Supporting Information

Gosselin-Badaroudine et al. 10.1073/pnas.1217990109

SI Materials and Methods

Construct Generation and Sodium-Channel Mutagenesis. We used a cDNA construct encoding the rat $Na_v1.4$ sodium channel. Mutant and wild-type $Na_v1.4$ VGSC were inserted in pGEMHE, an oocyte expression vector containing the T7 promoter (5′ to 3′), the *Xenopus laevis β*-globin 5′-untranslated region, a multiple cloning site, the *Xenopus laevis β*-globin 3′-untranslated region, a polyA tract, and a linearizing site. This construct is a generous gift from S. C. Cannon, University of Texas Southwestern Medical Center, Dallas. It was amplified in *Escherichia coli* XL2 Blue (Stratagene) and purified using GenElute HP Plasmid Maxiprep kits (Sigma). Mutations were generated using QuikChange site-directed mutagenesis kits according to the manufacturer's instructions (Stratagene). The construct was linearized with NheI, and T7 RNA polymerase was used to make sense RNA using mMESSAGE mMACHINE T7 kits (Ambion).

Data Analysis and Statistics. The electrophysiological data were analyzed using macros in Campfit (pCLAMP v10.0, Molecular Devices) and custom programs written using MATLAB (The MathWorks Inc.). The results are expressed as means ± SEM. The number of measurements (n) is indicated in parentheses. Statistical comparisons were performed using a one-way ANOVA with Bonferroni's post hoc test. Differences were deemed significant at $P < 0.05$. The P values are indicated in the text or figure legends. The Student t test was used to determine the voltages at which the amplitude of the proton current recorded at pH 6 is significantly different from the amplitude of the current measured at pH 8.6.

Sequence Alignment and Homology Modeling. The best template available to build a model of the transmembrane domains of $Na_v1.4$ was identified by a PSI-BLAST search over the Protein Data Bank database. Among the hits, the Na_vAb high-resolution structure released in 2011 (1) was the candidate bearing the transmembrane domains with the highest max score. To build the pairwise alignment between $Na_v1.4$ and Na_vAb, the 100 first human homologous sequences to Na_vAb among the National Center for Biotechnology Information reference protein database were identified using the PSI-BLAST server (2). Rather than building a pairwise alignment of the $Na_v1.4$ and Na_vAb pairs, a multiple sequence alignment of these 100 sequences was constructed using the ClustalW2 online server (3), ensuring a higher quality alignment. The blocks of amino acid substitution matrix was used, together with standard parameters (opening gap penalty of 10, gap extension penalty of 0.20, and gap distance of 5). The pairwise alignment of Na_vAb and $Na_v1.4$ was extracted and further used to build a 3D atomistic model of the mammalian channels. Before building the model, the highly disordered intra- and extracellular loop regions between the four channel subunits, for which no structural template is available, were removed from the alignment. The alignment of the voltage sensor domain transmembrane segments used for the structural modeling is presented in Fig. 1.

A standard MODELLER (4) routine was then used to build a comparative model of the full-length $Na_v1.4$ channel, comprising the pore domain and the four voltage sensor domains.

The homology model provides a low-resolution starting structure and, considering the high structural similarity between the VSD domains of all possible templates of the Protein Data Bank ($K_v1.2$, $K_v1.2/2.1$, Na_vAb, and Na_vRh), the choice of the reference structure is not critical: the relaxation using MD simulations (see the following section) ensures relaxation of the model in its membrane/solution environment.

MD Simulations. The $Na_v1.4$ channels were then inserted in a fully hydrated POPC bilayer. The systems were then equilibrated under normal constant temperature and pressure conditions (298 K, 1 atm) in a 150-mM NaCl solution. To ensure correct reorganization of the lipids and solution, the positions of all of the atoms of the channel were constrained during the first nanosecond. In a second step, the side chains were allowed to reorganize while the backbone was kept restrained during 8 ns. Finally, a 30-ns unrestrained MD simulation of the entire channel was conducted, enabling relaxation of the system.

The MD simulations were carried out using the program NAMD2.9 (5). Langevin dynamics was applied to keep the temperature (300 K) fixed. The equations of motion were integrated using a multiple time-step algorithm (6). Short- and long-range forces were calculated every 1 and 2 time-steps respectively, with a time step of 2.0 fs. Chemical bonds between hydrogen and heavy atoms were constrained to their equilibrium value. Long-range electrostatic forces were taken into account using the particle mesh Ewald approach (7). The water molecules were described using the TIP3P model (8). The simulation used the CHARMM22-CMAP force field with torsional cross-terms for the protein (9) and CHARMM27 for the phospholipids (10). A united-atom representation was adopted for the acyl chains of the POPC lipid molecules (11). The simulations were performed on the Cray XT5 Jaguar supercomputer at the Oak Ridge National Laboratory and on the Cray XT5 Kraken supercomputer at the National Institute for Computer Science.

Biased MD Using Contact Map Collective Variables. Because the template corresponds to a "preactivated" state of the Na_vAb, in which the pore is closed but the VSDs are in a "partially activated" state (1), the corresponding $Na_v1.4$ homology model also features the VSDs in a "partially activated" state. The resting state was reached by applying external forces in a procedure comparable to the one used in our study of $K_v1.2$ (12, 13). This procedure was done to obtain a structure compatible with the mutagenesis results described herein (i.e., the residue substituted to measure current must be located at the level of the conserved F residue, whereas the VSD bears a maximum number of interactions between positive and negative charges of the VSD and the lipid headgroups). Accordingly, we have biased our simulation in a stepwise procedure to drag DI/R1, DII/R1, and DIII/R2 toward the constriction site while maintaining a maximal number of interactions between basic and acidic residues within the VSD. This parameter is necessary for the stability of the states. Because the data for domain DIV seem inconclusive, we have chosen to bias DIV/R2 toward the constriction site based on other experimental results (14, 15). However, uncertainties remain at this point (*Results and Discussion*).

Contact maps between positive and negative residues were defined (Fig. S1) for each state: the activated state derived from the crystal structure (called herein α), of the supposed resting state (called γ) and of an intermediate state along the pathway linking the two states (called β). Expression of these contact maps were translated in terms of coordination numbers using the collective variables module of NAMD2. A first deactivation step was conducted to bias the MD simulation of the initial α structure toward an intermediate state β, using steered MD acting on the coordination number, with a force constant of 1000 kcal/mol/Å. Similarly, a second deactivation step was then

conducted to bias the β structure toward the final state (γ). This final state was equilibrated for 30 ns in its membrane/solution environment to reach a stable RMSD, indicative of an equilibrated structure.

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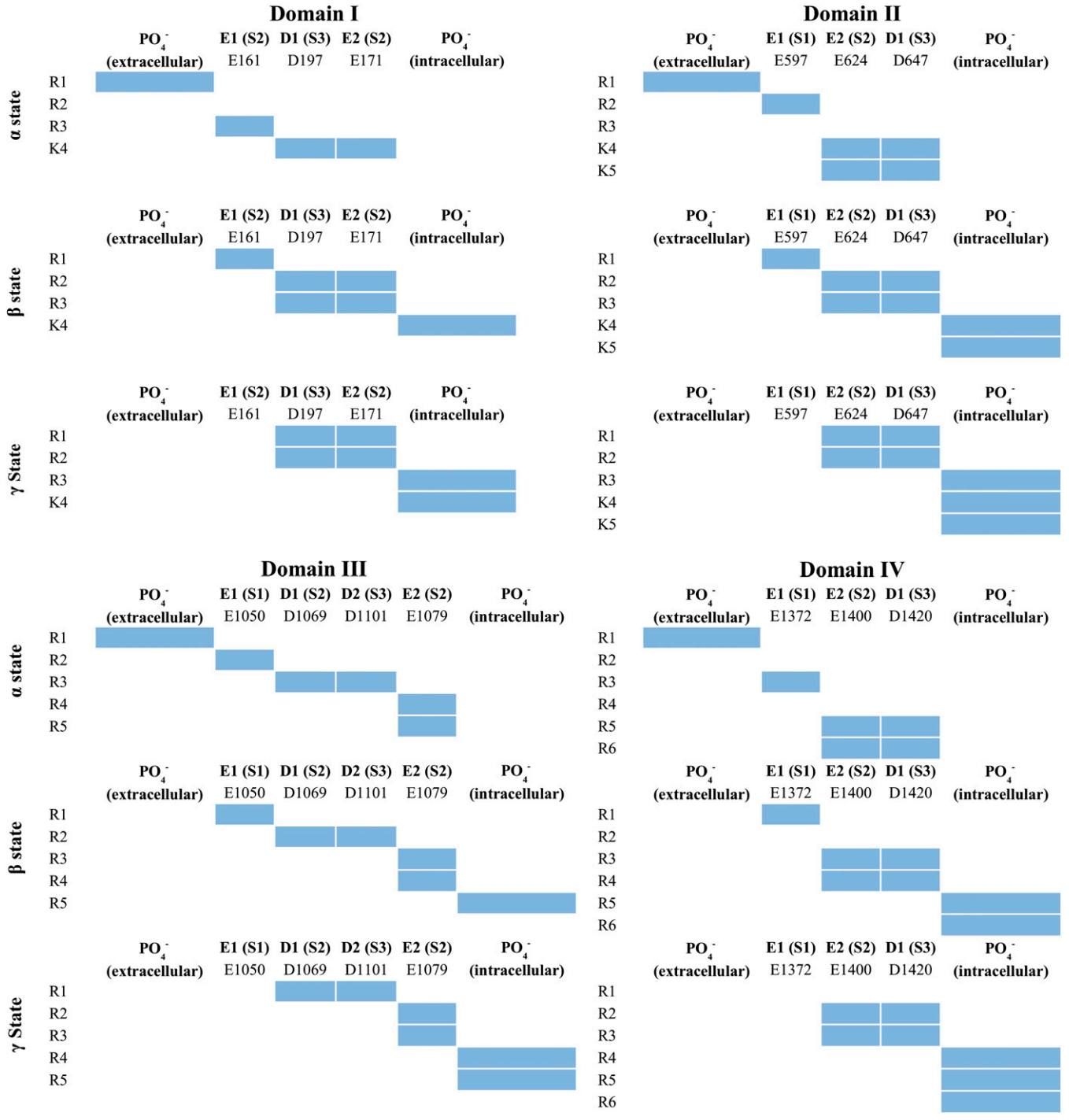

Fig. S1. Interactions of the positively charged residues of S4 with the corresponding negatively charged binding sites in the different conformations. The stepwise procedure used to reach the resting state from the activated state derived from the Na_vAb involved three VSD conformations (α, β, γ). In each of them, a maximal number of salt bridges between the S4 basic residues (Rs or Ks) and their binding sites (Es, Ds, top PO_4^-, or bottom PO_4^-) is maintained. Each of the two transitions involves a zipper-like motion in which successive ion pairs are formed.

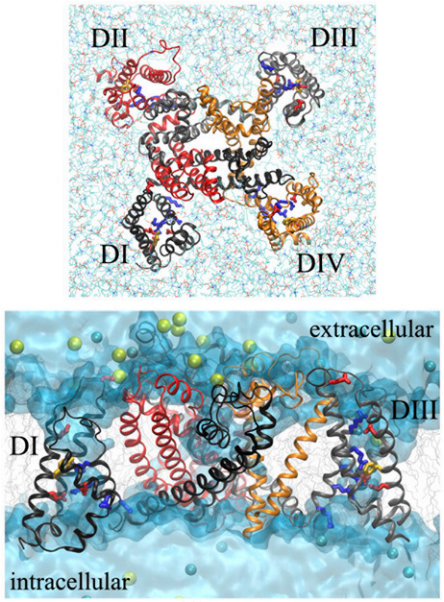

Fig. S2. Overall arrangement of the $Na_v1.4$ resting state model embedded in a POPC membrane. (*Upper*) Top view highlighting the arrangement of the four subunits (each depicted as ribbons of a distinct color, DI black, DII red, DIII gray, and DIV orange). Note especially the position of the VSDs as peripheral domains to the central pore. (*Lower*) Lateral view, with the extracellular medium above and the intracellular medium below. The protein backbone is represented as in the top view; lipids are represented as gray lines, ions as colored spheres (chloride cyan, sodium yellow), and the solvent accessible surface as a transparent blue surface. Charged residues of the voltage-sensor domains are represented as blue and red sticks and the conserved bulky residue defining the gating charge transfer center is represented in yellow.