

Project title: „Ion Selectivity and Conduction Mechanism of Cation Channels Investigated by Molecular Dynamics Simulations and Solid-State NMR Spectroscopy“

Project number: K305/2020

Executive Summary

Ion channels are essential for all living organisms due to their involvement in different physiological processes such as synaptic transmission, muscle contraction and cell signalling. Better understanding of how ion channels function will aid in the development of new drugs against pathologies related to those processes. Ion channels are embedded in the cell membrane, and their function depends on conformational dynamics that occur across multiple time scales. This inherent complexity makes them difficult to study using a single structural biology approach. In this SAW project, we approached the question of ion selectivity and conduction mechanism by a combination of solid-state NMR and MD simulations with a particular focus on cation channels. Although the project was initially slowed down slightly due to the corona pandemic situation that e.g. limited access to the experimental labs and complicated the hiring process, we successfully overcame these challenges. As reported below, we have essentially completed all components of the proposed work packages. The resulting data have been published in leading scientific journals (e.g. *JACS*), including a joint publication on the mechanism of calcium block in the potassium channel MthK, which was recently submitted. The findings have been also presented at major international conferences and have contributed to the scientific and career development of young researchers in the team.

1. Achievement of objectives and milestones

Overall, we have achieved a major part of the originally proposed objectives and milestones. In **WP1**, we aimed to study ammonium (NH_4^+) ions in cation channels by a combination of solid-state NMR and MD simulations. Our initial study on the non-selective and selective cation channels NaK and NaK2K was published in the leading chemistry journal *JACS* (Öster et al., 2022). Based on these results, we were invited to submit a chapter for the book “Potassium Channels – Methods and Protocols”, published in *Methods in Molecular Biology* (Öster et al., 2024). Subsequently, we applied this technique to the more complex channel MthK (see below for further information). In **WP2** we wanted to use thallium (Tl^+) as a substitute for potassium (K^+). This work faced some technical problems and still needs to be performed in the future. NH_4^+ has turned out to be a very good mimic for K^+ , enabling experiments to be performed using standard equipment, but there are some drawbacks. Since NH_4^+ is made up of two different atom types, it requires additional magnetization transfers in NMR experiments (leading to lower sensitivity) compared to an ion that only consists of one atom (e.g. Tl^+). In **WP3**, we wanted to expand our previous work (as published in Shi, *Nat Comm* 2018 with a focus on NaK) on the response of the selectivity filter (SF) to different ion types by including a variety of K^+ -selective mutants of NaK. We successfully finished this work, resulting in a publication (Hendriks et al., 2021). Lastly, in terms of solid-state NMR we wanted to study the allosteric coupling between the helix bundle gate and the SF (**WP4**). With the successes we obtained with the NH_4^+ approach we decided to slightly shift the focus of this WP and study the allosteric coupling between the calcium (Ca^{2+})-binding RCK domains in MthK and the ion configuration in the SF. In **WP5** we aimed to investigate molecular determinants of sodium (Na^+)/ K^+ selectivity in NaK2K mutants using MD simulations. Here, we have not performed the simulations of the originally planned mutants, but rather focused on the simulations of the wt-NaK and the NaK C-DI mutant. The reason behind it is that our external collaborators have recently solved a series of high-resolution X-ray structures of these channels. Using a combination of MD simulations and different structural biology approaches, we could show plasticity as a key determinant for ion non-selectivity in NaK-related channels. These works have been published (Roy et al., 2021; Minniberger et al., 2023). In **WP6** our initial goal was to study Na^+ , K^+ and Ca^{2+} conduction mechanism and ion non-selectivity in NaK2CNG channels using MD simulations. However, we have

revised our original plan after high-resolution cryo-electron (cryo-EM) microscopy structures of the native cyclic nucleotide-gated (CNG) channels and the structurally related hyperpolarization-activated cyclic nucleotide-gated (HCN) channels became available during the funding period. As a result, the NaK2CNG mutant was no longer needed as a model system. Within this project, we therefore focused on MD simulation studies of ion conduction and selectivity mechanisms in both native HCN and CNG channels. In **WP7** efforts are ongoing to simulate full-length MthK in both the open and closed states in the presence and absence of Ca^{2+} . Calcium binding was found to affect the conformational dynamics of the calcium binding RCK domains. In addition, calcium binding in the cavity below the selectivity filter was observed. Here, a key role of residue E92 was identified, with mutations at this position leading to conformations closer to the closed state of the channel. Ca^{2+} binding near F87 just below the selectivity filter was found to explain the experimentally observed calcium block of MthK. In **WP8**, our goal was to study hydrophobic gating in MthK and NaK2K channels. MD simulations of the closed conformation of MthK showed indeed a rapid cavity dehydration, unexpectedly assisted by the lipid tails, suggesting a novel and hitherto unexplored role of the lipid membrane in MthK gating. This observation will be followed in the future by experimental validation and further simulations with varying membrane composition.

2. Activities and obstacles

Our solid-state NMR work became more focussed on the Ca^{2+} -gated K^+ -selective channel MthK in the last two years. The ion channels we originally worked on, NaK and its K^+ -selective mutant NaK2K, are good model systems for non-selective and K^+ -selective ion channels. MthK represents another level of complexity and it is more similar to eukaryotic large conductance, voltage- and Ca^{2+} -gated K^+ (BK) channels and therefore provides an excellent model system for this important class of ion channels.

We have investigated the full-length MthK ion channel and a truncated version containing only the pore domain. First, we performed chemical shift assignments of these different constructs, which is the first step necessary for any solid-state NMR investigation of a protein. We then used the experiments we developed during the initial years for detection of bound NH_4^+ ions, as replacement for K^+ , in the SF of the different MthK constructs. In full-length MthK that is activated by Ca^{2+} we could not detect any NH_4^+ signals when Ca^{2+} was absent. After adding Ca^{2+} to the sample we could detect bound NH_4^+ ions in the SF. These results confirm that Ca^{2+} binding in the RCK domains causes allosteric effects that open the channel and allow ions to enter the SF.

Regarding the MD work, the team of HS performed a series of simulations on the NaK channel and its mutant NaK C-DI. This work benefited from a series of high-resolution X-ray structures solved by our external collaborators (Dr. Leighton Coates, Oak Ridge National Laboratory, USA; Prof. Andrew Plested, Humboldt University of Berlin). We further expanded our MD work on the simulations of the homomeric and heteromeric human CNG channels. Here, we simulated the conduction of different cations, such as K^+ , Na^+ , and Ca^{2+} . Our simulation work has benefited from careful validation using single-channel electrophysiology data on HCN and CNG channels, performed in Prof. Klaus Benndorf's lab (University Hospital of Jena).

The team of BdG and WK focused on simulations of the MthK channel, closely mimicking experimental setups, i.e. investigating the effect of Ca^{2+} ions on the channel behaviour. These simulations revealed two binding sites for Ca^{2+} below the SF, that led to the Ca^{2+} -mediated channel block, when occupied. Moreover, they further enabled a detailed analysis of the effect of voltage and Ca^{2+} ions on the SF occupancy by K^+/NH_4^+ , in a broad agreement with ssNMR experiments. The team has further established a collaboration with Prof. Crina Nimigean (Cornell University, USA) to study MthK gating and WT and mutated channels, using a combination of MD and cryoEM methods, which revealed a prominent role of cavity dehydration coupled with lipid binding in MthK gating.

3. Results and successes

An important discovery we have made during the SAW project concerns the detection of bound ions in the ion binding sites of cation channels. K^+ ions can in principle be detected in NMR experiments, but they give weak and broad signals and are therefore not suitable for studies of ion binding in proteins where the ions only make up a tiny fraction of the sample. Using NH_4^+ ions, which are very similar to K^+ ions, allows for detection of the commonly used nuclei 1H and ^{15}N . In this study we evaluated the new method on three different model systems, two of them being K^+ -selective (NaK2K and KcsA) and one being a non-selective cation channel conducting both K^+ and Na^+ (NaK). We combined the solid-state NMR data with MD simulations, which fitted very well to the differences we saw in the NMR data between the ion channels. The work was published at the beginning of 2022 (Öster et al., *JACS* 2022) and later presented in talks at several major NMR conferences (e.g. Chamonix solid-state NMR conference 2022, EUROMAR in Utrecht 2022).

Importantly, it was possible to distinguish between ions bound in different ion binding sites. Because of this, we could show that only an ion bound in an outer ion binding site is in close proximity to water. This is interesting since there are conflicting models for how ions are conducted through the SF of K^+ -selective ion channels. In the previously accepted mechanism, water molecules and K^+ are conducted in an alternating fashion, whereas in a more recently proposed (by some of us) mechanism, K^+ is conducted by direct interactions between the ions without water molecules in-between. In collaboration with the lab of Colin Nichols, a comprehensive review was recently completed on these findings (Lee et al, *Function*, in revision, 2025) Our new results strongly support that no water molecules are present between the ions and further confirm the results from our previous solid-state NMR study, where we investigated the ion conduction mechanism from the point of view of water molecules (Öster et al., *Sci Adv* 2019).

Additional work on NaK and NaK mutants resulted in a total number of four publications: (i) Solid-state NMR revealed substantial differences in the SF stability of NaK2K mutants by variation of ionic conditions (Hendriks et al. 2021); (ii) High-resolution X-ray, solid-state NMR and MD simulations suggest plasticity of the SF as a key determinant for ion non-selectivity in the NaK channel (Roy et al. 2021); (iii) Plasticity was also revealed for NaK C-DI and NaK S-DI, which are non-selective NaK mutants derived from ionotropic glutamate receptors (Minniberger et al. 2023). (iv) Ca^{2+} simulations in the NaK C-DI mutant were performed, where the derived Ca^{2+} occupancy in the SF matches very well with the X-ray data (Schackert et al. 2023). The results of some this work were presented by HS at several international conferences (e.g. Europhysiology Conference 2022 in Copenhagen, the 9th International Ion channel Conference in Nanjing, China).

The major findings from the CNG channel simulations have been recently published (Liu et al., 2025). Furthermore, we conducted a collaborative study on a structurally related ion channel, the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, with Prof. Klaus Benndorf's lab. The major findings of this study have been recently published (Benndorf et al., 2025), and a comparative analysis of ion conduction and selectivity mechanisms between CNG and HCN channels is currently underway.

A breakthrough has been achieved in the calculation of ionic currents through potassium channels with MD simulations. Traditionally, both currents as well as ion occupancies have been severely underestimated in MD. Comparison with quantum chemical simulations have suggested that the reason is the lack of explicit electronic polarizability in MD. Indeed, implementation of the Electronic Continuum Correction (ECC) has led to a dramatic increase in current and quantitative agreement between simulated and experimentally measured currents for a wide range of voltages for the channels MthK, TRAAK, Kv1.2, NaK2K, and KcsA (Hui et al, *PNAS*, 2025).

In close collaboration between the Berlin and Göttingen teams, the mechanism of calcium block in MthK has been resolved in detail. Near the activation gate, binding of calcium near residue E92 affects the open probability of the pore, whereas binding of calcium near F87, near the cytosolic side of the selectivity filter, reduces inward current of potassium (Öster et al, *JACS*, under review, 2025). These results have also been presented in a talk at EUROMAR in Oulu, 2025.

4. Equal opportunities, career development and internationalisation

We have emphasized in our job advertisements that female students and scientists are especially welcome to apply. Except one female PI in the research teams, two out of seven employed PhD students were female. All female members of this project benefitted from the opportunities of taking part in mentoring programs, such as the Leibniz mentoring program for female scientists in Berlin and the Minerva-FemmeNet program of the Max Planck Society. The PhD students were recruited internationally: one from the Netherlands, one from Greece, one from Colombia, one from Great Britain and two from Kazakhstan.

Regarding the PIs, two were on the junior level at the start of the project. Among those HS accepted a professorship position at Technical University of Berlin and she was at the same time promoted to lead the Chemical Biology Platform at FMP Berlin. In summer 2021, WK received a prestigious Human Frontier Science Programme Early Investigator Grant. In summer 2023, WK started an independent Lecturer position in Computational Pharmaceutical Chemistry at the Queen Mary University of London, UK.

5. Structures and collaboration

The structure of existing collaborations during the reporting period was as initially planned. This also included Prof. Thomas Baukrowitz from Kiel, Germany and Dr. Leighton Coates who were already indicated in the original proposal as long-term collaborators.

Additional national and international collaborators were involved in different sub-projects. HS was collaborating with Prof. Chen Song, Prof. Paolo Carloni and Prof. Andrew Plested on the evaluation of the multi-site Ca^{2+} model, which was subsequently employed in the Ca^{2+} simulations of the CNG channels. Her team also collaborated with Prof. Klaus Benndorf, an expert in single-channel electrophysiology of CNG and HCN channels. BdG and WK started a collaboration with Prof. Crina Nimigean, a world-class expert in structural characterization of potassium channels, focusing on hydrophobic gating and protein-lipid interactions in MthK, combining long-scale simulations and cryoEM.

6. Quality assurance

Animal testing has not been conducted for this project.

For the 15 publications that have resulted overall from this project (see accompanying Excel file), the overwhelming majority (i.e. 11) were published in an open access format.

Complying with good scientific practice, NMR and simulation data are archived in long time storage (>10 years) for future reference. In addition, electronic lab notebooks are kept and stored with the project. For most of the recently performed MD projects, the necessary files required to perform the simulations have been deposited on Zenodo. For all publications from FMP, a separate folder is allocated in long term storage with all the primary and derived data relevant to that publication.

7. Additional resources

The solid-state NMR part of the SAW project relies heavily on the production of NMR samples. Here it was instrumental that the wet lab of Research Unit Molecular Biophysics (lead by AL) was strongly supporting the SAW project. The involved personnel comprised Dr. Sascha Lange (head of the wet lab) and one technician, Dagmar Michl, who were both involved with approximately 30% of their time in the SAW Cation project and who are both financed by FMP Berlin.

In the HS team, computing power for this project was partially covered by an FMP internal CPU/GPU cluster, which was maintained by Dr. Tillmann Utesch, who devoted 15% of his time for this task. The position of Dr. Utesch is financed by FMP Berlin.

In the WK/BdG team, utilized compute resources were primarily provided by two in-house dedicated CPU/GPU clusters. These are maintained by Martin Fechner and Ansgar Esztermann, who both spent approx. 10% of their time on CATION related activities.

8. Outlook

The results we have obtained over the last years on MthK have given a lot of insights into the ion conduction mechanism. Now it would be interesting to look into the gating mechanism. So far we have been able to see changes in the SF upon Ca^{2+} binding to the cytoplasmic RCK domains, but we could not detect any structural changes in other regions of the protein. This is because so far, our samples had been produced in deuterated media and subsequently exposed to water, meaning that we can only detect residues that are exposed to water. In order to detect structural changes in the transmembrane regions, that are protected from water, we would need to introduce additional protons. For this purpose we can either produce fully protonated samples, which we can study using faster (100 kHz) magic angle spinning, or introduce specific labels (e.g methyl groups).

Our MD simulations of the MthK channel in both conformations have already provided a number of interesting observations that we are currently following up with additional simulations and analyses. Specifically, we plan to obtain a full gating pathway, induced by (un)binding of Ca^{2+} ions to the RCK domains. Further, the mechanism of signal transmission from RCK domains to the channel pore domain will be studied, which is coupled to the cavity dehydration and lipid tail entering. Here, a number of mutants of the key E92 residue, known to regulate MthK gating, will be simulated as well. Finally, the mechanism of Ca^{2+} block of MthK will be investigated in detail. Our simulations suggest that Ca^{2+} can bind in at least two positions - directly below the SF and at the E92 residue. Free energy calculations will be employed to quantify which of these two positions is more likely and physiologically relevant. The AlphaFold-predicted model of the NaK2K closed conformation will be simulated and compared with experimental ssNMR data, to assess its plausibility. In a separate project, we have established a method to calculate the free energy of pore dehydration, which will be used for both channels.

The calcium block permeation and gating studies in the SAW project have paved the way for future gating and inactivation studies that rely on accurate current readout, The achieved high permeation rates that closely match experimentally measured currents allow to study the subtle conformational changes in the selectivity filter of potassium channels that lead to C-type inactivation. This electrophysiologically well studied phenomenon is poorly understood structurally. Based on the progress made in this SAW project, we should be well positioned to tackle the mechanisms underlying C-type inactivation using solid-state NMR and MD simulation.