Voltage-gated sodium channel (NaV) protein dissection creates a set of functional pore-only proteins

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Many voltage-gated ion channel (VGIC) superfamily members contain six-transmembrane segments in which the first four form a voltage-sensing domain (VSD) and the last two form the pore domain (PD). Studies of potassium channels from the VGIC superfamily together with identification of voltage-sensor only proteins have suggested that the VSD and the PD can fold independently. Whether such transmembrane modularity is common to other VGIC superfamily members has remained untested. Here we show, using protein dissection, that the Silicibacter pomeroyi voltage-gated sodium channel (NaVSp1p) PD forms a stand-alone, ion selective pore (NaVSp1p) that is tetrameric, α-helical, and that forms functional, sodium-selective channels when reconstituted into lipid bilayers. Mutation of the NaVSp1p selectivity filter from LE5WSW to LDDWSD, a change similar to that previously shown to alter ion selectivity of the bacterial sodium channel NaVBh1 (NaChBac), creates a calcium-selective pore-only channel, CaVSp1p. We further show that production of PDs can be generalized by making pore-only proteins from two other extremophile NaVs: one from the hydrocarbon degrader Alcanivorax borkumensis (NaVAe1p), and one from the arsenite oxidizer Alkalimimica ehrlichii (NaVAe1p). Together, our data establish a family of active pore-only ion channels that should be excellent model systems for study of the factors that govern both sodium and calcium selectivity and permeability. Further, our findings suggest that similar dissection approaches may be applicable to a wide range of VGICs and, thus, serve as a means to simplify and accelerate biophysical, structural, and drug development efforts.

Voltage-gated sodium channels (NaVs) are large polytopic membrane proteins involved in action potential generation in excitable cells and belong to an ion channel superfamily that includes voltage-gated calcium channels (CaVs), voltage-gated potassium channels (KVs), and transient receptor potential (TRP) channels (1, 2). Within the voltage-gated ion channel (VGIC) superfamily, NaVs and CaVs are close relatives (1–3) that share a topology of 24 transmembrane segments organized in four homologous six-transmembrane repeats. These two families are also thought to share some common structure in the ion selectivity filter despite having markedly different ion permeation properties (4). Both are central to human neuromuscular, cardiovascular, and neural physiology. Consequently, they are targets for a host of pharmaceuticals used to treat a diverse set of disorders and remain active targets for drug development (5–7). Recently, single subunit, six-transmembrane segment NaVs have been identified in a large number of bacteria from diverse environments (8, 9). These channels show clear similarities to eukaryotic NaVs and CaVs (2, 9, 10), suggesting that the prokaryotic channels may have been ancestors of the more complex vertebrate channels.

Despite the central importance of NaVs, nothing is known about the high-resolution structure of the NaV transmembrane portions. Present knowledge is limited to low-resolution electron microscopy studies (19 Å) that have suggested some general features of NaVs purified from eel electric organs (11) but that lack the resolution for detailed mechanistic insight. Study of bacterial NaVs provides a simplified system for understanding basic aspects of both NaV and CaV function and holds promise as a template for guiding small molecule modulator development (6). Although there have been ongoing efforts to produce bacterial NaVs samples that can be used for biochemical and structural studies (12–14), these have yet to achieve the multi-milli-gram amounts required for extensive structural studies.

Here, we show that a protein dissection approach produces folded, electrophysiologically active pore domains (PDs) from a set of bacterial NaVs. These PDs are well-behaved, biophysically tractable, stand-alone pores that can be produced in multi-milli-gram amounts. Application of a battery of biochemical and biophysical characterizations demonstrates that the PD-only proteins self-assemble as tetramers having α-helical structure and form functional channels when incorporated into lipid bilayers. We further show that introduction of aspartic acid residues at key selectivity filter positions in the Silicibacter pomeroyi PD-only channel NaVSp1p creates a channel, CaVSp1p, in which ion selectivity is changed from sodium to calcium. Taken together, our data demonstrate that the NaV pore-only proteins are active and biophysically accessible ion channels. Because of their favorable biochemical properties, these NaV PDs should provide excellent model systems for the structural study of the factors that govern sodium selectivity and permeability. Further, the dissection strategy eliminates the potential complications that arise from voltage-sensing domain (VSD) conformational heterogeneity and should be a generally applicable means to simplify studies of the PDs of other VGIC superfamily members.

**Results**

Creation of a Pore-Only NaV. We previously identified an NaV from the marine sulfur-reducing bacterium *S. pomeroyi* (NaVSp1), which is functional when expressed in mammalian cells (8), as a protein that could be highly expressed (approximately 20 mg L−1) in *Escherichia coli* membranes (15). Despite the high expression level, size-exclusion chromatography analysis of purified, detergent-solubilized NaVSp1 revealed a broad elution profile indicative of a polydisperse sample that was not well suited to further


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Characterization (Fig. 1A). Thus, we sought alternative strategies to produce biocatalytically tractable NaVSp samples. Three lines of evidence have suggested that VGIC members are composed of two domains that can fold independently, the VSD and PD (16–18): comparison of potassium channel gene families (19), structural and chimera studies of potassium channels (16, 20–25), and identification of both pore-only (26) and voltage-sensor-only proteins (27–29). In further support of this idea, the independence of potassium channel PDs have been demonstrated by the functional reconstitution of a PD-only potassium channel from Listeria monocytogenes following genetic deletion of the VSD (30). We were inspired by these potassium channel examples and reasoned that the poor size-exclusion chromatography behavior of NaVSp was most likely caused by VSD conformational heterogeneity. Therefore, we decided to execute a protein dissection strategy that would remove the likely problematic region, the VSD, and create a pore-only sodium channel, NaVSp1p, by cutting the protein in the S4–S5 linker region that connects the VSD to the PD (Fig. 1B and Fig. S1A). In line with our expectations, the pore-only construct NaVSp1p was expressed in the membrane fraction of E. coli to very high levels as a hexahistidine tag, maltose binding protein fusion (>25 mg L⁻¹), and yielded material that displayed greatly improved size-exclusion behavior relative to full-length NaVSp1 following removal of the affinity tags and purification (Fig. 1A and C). These properties made NaVSp1p very suitable for further biochemical and biophysical characterization.

**Biophysical Characterization Demonstrates That NaVSp1p Is Folded and Forms Tetramers.** Structurally characterized VGIC members have high α-helical content (16, 24, 31, 32). To examine NaVSp1p secondary structure, we used circular dichroism (CD) and measured spectra in two different detergents, n-dodecyl-β-D-maltopyranoside (DDM) and n-decyl-β-D-maltopyranoside (DM). In both, NaVSp1p showed the characteristic hallmark double minima of helical proteins (Fig. 2A) (33). These spectral signatures are similar to those reported for purified, detergent-solubilized full-length NaVb1 (NaChBac) (12, 13), electric eel NaV (34), and the pore-only potassium channel KcsA (35, 36) and indicate that NaVSp1p has the high α-helical content characteristic of the VGIC superfamily. Thermal denaturation experiments monitored by CD indicate that NaVSp1p undergoes a cooperative loss of secondary structure (Fig. 2B) that is characteristic of a folded protein and that resembles that seen in the full-length electric eel NaV (34). Moreover, NaVSp1p has different degrees of stability in DDM and DM (apparent melting temperatures, Tm, of 44 °C and 52 °C, respectively) and the thermal transition in DDM can be made largely reversible (90% recovery of the signal at 222 nm) by the inclusion of 8% glycerol (Fig. S1B). Together, these spectral and thermal denaturation properties suggest that NaVSp1p is a well-folded, α-helical protein that has similar secondary structure to other members of the VGIC superfamily.

Prior studies of purified full-length NaVb1 indicate that bacterial NaVs are tetramers (14). We expected that this property should be maintained in NaVSp1p and used two independent methods to probe NaVSp1p oligomerization (Table 1): glutaraldehyde (GTA) chemical cross-linking (37, 38) and size-exclusion chromatography coupled with triple detector analysis (SEC-TDA) (39). SDS-PAGE analysis of GTA cross-linked NaVSp1p solubilized in DDM revealed the presence of three cross-linked products that corresponded to dimers, trimers, and tetramers, but did not indicate higher-order species (Fig. 2C and Table 1). Altering the conditions to favor more extensively cross-linked species showed a reduction of lower-order cross-linked species and enhancement of the tetrameric species, thereby indicating that NaVSp1p assembles into a complex containing four subunits. This behavior was recapitulated in DM (Fig. S1C).

We further examined the association state of NaVSp1p by SEC-TDA. This methodology, in which changes in refractive index, ultraviolet absorption, and static angle light scattering are...
measured, allows determination of the absolute molecular weight of the protein component of the protein-detergent micelle (39, 40) and has been recently applied to determine the association states of a variety of membrane proteins in different detergents (41–43). SEC-TDA of NaVSp1p (Fig. 2D) indicates that the protein complex is a monodisperse entity that contains four monomers (Table 1). The excellent agreement between these experiments and the GTA experiments provides strong evidence that NaVSp1p is a tetramer.

**Functional Characterization of Reconstituted NaVSp1p.** Although the biochemical evidence provided strong indications that NaVSp1p is a folded, tetrameric protein, it was essential to determine whether NaVSp1p retained any functional ion channel properties. Therefore, we reconstituted purified NaVSp1p into 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) giant unilamellar vesicles (GUVs) created by electroformation (44, 45) and assayed the properties of the reconstituted channels using planar patch clamp technology (46, 47). Measurement of the reversal potential of bilayers containing hundreds of channels under asymmetric NaCl conditions yielded \( E_{\text{rev}} = 53.69 \pm 8.05 \text{ mV} \) (\( n = 6 \)) (Fig. S2) in good agreement with the expected Nernst potential for sodium, 58.5 mV, and indicated that NaVSp1p (Fig. S3). As expected from the absence of the VSD, NaVSp1p activity lacked a strong voltage dependence (Fig. 3B and Fig. S3). Importantly, application of mibebradil, a CaV blocker that blocks bacterial NaVs (9, 13), inhibited the activity of the purified, reconstituted NaVSp1p (Fig. 3C and D) and provided evidence that the ion channel activity was the result of a reconstituted sodium channel.

To characterize the biophysical properties of the NaVSp1p channel further, we measured its selectivity properties. Comparison of the selectivity for sodium, potassium, and calcium ions by measuring the reversal potentials in a set of asymmetric ion conditions indicated that NaVSp1p has a preference for permeant ions of \( \text{Na}^+:\text{K}^+:\text{Ca}^{2+} = 1:0.22:0.08 \) (Fig. 3B and E and Table 2). The measured preference for sodium over calcium \( (P_{\text{Ca}}/P_{\text{Na}} = 0.08) \) is in good agreement with the reported value for the related bacterial channel NaVbH1 \( (P_{\text{Ca}}/P_{\text{Na}} = 0.15) \) (10), which bears strong similarity to NaVSp1p in the selectivity filter (Fig. S4A and Fig. S4B). These selectivity properties, together with the sensitivity to mibebradil, indicate that the core functions of the PD, ion selectivity and conduction, remain intact and provide strong evidence that the folded, tetrameric NaVSp1p forms a functional, stand-alone ion selective channel.

**Selectivity Filter Mutations Convert NaVSp1p into a Calcium-Selective Channel, CaVSp1p.** It has long been thought that the selectivity filters of NaVs and CaVs are related (4). In accord with this view, bacterial NaVs bear sequence similarity to both NaVs and CaVs (9). Further, prior studies of bacterial NaVs selectivity filter elements have shown that a triple aspartate mutation in the NaVSp selectivity filter positions to create a mutant channel “CaVSp1p” (Fig. 4A). Characterization of purified CaVSp1p by CD, GTA cross-linking, and SEC-TDA (Fig. 4B–D and Table 1) showed that CaVSp1p had physical properties that were identical to NaVSp1p. Importantly, incorporation of CaVSp1p into DPhPC GUVs and subsequent electrophysiological recording by planar patch clamp revealed that CaVSp1p forms functional channels (Fig. 4E and F). In stark contrast to NaVSp1p, CaVSp1p channels were calcium-selective (Fig. 4G and Table 2) and displayed an approximately 30-fold increase in single channel conductance in calcium compared to NaVSp1p (335.3 ± 6.2 pS versus 31.1 ± 1.4 pS) for CaVSp1p and NaVSp1p, respectively, Table 2) and approximately 39-fold change in \( P_{\text{Ca}}/P_{\text{Na}} \) (3.13 ± 0.16 versus 0.08 ± 0.01 for CaVSp1p and NaVSp1p, respectively, Table 2).

**Table 1. Oligomeric states of pore-only proteins**

<table>
<thead>
<tr>
<th>GTA cross-linking</th>
<th>SEC-TDA</th>
<th>Monomer mass, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subunits</td>
<td>Number of subunits</td>
<td>Mass, kDa</td>
</tr>
<tr>
<td>NaVSp1p</td>
<td>4 (DDM)</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>N2Ab1p</td>
<td>4 (DDM)</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>NaVAb1p</td>
<td>4 (DM)</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>CaVSp1p</td>
<td>4 (DM)</td>
<td>4.3 ± 0.1</td>
</tr>
</tbody>
</table>

**Fig. 3.** Electrophysiological characterization of reconstituted NaVSp1p. (A) Representative traces recorded from a planar lipid bilayer containing single NaVSp1p channels at the indicated holding potentials using 110 mM KCl, 10 mM Hepes, pH 7.0 and 200 mM NaCl, 10 mM Hepes, pH 7.0 internal and external solutions, respectively. Closed channel current level is indicated. (B) Single channel I–V relationships for NaVSp1p channels in 110 mM KCl internal, [K]o, and NaCl 200 mM external, [Na]o solutions. (C and D) Bilayer recordings of NaVSp1p at +60 mV in symmetrical 500 mM NaCl before (C) and after (D) addition of 100 µM mibebradil. (E) Single channel I–V relationships for NaVSp1p channels in 200 mM NaCl internal, [Na]o, and 160 mM CaCl2 external, [Ca]o solutions. I–V curves in B and E are derived from single channel recordings of multiple bilayers.
fig. 4. Characterization of a calcium-selective NaVSp1 mutant. (A) Pore region sequence comparison. Residues changed to alter CaVSp1 and CaVSp1p ion selectivity are highlighted. (B) Comparison of CaVSp1p (open circles) and NaVSp1p (blue circles) CD spectra at 4 °C in 150 mM NaCl, 2.7 mM DM, 10 mM naphosphate, pH 7.4. (Inset) Molecular weight distribution across the main peak. (E) Representative trace at -80 mV holding potential from a planar lipid bilayer containing CaVSp1p using 200 mM NaCl, 10 mM Hepes, pH 7.0; and 110 mM KCl, 10 mM Hepes, pH 7.0 external and internal solutions, respectively. Closed channel current level is indicated. (F) Single channel I–V relationships for CaVSp1p in the solutions from E indicated as [K+]i, internal and [Na+]o, external. Solid line was obtained by a nonlinear fit. (G) Single channel current voltage relationships for CaVSp1p channel measured using 200 mM NaCl internal, [Na+]i, and 160 mM CaCl2 external, [Ca2+]o, solutions. Solid line was obtained by a nonlinear fit. These data are consistent with the previous studies of CaVSp1 (10) and show definitively that the measured single channel currents must arise from the reconstituted channels.

Generation of Pore-Only Proteins from Other Extremophile NaVs. To test whether the ability to make folded PDs could be generalized to other NaVs, we investigated the application of the dissection approach to three related extremophile bacterial NaVs. To examine a diverse set, we picked NaVs that have sequence identities of approximately 50% or less with NaVSp1p. These included the well-characterized Bacillus halodurans NaVSp1p (9) (40% identical to NaVSp1p), an NaVAb from the hydrocarbon degrading bacterium Alcanivorax borkumensis (51% identical to NaVSp1p), and the NaAe from the arsenite oxidizing bacterium Alkalilimnicola ehrlichei (48% identical to NaVSp1p) (Fig. 4 A and B). Attempts to purify NaVSp1p were unsuccessful. However, we were able to express, purify, and characterize pore-only proteins from NaVAb1 and NaVAe1lp. Both showed CD spectra that were very similar to that of NaVSp1p (Fig. 4 A and B). Additionally, GFA cross-linking (Fig. 6 C and D) and SEC-TDA analysis (Fig. 6 E and F) indicated that NaVAb1p and NaVAe1lp form tetramers. Further, reconstitution of NaVAb1p into GUVs and measurement by planar patch clamp revealed the activity of single channels lacking a strong voltage dependence (Fig. 6 G and H and Fig. S3) and having a single channel conductance very similar to that of NaVSp1p, 37.3 ± 1.5 pS. Interestingly, NaVAb1p has different behavior than NaVSp1p and displays a lower open probability (e.g., Po at -80 mV of 0.5 versus 0.25 for NaVSp1p and NaVAb1p, respectively, Fig. S3) but substantially longer channel openings. These differences must arise from divergent elements in the PDs that affect the intrinsic properties of the PD. Similar functional studies with NaVAb1p did not yield clear single channel activity and were not analyzed further. Taken together, the similarity of the biophysical properties of NaVAb1p and NaVAe1lp to NaVSp1p demonstrate that NaVAb1p and NaVAe1lp are well-folded, stand-alone pore domains. These results show that the protein dissection approach to create PD-only proteins can be generalized and should be applicable to a range of VGICs.

Discussion

Developing a detailed understanding of the structural principles that underlie VGIC superfamily transmembrane architecture is an important goal that has a direct bearing on elaboration of channel gating and ion permeation mechanisms, design of ion channels having unique function, drug development, and understanding channel evolution. Within the VGIC superfamily, a number of lines of evidence have suggested that the transmembrane segments of KV channels are arranged in distinct domains comprising the VSD and PD. This possibility was first noted from comparison of KV channels, which have six-transmembrane segments that include a VSD, and inward rectifiers, which contain only a two-transmembrane pore-forming unit, as both contain common pore elements (19). A variety of structural and chimera studies of different potassium channels have further supported this intramembrane domain segregation idea (16, 20–25). Most notably, independent X-ray and NMR structures of the VSD from the Aeropyrum pernix voltage-gated potassium channel KvAP show that the VSD can fold separately from the pore domain (23, 25); however, this folding appears to be subject to strong context-dependent effects (23, 48). Additional evidence for potassium channel transmembrane modularity comes from the recent purification and reconstitution of a PD-only potassium channel from Listeria monocytogenes following genetic removal of the VSD (30). Besides these potassium channel studies, the idea

**Table 2. Ion conduction and selectivity properties for NaVSp1p and LDDWSD mutant CaVSp1p**

<table>
<thead>
<tr>
<th>Pore sequence</th>
<th>Ion Conductance, pS</th>
<th>GNa/GK</th>
<th>PNa/PK</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaVSp1p</td>
<td>FIOMTL6SWMGIV</td>
<td>Na+</td>
<td>31.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K+</td>
<td>18.4 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca2+</td>
<td>10.7 ± 1.7</td>
</tr>
<tr>
<td>CaVSp1p</td>
<td>FIOMLDDWSDGIV</td>
<td>Na+</td>
<td>74.4 ± 4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K+</td>
<td>30.3 ± 2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca2+</td>
<td>335.3 ± 6.2</td>
</tr>
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</table>

n = 7 except for CaVSp1p PNa / PPK where n = 5. Values are mean ± SD. Ratios were measured using 200 mM NaCl, 110 mM KCl, and 160 mM CaCl2.

Fig. 5. Sequence relationships of bacterial NaVs. (A) Phylogenetic tree of the pore regions of from A. borkumensis (NaVAb1p), A. ehrlichei (NaVAe1lp), S. pomeroyi (NaVSp1p), and B. halodurans (NaVSp1). (B) Sequence alignment of NaVAb1p, NaVAe1lp, NaVSp1p, and NaVSp1p. Absolutely conserved residues are shown in orange. Blue shading and lines indicate the predicted transmembrane segments S5 and S6 and the pore region, P.
Larity is present in members of the NaV family. We show that branches of the VGIC superfamily have been reported. Detox, either DDM or DM. Importantly, both NaVSp

Sensitive to block by a known bacterial NaV pore blocker, mibefradil NaVAb

S. pomeroyi

and characterization (approximately 25 different extremophile NaVs by genetic excision of the VSD: it is possible to generate pore-domain-only proteins from three (9, 13) (Fig. 3

A planar lipid bilayer containing NaVAb

p at different holding potentials (Table 2) and is sensitive to dehydrated by Montal and colleagues for KVs (50) suggests that it is generally feasible to create folded and functional PDs from a variety of extremophile NaVs, including channels that are either sodium- or calcium-selective, opens the possibility that this strategy may lead to the crystallization and structure determination of both sodium- and calcium-selective pores. Further, the biochemical tractability of the PD-only proteins may permit them to be used as platforms for screening and characterization of previously undescribed peptide or small molecule pore blockers.

The PDs display an apparent general conformational and functional robustness that contrasts with the context-dependent folding and conformational heterogeneity of the VSD. Such robustness is consistent with the high stereochemical demands required to make a channel that is selective for particular permeant ions. The independent folding of PDs as demonstrated here for NaVs and by Montal and colleagues for KVs (50) suggests that similar strategies may be applicable for other members of the VGIC superfamily. Such efforts may facilitate attempts to obtain structural information about the pore regions of homomeric members of the VGIC that presently lack structural data such as TRP channels.

Taken together with the previous work on potassium channels (16, 20–26) and voltage-sensor only proteins (27–29), our results provide support for the general independent nature of the PD and VSD within the VGIC superfamily. This concept is also supported by recent studies from McCusker et al. that reported the creation of an NaVBh pore-only protein (51) similar to the

NaVBh

part with the three pore-only NaVs we describe establishes a set of biochemically tractable proteins that should allow the exploration of the atomic basis for both sodium and calcium selectivity.

The complexity and transmembrane nature of VGIC superfamily members remains a barrier to understanding their function from a high-resolution structural standpoint. The demonstration that it is generally feasible to create folded and functional PDs from a variety of extremophile NaVs, including channels that are either sodium- or calcium-selective, opens the possibility that this strategy may lead to the crystallization and structure determination of both sodium- and calcium-selective pores. Further, the biochemical tractability of the PD-only proteins may permit them to be used as platforms for screening and characterization of previously undescribed peptide or small molecule pore blockers.

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with a final concentration of 100 mM Tris pH 8.0 at room temperature and boiled for 10 min in a reducing SDS-sample buffer prior to analysis by 15% SDS-PAGE and Coomassie brilliant blue R-250.

**Size-Exclusion Chromatography-Tridentector Analysis.** Simultaneous detection of UV, refractive index, and right angle light scattering signals from SEC of pore-only domains was performed using a Visocetek 302 Tetra Detector Array (TDA, MalvernVScotix) in a Superdex 200 10/30 GL (GE Healthcare) column.

**Functional Characterization of Reconstituted Pore-Only Proteins.** Lipid bilayer experiments performed using a planar patch clamp system (Port-a-Patch, Nanion Technologies GmbH) on Na₅p1p, Na₅Aep1, or Na₅A1p were incorporated into the planar phosphatidylcholine (PC) Unipor procedures described previously [44, 45]. To determine Na⁺, K⁺, and Ca²⁺ permeabilities, the reversal potential was measured in asymmetric conditions where the internal solution contained 10 mM Na-Hepes, 200 mM NaCl, pH 7.0 (adjusted with NaOH) and the external solution contained 10 mM Hepes, 160 mM CaCl₂, pH 7.0 (adjusted with HCl) or 10 mM Hepes, 110 mM KCl, pH 7.0 (adjusted with KOH). The permeability ratio of ions was estimated according to the following equation: 

\[ P_i/P_K = \frac{a_i}{a_K} \exp(E_{rev,K}/RT) \frac{\exp(E_{rev,i}/RT) + 1}{\exp(E_{rev,i}/RT) + 1/a_K} \]

\( R, T, F, \) and \( E_{rev} \) are the gas constant, absolute temperature, Faraday constant, and reversal potential, respectively; "i" represents K⁺ or Ca²⁺. Activity coefficients for Na⁺, K⁺, and Ca²⁺ were estimated as follows: 

\[ \gamma_i = \frac{z_i \gamma_{rev,i}}{1 + 3.5z_i \gamma_{rev,i}} \]

where \( \gamma_i \) is the effective concentration of an ion in solution, \( s \) is related to the nominal concentration \( |X| \) by the activity coefficient, \( \gamma_r \), \( \gamma_r \) was calculated from the Debye–Hückel equation: 

\[ \log_{10} \gamma_r = -0.51 + \frac{1}{\sqrt{1 + 3.5z_i \gamma_{rev,i}}} \]

where \( \gamma_{rev,i} \) is the ionic strength of the solution, \( z_i \) is the charge on the ion, and \( c_i \) is the effective diameter of the hydrated ion in nanometers. Detailed procedures can be found in SI Text.

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