**Response to Reviews**

I thank all the reviewers for carefully reading the manuscript and providing thoughtful comments. I have incorporated most of these into the revised manuscript and believe that this has significantly improved the paper. To account for some of the comments, I have included three new sets of results. The single Ca2+ PMF has been obtained from 3 independent simulations to get an idea of the reproducibility of the results. A new2 ion Ca2+ - Ca2+ PMF (figure 7 in the revised manuscript) and a new analysis of the energy required to partially dehydrate the ions (table 1) have also been included. Specific responses to each of the reviews are listed below.

**Reviewer 1**

*The background and current pertinant literature are well described, except for some neglect of early theory on space-charge competition in the charged selectivity ... and of some similar simulations with similar results done by Yan Yang under my supervision...*

References have been included to the space-charge competition model of Nonner et al in the introduction. The work of Yang et al is particularly interesting in understanding what changes may make the pore Ca2+selective and so discussion of this work has been included in the results section and at the end of the discussion.

*First, the glutamates are not free to follow the ions during translation through the filter, and if they were it would probably affect a point that receives a lot of emphasis from the author: that the high barrier to Ca++ at 0 Angstroms may be the source of the preference for Na+ transport...*

In our simulations there are no constraints on the glutamate residues that prevent them from following the ion other than the interactions with other parts of the protein. In some cases we do see the glutamate residues swing to follow the ion as it passes through the pore, something which occurs more often with Ca2+ than Na+ as can be seen from the extended range of coordination by Glu in the plots of coordination number (figure 4). However, the Glu residues do also form hydrogen bonds with Ser 1180 and Met 1181 on the helix further from the pore. These hydrogen bonds do prevent excessive motion of the Glu residues and probably explains why Ca2+ is typically only coordinated by one Glu side chain rather than multiple side chains as in the work of Yang et al. Discussion of this point has been included in the revised manuscript.

*Secondly, and far more critical, the real barrier to Ca++ transport is really the barrier to leaving the filter and getting into the central cavity, a full 11 kcal/mol between 5 Ang and -5 Ang. The barrier at 0 Ang is of little importance in comparison.*

The second point here probably arises from a lack of clarity in the original manuscript. The left hand edge of figure 2 which shows a large and increasing barrier to Ca2+ represents the ion moving to the intracellular gate of the channel which is closed in the present structure. As the cavity closes down tightly at the left hand of the figure, the energy profile would likely be very different at this point in a fully open channel. A new paragraph discussing this point has been added when the single ion OMFs are first described in the results section. The barrier for Ca2+ to move from the filter into the central cavity can be determined from figure 2 using the right hand end of the central cavity (shaded gray in the figure) which is less likely to be affected by the closed gate. This shows a barrier of only 3kcal/mol. However, the reviewer is correct to say that Ca2+ binds strongly in the filter – the fact that it binds more strongly than Na+ is the reason why Na+ is more likely to pass a resident Ca2+ ion than for the Ca2+ to be pushed through the pore. We hope these issues have been clarified in the revised manuscript.

*Why is ASP in the label in Figure 4A?*

ASP has been removed.

*Why not remove the lipid tails from the channel? Aren't they likely to be artefacts of crystal formation?*

The lipid tails do not seem to simply be an artefact of the crystallization as they remain present even in completely unrestrained simulations – although there is seen an occasional exchange of lipids. This point is mentioned briefly in the revised manuscript, but will be a greater focus of future work.

*It may be that the asymmetric positions of the 4 Glu side chains in eukaryotic voltage-gated calcium channels, pointed out in the early site-directed mutagenesis/electrophysiology literature of the mid-1980's, helps reduce Ca++ binding affinity to allow higher Ca++ permeability.*

The influence of the asymmetric positions of the glutamate residues in calcium channels has been mentioned on page 9.

Other minor changes have been corrected.

**Reviewer 2**

*Another qualification that should be mentioned, is that the calculations are based on what Payandeh et al identify as “pre-open” state – closed at S6 bundle crossing...*

A new paragraph has been added early in the results section to discussion of the possible effects of using the ‘pre-open’ rather than open state of the pore.

*Figure 5B. At -4 Angstrom, in 5A, the sum of the points is about -700 kcal/mol, but the first point in 5B is about -1 kcal/mol. How can the curves in 5B be derived as a simple sum? What am I missing?*

The curved in figure 5B were shifted vertically so that the results for Na+ and Ca2+ could be plotted on the same figure. To do this, both graphs were set to zero at the left hand edge. As we were interested only in changes in the total energy with position, this does not alter the final conclusions. A note explaining this has been added to the figure legend and in the main text.

*Figures 6 & 7 I find the arrows confusing. Why not indicate the locations of the individual snapshots on the 2D surfaces by numbers 1, 2, 3, etc., and, in addition, plot the energetically favored path, through the selectivity filter for the selected ion, connecting the snapshot locations (Figs 6 & 7)? – subject, of course, to the reservations that you have stated regarding interpretation of quantitative details.*

Following the suggestion of the referee, the arrows have been replaced by numbers on the 2D plots. I have also indicated the lowest energy pathway for ion conduction by a dotted line in the revised figure.

*Discussion and conclusion: A suggestion - The immediately following material may be better used to expand the last paragraph of the Discussion. This would leave the Conclusions to focus specifically on factors, directly identified in your calculations, which may contribute to selectivity of Na over Ca.*

Additional text has been added to the discussion to highlight the importance of the additional negative charges in creating Ca2+ selectivity. The suggested references have also been added and the final sentence of the conclusions removed.

**Reviewer 3**

*Although a new 2 ion PMF is presented, the study focuses primarily on the single ion movement through the channel, which is of some but limited relevance, and claims to explain Na/Ca selectivity in terms of barrier heights...*

To get a better idea of the validity of the results we have included two new sets of results. Firstly we have calculated the single Ca2+ PMF two more times from independent simulations to get an idea of the reproducibility of the results. Secondly, we calculated the PMF with 2 Ca2+ ions in the pore. In all cases the maximum barrier for conduction arises at the same position near z=0.5A. These new results highlight that the presence of this barrier is not dependent on the conduction mechanism (1 ion vs 2 ion) or an artefact occurring in only a single simulation. The size of the barrier is reduced in the presence of two ions which may explain why the relative size of the Ca2+ current compared to Na+ is not as small as would be deduced from the single ion PMF.

*Is 1 nanosecond sampling for the calculation of the free energy sufficient and why? I would need to see convergence of the free energies and reproducibility of the results. Unfortunately, a block analysis of such short simulations might give misleadingly low errors. Perhaps a better approach would be to carry out completely independent calculations to demonstrate reproducibility.*

Following the suggestion of the referee we have conducted 3 independent calculations of the single Ca2+ PMF, the standard deviations of which are shown in the revised figure 2. This does show a standard deviation of up to 1.5 kcal/mol, highlighting the degree of uncertainty in the quantitative values of the PMF, but the barriers to conduction are located at the same positions in all cases lending support to the overall conclusion of the manuscript that these are the rate limiting steps to conduction.

*On page 5, the author states that it is because of the increased charge that Ca2+ binds more strongly to the protein. This is not self-evident.*

The reason for the stronger binding has been removed from the text.

*On page 5 the author claims that it is one position in the plane of the side chains Glu where “selectivity of Na over K arises”, which has yet to be proved.... Unless there is quantitative proof, the wording should be modified.*

The wording has been modified at the noted sections on page 2 and 5.

*Author claims a “significant drop” in the hydration of Ca below 6.5. But is this 0.3 or so water really important? What is disappointing is that the author digs up old analysis on the free energy costs of removing waters from Na, but then claims those energetics might also explain the barrier for Ca. But those calculations should be done on Ca2+ to be convincing. The statement “the barrier seen for Ca2+ seems to be due to a less than ideal combination of ion-protein and ion-water interactions” on the same page is vague.*

Additional analysis of the energy costs in partly dehydrating the ions has been done as suggested. The results are included in a new table added to the manuscript. A discussion of this new data is presented in the manuscript assessing whether the partial dehydration can account for the Ca2+ barrier.

 *I question the statement (page 6) that “we would expect Ca2+ to attenuate Na+ currents, but not block them”. The author should be careful not to misuse the term “block”, which of course can account for attenuated current.*

This statement has been clarified so that the word ‘block’ is not used (it is true that temporary blockage can lead to an attenuated current), along with some of the discussion in the following paragraph.

*Related on page 7, the author offers his idea that “Ca selectivity could be obtained by preventing Na from being able to pass a resident Ca ion”. Does this proposed mixed-ion mechanism relate to the experimental setup used for selectivity measurements?*

The possible mechanism suggested for creating ion selectivity by making it difficult for ions to pass in the channel is not specific to a particular experimental setup. All that is required for this mechanism to work is for Ca2+t be able to get into the selectivity filter – something that could arise from either side and thus in many experimental systems. The idea that a resident Ca2+ can guard the channel to prevent permeation of other ions has been discussed many times in the literature – see for example Hess and Tsien Nature 1984 or Corry et al Biophys. J. 2001.

*In terms of the model, how much should we believe the 5 kcal/mol bigger barrier for Ca? What are the likely pitfalls in the model that were not as big an issue for the monovalent ions and is there any precedent that might allow us to judge the severity of the problems? The author’s incorrect statements such as that divalent ions are problematic in a “non-polar force field” and “Ca can be expected to polarize surrounding molecules which is an effect not considered in the present study” either reveal careless writing or a lack of familiarity with the subject. Also, the brief mention of reproduction of hydration free energies on page 7 also does not bring to the reader’s attention the more extensive testing that would be needed for accurate results.*

The question raised by the reviewer is a very difficult one to answer. The calcium parameters used have been determined to reproduce experimental hydration free energies, but I am not aware of any studies that carefully compare free energies, interaction energies with proteins or ion-ligand structures derived from the classical force field with more detailed calculations, making it hard to estimate the likely uncertainty in the results described in the present manuscript. Having said this, there have been a number of ab-initio MD studies into hydration calcium ions which do give an indication of likely coordination structures and polarisation which may form the basis of future comparisons. I have extended the discussion of these aspects on page 8 of the revised manuscript and given some direct justification for the statement that divalent ions are more likely to polarise their environment than monovalent ions. The degree of testing of the ion parameters required to gain reliable results has also been clarified.