Two Alternative Conformations of a Voltage-Gated Sodium Channel

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Abstract

Activation and inactivation of voltage-gated sodium channels (Navs) are well studied, yet the molecular mechanisms governing channel gating in the membrane remain unknown. We present two conformations of a Nav from Caldalkalibacillus thermarum reconstituted into lipid bilayers in one crystal at 9 Å resolution based on electron crystallography. Despite a voltage sensor arrangement identical with that in the activated form, we observed two distinct pore domain structures: a prominent form with a relatively open inner gate and a closed inner-gate conformation similar to the first prokaryotic Nav structure. Structural differences, together with mutational and electrophysiological analyses, indicated that widening of the inner gate was dependent on interactions among the S4–S5 linker, the N-terminal part of S5 and its adjoining part in S6, and on interhelical repulsion by a negatively charged C-terminal region subsequent to S6. Our findings suggest that these specific interactions result in two conformational structures.

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Legend: Crystal structure of the voltage-gated sodium channel NavCt in two-dimensional crystals by cryo-electron microscopy. The background is tiled by the original and reverse contrast projection map of the crystal, which clearly shows two types of tetramers in one unit cell. Each cartoon model is viewed parallel to the membrane plane.
Introduction

Voltage-gated ion channels are crucial for the propagation of electrical signals that regulate a variety of physiological processes [1]. All tetrameric voltage-gated cation channels have six-transmembrane helices divided into a voltage sensor domain (VSD) formed by the S1–S4 segments and a pore domain (PD) formed by the S5–S6 segments. The S4 segments of the VSD carry positively charged residues that are essential for sensing the membrane potential and opening the PD for Na+ conduction upon depolarization [1–3]. Activation of voltage-gated sodium channels (Navs) triggers action potentials in neuronal signal transduction [1]. Gating, namely, activation and inactivation caused by changes in the membrane potential, is strictly regulated to generate an action potential with the proper shape. Therefore, Navs are principal targets of local anaesthetic drugs and neurotoxins, and mutations of these channels cause multiple inherited diseases known as channelopathies, including periodic paralysis, cardiac arrhythmia, epilepsy, and neuropathic pain [4–6].

Structural information associated with multiple function-related conformations of voltage-gated ion channels is indispensable for understanding the underlying molecular mechanism of gating. High-resolution potassium channel structures together with richly documented functional data have clarified the underlying molecular mechanisms of potassium selectivity and gating [7–10]. Compared with potassium channels, eukaryotic Navs are huge proteins and are thus difficult targets for structural experiments. The discovery of prokaryotic Navs (NavBacs), which are simple homotetrameric channels, provides an important opportunity to study the structure–function relationship of eukaryotic Navs [11–14]. NavBacs play multiple physiological roles in prokaryotes, such as motility, chemotaxis, and pH homeostasis [15,16]. Moreover, their physiological and pharmacological characteristics resemble those of eukaryotic Navs [11–13]. In the past few years, only two NavBacs structures, NavAb and NavRh, have been analyzed by X-ray crystallography [17–19]. The first NavBac structure determined from NavAb, which was cloned from Arcobacter butzleri, revealed that NavBacs have two short helices (pore and P2 helices) between the outer helix S5 and inner helix S6, and the loop between these two short helices forms the selectivity filter (SF) [17]. The conformational changes of NavBacs have also been discussed on the basis of a structural comparison between NavAb and NavRh [20]. The structural information of Nav in the absence of detergent, however, has not been

Fig. 1. Functional expression of NavCt in Chinese hamster ovary cells. (a) Traces of NavCt resulting from the voltage protocol shown below. \( V_{\text{hold}} \) indicates the holding potential. (b) Mean peak current–voltage (\( I/V \)) relation of NavCt normalized by the peak current. (c) Summary of mean relative NavCt current in cells exposed to solutions containing the indicated ions. Recordings in each test ion-containing solution are flanked by recordings in Na-containing solution; recordings in the test ion-containing solution were normalized to the mean of the flanking recordings in the presence of sodium \( (n = 5) \).
reported. We thus used a method for two-dimensional (2D) crystallization of NavCt, a NavBac homologue from thermoalkaliphilic bacteria, and performed electrophysiological measurements together with mutational experiments guided by structural information on NavCt.

Results

Functional and structural analyses

NavCt was cloned from *Caldalkalibacillus thermarum* strain TA2.A1, which was obtained from an alkaline thermal bore at Mount Te Aroha, New Zealand [21]. Therefore, NavCt should have high thermostability, and indeed NavCt expressed in mammalian cells generated an inward sodium current at room temperature (Figs. 1 and 2). The amplitude of the generated current was smaller than that of other prokaryotic homologues. The NavCt current amplitude was not decreased by a repeated pulse protocol with a significantly longer interval (Fig. 2d); therefore, NavCt did not exhibit the late slow inactivation observed in NavAb [18]. The time constant of the inactivation ($\tau_{\text{inact}}$) of NavCt at a membrane potential of $-20$ mV was $560 \pm 67.2$ ms ($n = 6$), which was larger than that of other homologues. To examine the NavCt structures in lipid bilayers, we used a 2D crystallization approach. NavCt molecules in the 2D crystals would more likely adopt their natural conformation in the lipid bilayer.

![Fig. 2. Voltage-dependent activation and inactivation of NavCt. (a) $I_{\text{NavCt}}$ deactivation tail currents. After prepulses of varying depolarization (from $-120$ to $+30$ mV, in $+10$-mV increments), tail currents were measured at $-120$ mV. (b) $I_{\text{NavCt}}$ steady-state inactivation currents after a 3-s prepulse. (c) Mean voltage dependence of activation (open circles) and inactivation (filled circles). Values from individual experiments of deactivation tail currents were normalized to the maximum conductance. Individual normalized conductance voltage curves were fit with a Boltzmann equation. Mean $V_{1/2}$ of activation was $-64.9 \pm 1.5$ mV, mean $\kappa$ was $6.65 \pm 1.26$ mV, $n = 6$. Values from individual cells of steady-state inactivation currents were normalized to the largest currents following prepulses to strongly negative potentials. Individual normalized inactivation curves were fit using the Boltzmann equation. Mean $V_{1/2}$ of inactivation was $-86.4 \pm 0.58$ mV, mean $\kappa$ was $-6.76 \pm 0.49$ mV, $n = 5$. (d) Recovery of the NavCt current. Depolarizations from a holding potential of $-140$ mV to $-20$ mV, 2.5 s in duration, were applied at 1-min intervals, and the peak current elicited by each pulse was measured ($n = 3$). Currents were normalized to the peak inward current during the first pulse.](image-url)
without the interference of detergents. The 2D crystals of full-length NavCt were analyzed by electron crystallography using cryo-electron microscopy (EM) (Fig. S1) and we confirmed that the 2D crystal has $P_4$ symmetry (Table S1, and see Materials and Methods). One asymmetric unit contained two monomers (Fig. 3a). The three-dimensional (3D) map of NavCt was finally calculated at 9 Å resolution (Table 1) based on electron crystallography. Cryo-EM structural analyses, even at 6–10 Å resolution, provided precise structural information due to the high experimental phase quality [22–24], thereby allowing us to describe the conformational significance using fitted models under the current resolution.

Until now, only the structures of NavAb and NavRh have been determined [17–19], and their sequence identities with NavCt are 42% and 35%, respectively. Although NavRh has the minimal border identities for homology modeling, the amino acid sequence alignment between NavCt and NavAb displayed high identity with only three gaps, suggesting that a reliable homology model can be built on the basis of the NavAb structure [Protein Data Bank (PDB) code: 3RVY] [17] (Fig. S2). The homology model, split into each domain, can be fitted using a collage in Situs.
with optional enforcement of $P4$ symmetry in accordance with the observed density map (Figs. 3 and 4). The low noise level in the final analyzed map enabled us to assign the model based on the NavAb structure to the experimental density map (Fig. 3c). One unit cell of the 2D crystal contained two NavCt channel molecules, each with a fourfold symmetric axis (Fig. 3a). The molecules, indicated by the letters A (Mol A, marine in Figs. 3 and 4a–d) and B (Mol B, magenta in Figs. 3 and 4e–h), were packed in a configuration opposite one another in the lipid bilayer (Fig. 3a and b).

The crystal packing interactions were different between Mol A and Mol B. The N-termini of Mol A and Mol B interacted with the N-terminus and the P2-helix extracellular sides of the channel adjacent to Mol A, respectively (Fig. 5a). On the other hand, no interaction was observed on the extracellular side of Mol B (Fig. 5b). As a result, each PD of Mol B was individually isolated by the molecular lattice formed by the Mol A channel in the 2D crystal (Figs. 3a and 5b). Although the crystal-packing environment was different, the VSD arrangements of Mol A and Mol B were highly similar to each other and also similar to those of NavAb [17] and the Kv1.2 chimera channel [10] (Fig. 3d). In contrast, they were very distinct from those of NavRh [19] (Fig. 3d). Therefore, structural comparison with NavAb rather than NavRh would allow us to better clarify the underlying mechanism of the different NavCt conformations, despite the similar VSD arrangements.

### Two conformations

The crystallographic interactions contributed to remarkable differences in the structures of the two molecules (Fig. 4). While the PD of Mol B was relatively free from crystal contacts (Fig. 5b), the PD position of Mol A was stabilized by several
interactions dependent on crystal packing (Fig. 5a). These differences seemed to allow for clear visualization of the density map near the SF of Mol A and the disordered SF of Mol B, especially around the position corresponding to Arg202 in the P2-helix (Fig. 6a and b). The helical structure of Mol A at the P2-helix might particularly be stabilized with the N-terminus of Mol B in 2D crystals (Fig. 5a).

To test the functional role of Arg202 in the P2-helix based on the structural observations, we introduced Arg202 mutations to probe their effects on channel activity (Fig. 6). Although substitution with positively charged hydrophilic amino acids (His or Lys) did not significantly alter channel activity, substitution of Arg202 with large hydrophobic residues (Leu, Phe, or Trp) increased the inactivation time constant. In particular, the R202W mutant produced a larger current with a significantly larger inactivation time constant (1010 ± 116 ms at −20 mV membrane potential; n = 10; Fig. 6d) than the wild-type channel (P = 0.014). These results indicated that substitutions of this residue with a different size, hydrophobicity, and neutralization always allowed for channel activity, even though some led to altered inactivation kinetics. Taking into account the variation at the corresponding position in eukaryotic Navs [27] as well as its being noncritical for channel activity, this residue must only tune channel gating properties. The visible change of the part from the SF to the P2-helix between the two conformations might not be explained only by the change in the stability of the P2-helix that we deduced from the Arg202 substitution studies. Therefore, it is likely that their inherent different conformation causes such structural differences around the SF region (see subsequent sections).

A negatively charged C-terminal region subsequent to helix S6

The most remarkable difference between Mol A and Mol B was in the cytosolic C-terminal region of helix S6 (Fig. 7a–d). The inner gate of Mol B was wider than that of Mol A (arrowheads in Fig. 7a–d), and the electron density of the following region of the inner helix S6 was clearer in Mol B than in Mol A (Figs. 4 and 7a–d). Interestingly, this region corresponds to the characteristically negatively charged region (NCR) from Glu239 to Glu255 of the NavCt channel sequence. Such negatively charged residues are also observed in the following C-terminal end of the inner helix S6 in all NavBacs whose channel activities are observed (Fig. 7e and Fig. S2). The C-terminal structure of Mol B contains a negatively charged surface and has the potential to form a helix in a hydrophilic environment of the NCR extending from helix S6 (Fig. 7c, e, and f). To evaluate the role of the NCR, we constructed a
mutant with the C-terminal deletion beginning at Glu254 (NavCt ΔC254). Glu254 is the C-terminal end of the NCR. The current amplitude of NavCt ΔC254 was too small, however, to characterize the channel properties. Fortunately, we found that an R202W mutation in the P2-helix increased the channel current (see Two conformations) and we introduced this mutant in addition to the ΔC254 mutant. The NavCt R202W-ΔC254 mutant produced enough current to evaluate voltage-dependent activation. The voltage dependency of NavCt R202W-ΔC254 was comparable to that of NavCt R202W (Fig. 8 and Table 2). NavBacs have a four-helix bundle after the NCR at the C-terminus, which stabilizes the channel tetramer [29–31]. NavCt R202W-ΔC254, which has the NCR in the cytoplasmic C-terminal part, could produce the current without the C-terminal four-helix bundle.

A series of C-terminal deletion mutants (ΔC241, ΔC243, ΔC246, ΔC248, and ΔC254) with R202W mutations were designed to determine the minimum length of the NCR C-terminal tail required for NavCt activity. The ΔC246, ΔC248, and ΔC254 mutants produced the typical current, but the current was completely abolished in the ΔC241 and ΔC243 mutants (Figs. 7e and 8). Further mutational experiments of the NCR showed that the current characteristics of the ΔC248 mutant with E239A/E242A mutations (ΔC248 AA-ED-E) were similar to those of the ΔC248 mutant (Fig. 8 and Table 2). The current of the ΔC248 mutant with E244A/D245A/E247A mutations (ΔC248 EE-AA-A) was too weak...
and unstable. The ΔC248 mutant with all Glu or Asp from Glu239 to Glu247 substituted with Ala (ΔC248 AA-AA-A) had non-functional channels similar to the ΔC241 and ΔC243 mutants. We confirmed that all C-terminal deletion mutants were expressed in mammalian cells at levels similar to those in the wild-type channel (Fig. 8h). These results suggested that the negatively charged residues in the NCR of NavCt, namely, from Glu244 to Glu247 (244-EDAE-247), are essential for maintaining the channel activity. These mutagenesis results, together with the differences in the density maps between Mol A and Mol B in the NCR (Fig. 7), suggest that the NCR is necessary for channel function.

The interaction of helix S5 with helix S6

Mol A showed an S4–S5 linker with a clearer electron density and a less visible C-terminal end than Mol B (Figs. 4, 7a and c, and 9a). The S4–S5 linker connected to helix S5 at Leu147 (open red arrowhead in Fig. 9a) in our model. Leu147 and Ile150 at the N-terminal end of helix S5 tightly interacted with Leu229 of helix S6 near the activation gate in the structure of Mol A (Fig. 9b and c). Importantly, in Mol B, this interaction almost disappeared (Figs. 4 and 9a). We examined the importance of this interaction by mutational analyses. Only the L147A mutant had a very small current in six single-point mutants of Leu147, Ile150, or Leu229 to either alanine or phenylalanine. Therefore, the interaction of Leu147 and Ile150 of helix S5 to Leu229 of helix S6 seemed to require a strict size configuration. To further investigate the size configuration, we tested the L147V, L147I, and L147M mutations. These three mutants had a similar positive shift of voltage-dependent activation (Fig. 9d and Table 2). These results suggested that the hydrophobic interaction of the N-terminal part of the outer helix S5 with the inner helix S6 is important for activation gating and thus could be a critical element to widen the inner gate (arrowheads in Fig. 7a–d).

The direct interaction identified between the outer and inner helices of the PD is also observed in the structure of the KcsA open mutant [32,33]. Opening of the inner helix correlated with disorder of the N-terminal end of the outer helix (Fig. S3). The Shaker 

Fig. 7. Structural insights at the cytoplasmic side. Meshes represent the density map (1.0 σ) of Mol A (a and b) and Mol B (c and d). The side views parallel to the membrane plane are shown in (a) and (c), and horizontal sectioned images viewed from the intracellular side are shown in (b) and (d) for Mol A and Mol B, respectively. The range of the horizontal view is indicated by the dashed box of (a) and (c). The density map of Mol A near the inner helix bundle crossing showed a closed inner-gate conformation (arrowhead in a and b). On the other hand, the elongated cylindrical densities corresponding to S6 helix could be clearly distinguished from each other in Mol B (arrowhead in c and d). The negatively charged residues in NCR (E239, E242, E244, D245, E247, E249, and E250) are indicated as spheres. (e) The sequence alignment at the C-terminus between NavBacs. Black arrows indicate the deletion site for the mutational analysis. (f) Electrostatic potential surface map of the PD model visualized with ±10 kT/e generated by the program APBS [28]. For clarity, only two of four monomers are shown. The dashed rectangular regions indicate the NCR and show the highly negative surfaces beginning from E239.
and KcsA chimeric channels also require the contribution of the S4–S5 linker to regulate the activation gate, especially at the proximal part of both the helix S4 and outer helix S5 [34]. In addition, mutational analyses of Kv also suggested the importance of the helical interaction between the cytoplasmic part of helices S5 and S6 for channel gating [35,36]. Therefore, regulation of the inner-gate opening by the S4–S5 linker and N-terminal end of the outer helix S5 might be a conserved mechanism in the tetrameric cation channel family.

**Discussion**

Our results suggest the functional importance of the NCR, the negatively charged cytoplasmic region succeeding helix S6, in channel function. Mammalian Navs have negatively charged residues in the C-terminal EF-hand domain after helix S6 of domain IV [37–39]. The EF-hand domain forms the helix–loop–helix structure, and negatively charged residues might be involved in modulating the inactivation gating by coordinating Ca$^{2+}$ [38]. In contrast, the
NCR of NavCt simply forms a helical structure across almost all regions (Fig. 7a and c). Sequence analysis showed that there is no significant similarity between the NavCt C-terminus and EF-hand helix–loop–helix motifs. Therefore, the C-terminal NCR of NavBacs and the C-terminal EF-hand domain might differentially contribute to channel function. Analysis of the Mol A and B structures suggested that the NCR, as the charged helical linker, adopts multiple conformations adjacent to the activation gate under physiological conditions. The closed cytoplasmic part of the inner helix S6 of Mol A was caused by smearing out the electron density of the NCR.

Table 2. \( V_{1/2} \) for activation of NavCt mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>( V_{1/2} ) for activation (mV)</th>
<th>( \kappa ) (mV)</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NavCt WT</td>
<td>−64.9 ± 1.5 (mV) ( k = 6.65 \pm 1.26 )</td>
<td>( n = 6 )</td>
<td></td>
</tr>
<tr>
<td>R202W</td>
<td>−65.3 ± 1.5 (mV) ( k = 7.11 \pm 1.27 )</td>
<td>( n = 4 )</td>
<td></td>
</tr>
<tr>
<td>ΔC254</td>
<td>−58.2 ± 1.5 (mV) ( k = 8.34 \pm 1.29 )</td>
<td>( n = 6 )</td>
<td></td>
</tr>
<tr>
<td>ΔC248</td>
<td>−67.6 ± 1.4 (mV) ( k = 6.37 \pm 1.23 )</td>
<td>( n = 4 )</td>
<td></td>
</tr>
<tr>
<td>ΔC246</td>
<td>−73.3 ± 2.3 (mV) ( k = 8.85 \pm 2.05 )</td>
<td>( n = 5 )</td>
<td></td>
</tr>
<tr>
<td>ΔC248 E239A/E242A</td>
<td>−74.4 ± 1.1 (mV) ( k = 6.85 \pm 0.91 )</td>
<td>( n = 6 )</td>
<td></td>
</tr>
<tr>
<td>L147V</td>
<td>−40.9 ± 0.8 (mV) ( k = 6.69 \pm 0.67 )</td>
<td>( n = 4 )</td>
<td></td>
</tr>
<tr>
<td>L147I</td>
<td>−38.6 ± 0.9 (mV) ( k = 8.67 \pm 0.82 )</td>
<td>( n = 6 )</td>
<td></td>
</tr>
<tr>
<td>L147M</td>
<td>−38.6 ± 3.2 (mV) ( k = 12.20 \pm 2.97 )</td>
<td>( n = 4 )</td>
<td></td>
</tr>
</tbody>
</table>

All C-terminal deletion mutants also had an R202W mutation.

Fig. 9. Overall conformational changes of NavCt. (a) For clarity, each VSD (S1–S4) with an S4–S5 linker and PD of a neighboring subunit (S51–S61) are represented by a ribbon diagram. The arrangement of the VSD and PD viewed from the extracellular side is shown as an inset in which the transparent parts are indicated as the excluded regions for clarity. Each molecule is aligned by the superimposition of Mol A and Mol B of NavCt and NavAb, based on the tetrameric pore module (color codes are the same as in Fig. 3). The conformation with the highly disordered S4–S5 linker (such as Mol B) seems to open the inner gate and to increase the observable cytoplasmic region (indicated as each colored broken line). Instead of enlarging the cytoplasmic region, the initial outer helix S5 disappeared (indicated as red unfilled and filled arrowheads of Mol B, corresponding to Leu147 and Ile150, respectively). The disordered SF of Mol B is represented by translucent magenta. (b and c) Interaction of Leu147 and Ile150 of the outer helix S5 with Leu229 of the inner helix S6 is focused on in (b) and viewed from the extracellular side in (c). The C\(^\alpha\) of these residues (L140, I150, and L229 of NavCt and M130, V133, and L212 of NavAb) is indicated as a sphere in (a), and the color code is the same as that in (b) and (c). Orange broken lines indicate the disordered region of the S4–S5 linker. (d) Voltage-dependent activation of the L147 mutants of NavCt. The voltage dependency of activation of wild type (filled circles: \( n = 5 \)), L147V (open circles: \( n = 4 \)), L147M (open inverted triangles: \( n = 4 \)), and L147I (open triangles: \( n = 6 \)) is represented as mean ± standard error.
Furthermore, our mutational analysis indicated that the negatively charged cluster, namely, from Glu244 to Glu247 (244-EDAE-247), is required to maintain channel activity (Fig. 7e). The NCR might generate a negative repulsive force at the C-terminal end of the activation gate, thus supporting channel function.

The two conformations in one 2D crystal of the NavCt channel revealed different features of the PD. They were mainly attributed to the local environments around each PD in the 2D crystal. The PD of Mol A was fixed by the interaction between its P2-helix and the N-terminus of Mol B (Fig. 10), but the PD of Mol B was isolated from crystal contact (Fig. 5). Furthermore, these differences would also represent the conformational transition of the PD after VSD activation, because both VSD structures were similar to the previously determined activated VSD structures [10,17]. In the two conformations, both cytoplasmic parts of the inner helix S6 were free from crystallographic packing (Figs. 4 and 5), but Mol A showed a relatively closed cytoplasmic part of the inner helix S6 (an arrowhead in Fig. 7a and b) and a visible SF (Fig. 6a). In comparison, Mol B showed a relatively but not fully opened C-terminal part of the inner helix S6 (an arrowhead in Fig. 7c and d) and a disordered SF (Fig. 6b). Although the differences in the two structures are small, these changed parts were obviously distinct in comparison with the first structure of NavAb [17] (Fig. 9a). Even such small changes in the two PD structures in our crystal are indicative of functional conformation changes as distinctive structural features. The NavAb structures [17,18] were actually assumed to be in a different state with very little movement of the inner helix of the PD. As another example, in nicotinic acetylcholine receptors, very small movements of the transmembrane helix regulate channel gating [40].

On the basis of our results, we propose the following schemes for converting one conformation to the other in NavCt (Fig. 10). In Mol A, the S4–S5 linker forces helix S5 to fix at a closed position and the pressure of S5 transfers to inner helix S6 through Leu147 and Ile150 (from step 1a to 3a in Fig. 10). Then, the closing of helix S6 enhances the intersubunit repulsive force at the NCR by getting close to its negative charges and then destabilizes the C-terminal part of the NCR (Fig. 7f and step 4a in Fig. 10). On the other hand, in the case of Mol B, the density of the S4–S5 linker is blurred, and helices S5 and S6 can move freely to open the inner gate as well as to reduce the repulsive force of the NCR (Fig. 9a and from step 1b to 4b in Fig. 10). The opening of the activation gate leads to the collapse of the SF in the inactivation scheme of NavBacs [41] (step 4b’ in Fig. 10). By contrast, the conformation to keep the C-terminal part of the inner helix S6 of Mol A closed induced the stabilization of the SF as shown in the closed form of KcsA [32,33] (step 4a’ in Fig. 10), while the NCR of Mol A had a disordered structure to compensate for the repulsive force of

**Fig. 10.** A proposed diagram of two alternative conformational changes of NavCt. We propose the sequential events of Mol A and Mol B based on our findings. Detailed descriptions are provided in the Discussion. The description of the ribbon model is the same as for Fig. 9, except for the dimer model. The depicted models of both Mol A and Mol B are labeled with a number corresponding to the step number in each scheme respectively. Each step is connected by continuous or broken lines for Mol A and Mol B, respectively.
the NCR (step 4a in Fig. 10). Our two conformations may provide new structural insights into the alternative conformations of sodium channels in the depolarized condition.

Materials and Methods

Plasmid constructs and protein expression

The hexahistidine-tagged NavCt (ZP_08532019) was expressed using the expression vector pTrc99A. Primers HisNavCtFwd (5′-AAATTTCCATGGGCACCCACCACTCCACATCCCCAGCGCCA-3′, NcoI site underlined) and NavCtRev (5′-AAATTTTCTAGTCAGTTGGAAGATTGGGAGTGGT-3′, XbaI site underlined) were used to amplify NavCt by polymerase chain reaction. The amplicon was digested with Ncol and Xbal and cloned into pTrc99A digested with the same restriction enzymes. All amplified regions were verified by DNA sequencing. Plasmid DNA was transformed into the competent Escherichia coli strain C43. A single colony was picked and inoculated into 2 ml of growth medium at 37 °C for 6 h, followed by amplification to 300 ml at 30 °C overnight. A 15-ml aliquot of overnight culture was grown at 600 nm) of 0.8 β-D-thiogalactopyranoside (0.5 mM) was added. The culture density reached an OD600 of 0.8–1.1. E. coli cells were harvested by centrifugation after 3 h of induction. Luria broth medium containing 0.1 mg/ml ampicillin was used as the growth medium in all culture growth and protein expression steps.

Protein purification

E. coli cells were homogenized in the basal buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, and 10 mM imidazole) and passed through a French press 5 times at a maximal pressure of 6895 kPa. Unbroken cells and cell debris were removed by centrifugation at 29,000 g for 30 min. Cell membranes were harvested by ultracentrifugation at 235,000 g for 2 h; about 10 g of pelletized cell membranes was obtained from 12 L of E. coli culture. The 10-g E. coli membrane pellet was homogenized and solubilized for 1 h in 100 ml of basal buffer containing 1% dodecylmaltoside (Sol-grade, Anatrace), corresponding to a membrane-to-detergent ratio of 10:1 (w/w). After centrifugation at 235,000 g for 30 min, the solubilized fraction was gently agitated with 1 ml of 100% Ni Sepharose high-performance resin (GE Healthcare) for 1 h. The Ni resin was collected and washed stepwise with imidazole from 20 mM to 300 mM, each step using 2 column volumes of basal buffer containing 0.12% dodecylmaltoside (Anagrade, Anatrace) and an increase of 20 mM imidazole. The protein was eluted with 10 column volumes of basal buffer containing 0.12% dodecylmaltoside and 500 mM imidazole. Fractions above 160 mM imidazole were combined and concentrated to less than 1 ml using a VIVASPIN concentrator with a 100-kDa-cutoff membrane filter. The concentrated fraction was loaded onto a desalting column (Illustra NAP column, GE Healthcare) equilibrated with a buffer containing 50 mM Na-phosphate, pH 8.0, 100 mM NaCl, 10% glycerol, 0.12% decylmaltoside, and 0.5 mg/ml 1-hexadecanoyl-2-(9Z-octadecenooyl)-sn-glycero-3-phosphoethanolamine (POPE, PE 16:0–18:1, Avanti Polar Lipids). The same buffer was used for protein elution. The protein concentration was measured by absorption at 280 nm using a Nanodrop machine. All of the procedures for membrane preparation and protein purification were performed on ice.

2D crystallization

Eluted protein was mixed with additional decylmaltoside and POPE (MW = 717.996) to a final protein–lipid–detergent mixture containing ~4 mg/ml protein, 0.25% decylmaltoside, and a lipid-to-protein ratio of 0.05–0.3 (w/w; molar ratio: 6–60) and subsequently incubated at room temperature for 0.5 to 1 h. The protein–lipid–detergent mixture was dialyzed against a detergent-free buffer (50 mM glycine–NaOH, pH 9.0, 200 mM NaCl, 4 mM MgCl2, 5% glycerol, 5% methyl-2,4-pentanediol, and 1.5 mM NaN3) at 37 °C for 1 week using 10-kDa-cutoff mini slide-A-lyzers (Thermo Scientific) in an incubator with 5% CO2 supply.

Electron microscopy

Dialysates were negatively stained using 1% uranyl acetate to check crystal quality (Fig. S1a and b). Specimen preparation for cryo-EM was performed using the back-injection method by mixing 2D crystals with an equal amount of buffer containing 50 mM glycine–NaOH, pH 9.0, and 23.75% trehalose on a molybdenum EM grid covered with a thin layer of carbon film. Excess material was removed by blotting the EM grid on filter paper. EM grids were plunged into liquid nitrogen and transferred into a JEOL 3000 SFF electron microscope equipped with a top-entry liquid helium stage and operated at an acceleration voltage of 300 kV [42,43]. Images were taken from 2D crystals tilted at 0°, 20°, 45°, and 60°, respectively, at 40,000× magnification on Kodak SO-163 micrographs with an electron dose of ~25 electrons/Å 2. The micrographs were developed in Kodak D-19 developed for 14 min, followed by washing with deionized water for 30 s and fixation for 5 min.

Data processing

The crystal areas on micrographs were selected using an optical diffractometer and digitized using a Zeiss SCAI scanner with a scanning pixel size of 7 μm. Scanned images were transformed to MRC format. All images were processed with the MRC 2D crystal processing package [44] to correct lattice distortions. The contrast transfer function was corrected using square frequency filtering in combination with periodogram averaging [45]. Initial symmetry assignments were performed using ALLSPACE [46] (Table S1). The results clearly demonstrated that the two tetramers could not be superimposed, because phase residuals for p42;2 symmetry were ~1.7 times higher than those for p4. After reconstruction of the 3D map with P4 symmetry, there was no screw axis in the unit cell, even if the two tetramers adopted an identical conformation (Fig. S4). Therefore, P4 symmetry was confirmed and consistent with two different conformations in one
crystal. The 3D map and models were visualized using the program Chimera [47] or PyMOL.† The electrostatic potential surface map was visualized with PyMOL and APBS [28].

3D map generating, model building, and validation

The homology model for the NavCt structures was built with MODELLER v9.8 [48] using the atomic model of a Nav, NavAb (PDB code: 3RVY), as a starting template, because NavCt had 42% homology with NavAb. Because the density corresponding to the S4–S5 linker was disordered, we used the VSD and PD portions of the model. These two domains were split, and then model fitting was performed as described previously [24] with minor modifications by refinement using the multi-fragment correlation-based rigid-body refinement and symmetry enforcement functions in Situs v2.6 [25]. The fitted model was further refined by CNS v1.3 [49] without experimental data, and the refined model was assessed by PROCHECK [50]. The primary sequence alignment was generated by the ALINE program [51].

Electrophysiology using mammalian cells

All electrophysiological experiments were performed as described previously [13,31,52]. All experiments were conducted at 25 ± 2 °C. The inactivation time constant (τinact) was calculated from the current trace fitted with I = I0 + a[exp(−bt)]. All results are presented as mean ± standard error. The intracellular pipette solution contained the following (in millimolar): 35 NaCl, 105 CsF, 10 EGTA [ethylene glycol bis(β-aminoethyl ether) N,N′-tetraacetic acid], and 10 Hepes, pH 7.4 (adjusted with CsOH). The extracellular NaCl solution contained the following (in millimolar): 150 NaCl, 1.5 CaCl2, 1 MgCl2, 10 glucose, and 10 Hepes, pH 7.4 (adjusted with NaOH). For extracellular solutions containing K+, Cs+, and NH4+, NaCl was replaced with KCl, CsCl, and NH4Cl, respectively. The extracellular N-methyl-d-glucamine solution contained the following (in millimolar): 150 N-methyl-d-glucamine, 1.5 CaCl2, 1 MgCl2, 10 glucose, and 10 Hepes, pH 7.4 (adjusted with HCl). Voltage clamp pulses were generated and currents were recorded using Pulse software. Data were analyzed using Igor Pro 6.2 (WaveMetrics).

Data deposition

The EM density map was deposited in the EMDataBank§ (accession code EMD-2347). The coordinates of the homology model were also deposited in the PDB (accession code 4BGN).

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Supplementary Data

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Abbreviations used:
Nav, voltage-gated sodium channel; VSD, voltage sensor domain; PD, pore domain; SF, selectivity filter; 2D, two-dimensional; 3D, three-dimensional; EM, electron microscopy; NCR, negatively charged region; PDB, Protein Data Bank.

References


