resolution structural data on human sodium channels, the publication of a number of crystal structures of related, bacterial voltage-gated sodium channel in the last 2 years has offered the opportunity to explore the mechanism of entry and location of binding sodium channel blockers. Here, molecular dynamics simulations were used to determine the binding site of two channel blockers, benzocaine and phenytoin in the bacterial channel NavAb. We find that that binding involves nonspecific hydrophobic interaction in the channel lumen, relate this to measured binding affinities and speculate how sequence differences in this region could alter the affinity for anaesthetics. We also show the feasibility of drug entry from the membrane via the lateral fenestrations. By calculating the free energy for this process we show that these fenestrations are likely to represent the long postulated hydrophobic route of entry leading to tonic block of resting channels. While there are many differences between bacterial and human sodium channels, these results provide a first step in understanding the mechanism of action of local anaesthetics and can aid the rational search for subtype-specific drugs for use in mammalian sodium channels.

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A Novel Gating Mechanism of the NaVMs Selectivity Filter Suggested by Molecular Dynamics Simulations

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Rapid and selective ion transport is essential for the generation and regulation of electrical signaling pathways in living organisms. Here, we use molecular dynamics (MD) simulations with applied membrane potential to investigate the ion flux of bacterial sodium channel NaVMs (Mccusker et al., 2012). 4-microsecond simulations with 500mM NaCl suggest different mechanisms for inward and outward flux.

The predicted inward conductance rate of ~26 pS agrees with experiment (~33 pS, Ulmschneider et al., 2013). The estimated outward conductance rate is 14 pS, which is considerably slower. The mean ion dwell time of the selectivity filter is prolonged from 14.8 ± 0.8 ns to 21.4 ± 1.1 ns. Analysis of the Na^+ distribution revealed distinct patterns for influx and efflux events.

During influx, site-HFS (High Field Strength) (Payandeh et al., 2012) is the dominant site with highest ion density. Ions are directly coordinated by GLU53 and SER54 sidechains in an off-axis manner. Site-CEN and Site-IN are less populated. In contrast to site-HFS, ions are distributed in the middle of the selectivity filter.

The distribution of the outward current displays a different pattern: site-CEN and site-IN are instantaneously occupied with Na^+, while site HFS is populated less frequently.

Examining the structural fluctuation of the trajectories suggests subtle differences at the selectivity filter. Translocation of ions from cytoplasma to periplasma induces structural changes of the GLU53 sidechains from an out-facing to a lumen-facing conformation. Thereby, it slows down the flux rate by creating a narrower constriction ''mouth''. During influx this residue remains rigid and potentially energetically more favorable for ion passage. We hypothesize that these structural changes at the selectivity filter glutamic acids represent a novel gating mechanism decreasing outward current.

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Congruent Pattern of Accessibility within the Pore of a Voltage-Gated Na^+ Channel

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Voltage-gated ion channels open a gate in response to membrane depolarization which allows ions to pass through the transmembrane pore of the channel. MTSET accessibility studies within the Shaker potassium channel revealed that K^+ permeation is controlled by an intracellular gate, whereas $Cd2^+$ and Ag^+ accessibility measurements in the cyclic nucleotide-gated channel suggests that this process occurs at the selectivity filter. Modification data of cysteine residues introduced into the DIV S6 of a fast-inactivation removed voltage-gated sodium channel (VGSC) by MTSET indicate that sodium channel gating is also regulated by an intracellular gate. However, unlike the Shaker potassium channel, VGSCs are not composed of four identical subunits. Despite sequence similarity between each S6 helix, the multi-domain nature of VGSCs implies that there may be asymmetry within the pore that may have consequences in regard to channel gating. Thus, we sought to determine if analogous positions within the S6 helices of a VGSC act together to form an intracellular gate which occludes the pore while channels are closed. We scanned the MTSET accessibility of substituted cysteines in the pore lining helices of the first three domains (DI-DIII) in the rat skeletal muscle sodium channel. The modification data at these sites, selected on the basis of sequence alignment and MTSET accessibility data of DIV-S6, confirms that DII and DIII follow the same pattern of accessibility as that of DIV; however, the extent of block is quite varied across each domain. Along with DI accessibility data, these findings will be discussed in the context of pore gating in related voltage-gated ion channels.

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Catalysis and Selectivity of Na^+ Permeation in Bacterial Sodium Channel NaVAb

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The determination of a high-resolution 3D structure of voltage-gated sodium channel NaVAb opens the way to elucidating the mechanism of ion conductance and selectivity. To examine the selective permeation of Na^+ over K^+ through the selectivity filter of the channel, we performed large-scale molecular dynamics simulations of NaVAb in an explicit, hydrated lipid bilayer at 0mV successively in 150mM NaCl, 150mM KCl, and a combination of both, for a total simulation time of more than 70 microseconds. Although the cytoplasmic end of the pore is closed, reversible influx and efflux of Na^+ and K^+ through the selectivity filter occurred spontaneously during simulations, leading to equilibrium movement of these cations between the extracellular medium and the central cavity of the channel. Analysis of Na^+ dynamics reveals a knock-on mechanism of ion permeation characterized by alternating occupancy of the channel by 2 and 3 Na^+ ions, with a computed rate of translocation of $(6 \pm 1) \times 10^6$ ions per second that is consistent with expectations from electrophysiological studies. The binding of Na^+ is intimately coupled to conformational isomerization of the four E177 side chains lining the extracellular end of the selectivity filter. The reciprocal coordination of variable numbers of Na^+ ions and carboxylate groups leads to their condensation into ionic clusters of variable charge and spatial arrangement. By stabilizing multiple ionic occupancy states while helping Na^+ ions diffuse within the selectivity filter, the conformational flexibility of E177 side chains underpins the knock-on mechanism of Na^+ permeation. K^+ also forms ionic complexes with E177 but, contrary to Na^+, K^+ ions pass through the selectivity filter in single file. The analysis of competitive binding of Na^+ and K^+ in mixed-cation simulations provides detailed mechanistic insight into the molecular basis of ion selectivity in NaVAb.

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The Origins of Ion Selectivity in a Bacterial Sodium Channel Revealed by μS-Long Simulations

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The recent solution of X-ray structures for the bacterial channel NavAb has provided the first opportunity to study functional mechanisms of voltage-gated sodium channels at the atomic level. NavAb is reminiscent of voltage gated K^+ channels but present a wider selectivity filter, lined by the side chains of 4 glutamates, usually indicative of a Ca^{2+} - selective mammalian channel. Despite this contradiction, nominal selectivity of Na^+ over Ca^{2+} is observed experimentally, and has been proposed to arise from the ability of the channel to accommodate and efficiently conduct a specific number of charges, while the difference between Na^+ and K^+ is generally attributed to the stronger affinity of carboxylate oxygens for smaller ions (high field strength). We carry out multi-μs fully-atomistic simulations, using the purpose-built Anton supercomputer, to extract representative conformations for all occupancy states of the pore and carry out Free Energy Perturbation calculations to determine the relative free energies of Na^+, K^+ and Ca^{2+} throughout the selectivity filter. We then explore the movements of multiple ions on the multi-μs timescale to construct unbiased free energy landscapes for conduction for each of the 3 ionic species. Our simulations provide new understanding of the fundamental interactions governing selective ion conduction in sodium channels.

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Characterizing Na^+/K^+ Permeation Rates through the Bacterial NavAb Sodium Channel

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The NavAb crystal structure displays a wider selectivity filter (SF) compared to the one observed in the well characterized K^+ channels, therefore raising

relevant questions concerning the conduction and selectivity mechanism in the former. So far various efforts have been made towards characterizing these processes, yet there are still aspects that remain to be clarified. Previous metadynamics analysis, as well as other independent studies, of the equilibrium conduction mechanism in NavAb point to a two-ion mechanism, decoupled from other incoming ions and water molecules. From the 0 mV potential of mean force (pmf) relative to the two-ion conduction through the channel SF, we analytically evaluate the pmf's dependence on applied voltage potential (V). When small V is applied biased and unbiased pmfs are overall similar. Contrastingly, under higher V conditions, an excess free energy arising from the applied external potential causes an asymmetry between hyper ($V<0$) and depolarized ($V>0$) conduction free energy surfaces and hence alters conduction mechanism. These results agree with MD studies explicitly considering the influence of transmembrane potentials. Same analysis has been applied to a similar system containing two K^+ inside the NavAb SF. We next aimed at further glancing at the channel conduction and selectivity, by characterizing the kinetics of the process by means of transition state theory. Surprisingly however, under higher voltages, the kinetic model highly overestimate the expected conductance of the channel. We are now faced with the question as to whether the TST model is inaccurate (though it has been previously shown to work), or whether deeper issues, such as mistaking the heights of barriers in the relatively confined SF environment are also playing a role.

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Negative Countercharges and S4 Interaction in Domain IV of Nav1.4

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The voltage sensor module comprises the positively charged S4 residues and negative countercharges in S1 to S3 segments. In the present study the putative interaction of these charges in domain IV during fast inactivation of the human voltage gated sodium channel of skeletal muscle was investigated. The effects of charge reversing mutations of R1448 and R1457 were compared to those for mutations at N1366, E1373, N1389 and D1420. Mutations slowed the entry of channels into fast inactivation, slowed or accelerated the recovery of channels from fast inactivation, and reduced the gating charge. Charge swapping mutations had a more pronounced effect to rescue the wild type phenotype on recovery from fast inactivation than for entry into fast inactivation. These results suggest specific residue interactions that promote S4 translocation during the deactivating transition leading to recovery from the fast inactivated state.

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Modulation of Inactivation Kinetics of the Bacterial Sodium Channel Nachbac Suggests a Complex Mode of Inhibition by Isoflurane

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The bacterial sodium channel NaChBac is a prokaryotic ancestor of eukaryotic voltage-gated sodium channels (Nav), which are critical for action potential generation and propagation in nervous and cardiac tissue. Like all mammalian Nav isoforms examined thus far, NaChBac is inhibited by clinically relevant concentrations of the inhaled volatile anesthetic isoflurane, and inhibition is accompanied by enhancement of slow or C-type inactivation (Ouyang et al., 2007). However, a detailed mechanistic explanation of the interplay between C-type inactivation and isoflurane inhibition is lacking. To shed light on the relationship between inactivation and inhibition, we introduced point mutations known to alter inactivation in NaChBac (G219A, G219P, G229A, and S195E), expressed the channels in HEK293FT cells, and applied isoflurane during whole-cell patch clamp recording. Preliminary data support the idea that isoflurane acts by multiple mechanisms invovling multiple sites on NaChBac. Channel mutations that enhance inactivation show greater current reduction by isoflurane, suggesting that isoflurane binds more favorably to inactivated channels. However, isoflurane exhibits both tonic and use-dependent block of the essentially non-inactivating NaChBac G219P mutant, indicating that binding also occurs in the closed/resting and open/conducting states. A detailed biophysical and pharmacological profile of these NaChBac inactivation variants together with a recent molecular dynamics simulation study showing isoflurane interacting with NaChBac at three distinct binding sites (Carnavale et al, 2013) supports multiple mechanisms of state-dependent inhibtion. Such functional data help clarify the complex pharmacological effects of volatile anesthetics on Nav channels, and contribute to better understanding of C-type inactivation in these proteins.

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Pharmacological Properties of Cinnamaldehyde on NaChBac

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Recent breakthroughs in the structure determination of the bacterial voltage-gated sodium channel NavAb have revealed the full length Nav channel for the first time at atomic resolution. However, additional structures of Nav channels in different conformational states are still needed for further understanding of the conformational changes that take place in Nav channels during channel gating. Specifically, full-length structures of Nav channels in the resting state, open state, inactivated state, and drug-bound conformations are still missing. In our search for ligands that confine Nav channels in one of these conformational states we have turned our attention to cinnamaldehyde. We tested cinnamaldehyde, a compound that affects different subtypes of eukaryote Nav channels, on the bacterial Nav channel NaChBac expressed in Xenopus oocytes. Our results indicate that cinnamaldehyde has a dual effect on NaChBac: cinnamaldehyde not only decreases the peak current but also accelerates channel inactivation of this bacterial Nav channel in a concentration-dependent manner. Our recordings indicate that cinnamaldehyde stabilizes NaChBac in an inactivated state. This opens up perspectives to employ cinnamaldehyde as a molecular tool to aid crystallization of bacterial Nav channels in an inactivated conformation. We expect that such a structure could give important insight into the conformational changes that contribute to channel inactivation.

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Automated Patch Clamping of 384 Cells at Once for Massively Parallel Ion Channel Screening

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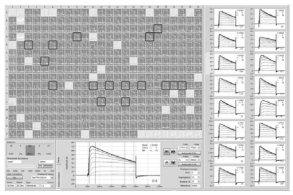
Automated patch clamping is well established within academic research and drug discovery efforts. Still, there is a longstanding desire to have gold standard electrophysiology compatible with primary ion channel drug screening requirements. We here present a chip-based approach for massively parallel patch clamp recordings. Using microstructured glass bottom microtitre plates, recordings from 384 cells are performed in an automated fashion. The recording system contains 384 patch clamp amplifiers and is integrated into a liquid handling robot with 384 channel pipettor, so all experiments are done completely in parallel. Success rates achieved are routinely over 85%. A full run of 384 cells for dose response analysis takes less than 15 minutes, delivering several thousand data points per hour.



The figure below shows a screenshot of an experiment on 384 CHO cells expressing Kv1.3 channels. (Raw current responses to a voltage step protocol). In this presentation, recordings from various ligand- and voltage-gated ion channels on the 384 channel platform will be shown.

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Sodium Channel Peptide Neurotoxin Studies Using a High Throughput Electrophysiology Platform and Very Long Assay Windows

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Neurotoxins identified in various animal venoms have long been used for studying ion channel structure and function, due to the high binding affinity of toxins to ion channels. Over the years, peptide neurotoxins have also attracted great interest as potential strategies for treatment of ion channel-related diseases. Despite the growing need of developing peptide toxins as drug candidates, the high-throughput analysis of peptide toxin can be challenging, as the toxins are typically large molecules with relatively slower binding-rate to the channel targets (compared to small molecule compounds). In addition, the binding affinities of these toxins to the channel are often state-dependent. Taken together, to successfully assay these toxins an assay platform needs to provide: 1) A long and stable assay window to capture the full association kinetics of the toxins, 2) Sophisticated voltage protocols to drive channels into desired conformational states, and 3) Adequate throughput and low running cost requirements of a drug screening environment. In this