Structural models of TREK channels and their gating mechanism

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Abbreviations: TM, transmembrane; MD, molecular dynamics; K2P, two-pore-domain background potassium; P segment, pore-forming segment; MSA, multiple sequence alignment; MI, mutual information

Mechanosensitive TREK channels belong to the family of K2P channels, a family of widely distributed, well-modulated channels that uniquely have two similar or identical subunits, each with two TM1-P-TM2 motifs. Our goal is to build viable structural models of TREK channels, as representatives of K2P channels family. The structures available to be used as templates belong to the 2TM channels superfamily. These have low sequence similarity and different structural features: four symmetrically arranged subunits, each having one TM1-P-TM2 motif. Our model building strategy used two subunits of the template (KcsA) to build one subunit of the target (TREK-1). Our models of the closed channel were adjusted to differ substantially from those of the template, e.g., TM2 of the second repeat is near the axis of the pore whereas TM2 of the first repeat is far from the axis. Segments linking the two repeats and immediately following the last TM segment were modeled ab initio as α-helices based on helical periodicities of hydrophobic and hydrophilic residues, highly conserved and poorly conserved residues and statistically related positions from multiple sequence alignments. The models were further refined by 2-fold symmetry-constrained MD simulations using a protocol we developed previously. We also built models of the open state and suggest a possible tension-activated gating mechanism characterized by helical motion with 2-fold symmetry. Our models are consistent with deletion/truncation mutagenesis and thermodynamic analysis of gating described in the accompanying paper.

Introduction

Two-pore-domain background potassium (K2P) channels form a distinct gene family of widely distributed background channels modulated by numerous physical and chemical factors that influence resting membrane potential and cell excitability.1 The family of mammalian K2P channels now include 15 members classified into six main structural classes2 based on sequence similarity and the stimuli to which they respond, as follows: (1) TWIK1, TWIK2 and KCNK7 channels (functional expression of KCNK7 has not yet been reported)-weak inward rectifiers; (2) TREK-1, TREK2 and TRAAK channels-mechanosensitive gated; (3) TASK1, TASK3 and TASK5 channels-acid inhibited; (4) THIK1 and THIK2 channels-halothane inhibited;3 (5) TALK1, TALK2 and TASK2 channels-alkaline-activated; and (6) TWIK-related spinal cord K+ (TRESK) channel, which is regulated by intracellular calcium. Despite low sequence similarity among the different classes and broad range of stimuli to which they respond, K2P channels share common structural features like two subunits per channel, each with two TM1-P-TM2 motifs. In one-pore-domain K+ (K1P) channels, four matching P loops are assembled in homo- or heterotetramers (all subunits have a similar P domain sequence, which contains the residues GYG or GFG), whereas in the dimeric K2P channels, the first and the second pore domains (P1 and P2) can have substantially different sequences. For example, many K2P channels have a F or L in the GXG motif (where X represents any amino acid) of the selectivity filter in the P2 domain instead of a Y.4,5 Therefore, in K2P channels, the pore is predicted to have a 2-fold symmetry rather than the classical 4-fold arrangement of other K+ channels. Although the selectivity of K2P channels for K+ over Na+ is high (permeability ratio (P Na/P K) < 0.03), these structural differences suggest a more varied permeation and gating compared with K1P channels.6,7

Members of the TREK subfamily, TREK-1, TREK-2 and TRAAK, are best known as background K+ channels exhibiting a low, weakly voltage-dependent, open probability that is strongly modulated by both chemical and physical stimuli.8 This subfamily is notable not only for their role in maintaining the resting potential and controlling membrane excitability, but...
also for contributing to diverse sensory transduction processes and metabolic regulation, including contributions to: mechano-
sensitivity,\textsuperscript{9,12} thermosensitivity,\textsuperscript{13,14} chemosensitivity,\textsuperscript{15} nociception\textsuperscript{16} and neuroprotection.\textsuperscript{17-19} Human TREK-1 is highly expressed in the brain, where it is particularly abundant in GABA (\textgamma-aminobutyric acid)-ergic interneurons of the caudate nucleus and putamen.\textsuperscript{20} TREK-1 is also expressed in the prefrontal cortex, hippocampus, hypothalamus, midbrain seroto-
nergic neurons of the dorsal raphé nucleus and sensory neurons of the dorsal root ganglia.\textsuperscript{21} TREK-1 is also found in peripheral tissues such as the gastrointestinal tract.\textsuperscript{22} TREK channels have also been identified as important drug targets for general anesthesia\textsuperscript{23} and potentially for treatment of depression.\textsuperscript{24}

Effective drug design is hampered by lack of structural information and limited understanding of activation mechanism. Our goal is to build structural models of TREK-1 channel that can be used as a starting point for hypothesis-driven structural and functional experiments. If experimental tests confirm crucial aspects of the models, they can further be used as templates for modeling other channels in K2P family.

Predicting the structure of TREK channels is a challenging task, since available templates are distantly related KcsA channels, members of the voltage-gated channels subfamily. Therefore we used a complex approach combining homology-based and ab initio modeling incorporating additional constraints derived from sequence conservation patterns in multiple sequence alignments (MSA).

**Results**

**Models of the closed, low-conductive and open states.** The first step in our modeling process was to use the KcsA crystal structure in the closed conformation to develop a homology model of TREK-1 (Fig. 1A). The alignment in Figure 1B was used to develop these models. KcsA and TREK-1 share only low sequence similarity, which makes alignment of some segments, especially the more peripheral TM1 segments, ambiguous when only two sequences are used. Much of this ambiguity was eliminated by choosing alignments in which highly conserved residues interact with other protein residues and align in both sequences, whereas poorly conserved residues are on the surfaces of the protein and interact primarily with either lipids in the TM region or water (Fig. 2).

The extracellular TM1-P loop of the first repeat is much longer in TREK-1 than in KcsA. The additional TREK-1 resi-
dues indicated in Figure 1B as an insert above the continuous sequence was not included in the models; helical segments are underlined using the same color scheme as in (A). (C) Bottom view and (D) Side view of structural models for the closed (left part), low-conductive (central part) and open (right part) conformation, in ribbon representation; helical segments are labeled and colored using the same color scheme as in (A and B).
positively charged sidechains that may interact with lipid head groups and residues conserved among TREK and TRAAK subfamilies that may interact with similarly conserved residues of other protein segments (see below). This pattern is also supported by a statistical analysis involving mutual information and graph theory discussed later. It was thus modeled in this way, with its highly conserved residues interacting with highly conserved residues near the N-terminus of TM1b. TM1b was selected for the postulated interactions with conserved residues of the linker because (a) the linker must be positioned near TM1b to connect the repeats, and (b) several conserved residues are on the “back” side of TM1b in our alignment where they would be exposed to lipid unless they interacted with the linker, and (c) the postulated interactions among similarly conserved residues of the two segments could be made in a complementary way that involved energetically favorable interactions among conserved hydrophobic residues and between conserved oppositely charged residues.

Some features of the initial KcsA-based model were not completely consistent with the TREK-1 sequence. TM2a has a highly conserved proline residue near the “hinge” region of other K+ channels where TM2 bends when the channel opens. Introduction of a proline in this region of the NaChBac channel stabilizes the open conformation. Thus, it seems likely that TM2a of TREK-1 may have a bent open-like conformation even when the channel is closed. The only residue on TM2b predicted to be exposed to lipid alkyl chains on the basis of mutability (poorly conserved blue in Fig. 2 and hydrophobic) is F269 near the N-terminus; whereas three (I161, L165 and I184) occur in TM2a. These patterns suggest that TM2b is more buried than TM2a and the TREK-1 structure deviates somewhat from the 4-fold symmetry of KcsA. We adjusted our model to be consistent with these features while maintaining 2-fold symmetry of the two subunits. In our closed conformation, TM2b is positioned nearer the central axis of the pore than TM2 of KcsA and TM2a is farther from the axis, in a position and conformation more like analogous segments of open K+ channel crystal structures (K VAP, MthK and K V1.2). This asymmetric collapse of the structure reduces the size of the central cavity and places side chains of the highly conserved L289 near the axis of the pore where they would block permeation of ions.

Channel opening was modeled in two ways. In the first approach the conformation of Repeat A was not altered much since it already had an open-like conformation and TM1b and TM2b were displaced radially outward, which confers approximate fourfold symmetry for the TM portion of the channel’s backbone (see low-conductive model of Fig. 1C and D). In this model most of the interactions among small residues at regions of close contact between helices are maintained. The difficulties with this type of model for the open conformation are that after MD simulations the radial in-plane expansion of the protein was -0.7 nm² and after symmetry refinement it reduced to -0.1 nm². Both of these values are much smaller than the -4 nm² calculated

![Figure 2. Residue conservation patterns from multiple sequence alignments, used as a modeling criteria. (A) Target-template sequence alignment in which residues are highlighted according to their degree of conservation, on a scale from Red (highly conserved) to Blue (not conserved). (B) Top view and (C) Bottom view of the models for closed (left), low-conductive (center) and open (right) conformations, colored according to the same degree-of-conservation scheme.](https://example.com/figure2.png)
from experimental measurements and the 0.75 nm² cross-section area of the inner pore (-0.6 x 1.2 nm at narrowest region). For comparison, the pore cross-section falls between ~1 nm² in the open structure of KcsA (pdb code 35fw20) and ~0.6 nm² in K1,1.2 (pdb code 2a79,23) with conductances -75 pS² and -13 pS² respectively, expected at 150 mM KCl concentration. Although the relationships between the pore geometry and conductance for such systems are rather complicated and the conductance is strongly influenced by charge at the channel’s intracellular mouth, pore size in the above TREK model appears too small to allow a high rate of permeation. However, TREV channels have two conductances, a small (~60 pS) and a large (~130 pS). Thus it is feasible that this model approximates the low-conductive state.

We thus developed another model for the open conformation with the inner portions of the TM helices located farther from the pore. This additional displacement required repositioning of the linker helix; it was modeled as a continuous extension of the TM2b helix (see open model of Fig. 1C and D). The expansion area for this model after symmetry-restrained MD simulations was calculated to be ~5.2 nm², in close agreement with the value estimated from experimental analysis of dose-response curves (see Maksaev et al., pp. 34–42).

Due to insufficient information from template-based modeling alone, complementary strategies were used to derive additional constraints. Thus, several factors were considered in developing these models. The first was interactions among conserved residues. Functionally and/or structurally important sites or regions are typically composed of residues with similar degrees of conservation interacting among multiple segments. For example, the only residues conserved among all K⁺ channels located in the selectivity filter where they interact with their counterparts of other subunits to determine the selectivity of the channel. These residues are not conserved between K⁺ channels and homologous channels with different selectivities. In contrast residues that are poorly conserved within closely related families or subfamilies tend to reside on the surfaces of proteins away from functionally important sites. In the analysis presented here, residues are classified into three categories: (1) highly conserved among all 2P sequences (red and orange in Fig. 2), (2) conserved among TREV and TRAAK mechanosensitive channels but not between these and other 2P families (yellow and green), and (3) poorly conserved among TREV and TRAAK sequences (cyan and blue). The outer half of the TM region that includes the K⁺ selectivity filter contains about 70% of residues that are well conserved among all 2P channels (see red and orange residues of Fig. 2). In contrast, only less than 10% of residues of the inner pore or inner half of the TM region are conserved among all 2P families; however, many are conserved among mechanosensitive TREV and TRAAK sequences (see yellow and green residues of Fig. 2). Supplementary Table 2 lists all of the interactions among residues of this region that are highly conserved at this level (red, orange and yellow residues of Fig. 2). All but two (M42 and W295) interact with at least one other equally conserved residue in the closed and/or open models. M42 may be conserved as an alternative initiation residue31 and W295 interacts with the cytoplasmic surface of the membrane, where it may serve as a lipid anchor.32 All poorly conserved residues of the transmembrane region (blue in Fig. 2) reside primarily on the outer surface of the protein.

The second factor was the relative positions of small residues. Small residues are frequently located at positions of closest contact between helices that do not cross at angles typical of “knobs-into-holes”33,34 or “ridges-into-grooves”35 helix packing patterns. In our closed model, residues S290 and G293 are near the crossing contact of the pore-lining TM2b helices; A175 and G178 of TM1a interact with A283, A286 and A287 of TM2b; P168 and G171 of TM1a are near TM2b of the other subunit; G182 of TM2a is near S210 and V211 of TM1b; and G188 of the linker is near S210 of TM1b (see blue residues of closed model in Fig. 3A).

In open model G178 of TM2a packs next to A283 of TM2b, P168 of TM2a packs next to G293 of TM2b of the other subunit, G178 of TM2a is next to V47 of TM1a: and S210 of TM2a is near the linker helix (see blue residues of open model in Fig. 3A). Energies required to transfer these types of small residues from an inaccessible environment inside a protein to an aqueous environment are relatively small.36 Thus, they are ideally suited to serve as residues that are relatively buried in a closed conformation but that become exposed to water inside the pore when the channel opens. G171, A175, G182, A286, A287 and S290 (red residues of Fig. 3A) of the TM2 segments have this property in our models.

The third factor identified statistically related positions from our MSA of all 2P channels. To characterize evolutionary relationships among residues within MSA columns, mutual information (MI) of pairs of MSA columns (j,k) was obtained. That MI was denoted as $M_{I}(j,k)$. For $N_{total}$ (=1,557) positions in a MSA, a total of $N_{total}(N_{total} - 1)/2$ unique MI(j,k) values (=1,211,346 pairs) were calculated using Equation 1 (described in Materials and Methods). The distribution of $M_{I}(j,k)$ values is shown in Supplementary Figure S1. From the over one million $M_{I}(j,k)$ pairs, the leading 1,000 high MI pairs were used to compute a MI graph that represented the statistical association of the high MI pairs with each other. The degree (or connectivity) of the leading thirty-one positions is shown in the last column of Supplementary Table 1. Residues from TREV-1 human sequence that correspond to the MSA positions with high connectivity tend to form clusters at functionally important regions. In previous work on other membrane proteins such clusters differed somewhat among analyzed subfamilies.37 The analysis excludes residues, such as those of the selectivity filter, that are conserved among all sequences and surface residues where mutations can occur somewhat randomly in an uncoordinated manner. Twenty-one of the highest thirty one scoring residues are in the inner pore region near the cytoplasmic surface of the membrane in our models (Fig. 3B), six are in the deleted TM1a-Pa loop, three are in the outer TM portion of the protein and one is in the C-terminus cytoplasmic domain. The twenty-one interior residues are (in rank order for each segment, top six in bold) K43, V41, K45, W44 and V47 near the N-terminus of TM1a; G176 and G182 near the C-terminus of TM2a; E193, F196 and I189 on the conserved face of the helix linking the two repeats, S210, T211, R207, I208 and I209 near the N-terminus of TM1b and S300, V298, K302, G293, E306...
and E305 near the C-terminus of TM2b. These residues form clusters in our models. The residue, corresponding to MSA position with the highest degree, S210 near the N-terminus of TM2b, interacts with a putative hinge region connecting the end of TM2a to the linking helix that changes conformation during gating. More precisely, in the closed conformation S210 interacts with I189, T211 with G182, R207 with I189 and I209 with F196; and in the open conformation S210 interacts with E193 and F196, T211 with I189 and R207 with E193 and I189. The N-terminus of TM1a interacts with the C-terminus of TM2a and C-terminus of TM2b and in the closed conformation; i.e., K43 with S300 and V47 with G176. The C-termini of TM2b also self associate in the closed conformation; i.e., G293, E305 and E306 self associate and K302 of one subunit interacts with E305 of the

Figure 3. Ribbon representations of the inner pore region of closed (left) and open (right) models viewed from the inside. (A) Small residues located at positions of close contact between helices are colored blue. Small residues that become exposed in the lining of the pore when the channel opens are colored red. (B) High degree, high MI positions obtained from the MSA of TREK homologs, used as a modeling criteria. Residues with the highest degree values are colored according to the color spectrum scheme used in Supplementary Table 1, red for the highest score and cyan for the lowest. (C) Charged residues of the inner pore segments. Side chains are colored by element (gray = carbon, blue = nitrogen, red = oxygen, white = hydrogen). Positively charged residues are labeled in blue, negatively charged residues in red.
For closed, low-conductive and open conformations we modeled the two putative cytoplasmic helices to form a parallel dimer (dimeric coiled-coil) in which the axis of the dimer corresponds to the axis of the pore and the highly conserved hydrophobic residues self-associate (Fig. 4C). Similar types of cytoplasmic coiled-coils have been reported for other K⁺ (tetrameric coiled-coil in KcsA) and mechanosensitive (pentameric coiled-coil in MscL) channels, and our groups correctly modeled this type of interaction for MscL channels before the corrected conformation was published. These putative interactions remained relatively stable during MD simulations.

Post TM2b cytoplasmic segment. Segment 306–326 has a sequence consistent with formation of an amphipathic α-helix. This sequential pattern is unique to TREK and TRAAK subfamilies; sequences of other 2P channels cannot be aligned unambiguously with these segments and do not exhibit helical periodicities of hydrophobic and hydrophilic residues. Residues beyond this segment are not conserved among TREK and TRAAK subfamilies, and can be deleted in TREK-1 channels without affecting channel properties substantially (see Maksaev et al., pp. 34–42). The helical wheel representation of Figure 4A shows that the polar face (top) consists primarily of negatively charged residues, the hydrophobic face (right side) consists primarily of alkyl and aromatic residues, an “alanine” face (left side) consists primarily of small alanines and a glycine, and a “bottom” faces has two threonines flanking a positively charged arginine. The segment has five residue positions highly conserved (yellow) among TREK and TRAAK subfamilies; four on the hydrophobic face and one on the alanine face (Fig. 4B).
not usually inactivate when the patch is excised from the cell and the normal cytoplasmic environment is lost. Thus, inactivation appears to involve interactions with some cytoplasmic component, likely mediated by the C-terminus. If so, the cytoplasmic component may interact with the highly-conserved alanine faces of the putative C-terminus coiled-coils. We have not attempted to model this interaction since the cytoplasmic component is unknown. The second possibility is that tension increases within the segment linking the C-terminus of TM2b to the N-terminus of the coiled-coil when the channel opens, causing the cytoplasmic helices to eventually pull apart in a scissor-like motion to form an antiparallel dimer in which the “alanine” faces pack next to each other (Fig. 4D) when the channel inactivates. In our highly tentative models of this conformation, the highly-conserved hydrophobic faces interact with other highly-conserved residues of surrounding TM segments, the arginine on the “bottom” face extends through the pore where it salt-bridges to D294 and inhibits permeation through the inner pore, and the negatively-charged polar face is exposed to the cytoplasm. Thus, virtually all sequence characteristics of this segment are explained by this model. Preliminary MD simulations of this model indicated only marginal stability of the assembly; interactions among the alanines were maintained in only some symmetry-restrained simulations, but deviations from this motif were not large in any of the simulations.

**Refinement of the models using symmetry-restrained MD simulations.** Although homology modeling is probably the most reliable structure prediction method,41 the accuracy of the resulting model depends on several factors: (a) accuracy of the template; (b) the level of sequence identity (above 30% typically yields a model with accuracy comparable to a low resolution crystal structure); (c) the accuracy of the target-template sequence alignment (the most important factor); and (d) the number of available templates.42 Given that our models were built based on a distant template and even certain regions were modeled in the ab-initio manner, they probably contain errors. Traditional MD simulations of this model indicated only marginal stability of the assembly; interactions among the alanines were maintained in only some symmetry-restrained simulations, but deviations from this motif were not large in any of the simulations.

We have developed structural models of TREK-1 channels in closed, low-conductive and open conformations, the least ambiguous of which are the closed and open models. These models are consistent with the following modeling criteria: (1) they are stable during MD simulations with RMSD values similar to those of crystal structures of K+ channels, (2) identical subunits have identical conformations, (3) almost all hydrophobic residues are in apolar environments while polar residues are in hydrophilic environments, (4) almost all charged residues form salt bridges, (5) numerous positively charged residues interact with cytoplasmic lipid head groups, (6) conserved residues interact with other comparably conserved residues, (7) residues from the human TREK-1 sequence that correspond to MSA positions with high degree of connectivity and high MI form spatial clusters, (8) regions of closest contact between helices that cross at apical angles contain small residues, and (9) most residues that are buried in the closed conformation and exposed in the open conformation have intermediate polarities.

This family of K+ channels has numerous unique features. The tandem arrangement of two homologous but not identical repeats and 2-fold symmetry allow for more variation in sequence, and hence more conformational freedom than in K+ channels made of four identical subunits which are expected to have identical average conformations. These differences are most apparent in our model of the closed conformation in which the inner pore has a collapsed conformation due to the location of the TM2b helix near the axis of the pore. A highly conserved proline 165 in TM2a is proposed to contribute to this asymmetry between the two repeats by constraining TM2a to an open-like configuration.
The inactivation mechanism is much more ambiguous and may involve interactions with cytoplasmic moieties not present in our models, and/or changes in the selectivity filter\textsuperscript{46} that we did not attempt to model. The model in which the cytoplasmic helices form an antiparallel dimer in the inactivated conformation is a rather speculative working hypothesis to provide a rationale for why the helix has alanines along one face. Although the models presented here are still tentative, they can be tested experimentally. One way is to introduce cysteine residues at positions distant from the pore’s axis in all conformations. The segment linking the two repeats is proposed to form a cytoplasmic membrane surface helix that changes conformation during gating. The cytoplasmic domain formed by the post TM2b segment of mechanosensitive TREK and TRAAK channels differ from other 2P channels in that they can form an amphipathic $\alpha$-helix. In our models these form a parallel dimeric coiled-coil in closed and open conformations. Intersubunit interactions for the parallel interaction involve highly conserved hydrophobic residues.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Refinement of structural models by symmetry-restrained MD simulations. (A) Simulation protocol: models were subject to 30 ns unrestrained MD simulation (black line); after 5, 10, 15, and respectively 20 ns, a symmetry annealing step was applied (blue and green thick lines) followed by 10 ns unrestrained simulation (blue and green thin lines); the model annealed after 15 ns was subject to a second step of symmetry annealing followed by unrestrained simulations (red lines). (B) RMSD plots of the C$\alpha$ trace of each model, during the first 10 ns of unrestrained simulation, using as reference the models at the beginning of each simulation step. (C) Root-mean square fluctuations (RMSF) of each model, during the last step of unrestrained simulation (thin red lines in A).}
\end{figure}
predicted to be proximal in some conformations but distant in others. Distances between the residues can be analyzed in several ways; by oxidizing the cysteines to form disulfide bridges, by determining whether Ca bridges can form between the sulfhydryl groups, by using reagents to cross-link the sulfhydryls or by attaching probes (e.g., EPR, FRET or LRET) to measure distances between the groups in the differing conformational states. Initial steps toward these types of experiments are described in the accompanying manuscript.

**Materials and Methods**

**Multiple sequence alignment.** Sequences within K2P channels family were identified using PSI-BLAST against RefSeq database. The multiple sequence alignment (MSA) of the resulting 235 sequences of K2P channels was generated using BioEdit then manually adjusted in the loop and terminal regions.

**Calculation of mutabilities.** The method by which mutabilities were calculated is described in Shrivastava et al. Calculations were performed for all 2P sequences of the initial alignment and for only the TREK and TRAAK subfamilies of this alignment. Mutabilities calculated from all sequences were used for the most highly conserved red and orange colored residues of Figure 2, mutabilities calculated for only the TREK and TRAAK subfamilies were used for all other residues.

**Identification of statistically related MSA positions.** The analysis for computing statistically related MSA positions is described in Fatakia et al. The independent probabilities of obtaining AAs x and y at positions j and k were represented as p_j(x) and p_k(y). The joint probability of AA pairs x and y at positions j and k was designated as p_{j,k}(x,y). The MI of pairs of columns j and k was calculated using:

\[ MI(j,k) = \sum_{x=1}^{20} \sum_{y=1}^{20} p_{j,k}(x,y) \log_2 \left( \frac{p_{j,k}(x,y)}{p_j(x)p_k(y)} \right). \]

Double counting was omitted because \( MI(j,k) = MI(k,j) \). Here \( N_{AA} (= 20 + 1) \) indicates the twenty naturally occurring amino acid residues and the "-" symbol that exists as gap in the MSA. The symbol "X" from the MSA was not considered separately (there were four such instances) and was computed using the gap "-" instead.

**MI graphs.** To associate high MI(j,k) pairs with all other statistically significant high MI(j,k) pairs, we constructed a MI graph. The vertices of the MI graph represented positions from the MSA and the edges represented the statistically significant high MI pairs as demonstrated previously. From over one million MI pairs of MSA columns, the leading 800–1,000 MI pairs resulted in a consistent cohort of positions represented in the MSA (results not shown). Therefore, MI graph with leading 1,000 MI(j,k) pairs was constructed. The degree (connectivity) of the 31 leading vertices is reported in the last column of Supplementary Table 1.

**Homology modeling.** The closed state model of the human TREK-1 channel (gb|AAD47569.1) was built using as template the K+ Channel KcsA at 2 Å resolution, pdb code 1k4c for each of the two repeats in one TREK subunit (Fig. 1A). Sequence alignment (Fig. 1B) was initially generated using Clustalx and refined by incorporating additional information from secondary structure prediction using JPred, Porter, Prof, Psipred and Sable and patterns of residue conservation. All initial models were generated using Modeller 9v1. Models were then adjusted manually to improve interactions among conserved residues and to bend TM2A just before a proline. The segment linking repeat A to repeat B was constructed as an α helix and then positioned and adjusted manually to connect the repeats while also optimizing interactions among conserved residues and placing the poorly-conserved hydrophobic face of the helix to interact with lipid alky chains at the inner surface of the membrane.

Two models of the open conformation were constructed from the closed conformation. The first was constructed by manually swinging only the TM2B and to a lesser extent TM1B helices radially outward to expand the inner portion of the pore. The TM region of this model was very similar to that of the “inactivated” model illustrated here. The second model was constructed by expanding the inner portion of the pore more by also swinging TM1A and TM2A radially outward slightly. These models were evaluated with MD simulations to determine which was more stable. The first model tended to return toward the closed conformation; whereas, the second remained stable as a highly open structure.

The cytoplasmic Post TM2B region was noted to have an amphipathic helical pattern. It was modeled to self-associate in two ways: as parallel and antiparallel dimers.

**Molecular dynamics simulations.** The models for the open and closed states were used as starting structures for MD simulations and were processed according to the following protocol:

(a) Hydrogen atoms were added; ionizable residues were in their default protonation state.

(b) The structures were oriented so that the 2-fold rotational symmetry axis coincided with z, with extracellular and selectivity filter side facing the positive direction. The channel was positioned along z coordinate to have the best match of the surface hydrophobicity pattern to the lipid membrane patch.

(c) Ions were positioned in the selectivity filters of HM the same way as in the corresponding template x-ray structures and with water molecules placed between the ions, since water is essential for ion conduction.

(d) Channels were embedded into a patch of 1-palmitoyl-
2-oleoyl-phosphatidylethanolamine (POPC) lipid bilayer fully hydrated with TIP3P water molecules. The lipid bilayer containing on average 228 molecules of POPC was pre-equilibrated in a solvated flexible simulation cell. The dimensions of the rectangular solvent box were chosen so that the minimum distance from the box boundaries to the protein was 25 Å. The simulation cell area was set at 112 x 116 A for all the systems, thus leaving ~50 Å (about 5–7 layers of lipid) between the copies of the channel in the periodic cells. To facilitate conformational rearrangements of channels in the membrane, the system was simulated with an anisotropic flexible cell maintaining 1 bar pressure in z direction and membrane tension of 40 dyne/cm in x-y plane (fixed x to y sizes ratio). Relatively high value of the tension...
was necessary to maintain an equilibrium area per POPC lipid matching the experimentally measured value of 68.3 Å².63,64

(e) K⁺ and Cl⁻ ions were added in order to maintain electro-neutrality. Structure file was generated using the psfgen plugin in VMD.65 The total number of water molecules, lipids and ions contained in the systems are indicated in Supplementary Table 3.

Unrestrained MD simulations. All MD simulations were performed in the NPT ensemble using NAMD2 package66 developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign, with the CHARMM27 force field parameters67 including the grid-based CMAP correction68 for the φ-, ψ-angular dependence of the energy. This is especially important for α-helical proteins (as the systems simulated in this work), since CMAP not only yields significant improvements in the distribution of dihedral angles, but also corrects the tendency of CHARMM force field to distort α-helix structures to π-helix structures.69,70

The simulation systems were subject to a stepwise equilibration before the production phase, involving initial 1,000 steps of energy minimization, followed by 100 ps of gradual heating to 303 K with the protein constrained to the initial coordinates and 4 ns of simulation in which protein backbone was constrained but lipids were allowed to pack around the protein.

After the initial minimization, heating and equilibration stages, the production phase of each MD unrestrained simulation used a time step of 1 fs for the bonded interactions, with coordinates saved every 1 ps. The Langevin piston method71,72 was used to maintain a constant pressure of 1 atm. The temperature, set to 303 K, was controlled by using Langevin dynamics with a coupling coefficient of 1 ps⁻¹. We used periodic boundary conditions and the particle mesh Ewald method68 with a real-space cutoff distance of 12 Å and a grid width of 0.97 Å. The switching distance for nonbonded electrostatics and van der Waals interactions was 10 Å and the integration time step was 4 and 2 fs respectively. Initial models of open, low-conductive and closed states were subject to 30 ns of unrestrained MD simulations.

Model refinement by symmetry annealing. Structural models of the open, inactivated and closed states were refined by using 2-fold symmetry restrained MD simulations (“symmetry annealing”) according to a protocol we previously developed.45 During the symmetry annealing procedure all protein atoms were gradually driven towards the frequently (every 1 ps) updated 2-fold symmetric average positions using harmonic restraints with spring constant slowly increasing from 0.001 to 10 kcal/mol Å². The rest of the system (lipids, water and ions) was unrestrained.

Simulation protocol. The models were first subject to 30 ns of unrestrained MD simulations. During the unrestrained simulation stages all medium and protein atoms were free to move. Symmetry annealing was applied at different timesteps during the unrestrained simulation: after 5 ns, 10 ns, 15 ns and 20 ns (Fig. 5A) and each annealing step was followed by 10 ns unrestrained MD simulation to test the stability of the resulting model. Our previous experiments45 indicated that the accuracy of the model is well correlated with its stability, therefore we selected the most stable model (after annealing at 15 ns) and attempted further refinement by a second step of symmetry annealing.

Data analysis. The size and shape of channels pore was estimated using the program HOLE.74

Protein secondary structure was estimated using the STRIDE algorithm75 embedded in VMD (http://www.ks.uiuc.edu/Research/vmd). RMSD relative to the starting structure, target RMSD structure, the symmetric average and RMSF were calculated using custom-written Tcl scripts in VMD.

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Note

Supplementary materials can be found at: http://www.landesbioscience.com/journals/channels/article/13905/
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