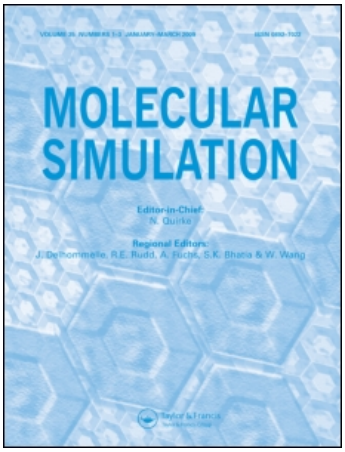

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Calcium Block of Sodium Current in a Model Calcium Channel: Cylindrical Atomistic Pore with Glutamate Side Chains

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Calcium Block of Sodium Current in a Model Calcium Channel: Cylindrical Atomistic Pore with Glutamate Side Chains

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Biological calcium channels are known to have permeation selectivity governed by the acidic groups on four conserved glutamate side chains, one each from the P regions of the four homologous domains. The binding selectivity of this filter region is revealed experimentally by the fact that at low external [Ca^{2+}] (1 μM), current carried by Na^+ ions is blocked completely. In our previous applied field non-equilibrium molecular dynamics (AF NEMD) simulations with confined but mobile half-charged oxygen atoms, there was no evidence of high affinity calcium block of sodium current [Yang, Y., Henderson, D. and Busath, D. (2003) "Applied-field molecular dynamics study of a model calcium channel selectivity filter", *J. Chem. Phys.* 118, 4213]. Here we report similar simulations with a similar model in which the glutamate side chains are explicitly represented. To efficiently optimize ion chelation in the filter, the channel was initiated with an ion in the filter. With the right channel diameter and sufficient channel length, AF NEMD simulations with such preloaded channels yield significant evidence of calcium-block of sodium current. In 9 out of 10 simulations, a calcium remains positioned in the filter coordinated simultaneously by three or four glutamate side chains throughout the 2 ns simulation, completely preventing Na^+ passage in all (but one) case. In contrast, with a Na^+ ion preloaded in the filter and no calcium in the bath or channel, Na^+ flows smoothly through the filter region. The Na^+ ion positioned in the filter at the outset escapes the glutamate side chains in 9 out of 10 cases within 470(±470) ps. On the assumption that the relative exit rates for the two ions are closely related to their relative binding affinities the results indicate that the binding constant for calcium is higher than for sodium. However, the second part of this permeation phenomenon responsible for calcium currents in calcium channels, namely calcium-relief of calcium-block, could not be observed on the time scale accessible to this approach.

Keywords: Calcium block; Sodium current; Cylindrical Atomistic Pore; Glutamate side chain

INTRODUCTION

Calcium channels are important membrane proteins in heart, nerve, and muscle cells. We focus here on the L-type calcium channel, which is one of many subtypes. Crystal structures are available for a somewhat related protein, the KcsA potassium channel [2,3], which is a homotetramer, each subunit comprised of two transmembrane helices linked by a P region that forms the channel selectivity filter. The α subunit of the L-type calcium channel consists of four homologous domains linked into a single chain. Like KcsA, it has a selectivity filter formed by four linkers, but in addition to the two structural transmembrane helices, each domain has an additional four transmembrane helices thought to be responsible for voltage gating [4].

Structural information about the L-type calcium channel must so far be inferred from site-directed mutagenesis, sequence homology, and other phenomenological data. To summarize briefly, the L-type calcium channel is permeable to

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tetramethylammonium (TMA) and Na^+ in the absence of Ca^{2+} [5]. Na^+ current is blocked by 1 μM Ca^{2+} external to the cell, and current is recovered when external [Ca^{2+}] exceeds ~1 mM [6]. The TMA permeability demonstrates that the internal diameter of the so-called selectivity filter region of the pore can be stretched easily to exceed 5.6 Å. The Ca^{2+}-block of Na^+ current (CBSC) is thought to demonstrate that a calcium ion can cause rearrangement of the pore interior to occlude the pore. The Ca^{2+}-relief of Ca^{2+}-block (CRCB) is thought to imply that multiple Ca^{2+} ions in the selectivity filter can disrupt the tight binding of one Ca^{2+} ion and allow Ca^{2+} mediated current flow. These interpretations were fortified by the discovery that conserved glutamate residues in each of the four repeats of a calcium channel were responsible for CBSC and CRCB [7]. In particular, CBSC is shifted to much higher [Ca^{2+}] in mutants approximating the homologous sodium channel [8–11], as well as in the native sodium channel itself [7], where instead of four glutamates at the homologous positions, there are aspartate, glutamate, lysine, and alanine residues.

A few simple energy computations of model calcium channels were performed quite early after this discovery [12,13], and recently several chemical physics computations have been carried out to more carefully analyze the electrostatic basis of CBSC and CRCB. Namely CBSC was predicted from properties of homogeneous solutions [14] and with Monte Carlo simulations of an infinite cylinder and a finite pore using the primitive model of electrolyte with confined half-charged oxygen atoms representing the glutamate side chains [15–17]. However, our initial attempts to simulate selective current flow with explicit-solvent applied field non-equilibrium molecular dynamics (AF NEMD) through a simple model calcium channel consisting of a cylindrical pore with eight confined half-charged oxygen atoms in the external (with respect to the biological cell) portion of the channel showed no CBSC or CRCB [1].

On the other hand, recent studies with more biologically realistic channels, modeled after the potassium channel, showed statistical behavior of ion exit rates from the selectivity filter consistent with CBSC and CRCB [18,19]. Here we report subsequent AF NEMD simulations with a more realistic (but still simple) model of a calcium channel consisting of a cylindrical atomistic pore with inward projecting glutamate side chains attached near one end (corresponding to the external end of the biological channel) of the 25 Å channel. As before, we use a rigid cylindrical channel and membrane comprised of generic neutral atoms. The side chains are anchored at their alpha carbons and are free to rotate about dihedral angles, but have rigid bond angles and bond lengths. However, for this initial report, we followed the approach in Ref. [18], focusing more on the statistics of ion exit from the filter rather than holistic current flow. For each of three ionic systems, 20 Na^+, 1 Ca^{2+} + 18 Na^+, or 10 Ca^{2+}, (in each case with 16 Cl^- so that the entire system would be electroneutral) there was an applied membrane potential of 2.2 V driving inward cation current. This is about 10-times the physiological membrane potential, but has been found for the filter-less version of this channel to be in the electrodiffusive (non-ballistic) regime [20]. We evaluated the probability of exit during each of ten different 2-ns trajectories for a prepositioned ion in the selectivity filter (Na^+ in the first system and Ca^{2+} in the other two) and whether the presence of that ion prevents the other ions in the system from entering the filter and passing through the channel. A simulation of 20 ns is inadequate to detect Ca^{2+} ion passages in a 7.5 pS channel like the L-type calcium channel [5], even at the high membrane potential used here (16.5 pA corresponds to ~2 passages/20 ns). However, using this approach, we can estimate the ratio of the single-Ca^{2+} and (multiple) Na^+ exit rate constants.

METHODOLOGY

AF NEMD simulations were performed using the Gaussian mechanics program [21] that we have used previously [1,20,22,23] with constant N, V, and T. Periodic boundary conditions in all directions provided recycling of ions being driven through the channel. Temperature was maintained with a holonomic constraint.

The system consisted of two 15-Å thick slabs of water molecules separated by a membrane that contained a single channel. The membrane consisted of impermeable layers of neutral Lennard-Jones (LJ) atoms ($\sigma = 2.5$ Å) forming a 10×10 array with total dimensions of 25 Å × 25 Å. The distance between monolayer centers was 25 Å. Squares of 36 atoms in the center of the membrane were removed from the intracellular and extracellular monolayers respectively as openings to the channel. The cylindrical channel walls consisted of 11 rings of 20 charged LJ atoms ($\sigma = 2.5$ Å) with a repeating pattern of partial charges, +0.35, −0.35, +0.50, −0.50 as defined previously [20]. The diameters of the rings (center-to-center) were 16.625 Å for the most extracellular ring, 14.625 Å for the next ring deeper, and 12.625 Å for the remaining 9 rings. Atom overlap was sufficient in the larger rings to prevent permeation through the channel wall. The four glutamate side chains were anchored to wall atoms at square-corner atoms in the third ring. Bond lengths and angles were taken from the CHARMM 19 force field, whereas LJ parameters and partial charges were taken from the CHARMM

TABLE I Parameters used in the simulations

Site	σ (Å)	ϵ/k (K)	q (e)
Na^+–Na^+	2.583	50.3	+1
Ca^{2+}–Ca^{2+}	2.869	50.3	+1
Cl^-–Cl^-	4.401	50.3	− 1
O–O (H_2O)	3.169	78.2	− 0.8476
H–H (H_2O)	0.0	0.0	+ 0.4238
$C_{\alpha,\beta}$–$C_{\alpha,\beta}$ (Glu)	3.982	60.4	0.0
$C_{\beta,\gamma}$–$C_{\beta,\gamma}$ (Glu)	3.982	60.4	− 0.1
C_δ–C_δ (Glu)	3.564	60.4	0.62
O–O (Glu)	3.029	60.4	− 0.76

22 force field. Bond lengths and angles were kept constant through the use of Gaussian mechanics. Side chain dihedrals (χ_1, χ_2, and χ_3) were freely mobile, constrained only by non-covalent interactions. There were no dihedral energy terms in the potential energy function. The wall atoms represented the C_α of the amino acid; other backbone atoms were not represented explicitly.

Atom parameters are shown in Table I. Lorentz-Berthelot rules were used for mixed interactions. 584 SPC/E water molecules were used to get ~55 M water in the equilibrated system. Dissolved in the water were either 20 Na^+ and 16 Cl^- ions (solution A), 1 Ca^{2+}, 18 Na^+, and 16 Cl^- ions (solution B) or 10 Ca^{2+} and 16 Cl^- ions (solution C). Because the glutamate side chains each bore a net charge of −1, the total system charge was 0.0. The number of ions in the pure solutions was designed to yield nominal concentrations of 2 M Na^+ and 1 M Ca^{2+}. The mixed solution represents 0.1 M Ca^{2+} and 1.8 M Na^+. The channel contents varied considerably during the runs. Because the results presented here focus on exit from the filter, rather than current flow, we consider the precise concentration of ions in the bath to be of minor consequence.

Ten simulations, differing in the initial positions and random velocities for the ions and water molecules, were carried out for each ionic solution. In each run, one ion (Na^+ in solution A, Ca^{2+} in solutions B and C) was prepositioned at a certain location (at the center of the channel, $Z = 17.5$ Å) near the four glutamate side chain carboxylates with the channel otherwise empty. This was done to facilitate binding of the ion to the glutamate side chains because, in the preliminary studies, we found that glutamate mobility decreases dramatically after ions and water molecules enter the filter and were generally poorly disposed to strongly ligate a single cation in the resulting configurations. As usual, an electric field of 0.4 V/nm along the z axis was applied to all mobile atoms, temperature was regulated, and the current was computed using methodology developed previously [1,20,22]. The applied field leads quickly to a voltage drop of 2.2 V across the membrane. For simplicity, no heating or equilibration periods were used. This contrasts with our preliminary and previous [1,20,22,23] methodology, where no prepositioning was used and steady state structure of the channel and bath were obtained before beginning production simulation.

In addition to the 30 2-ns runs reported here, several other channel configurations with realistic glutamate side chains were studied preliminarily, generally using 30 10-ns runs in each case. These included a 15-Å long cylindrical 11.625-Å diameter channel, a 25-Å long cylindrical channel with diameter 12.625-Å, and a 25-Å long channel with an outer vestibule leading to a diameter of 12.625 Å (like the principal study, but without ion prepositioning).

RESULTS

Because of the addition of realistic glutamate side chains to our simple channel, it was necessary to experiment with the structure a bit. The preliminary studies with shortened channels, narrow diameters, and no prepositioning of ions allowed us to identify conditions where there was adequate room for side chain rotations and ion passages, adequate separation of the selectivity filter anions from exit bath counterions, and adequate coordination of prepositioned ions by the side chains. We start with a description of the spontaneous development of ion coordination by the side chains resulting from our method of ion seeding.

Typically, for the first 10–20 ps of the simulations, before the channel had time to fill with ions or water molecules, the side chains each rotated dramatically about all three rotatable bonds while the prepositioned ion moved around throughout the filter region. Then, over a few ps, the prepositioned cation developed a complex with the side chains. For prepositioned Ca^{2+}, three or four of the glutamate carboxylates oriented near the channel axis, simultaneously coordinating the cation. This effect was stronger with Ca^{2+} than with Na^+ as is shown in Fig. 1, where a frame from the solution A run is shown on top and a frame from solution B on the bottom.

While complexation of the prepositioned ion was occurring, the bath structure (water orientations and ion positions) also evolved. Within a few hundred ps, other cations approached the entry. In the runs with solution A (a prepositioned Na^+ ion), these would usually enter the filter region readily and pass through, whereas for solutions B and C (a prepositioned Ca^{2+} ion), one to four other cations aggregated just outside the channel for the entire run, never entering nor regressing. Na^+ ions in solutions A and B penetrated deeper into the filter region than Ca^{2+} ion in solution C, evidencing a reduced penalty for dehydration, but in solution

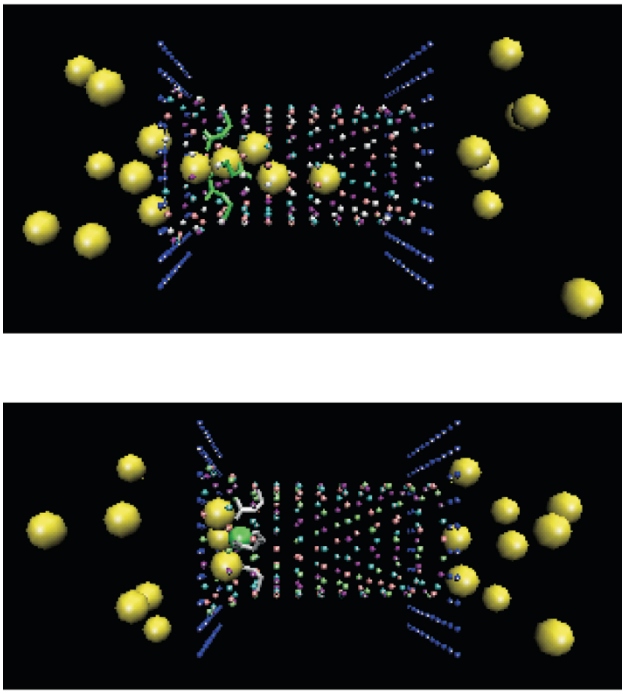

FIGURE 1 Snap shots of the simulations. (Top) Solution A: 20 Na^+, 16 Cl^-. Yellow atoms represent Na^+ ions and the four glutamate side chains are represented in green (bottom). Solution B: 1 Ca^{2+}, 18 Na^+, 16 Cl^-. Yellow atoms represent Na^+ ions, the green atom represents Ca^{2+} ion, and the four glutamate side chains are represented in white. Membranes and channel are also shown here in blue, pink and purple spheres, but water molecules and Cl^- ions are left out for clarity.

B the approaching Na^+ ions did not disrupt the complex with the prepositioned Ca^{2+}.

Figure 2 shows the time courses of ion coordination by the filter for solution A. The minimum distance of the prepositioned Na^+ ion from any of the filter carboxyl oxygen atoms is plotted as a function of time for each of the ten simulations. In all but one case, the Na^+ left

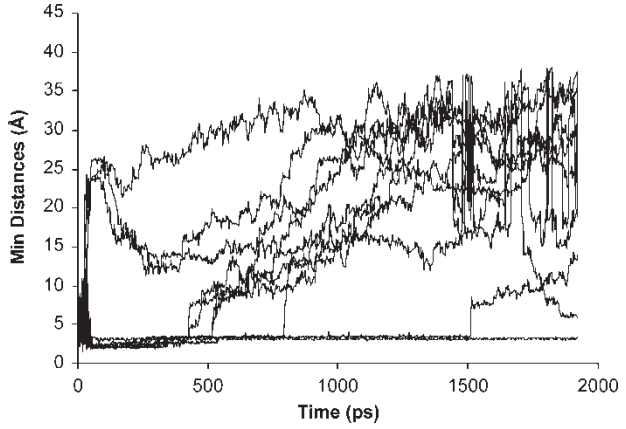

FIGURE 2 Time courses of prepositioned Na^+ ion coordination by the filter for solution A (all Na^+). The minimum distance between the prepositioned atom and the glutamate oxygen atoms is plotted against simulation time. Each of the ten simulations is represented as single trajectory.

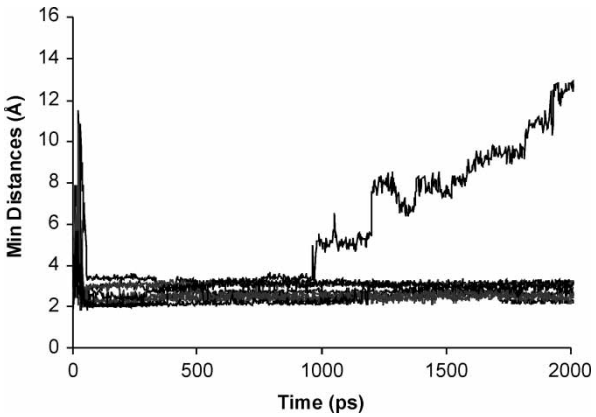

FIGURE 3 Time courses of prepositioned Ca^{2+} ion coordination by the filter for solution B (one Ca^{2+}, remainder Na^+), as in Fig. 2.

the filter within 2 ns, often to pass completely out of the channel. The residence time in the nine cases was 470 ± 470 ps, a reasonably short period. The standard deviation is consistent with an exponentially distributed dwell time. In contrast, Fig. 3 shows the same data for the runs with solution B. In all but one case, the prepositioned Ca^{2+} remained ligated to the glutamate(s) for the entire 2 ns period. For 8 of these cases, no Na^+ ion was able to pass the Ca^{2+} in the channel, clearly indicating that when three or four glutamates project axially they sterically occlude the channel for this geometry, as had been observed previously for 8-stranded β-barrel and KcsA-based homology protein models of the L-type calcium channel [18].

In Fig. 4 the time course of Ca^{2+} association with the filter in the runs with solution C (10 Ca^{2+}) is shown. Here, we hoped that Ca^{2+} ions would be able to dislodge the prepositioned Ca^{2+} from its site, but unfortunately that did not occur in 9 of 10 cases. From the animations of these simulations, we deduced that Ca^{2+} ions in the bath did not dehydrate during the 2 ns period, consistent with the fact that the experimental inner shell water molecule residence time is ~4 ns [24].

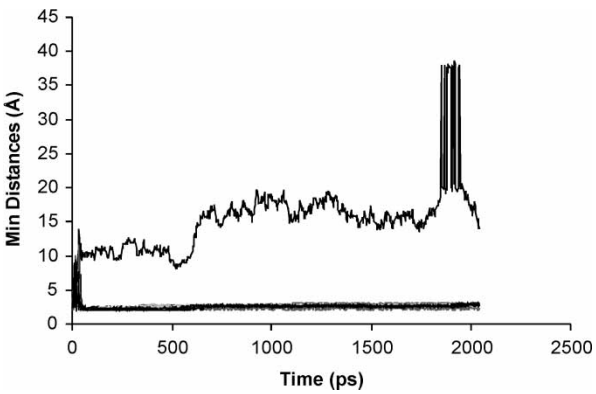

FIGURE 4 Time courses of prepositioned Ca^{2+} ion coordination by the filter for solution C (all Ca^{2+}), as in Fig. 2.

From these results, it is clear that the exit rate constant for a prepositioned Ca^{2+} ion is much lower than for a Na^{+} ion. An upper limit on the exit rate constant can be roughly estimated as the inverse of the aggregate dwell time in the simulations. This is an upper limit because in some cases the simulation is truncated before an exit occurs, particularly for the Ca^{2+} simulations where the estimate is probably much more skewed by this factor. Using this crude statistic, the ratio of exit rate constants is below 0.2, qualitatively consistent with experimental CBSC.

DISCUSSION

This result indicates that, at least for the level of membrane potential utilized here, poly-ligation of the Ca^{2+} ion in the filter organizes the glutamate side chains so as to occlude the channel similar to an iris. This notion of the mechanism is quite different from that envisioned in the charge-space competition model, where it is supposed that selective occupancy of the filter region by Ca^{2+} will suffice to exclude Na^{+} from the region and thus block Na^{+} flow. Although the charge-space competition appears to suffice at zero driving force, which is the assumption in the Nonner *et al.* [14] study and the Monte Carlo simulations [14–16], it is not clear whether at physiological voltages there is enough driving force on the Na^{+} ions in the bath to drive the Ca^{2+} out. Indeed, considering the present simulations together with the previous AF NEMD simulations done at 10-times the physiological voltage (Ref. [1] and preliminary studies), it appears that Na^{+} can readily knock Ca^{2+} out of the filter unless it is tripley or quadrupley coordinated by anchored side chains [18]. Furthermore, it appears from the current study that multiple coordination of Ca^{2+} provides more specific, stronger binding than is obtained with half-charged spheres. This could partly simply reflect the strong charges on the carboxylate oxygen atoms. The steric block occasioned by poly-coordination is thus an alternative or an extension to the charge-space competition model, in that it invokes two additional features that selective binding alone does not include: steric closure of the channel and stronger specific binding by anchored carboxylates than by free oxygen atoms. However, determining whether these factors are physiologically relevant in biological calcium channels will require both knowledge of the structure and computational studies at physiologically voltages (-30 to -75 mV), which neither the MC nor the AF NEMD have yet been able to examine.

Also, CRCB will require more work, at least with this model. Previous studies show that if 2nd and 3rd Ca^{2+} ions are forcibly inserted into the filter, that they successfully dislodge the primary Ca^{2+} within 2 ns [18]. Here we had hoped to show spontaneous entry of 2nd and 3rd ions into the filter and dislodging. Ca^{2+} channel currents require that Ca^{2+}'s pass through the channel on the microsecond time scale, so our results may not be inconsistent with physiology if first shell residence times can be overcome on that longer time scale. One approach to this challenge could be to use umbrella sampling MD simulations to estimate the free energy along the transport reaction coordinate for use with phenomenological theories or simulations (i.e. rate theory, Poisson-Nernst-Planck equations, the Smoluchoswki equation, random walk, Brownian dynamics, etc.). However, from the results presented here it appears that this will have to be done with care because the aggregation of ions and water molecules in the channel vestibule and selectivity filter may be quite diverse.

In summary, CRCB at high voltages appear to involve novel filter mechanism, steric block and high affinity specific multi-carboxylate coordination.

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