side, constrained to be > 8 RT units (equivalent to an entry rate < \(2 \times 10^9\) M\(^{-1}\)s\(^{-1}\)), given our data on the time course of block by 3-10 µM Ca\(^{2+}\), as discussed further below.

We attempted to fit our data to a 3S4B model without ion-ion repulsion (Dang and McCleskey, 1998), but were not able to obtain reliable parameter estimates, probably resulting from the increased number of free parameters.

Calculations were also done with the SCoP simulation package v. 3.52 (www.simresinc.com), using numerical integration to calculate the steady-state occupancy of each state, and then the resulting fluxes. Fitting with the SCoPFit
program gave similar results to MatLab (not shown), although 3 data sets had to be excluded (0.5 and 8 mM Ca\textsuperscript{2+}; 8 mM Ba\textsuperscript{2+}), and two others averaged (100 nM Ca\textsuperscript{2+} and 100 nM Ba\textsuperscript{2+}), because that program is limited to 20 data sets.

Another program, written in VisualBasic v. 6.0, was based on the analytic solution to state occupancy for the 2S3B model with two ions present. The three methods (MatLab, SCoP, and VisualBasic) calculated equivalent currents for a given parameter set. The VisualBasic program could also display a diagram of state occupancies, rate constants, and fluxes through each pathway for a given voltage and ionic condition, useful for developing intuition regarding the inner workings of the model.

The current through an open calcium channel changes ‘instantaneously’ upon step changes in voltage, except at low (1-10 µM) Ca\textsuperscript{2+}, where time-and voltage-dependent block by Ca\textsuperscript{2+} introduces fast time-dependent relaxations (Khan et al., 2008). Time-dependent block was evaluated in two ways. First, a program (written in VisualBasic) calculated the currents resulting from stochastic transitions of ions among channel states, based on the 2S3B model. This simulates single-channel currents. Briefly, given an initial channel state, one pseudorandom number determines which of the allowed transitions occurs (weighted by the rate constants for each transition), and a second pseudorandom number determines the time to the next transition (by sampling an exponential distribution with time constant = the reciprocal of the sum of all rate constants leading away from the current state). This method (Gillespie, 1977) has also been used (e. g.) for stochastic channel gating models (Chow and White, 1996). For the parameters sets with Q=1 or Q=11.89 (Supplementary Tables 2-3), one step (movement of Na\textsuperscript{+} over the central barrier) was extremely fast, but was not rate limiting for current flow, so the central barrier was adjusted (to +4.0 or +8.0, respectively), which did not markedly affect the
deterministically calculated currents. Second, MatLab was used to calculate the time-resolved response to a voltage step (from +60 mV to the desired voltage, as for the experimentally used IIV protocol). This simulates the fast component of tail currents seen with low $\text{Ca}^{2+}_o$ (Khan et al., 2008).

2.4: RESULTS

2.4.1 Effect of ion-ion repulsion

Electrostatic repulsion between ions at high-affinity binding sites within the pore was fundamental to the initial explanation of $\text{Ca}^{2+}$ permeation using 2S3B models (Almers and McCleskey, 1984; Hess and Tsien, 1984). So we were surprised to find that a wide range of ion-ion repulsion factors ($Q$) can account for the main qualitative features of $\text{Ca}^{2+}$ channel permeation. This does not mean that $Q$ is not important, as $Q$ strongly affects the other parameters of the model, and indeed the mechanisms of $\text{Ca}^{2+}$ permeation and selectivity. Fig. 1 compares three parameter sets, with no ion-ion repulsion at all ($Q=1$), with our best overall fit ($Q=5$), and with $Q=11.89$ (as in Almers & McCleskey, 1984). Parameters are given in Table 1 (and Supplementary Tables 1-2). Features common to all three parameter sets are similar energy profiles for $\text{Ca}^{2+}$ and $\text{Ba}^{2+}$, a high central barrier for $\text{Mg}^{2+}$ (explaining its blocking action), and weak binding of $\text{Na}^+$ to both sites (Fig. 1A). However, the models differ in other key respects.

With $Q=1$, the outer well is considerably deeper for $\text{Ca}^{2+}$ and $\text{Ba}^{2+}$, effectively producing one high affinity site flanked by a low affinity site. A $\text{Ca}^{2+}$ ion at the high affinity site can permeate by two relatively small steps, rather than one large leap. This is reminiscent of the 3S4B model of Dang & McCleskey (1998), but apparently
the outer low affinity site in that model is not necessary. In mixtures of Ca\(^{2+}\) and Na\(^{+}\), Ca\(^{2+}\) permeation usually requires double occupancy of the pore by Ca\(^{2+}\), reflected in a large variation in the summed charge of all ions in the pore as a function of concentration and voltage (Fig. 1B, left). That is, saturation of Ca\(^{2+}\) current through the channel parallels saturation of the second binding site with Ca\(^{2+}\) ions. The energy wells are slightly deeper for Ca\(^{2+}\) vs. Ba\(^{2+}\), but the inner barrier for Ca\(^{2+}\) is slightly lower, explaining the paradox that T-channels are selective for Ca\(^{2+}\) over Ba\(^{2+}\) by the criterion of reversal potential (reflecting binding), but Ca\(^{2+}\) and Ba\(^{2+}\) currents are closely similar.

In contrast, with Q=11.89, there clearly are two high affinity sites near the middle of the pore (Fig. 1A, right). At most Ca\(^{2+}\) concentrations and voltages, the summed charge of all ions in the pore is approximately 2 (Fig. 1B, right). Most of the time, there is a single Ca\(^{2+}\) ion in the pore, and entry of a second Ca\(^{2+}\) ion rapidly expels the first into the cytoplasm, a ‘knock-off’ mechanism. For this model, Ca\(^{2+}\) prefers the outer site while Ba\(^{2+}\) prefers the inner site (Fig. 1C), not true for Q=1.

The model with mild electrostatic repulsion (Q=5) uses a hybrid mechanism for selectivity. The outer energy well is lower than the inner well for Ca\(^{2+}\) and Ba\(^{2+}\), but not by as large an amount as with Q=1 (Fig. 1A). The net ionic charge in the pore is closer to 2 than with Q=1, but there is more variation than with Q=11.89 (Fig. 1B). The difference in preference of Ca\(^{2+}\) vs. Ba\(^{2+}\) for inner vs. outer sites is also intermediate (Fig. 1C).

The parameter set with Q=5 was the best fit overall to the IIV relationships. However, the error in the fit was only 1% higher with Q=11.89. Indeed, the error was only 1-11% higher for the best fit parameters with Q fixed to 2-1000 (not shown). With Q=1, the error was considerably higher (48%), so we conclude that our particular
data set cannot be quantitatively described by a 2S3B model without incorporating some degree of ion-ion repulsion. However, if we allow somewhat more rapid influx of extracellular Ca\(^{2+}\) (outer barrier ~6 RT), the error with Q=1 was only 5% higher than with Q=5 (parameters given in Supplementary Table 3).

2.4.2 Time-dependent block by Ca\(^{2+}\)

The time course of Ca\(^{2+}\) block of current carried by Na\(^{+}\) through T-channels can be observed either as fast relaxations in whole-cell currents, or as fast transitions at the single-channel level (Khan et al., 2008; Lux et al., 1990). Our estimate of the channel blocking rate, depending on the method used, was 2-6 × 10\(^8\) M\(^{-1}\)s\(^{-1}\), near the diffusion limit (Khan et al., 2008). Therefore, we wanted to determine whether the models truly reproduce time-dependent block.

First, we simulated single-channel behavior using a stochastic version of the 2S3B model, where ions were allowed to randomly move in and out of the channel (and between the two binding sites within the channel) according to the rate constants calculated from the fitted parameters (see Materials and Methods). Fig. 2 shows simulated channel block with 3 µM Ca\(^{2+}\) at -70 mV. The current integrated at 10 µs intervals revealed very rapid blocking and unblocking events (upper records). When filtered at 2 kHz (to simulate the filtering used experimentally), it is clear that many events were only partially resolved. For the parameter set with Q=1, block is so rapid that there is only a decrease in the apparent single channel current, with individual blocking events unresolved. With higher Q, the rapid, flickery behavior qualitatively resembles the experimentally observed channel gating in 3 µM Ca\(^{2+}\) (Khan et al., 2008). Quantitatively, with Q=5, the mean open time (measured by standard 50% amplitude crossing) is 0.15 ms at 100 kHz resolution, but 0.47 ms at 2 kHz. The latter value agrees well with the experimental open time of 0.64 ms (corrected assuming that
the observed mean open time of 0.85 ms in 0.1 µM Ca\textsuperscript{2+} reflects the normal channel closing rate, and that closing and block are independent) (Khan et al., 2008).

Next, we calculated the expected whole-cell current relaxation resulting from block by 3 µM Ca\textsuperscript{2+} at -50 mV (see Materials and Methods). The time constant was 0.05 ms with Q=5 and 0.08 ms with Q=11.89, compared to 0.53 ms experimentally (Khan et al., 2008). The experimentally observed relaxation will be slowed by series resistance error and filtering, but that may not fully account for the discrepancy. At any rate, it is clear that the model does predict a submillisecond relaxation in the macroscopic current resulting from Ca\textsuperscript{2+} block, as observed experimentally.

2.4.3 Effect of Ca\textsuperscript{2+} and Ba\textsuperscript{2+}

In summary, given our experimental data, we can exclude Q=1, while Q=5 and Q=11.89 remain plausible. The remainder of the Results section presents simulations with the best fit parameter set (Q=5).

Fig. 3 shows a fit of the 2S3B model to experimental IIV relationships recorded in 100 nM to 110 mM Ca\textsuperscript{2+} or Ba\textsuperscript{2+}. In 100 nM Ca\textsuperscript{2+} or Ba\textsuperscript{2+}, currents are carried by Na\textsuperscript{+}, and the IIV relationship is nearly linear for inward currents, with weak rectification for outward currents, resulting from block by Mg\textsuperscript{2+} (Khan et al., 2008; discussed further below). Currents carried by Na\textsuperscript{+} were progressively blocked by addition of 0.01 – 0.5 mM Ca\textsuperscript{2+} (Fig. 3A) or Ba\textsuperscript{2+} (Fig. 3C). At higher concentrations, inward currents increased with Ca\textsuperscript{2+} (Fig. 3B) or Ba\textsuperscript{2+} (Fig. 3D), reflecting currents carried by those ions, while outward Na\textsuperscript{+} currents were blocked further. The model reproduced these qualitative features of the data, and provided a reasonably good quantitative description. Note that currents were only subtly different between Ca\textsuperscript{2+} vs. Ba\textsuperscript{2+} (Fig. 3).
However, increases in Ca$^{2+}$ vs. Ba$^{2+}$ differentially shifted the reversal potential (V$_R$). The symbols in Fig. 4 are V$_R$ calculated from the 2S3B model for Ca$^{2+}$ and Ba$^{2+}$. The concentration dependence of V$_R$ was described well by Goldman-Hodgkin-Katz (GHK) theory (curves, Fig. 4), with subtle deviations in the 0.1-0.5 mM range. The best-fit GHK permeability ratios were P$_{Ca}$/P$_{Na}$ = 111 (solid curve), and P$_{Ba}$/P$_{Na}$ = 67 (dashed curve). Those values compare reasonably well to the experimental values (P$_{Ca}$/P$_{Na}$ = 87 and P$_{Ba}$/P$_{Na}$ = 44) (Khan et al., 2008).

These results are consistent with the classical view that calcium channels are intrinsically highly conductive to Na$^+$, but low concentrations of Ca$^{2+}$ (and Ba$^{2+}$) block, while high (mM) concentrations result in inward currents carried by Ca$^{2+}$ or Ba$^{2+}$. In addition, it is apparent that the strength of block, and the transition from block to permeation, depends on voltage as well as on concentration (Fig. 3). However, direct comparison of currents recorded at different voltages is complicated by changes in driving force, resulting from changes in V$_R$. This can be avoided by calculation of chord conductances (Fig. 5A-B and D-E). Since block is negligible at 100 nM Ca$^{2+}$ or Ba$^{2+}$ for the 2S3B model, the voltage-dependence of block can be calculated from chord conductance ratios (Fig. 5C,F). The 2S3B model again captures the main qualitative features, notably the U-shaped voltage-dependence (i.e., block is relieved either by strong depolarization or by strong hyperpolarization). Quantitative discrepancies, especially at the lower concentrations, result primarily from ~20% larger currents recorded experimentally in 100 nM Ba$^{2+}$ vs. 100 nM Ca$^{2+}$ for this data set (see Fig. 3A,C; Fig. 5A,D). Note that the data and 2S3B model both indicate slightly weaker block by 10 µM Ba$^{2+}$, compared to Ca$^{2+}$.

The voltage- and current-dependence of permeation and block were evaluated by fitting the conductance-concentration relationship to a simple empirical model, the sum of a conductance to Na$^+$ (G$_{Na}$, with voltage-dependent block by Ca$^{2+}$ or Ba$^{2+}$
reflecting saturable binding to a single site following mass action) plus a conductance to Ca\textsuperscript{2+} or Ba\textsuperscript{2+} (reflecting occupancy of a single voltage-dependent binding site), as described by Khan et al. (2008). The fit is shown for representative voltages in Fig. 6A-D. Chord conductances calculated from the 2S3B model (symbols) were described well. Note that the decrease in current at lower concentrations was clearly voltage-dependent, strongest near -30 mV.

The conductances and apparent dissociation constants (K\textsubscript{D}) are shown as a function of voltage in Fig. 6E-F. Here, symbols are the experimental values, and the curves are the values from analyzing the 2S3B simulated data in the same manner. With depolarization, G\textsubscript{Na} decreased weakly, while G\textsubscript{Ca} (or G\textsubscript{Ba}) decreased more strongly but did not reach zero (Fig. 6E). The 2S3B model reproduced the strong U-shaped voltage dependence of block, but the K\textsubscript{D} for permeation was ~2 mM with little voltage dependence.

2.4.4 Effect of Mg\textsuperscript{2+\textsubscript{o}}

The 2S3B model described block by Mg\textsuperscript{2+\textsubscript{o}} much less well (Fig. 7). The fit to the IIV relationships appeared reasonable for Ba\textsuperscript{2+}, but block was clearly overestimated in Ca\textsuperscript{2+} (Fig. 7A,D). Analyzed as chord conductances, the voltage dependence of block was not well described (Fig. 7B,E). The discrepancies are even more obvious in plots of the fractional block (Fig. 7C,F), where the 2S3B model produced a complex voltage dependence, deviating strongly from the data. Actually, a simple Woodhull model provided a more accurate description (dashed lines, Fig. 7C,F), but still exhibited subtle systematic deviations from the data (Khan et al., 2008). With the 2S3B model, Mg\textsuperscript{2+\textsubscript{o}} block was stronger in Ba\textsuperscript{2+\textsubscript{o}} vs. Ca\textsuperscript{2+\textsubscript{o}}, but the difference was less than observed experimentally.

2.4.5 Effect of Mg\textsuperscript{2+\textsubscript{i}}
The intracellular (pipet) solution for whole-cell recording typically includes MgATP, which results in significant free Mg\(^{2+}\) \(_{i}\) (1 mM in our experiments). Reduction of Mg\(^{2+}\) \(_{i}\) led to larger outward currents, nearly linearizing the IIV relationship with Na\(^{+}\) as the charge carrier, resulting from voltage-dependent block by Mg\(^{2+}\) \(_{i}\) (Khan et al., 2008). Block by Mg\(^{2+}\) \(_{i}\) was described reasonably well by the 2S3B model (Fig. 8).

2.5: DISCUSSION

2.5.1 How Well Does the 2S3B Model Fit the Data?

A 2S3B Eyring model described selectivity of Cav3.1 among Ca\(^{2+}\), Ba\(^{2+}\), and Na\(^{+}\) over a wide range of concentration and voltage, although the fit to Mg\(^{2+}\) \(_{o}\) block was less satisfactory. Notable features that the model described well include the overall shape of IIV relations over a very wide range of Ca\(^{2+}\) \(_{o}\) and Ba\(^{2+}\) \(_{o}\) (Fig. 3), the effects of Ca\(^{2+}\) \(_{o}\) and Ba\(^{2+}\) \(_{o}\) on V\(_{R}\) (including the more positive V\(_{R}\) with Ca\(^{2+}\) \(_{o}\); Fig. 4), the dramatic difference in voltage-dependence of K\(_{D}\) for permeation vs. block (Fig. 6F), block by Mg\(^{2+}\) \(_{i}\) (Fig. 8), and time-dependent block by ~3 µM Ca\(^{2+}\) \(_{o}\) (Fig. 2). It is noteworthy that our 2S3B model can generate a wide range of IIV shapes, ranging from nearly linear (with 100 nM Ca\(^{2+}\) \(_{o}\)) to highly nonlinear (0.1 – 0.5 mM Ca\(^{2+}\) \(_{o}\) or Ba\(^{2+}\) \(_{o}\)). However, the model produced unnecessarily complex IIVs for Mg\(^{2+}\) \(_{o}\) block, especially visible when converted to chord conductances or fractional block (Fig. 7). We conclude that this model is not a complete description of permeation in Cav3.1, but it does reproduce many quantitative and qualitative features of the data.

The experimental data (whole-cell IIV relationships) should equal the single-channel current times the number of channels per cell. Assuming 8000 channels per cell, the 2S3B model predicted a slope conductance of 28 pS for current carried by Na\(^{+}\) \(_{o}\) (-90 to -40 mV), compared to the experimental value of 33 pS (recorded with 105 mM Na\(^{+}\) \(_{o}\), and K\(^{+}\); Khan et al., 2008). The model’s slope conductance was 11 pS in
110 mM Ca\textsuperscript{2+}\textsubscript{o} and 12 pS in 110 mM Ba\textsuperscript{2+}\textsubscript{o} (-50 to -10 mV), comparable to experimental values: 7.5 pS in Ba\textsuperscript{2+} (Perez-Reyes et al., 1998), or 9.5 pS in Ca\textsuperscript{2+} and 10.3 pS in Ba\textsuperscript{2+} (Bittner and Hanck, 2008). As we noted previously (Khan et al., 2008), the commonly reported slope conductance is problematic as a measure of Ca\textsuperscript{2+} channel permeation, as it does not reflect the reversal potential. For example, the actual \textit{currents} were 10-20\% lower in 110 mM Ba\textsuperscript{2+} than in 110 mM Ca\textsuperscript{2+}, even though the \textit{slope} was 10\% larger in Ba\textsuperscript{2+}.

The model predicted a very small anomalous mole fraction effect between Ca\textsuperscript{2+}\textsubscript{o} and Ba\textsuperscript{2+}\textsubscript{o}, maximally a \textasciitilde{}1\% decrease in current (compared to the smaller of the two currents calculated for Ca\textsuperscript{2+}\textsubscript{o} or Ba\textsuperscript{2+}\textsubscript{o} alone) at -140 mV for a Ca\textsuperscript{2+}\textsubscript{o} mole fraction of 0.34 with 2 mM total Ca\textsuperscript{2+}\textsubscript{o} + Ba\textsuperscript{2+}\textsubscript{o}.

For this model, a small fraction of the inward current is carried by Na\textsuperscript{+} in the presence of 2 mM Ca\textsuperscript{2+}\textsubscript{o} (1\% at -50 mV, 6\% at -100 mV, 24\% at -150 mV), and removal of Na\textsuperscript{+}\textsubscript{o} shifted V\textsubscript{R} by -3.2 mV. This is consistent with our data, but the contribution of Na\textsuperscript{+} entry was difficult to resolve experimentally since impermeant cations (e. g., N-methyl-D-glucamine) seem to block calcium channels (Khan et al., 2008; Kuo and Hess, 1992).

Also consistent with our data, the model predicts negligible changes in V\textsubscript{R} upon addition of Mg\textsuperscript{2+}\textsubscript{o}. For example, 10 mM Mg\textsuperscript{2+}\textsubscript{o} shifted V\textsubscript{R} by +0.5 mV in 2 mM Ca\textsuperscript{2+}\textsubscript{o} and +0.4 mV in 2 mM Ba\textsuperscript{2+}\textsubscript{o}.

Our experimental data set did not examine effects of varying Na\textsuperscript{+}, but our model qualitatively reproduced effects of varying Na\textsuperscript{+} on lymphocyte T-channels (Yamashita et al., 1990) (simulations not shown). Specifically, changes in Na\textsuperscript{+}\textsubscript{o} have little effect (in the presence of Ca\textsuperscript{2+}\textsubscript{o}), but changes in Na\textsuperscript{+}\textsubscript{i} strongly shift V\textsubscript{R}, and change the amplitudes of outward currents at positive potentials. The supralinear
increase in outward currents with Na$^{\text{+}}$, measured at +65 mV (Fig. 7 of Yamashita et al., 1990), is subtly visible with chord conductances, but results primarily from the change in driving force. Our model does allow double occupancy of the pore by Na$^{\text{+}}$ ions, but the calculated probability is low (<1% with 2 mM Ca$^{2+}$). It seems that constraining the IIV relationships with constant 145 mM Na$^{\text{+}}$ (but with varied Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$) was sufficient to describe Na$^{\text{+}}$ permeation.

2.5.2 ‘Inner Workings’ of the 2S3B Model

To our knowledge, this study is the first attempt to fit a 2S3B model using automated parameter estimation procedures, with a data set sufficiently extensive to strongly constrain most of the model parameters. Serrano et al. (2000) used automated parameter estimation, but for a more limited data set (Mg$^{2+}$ block at 1-2 concentrations, with 2 mM Ca$^{2+}$ or Ba$^{2+}$). The parameters from that model do not describe well the full Ca$^{2+}$ or Ba$^{2+}$ concentration dependence, as might be expected.

We were surprised to find that the 2S3B model could fit the IIV relationships with essentially any value of Q, the ion-ion repulsion factor. For our data, we can exclude Q=1, as the flickery block by Ca$^{2+}$ of current carried by Na$^{\text{+}}$ was not described (Fig. 2). However, it is interesting as a theoretical point that a 2S3B model does not absolutely need ion-ion repulsion to explain key features of calcium channel permeation.

For all parameter sets considered here, the middle portion of the energy profile for Na$^{\text{+}}$ is not well defined (Table 1; Supplementary Tables 1-3). It appears that the data can be described well as long as Na$^{\text{+}}$ binding is weak, and movement of Na$^{\text{+}}$ between the two binding sites is not rate-limiting. Other parameters appear to be well constrained (for a given Q value).
For an Eyring permeation model, at a given voltage and set of concentrations, the calculated current depends both on the rate constants, and on the steady-state probability of observing each state of ion occupancy. It is noteworthy that the total charge of all ions bound within the channel can be relatively constant (Fig. 1B), even though the chemical identity of the ions in the pore varies substantially. This implies that saturation of Ca$^{2+}$ current through the calcium channel does not require full occupancy by Ca$^{2+}$ of both sites in the pore (z = 4). For high Q values, the rate-limiting step for Ca$^{2+}$ influx at high concentrations is the rate of Ca$^{2+}$ movement from the outer to the inner binding site (analysis not shown). This amounts to a ‘knock-off’ mechanism for permeation, where entry of a second ion rapidly ‘pushes’ the first ion into the cytoplasm, without need for a high steady-state probability of double occupancy. This contrasts with the Hess & Tsien (1984) model, proposed for L-type calcium channels (simulations not shown). That 2S3B model predicts very substantial changes in net pore occupancy, z=0.6 at 100 nM Ca$^{2+}$ (mostly Na$^+$), z=2 at 0.1 mM Ca$^{2+}$ (1 Ca$^{2+}$ ion in pore), and z=4 at 110 mM Ca$^{2+}$ (2 Ca$^{2+}$ ions in pore). For that model, saturation of Ca$^{2+}$ current through the pore parallels saturation of Ca$^{2+}$ occupancy. Ion occupancy in the Almers & McCleskey (1984) model is closer to our situation with Q=5 or Q=11.89.

The summed charge of ~+2 for models with high Q is intriguing, given the -4 charge of the glutamates and aspartates at the presumed selectivity filter of a Cav3.1 pore. It is possible that nearby ions in the inner and/or outer vestibules (not considered in a 2S3B model) make the net charge in the pore closer to zero at any given time, as expected from PNP theory (Nonner and Eisenberg, 1998).

How does the 2S3B model describe the differences (and similarities) between Ca$^{2+}$ and Ba$^{2+}$? At first glance, the energy profiles are quite similar (Fig. 1). For Q=5 or Q=11.89, one striking difference is that Ba$^{2+}$ prefers the inner site while Ca$^{2+}$
prefers the outer site (Fig. 1C; note difference in energy wells, Fig. 1A). That may explain the higher entry rate of some blocking ions into Ba\(^{2+}\)-occupied pores (Obejero-Paz et al., 2004; Obejero-Paz et al., 2008), as well as the more positive \(V_R\) (Fig. 4) and weaker block by Mg\(^{2+}\)o with Ca\(^{2+}\)o vs. Ba\(^{2+}\)o (Fig. 7). This is reminiscent of the differential dependence of Ca\(^{2+}\) vs. Cd\(^{2+}\) block on specific glutamate residues in L-channels, suggesting an asymmetrical arrangement of the 4 negative charges in the pore (Yang et al., 1993). A T-channel, with 2 glutamates and 2 aspartates at the presumed selectivity filter (Perez-Reyes et al., 1998), is clearly asymmetrical. This aspect of our model also differs from previous suggestions for Ba\(^{2+}\)-Ca\(^{2+}\) selectivity, which used symmetrical profiles, but with differences in rates of ion entry and/or exit (Almers and McCleskey, 1984; Hess and Tsien, 1984; Serrano et al., 2000).

### 2.5.3 Models and Mechanisms

Our model is based on the 2S3B model of Almers & McCleskey (1984), where ion-ion repulsion affects only exit, instead of Hess & Tsien (1984), where both entry and exit rates are affected. We can fit the data if ion entry is slowed by occupancy (not shown), but we prefer the idea that ion-ion repulsion primarily affects ion exit, since the rates for ion entry are close to the diffusion limit, and are only weakly voltage-dependent. That is, the ‘barriers’ for ion entry mainly reflect diffusion into the mouth of the pore, not interactions with the pore. If the ion does not ‘see’ an ion at the opposite binding site until it has passed the transition state for ion entry, the entry rate should be unaffected.

Is a 2S3B model inconsistent with the view that a calcium channel pore contains a single high-affinity Ca\(^{2+}\)-binding site? Perhaps there is a single, complex site within the pore that can either bind one calcium ion with high affinity, or two with low affinity. Yang et al. (1993) proposed that the four carbonyl groups move flexibly
to coordinate ions as necessary. Strictly speaking, such flexibility is beyond the scope of a 2S3B model, but the idea of two preexisting sites (which cannot simultaneously bind two ions with high affinity) is not far from the contemporary view. Also note that one site is higher affinity than the other in our preferred model (Q=5, Fig. 1A).

If 2S3B (and other Eyring) models are to be abandoned, it is not entirely clear what should replace them. Full-scale molecular dynamic simulations of channel permeation remain far too slow to generate I-V relationships over a range of experimental conditions comparable to those reported here, and the lack of calcium channel structure at atomic resolution would require reliance on homology models based on the quite distantly related K\(^+\) channel family. Poisson-Nernst-Planck (PNP) theory can account for an impressive number of features of calcium channel permeation (Nonner and Eisenberg, 1998). We previously were unable to obtain a satisfactory quantitative description of a more limited data set for Ca\(_{\text{v}}\)3.1 using PNP (Serrano et al., 2000), but we have not yet explored more sophisticated versions of that theory (Boda et al., 2006; Boda et al., 2009; Nonner et al., 2000). We expect that the data set explored here, combined with data on other di- and trivalent cations (Obejero-Paz et al., 2004; Obejero-Paz et al., 2008), will be useful to constrain and test future theoretical descriptions of calcium channel permeation. At the very least, the model presented here is a challenge to the field to present a plausible model that can provide a better description of our experimental data.
### Table 2.6.1 Parameters for 2S3B permeation model with $Q=5$

<table>
<thead>
<tr>
<th></th>
<th>Electrical distance</th>
<th>$Ca^{2+}$</th>
<th>$Ba^{2+}$</th>
<th>$Mg^{2+}$</th>
<th>$Na^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer barrier</td>
<td>0.095 ± 0.032</td>
<td>8.12 ± 0.61</td>
<td>8.74 ± 0.62</td>
<td>10.47 ± 1.20</td>
<td>10.99 ± 0.37</td>
</tr>
<tr>
<td>Outer well</td>
<td>0.501 ± 0.033</td>
<td>-13.45 ± 0.53</td>
<td>-12.82 ± 0.51</td>
<td>-9.62 ± 1.05</td>
<td>-2.00 ± 0.63</td>
</tr>
<tr>
<td>Central barrier</td>
<td>0.553 ± 0.043</td>
<td>0.96 ± 0.76</td>
<td>1.46 ± 0.85</td>
<td>9.68 ± 0.79</td>
<td>6.49 ± 16.06</td>
</tr>
<tr>
<td>Inner well</td>
<td>0.644 ± 0.046</td>
<td>-11.25 ± 0.31</td>
<td>-11.19 ± 0.28</td>
<td>-6.44 ± 1.07</td>
<td>-2.90 ± 0.54</td>
</tr>
<tr>
<td>Inner barrier</td>
<td>0.999 ± 0.062</td>
<td>10.64 ± 0.37</td>
<td>11.02 ± 0.40</td>
<td>6.30 ± 1.28</td>
<td>10.16 ± 0.73</td>
</tr>
</tbody>
</table>

Parameters were estimated for the Almers & McCleskey (1984) model as described in Materials and Methods. The ion-ion repulsion factor ($Q$) was fixed to 5. See middle column in Figs. 1-2. Error bars are the 95% confidence intervals (max or min) minus the mean. Positive and negative errors were symmetrical. Note that the central barrier for $Na^+$ was not well constrained.
Table 2.6.2 Parameters for 2S3B permeation model with Q=11.89

<table>
<thead>
<tr>
<th></th>
<th>Electrical distance</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Ba&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Na&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer barrier</td>
<td>0.127 ± 0.033</td>
<td>8.77 ± 0.57</td>
<td>9.56 ± 0.54</td>
<td>12.64 ± 1.17</td>
<td>11.02 ± 0.27</td>
</tr>
<tr>
<td>Outer well</td>
<td>0.465 ± 0.049</td>
<td>-15.12 ± 0.82</td>
<td>-14.05 ± 0.80</td>
<td>-10.64 ± 1.09</td>
<td>-3.49 ± 0.42</td>
</tr>
<tr>
<td>Central barrier</td>
<td>0.562 ± 0.044</td>
<td>-0.12 ± 0.70</td>
<td>0.82 ± 0.20</td>
<td>10.12 ± 0.65</td>
<td>-2.49*</td>
</tr>
<tr>
<td>Inner well</td>
<td>0.562 ± 0.083</td>
<td>-13.90 ± 0.53</td>
<td>-14.61 ± 0.40</td>
<td>-6.44 ± 1.15</td>
<td>-4.60 ± 0.36</td>
</tr>
<tr>
<td>Inner barrier</td>
<td>0.999 ± 0.059</td>
<td>10.90 ± 0.64</td>
<td>10.88 ± 0.88</td>
<td>7.56 ± 0.74</td>
<td>9.44 ± 0.83</td>
</tr>
</tbody>
</table>

Parameters were estimated for the Almers & McCleskey (1984) model as described in Materials and Methods. The ion-ion repulsion factor (Q) was fixed to 11.89, as in Almers & McCleskey (1984). See right column in Figs. 1-2. Error bars are the 95% confidence intervals (max or min) minus the mean. Positive and negative errors were symmetrical. The summed fitting error for instantaneous I-V relationships (data shown in Figs. 3A-D, 7A, 7D, and 8A) was 1% higher than for the preferred parameter set (with Q=5).

*Constrained to be at least 1 RT above the outer well.
### Table 2.6.3 Parameters for 2S3B permeation model with Q=1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Electrical distance</th>
<th>Ca(^{2+})</th>
<th>Ba(^{2+})</th>
<th>Mg(^{2+})</th>
<th>Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer barrier</td>
<td>0.229 ± 0.050</td>
<td>8.07 ± 0.49</td>
<td>8.18 ± 0.45</td>
<td>8.14 ± 0.88</td>
<td>11.65 ± 0.26</td>
</tr>
<tr>
<td>Outer well</td>
<td>0.644 ± 0.060</td>
<td>-11.52 ± 0.79</td>
<td>-10.83 ± 0.74</td>
<td>-8.18 ± 0.77</td>
<td>3.98 ± 4.20</td>
</tr>
<tr>
<td>Central barrier</td>
<td>0.645 ± 0.055</td>
<td>2.05 ± 0.67</td>
<td>2.63 ± 0.73</td>
<td>9.11 ± 0.49</td>
<td>5.98 ± 172</td>
</tr>
<tr>
<td>Inner well</td>
<td>0.758 ± 0.060</td>
<td>-7.57 ± 0.65</td>
<td>-7.10 ± 0.55</td>
<td>-6.85 ± 2.81</td>
<td>1.72 ± 17.37</td>
</tr>
<tr>
<td>Inner barrier</td>
<td>0.999 ± 0.075</td>
<td>7.61 ± 0.73</td>
<td>8.25 ± 0.72</td>
<td>6.09 ± 2.83</td>
<td>10.67 ± 0.57</td>
</tr>
</tbody>
</table>

Parameters were estimated for the Almers & McCleskey (1984) model as described in Materials and Methods. See left column in Figs. 1-2. The ion-ion repulsion factor (Q) was fixed to 1 (i.e., no repulsion). The outer barrier for Ca\(^{2+}\) was constrained to be ≥ 8. Error bars are the 95% confidence intervals (max or min) minus the mean. Note that the central barrier and wells for Na\(^+\) were not well constrained. Positive and negative errors were symmetrical. The summed fitting error for instantaneous I-V relationships (data shown in Figs. 3A-D, 7A, 7D, and 8A) was 48% higher than for the preferred parameter set (with Q=5). The description of block by 3 μM Ca\(^{2+}\) was also poor (Fig. 2).
Table 2.6.4 Parameters for 2S3B permeation model with Q=1

<table>
<thead>
<tr>
<th></th>
<th>Electrical distance</th>
<th>Ca(^{2+})</th>
<th>Ba(^{2+})</th>
<th>Mg(^{2+})</th>
<th>Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer barrier</td>
<td>0.071 ± 0.033</td>
<td>6.09 ± 0.26</td>
<td>6.67 ± 0.25</td>
<td>11.19 ± 3.67</td>
<td>10.55 ± 0.43</td>
</tr>
<tr>
<td>Outer well</td>
<td>0.549 ± 0.546</td>
<td>-11.84 ± 0.34</td>
<td>-11.19 ± 0.33</td>
<td>-8.70 ± 3.59</td>
<td>5.50 ± 8.17</td>
</tr>
<tr>
<td>Central barrier</td>
<td>0.549 ± 0.482</td>
<td>1.85 ± 0.36</td>
<td>2.35 ± 0.40</td>
<td>12.00 ± 3.70</td>
<td>6.50 ± 0.89</td>
</tr>
<tr>
<td>Inner well</td>
<td>0.644 ± 0.416</td>
<td>-7.69 ± 0.32</td>
<td>-7.42 ± 0.29</td>
<td>-5.18 ± 4.68</td>
<td>-2.04 ± 0.33</td>
</tr>
<tr>
<td>Inner barrier</td>
<td>0.998 ± 0.033</td>
<td>7.88 ± 0.42</td>
<td>8.48 ± 0.43</td>
<td>8.10 ± 4.55</td>
<td>10.55 ± 0.23</td>
</tr>
</tbody>
</table>

Parameters were estimated for the Almers & McCleskey (1984) model as described in Materials and Methods. The ion-ion repulsion factor (Q) was fixed to 1 (i.e., no repulsion). The outer barrier for Ca\(^{2+}\) was constrained to be ≥ 6. This allows ~8-fold faster Ca\(^{2+}\) entry than the other parameter sets considered in this study. Compare especially to Supplementary Table 2. Error bars are the 95% confidence intervals (max or min) minus the mean. Positive and negative errors were symmetrical. Note that several parameters were not well constrained. The summed fitting error for instantaneous I-V relationships (data shown in Figs. 3A-D, 7A, 7D, and 8A) was only 5% higher than for the preferred parameter set (with Q=5), but the time course of block by 3 μM Ca\(^{2+}\) (examined as in Fig. 2) was too fast (reducing the apparent single channel current, with 2 kHz filtering).
2.7: FIGURE LEGENDS

**Fig. 2.1.** Models for permeation and block, based on Almers & McCleskey (1984), as described in Materials and Methods.  
(A) Energy level diagrams for Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, and Na$^+$. Assuming a preexponential factor of $6.1 \times 10^{12}$ for all rate constants (including ion entry into the pore), an ‘energy’ of 5 RT corresponds to a rate constant representation (RCR) of 3.15 (Andersen, 1999). Parameters are the best fits obtained for 3 values of the ion-ion repulsion factor (Q), with numerical values in Table 1 (Q=5) and Supplementary Tables 1-2 (Q=1, Q=11.89). The energy profiles are for a single channel, fitted assuming that experimental macroscopic currents result from 8000 channels per cell.  
(B) The predictions of the 2S3B model for the total charge of all ions in the pore, calculated for the Ca$^{2+}_o$ concentrations examined experimentally.  
(C) Probability of occupancy of outer vs. inner binding sites by Ca$^{2+}$ or Ba$^{2+}$, calculated for 2 mM Ca$^{2+}_o$ or 2 mM Ba$^{2+}_o$.

**Fig. 2.2.** Simulated block by 3 µM Ca$^{2+}_o$ of currents carried by Na$^+$, at -70 mV, for the same three parameter sets as in Fig. 1. The upper records show the calculated current, with each point being the integrated current over a 10 µs interval. The lower records show the same simulations after digital Gaussian filtering at 2 kHz. Zero current is indicated with dashed lines. Same time and current scale for all records. Compare to Fig. 8A of Khan et al. (2008), noting that experimentally observed burst durations are limited by the mean open time of ~0.8 ms in the absence of Ca$^{2+}_o$.

**Fig. 2.3.** IIV relationships. Symbols are the experimental data (Fig. 2 of Khan et al., 2008), and solid curves are the currents calculated from the 2S3B model.
**Fig. 2.4.** Reversal potentials calculated from the 2S3B model. $V_R$ was interpolated from IIV relations (Fig. 3) calculated in 1 mV intervals, for the indicated concentrations of Ca$^{2+}$ and Ba$^{2+}$. Symbols are $V_R$ values from the 2S3B model; curves are the best fit to GHK theory. Note that the symbols in this figure are not experimental data. Compare to Fig. 3 of Khan et al. (2008).

**Fig. 2.5.** Conductance-voltage relationships. Chord conductances were calculated from the IIV relationships in Fig. 3, for experimental values (symbols), and 2S3B model (solid curves). Compare to Fig. 4 of Khan et al. (2008).

**Fig. 2.6.** Concentration- and voltage-dependence of block and permeation. (A-D) Chord conductances vs. [Ca$^{2+}$], for the indicated voltages. The symbols are chord conductances calculated from the 2S3B model (theoretical curves in Fig. 5A,B,D,E), for the Ca$^{2+}$ and Ba$^{2+}$ concentrations used experimentally. The curves are fits to the sum of independent components for block (of current carried by Na$^+$) and permeation (see text and Eq. 4 of Khan et al, 2008). Parameters ($G_{Na}$, $G_{Ca}$ or $G_{Ba}$, and apparent $K_D$ values for block and permeation) were estimated by minimizing the sum of squared errors using the Solver function in Microsoft Excel. The symbols defined in A and B also apply to C and D (respectively). (E-F) Voltage dependence of conductances and apparent $K_D$ values. Symbols are experimental values (Khan et al., 2008), and the curves are calculated from the 2S3B model. Parameters (G’s and K$_D$’s) were estimated separately for each voltage, as shown for representative voltages in A-D. Solid curves are for Ca$^{2+}_o$, dashed curves for Ba$^{2+}_o$. From +40 to +100 mV, the model
conductances were described by the sum of a constant permeation term (with no $K_D$) and a blocking term (note slight discontinuity in the curves). Compare to Fig. 5 of Khan et al. (2008).

**Fig. 2.7.** Block by $\text{Mg}^{2+}_o$, with 2 mM $\text{Ca}^{2+}_o$ (A-C) or 2 mM $\text{Ba}^{2+}_o$ (D-F). *Symbols* are experimental values (Fig. S12A-D; Khan et al, 2008), and the *solid curves* are the corresponding values calculated from the 2S3B model. The effect of $\text{Mg}^{2+}_o$ is shown as IIV relationships (A,D), chord conductances (B,E), and as conductance ratios (C,F). The *dashed lines* in C and F are fits to the experimental data assuming voltage-dependent block by $\text{Mg}^{2+}_o$, with no $\text{Mg}^{2+}$ permeation (Woodhull, 1973), from Fig. S12E-F of Khan et al. (2008).

**Fig. 2.8.** Block by $\text{Mg}^{2+}_i$. *Symbols* are the experimental data (Fig. S19 of Khan et al., 2008) and *curves* are 2S3B model calculations, comparing cells with 1 mM $\text{MgATP}_i$ (*solid curves*) vs. 0-0.2 mM $\text{MgATP}_i$ (*dashed curves*). The “$10^{-4}$ mM $\text{Ca}^{2+}$” data actually include cells recorded in either 100 nM $\text{Ca}^{2+}$ or 100 nM $\text{Ba}^{2+}$ (n=8, for both normal and low $\text{MgATP}_i$). Currents (A) and chord conductances (B). Symbols in A also apply to B. (C) $\text{Mg}^{2+}_i$ block, calculated as chord conductance ratios, from the data and simulations in A-B.
FIGURE 2.1
FIGURE 2.3
FIGURE 2.6

A 2 mM Ca²⁺

B 2 mM Ba²⁺

C

D

E G(Mg²⁺) / G(Control)

F G(Mg²⁺) / G(Control)
FIGURE 2.7
Cd\textsuperscript{2+} Block and Permeation in Ca\textsubscript{v}3.1 (\(\alpha1G\)) T-type Calcium Channels.

A Candidate Mechanism for Cd\textsuperscript{2+} Influx

\textsuperscript{1}Kyle V. Lopin, \textsuperscript{1}Frank Thévenod, Jessica C. Page, and Stephen W. Jones


Cd\textsuperscript{2+} block experiments were performed by Kyle V. Lopin, Frank Thévenod, and Jessica C. Page. Incubation experiments were performed by Frank Thévenod. All other experiments were performed by Kyle V. Lopin

\textsuperscript{1}These authors contributed equally to the study.
3.1 ABSTRACT

Cd\textsuperscript{2+} is an industrial pollutant that can cause cytotoxicity in multiple organs. We have examined the effects of Cd\textsuperscript{2+} on permeation and gating in Ca\textsubscript{\textit{v}3.1 (α1G) channels, stably transfected in HEK 293 cells, using whole-cell recording. Using instantaneous I-V currents (measured following strong depolarization) to isolate effects on permeation, Cd\textsuperscript{2+} rapidly blocked currents with 2 mM Ca\textsuperscript{2+} in a voltage-dependent manner. The block caused by Cd\textsuperscript{2+} is relieved at more hyperpolarized potentials, suggesting that Cd\textsuperscript{2+} can permeate through the selectivity filter of the channel into the cytosol. In the absence of other permeant ions (Ca\textsuperscript{2+} and Na\textsuperscript{+} replaced by N-methyl D-glucamine) Cd\textsuperscript{2+} carried sizable inward currents through Ca\textsubscript{\textit{v}3.1 channels (210 ± 20 pA at -60 mV with 2 mM Cd\textsuperscript{2+}). Ca\textsubscript{\textit{v}3.1 channels have a significant ‘window current’ at this voltage (P\textsubscript{open} ~ 1%) making them a candidate pathway for Cd\textsuperscript{2+} entry into cells during Cd\textsuperscript{2+} exposure. Incubation with radiolabeled \textsuperscript{109}Cd\textsuperscript{2+} confirmed that Ca\textsubscript{\textit{v}3.1 channels can lead to the uptake of Cd\textsuperscript{2+} into cells.
3.2 INTRODUCTION

Increasing industrial use of Cd\textsuperscript{2+} has led to widespread contamination of the environment that threatens human health (ATSDR, 2008). The main challenge in the 21\textsuperscript{st} century in a global setting seems to be not acute toxicity but chronic low Cd\textsuperscript{2+} exposure, mainly from dietary sources (Jarup and Akesson, 2009). Ubiquity of Cd\textsuperscript{2+} makes it a serious environmental health problem that needs to be thoroughly assessed because it already affects, or will affect, large proportions of the world’s population.

A variety of pathways have been suggested to allow Cd\textsuperscript{2+} entry in excitable and non-excitable cells (Thévenod, 2010). Candidates are DMT1 with a $K_m$ for Cd\textsuperscript{2+} of $\sim$1 µM (Gunshin et al., 1997; Okubo et al., 2003), ZIP8 with a $K_m$ for Cd\textsuperscript{2+} of $\sim$ 0.5 µM (Liu et al., 2008) and ZIP14A/B with a $K_m$ for Cd\textsuperscript{2+} of 0.1-1 µM (Girijashanker et al., 2008). In this context, it is crucial to be aware that blood Cd\textsuperscript{2+} concentrations in the general population are in the range of 1-10 nM (Elinder et al., 1983) and may exceed 100-300 nM in occupationally exposed workers (Hassler et al., 1983). The free Cd\textsuperscript{2+} concentrations in the extracellular fluid that cause tissue damage are unknown but are likely to be in the submicromolar range: Even acute poisoning with oral intake of a high dose of Cd\textsuperscript{2+} result in a Cd\textsuperscript{2+} concentration in the blood of merely $\sim$200 nM (Hung and Chung, 2004). It is not clear whether most studies describing transport of Cd\textsuperscript{2+} have only \textit{in vitro} or mechanistic relevance, or could significantly contribute to \textit{in vivo} toxicity of Cd\textsuperscript{2+}.

T-type calcium channels are blocked by Cd\textsuperscript{2+} (Diaz et al., 2005a; Lacinova et al., 2000) but their role in Cd\textsuperscript{2+} transport has not been investigated so far. Ca\textsubscript{v}3.1 channels may be suitable for Cd\textsuperscript{2+} transport since they have a well-defined window current at
negative membrane potentials where the driving force for divalent cation entry is high (Serrano et al., 1999), and CaV3.1 channels are ~2-fold less selective for Ca\(^{2+}\) than L-type calcium channels (Perez-Reyes, 2003) suggesting that Cd\(^{2+}\) has an increased chance of permeating the channel in the presence of competing Ca\(^{2+}\). CaV3.1 calcium channels are expressed in excitable cells, such as neurons, heart, smooth and skeletal muscle and endocrine cells (Perez-Reyes, 2003). Surprisingly, Ca\(_{v}3.1\) is also expressed in the distal nephron of the kidney (Andreasen et al., 2000), where it may be involved in Ca\(^{2+}\) reabsorption (Leclerc et al., 2004). In this paper we examine Cd\(^{2+}\) effects on gating and permeation of Ca\(_{v}3.1\) channels, and use a model of permeation to estimate the amount of Cd\(^{2+}\) that can permeate through the channels at levels seen during chronic Cd\(^{2+}\) exposure.
3.3 MATERIALS AND METHODS

3.3.1 Electrophysiology.

Whole-cell patch clamp recordings were made from HEK 293 cells stably transfected with CaV3.1 calcium channels as described previously (Khan et al., 2008). Electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) and had open-tip resistances of 1.1 to 2.0 MΩ and access resistances upon going whole-cell of 2.0 to 5.0 MΩ. Currents were recorded at room temperature (~21-24 °C), compensated at 90%, filtered at 10 kHz, and sampled at 50 kHz. Leak and capacitative currents were subtracted using a –P/4 protocol. A holding potential of -100 mV was used to prevent inactivation of the channel. Only cells that had a resting leak less than 200 pA and rundown less than 25% were used. Currents were acquired using an Axopatch 200A amplifier and pClamp 8.2 software (Molecular Devices, Sunnyvale, CA) and analyzed using Clampfit and Matlab.

3.3.2 Recording solutions.

The intracellular solution used for all experiments contained (in mM): 2 CaCl₂, 1 MgCl₂, 120 NaCl, 10 HEPES, 11 EGTA, and 4 Mg-ATP. The pH was adjusted to 7.2 using NaOH. The free Ca²⁺ was calculated as 70 nM. The standard extracellular solution contained (in mM): 2 CaCl₂, 135 NaCl, 10 HEPES, and 10 glucose. The pH was adjusted to 7.2 using NaOH. Where indicated CdCl₂ was added to the extracellular solution. For the Ba²⁺ data set BaCl₂ replaced CaCl₂. For the Cd²⁺ permeation data with NMDG⁺, Na⁺ was replaced by NMDG⁺ and the pH was adjusted using HCl. All chemicals used in the electrophysiology experiments were purchased from Sigma (St. Louis, MO).

3.3.3. Data Analysis.

The current through ion channels is affected by two processes, gating and permeation. To separate these two we used an ‘instantaneous’ current voltage (IIV) protocol to
isolate the gating of Ca\textsubscript{v}3.1 from permeation (Hodgkin and Huxley, 1952a). The IIV protocol uses a short (2 msec) pulse to a voltage that will maximally open the channels (+60 mV). The potential is then reset to a wide range of voltages and the initial current measured, see Figure 1A. As the initial pulse will open the same number of channels for each recording, the current-voltage relation is directly proportional to the permeation of ions through a single channel. For determining the effect of added Cd\textsuperscript{2+} on the IIV relationship (‘Cd\textsuperscript{2+} block’), currents were converted to chord conductances (Khan et al., 2008) since Cd\textsuperscript{2+} affected the reversal potential and thus the driving force at a particular voltage.

In a second protocol (IV), steps were given from the holding potential to a variable depolarizing potential and the currents were recorded, see Fig. 1B. The currents recorded with the IV protocol are affected by both gating and permeation. Dividing the IV current by the IIV current for each voltage gives the relative open probability (P\textsubscript{o,r}) (Serrano et al., 1999).

Inward currents when Cd\textsuperscript{2+} was the charge carrier were too small to measure. To measure any change in gating in those conditions, channels were activated by a short (2 msec) pulse to varying voltages, and tail currents were measured after the potential was reset to -100 mV, see inset Fig. 9A. As the driving force of the tail currents are the same for each recording, any difference in the currents are proportional to how many channels are opened during the 2 msec voltage step.

For Cd\textsuperscript{2+} block, cells were recorded in control solution (2 mM Ca\textsuperscript{2+}), after the addition of Cd\textsuperscript{2+}, and after return to control solution (washout), Fig. 1A-B. The control and washout currents were averaged to offset any rundown that may have occurred for data on Cd\textsuperscript{2+} block of Ca\textsuperscript{2+} currents. Averaging controls and washout was not done for Cd\textsuperscript{2+} permeation because the cells became very leaky when switched back to Ca\textsuperscript{2+} after being in high levels of Cd\textsuperscript{2+} without Ca\textsuperscript{2+} for periods greater than 2 minutes. To evaluate rundown, briefer Cd\textsuperscript{2+} applications (~30 sec) were performed.
and, tail currents at -100 mV were measured following brief test pulses, before, during, and after recovery from Cd\(^{2+}\). We estimate that currents reported here for 10 mM Cd\(^{2+}\) were reduced by 14 ± 2 % by rundown, and there was no significant change for 2 mM Cd\(^{2+}\) (data not shown).

In each condition, data were scaled based on the sum of the IIV currents from +80 mV to -80 mV, to reduce variability from variations in channel expression from cell to cell (Eq. 3 of Khan et al., 2008). For the IIV protocol, the initial amplitudes were estimated from fits to a single exponential equation

\[
A \cdot e^{-t/\tau} + C
\]

where \(A\) is the initial amplitude, \(\tau\) is the time constant of decay and \(C\) is a constant offset. Inward currents for the IV protocol were too small to accurately fit to exponential equations in all but control and 0.3 mM Cd\(^{2+}\) conditions. For the IV data the peak currents were measured by averaging between 2 cursors placed by eye around the peak.

### 3.3.4 Permeability ratios.

Reversal potentials were calculated by linear interpretation between the data points on either side of reversal. Permeability ratios were calculated from the reversal potentials assuming Goldman-Hodgkin-Katz (GHK) theory. For two ions

\[
\frac{P_A}{P_B} = -\frac{z_A^2}{z_B^2} \cdot \left( \frac{[B]_\text{in} - [B]_\text{out} e^{-\nu_i \tau}}{[A]_\text{in} - [A]_\text{out} e^{-\nu_i \tau}} \right) \frac{(1 - e^{-\nu_i \tau})}{(1 - e^{-\nu_i \tau})}
\]

where \(\nu_i = z_i V_r F/RT\) (Frazier et al., 2000). With 3 permeant ions (Cd\(^{2+}\), Ca\(^{2+}\), Na\(^+\)) the following equation can be derived.
This equation predicts that adding a permeant extracellular ion should cause a more positive reversal potential.

3.3.5 Gating.

To measure the effect of Cd\(^{2+}\) on gating, channel activation was measured by fitting currents from the IV protocol to a fourth power Boltzmann

$$P_{\alpha \sigma}(V) = \left(\frac{1}{1+e^{-(V-V_{0.5})/k}}\right)^4$$

where \(V_{0.5}\) is the halfway point of activation for an individual voltage sensor and \(k\) is the voltage sensor sensitivity. Voltage shifts in gating caused by Cd\(^{2+}\) were calculated (Zhou and Jones, 1995) by subtracting the \(V_{0.5}\) for Cd\(^{2+}\) activation from the average \(V_{0.5}\) for control (2 mM Ca\(^{2+}\)) and washout. The Grahame equation was used to calculate voltage shifts from charge screening according to Gouy-Chapman theory (Grahame, 1947).

$$\sigma^2 G^2 = \sum C_i \left\{ e^{-\sigma \Phi/kT} - 1 \right\}$$

where \(G\) is a constant equal to 270 Å\(^2\)e\(^{-1}\)M\(^{1/2}\) at room temperature, \(C_i\) is the concentration of the \(i^{th}\) ionic species in solution, \(z\) is the valance, \(k\) is Boltzmann’s constant, \(T\) is temperature and \(\sigma\) is the planar charge density. For charge screening without binding \(\sigma\) was set to 1 e/98 Å\(^2\), as estimated previously (Khan et al., 2008). Binding of Cd\(^{2+}\) to the planar charge followed Gouy-Chapman-Stern theory

$$\sigma = \sigma_i \left[ 1 + K_{Cd} \left( Cd \frac{z^2 \Phi}{kT} \right)^{-1} \right]$$
where $\sigma_t$ was set to $1 \ e^\prime / 98 \ \text{Å}^2$, $K_{Cd}$ is the association constant for $\text{Cd}^{2+}$ in $\text{M}^{-1}$ and $\sigma$ is the surface charge not neutralized by binding.

### 3.3.6 Permeation model.

We previously reported a 2-site 3-barrier (2S3B) Eyring model for permeation in CaV3.1 (Lopin et al., 2010b). In short, the IIIV data collected in this study were normalized to the original data. The parameters for the electrical distances and the energy parameters for $\text{Ca}^{2+}$, $\text{Ba}^{2+}$, $\text{Na}^+$, and $\text{Mg}^{2+}$ were fixed to the parameters fitted previously. Parameters for $\text{Cd}^{2+}$ were fitted by a least sum of absolute error to all the data points using the Levenberg-Marquardt algorithm.

### 3.3.7 $^{109}\text{Cd}^{2+}$ transport.

Cellular $^{109}\text{Cd}^{2+}$ uptake (specific activity 1.5 MBq/µg $\text{Cd}^{2+}$; QSA Global, Braunschweig, Germany) was performed according to a previously described protocol (Erfurt et al., 2003), with some modifications. Briefly, confluent monolayers of HEK293 cells (control, or stably transfected with CaV3.1) were washed twice with Hank’s balanced salt solution with 5.55 mM glucose (HBSS-glucose) before $\text{Cd}^{2+}$ incubation. The concentration of $\text{CdCl}_2$ (10 mM stock solution in water) was adjusted in HBSS-glucose and labeled with $^{109}\text{Cd}^{2+}$ to give a final activity of 18.5 kBq/ml. At specific time points, monolayers were washed with HBSS-glucose containing 2 mM EGTA (pH 7.0, adjusted with Tris) and solubilized in 1 N NaOH overnight. $^{109}\text{Cd}^{2+}$ content was determined using a Cobra II Auto-Gamma counter (Packard Instrument Company, Meriden, CT). Experiments were performed in the absence or presence of 25 µM NNC 55-0396 (Sigma; 2.5 mM stock dissolved in water), a selective inhibitor of T-type calcium channels (Huang et al., 2004), to obtain CaV3.1-specific $^{109}\text{Cd}^{2+}$ uptake.

Throughout the paper data are shown as mean ± sem.
3.4 RESULTS

Cd²⁺ is commonly used to block Ca²⁺ currents. To characterize this effect most studies have used voltage steps (Lacinova et al., 2000) and measured the current voltage (IV) relationships, but this reflects effects of Cd²⁺ on both gating and pore block simultaneously. To separate these two effects we have used an ‘instantaneous’ current voltage (IIV) protocol where a short prepulse is given to maximally open all the channels and the voltage is ‘instantaneously’ reset and the current measured, as shown in Fig 1A,C. Using the IIV protocol the effect of Cd²⁺ on the permeation pathway can be examined in isolation from effects on gating.

3.4.1 Cd²⁺ blocks and permeates through Ca₃.1 channels.

Fig. 1A-B shows current records from the IIV and IV protocols, in control (2 mM Ca²⁺ with 145 mM Na⁺), with the addition of 300 µM Cd²⁺, and following wash out. The large voltage range allows both outward Na⁺ currents and inward currents carried mainly by Ca²⁺ to be measured. The peak currents for each protocol are shown in Fig. 1C-D for controls and in the presence of three different concentrations of [Cd²⁺] (0.3, 1, and 3 mM). To determine the voltage dependence of block by Cd²⁺ the chord conductances in the presence of Cd²⁺ were divided by the control value (Fig. 2A). Here it can be clearly seen that as the cell is hyperpolarized the fraction of channels blocked by Cd²⁺ decreases. The rate of Cd²⁺ exit out of the pore to the extracellular side will slow as the cell is hyperpolarized, but if the divalent blocker can permeate, then the rate of Cd²⁺ exiting the pore at hyperpolarizing potentials will increase, relieving pore block. As was already noted for Cd²⁺ on Ca,3.1, “Taken together, these results suggest that extreme hyperpolarization appears to attract Cd²⁺ into the cell” (Diaz et al., 2005a). Fig. 2B shows the conductance as a function of Cd²⁺ concentration and voltage. The block saturates at ~85%, as there is an appreciable
amount of current that is observed in 3 mM [Cd\(^{2+}\)]\(_o\). The current remaining could be due to incomplete block of Ca\(^{2+}\) currents and/or Cd\(^{2+}\) permeation. Given the voltage dependence of block it is likely that Cd\(^{2+}\) decreased currents by getting into and obstructing the pore. To confirm this we changed the charge carrier from Ca\(^{2+}\) to Ba\(^{2+}\), shown in Fig. 3. It has been shown previously (Serrano et al., 2000) that due to ion-ion interactions in the pore blockers are more potent when Ba\(^{2+}\) is the charge carrier, compared to Ca\(^{2+}\), if the effect of the blocker is on the selectivity filter of the pore. This can clearly be seen in Fig. 3D where Ba\(^{2+}\) currents are blocked appreciably more by 300 µM Cd\(^{2+}\) than Ca\(^{2+}\) currents.

Since the voltage dependence of block suggested Cd\(^{2+}\) permeation, we examined the reversal potentials of the currents after the addition of Cd\(^{2+}\). According to GHK theory, a permeant ion added to the extracellular side should cause a more positive reversal potential. As shown in Fig. 4A, adding [Cd\(^{2+}\)]\(_e\) actually caused a less positive V\(_r\).

### 3.4.2 Currents carried by Cd\(^{2+}\).

The voltage dependence of block by Cd\(^{2+}\) suggests that Cd\(^{2+}\) can permeate through the pore. To test this more directly, we recorded currents after replacing 2 mM Ca\(^{2+}\) with 2 mM Cd\(^{2+}\) (Fig. 5) and saw sizeable inward currents. To confirm that the currents observed were being carried by Cd\(^{2+}\) and not Na\(^+\) we increased the Cd\(^{2+}\) concentration to 10 mM. This did not lead to an increase in the current, which might be expected if the currents were being carried by Cd\(^{2+}\). In fact, at very hyperpolarizing potentials (-120 mV to -150 mV), the current was less in 10 mM Cd\(^{2+}\). One possibility is that some of the inward current is carried by Na\(^+\), and the higher concentration of Cd\(^{2+}\) simply blocks the Na\(^+\) current more effectively.

To eliminate any Na\(^+\) currents that may be mixing in with the Cd\(^{2+}\) currents we replaced the extracellular Na\(^+\) with NMDG\(^+\), an impermeant cation. Fig. 6 compares currents in 0.2, 2, and 10 mM Cd\(^{2+}\) to 2 mM Ca\(^{2+}\). Inward currents increase
monotonically with \([\text{Cd}^{2+}]\), approaching saturation at 10 mM (Fig. 3B-C). Currents carried by \(\text{Cd}^{2+}\) were rather large, >200 pA at -60 mV in both 2 and 10 mM \([\text{Cd}^{2+}]_e\) (Fig. 6B). This level of current carried through a calcium channel by a ‘blocker’ is surprising. With the IV protocol (Fig. 6D) inward currents were very small (~30 pA). Using the reversal potentials for \(\text{Cd}^{2+}\) permeation with NMDG (Fig. 4B) a permeability ratio of \(\text{Cd}^{2+}\) to \(\text{Na}^+\) \((P_{\text{Cd}/\text{Na}})\) was calculated as 57.1 using GHK theory (Fig. 4C). This compares to the \(P_{\text{Ca}/\text{Na}}\) ratio of 87 (Khan et al., 2008). Together this would give a \(P_{\text{Cd}/\text{Ca}}\) of 0.66 where \(\text{Cd}^{2+}\) is only slightly less permeable than \(\text{Ca}^{2+}\), defined using GHK theory.

### 3.4.3 \(\text{Cd}^{2+}\) uptake through Ca,3.1.

Ca,3.1 channels are known to have a substantial ‘window’ current, where partial activation combined with incomplete inactivation allows a steady inward \(\text{Ca}^{2+}\) current near the resting potential (Chemin et al., 2000; Serrano et al., 1999; Williams et al., 1997). The window current is classically measured as the product of the activation curve and the inactivation curve, usually assuming that inactivation is 100% at depolarized potentials. However, inactivation is incomplete for CaV3.1 channels, so 1-2% of channels remain open and will conduct current at all voltages where channels open (Serrano et al., 1999). The ‘window current’ (Po) predicted by the Serrano et al (1999) model is shown in Fig.8F. This window current could be an important source of \(\text{Cd}^{2+}\) entry into cells. To study if \(\text{Cd}^{2+}\) can permeate via the window current of Ca,3.1 channels incubation studies were conducted with radiolabeled \(^{109}\text{Cd}^{2+}\). Experiments were conducted for 30 minutes at varying concentrations of \(\text{Cd}^{2+}\) in the presence of physiological levels of \(\text{Ca}^{2+}\). \(\text{Cd}^{2+}\) uptake by CaV3.1 was measured as the difference between cells incubated with \(\text{Cd}^{2+}\) and cells incubated with \(\text{Cd}^{2+}\) and the CaV3 blocker NNC 55-0396 (Huang et al., 2004). Fig. 7 shows that CaV3.1 can
transport Cd\textsuperscript{2+} into cells at the resting membrane potential in a dose-dependent manner.

3.4.4 Permeation model.

To estimate how Ca\textsubscript{V3.1} transports trace amounts of Cd\textsuperscript{2+} in physiological conditions, a 2-binding site 3-barrier (2S3B) model was used (Fig. 8). Parameters were estimated by fitting the data on Cd\textsuperscript{2+} block and permeation (Figs. 1, 3, 6) The electrical distances and parameters for Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, Na\textsuperscript{+} and Mg\textsuperscript{2+} were fixed to the values used by Lopin et al. (2010). The model was able to effectively describe Cd\textsuperscript{2+} block of Ca\textsuperscript{2+} currents (Fig. 8A), Ba\textsuperscript{2+} currents (Fig 8D) and the permeation of Cd\textsuperscript{2+} (Fig. 8B). However, there is a deviation between the model and data at the most hyperpolarizing potentials (-120 to -150 mV) for Cd\textsuperscript{2+} permeation. When Na\textsuperscript{+} is replaced by NMDG the current levels off for potentials < -120 mV even when Ca\textsuperscript{2+} is the carrier (Khan et al., 2008, Fig. S20; and compare control currents in Fig 6C to Fig. 1C). It is unclear if this is caused by voltage dependent block of NMDG\textsuperscript{+} or if there are Na\textsuperscript{+} currents in addition to Ca\textsuperscript{2+} currents at strong negative voltages (Khan et al., 2008).

The model was used to estimate the transport rate of Cd\textsuperscript{2+} through Ca\textsubscript{V3.1} channels as a function of [Cd\textsuperscript{2+}]\textsubscript{o} (Fig. 8E). The model predicts that with 3-10 nM Cd\textsuperscript{2+} Ca\textsubscript{V3.1} channels can transport on the order of 1 Cd\textsuperscript{2+} ion per second through an open channel. To evaluate the steady-state Cd\textsuperscript{2+} entry rate, the expected steady-state Po is shown as a function of voltage in Fig. 8F (dashed line, calculated from the model in Serrano et.al., 1999). Notice that the window Po is constant at depolarized potentials due to incomplete inactivation. Fig. 8F also shows the transport rate through an open channel multiplied by the steady-state Po; this is the calculated Cd\textsuperscript{2+} transport rate via the window current. The model can also be directly compared to the incubation data by multiplying the transport rate by estimates for the number of cells.
per well (200,000), number of channels per cell (8,000) (Lopin et al., 2010b), and the open probability at rest taking into account slow inactivation* (0.975%), calculated assuming 98.5% fast inactivation (Serrano et al., 1999) and 35% slow inactivation (Hering et al., 2004) at steady-state. All values were taken at -35 mV (Chemin et al., 2000). As shown in Fig. 7, the uptake calculated from the model is very similar to the experimentally observed rate (see Discussion).

3.4.5 Shifts in gating by Cd\textsuperscript{2+}.

To determine whether Cd\textsuperscript{2+} affects gating, activation curves were calculated from the Cd\textsuperscript{2+} block data (Fig. 1) using the relative open probability (P\textsubscript{o,r}), calculated by dividing the IV current by the IIV current at each voltage (Serrano et al., 1999) (Fig. 9B). This measurement could not be used for the Cd\textsuperscript{2+} permeation data, as the IV currents were too small to measure accurately (Fig. 6D), so currents were measured from tail currents following brief (2 msec) depolarizations (Fig. 9A). Activation was shifted to more positive voltages in 2 mM Cd\textsuperscript{2+} compared to 2 mM Ca\textsuperscript{2+}. As described previously (Khan et al., 2008), Ca\textsuperscript{2+} causes a voltage shift by interacting with the negatively charged head groups on the cell membrane without binding, i.e. by charge screening or a Gouy-Chapman mechanism (Hille et al., 1975). The additional shift caused by Cd\textsuperscript{2+} compared to an equimolar concentration of Ca\textsuperscript{2+} therefore requires some additional mechanism of action, most likely binding of Cd\textsuperscript{2+} to the channel or cell surface. The simplest such model is a Gouy-Chapman-Stern mechanism, which allows cations to bind to the surface charges in addition to screening. The voltage shifts caused by Cd\textsuperscript{2+} are shown in Fig. 10. It can be seen that using K\textsubscript{A} = 0.4435 M\textsuperscript{-1} both the permeation and block data are described fairly well. Binding to surface charge should also shift the time constants for channel closing to the same degree as the P\textsubscript{o,r} data. The time constants for the tail currents of Ca\textsuperscript{2+} currents, and Ca\textsuperscript{2+} currents with the addition of Cd\textsuperscript{2+}, are shown in Fig. 9C. Cd\textsuperscript{2+} caused no change in the
inactivation rate (e. g., above 0 mV). There was no clear shift in the voltage-
dependence of channel closing (e. g., below -50 mV), but a slight change in slope at 1
mM or 3 mM Cd$^{2+}$ . This implies that effects of Cd$^{2+}$ on gating cannot be explained
fully by Gouy-Chapman-Stern theory. The effects of Cd$^{2+}$ on gating in Cav3.1 should
be negligible at Cd$^{2+}$ concentrations found in the body.
3.5 DISCUSSION

In this study, we demonstrate that Cd\(^{2+}\) can permeate directly through Cav3.1 calcium channels. The voltage dependence of Cd\(^{2+}\) block of Ca\(^{2+}\) currents strongly suggested that Cd\(^{2+}\) is a permeant ion, and inward currents were carried by Cd\(^{2+}\) in the absence of other extracellular permeant ions. To calculate the rate that Cd\(^{2+}\) can permeate Cav3.1 calcium channels at concentrations seen during Cd\(^{2+}\) exposure (3-10 nM) we used a model of permeation, and estimated that \(\sim 1\) Cd\(^{2+}\) ion per second can pass through an open channel.

3.5.1 Cd\(^{2+}\) permeation and block.

Cd\(^{2+}\) is classically considered a calcium channel blocker, but previous studies have demonstrated relief of block by hyperpolarization, strong evidence that Cd\(^{2+}\) can enter cells via voltage-dependent calcium channels (Brown et al., 1983). This is not surprising in principle, as many divalent cations (including Ca\(^{2+}\) itself) can either act as pore blockers or permeant cations, depending on conditions (Almers and McCleskey, 1984; Hess and Tsien, 1984). However, the size of currents carried by Cd\(^{2+}\) was surprising, 5-17\% of the current carried by Ca\(^{2+}\) (comparing both ions at 2 mM; Fig. 6). From reversal potentials with Cd\(^{2+}\) (in the absence of Ca\(^{2+}\)), the permeability ratio is \(P_{Cd/Ca} = 0.66\). Note that the permeability ratio primarily reflects the strength of binding, but the actual observed current is also affected by the rate of ion movement through the pore.

Block of Cav3.1 by Cd\(^{2+}\) decreased with hyperpolarization, the reverse of the voltage dependence observed for many other divalents, including Mg\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) (Diaz et al., 2005a; Obejero-Paz et al., 2008; Serrano et al., 2000). This reflects the relatively strong permeation observed for Cd\(^{2+}\). The relief of Cd\(^{2+}\) block by hyperpolarization has also been seen for Ca\(^{2+}\) channels in chicken sensory neurons (Swandulla and Armstrong, 1989), suggesting that it could be a common feature of
Ca\textsuperscript{2+} channels. Furthermore, based on data with a range of Cd\textsuperscript{2+} concentrations, Cd\textsuperscript{2+} block saturated at 80-85\% (Fig. 2), and thus does not completely block currents through Ca\textsubscript{V3.1}. The remaining current is carried in part by Cd\textsuperscript{2+} and in part by Ca\textsuperscript{2+}; our permeation model predicts that 50-60\% of the inward current is carried by Cd\textsuperscript{2+}, with 2 mM Ca\textsuperscript{2+\textsubscript{o}} and 3 mM Cd\textsuperscript{2+\textsubscript{o}}.

It does not appear that the incomplete block of calcium channels by Cd\textsuperscript{2+} has been noted previously. One factor is that most previous studies have measured inhibition using the IV protocol, instead of the IIV used here. Since Cd\textsuperscript{2+} shifts gating to more positive voltages, inhibition measured by the IV protocol will include both inhibition by pore block and inhibition by lowering channel open probability, which will exaggerate the potency of Cd\textsuperscript{2+} as a pore blocker, and can also exaggerate the maximal extent of pore block (see Fig. 1D).

From the experiments examining Cd\textsuperscript{2+} block of Ca\textsuperscript{2+} currents, the reversal potential was shifted to more negative potentials with Cd\textsuperscript{2+}, opposite to expectations from GHK theory. Deviations from GHK behavior are expected for multi-ion pores where ion-ion interactions are important such as calcium channels.

3.5.2 Permeation model.

For the nanomolar concentrations observed in chronic Cd\textsuperscript{2+} exposure in vivo, the rate of Cd\textsuperscript{2+} permeation through Ca\textsubscript{V3.1} channels cannot be directly measured using electrophysiology. We estimated the transport rate using an Eyring rate theory model (Eyring, 1935) of permeation in calcium channels. The model we propose in this paper is a refinement of the original 2-site 3-barrier models of calcium channels (Almers and McCleskey, 1984; Hess and Tsien, 1984) fit to a large data set over various voltages and concentrations of Ca\textsuperscript{2+}, Ba\textsuperscript{2+}, Mg\textsuperscript{2+}, and Na\textsuperscript{+} (Lopin et al., 2010b). Parameters for Cd\textsuperscript{2+} were estimated by including our data on Cd\textsuperscript{2+} block and
permeation. The model can then translate electrophysiological data into transport rates of trace metals through an ion channel under pathophysiological conditions. The model was able to reproduce the $^{109}\text{Cd}^{2+}$ uptake data fairly well using previously reported values of the window current and membrane potential. Uncertainties in the resting potential of HEK293 cells, especially with CaV3.1 channels active (or partially blocked by Cd$^{2+}$) limit quantitative comparison, but the fraction of CaV3.1 channels active at steady-state near the assumed resting potential (-35 mV; Chemin et al., 2000) does not depend strongly on voltage (Serrano et al., 1999).

3.5.3 Calcium channels and Cd$^{2+}$ uptake.

It is unlikely that there is a dedicated protein to transport Cd$^{2+}$ as it is not a biologically essential metal. Instead Cd$^{2+}$ is transported into cells using mechanisms for other naturally occurring cations such as Ca$^{2+}$ (i.e. ‘ionic mimicry’ (Bridges and Zalups, 2005; Clarkson, 1993). Cd$^{2+}$ uptake via CaV3.1 in our study (Fig. 7) is comparable to a previous study with ZIP14 (Girijashanker et al., 2008), but uncertainty in expression levels of different channels and transporters prevents definitive conclusions regarding the relative importance of pathways for Cd$^{2+}$ influx. Previous studies have also linked L-type calcium channels (Hinkle et al., 1987) and the calcium selective TRPC6 channel (Kovacs et al., 2011) to Cd$^{2+}$ uptake. Development of resistance to Cd$^{2+}$ in cell culture has been linked to downregulation of CaV3.1, suggesting involvement of this channel in Cd$^{2+}$ toxicity (Leslie et al., 2006). Given the large number of calcium channels expressed throughout the body, the importance of Ca$^{2+}$ signaling, and the large number of ions a channel can transport ($\sim 10^5$ s$^{-1}$), if a calcium channel is even slightly permeable to Cd$^{2+}$ this could lead to a significant Cd$^{2+}$ entry. This is especially true for CaV3.1, which has a substantial ‘window current’ near the resting potential.
3.6 FIGURE LEGENDS

**Fig. 3.1.** Cd\textsuperscript{2+} block of currents through Ca\textsubscript{3.1} channels. A-B, sample IIV and IV records. The protocols are shown below the middle traces. Control currents in 2 mM Ca\textsuperscript{2+} (left), with the addition of 300 µM Cd\textsuperscript{2+} (middle), and after washout (right) are shown. Currents were Gaussian filtered offline to a final -3 dB cutoff of 5 kHz and shown in 20 mV increments. C, ‘instantaneous’ currents from IIIV protocols shown in A, for control and in the presence of 3 concentrations of Cd\textsuperscript{2+} (n=4 for all concentrations). D, peak currents for the IV protocol shown in B (n=4). Symbols in C apply to D.

**Fig. 3.2.** Fraction of the chord conductance remaining in Cd\textsuperscript{2+}. A, ratio of the conductance in the presence of Cd\textsuperscript{2+} compared to control as a function of voltage. Data were from IIIV measurements as in Fig. 1C (n=4). Symbols as defined in Fig. 1C. B, conductance ratios as a function of [Cd\textsuperscript{2+}]. Curves drawn are fits to a single-site model with variable maximal inhibition: 0.11 mM, 84% inhibition (-20 mV); 0.15 mM, 84% inhibition (-40 mV); 0.23 mM, 80% inhibition (-60 mV); 0.36 mM, 80% inhibition. (-80 mV) Data were not well described with a single-site model with 100% maximal inhibition.

**Fig. 3.3.** Cd\textsuperscript{2+} block of Ba\textsuperscript{2+} currents. A-B, IIIV and IV current records, using the protocol shown below the middle trace. Control currents in 2 mM Ba\textsuperscript{2+} (left), with the addition of 300 µM Cd\textsuperscript{2+} (middle), and after washout (right) are shown. Currents were Gaussian filtered at 5 kHz and shown in 20 mV increments. C, ‘instantaneous’ currents from the IIIV protocol shown in A. D, the fraction of control conductance remaining in 300 µM Cd\textsuperscript{2+} when 2 mM Ca\textsuperscript{2+} or Ba\textsuperscript{2+} was the charge carrier. E, peak
currents from the IV protocol shown in B. F, expanded view of the inward currents with the IV protocol. For experiments with Ba\(^{2+}\), n=4.

**Fig. 3.4.** Effects of Cd\(^{2+}\) on reversal potentials. A, effect of addition of Cd\(^{2+}\) (to 2 mM Ca\(^{2+}\)) on the reversal potential. B, reversal potentials with extracellular Cd\(^{2+}\) (0 Ca\(^{2+}\), NMDG replacing Na\(^{+}\)) and intracellular Na\(^{+}\). C, fits of P\(_{Cd}/P_{Na}\) to GHK theory. The solid line is the best fit, with P\(_{Cd}/P_{Na}\) = 57.1, and the dashed lines are GHK fits with P\(_{Cd}/P_{Na}\) increased or decreased by 50% (85.7 and 28.6). n=4.

**Fig. 3.5.** IIV relationships with extracellular Cd\(^{2+}\) and Na\(^{+}\). A, currents recorded when Ca\(^{2+}\) was replaced with Cd\(^{2+}\). B, expanded view of A. Note that the currents were larger in 2 mM Cd\(^{2+}\) than in 10 mM Cd\(^{2+}\) at the most hyperpolarized potentials. n=4.

**Fig. 3.6.** Permeation by Cd\(^{2+}\). A, sample current records from the IIV protocol with the extracellular solution containing 2 mM Cd\(^{2+}\) and NMDG\(^{+}\). Currents shown were measured between +80 mV and -100 mV in 20 mV increments and are shown after 2 kHz Gaussian filtering offline. B, ‘instantaneous’ currents from the IIV protocol (as in A) on an expanded scale to show inward currents carried by Cd\(^{2+}\) (n=4 for all concentrations). C, same as B but scaled to compare currents carried by Cd\(^{2+}\) to Ca\(^{2+}\). D, peak currents measured using the IV protocol (n=4), symbols as in B and C.

**Fig. 3.7.** Uptake of Cd\(^{2+}\) by HEK cells. NNC 55-0396 (25 µM) sensitive uptake of \(^{109}\)Cd\(^{2+}\) in untransfected HEK cells, and HEK cells stably expressing Ca\(_{v}\)3.1 calcium channels. Cells were incubated for 30 minutes at the indicated concentration of Cd\(^{2+}\). The dashed line is the calculated transport rate for the 2S3B model using the assumptions described in the text. n= 5-9.
**Fig. 3.8.** 2-binding site 3-barrier Eyring rate model for Cd$^{2+}$ block and permeation in CaV3.1 channels. A, fit of the model to IIV relationships where Cd$^{2+}$o was added to solutions containing 2 mM Ca$^{2+}$o (and Na$^{+}$i,o). B, fit to IIVs in the absence of Ca$^{2+}$o and Na$^{+}$o. C, fit to IIVs with Cd$^{2+}$o, zero Ca$^{2+}$o, but normal Na$^{+}$o. D, fit to IIVs with 2 mM Ba$^{2+}$o, and with added 0.3 mM Cd$^{2+}$o. For A-D, symbols are experimental measurements, and curves are model calculations. E, calculated rate of Cd$^{2+}$ influx through an open CaV3.1 channel as a function of voltage for different [Cd$^{2+}$]o. F, calculated rate of Cd$^{2+}$ influx through the window current of Cav3.1 channel as a function of voltage (same symbols as E), dashed line is the open probability of Cav3.1 calculated from the model in Seranno et al. 1999. G, energy profiles for the ions included in the model. The energy levels for Cd$^{2+}$ in kT units, from outside to inside, are 8.56, -14.46, 2.17, -11.10, and 14.49. Other parameters are from Table 1 of Lopin et al. (2010).

**Fig. 3.9.** Effects of Cd$^{2+}$ on gating. A, activation measured from tail currents after 2 msec prepulses (n=4), normalized to the tail currents following steps to +100 mV. B, activation curves calculated by dividing the peak IV current by the IIV current (n=4). C, time constants of the tail currents using the IIV protocol (n=4).

**Fig. 3.10.** Voltage shifts induced by Cd$^{2+}$, fitted to Gouy-Chapman-Stern theory using the Grahame equation. The solid curves are the fit of both Cd$^{2+}$ block and permeation data to the same $K_A$ (0.4435 M$^{-1}$). The fit is fairly good to both data sets considering different methods were used to measure the $P_{o,f}$ (Fig. 9). The best fits to the data sets separately were 0.85 M$^{-1}$ for block, and 0.26 M$^{-1}$ for permeation (dashed curves).
FIGURE 3.1

A

B

C

D

2 mM Ca²⁺

2 mM Ca²⁺ + 300 μM Cd²⁺

2 mM Ca²⁺

2 nA

-100 mV

-120 mV

-80 mV

+80 mV

5 msec

-100 mV

-90 mV

+80 mV

0.5 nA

5 msec

-160

0

1

3

[Graphs showing current-voltage relationships and concentration effects on Ca²⁺ and Cd²⁺ interactions]
FIGURE 3.2

A

\[ \frac{\alpha_{\text{tot}}}{\alpha_{\text{max}}} \]

-100 -100 -50 0 50 100 mV

B

\[ \frac{\alpha_{\text{tot}}}{\alpha_{\text{max}}} \]

-20 mV
-40 mV
-60 mV

[Co²⁺]ₜ
FIGURE 3.4

A. Cdp⁺ block of Ca²⁺ currents

B. Cdp⁺ current

C. GHK theory fit to Cdp⁺ current
FIGURE 3.5
FIGURE 3.8

A

B

C

D

E

F

G

2538 model energy profile

Fractional electrical distance from the outside (R)

[Graphs and data representations as per the figure 3.8]
FIGURE 3.10
CHAPTER 4

Fe\(^{2+}\) block and permeation in Ca\(_{\text{v}}\)3.1 (\(\alpha1\)G) T-type calcium channels.

A candidate mechanism for non-tranferrin-mediated Fe\(^{2+}\) influx

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Fe\(^{2+}\) permeation with gating current experiments were performed by I. Patrick Gray with assistance from Carlos A. Obejero-Paz. All other experiments were performed by Kyle V. Lopin with assistance from Carlos A. Obejero-Paz
4.1 ABSTRACT

Iron is a biologically essential metal, but excess iron can cause damage to the cardiovascular and nervous systems. We have examined the effects of Fe\(^{2+}\) on permeation and gating in Cav3.1 channels, stably transfected in HEK 293 cells, using whole-cell recording. Precautions were taken to maintain iron in the Fe\(^{2+}\) state (e.g., extracellular ascorbate). Using instantaneous I-V relations (measured following strong depolarization) to isolate effects on permeation, Fe\(^{2+}\) rapidly blocked currents with 2 mM Ca\(^{2+}\) in a voltage-dependent manner, described by a Woodhull model with K\(_D\) = 2.5 mM at 0 mV and an apparent electrical distance \(\delta = 0.17\). Fe\(^{2+}\) also shifted activation to more depolarized voltages (by ~10 mV at 1.8 mM Fe\(^{2+}\)), somewhat more strongly than Ca\(^{2+}\) or Mg\(^{2+}\), consistent with a Gouy-Chapman-Stern model with a surface charge density \(\sigma = 1\ e^-/98\ \text{Å}^2\) and \(K_{Fe} = 4.5\ \text{M}^{-1}\) for Fe\(^{2+}\). In the absence of Ca\(^{2+}\) (and with Na\(^{+}\) replaced by tetraethylammonium), Fe\(^{2+}\) carried detectable whole-cell inward currents at millimolar concentrations (73 ± 7 pA at -60 mV with 10 mM Fe\(^{2+}\)). From a 2-site 3-barrier Eyring model for permeation in Cav3.1, we estimate a transport rate for Fe\(^{2+}\) of ~20 ions per second per open channel at -60 mV and pH 7.2, in 1 \(\mu\text{M}\) Fe\(^{2+}\) (with 2 mM Ca\(^{2+}\)). Since Cav3.1 exhibits a significant ‘window current’ in that voltage range (P\(_O\) ~1%), Cav3.1 channels are a likely pathway for Fe\(^{2+}\) entry into cells, at clinically relevant concentrations of Fe\(^{2+}\).
4.2 INTRODUCTION

Iron enters cells not only via the well-characterized transferrin receptor-endocytosis pathway for ferric iron, but also the ill-defined non-transferrin bound iron (NTBI) mechanism for both ferric and ferrous iron entry (Anderson and Vulpe, 2009). Candidate mechanisms for NTBI include the divalent metal-ion transporter DMT1 (Gunshin et al., 1997), Zip14 (Liuzzi et al., 2006), the non-selective TRPC6 channel (Mwanjewe and Grover, 2004), and voltage gated calcium channels. Both L-type (Oudit et al., 2003; Tsushima et al., 1999) and T-type (Kumfu et al., 2011) calcium channels have been implicated in Fe\(^{2+}\) uptake using selective blockers. Currents carried by Fe\(^{2+}\) through L-channels have been recorded electrophysiologically for L-channels (Tsushima et al., 1999) but not T-channels.

Iron overload is typically caused by excess dietary absorption of iron in genetic hemochromatosis (Clark et al., 2010) or repeated blood transfusions when red blood cells are broken down and the heme-bound iron is released (Kwiatkowski, 2011). Plasma NTBI can reach ~5 \(\mu\)M (Loreal et al., 2000). NTBI is highly reactive and can cause the formation of damaging free radicals. Excess iron accumulates mainly in the liver and the heart (Andrews, 1999). In myocardial cells, iron overload affects cellular structure (Iancu et al., 1987), gene expression (Parkes et al., 2000), Ca\(^{2+}\) handling (Kim et al., 1995), and ion channel properties (Kuryshev et al., 1999). Because Ca\(_{\alpha}3.1\) channels are widely expressed in excitable and nonexcitable cells including brain, ovary, placenta, heart, liver and vascular smooth muscle (Perez-Reyes, 2003; Rodman et al., 2005a; Yunker and McEnery, 2003) understanding the mechanism of Fe\(^{2+}\) transport by these channels is necessary to understand their role in organ damage in conditions associated with iron overload.

Besides a role as pore blocker and permeant ion (Tsushima et al., 1999; Winegar et al., 1991), Fe\(^{2+}\) might have significant effects on channel gating as is the case for other
divalent cations (Elinder and Arhem, 2003). Gating changes induced by divalent cations may rise from pore occupancy, allosteric effects of binding to sites outside the pore (Beedle et al., 2002; Kang et al., 2005; Traboulsie et al., 2007), or by screening or binding to surface charge (Zhou and Jones, 1995).

We find that Fe$^{2+}$ blocks currents carried by Ca$^{2+}$ or Ba$^{2+}$ by voltage-dependent block within the pore. Fe$^{2+}$ also permeates, less well than Ca$^{2+}$ or Ba$^{2+}$. Effects of Fe$^{2+}$ on gating are consistent with a surface charge mechanism, where Fe$^{2+}$ both screens and binds to surface charge. The effects of Fe$^{2+}$ to block and shift gating would be minimal at clinically observed concentrations of Fe$^{2+}$. However, the estimated rates of Fe$^{2+}$ permeation suggest that Ca$\nu3.1$ may be a significant source of Fe$^{2+}$ entry into cells even at the resting potential.
4.3 MATERIALS AND METHODS

4.3.1 Electrophysiology.

Patch clamp experiments were performed in the whole-cell configuration using HEK 293 cells stably transfected with CaV3.1 (α1G) calcium channels, as described (Khan et al., 2008). Electrodes were made from borosilicate glass, with open pipet resistances of 1.8-2.3 MΩ, and access resistances of 5 ± 1 MΩ before compensation (80%). Currents were digitally sampled at 50 kHz after 10 kHz analog filtering using an Axopatch 200 amplifier and pClamp 8.2 software. Leak and capacitative currents were subtracted online using a P/-4 protocol. Experiments were performed at room temperature (~22 °C).

We evaluated the effect of Fe²⁺ using two basic voltage protocols, IV (direct depolarization to a range of voltages) and IIV (preactivating channels by strong, brief depolarization, followed by steps to a range of voltages) (Figs. 1-2). Assuming effects of Fe²⁺ are effectively instantaneous (as we conclude below), this approach allows separation of effects on permeation vs. gating (Hodgkin and Huxley, 1952a; Khan et al., 2008; Lopin et al., 2012b; Obejero-Paz et al., 2008; Serrano et al., 1999). Currents measured immediately following repolarization with the IIV protocol (Fig. 1A) should be directly proportional to the current through a single open channel. Thus, effects of Fe²⁺ on the IIV relationship should reflect inhibition of current through open channels. Effects on the IV relationship, in contrast, reflect the net effect of the ion both on permeation and on gating.

4.3.2 Standard recording solutions.

The intracellular solution contained (in mM) 2 CaCl₂, 1 MgCl₂, 120 NaCl, 10 HEPES, 4 MgATP, and 11 EGTA, adjusted to pH 7.2 with NaOH (total Na⁺ 145 mM,
calculated free Ca\(^{2+}\) 70 nM). The normal extracellular solution contained 2 CaCl\(_2\), 128 NaCl, 5 ascorbic acid, 10 glucose, 20 HEPES, adjusted to pH 7.2 with NaOH (total Na\(^+\) 145 mM). When noted, CaCl\(_2\) was replaced by BaCl\(_2\).

### 4.3.3 Extracellular solutions containing Fe\(^{2+}\).

Extreme care must be taken to maintain iron in the soluble Fe\(^{2+}\) state. To do this FeCl\(_2\) was added to the solution only after cells were patched and control currents were being recorded to reduce the amount of time Fe2+ could oxidize. Solutions were made 1-2 minutes before they were applied to the cells and were used within the 6 minutes of being made. Fe\(^{2+}\) was added to the final desired concentration from a 200 mM stock solution of FeCl\(_2\)·4 H\(_2\)O in 1% HCl (v/v). Each solution was measured for free Fe\(^{2+}\) using the ferrozine method (Dorey et al., 1993; Viollier et al., 2000) while the electrophysiological experiments were being performed. To this end a sample of the extracellular solution was diluted to a final concentration of 100 µM Fe\(^{2+}\) with a solution containing the same components (except FeCl\(_2\)), or a solution containing 5 mM ascorbate (pH 3.3) to reduce all iron forms. 0.75 ml of those samples were mixed with the same volume of 2 mM ferrozine, and absorbance was measured (at 562 nm) in a Beckman DU640B spectrophotometer. Standards in the range from 10 µM and 100 µM Fe\(^{2+}\) were prepared by dilution of a stock solution of 20 mM FeCl\(_2\)·4 H\(_2\)O, with a final concentration of 5 mM ascorbic acid. Measured free Fe\(^{2+}\) concentrations ranged from 28 – 95% of the nominal value. Throughout this paper, the values for Fe\(^{2+}\) concentration are the actual values measured by this procedure.

For experiments examining block by Fe\(^{2+}\), Fe\(^{2+}\) was added to the normal extracellular solutions (2 mM Ca\(^{2+}\) or 2 mM Ba\(^{2+}\)). To investigate whether Ca\(_V\)3.1 currents allow Fe\(^{2+}\) influx, we designed extracellular solutions where Fe\(^{2+}\) was the only charge carrier. To this end extracellular NaCl was replaced by TEA-Cl, Ca\(^{2+}\) by Fe\(^{2+}\),
and solutions were maintained at pH 7.0 to reduce the rate of iron oxidation. A pH of 6.8 – 7.05 measured at the end of the experiment was considered acceptable. A control solution containing 2 mM Ca$^{2+}$ was applied to the cell before and after the test solution.

Since inward currents were small with Fe$^{2+}$, we performed experiments to evaluate the contribution of gating currents, using an extracellular solution containing (mM) 140 NaCl, 2 CdCl$_2$, and 1 mM LaCl$_3$.

4.3.4 Data Analysis.

Most methods were as described (Lopin et al., 2012b). Through the paper, data are shown as mean ± sem. We used the paired t-test implemented in Origin 7.0 (OriginLab corporation) to assess differences between means when controls were from the same cell. We used one way ANOVA to investigate differences between means from different groups. A two tailed $p<0.05$ was considered statistically significant.

4.3.5 Fe$^{2+}$ block.

The voltage dependence of block by Fe$^{2+}$ was described by a model that assumes that Fe$^{2+}$ binds within the electrical field of the membrane, with Fe$^{2+}$ entry and exit exclusively from the extracellular solution (Woodhull, 1973)

$$ f' = 1 / \left[ 1 + \left[ \frac{[Fe^{2+}]}{K_{D,0} e^{\frac{zFV}{RT}}} \right] \right] $$

(1)

where $f'$ is the fraction of peak tail current remaining in the presence of Fe$^{2+}$, and $K_{D,0}$ is the $K_D$ at 0 mV.

4.3.6 Permeation model.
The classical 2-site 3-barrier model of channel permeation (2S3B) (Almers and McCleskey, 1984; Hess and Tsien, 1984), was extended to Fe$^{2+}$, as for Cd$^{2+}$ (Lopin et al., 2010b). In brief, parameters for the energy profile of Fe$^{2+}$ were chosen that minimized the sum of absolute error to currents recorded due to Fe$^{2+}$ block and permeation. The parameters for Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, and Na$^{+}$ were fixed to the parameters from Lopin et al., 2010 where they were fit to a wide range of ionic conditions. The parameters chosen were the best fit but other parameter sets also fit the data well (7 parameters sets were within 10% of the total error of the best fit parameter set). Because control currents in Fe$^{2+}$ permeation experiments were significantly larger than controls in experiments from Khan et al. (2008), we assumed 18000 per cell as opposed to 8000. We expect that Fe$^{2+}$ currents are minimally affected by the 0.2 pH unit difference between solutions. Because it was not always possible to record control currents after the test solution we used the first control for normalization, a procedure that will underestimate the iron currents in the presence of current run-down.

4.3.7 Gating.

To investigate the effect of Fe$^{2+}$ on channel activation we fitted simultaneously relative open probability ($P_{OR}$) measured in controls and Fe$^{2+}$ to a fourth-power Boltzmann function

$$P_{OR}(V) = \frac{1}{1 + e^{\frac{-[V-(V_{50}+\delta_{f}A_{50})]}{k}}}$$

(2)
where $V_{0.5}$ is the half point of activation of each individual voltage sensor, $k$ is the voltage sensitivity, and $\Delta V_{0.5}$ is the shift in $V_{0.5}$ induced by Fe$^{2+}$ and $\delta$ is the Kronecker delta ($\delta = 1$ when $i=j$ and 0 else) function where it takes the value of 1 for Fe$^{2+}$ and 0 for controls.

The effect of Fe$^{2+}$ on the rate of channel opening was addressed indirectly studying changes in the time to peak (TP). To this end we simultaneously fitted data from controls and Fe$^{2+}$ to equation 4

$$TP(V) = e^{(-[V-(V_{1-TP\infty}-\Delta V_{1-TP\infty})]/k)} + TP_{\infty}$$  

(3)

where $V_{1-TP\infty}$ is the voltage at which the time to peak is equal to one minus the asymptotic value of TP ($TP_{\infty}$), $k$ is the voltage sensitivity and $\Delta V_{1-TP\infty}$ is the shift along the voltage axis.

The effect of Fe$^{2+}$ on the closing rate was investigated fitting simultaneously deactivating time constants between -70 and -120 mV to equation 4

$$\tau(V) = e^{\left[\ln(2)\cdot(V-(V_{\tau2ms}-\Delta V_{\tau2ms})/k)\right]}$$  

(4)

where $V_{\tau2ms}$ is the voltage where the time constant equals 2 ms. $\Delta V_{\tau2ms}$ is the displacement induced by Fe$^{2+}$ along the voltage axis, and $k$ is the slope factor.

Gating shifts were calculated using the Minerror procedure (Mathcad) to calculate the values of $\sigma_i$ and $K_{Fe}$ that minimize $\chi^2$ for $\Delta V_{0.5}, \Delta V_{1-TP\infty}$, and $\Delta V_{\tau2ms}$.  

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Through the paper, data are shown as mean ± sem. We used the paired t-test implemented in Origin 7.0 (OriginLab corporation) to assess differences between means when controls were from the same cell. We used one way ANOVA to investigate differences between means from different groups. A two tailed $p<0.05$ was considered statistically significant.
4.4 RESULTS

4.4.1 Effects of Fe$^{2+}$ on permeation.

Extracellular application of Fe$^{2+}$ reversibly inhibited currents through CaV3.1 channels evaluated using the IIV protocol (Fig. 1A). In these ionic conditions (2 mM Ca$^{2+}$ and 145 mM Na$^{+}$), CaV3.1 channels exhibited inward currents carried mostly by Ca$^{2+}$ and outward currents carried by Na$^{+}$. Note that the inward tail currents in Fe$^{2+}$ were smaller and faster. Peak tail currents were reduced immediately after repolarization, suggesting that Fe$^{2+}$ reached steady-state block rapidly. Block was concentration- and voltage-dependent, with strong inhibition at negative voltages, but little effect on outward currents (Fig. 1B). Fe$^{2+}$ had no clear effect on the reversal potential (Fig. 1C). The voltage dependence of block is best illustrated by chord conductances, especially around the reversal potential (Fig. 1D). The fractional inhibition, measured from chord conductances, was well described by a Woodhull (1973) model (Fig. 1E), suggesting negligible relief of block by hyperpolarization. The data were fitted best with $K_D = 2.5$ mM at 0 mV, and electrical distance $\delta = 0.17$.

4.4.2 Effects of Fe$^{2+}$ on gating.

Fe$^{2+}$ also inhibited currents examined with the IV protocol, evoking currents by direct depolarizations from the holding potential (Fig. 2A). The peak current at each voltage is shown in Fig. 2B, on an expanded scale in Fig. 2C, and as chord conductances in Fig. 2D. With this protocol, inhibition by Fe$^{2+}$ was also voltage-dependent, stronger at more negative voltages, and the voltage producing peak inward current was shifted to more positive voltages (Fig. 2C). Inhibition of peak current could also be described by a Woodhull (1973) model, with $K_D = 1.4$ mM at 0 mV and $\delta = 0.33$.

Why does the effect of Fe$^{2+}$ appear to be more potent and more voltage-dependent with the IV protocol? The currents recorded in that manner are affected not

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only by permeation (e.g., channel block), but also by gating (e.g., surface charge effects of Fe$^{2+}$).

We examined the effects of Fe$^{2+}$ on activation by three measures: effects on the time course of channel activation (Fig. 3A) and deactivation (Fig. 3B), and on the voltage-dependence of peak activation (Fig. 3C). Activation curves were measured as the relative open probability ($P_{O,r}$), calculated as the ratio of peak current from the IV protocol divided by the current at that same voltage from the IIV protocol (Fig. 3C) (Khan et al., 2008; Serrano et al., 1999). This is a more accurate reflection of channel open probability than the more commonly used chord conductance, since the open channel conductance is not constant with voltage (Fig. 1D), so the chord conductances measured with the IV protocol (Fig. 2D) are not pure measures of channel activation (Khan et al., 2008). All three measures of the voltage-dependence of channel gating were affected by Fe$^{2+}$ in a similar manner (Fig. 3D), demonstrating positive shifts along the voltage axis with Fe$^{2+}$. It is noteworthy that Fe$^{2+}$ did not affect the limiting rates for channel activation (Fig. 3A) or inactivation (Fig. 3B) at strongly depolarized voltages.

Qualitatively, the observed voltage shifts were as expected from a surface charge mechanism, where cations screen a negative surface charge on the extracellular side. Quantitatively, the effect was approximately twice as large as previously observed for Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ (Khan et al., 2008; dashed curve in Fig. 3D). Since simple charge screening (Gouy-Chapman theory) assumes all divalent cations are equivalent, we considered the possibility that Fe$^{2+}$ can bind to the surface charge, as well as screening it (Gouy-Chapman-Stern theory), as observed for Cd$^{2+}$ (Lopin et al., 2012b). Fig. 3D demonstrates that the data can be described well by the same surface charge density previously determined for effects of Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ ($\sigma = 1$ e$/98$
Å², Khan et al. 2008), but allowing binding of Fe²⁺ to the surface charge with $K_F = 4.5 \text{M}^{-1}$.

4.4.3 Effects of Fe²⁺ with 2 mM Ba²⁺.

CaV3.1 calcium channels are selective for Ca²⁺ over Ba²⁺ by the classical criterion of permeability ratios, reflecting a more positive reversal potential with Ca²⁺, indicating greater selectivity vs. monovalent cations (Serrano et al., 2000). Correspondingly, di- and trivalent cations block more rapidly and/or strongly when Ba²⁺ is the charge carrier, reflecting stronger competition vs. the less permeant Ba²⁺ ion (Mg²⁺, Serrano et al., 2000; Ni²⁺, Obejero-Paz et al., 2008; Y³⁺, Obejero-Paz et al., 2005; Cd²⁺, Lopin et al. 2012). That was also observed for Fe²⁺ (Figs. 4-5). 0.13 mM Fe²⁺ blocked strongly at hyperpolarized voltages, using either the IIV (Figs. 4A, 5A) or IV protocols (Figs. 4B, 5C). Inhibition measured from chord conductances, using the IIV protocol, was described by a Woodhull model with $K_D = 0.33 \text{mM}$ at 0 mV and $\delta = 0.21$ (Fig. 5B). Block was slightly overestimated by the model at the most negative voltages, suggesting relief of block by exit of Fe²⁺ into the cell. That low concentration of Fe²⁺ had minimal effect on channel activation (Fig. 5D). The activation curve was shifted by +2.8 ± 1.0 mV, and time constants for activation and deactivation by 2.9 ± 0.5 mV and -1.0 ± 1.6 mV respectively.

4.4.4 Permeation by Fe²⁺.

When extracellular Ca²⁺ and Na⁺ were replaced by Fe²⁺ and TEA (respectively), inward currents were small but clearly detectable (Fig. 6). Currents were larger in 9 mM vs. 1 mM Fe²⁺ (Fig. 6C), as expected for permeation by Fe²⁺. The chord conductance in 9 mM Fe²⁺ was 1.5 ± 0.2 -fold larger than in 1 mM Fe²⁺, averaged from -150 to -50 mV ($p < 0.01$).
An alternative interpretation is that the inward currents observed in Fe\textsuperscript{2+} might be "off" gating currents. To evaluate that possibility, we compared the integrated tail current amplitudes to gating currents, isolated using a combination of 0.1 mM La\textsuperscript{3+} and 2 mM Cd\textsuperscript{2+} to block ionic currents (Fig. 7). The insets in Fig. 7A show that the inward currents were larger in Fe\textsuperscript{2+}. Quantitatively, the integrated Fe\textsuperscript{2+} tail current greatly exceeded the gating currents at voltages where tail currents were relatively large and slowly decaying (Fig. 7B). The amplitude of the integrated tail current increased with [Fe\textsuperscript{2+}]\textsubscript{o} (Fig. 7C). There was considerable scatter in the data, presumably reflecting cell-cell variation in channel expression levels, so the apparent K\textsubscript{D} of 4.7 mM for saturation of current with Fe\textsuperscript{2+} should be considered a rough estimate.

Reversal potentials were less positive in Fe\textsuperscript{2+} than in Ca\textsuperscript{2+} (Fig. 6C), -26.1 \pm 4.7 mV (n=4) in 1.1 mM Fe\textsuperscript{2+}, and -9.0 \pm 3.7 mV (n=5) in 8.9 mM Fe\textsuperscript{2+}. Those values correspond to permeability ratios P\textsubscript{Fe}/P\textsubscript{Na} = 16 or 5, respectively. That compares to P\textsubscript{Ca}/P\textsubscript{Na} = 87 and P\textsubscript{Ba}/P\textsubscript{Na} = 44 (Khan et al., 2008), and imply P\textsubscript{Fe}/P\textsubscript{Ca} = 0.06-0.18.

Incubation studies with \textsuperscript{59}Fe\textsuperscript{2+} were performed and showed a trend towards increased Fe\textsuperscript{2+} uptake by Ca\textsubscript{v} 3.1 channels but were inconclusive due to large background uptake of Fe\textsuperscript{2+} and increased cell death (data not shown).

4.4.5 Model for Fe\textsuperscript{2+} permeation and block.

We fitted the data on Fe\textsuperscript{2+} permeation and block to a 2-site 3-barrier (2S3B) Eyring rate theory model (Almers and McCleskey, 1984). The model parameters include a high central barrier for Fe\textsuperscript{2+} (Fig. 8A), explaining the lower rate of permeation for Fe\textsuperscript{2+} compared to Ca\textsuperscript{2+}. The fit of the model to the data is shown for Fe\textsuperscript{2+} permeation (Fig. 8B), and for block of current carried by Ca\textsuperscript{2+} (Fig. 8C) or Ba\textsuperscript{2+} (Fig. 8D).
We used the model to estimate the extent of Fe$^{2+}$ permeation at concentrations more relevant to physiological or pathophysiological conditions (Fig. 8E-F). Simulated addition of 1-10 µM Fe$^{2+}$ (to extracellular solutions also containing 2 mM Ca$^{2+}$) yielded predicted Fe$^{2+}$ influx at rates up to several hundred ions per second through a single open channel (Fig. 8E). The mechanism of Fe$^{2+}$ permeation predicted by the model is similar to the permeation of Ca$^{2+}$ with 3 main differences. Both ions seem to get into the pore and bind to the first site similarly but Fe$^{2+}$ is slower to move over to the second site, binds less tightly to second site, and the energy barrier to Fe$^{2+}$ exit from the pore is higher. Structurally this would suggest the binding site deeper in the pore binds Fe$^{2+}$ less favorably than Ca$^{2+}$.

Ca$\text{v}$3.1 channels inactivate rapidly and strongly, but inactivation is incomplete, with 1-2% of channels remaining open even after 0.3 sec, 20× the time constant for inactivation (Serrano et al., 1999). This produces a ‘window current’ that can potentially allow maintained entry of divalent cations into the cell even near the resting potential. When the 2S3B model for permeation was combined with the Serrano et al. (1999) model for gating of Ca$\text{v}$3.1, the predicted steady-state Fe$^{2+}$ influx peaked at 6 ions/sec near -60 mV, in 10 µM Fe$^{2+}$ (Fig. 8F). Correction for slow inactivation would lower these values by ~35% (Hering et al., 2004).

It should be noted that 7 other sets of parameters fit the data qualitatively the same with only slightly higher absolute errors, all alternative parameters predicted high Fe$^{2+}$ transports rate up to twice the rate shown.
4.5 DISCUSSION

We conclude that Fe$^{2+}$ affects currents through CaV3.1 channels by three mechanisms: block of the open pore by Fe$^{2+}$, shifts in channel activation, and permeation by Fe$^{2+}$. Fe$^{2+}$ permeates CaV3.1 pores poorly, compared to Ca$^{2+}$ or Ba$^{2+}$ or even Cd$^{2+}$ (Lopin et al., 2012b), but the estimated rate of Fe$^{2+}$ entry suggests that CaV3.1 is a strong candidate for Fe$^{2+}$ influx in conditions where free Fe$^{2+}_o$ is present at micromolar concentrations. We discuss first the biophysical mechanisms of Fe$^{2+}$ interaction with calcium channels, and then the potential implications for iron overload.

4.5.1 Block by Fe$^{2+}$.

The effect of Fe$^{2+}$ on the IIV relationship is consistent with block by occupancy of the pore, presumably at the ‘selectivity filter’ responsible for selectivity for Ca$^{2+}$ and other di- and trivalent cations. First, block is voltage-dependent, well approximated by a Woodhull (1973) model assuming binding to a site within the electrical field of the membrane. Second, block was ~4-fold stronger when 2 mM Ba$^{2+}$ was the charge carrier (compared to 2 mM Ca$^{2+}$), suggesting ion-ion competition within the pore. Reduction of the current measured ‘instantaneously’ implies that Fe$^{2+}$ equilibrates rapidly with the open pore, on the time scale of the voltage clamp (~0.1 ms). For the lowest concentration used (0.13 mM Fe$^{2+}$, for experiments with Ba$^{2+}$), that implies binding with a bimolecular rate constant of $10^8$ M$^{-1}$s$^{-1}$ or faster, near the diffusion limit.

4.5.2 Effects of Fe$^{2+}$ on gating.

The effect of Fe$^{2+}$ on the peak current measured with the IV protocol was stronger and more voltage-dependent than with the IIV protocol. Since the current measured with the IV protocol is affected both by permeation and gating (i. e., changes in the
probability that a channel is open at a particular voltage and time), this suggests that Fe\(^{2+}\) affects the response of the channel to voltage. Fig. 3 shows that the effect of Fe\(^{2+}\) can be attributed to screening and binding to surface charge. We assumed a Guoy-Chapman-Stern for simplicity but a specific binding site on the channel can not be excluded.

### 4.5.3 Fe\(^{2+}\) permeation.

In the absence of Ca\(^{2+}\)\(_o\) and Na\(^{+}\)\(_o\) Cav3.1 channels carry a significant Fe\(^{2+}\) current that saturates in the millimolar range. The currents measured in Fe\(^{2+}\) were small, showing Fe\(^{2+}\) is a poorly permeant ion, but were noticeable larger than could be attributed to gating charge movement (Fig 7B). This current increased as external Fe\(^{2+}\) was increased, suggesting that the current is carried by Fe\(^{2+}\) and not contaminating cations.

### 4.5.4 Cav3.1 as a pathway for Fe\(^{2+}\) entry.

To explain the effects of Fe\(^{2+}\) on permeation we expanded a model of permeation for Cav3.1 channels to account for Fe\(^{2+}\). The model fit the data well, although block of outward currents by 1.1 mM Fe\(^{2+}\) was underestimated (Fig. 8B). The model can assess Fe\(^{2+}\) permeation when Ca\(^{2+}\) and Mg\(^{2+}\) are present in physiological concentrations. Fig 8E and F show calculated transport rates for Fe\(^{2+}\) for external concentrations in the range of 1-10 μM and membrane potentials encountered at rest and during action potentials. Cav3.1 channels have a window current due to incomplete inactivation (Serrano et al., 1999) that leaves ~1-2% of channels open at resting membrane potentials. Because channels are open even at resting membrane potentials, we used our model of Cav3.1 channel gating to calculate the fraction of channels expected to be open in the steady state (Serrano et al., 1999). This value, times the transport rate calculated in Fig 8F, is the number of Fe\(^{2+}\) ions transported per second and channel (Fig 8D).
4.5.5 Comparison of Fe$^{2+}$ to other divalent cations.

We have now examined effects of several divalent cations on permeation and gating of CaV3.1: Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, Ni$^{2+}$, and Cd$^{2+}$ (Khan et al., 2008; Lopin et al., 2012b; Obejero-Paz et al., 2008). To a surprising extent, each ion is an individual that the channel can easily distinguish. As previously established for L-type calcium channels, there is a spectrum from highly permeant ions to strong blockers. Ca$^{2+}$ and Ba$^{2+}$ are the most permeant at millimolar concentrations, although they also potently block currents carried by Na$^{+}$ at micromolar concentrations. Mg$^{2+}$ is nearly impermeant, but blocks currents carried by Ca$^{2+}$ from either side of the membrane (Khan et al., 2008). Fe$^{2+}$ appears at first to be a Mg$^{2+}$-like blocker, but can carry small inward currents. The classical calcium channel blocker Cd$^{2+}$ produces surprisingly large inward currents, and has a reversed voltage-dependence of channel block (Lopin et al., 2012b). Ni$^{2+}$ seems to be unique in blocking rapidly at an extracellular site (that cannot distinguish Ca$^{2+}$ from Ba$^{2+}$), in addition to slow block at at the selectivity filter (Obejero-Paz et al., 2008). Except for the fast block site for Ni$^{2+}$, these effects can be explained by a 2-site 3-barrier model as subtle quantitative changes in the energetics of ion binding, as opposed to distinct biophysical mechanisms.

4.5.6 Iron overload.

Iron is normally tightly regulated in the body (Zhang and Enns, 2009). Whether a cause or consequence of the pathological process, increased cellular iron content has been associated with disorders in both the heart (Horwitz and Rosenthal, 1999; Kremastinos and Farmakis, 2011) and the brain (Stankiewicz and Brass, 2009) where accumulation has been associated with a number of neurological disorders including amyotrophic lateral sclerosis (ALS), Parkinson’s, and Alzheimer’s disease (Oshiro et al., 2011). Intracellular iron build up leads to the production of reactive oxygen
species through Fenton-type reactions that causes oxidative damage to proteins, lipids, and DNA (Giorgio et al., 2007).

4.5.7 Pathways for NTBI iron influx.

In neurons, voltage-gated calcium channels (Gaasch et al., 2007b) and NMDA receptors (Pelizzoni et al., 2011) have been implicated in NTBI. Studies with calcium channel blockers implicated both L-type channels and non-L high voltage-activated channels in Fe$^{2+}$ entry into hippocampal neurons (Pelizzoni et al., 2011). The cerebrospinal fluid has levels of iron that saturate transferrin, leaving ~1 µM free iron (Bradbury, 1997). This iron should be maintained in its ferrous form (Fe$^{2+}$) by high levels of ascorbate (Bradbury 1999) and ferrireductases (Lane et al., 2010; Mills et al., 2010). In the case of cerebral hemorrhage free iron levels were measured to peak at >10 µM and to remain above 5 µM for 28 days (Wan et al., 2006). This could provide sufficient free Fe$^{2+}$ for entry into neurons via calcium channels.

The mechanism of Fe$^{2+}$ uptake into cardiomyoctes is still debated (Chattipakorn et al., 2011). Block of L-type and T-type calcium channels in cardiomyocytes in vivo (Kumfu et al., 2012; Oudit et al., 2003) can decrease iron uptake into the heart, an indication that sufficient free Fe$^{2+}$ is available in the plasma for calcium channels, and it can permeate in the presence of physiological levels of Ca$^{2+}$.

While the main mechanism of preventing excess iron in cells is to prevent iron uptake there are mechanisms cells use to efflux excess iron out of the cell, the main protein being ferroportin1 (Fpn1) (Donovan et al., 2000). To maintain iron homeostasis, when iron levels are increased the liver releases hepcidin (Park et al., 2001) which binds to Fpn1 causing its endocytosis and degradation (Nemeth et al., 2004). Normally this reduces plasma iron levels by decreasing Fpn1 in intestinal cells, decreasing iron absorption from the diet (Ganz, 2011). In iron overload conditions where iron
absorption is unregulated in this way due to repeated transfusions to treat a blood disorder the mechanism of hepcin downregulating Fpn1 could cause iron handling problems. In cells with unregulated Fe$^{2+}$ entry, such as cardiomyocytes and neurons which have a large number of calcium channels, hepcidin release will cause Fpn1 to decrease (Wang et al., 2010), lowering the capacity of cells to export Fe$^{2+}$ while calcium channels continue to allow unregulated Fe$^{2+}$ entry.

Our results indicate that Ca$_{v}$3.1 channels can constitute a pathway for iron entry at resting membrane potentials, and possibly during the course of action potentials, when extracellular Fe$^{2+}$ reaches concentrations in the micromolar range.
4.6 FIGURE LEGENDS

Fig. 4.1. Block by Fe$^{2+}$ of currents carried by Ca$^{2+}$ with the IIV protocol. A, sample records of currents, using the protocol illustrated below the middle record, in control (left), after addition of Fe$^{2+}$ (middle), and after washout of Fe$^{2+}$ (right). 3 kHz Gaussian filter. Currents shown here are in 40 mV increments. B, IIV relations from the protocol of A, in control (0 Fe$^{2+}$), and in two concentrations of Fe$^{2+}$ (n=4 for each concentration). C, expanded view of IIV relations. D, chord conductances calculated for the data from B. E, Inhibition by Fe$^{2+}$, expressed as the ratio of the chord conductance in Fe$^{2+}$ to the chord conductance in control conditions. Data not shown near the reversal potential, where errors in calculating conductances can be large. Solid curves are fits to a Woodhull (1973) model. Symbols in B apply to panels B-E.

Fig. 4.2. Block by Fe$^{2+}$ of currents carried by Ca$^{2+}$ using the IV protocol. A, sample records, shown in 20 mV increments, with 3 kHz Gaussian filter. B, IV relations from the protocol of A, for the same cells as Fig. 1. C, IV relations on an expanded scale. D, chord conductances from the data of B. E, inhibition by Fe$^{2+}$, as the chord conductance ratio, fitted to a Woodhull (1973) model. Data not shown near the reversal potential. Symbols in B apply to panels B-E.

Fig. 4.3. Effects of Fe$^{2+}$ on gating. A, effect of Fe$^{2+}$ on the time-to-peak of currents, using the IV protocol. B, effect of Fe$^{2+}$ on the time constant for deactivation, using the IIV protocol. C, effect of Fe$^{2+}$ on channel activation, as the ratio of the peak IV current to the IIV current at the same voltage. D, voltage shifts for the data shown in A-C, for the activation curve ($\Delta P_{O_{1/2}}$), for deactivation ($\Delta \tau_{IIV}$), and for time to peak
(\Delta t_{\text{peak}}). The solid curve is a fit to Gouy-Chapman-Stern theory. The dashed curve is the fit to voltage shifts induced by Ca\(^{2+}\), Ba\(^{2+}\), and Mg\(^{2+}\), from Khan et al. (2008). Symbols in A apply to panels A-C. Data not shown near the reversal potential in A-C. Same cells as Figs. 1-2.

**Fig. 4.4.** Effects of Fe\(^{2+}\) with 2 mM Ba\(^{2+}\) as charge carrier. A, sample records with the IIV protocol, 40 mV increments. B, sample records with the IV protocol, 20 mV increments. 3 kHz Gaussian filter.

**Fig. 4.5.** Analysis of effects of Fe\(^{2+}\) in 2 mM Ba\(^{2+}\). A, IIV relations in control and in 0.13 mM Fe\(^{2+}\). B, inhibition by Fe\(^{2+}\), from the ratio of chord conductances (Fe\(^{2+}\)/control), fitted to a Woodhull (1973) model. C, IV relations (currents from +50 to +100 mV not shown). D, activation curves, from IV/IIV current ratios. Data not shown near the reversal potential in B and D. n=4 for all panels.

**Fig. 4.6.** Permeation by Fe\(^{2+}\). A, sample records with the IIV protocol, in 40 mV increments, with 3 kHz Gaussian filter. The inset below the middle record shows currents from -100 to +20 mV, on a 5-fold expanded scale. B, IIV relations. C, IIV relations on an expanded scale. Symbols in C also apply to B. Data in B-C are from 7 cells, 4 in 1.1 mM Fe\(^{2+}\), 5 in 8.9 mM Fe\(^{2+}\).

**Fig. 4.7.** Comparison of tail currents in Fe\(^{2+}\) to gating currents. A, sample records with the IIV protocol, in 40 mV increments. Note partial recovery (right panel).
following superfusion with the solution used to isolate gating currents (La$^{3+}$ + Cd$^{2+}$). The insets below the middle two records are on a 5× expanded scale, to show tail currents in Fe$^{2+}$ and on- and off gating currents in La$^{3+}$ + Cd$^{2+}$. 3 kHz Gaussian filter.

B, integrated tail currents in Fe$^{2+}$ compared to $Q_{on}$ (measured during depolarization to +60 mV) and $Q_{off}$ (measured following repolarization). n=5 (Fe$^{2+}$ tails) and n=4 ($Q_{on}$ and $Q_{off}$).

C, the portion of the integrated tail current amplitude attributable to Fe$^{2+}$ entry, as a function of Fe$^{2+}$, for the 5 cells in B, and 2 cells tested at ~1 mM Fe$^{2+}$. The solid curve is a fit to a single saturable binding site with $K_D = 4.7$ mM and maximal Q = 0.78 pC.

**Fig. 4.8.** A 2S3B Eyring model for permeation and block by Fe$^{2+}$. A, energy levels and electrical distances for barriers and wells, for Ca$^{2+}$ and Fe$^{2+}$. Energy levels (outside to inside) were 9.32, -12.73, 5.26, -6.97, and 15.65 RT. The electrical distances and energy profiles for Na$^+$, Mg$^{2+}$, and Ba$^{2+}$ are from Lopin et al. (2011).

B to D, fits of the model to experimental IIV data are shown for permeation (B), block of current carried by 2 mM Ca$^{2+}$ (C), and block with 2 mM Ba$^{2+}$ (C).

E, calculation of the rate of Fe$^{2+}$ entry for the indicated Fe$^{2+}_o$ concentrations, in the presence of 2 mM Ca$^{2+}_o$ for a single open channel. F, calculation of the steady-state rate of Fe$^{2+}$ entry considering channel gating (activation and inactivation). The steady-state $P_{o-V}$ relationship calculated from the Serrano et al. (1999) model was convolved with the curves in panel E.
FIGURE 4.1

A. 2 mM Ca²⁺  
2 mM Ca²⁺ + 1.4 mM Fe²⁺  
2 mM Ca²⁺  

B. [Fe³⁺]₂⁺ (mM)  
-150, -100, -50  

C.  
-150, -100, -50  

D. G (nS)  
-150, -100, -50  

E. G/F²⁺/G Control  
-150, -100, -50  

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FIGURE 4.3
FIGURE 4.4

A

2 mM Ba²⁺  +0.14 mM Fe²⁺  2 mM Ba²⁺

+80
-100 mV

+100
-140

3 nA

5 ms

B

-100 mV

-80

1 nA

5 ms
FIGURE 4.5

A

-150 -100 -50

-16 -12 -8

-4 0 4

nA

mV

B

Fe²⁺/Control

-150 -100 -50

-2 -0.5 0

0 0.5 1

mV

D

2 mM Ba²⁺ □

+0.13 mM Fe²⁺ ●

-50 0 50 100

mV

C

-80 -40 40

-1.0 -0.5 0 0.5 1.0

nA

mV
FIGURE 4.6
FIGURE 4.8
CHAPTER 5

Conclusions and Future Directions
5.1 Block and Permeation of Fe\textsuperscript{2+} and Cd\textsuperscript{2+} on \(\alpha_1G\)

It was clearly shown that Fe\textsuperscript{2+} could block and permeate through \(\alpha_1G\) calcium channels (Lopin et al., 2012a). The block was voltage and charge carrier-dependent suggesting it is caused by pore block. The block of Fe\textsuperscript{2+} was stronger at hyperpolarized potentials suggesting that Fe\textsuperscript{2+} either can not permeate or can only slowly permeate through the pore. However, a small inward current was observed in the presence of Fe\textsuperscript{2+} and the absence of other permeable ions. This current was ionic current because it was larger and slower than the gating current. The current also increased as the concentration of extracellular Fe\textsuperscript{2+} was increase, a sign that the current was carried by Fe\textsuperscript{2+}. If Fe\textsuperscript{2+} was not permeable to \(\alpha_1G\) channels and the current was carried by protons or contaminating cations then the current would decrease as the Fe\textsuperscript{2+} concentration was increased and more channels were blocked. This study showed that \(\alpha_1G\) channels make cell membranes permeable to Fe\textsuperscript{2+}.

Cd\textsuperscript{2+} is classically used as a calcium channel blocker; though earlier studies showed that hyperpolarization leads to relief of Cd\textsuperscript{2+} block (Diaz et al., 2005b; Swandulla and Armstrong, 1989) suggesting that Cd\textsuperscript{2+} is permeable to calcium channels. In this study we showed that Cd\textsuperscript{2+} does block \(\alpha_1G\) channels but it does not block them completely as there was \(\sim 20\%\) of the current remaining even at 3 mM Cd\textsuperscript{2+}. It was also shown that Cd\textsuperscript{2+} could carry large inward currents through \(\alpha_1G\) channels in a dose-dependent manner showing that \(\alpha_1G\) channels are permeable to Cd\textsuperscript{2+}.

5.2 2 Binding Site 3 Barrier Model Fit to Block and Permeation
The 2S3B model of permeation was able to fit a large amount of permeation and block data in α1G calcium channels. Permeation by Na\(^+\), Ca\(^{2+}\), Ba\(^{2+}\), Cd\(^{2+}\), and Fe\(^{2+}\) were fit well. The model was able to describe well the block of Fe\(^{2+}\) and Cd\(^{2+}\) on Ca\(^{2+}\) and Ba\(^{2+}\) currents and captured the increased affinity of divalent block on Ba\(^{2+}\) currents compared to currents carried by Ca\(^{2+}\). The model was able to qualitatively reproduce stronger block of Mg\(^{2+}\) on Ba\(^{2+}\) currents but was not able to quantitatively fit the differences of the block of Ca\(^{2+}\) and Ba\(^{2+}\) currents by Mg\(^{2+}\) (Lopin et al., 2010a). Mg\(^{2+}\) has a large dehydration energy for its first shell waters in solution. This makes Mg\(^{2+}\) have a binding rate that is highly dependent on the charge of the ligand it is binding to, i.e. the rate for Mg\(^{2+}\) binding to ATP\(^{4-}\) is faster than binding to ADP\(^{3-}\) which is faster than AMP\(^{2-}\). There may be a difference in the conformation of the acidic residues in the selectivity filter of α1G channels when a Ca\(^{2+}\) is bound versus a Ba\(^{2+}\) bound that is not reflected in the 2S3B model. This conformation may change the charge seen by the Mg\(^{2+}\) ion affecting the rate of Mg\(^{2+}\) entry. The 2S3B model has the same entry rate for Mg\(^{2+}\) no matter what ion is in the second binding site.

The model has many free parameters that could lead to multiple parameters sets fitting the data, but the large data set the model had to fit helps to confine the parameters. For example, the ion repulsion factor Q could fit the whole cell currents well with a large range of values from 1 to 25. The use of single channel recordings could eliminate Q factors in the lower range (1-2) because the model could not reproduce the relatively slow kinetics of Na\(^+\) currents blocked by 3 µM extracellular Ca\(^{2+}\). Similarly, a high Q value of 11.89 could fit the Ba\(^{2+}\) and Ca\(^{2+}\) permeation but
not the Fe\(^{2+}\) data. This shows that for a model with a large number of parameters a large data set is needed to confine the parameters.

Parameters for Cd\(^{2+}\) were well defined but the fits to the Fe\(^{2+}\) data gave multiple parameter sets that fit the data well (errors within \(\pm 10\%\)). This could be due to multiple reasons including that the concentration of Fe\(^{2+}\) could not be held constant for different experiments because of its redox dynamics so multiple concentrations of Fe\(^{2+}\) had to be pooled to create data sets that could be fit, leading to errors in the conditions the model fit. Also the concentration of Fe\(^{2+}\) had to be measured during each experiment from a sample of the solution being applied to the cell; this should give an appropriate Fe\(^{2+}\) concentration but the concentration of Fe\(^{2+}\) changes over the 2-5 minutes it takes to gather the electrophysiological data. Fe\(^{3+}\) was also ignored in the model but it could affect the data in ways not reflected in the model. While Fe\(^{3+}\) is often thought of as insoluble in water, due to its propensity to hydrolyze water, chelators such as the citrate used in our solution can bind and make ferric iron soluble and available for uptake by cells (Kumfu et al., 2013). The data set was not as large as for Cd\(^{2+}\) because of the difficulties in performing experiments with Fe\(^{2+}\) so the parameters were not as well defined. Alternatively, using a 2S3B model of permeation to describe Fe\(^{2+}\) block and permeation through \(\alpha1\)G channels may just give multiple solutions to the data.

5.3 Rate Theory Models

The original rate theory model developed by Svante Arrhenius was a purely phenomological models where the average transition rates were observed empirically
and the underlying physical process were ignored (Dougherty, 2006). The Eyring equation was an attempt to provide a mechanistic understanding to the Arrhenius equation by computing the rates as a pre-exponential factor (A) and an activation energy (Ea), i.e.

\[
\text{rate} = A \cdot e^{-\frac{E_a}{kT}}
\]

Where k is the Boltzmann constant and T is the temperature. There are two main assumptions used in the model that have been raised that could be improved in the future to refine the 2S3B model (see following paragraphs). The first assumption is that pre-exponential factor which was first derived in 1919 by Karl Ferdinand Herzfeld using statistical mechanics for the reversible reaction of a diatomic molecule (Dougherty, 2006) to calculate the pre-exponential factor as the universal frequency kT/h, where h is Planck’s constant. This factor is applied generally, including in our model, even though it was derived from a specific reaction and it has been suggested that it be modified in the case of modeling permeation (Hill, 1976). The second objection to Erying rate theory is that it assumes a quasi-equilibrium between the system and the transition state (Winzor and Jackson, 2005). To overcome these objections it has been proposed to remove the mechanistic interpretation of the Erying equation and use the Arrhenius rate equation on a purely empirical bases (Winzor and Jackson, 2006). A future direction of the 2S3B model would be to replace the rates calculated using the activation energies and Eyring rate equations with just the rates of ion movement (see Fig 5.1). One of the rates shown in the figure would have to be constrained so that there is no ion flux in isotonic solutions at 0 mV. This model would make no mechanistic assumptions about how ions permeate through an ion
channel but may be able to quantitatively describe ion transport through a channel better than the Erying rate theory model used in the current study.

Another assumption used in the model that could be improved is the ion-ion repulsion factor Q. In the model, it is calculated as simple electrostatic repulsion between 2 charges when it is most likely that counter charges of the acidic residues would be between the ions and screening their interactions. Incorporating counter charges into the Q factor could be simple or difficult depending on the geometry and complexity of the counter charges incorporated but could yield valuable structural information about the pore including the physical distance between 2 ions in a pore and the configuration of the acidic residues in the pore.

The other short coming of applying Erying rate theory to ion channels is the assumption that the transporting particles are in equilibrium and applying Boltzmann statistics to the particles to calculate the rates. If the first binding site is 25% of the way through the electric field then a divalent entering the channel moving to the site will pick up the energy of $z\cdot q\cdot \delta \cdot V = 2\cdot q\cdot 0.25\cdot 100 \text{ mV} = 50 \text{ meV}$, where $z$ is the valance charge, $q$ is the elemental charge, $\delta$ is the fraction of the electrical distance, and $V$ is the voltage. This energy of 50 meV is two times $kT$, the average energy of a particle so that an ion that is entering a channel will have much more kinetic energy than is assumed in the model. This increase kinetic energy will increase the velocity of the cation traveling through the pore. Making this situation more complicated is that the energy of the ion will dissipate back to a Boltzmann distribution of energy with time if the ion does not cross the following energy barrier right away.
Incorporating this into the model would be difficult but would highlight the ballistic nature of permeation ions missing from rate theory models.

The use of the 2S3B model also raises the question of how are ions bound in the pore of calcium channels, in two separate binding sites or one site that can bind two ions. The model used in this thesis assumes movement between two distinct rigid binding sites. Another model of permeation is the ‘car wash’ model of Ca$^{2+}$ permeation (Sather and McCleskey, 2003). This model incorporates a single high affinity site with two low affinity sites, mimicking a 3-site 4-barrier model, but greatly increases the number of free parameters that have to be fit, see figure 5.2. The matrix for this model has been written but the outer data handling structure has not. Comparing how the two models fit the data would help to understand the structure of the pore of calcium channels and how far apart the acidic residues are.

Mutation studies performed on L-type calcium channels showed that mutating the glutamates of different domains caused different effects on how divalent blocked the channel (Yang et al., 1993). A future direction to test the validity of a 2S3B or a ‘car wash’ model of permeation would be to mutate the acidic residues in the pore and fit the IIV curves of the mutated channels to the model and see if single mutations can be explained by changing the binding energy of just one binding site. Changing the acidic residues may cause too big of a change to the channel and may create other confounding effects such as causing structural changes. Subtler mutations to surrounding amino acids could also be performed to see how they affect the parameters of the model. By substituting amino acids in the p-loop with slightly larger analogs and fitting the new currents to a model of permeation and observing what
parameters change, whether binding sites or transition sites, could give structural and topological data about the permeation pathway of α1G calcium channels.

The three dimensional structure of ions channels may allow ions to permeate through the pore in a way that can not be captured in a one dimensional energy profile used in rate theory models (Allen et al., 2006). Other methods of permeation are also used to study permeation such as Poisson-Nernst-Planck, Brownian Dynamics, and Molecular dynamics (Chung and Kuyucak, 2002; Roux et al., 2004; Sugioka, 2012). These methods use a three dimensional structure of the pore and model the interaction of the ions with the protein during permeation through the channel. To save computational space the permeating ions’ valance electrons are not modeled and the ions are treated as spheres. This will ignore the coordination chemistry of ions, which become important in selecting between ions with the same valence number. The methods also need a three dimensional structure of the protein, something which is not available for a eukaryotic calcium channel. The methods have been used to study ion permeation through potassium channels for over a decade with multiple potassium channel structures (Doyle et al., 1998; Roux and MacKinnon, 1999; Yu and Roux, 2009). Still many controversies remain over fundamental questions of permeation in these channels (Miloshevsky and Jordan, 2004; Varma and Rempe, 2010). These methods, like rate theory models, make many assumptions to make them easier to use. For example Poisson-Nernst-Planck theory lacks a method to handle induced surface charges due to an ion approaching a low dielectric region (Feynman et al., 1963), a force that becomes large in narrow channels such as ion channels (Krishnamurthy and Cornell, 2012).
A way to get around these problems would be to implement a quantum mechanical treatment of the channel. As noted by Richard Feynman 30 years ago, “I'm not happy with all the analyses that go with just the classical theory, because nature isn't classical, dammit. And if you want to make a simulation of nature, you'd better make it quantum mechanical, and, by golly, it's a wonderful problem because it doesn't look so easy” (Feynman, 1982). Doing this would involve solving the multi-body Schrödinger equation in a self-consistent manner, a Hartree-Fock method (Cramer, 2005). This method is not currently used, as it is extremely difficult and time consuming to implement on digital computers. New computers such as optical, quantum, and analog computers (Marinica et al., 2012; Temme et al., 2011) being developed now have the promise of being able to perform complex mathematical functions found in Schrödinger’s equation much quicker than current computers and could be a method in the future of understanding ion permeation in the future.

5.4 Iron Transport

Iron is an essential biological ion but excess iron build up can cause problems. Iron overload is caused by two main conditions hereditary haemochromatosis where excess iron is absorbed from the diet and in patients that have to receive constant blood transfusions. These two different conditions have different challenges associated with them. For hereditary iron haemochromatosis, absence large scale genetic screening, patients and their physician will not know they are building up iron in their system for decades (Delatycki et al., 2004). Understanding where and how iron is deposited over these decades will help to explain the problems people with
hereditary haemochromatosis often have with their heart, pancreas, kidney and liver (Klopfleisch and Olias, 2012).

For iron overload due to blood transfusions it will be known that patients will have elevated iron levels so chelation treatment can be prescribed, but the chelation therapy does not completely remove all free iron (Kwiatkowski, 2011). This along with a high non compliance rate for chelation therapy means that patients with ongoing blood transfusions will have abnormal iron levels and iron trafficking (Porter et al., 2011). Understanding how this iron traffics will help understand complications that often rise from iron overload in thalassemia patients (Daar et al., 2010).

Recent studies have tried to discern the role different types of calcium channels play in iron uptake in the heart (Chattipakorn et al., 2011; Das and Oudit, 2012) and in neurons (Gaasch et al., 2007a) during iron overload. To do this they measure iron uptake in a mouse model or in cell cultures and look at the effects of channel ‘specific’ blockers, though some have questioned the specificity of some of the blockers used in these studies (Das and Oudit, 2012). Others have also questioned the validity of using animal models given the differences in how they store iron (Klopfleisch and Olias, 2012). The dynamics of iron overload depends on the rates of iron entry and exit from all sources of transport in each cell type. Iron could enter cells from multiple pathways, including transferrin mediated iron entry, divalent metal transporter 1 (DMT1) and ion mimicry of iron going through other cation channels and transporters. This thesis dealt with quantitatively calculating the rate of Fe$^{2+}$ entry through α1G T-type calcium channels. Given the lack of quantitative data on other cation channels and transporters it is still difficult to accurately describe what channel or transporters
are responsive for non-transferrin mediated iron entry. Calculating the rate of iron entry by building a model of permeation for other calcium channels would require a hefty amount of work and any uptake rates would be model dependent not direct uptake measurements. A faster way to determine the rate of iron entry though channels and transporters would be to directly measure iron uptake through channels and transporters. This could be done with high throughput using an iron sensitive fluorophore such as PhenGreen SK (Illing et al., 2012), X-ray spectrometry (Milman et al., 1983), or radiolabeled Fe\(^{2+}\). An attempt was made to measure iron uptake into HEK cells expressing α1G but was inconclusive because of a large background iron uptake (data not shown). This is most likely due to endogenous iron transporters in HEK cells masking any uptake that may be occurring through α1G channels. To get around this channel/transporter expression and iron uptake experiments could performed on cells that show low iron permeability such as Xenopus oocytes (Illing et al., 2012) or in artificial liposomes. These experiments would give the rates that different channels and transporters allow iron to permeation through membranes. This would allow a better understanding of what proteins are responsible for non-transferrin bound iron entry.

A more physiological approach to understanding how iron is transported would be to measure where iron accumulates in an organism under physiological or pathological conditions. This is often done in mouse models or when it is done on human cells, on cultured cells. Both of these models systems have raised concerns because they do not accurately describe the physiology of human patients (Huh et al., 2011; Klopfleisch and Olias, 2012). Knockout mice are often used but they can have
confounding effects, such as upregulation of a channel isoform or behavioral changes that obscure the results (Gerlai, 1996), and some channels do not have specific blockers so that individual channels can be tested (Das and Oudit, 2012). To get around this issue groups have developed ‘organ’ and ‘human on a chip’ techniques to use primary human cells to grow organs in bioreactors where drugs can be tested and the physiology of the organs can be better understood (Marx et al., 2012). Organs on a chip could be used to study how iron is taken up by different organs. The most important organs effected by iron overload are the liver, pancreas, heart, kidneys, and the brain (Siddique and Kowdley, 2012). There is now work on producing all of these organs on chip (Carraro et al., 2008; Jang and Suh, 2010; Lee et al., 2007; Park et al., 2009; Shackman et al., 2012). Using the individual organs on a chip, it can be observed how the different cell types in those organs take up iron during iron overload conditions seen in patients with haemochromatosis. To measure of the uptake of iron into the cells can be measured using a fluorescent dye, but this would introduce another intracellular iron binding pool, which may change how iron is handled in a cell. An alternative approach is to use a fluorescent reporter tagged to the iron responsive elements (IREs) (Li et al., 2004). The IREs respond to increased or decreased cellular iron so tagging them with a fluorescent marker would allow real time measurement of intracellular iron changes. For longer term measurements of iron uptake, the organs on a chip could be biopsied and the iron levels measured.

With this model of measuring iron uptake, the mechanisms responsible can be screened for by using different channel or transporter blockers. If a specific blocker is not available small interfering RNA (SiRNA) can be used to knock out the protein,
SiRNAs can also be used to verify that the blockers used are specific and the effects seen are due to the protein in question and not some secondary effects. Understanding what pathways are most important for iron entry could allow for new therapeutic interventions in treating iron overload conditions.

**5.5 Cadmium transport**

Studies similar to iron uptake in organ on a chip could be done with Cd\(^{2+}\) uptake to understand what mechanisms are responsible. There will more difficulty in measure Cd\(^{2+}\) uptake though as there are no proteins or RNAs that naturally respond to intracellular Cd\(^{2+}\) changes that could be fluorescently tagged as is the case for iron. The use of Fura 2 and Quin 2 has been used as fluorescence markers of intracellular Cd\(^{2+}\) and could be used to measure Cd\(^{2+}\) uptake (Hinkle et al., 1992). Identifying what mechanism control Cd\(^{2+}\) influx and efflux from cells would help develop a therapeutic treatment for Cd\(^{2+}\) toxicity.
5.6 Figure Legends

Figure 5.1: 2-Binding Site 3-Barrier model will just rates, energy barriers show to distinguish the two sites.

Figure 5.2: ‘Car wash’ model of permeation (only half of the acidic residues are shown for clarity). Top panel is the interaction of a single ion with the selectivity filter of a calcium channel. The rates are modeled as k9, k10, k11, k12 and electrical distances are shown below. Middle panel is the energetic barriers for ion movement. Capital letters are the electrical distances between barriers and wells, lower case letters and Greek letter names are total electrical distances of both ions and acidic residues moving when one ion enters a channel already occupied by another. Bottom panel shows the method of permeation for when one ion enters a pore already occupied by another ion and how one ions exits the pore when it is occupied by two ions. The electrical distances traversed for the individual ions are shown, Greek letter names for ions moving to the right and lower case letters for moving to the left. Rates (k1-k8), not shown for clarity, are associated with each ion. Constraints on electrical distances are A+B+C+D=1; alpha+beta+gamma+delta+e+g+h+f=1; A+C=beta+delta+g+e; B+D=alpha+gamma+h+f.

Car wash model adopted from (Sather and McCleskey, 2003; Yang et al., 1993)
5.7 Figures

Fig. 5.1
Fig 5.2
REFERENCES


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