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Structure of a Prokaryotic Sodium Channel Pore Reveals Essential Gating Elements and an Outer Ion Binding Site Common to Eukaryotic Channels

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Abstract

Voltage-gated sodium channels (NaVs) are central elements of cellular excitation. Notwithstanding advances from recent bacterial NaV (BacNaV) structures, key questions about gating and ion selectivity remain. Here, we present a closed conformation of NaVAe1p, a pore-only BacNaV derived from NaVAe1, a BacNaV from the arsenite oxidizer Alkalilimnicola ehrlichei found in Mono Lake, California, that provides insight into both fundamental properties. The structure reveals a pore domain in which the pore-lining S6 helix connects to a helical cytoplasmic tail. Electrophysiological studies of full-length BacNaVs show that two elements defined by the NaVAe1p structure, an S6 activation gate position and the cytoplasmic tail “neck”, are central to BacNaV gating. The structure also reveals the selectivity filter ion entry site, termed the “outer ion” site. Comparison with mammalian voltage-gated calcium channel (CaV) selectivity filters, together with functional studies, shows that this site forms a previously unknown determinant of CaV high-affinity calcium binding. Our findings underscore commonalities between BacNaVs and eukaryotic voltage-gated channels and provide a framework for understanding gating and ion permeation in this superfamily.

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Introduction

Voltage-gated sodium channels (NaVs) are central elements of cellular excitation [1,2]. These channels are targets for drugs directed at neuropathic pain, migraine, arrhythmias, and epilepsy [3,4], as well as environmental toxins [5]. NaVs belong to the voltage-gated ion channel (VGIC) superfamily and are most closely related to voltage-gated calcium channels (CaVs) [6,7]. Despite ion selectivity differences, mutational studies [6–10] and sequence similarities [6,7] have suggested that NaVs and CaVs share similar selectivity filter architectures [2]. However, details of this presumed commonality are unknown.

Discovery of a large family of bacterial NaVs (BacNaVs) [11–13] that may be ancestors of eukaryotic NaVs and CaVs [14] has enabled delineation of structural principles shared by this VGIC superfamily branch. BacNaVs are tetramers. Each subunit has six transmembrane segments that comprise a voltage-sensing domain (VSD) composed of the S1–S4 segments and a pore domain (PD) formed from the S5–S6 segments [15–17]. This subunit architecture is
recapitulated in eukaryotic Na\textsubscript{v}s and Ca\textsubscript{v}s where four homologous six-transmembrane repeats occur in a single polypeptide [2,6,7]. Protein dissection studies have demonstrated a further modular aspect of BacNa\textsubscript{v} architecture within the membrane domains. BacNa\textsubscript{v}, “pore-only” constructs lacking the VSD have been demonstrated to fold [18–20], assemble [18–20], and form functional, selective ion channels [19]. These demonstrations of BacNa\textsubscript{v} modularity are in accord with various lines of evidence that support the independence of the VSDs and PDs. These include: the fact that within the VGIC family, potassium channels occur two forms, those that encompass a PD alone (K\textsubscript{ir} and K\textsubscript{2P} channels) and those having a VSD attached to the PD [6,7], results from VSD-PD chimera studies [21–24] and structural evidence indicating that VSDs and PDs lack extensive contacts [15–17,25–27]. Although recent BacNa\textsubscript{v} structures have revealed the basic transmembrane architecture [15–17,20], fundamental questions about gating, ion permeation, and ion selectivity have remained unanswered.

BacNa\textsubscript{v}s have a conserved ~40-residue C-terminal cytoplasmic tail [28,29] that is important for assembly [28] and function [29,30]. However, this domain is either unresolved [15,16] or absent from the crystalized constructs [17,20] of prior BacNa\textsubscript{v} structures. Hence, its structure, relationship to the PD, and key functional elements have remained enigmatic.

Ion permeation is fundamental ion channel property [2]. Original descriptions of the BacNa\textsubscript{v} Na\textsubscript{v}Ab suggested a single ion pore model [15]. In contrast, functional studies of Na\textsubscript{v}s [2,31] and Ca\textsubscript{v}s [2,32] support the presence of multi-ion pores as a means to affect ion selectivity and permeation [33,34]. To date, only a single BacNa\textsubscript{v} ion binding site has been observed at the inner vestibule of the Na\textsubscript{v}Rh selectivity filter [17]. Recent computational studies have suggested the possibility of other ion binding sites [35,36], but the absence of experimental data have left unresolved questions regarding the existence of such sites, their exact locations, and residues involved in ion binding.

Here, we present the structure of Na\textsubscript{v}Ae1p, a pore-only sodium channel derived from the Alkalilimnicola ehrlichei BacNa\textsubscript{v} Na\textsubscript{v}Ae1 [19]. The structure shows a closed conformation of a complete PD and cytoplasmic tail. Functional tests of key structural elements suggest that BacNa\textsubscript{v} opening involves changes at an S6 activation gate residue and a structural rearrangement in the neck region of the cytoplasmic tail. The structure also reveals an ion binding site in the selectivity filter that we term the “outer” ion site. We demonstrate that the ion coordination residue comprising this site has a previously unrecognized counterpart in mammalian Ca\textsubscript{v}s that is crucial to high-affinity calcium binding, a result that lends support to long-standing proposals regarding the presence of multiple ion binding sites in Ca\textsubscript{v}s [32–34]. Together, our results emphasize the deep evolutionary links between BacNa\textsubscript{v}s and mammalian channels and suggest that channels sharing this selectivity filter architecture have multiple ion binding sites.

Results

Structure of the pore-only channel Na\textsubscript{v}Ae1p

We determined the structure of Na\textsubscript{v}Ae1p, a “pore-only” protein bearing the PD and cytoplasmic tail of the BacNa\textsubscript{v} Na\textsubscript{v}Ae1 [19] (Table S1) using X-ray diffraction data obtained from a l222 crystal that diffracted to a resolution of 4.00 Å based on traditional measures such as $R_{pim}$ or $k/l$. However, as it has been shown recently that adding weak high-resolution data beyond the commonly arbitrarily defined cutoffs used to judge resolution limits may be beneficial [37–39], we used data to a resolution of 3.46 Å based on CC (correlation coefficient) evaluation ($CC_{1/2} > 0.1$) [37]. Molecular replacement using a PD ensemble from the Arcobacter butzleri (Na\textsubscript{v}Ab) [15,16] and Rickettsia sp. (Na\textsubscript{v}Rh) [17] BacNa\textsubscript{v}s revealed electron-density spanning from the beginning of the transmembrane segment S5 (Ile150) through the end of the cytoplasmic coiled-coil domain (Ser285) (Fig. S1a). Na\textsubscript{v}Ae1p shows the funnel-shaped architecture found in other BacNa\textsubscript{v}s [15–17,20] (Fig. 1a and b). Each tetramer subunit is composed of S5 and S6 transmembrane helices that form the outer and inner parts of the PD, respectively. The P1 and P2 pore helices bridge S5 and S6 and are connected by the selectivity filter (Fig. 1).

Initial electron density maps (Fig. S1a) revealed an element absent from prior BacNa\textsubscript{v} structures, a long helical cytoplasmic tail (Figs. 1a and 2a). The Na\textsubscript{v}Ae1p tetramer forms the asymmetric unit and is packed in the crystal lattice such that the crystallographic axes are not coincident with the channel 4-fold symmetry axis (Fig. S1b and Table S1). This arrangement leads to four, independent but similar channel protomers arranged around the channel central axis in the asymmetric unit. Model building and refinement ($R_{work}/R_{free}$ of 22.4/26.8: Table S1) showed that the pore-lining S6 helix continues for one turn after Met241, the intracellular pore constriction point (Fig. 2a). A 40° bend at His245 follows and leads to a continuous helix that terminates with a four-stranded, parallel coiled coil encompassing residues Leu265–Ser285 (Table S2). The clear quality differences between electron density maps calculated using data resolution cutoffs based on traditional (Fig. S1c) versus $CC_{1/2}$ metrics (Fig. S1d) support the choice of resolution cutoff based on $CC_{1/2}$ values. The obvious differences in map quality reinforce the assertion that
Fig. 1. Structure of the *A. ehrlichei* pore-only sodium channel protein NaVAe1p. (a) Side view showing two transmembrane region subunits and four cytoplasmic tail subunits. Transmembrane helices S5 and S6 are colored green and blue, respectively. Calcium ion is a white sphere. Gray lines show approximate lipid bilayer boundaries. (b) NaVAe1p tetramer extracellular view. Colors are as in (a). (c) NaVAe1p secondary structure and alignment with BacNaV PD sequences. NaVAe1p secondary structure elements are indicated and colored as in (a). Selectivity filter position numbers are indicated relative to position “0”, NaVAe1p Glu197. “+” indicates the activation gate residue. “*” indicates position of the NaVAe1p His245 bend. Black vertical broken line indicates division between “Neck” and “Coiled-coil”. Colored bars indicate the following: selectivity filter (+1) position, red; S6 pore lining, blue; and coiled-coil core residues, orange. Positions of 3Gly and 7Gly neck mutants are indicated in brown. Gray letters show NaVSulP portion used in the NaK chimera [29]. Red vertical lines show crystallized NaVMs pore-only construct boundaries [20]. Other sequences are as follows: NaVSulP, *S. pomoreyi* [19]; NaVAe1p, *Alcanivorax borkumensis* [19]; NaVBh1, *Bacillus halodurans* [11]; NaVAe1p, *A. butzleri* (GI: 157737984) [15]; NaVRh, *Rickettsia* sp. (GI: 262276647) [17]; NaVSulP, *Sulfitobacter pontiacus* (GI: ZP_00961826.1) [29]; and NaVMs, *Magnetococcus* sp. (UniProt ID A0L5S6) [20].
adherence to traditional metrics for defining resolution limits can result in the omission of useful diffraction data [37,38].

The cytoplasmic tail is considerably longer than the pore-forming region (~65 Å versus ~40 Å, respectively). Consequently, Na\textsubscript{v}Ae1p spans ~110 Å in the axial dimension and resembles the general architecture of a NaK-Na\textsubscript{v}SulP chimera [29] and the full-length KcsA potassium channel [40] (Fig. 2b and c, respectively). The coiled coil is common among BacNaVs [28] and is thought to participate in channel assembly [28,30]. Its location, C-terminal to a segment that trails S6, is reminiscent of similar domains from eukaryotic Kv7 (KCNQ) [41,42] and TRP channels.

\begin{image}
\centering
\includegraphics[width=\textwidth]{image.png}
\end{image}
A Prokaryotic Sodium Channel Pore Structure

[43] and agrees with predictions [28] and a similar structure in the NaK-Na,SulP chimera [29] (Fig. 2b).

The cytoplasmic tail arrangement resembles the stems of a flower bouquet. Individual helices interact extensively at the C-terminal base throughout the 18-residue coiled coil but splay apart above Met267 into individual helical stems that connect the coiled coil to S6 (Fig. 2d). This region, termed the “neck” (Figs. 1a and 2a), extends over six helical turns. The distance between the Cα positions and the superhelix axis widens from ~7 Å in the coiled coil to ~9 Å in the neck (Fig. 2d). The neck showed another unexpected feature, a large electron density that anomalous scattering indicated as a metal ion (Fig. S2a). B-Factors indicate that the neck is as well ordered as other parts of the structure with the exception of the region near the neck ion (Fig. S2b).

Comparison of NaVAe1p with a chimera between the nonselective NaK channel and the BacNaV Na,SulP coiled coil [29] shows good agreement in the coiled coils (Fig. 2b and e) and Table S3 (RMSD Cα = 1.2 Å for the tetramer). Contrastingly, the corresponding KcsA region, where there is no superhelical coil (Table S2), poorly matches the NaVAe1p coiled coil (Figs. 2c and f and Table S3) and reveals an unexpected diversity in how seemingly similar cytoplasmic domains can assemble. The essentially continuous helical conformation from S6 to the coiled coil is contrary to predictions from circular dichroism and sequence analysis suggesting that this BacNaV region is disordered [28]. Notably, the neck has an abundance of charged and polar residues (15/20 residues) (Figs. 1c and 2g). This density of hydrophilic residues may be important for neck function.

Comparison of NaVAe1p pore region with other BacNaV structures

NaVAe1p conforms to expectations for a closed conformation as the selectivity filter is not collapsed and the intracellular gate is closed (Fig. 3a and b and Fig. S2c). Overall, the PD superposes well with other BacNaVs (Fig. 3c and Table S3). Despite the VSD absence, S5 has position similar to that seen in NaVAb and is only substantially different from S5 of NaVRh, which is the outlier of currently known BacNaV structures. The main variations from other BacNaVs lie in the C-terminal ends of S6 from the putative inactivated NaVAb conformation [16] and NaVMs pore [20] (Fig. 3c and Fig. S3).

NaVAe1p has a wide extracellular funnel that connects through the selectivity filter to the central cavity (Fig. 3a). Side-chain oxygens of selectivity filter residue Ser198 form the narrowest extracellular constriction (pore radius, 1.6 Å) (Fig. 3b). This is larger than that seen at the analogous NaVRh position (pore radius, 1.1 Å), where the filter is closed [17], but is not as wide as that in NaVAb (pore radius, 2.3 Å) [15] (Fig. 3b and Fig. S4). It is important to point out that the structure of NaVMs has a diameter that is close to that of the other BacNaVs (Fig. 3b) due to the similarity of its backbone positions with the other BacNaV structures (Table S3). The remaining differences for NaVMs are largely due to the fact that the NaVMs structure has incomplete side chains at seven out of eight of the positions that correspond to the NaVAe1p residues that constrict the inner cavity, Phe233 and Ile237 (Fig. 3a), and it truncates in three of the subunits before the portion that corresponds to NaVAe1p Met241. The inner diameter of all of the solved structures contrasts to that of the open state model NaVRhMsOM that is generated from the most deviant subunit in the NaVMs structure (Fig. 3b) [20].

The inside of the selectivity filter abuts an aqueous cavity that includes lateral openings to the membrane and that ends in a constriction formed by S6 residues Phe223, Ile237, and Met241 (Fig. 3a and b), positions largely conserved among BacNaVs (Fig. 1c). Both the presence of F6−F6 electron density (Fig. 3d) and a side-chain sulfur difference anomalous signal (Fig. S2a) support the placement of Met241 as the site of intracellular pore closure. This position corresponds to the suggested NaVAb activation gate [15] and, strikingly, forms a closure point further along S6 than in all but the initial NaVAb structure (Fig. 2b). It is notable that evaluation of possible boundaries of the lipid bilayer hydrophobic portion [45] suggests that, unlike other BacNaV structures, the NaVAe1p activation gate region protrudes from the bilayer core into the zone comprising phospholipid head groups (Fig. S5).

Fig. 2. NaVAe1p structure comparison and analysis. (a) Side view of a single NaVAe1p monomer. Secondary structure elements are labeled and colored as in Fig. 1. Select residue positions are indicated. (b) Cα superposition of the tetrameric NaVAe1p region of the NaK-Na,SulP chimera [29] (Na,SulP portion, red; NaK portion, wheat) with equivalent parts of the NaVAe1p neck and coiled coil (colored as in Fig. 1). One NaVAe1p and NaK-Na,SulP chimera monomer is shown in cartoon representation in front of the surface of three remaining NaVAe1p subunits. (c) Cα superposition of the NaVAe1p coiled-coil tetramer with the equivalent KcsA residues. One NaVAe1p (colored as in Fig. 1) and KcsA (wheat) monomer is shown in cartoon representation in front of the surface of three remaining NaVAe1p subunits. (d) Plot of NaVAe1p coiled-coil superhelix radius versus residue number. Neck and coiled-coil elements are shaded as in (a), (e) Close-up of the coiled-coil regions of NaVAe1p (orange) and Na,SulP (red) used for superposition in (b). (f) Close-up of the cytoplasmic parts of KcsA (left panel, wheat) and NaVAe1p (neck, sand; coiled coil, orange). (g) NaVAe1p electrostatic surface potential [+4kT (blue) to −4kT (red)] mapped on the channel van der Waals surface. Gray lines show approximate lipid bilayer boundaries.
Identification of the “outer ion” binding site

We found a large positive electron density peak perched on the 4-fold axis of the channel at the selectivity filter outer mouth (Fig. 3e). Calculation of anomalous difference maps at 6.5 Å revealed a strong peak (11σ) indicating the presence of a non-protein anomalous scatterer (Fig. 3f and Fig. S6a–d). Recognizing that there could be challenges in identifying this peak due to the data resolution, we searched for other evidence that it represented an ion and not noise or some other possibility. We found a similar non-protein anomalous scatterer at the exact same location in a second lower-resolution (3.80 Å) data set (Na_{Ae1p}, crystal II; Table S1 and Fig. S6c) obtained from a crystal grown using the same high-calcium (200 mM CaCl$_2$) conditions as the crystal that yielded the 3.46-Å-resolution data set. By contrast, there was no anomalous peak at this location in maps calculated from crystals grown

![Diagram](a)

![Diagram](b)

![Diagram](c)

![Diagram](d)

![Diagram](e)

![Diagram](f)
without calcium (Figs. S1e and S6c). In all three cases, the maps, which are all calculated at the same resolution (6.5 Å), showed strong evidence for the neck ion. Finally, structural studies of the H245G mutant, in which crystals were grown in the absence of calcium and in the identical space group as NaVAe1p crystal I and crystal II (Fig. S6c), gave no evidence for the selectivity filter ion. Given these multiple lines of evidence that the anomalous peak at the selectivity filter outer mouth depends on the presence of calcium, we assigned this density as a calcium ion.

Inclusion of the ion alone in the refinement left substantial unaccounted electron density. Taking into account the ion position and likelihood that it is partly solvated, we modeled four waters using standard calcium coordination geometry (Fig. 3e). We also found additional positive difference (Fo – Fc) electron density extending from the outer ion position through the selectivity filter along the 4-fold channel axis; however, we were unable to model whether this arises from ions or solvent (Fig. S6e). The refined structure shows a partially hydrated calcium ion coordinated by four NaVAe1p serine oxygens and four water molecules, giving a coordination number common to protein–Ca2+ complexes [46] and serine oxygen–Ca2+ distances (2.9–3.5 Å) that are within those for calcium ions partially coordinated by protein ligands [46,47].

We denote the calcium ion position as the “outer ion” because it is separated from the previously reported selectivity filter inner ion position [17,35] by 10.7 Å (Fig. 3c). The outer ion site also does not correspond to the position of the unassigned selectivity filter density reported for NaVMs [20]. Observation of the outer ion binding site, together with the strong structural (Fig. 3c) and sequence similarity (Fig. 1c) of the region and previous identification of an inner ion site, establishes that BacNaV selectivity filters have more than one ion binding site. Sodium and calcium ions have similar radii (0.95 Å versus 0.99 Å, respectively) and coordination geometries [2]. Hence, the outer ion position appears to mark the site of entry and partial dehydration as the ion passes into the selectivity filter.

To facilitate comparison among NaV, CaV, and BacNaV selectivity filters, we denote the residue corresponding to the mammalian NaV “DEKA” motif [2] and the conserved glutamates in CaVs [2,33] and BacNaVs (Fig. 1c) as position “0”. Other residues are numbered positively or negatively relative to this residue (Fig. 4a). Hence, the NaVAe1p Ser198 (+1) side-chain oxygens coordinate the outer ion (Fig. 3e). Comparison of BacNaV and CaV selectivity filter sequences revealed that the (+1) position is strictly conserved as an acidic residue in CaV domain II selectivity filters (Fig. 4b). This (+1) position had not been previously implicated in CaV selectivity. As it plays a role in BacNaV calcium selectivity [19,48], the compelling similarities together with the observation of a bound calcium ion prompted us to examine the role of the (+1) position in a mammalian CaV.

**Outer ion site is important for mammalian CaV ion binding**

Calcium selectivity in CaVs is thought to arise from the interaction of permeant calcium ions with a ring of selectivity filter (0) position glutamates [33,49]. Following experiments that first demonstrated the importance of the (0) position glutamates [33], we used two-electrode voltage clamp to measure calcium block of lithium currents through human cardiac CaV1.2 channels expressed in Xenopus oocytes and examined how (+1) position mutations in domain II affect the high-affinity calcium site. In contrast to previous reports [33], we found that D707N channels exhibited a reduction in the apparent calcium affinity relative to wild type (IC50, 11.3 ± 2.0 μM and 1.9 ± 0.2 μM for D707N and wild type, respectively) (Fig. 4c and d). This change was equivalent to that caused by the charge neutralization E1115Q (IC50, 15.1 ± 1.9 μM), the (0) position glutamate neutralization having the largest reported impact on the high-affinity site [33,49]. Changing D707 to alanine to mimic the corresponding CaV domain IV position caused a reduction in apparent affinity similar to D707N (IC50, 13.6 ± 2.1 μM). Complete removal of the D707 side chain by D707G to mimic the equivalent position of CaV domains I and III caused an even greater

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**Fig. 3.** NaVAe1p pore region analysis. (a) NaVAe1p pore volume represented as a teal mesh calculated by CAVER [44]. In order to show the central cavity, dummy atoms closed the side vestibules. S6 residues forming the narrow constriction are shown as sticks and labeled. NaVAe1p elements are labeled as in Fig. 1. (b) Plot of channel radius versus distance along the central channel axis for closed NaVA [15] (black), inactivated NaVA [16] (light gray), NaVRh [17] (wheat), NaVMs (dark red) [20], NaVMs [20] (orange) open state model following [20], and NaVAe1p (blue). NaVAe1p constriction points caused by selectivity filter residue Ser198 and S6 residue Met241 are indicated. (c) Backbone superposition of PDs of NaVA [15] (black), NaVAe1p [16] (light gray), NaVAe1p [16] (medium gray), NaVRh [17] (wheat), NaVMs [20] (dark red), and NaVAe1p (colored as in a). Outer ion from NaVAe1p and inner ion from NaVRh are shown as white and wheat spheres, respectively. Two subunits are shown. (d) View from the intracellular side showing M241 side-chain Fo – Fc density. (e) Outer ion site side view. Ca2+ (white sphere) is surrounded by four water molecules (red spheres), and Ser198 (+1) is shown in sticks with 2Fo – Fc density surrounding the Ca2+ ion as a 1.0σ contoured blue mesh. (f) Side view of outer ion anomalous difference density (blue mesh) calculated at 6.5 Å and contoured at 6.5σ. Glu197 (0) and Ser198 (+1) are indicated. Ca2+ is shown as a white sphere. The front channel monomer is removed for clarity.
reduction in calcium binding (IC$_{50}$, 22.8 ± 6.7 μM). The magnitude of the effects of the D707 neutralizations are striking, as this position is much more exposed to bulk solvent than the (0) position glutamate, and strongly suggest that the effects of the D707 mutation are through direct interaction with Ca$^{2+}$ rather than an indirect consequence of electrostatic environment alteration. These data demonstrate the importance of a previously unrecognized calcium binding determinant of mammalian CaVs. These findings underscore the similarities between selectivity filters of homomeric BacNaVs and their more distant eukaryotic relatives, which have four non-identical selectivity filter repeats.

Fig. 4. Selectivity filter (+1) position is conserved in mammalian CaVs and important for ion binding. (a) Cartoon depiction of a single Na$_{AV}A_{1p}$ subunit selectivity filter colored as in Fig. 1. Selectivity filter residues are shown and indicated relative to the (0) position glutamate. Broken line shows the central pore axis approximate position. (b) Selectivity filter and pore helices sequence alignment for selected BacNaVs, mammalian CaV subtype exemplars, and mammalian Na$_{AV}A_{1.4}$. Ca$_{VBh1}$ and Ca$_{VSp1p}$ are calcium selective mutants of Na$_{VBh1}$ (NaChBac) [48] and Na$_{VSp1p}$ [19], respectively. Selectivity filter numbering is indicated. (c) Two-electrode voltage-clamp recordings from Xenopus oocyte expressing wild-type CaV$_{1.2}$ or the indicated mutants recorded in a buffer containing 100 mM Li$^+$ and either 3 nM or 10 μM free Ca$^{2+}$ and normalized to the 3-nM trace. Currents were elicited by a voltage step from −90 mV to −20 mV. (d) Dose–response curves for calcium block of lithium currents for CaV$_{1.2}$ (gray), E1115Q (black), D707N (blue), D707A (green), and D707G (red). Each data point at each calcium concentration is normalized to the current at 3 nM Ca$^{2+}$ and averaged for n = 5–7 oocytes. Error bars are standard error of the mean.
S6 activation gate residue and neck are important for BacNaV gating

Observation of a complete BacNaV cytoplasmic domain connected to a closed pore prompted us to test how the newly described channel elements (Fig. 5a) contribute to function. In line with the low success rate of BacNaV functional expression [12,17], our initial attempts to measure currents from full-length NaVAe1 using transfected mammalian cells or mRNA injected Xenopus oocytes failed. Therefore, we turned to NaVSp1, a previously characterized Silicibacter pomeroyi homolog [12] (Figs. 1c and 5b).

As prior structural studies have not achieved consensus regarding which S6 residues close the intracellular side of the pore (Fig. 3b), we first examined the NaVSp1 S6 positions equivalent to those that narrow the NaVAe1p intracellular side (Fig. 3a). Alanine substitution in each of the two helical turns above the constriction site, NaVAe1p F233 and I237 (NaVSp1 L212A and I216A) (Figs. 3a and 5a and Fig. S7a), did not affect the voltage dependence of activation (\(\Delta V_{1/2,act}\)) to more negative potentials (\(\Delta V_{1/2,inact} = -14.8 \pm 4.3\) and \(-14.0 \pm 3.8\) mV, respectively) (Fig. 5c and Table 1). However, these mutants did shift the voltage dependence of inactivation (\(V_{1/2,inact}\)) to more negative potentials (\(\Delta V_{1/2,inact} = -49.8 \pm 3.3\) mV and \(-40.0 \pm 3.5\) mV) (Fig. 5d and Table 1) but left the inactivation time constants and accelerated recovery from inactivation (Fig. S7b and c). By contrast, alanine substitution of the position equivalent to the pore occlusion point, NaVAe1p Met241 (NaVSp1 M220A) (Figs. 3a and 5a), caused dramatic negative shifts in both \(V_{1/2,act}\) and \(V_{1/2,inact}\) (\(\Delta V_{1/2,act} = -25.3 \pm 2.6\), \(-35.0 \pm 3.8\), and \(-40.7 \pm 3.9\) mV and \(\Delta V_{1/2,inact} = -4.5 \pm 3.2\), \(-18.1 \pm 3.6\), and \(-22.1 \pm 5.2\) mV for 2Gly, 3Gly, and 7Gly, respectively) (Fig. 5e and f and Table 1). The 2Ala mutant revealed that part of the shift caused by the 2Gly mutant arises from a synergistic effect of removing NaVSp1 E227 and D228 simultaneously (\(\Delta V_{1/2,act} = -15.7 \pm 3.4\) versus \(-25.3 \pm 2.6\) mV for 2Ala and 2Gly, respectively (Fig. 5e and Table 1)), which indicates an additional role for these charges.

Nevertheless, the 7Ala mutant had activation properties similar to wild type and that strongly contrasted the large negative shifts in \(V_{1/2,act}\) and \(V_{1/2,inact}\) caused by 7Gly (\(\Delta V_{1/2,act} = 4.7 \pm 4.4\) versus \(-40.7 \pm 3.9\) mV for 7Ala and 7Gly, respectively). The major negative shifts in \(V_{1/2,act}\) produced by neck substitutions that are detrimental to helix formation strongly support the idea that a structured neck is critical for closed state stabilization.

The majority of neck mutants causing negative shifts in \(V_{1/2,act}\) also elicited negative shifts in \(V_{1/2,inact}\) (Fig. 5e and Table 1). However, unlike previously reported mutations in the NaVSulP coiled coil that slowed \(\tau_{inact}\) by an order of magnitude or more [29], most of the neck mutants left \(\tau_{inact}\) unperturbed (Fig. S7f and g). Of the few that did not (E227G, D228G, 2G, and 7G; \(P < 0.001\)), none caused a perturbation larger than a factor of three. To examine this discrepancy further, we characterized NaVSp1 R242E (Fig. S8), a substitution at a conserved position at the N-terminal end of the coiled-coil region that was reported to cause a negative shift in \(V_{1/2,act}\) and slow NaVSulP inactivation by ~37-fold [29]. Although NaVSp1 R242E did cause a negative shift in \(V_{1/2,act}\) (Table 1), unlike its NaVSulP counterpart, this change caused only a modest (~2.7-fold, \(P = 0.04\) slowing of inactivation (Fig. S8d).

Finally, we tested the consequences of alanine and glycine substitutions at the bend, NaVAe1p His245 (NaVSp1, H224) (Fig. 5g and Fig. S9a). NaVSp1 H224A did not affect \(V_{1/2,act}\) or \(V_{1/2,inact}\) (\(\Delta V_{1/2,act} = 2.8 \pm 4.2\) and \(\Delta V_{1/2,inact} = -5.4 \pm 4.8\) mV) (Fig. 5g and Table 1) but did slow inactivation (~2-fold, \(P < 0.001\)) (Fig. S9b). In contrast, NaVSp1 H224G spared \(V_{1/2,act}\) and caused a large negative shift in \(V_{1/2,inact}\) (\(\Delta V_{1/2,inact} = -4.5 \pm 3.9\) mV and \(\Delta V_{1/2,inact} = -25.0 \pm 4.0\) mV) (Fig. 5g and Table 1). Although local disorder (Fig. S2b) precluded us from modeling the NaVAe1p His245 side chain, this residue could coordinate the neck ion. To test the structural consequence of loss of this potential ligand, we determined a 5.8 Å-resolution structure of NaVAe1p H245G crystallized from a
low-calcium condition (Table S1). The structure showed no major changes from wild type except for the loss of anomalous density for the neck ion and outer ion (Fig. S8c) (RMSD C\(^\alpha\) = 0.5 Å for the tetramer relative to wild type). Hence, the bend residue appears to be important for neck ion coordination but the neck ion is not essential for the bend structure or helical character of the neck. 

Our structure-based mutational studies uncovered two functional phenotypes. Mutations in the activation gate and neck having negative shifts in \(V_{1/2,\text{act}}\) ≥ −20 mV also caused negative shifts in \(V_{1/2,\text{inact}}\), suggesting that the two processes are strongly coupled. Mutations at S6 residues above the activation gate and bend residue H224 selectively impacted \(V_{1/2,\text{inact}}\) (Table 1). Together, these data support the ideas that (i) the Na\(_v\)AE1p structure represents a closed state, (ii) destabilization of the neck facilitates channel opening, and (iii) residues in S6 above the constriction site and at the bend are important for the molecular transitions underlying inactivation.
Neck destabilization allows Na\textsubscript{v}Ae1 functional characterization

Having established the importance of the neck helical structure for closed state stabilization, we revisited Na\textsubscript{v}Ae1 functional studies to test whether glycine substitution in the neck would permit us to record from full-length channels. Indeed, Na\textsubscript{v}Ae1 channels bearing the 3Gly mutation (residues 248–250) produced voltage-dependent channels (Fig. 6a and b). These had a \( V_{1/2,\text{act}} \) similar to Na\textsubscript{v}Sp1 but ~40 mV more positive than the equivalent Na\textsubscript{v}Sp1 3Gly mutant (\( V_{1/2,\text{act}} = 32.1 \pm 2.8, 27.4 \pm 1.1, \) and ~7.6 ± 2.1 mV for Na\textsubscript{v}Ae1 3Gly, Na\textsubscript{v}Sp1, and Na\textsubscript{v}Sp1 3Gly, respectively). These results further support the idea that the neck helical structure is important for closed state stabilization and suggest that wild-type Na\textsubscript{v}Ae1 has a very positive \( V_{1/2,\text{act}} \) of activation that had prevented functional characterization.

The ability to record from Na\textsubscript{v}Ae1 3Gly allowed us to test the functional properties of the Na\textsubscript{v}Ae1 selectivity.

### Table 1. Activation and inactivation properties of BacNa\textsubscript{v}s and mutants

<table>
<thead>
<tr>
<th></th>
<th>( V_{1/2,\text{act}} )</th>
<th>( \Delta V_{1/2,\text{act}} )</th>
<th>( n )</th>
<th>( P ) Value</th>
<th>( V_{1/2,\text{inact}} )</th>
<th>( \Delta V_{1/2,\text{inact}} )</th>
<th>( n )</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na\textsubscript{v}Sp1 S6</td>
<td>27.4 ± 1.1</td>
<td>—</td>
<td>13</td>
<td>n.a.</td>
<td>—</td>
<td>42.7 ± 1.1</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>L212A</td>
<td>26.4 ± 3.1</td>
<td>-1.0 ± 4.5</td>
<td>5</td>
<td>n.s.</td>
<td>-57.5 ± 2.7</td>
<td>-14.8 ± 4.3</td>
<td>6</td>
<td>***</td>
</tr>
<tr>
<td>H216A</td>
<td>27.0 ± 3.4</td>
<td>-0.4 ± 4.8</td>
<td>3</td>
<td>n.s.</td>
<td>-56.7 ± 1.6</td>
<td>-14.0 ± 3.8</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>M220A</td>
<td>-22.4 ± 1.6</td>
<td>-49.8 ± 3.3</td>
<td>7</td>
<td>***</td>
<td>-82.7 ± 1.8</td>
<td>-40.0 ± 3.5</td>
<td>7</td>
<td>***</td>
</tr>
<tr>
<td>Bend</td>
<td>30.2 ± 1.1</td>
<td>2.8 ± 4.2</td>
<td>3</td>
<td>n.s.</td>
<td>-48.1 ± 3.5</td>
<td>-5.4 ± 4.8</td>
<td>3</td>
<td>n.s.</td>
</tr>
<tr>
<td>Neck</td>
<td>22.9 ± 1.7</td>
<td>-4.5 ± 3.9</td>
<td>4</td>
<td>n.s.</td>
<td>-67.7 ± 2.3</td>
<td>-25.0 ± 4.0</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>A226G</td>
<td>11.1 ± 0.2</td>
<td>-16.3 ± 4.2</td>
<td>3</td>
<td>***</td>
<td>-51.4 ± 2.8</td>
<td>-8.7 ± 4.3</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>E227G</td>
<td>15.2 ± 1.5</td>
<td>-12.2 ± 3.3</td>
<td>6</td>
<td>***</td>
<td>-46.8 ± 1.1</td>
<td>-4.1 ± 3.6</td>
<td>4</td>
<td>n.s.</td>
</tr>
<tr>
<td>E227A</td>
<td>25.1 ± 2.3</td>
<td>-2.3 ± 4.1</td>
<td>4</td>
<td>n.s.</td>
<td>-47.1 ± 1.3</td>
<td>-4.4 ± 3.2</td>
<td>6</td>
<td>*</td>
</tr>
<tr>
<td>D228G</td>
<td>5.7 ± 1.9</td>
<td>-21.7 ± 3.9</td>
<td>4</td>
<td>***</td>
<td>-51.7 ± 1.2</td>
<td>-9.0 ± 3.6</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>D228A</td>
<td>28.4 ± 1.3</td>
<td>1.0 ± 4.3</td>
<td>3</td>
<td>n.s.</td>
<td>-45.7 ± 2.5</td>
<td>-3.0 ± 4.2</td>
<td>4</td>
<td>n.s.</td>
</tr>
<tr>
<td>2G</td>
<td>2.1 ± 2.4</td>
<td>-25.3 ± 2.6</td>
<td>6</td>
<td>***</td>
<td>-47.2 ± 2.6</td>
<td>-4.5 ± 3.2</td>
<td>4</td>
<td>n.s.</td>
</tr>
<tr>
<td>2A</td>
<td>11.7 ± 1.3</td>
<td>-15.7 ± 3.4</td>
<td>5</td>
<td>***</td>
<td>-52.8 ± 2.0</td>
<td>-10.1 ± 3.9</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>3G</td>
<td>-7.6 ± 2.1</td>
<td>-35.0 ± 3.8</td>
<td>5</td>
<td>***</td>
<td>-60.8 ± 1.9</td>
<td>-18.1 ± 3.6</td>
<td>10</td>
<td>***</td>
</tr>
<tr>
<td>7G</td>
<td>-13.3 ± 1.9</td>
<td>-40.7 ± 3.9</td>
<td>4</td>
<td>***</td>
<td>-64.8 ± 4.1</td>
<td>-22.1 ± 5.2</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>7A</td>
<td>32.1 ± 2.8</td>
<td>4.7 ± 4.4</td>
<td>4</td>
<td>n.s.</td>
<td>-67.3 ± 4.2</td>
<td>-24.6 ± 5.2</td>
<td>4</td>
<td>***</td>
</tr>
<tr>
<td>Coiled coil</td>
<td>7.8 ± 0.8</td>
<td>-19.6 ± 3.3</td>
<td>5</td>
<td>***</td>
<td>-44.4 ± 2.5</td>
<td>-1.7 ± 4.2</td>
<td>4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Na\textsubscript{v}Ae1 3G</td>
<td>32.1 ± 1.1</td>
<td>n.a.</td>
<td>6</td>
<td>5.3 ± 3.1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± standard error of the mean. \( \Delta V_{1/2} \) *\#* denotes 90% confidence interval. \( P \) Values are calculated relative to wild-type Na\textsubscript{v}Sp1. n.s., not significant; \( P > 0.05 \).

* *** indicates \( 0.001 < P < 0.05 \).

* **** indicates \( P < 0.001 \).

Fig. 5. Functional studies of Na\textsubscript{v}Sp1 structure-based mutants. (a) Na\textsubscript{v}Ae1p cartoon depicting positions investigated by patch-clamp. S6, bend, and neck positions are colored yellow, red, and light blue, respectively, and are indicated using the corresponding Na\textsubscript{v}Sp1 residues. (b) Left panel, exemplar Na\textsubscript{v}Sp1 Na\textsuperscript{+} currents in response to the activation protocol shown in the middle panel (300 ms depolarizations from \(-60 \) to \(+70 \) mV in \( 10 \) mV steps from a holding potential of \(-90 \) mV, sweep-to-sweep interval = \( 5 \) s). Right panel, protocol for examining steady-state inactivation voltage dependence (\( 5 \) s pre-pulse depolarization from \( 0 \) to \(-130 \) mV in \( 10 \) mV steps, followed by a \( 300 \) ms step to \(+30 \) mV, and repolarization to the holding potential, \(-90 \) mV; sweep-to-sweep interval = \( 10 \) s). (c) Left panel, exemplar Na\textsubscript{v}Sp1 L212A Na\textsuperscript{+} currents. Activation (middle panel) and inactivation (right panel) curves of Na\textsubscript{v}Sp1 (open circles). L212A (black triangle), and I216A (inverted black triangle). (d) Left panel, exemplar Na\textsubscript{v}Sp1 M220A Na\textsuperscript{+} currents. Activation (middle panel) and inactivation (right panel) curves of Na\textsubscript{v}Sp1 (open circles) and M220A (black squares). (e) \( V_{1/2,\text{act}} \) and \( V_{1/2,\text{inact}} \) for Na\textsubscript{v}Sp1 and indicated neck mutants. (f) Left panel, exemplar Na\textsubscript{v}Sp1 3Gly Na\textsuperscript{+} currents. Activation (middle panel) and inactivation (right panel) curves of Na\textsubscript{v}Sp1 (open circles), 2Gly (open diamonds), 3Gly (open squares), 7Gly (filled open squares), 3Ala (black diamonds), and 7Ala (black triangles). (g) Left panel, exemplar Na\textsubscript{v}Sp1 H224G Na\textsuperscript{+} currents. Activation (middle panel) and inactivation (right panel) curves of NavSp1 (open circles), H224G (black circles), and H224A (black squares). Activation curves are obtained by normalizing maximal amplitudes divided by the driving force. Inactivation curves are obtained by normalizing maximum amplitudes upon second pulse. NavSp1 activation and inactivation curve Boltzmann fits are shown in (c), (d), (f), and (g).
filter defined by our structure. In agreement with the strong selectivity filter conservation (Fig. 1c), bionic recording experiments (Fig. 6c and d) showed that Na\textsubscript{VAe1} has selectivity properties similar to other BacNaVs including Na\textsubscript{VSp1p} [19], the “pore-only” version of Na\textsubscript{VSp1} (\(P_{\text{Ca}}/P_{\text{Na}} = 0.07 \pm 0.02\) and 0.08 ± 0.01 for Na\textsubscript{VAe1} 3Gly and Na\textsubscript{VSp1p}, respectively), and Na\textsubscript{VAb1} (NaChBac) [47]. Due to the relatively low current amplitude of Na\textsubscript{VAe1} 3Gly, we were restricted to this extrapolation method of examining the permeability ratio. Hence, we validated the measurement by determining the ion selectivity of full-length Na\textsubscript{VSp1} 3Gly, which expresses much better than Na\textsubscript{VAe1} 3Gly, by two methods, the extrapolation method used to examine Na\textsubscript{VAe1} 3Gly and a tail current protocol (Fig. S10). Both methods gave the same \(P_{\text{Ca}}/P_{\text{Na}}\) ratio (0.05 ± 0.02 and 0.07 ± 0.02, respectively) and agree with the values for Na\textsubscript{VAe1} 3Gly and the “pore-only” Na\textsubscript{VSp1}. These results support the idea that the 3Gly mutation has minimal influence on selectivity and that Na\textsubscript{VAe1} is a sodium selective channel.

**Discussion**

Structure determination of the “pore-only” BacNa\textsubscript{V} Na\textsubscript{VAe1p} revealed previously uncharacterized BacNa\textsubscript{V} architectural features that are important for function. The presence of the long helical intracellular domain allowed the complete definition of S6, which extends into the intracellular side of the membrane (Fig. S5). Relative to all but the initial Na\textsubscript{VAb} structure [15], which contained pore-lining cysteine mutants near the activation gate that may have influenced S6 positioning, this structure places the intracellular gate more toward the S6 C-terminus (Fig. 3b). It seems likely that the absence of a consensus among the prior BacNa\textsubscript{V} structures in defining the site of this important channel element arises from the fact that they lack the intracellular domain. The structural plasticity of the intracellular gate seems a likely consequence of it being the central point of structural changes required for gating and inactivation. Our observations are not unlike those described for KcsA in which the full-length structure [40] showed that the activation gate closure point was more intracellular than defined from a structure lacking the intracellular domain [53]. The Na\textsubscript{VAe1p} activation gate and subsequent helical extension of S6 should protrude beyond the boundaries of the hydrophobic portion of the lipid bilayer into the lipid head groups (Fig. S5). This location may have important consequences as, based on voltage-gated potassium channel studies [26], it could allow the C-terminal portion of S6 to interact directly with the phospholipid head groups in a way that could influence function. It might also permit

**Fig. 6.** Na\textsubscript{VAe1} functional properties. (a) Left panel, exemplar Na\textsubscript{VAe1} 3Gly Na\textsuperscript{+} currents in response to an activation protocol, right panel (600 ms depolarizations from 0 to +70 mV in 10 mV steps from a holding potential of −90 mV, sweep-to-sweep interval = 5 s). (b) Left panel, protocol for examining steady-state inactivation voltage dependence (5 s pre-pulse depolarization from 50 to −30 mV in 10 mV steps, followed by a 300-ms step to +30 mV, and repolarization to the holding potential, −90 mV; sweep-to-sweep interval = 10 s). Right panel, Na\textsubscript{VAe1} 3Gly activation and inactivation curves. Boltzmann fits are indicated. (c) Left panel, exemplar Na\textsubscript{VAe1} 3Gly currents in the presence of 130 mM intracellular Na\textsuperscript{+} and 107.5 mM extracellular Ca\textsuperscript{2+}, in response to an activation protocol, right panel (600 ms depolarizations from 10 to +100 mV in variable steps, 10 mV and then 5 mV after 70 mV, from a holding potential of −90 mV; sweep-to-sweep interval = 5 s). (d) Normalized current–voltage curve from (c). Reversal potential can be obtained by linear regression, as indicated. The averaged value obtained by this method (\(n = 5\)) gives \(E_{\text{rev}} = -52.1 \pm 10.3\) mV, which corresponds to \(P_{\text{Ca}}P_{\text{Na}} = 0.07 \pm 0.02 (n = 5)\) when corrected for the liquid junction potential (−17 mV).
interactions with the proposed S4–S5 linker closed state pose [26].

Our studies demonstrate that destabilizing the helical structure of the neck causes negative shifts in \( V_{1/2,act} \). The largest perturbation, 7Gly in which two full helical turns of the neck are glycines, has effects that are of the same magnitude as activation gate disruption (Table 1). In both cases, there are parallel shifts in \( V_{1/2,inact} \) indicating that activation and inactivation are tightly coupled. Previous work with Na\(_v\)SuIP showed that coiled-coil disruption slowed inactivation kinetics by more than an order of magnitude, suggesting this structure as a role in accelerating inactivation [29]. Unlike these effects, which were caused by disruption further from the pore, destabilization of the Na\(_v\)Sp1 neck had minimal impact on inactivation time constants (Fig. S7f and g). Moreover, examination of a coiled-coil mutation, R242E, equivalent to one from Na\(_v\)SuIP that caused a dramatic slowing of inactivation, negatively shifted \( V_{1/2,act} \) but failed to produce a similar effect on Na\(_v\)Sp1 inactivation kinetics (Fig. S8d). Thus, the major role of the neck is to stabilize the channel closed state.

Our studies lead us to propose the following model for BacNa\(_v\) gating (Fig. 7). In the closed state, represented by the Na\(_v\)Ae1p structure (Fig. 1), the intracellular side of the channel central pore is occluded by the activation gate residue constriction (Na\(_v\)Ae1p M241). Opening would proceed with a radial expansion of this region [15] accompanied by an order \( \rightarrow \) disorder transition in the neck. The neck region is a site of potential disorder [28]. The abundance of polar and charged neck residues (Fig. 2g) may aid the transition to this state and assist in permeant ion escape into the cytoplasm (Fig. 7). Whether such a state resembles the proposed BacNa\(_v\) open state model [20] is unclear, as much of the end of S6, including the activation gate equivalent of Na\(_v\)Ae1p Met241, is absent from the Na\(_v\)Ms structure. Eukaryotic Na\(_v\)s and Ca\(_v\)s lack an equivalent of the C-terminal tail; however, the prevalence of similarly located C-terminal coiled-coil domains among diverse eukaryotic VGICs [41–43] and the importance of the intervening region that connects S6 to the coiled coils for channel regulation by a diverse factors [54,55] suggests that the essence of this proposed BacNa\(_v\) mechanism has parallels in eukaryotic VGICs.

The ionic radii and coordination geometries of sodium and calcium ions are similar [2]. Hence, the Na\(_v\)Ae1p outer ion position, revealed by calcium, appears to mark the site of entry and partial dehydration as the permeant ion interacts with the (+1) residues and passes into the selectivity filter. This role in ion coordination agrees with the observation that a single S \( \rightarrow \) D change at the NaChBac (Na\(_v\)Bh1) (+1) position alters selectivity for calcium over sodium by \( \sim 200 \)-fold [48] and with simulations suggesting that residues forming the outer ion site may be involved in ion recruitment [35]. The outer ion site may also participate in the divalent ion block described for NaChBac (Na\(_v\)Bh1) [11,17] and the NaChBac/Na\(_v\)Rh selectivity filter chimera [17] as it corresponds well with the predicted “site 1” blocking site from molecular dynamics simulations [35]. It is also striking that structural changes at the (+1) serine cause substantial alterations to the size of the selectivity filter entrance in the putative inactivated conformation of Na\(_v\)Ab [16] and in Na\(_v\)Rh, where the (+1) serine occludes the pore (Fig. S4). Thus, this outer ion site not only may be important for engagement of permeant and blocking ions but also may participate in rearrangements leading to slow inactivation [56].

Observation of an outer ion binding site in the selectivity filter together with the prior discovery of a selectivity filter inner ion site [17] (Fig. 3c) and

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**Fig. 7.** Cartoon model of BacNa\(_v\) gating. Activation of BacNa\(_v\)s is proposed to involve the expansion of the pore at the activation gate (Met241) and an order \( \rightarrow \) disorder transition in the neck region. Ions can escape into the cytoplasm through spaces created by the neck region. Channel elements are colored as in Fig. 1. VSDs are not shown.
strong sequence and structural conservation of this region (Figs. 1c and 3c) shows that BacNaV selectivity filters possess more than one ion binding site. It seems possible, especially given the ~10-Å separation between outer and inner ion sites, that multiple ions may occupy the selectivity filter simultaneously. This situation would be tantalizingly close to accepted ideas regarding multi-ion pores in mammalian NaV and CaV counterparts [2,31,32,34], not unlike that of potassium channels [57], in line with recent computational studies of BacNaV filters [58], and argues against the single ion pore model suggested in the initial BacNaV structure analysis [15].

Identification of the outer ion binding site uncovered a previously unknown role for the analogous conserved (+1) position in mammalian CaVs as an important determinant for calcium selectivity. Notably, despite its more exposed location, the impact of neutralization of the (+1) position is equivalent to that of neutralization of the (0) position glutamate that resides deeper in the selectivity filter (Fig. 4d), strongly suggesting that it may interact directly with the permeant ion. These results demonstrate a deep commonality between BacNaVs and eukaryotic voltage-gated channels that should facilitate understanding ion permeation and gating in the superfamily.

Materials and Methods

Crystallization

Na₉Ae1p was expressed and purified in β-dodecyl maltoside (DDM) as previously described [19]. For high-calcium-condition crystals, purified protein was concentrated to 15 mg ml⁻¹ by centrifugal filtration (Amicon® Ultra-15 100-kDa molecular mass cutoff; Millipore) and mixed with 5 M trimethylamine oxide (TMAO [59,60]) creating a solution of 13.5 mg ml⁻¹ protein, 0.25 M DDM, 0.5 M TMAO, 200 mM NaCl, and 20 mM Na–Hepes (pH 8.0). The protein was crystallized using hanging-drop vapor diffusion at 4 °C over a reservoir of 200 mM CaCl₂, 0.3 M DDM, 200 mM NaCl, 2 mM MgCl₂, and 15 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (pH 4.5). Crystals appeared in 2 days and grew to ~200 μm × 75 μm × 15 μm after 2 weeks.

Data collection, structure determination, and refinement

Crystals were frozen directly into liquid N₂ for data collection. Diffraction data were collected at Advanced Light Source Beamline 8.3.1, Lawrence Berkeley National Laboratory, integrated with MOSFLM 7.0.4 [61] and scaled with SCALA (3.3.20) [62]. Phase information was obtained by molecular replacement with an ensemble model based on the 3RVY, 4DXW, and 4EKW PDs using Phaser (2.1.4) [63]. Model was improved using cycles of manual rebuilding, Coot (0.7) [64], and refinement, Refmac (5.7.32) [65]. NCS-averaged maps improved apparent electron density and allowed placing of most of the side chains. For the high-calcium structure, initial tight NCS restraints were employed and later relaxed for all segments except for residues 183–208 and 214–220. For both the low-calcium structure and the Na₉Ae1p H245G mutant, tight NCS restraints were employed throughout.

Two-electrode voltage-clamp electrophysiology

Human Ca₉V₁.2 (α₁C77; GenBank CA843436), rat Caᵥβ₂α (GenBank NP 446303), and Caᵥα₂δ₁ (GenBank NM_00182276) were used for two-electrode voltage clamp experiments in *Xenopus* oocytes. Mutations were introduced using QuikChange (Stratagene, La Jolla, CA, USA). Linearized cDNA was translated into capped mRNA using the T7 mMessage mMachine kit (Ambion). We injected 50 nl of Caᵥ₁.2α₁, Caᵥβ₂α, and Caᵥα₂δ₁ mRNA at a 1:1:1 molar ratio into *Xenopus* oocytes. Two-electrode voltage-clamp experiments were performed 2–3 days post-injection. Oocytes were injected with 50 nl of 100 mM BAPTA 4’ before recording to minimize calcium-activated chloride currents. Recording solutions contained 100 mM LiOH, Ca(NO₃)₂ at the concentration indicated, and 10 mM Heps, adjusted to pH 7.4 using HNO₃. Ca²⁺ concentrations were verified using a Ca²⁺ electrode. The solution with a nominal free Ca²⁺ concentration of 3 mM contained 170 μM Ca(NO₃)₂ and 15 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetate acid (EGTA). Buffered solutions with nominal free Ca²⁺ concentrations of 100 mM, 500 mM, and 1 μM were also tested and gave results similar to the corresponding solutions in which Ca²⁺ was not buffered by EGTA. Electrodes were filled with 3 M KCl and had resistances of 0.3–1.0 MΩ. Recordings were conducted at room temperature from a holding potential of ~90 mV. Leak currents were subtracted using a P/4 protocol. Currents were analyzed with Clampfit 8.2 (Axon Instruments). All results are from at least two independent oocyte batches. Dose–response curves were calculated as follows: \( I/\text{IC}_{50} = 1/(1 + x/\text{IC}_{50}) \), where \( I_x \) is the current at the Ca²⁺ concentration \( x \) and \( \text{IC}_{50} \) is the half-maximal inhibitory concentration.
Patch-clamp electrophysiology

BacNaVs from S. pomeroyi (NavSp1) and A. ehrlichiae (NavAe1) were cloned into the pIRE2-EGFP vector (Clontech, Mountain View, CA, USA). All the NaVSp1 mutants were made using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene) and sequenced (University of California, San Francisco Cell Culture Facility). HEK 293 cells were transfected (in 35-mm-diameter wells) with LipofectAMINE™ 2000 (Invitrogen, Carlsbad, CA, USA) and plated onto coverslips coated with Matrigel (BD Biosciences, San Diego, CA, USA). We used 2 μg of DNA except for the L212A and I216A for which 4 μg of DNA was used to increase current amplitude.

Transfected cells were identified visually enhanced green fluorescent protein (EGFP) expression. Whole cell patch-clamp [66] was used to record Na+ current at room temperature (23 ± 2 °C) 48–72 h post-transfection. Acquisition and analysis of data were performed using pCLAMP 9 (Molecular Devices, Sunnyvale, CA, USA) and an Axopatch 200B amplifier (Molecular Devices). Pipettes were pulled from borosilicate glass capillaries (TW150F-3; World Precision Instruments, Sarasota, FL, USA) and polished (MF-900 microforge; Narishige, Tokyo, Japan) to obtain 2–3 MΩ resistances. Sixty to eighty percent of the voltage error due to the series resistance was compensated. Unless stated otherwise, pipette solution contained the following, in millimolars: 155, NaCl; 10, EGTA; 2, Mg-ATP; and 20, Hepes (pH 7.4 with NaOH). Bath solution contained the following, in millimolars: 120, Cs methane sulfonate; 8, NaCl; 10, EGTA; 2, Mg-ATP; and 20, Hepes (pH 7.4 with CsOH).

For reversal potential measurements determining the Na+ and Ca2+ relative permeabilities, pipette solution contained the following, in millimolars: 100, Na-Gluconate; 10, NaCl; 10, EGTA; and 20, Hepes (pH 7.4 adjusted with NaOH; total [Na] is 130). For tail protocol experiments on NaVSp1 3Gly, the pipette solution contained the following, in millimolars: 30, Na-Gluconate; 10, NaCl; 10, EGTA; 20, Hepes; and 70, NMDG-Cl (pH 7.4 adjusted with NaOH; total [Na] is 45). External solution in both was as follows, in millimolars: 5, NMDG-Cl; 100, CaCl2; and 20, Hepes (pH 7.4 adjusted with CaOH; total [Ca] is 107.5). The permeability ratio of Ca2+ over Na+ was estimated using the following equation:

$$P_{Ca}/P_{Na} = a_{Na\text{,int}} \times \exp(E_{rev\text{,F}/RT]) \times \exp(E_{rev\text{,F}/RT} + 1) / \sqrt{4a_{Ca\text{,ext}}},$$

where $R$, $T$, $F$, and $E_{rev}$ are the gas constant, absolute temperature, Faraday constant, and reversal potential, respectively (int, internal; ext, external) [2]. Ca2+ and Na+ activity coefficients were estimated as follows:

$$a_s = g_s |X_s|,$$

where activity, $a_s$, is the effective concentration of an ion in solution, $s$, related to the nominal concentration $|X_s|$ by the activity coefficient $\gamma_s$. $\gamma_s$ was calculated from the Davies equation. The calculated activity coefficients were $\gamma_{Na\text{,int}} = 0.76$ and $\gamma_{Ca\text{,ext}} = 0.33$. The liquid junction potentials were calculated by the JPCalc program (P. Barry) within Clampex (Molecular Devices) and taken into account to determine $E_{rev}$.

Accession codes

Crystallographic coordinates and structure factors are deposited with the PDB will be released immediately upon publication: 4LTO, NaV Ae1p, crystal I, high calcium; 4LTP, NaV Ae1p, crystal II, high calcium; 4LTQ, NaV Ae1p, low calcium; 4LTR, NaV Ae1p H245G, low calcium.

Acknowledgements

We thank Christine Rumpf and Ahmed Rohaim for expert molecular biology assistance and J. Holton and G. Meigs at Advanced Light Source Beamline 8.3.1 for data collection assistance. We thank K. Brejc, L. Jan, and E. Reuveny for helpful manuscript comments and Minor laboratory members for support throughout these studies. This work was supported by National Institutes of Health grants R01-HL080050, R01-D0007664, and U54-GM094625 and the American Heart Association grant 0740019N to D.L.M.; an American Heart Association Postdoctoral Fellowship to D.S.; and Fulbright Scholarship and a Monahan Foundation Fellowship to F.A.-A. G.L. was supported by a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Programme. D.L.M. is an American Heart Association Established Investigator.


Conflict of Interest: The authors declare that there are no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2013.10.010.
A Prokaryotic Sodium Channel Pore Structure

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Keywords:
voltage-gated sodium channel; X-ray crystallography; electrophysiology; ion binding; voltage-gated calcium channel

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† D.S. and F.F. contributed equally to this work.

Abbreviations used:
VGIC, voltage-gated ion channel; VSD, voltage-sensing domain; PD, pore domain; DDM, β–dodecyl maltoside; PEG, polyethylene glycol; EGTA, ethylene glycol-bis(2-aminoethylether)-N, N′,N′-tetraacetic acid.

References


[50] Bagriantsev SN, Clark KA, Minor DL. Metabolic and thermal stimuli control K(2P)2.1 (TREK-1) through modular sensory and gating domains. EMBO J 2012;31:3297–308.


Supplementary Material for:

Structure of a prokaryotic sodium channel pore reveals essential gating elements and an outer ion binding site common to eukaryotic channels

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Inventory of Supplementary Material:

Supplementary Figures S1-S10
Supplementary Tables S1-S3
Supplementary References
Supplementary Figure S1 A, Initial electron density map following molecular replacement using a model that only included the transmembrane domains. Map is contoured at 1.5σ. B, NaV1.2 crystal packing. The asymmetric unit, a channel tetramer, is shown in marine. Three tetramers are shown in grey to aid in lattice visualization. Remaining molecules are shown in orange. C and D, Representative 2Fo-Fc electron density contoured at 1σ for a portion of the electron density map including the P1 and P2 helices using data to C, 4.00Å and D, 3.46Å. E, NaV1.2 P42 crystal packing. The asymmetric unit, two channel tetramers, is shown in marine. Symmetry related molecules are shown in orange.
Supplementary Figure S2  

**A**  
Na\(\text{v}\)\text{Ae1p} NCS-averaged anomalous difference map calculated at 6.5Å and contoured at 2.5\(\sigma\). S6 and neck are colored blue and sand, respectively. Positions of Met241 and neck ion densities are indicated. Met241 sidechains are shown as sticks.

**B**  
Averaged Na\(\text{v}\)\text{Ae1p} main chain B-factors. Secondary structure elements are indicated: S5 (green), S6 (blue), P1 and P2 (tan), neck (brown), and coiled-coil (orange).

**C**  
Electrostatic surface potential mapped on the Na\(\text{v}\)\text{Ae1p} van der Waals surface, sliced open to show the central cavity and colored from -4 kT to +4 kT (red to blue). Grey lines indicate approximate lipid bilayer boundaries.
Supplementary Figure S3 Na\textsubscript{V}Ae1p and BacNa\textsubscript{V} pore domain comparisons

Residue by residue Cα RMSD plots comparing the Na\textsubscript{V}Ae1p pore with the pore domains from:

A, Na\textsubscript{V}Ab-I217C (3RVY) (Payandeh et al., 2011),
B, Na\textsubscript{V}Ab, chains A (blue) and B (orange) (Payandeh et al., 2012),
C, Na\textsubscript{V}Ab, chains C (blue) and D (orange) (Payandeh et al., 2012),
D, Na\textsubscript{V}Rh (Zhang et al., 2012), and
E, Na\textsubscript{V}Ms (McCusker et al., 2012).

Secondary structure elements are indicated as S5 (green), S6 (blue), P1 and P2 (tan).
Boundaries used in the superpositions are listed in Supplementary Table S3.
Supplementary Figure S4 Semitransparent surfaces showing the selectivity filter extracellular side for: A, Na\textsubscript{v}Ae1p, B, Na\textsubscript{v}Ab (Payandeh et al., 2011), C, Na\textsubscript{v}Ab ‘inactivated’ conformation of chains A/B (Payandeh et al., 2012), D, Na\textsubscript{v}Ab ‘inactivated’ conformation of chains C/D (Payandeh et al., 2012), E, Na\textsubscript{v}Rh (Zhang et al., 2012), and F, Na\textsubscript{v}Ms (McCusker et al., 2012). In all cases, select residues are labeled according to the scheme shown in Figure 4. Na\textsubscript{v}Ae1p is colored violet (selectivity filter) and olive (P2 helix) as in Figure 1. Na\textsubscript{v}Ab is grey, Na\textsubscript{v}Rh is wheat, and Na\textsubscript{v}Ms is dark red.
Supplementary Figure S5

Predicted membrane hydrophobic layer boundaries. Transmembrane layers were calculated for: A, $\text{Na}_\text{v}Ae1\text{p}$, B, $\text{Na}_\text{v}A b\text{ I217C (3RVY)}$ (Payandeh et al., 2011), C, $\text{Na}_\text{v}R h$ (4DXW) (Zhang et al., 2012), and D, $\text{Na}_\text{v}M s$ (4F4L) (McCusker et al., 2012), using the OPM server (http://opm.phar.umich.edu) (Lomize et al., 2006) and are represented by a red disk (boundary between hydrophobic layer and extracellular lipid head groups) and a blue disk (boundary between hydrophobic layer and intracellular lipid head groups).
Supplementary Figure S6 Na_{v}Ae1p difference maps A, High calcium Na_{v}Ae1p anomalous difference map calculated at 6.5Å and contoured at 6.5σ showing a strong peak at the pore center close to the side chains of Na_{v}Ae1p Ser198. B, Temperature factors for outer ion and associated ligands. C, NCS-averaged anomalous difference maps calculated for the indicated datasets at 6.5Å. Contours show 5σ (grey) and 10σ (pink). The anomalous signal at the site for the pore metal is present in both high Ca^{2+}-structures but is absent in both low Ca^{2+}-structures. Anomalous signal at the neck ion site is present in all but the H245G structure. Select residues are indicated for orientation purposes. D, Closeup of the anomalous signal for the outer ion site in the Na_{v}Ae1p, Crystal I, High calcium dataset. Contour levels are indicated. E, Fo-Fc map (green) of Na_{v}Ae1p selectivity filter region calculated using a protein model but lacking the outer ion and waters contoured at 3.0σ. Map shows electron density for the Ca^{2+} ion and extends through the entire selectivity filter length. Ca^{2+} is indicated as a white sphere, four water molecules are shown as red spheres. Na_{v}Ae1p is colored as in Figure 1.
Supplementary Figure S7  Functional studies of Na\textsubscript{V}Sp1, S6 mutants, and neck mutants

A, Representative Na\textsuperscript{+} currents from Na\textsubscript{V}Sp1, L212A, I216A, and M220A in response to an activation protocol from -60 to +70 mV in 10 mV steps (cf. Figure 5B middle panel). B, Inactivation time constants for Na\textsubscript{V}Sp1, L212A, I216A, and M220A (n =3-8; error bars are ± s.e.m.). C, Recovery from inactivation for Na\textsubscript{V}Sp1, L212A, I216A, and M220A. Time interval between the pre-pulse (+30 mV, 300 ms) and the test-pulse (+30 mV, 300 ms) was varied from 0.25 to 3 s. The ratios between currents elicited by the two pulses were used to construct the recovery from inactivation curve. (n =3 to 7; ± s.e.m.). Recovery curve of Na\textsubscript{V}Sp1 is highlighted with a single exponential fit. D, Representative Na\textsuperscript{+} currents from the indicated Na\textsubscript{V}Sp1 neck mutants in response to an activation protocol of -60 to +70 mV in 10 mV steps (cf. Figure 5B middle panel). E, Voltage dependences of activation (top) and inactivation (bottom) for Na\textsubscript{V}Sp1 and indicated mutants. F and G, Inactivation time constants for Na\textsubscript{V}Sp1 and indicated mutants (n = 4-8; error bars are ± s.e.m.).
Supplementary Figure S8

A, Representative Na⁺ currents from Na₉Sp1 R242E in response to an activation protocol of -60 to +70 mV in 10 mV steps. B, Voltage dependence of activation and C, Voltage dependence of inactivation for Na₉Sp1 and R242E. D, Inactivation time constants for NavSp1 and R242E (n =3-8; error bars are ±S.E.M).
Supplementary Figure S9 Functional and structural characterization of bend position mutants

A, Representative Na\(^+\) currents from Na\(_v\)Sp1, H224G, and H224A in response to an activation protocol of -60 to +70 mV in 10 mV steps. B, Comparison of inactivation time constants of Na\(_v\)Sp1 and indicated mutants (n = 3-8; error bars are ± s.e.m.).

C, Na\(_v\)Ae1p (left) and Na\(_v\)Ae1p H245G (right) NCS-averaged anomalous difference maps calculated at 6.5 Å contoured at 7σ (high calcium) or at 7.5 Å contoured at 4.5σ (H245G). The H245G structure shows no anomalous peak for either the neck ion or the pore Ca\(^{2+}\) ion site.
Supplementary Figure S10 Ion selectivity measurements for Na\textsubscript{v}Sp1 3Gly

**A** Exemplar Na\textsubscript{v}Sp1 3Gly current trace in presence of 130 mM intracellular Na\textsuperscript{+} and 107.5 mM extracellular Ca\textsuperscript{2+}, in response to an activation protocol, (400 ms depolarizations to -30 to +90 mV in variable steps (10 mV and then 5 mV after 60 mV) from a holding potential of -90 mV, sweep to sweep interval = 5 s).

**B** Normalized current-voltage curve from experiments performed as in ‘A’. Reversal potential can be obtained by linear regression, as indicated. The averaged value obtained by this method (n=5) yields $E_{\text{rev}} = -77.6 \pm 8.1$ mV, which gives $P_{\text{Ca}}/P_{\text{Na}} = 0.05 \pm 0.02$ (n=5) when corrected for the liquid junction potential (-17mV).

**C** Exemplar Na\textsubscript{v}Sp1 3Gly tail currents in presence of 40 mM intracellular Na\textsuperscript{+} and 107.5 mM extracellular Ca\textsuperscript{2+}, evoked by a step protocol (10 ms from -50 to 0 mV in 10 mV steps, after a 4 ms depolarizing step to 60 mV from a holding potential of -90 mV, sweep to sweep interval = 5 s).

**D** Tail current-voltage curve from ‘C’. Linear regression of data points from different experiments (n =4) shows a reversal potential of -33.0 ± 0.9 mV, when corrected for liquid junction potential (-18mV), which gives a permeability ratio $P_{\text{Ca}}/P_{\text{Na}}$ of 0.07 ± 0.02. All values are mean ± s.e..
Supplementary Table S1 Crystallographic data collection and refinement statistics

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* 200 mM calcium  
** 0 mM calcium  
§ Na⁺AE1p Crystal I using data resolution defined by CC₁/₂ > 0.1 (Karplus and Diederichs, 2012).  
† Na⁺AE1p, Crystal I using data resolution defined using a cutoff of Mn (I/σI) = 1.9
## Supplementary Table S2 Coiled coil parameters

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### Superhelical parameters

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### α-helical parameters

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<td>2.37</td>
</tr>
<tr>
<td>Residues/turn</td>
<td>3.57</td>
<td>3.59</td>
<td>3.61</td>
<td>3.68</td>
<td>3.67</td>
<td>3.69</td>
<td>3.76</td>
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<tr>
<td>Rise/residue (Å)</td>
<td>1.57</td>
<td>1.55</td>
<td>1.52</td>
<td>1.51</td>
<td>1.51</td>
<td>1.49</td>
<td>1.49</td>
</tr>
</tbody>
</table>

Comparison of coiled-coil parameters as determined using Twister (Strelkov and Burkhard, 2002)
### Supplementary Table S3 Comparisons of NaV pore structures

<table>
<thead>
<tr>
<th></th>
<th>PDB code</th>
<th>Monomer RMSD (Å)</th>
<th>Tetramer RMSD (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NaVAb I217C</strong></td>
<td>3RVY</td>
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<td>1.2</td>
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<tr>
<td><strong>NaVAb WT chains A/B</strong></td>
<td>4EKW</td>
<td>1.2/1.2</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>NaVAb WT chains C/D</strong></td>
<td>4EKW</td>
<td>1.2/1.2</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>NaVRh G208S</strong></td>
<td>4DXW</td>
<td>1.5</td>
<td>2.1</td>
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<tr>
<td><strong>NaVMs</strong></td>
<td>4F4L</td>
<td>1.0/1.0/1.1/1.1</td>
<td>1.3</td>
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<tr>
<td><strong>NaVSulP cc</strong></td>
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<tr>
<td><strong>KcsA four helix bundle</strong></td>
<td>0.6</td>
<td>4.6</td>
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</tr>
</tbody>
</table>

In cases where monomer/monomer superpositions deviated by more than 10%, values are shown for each independent chain.

For the NaV superpositions residues NaV Ae1p residues 150-239 were compared with the equivalent positions from the indicated BacNaV channels.

For NaVSulP cc comparison NaV SulP residues 115-139 are compared with NaV Ae1p 260-284.

For KcsA four helix bundle comparison, KcsA residues 137-151 were compared with compared with NaV Ae1p 266-280.
Supplementary References


