Roles for Countercharge in the Voltage Sensor Domain of Ion Channels

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Voltage-gated ion channels share a common structure typified by peripheral, voltage sensor domains. Their S4 segments respond to alteration in membrane potential with translocation coupled to ion permeation through a central pore domain. The mechanisms of gating in these channels have been intensely studied using pioneering methods such as measurement of charge displacement across a membrane, sequencing of genes coding for voltage-gated ion channels, and the development of all-atom molecular dynamics simulations using structural information from prokaryotic and eukaryotic channel proteins. One aspect of this work has been the description of the role of conserved negative countercharges in S1, S2, and S3 transmembrane segments to promote sequential salt-bridge formation with positively charged residues in S4 segments. These interactions facilitate S4 translocation through the lipid bilayer. In this review, we describe functional and computational work investigating the role of these countercharges in S4 translocation, voltage sensor domain hydration, and in diseases resulting from countercharge mutations.

Keywords: countercharge, crystallography, electrostatic, ion channel, molecular dynamics, channelopathy, sliding helix model, voltage sensor domain

VOLTAGE-GATED ION CHANNELS AND ELECTRICAL EXCITABILITY

The seminal work of Hodgkin and Huxley (1952) described voltage-dependent gating particles that determine membrane permeability to sodium and potassium ions during an action potential. Their experimental work culminated in a mathematical description of the action potential based on activation gates for sodium (m) and potassium (n) ions, and an inactivation gate for sodium (h). This paradigm describing the ionic basis for the action potential has since been upheld and augmented with biophysical explanations of the molecular phenomena through which voltage-gated ion channels (VGICs) dictate membrane excitability.

The description of the structure of DNA and the discovery of the genetic code allowed molecular biology to become an integral part of research into VGIC function. Genes for ion channels and other proteins involved in electrical signaling were cloned using strategies of peptide purification from a diversity of electrically excitable tissues [reviewed by (Dolphin, 2018)], and by employing unique phenotypes of Drosophila mutants to generate probes for gene cloning [i.e., Shaker potassium channel (Papazian et al., 1987)], and para sodium channel [reviewed by (Papazian et al., 1988; Ganetzky, 2000)].
A striking pattern emerged in the amino acid sequences predicted from gene sequences for VGICs. Regularly spaced positively charged amino acid residues were observed within the fourth segment (S4) of hydrophobicity (Noda et al., 1984; Figure 1). Subsequent models of channel structure that were put forth included postulates regarding the interaction of positive and negative charges, and a helical screw mechanism of S4 translocation in channel activation (Greenblatt et al., 1985; Guy and Seetharamulu, 1986). With functional reconstitution of ion channel proteins in mammalian cells or X. oocytes (Noda et al., 1986; Stuhmer et al., 1988; Timpe et al., 1988; Leonard et al., 1989), investigations of the structure to function relationships in VGICs focused first on the role of S4 segments in channel activation and fast inactivation (Stuhmer et al., 1989; Zagotta et al., 1989; Papazian et al., 1991). Progress in our understanding of the mechanisms of voltage-gating in ion channels has since been facilitated with advanced electrophysiological techniques, crystallography, and computational work. In this review, we examine the research efforts that describe the contributions made by negative countercharges of S1 to S3 segments to the voltage-gating of ion channels.

**Homology of Voltage Sensor Domains: Positively and Negatively Charged Residues**

Voltage-gated channels show considerable homology in S1–S4 segments that comprise the voltage sensor domain (VSD). Alignments for a sample of voltage-gated sodium, calcium, and potassium channel VSDs are shown in Figure 1. Loci of conserved negatively charged (acidic) or polar charges are observed in S1–S3 segments. These residues represent putative countercharges to conserved, positively charged residues observed in the S4 segment.

An extensive analysis of VSDs from over 6,500 sequence alignments across taxa (Figure 2A; Palovcak et al., 2014) highlights the conservation of acidic or polar charges in S1–S3, positive charges in S4, and the degree of sequence identity. Figure 2B shows the location of conserved countercharges in relation to a central hydrophobic constriction site (HCS) that separates the outer and inner vestibules of the VSD. Countercharges above the HCS are located in an extracellular negatively charged region (ENC), with those nearer to the cytoplasmic region located in the intracellular negatively charged region (INC). Also shown is a conserved S2 aromatic residue located in a putative, gating charge transfer center (GCTC); this aromatic residue may act as a steric barrier to S4 translocation (Tao et al., 2010; Schwaiger et al., 2013). Current models of that S4 movement postulate that S4 arginine residues pass through an aqueous gating pore in the HCS region of the VSD [reviewed by (Groome et al., 2018)]. Side chains of S1–S3 countercharges face toward the guanidyl groups of the conserved S4 arginine residues (Figure 2C). The high degree of conservation of countercharge residues suggests that they play an important role or roles in channel function.
these countercharges with S4 residues have been investigated in a set of functional experiments described below, that support roles for countercharges in protein folding and S4 translocation.

VSD S1–S3 Negative Charges Contribute to Voltage-Gating: Functional Studies

Experiments to describe gating charge movements associated with sodium channel function in the squid giant axon (Armstrong and Bezanilla, 1973; Armstrong and Bezanilla, 1977) were followed by characterization of gating charge movement in Shaker potassium channels (Perozo et al., 1992; Perozo et al., 1994; Bezanilla et al., 1994). The biophysical basis for displacement of the gating charge, and thus voltage-sensitivity, is S4 translocation in response to altered membrane potential [reviewed by (Bezanilla, 2000; Bezanilla, 2018)]. Investigations of the role of the S4 segment as the voltage sensor have employed mutagenesis including mutant cycle analysis, toxins to trap resting or activated states, thiosulfonate reagents to determine S4 residue accessibility, fluorescence measurements, and molecular dynamics simulations [reviewed by (Groome, 2014)]. Pertinent to this review, a series of investigations have supported the premise that sequential, salt-bridge (countercharge/S4 residue) interactions facilitate transitions from the resting to fully activated state of VGICs. Here, we describe the functional experiments that provide support for that role of countercharges in voltage-gating.

Pairwise Electrostatic Interactions of Countercharges and S4 Residues: Potassium Channels

Countercharge residues that interact with S4 arginines were first identified in potassium channels [Table 1, and reviewed by (Fedida and Hesketh, 2001; Papazian et al., 2002; Kuang et al., 2015)]. In a series of papers, Papazian and colleagues showed that electrostatic interactions between S4 residues and negative countercharges in S2 and S3 were crucial in the folding or maturation of the fully functional tetrameric Shaker channel (Papazian et al., 1995; Seoh et al., 1996; Tiwari-Woodruff et al., 1997; Schulteis et al., 1998). Protein maturation in Shaker was dependent on short-range electrostatic interactions between S2 ENC residue E283 and S4 residues R368 (R3) and R371 (R4), and between INC residues E293 (S2) and D316 (S3) and S4 residue K374 (K5).

Comparison of the effects of single (charge-reversing) versus double (charge-swapping) mutations revealed pairwise interactions in the functional Shaker channel between E283 and R368 (intermediate, closed state activation) and between E283 and R371 [open-state activation; (Tiwari-Woodruff et al., 2000)]. A closed, resting state interaction was suggested between E283 and R362 (R1) by comparing the amplitude of omega current in single and double mutations using an R1S construct (Tombola et al., 2005; Tombola et al., 2007). Studies of EAG potassium channels confirmed interactions of countercharge and S4 arginine residues during activation. Silverman et al. (2003) showed that a unique,
TABLE 1 | Specific countercharge/S4 residue interactions identified in potassium channels.

| Channel | Countercharge locus | S4 locus | Experimental approach | Functional interaction | Reference |
|---------|---------------------|----------|-----------------------|------------------------|-----------|
| Shaker | S2 ENC (E283) | R3 (R368) | Charge swapping | Folding (maturation) | Tiwari-Woodruff et al., 1997 |
|        | S2 INC (E293) | R4 (R371) | Charge swapping | Folding (maturation) | Papazian et al., 1995 |
|        | S3 ENC (D318) | K5 (K374) | Charge swapping | Intermediate (deactivated) state | Tiwari-Woodruff et al., 1997 |
|        | S2 ENC (E283) | R3 (R368) | Charge swapping | Activated state | Tiwari-Woodruff et al., 2000 |
|        | S2 ENC (E283) | R4 (R371) | Charge swapping | Resting state | Tiwari-Woodruff et al., 2000 |

For each channel, residues are identified in ENC (extracellular negatively charged) or INC (intracellular negatively charged) regions of S1–S3 segments, locus in S4 segment, and with interpreted role of their paired interaction.

**Pairwise Electrostatic Interactions of Countercharges and S4 Residues: Sodium Channels**

Cloning of the prokaryotic sodium channel NaChBac (Ren et al., 2001) provided an advantage to study specific electrostatic interactions in the VSD of sodium channels. Like potassium channels, functional NaChBac is comprised of a homotetramer from the channel gene coding for one domain, providing robust effect with mutagenesis and more direct interpretation of the effect of these mutations on channel function.

In studies with NaChBac, cysteine substitution of countercharge and S4 residue pairs allowed for interpretation of their putative interaction (Table 2). For cysteine substitutions in close proximity, disulfide bond formation resulted in loss of channel availability. A key element of these experiments was the observation that reducing agents such as beta-mercaptoethanol or TCEP (tris 2-carboxyethylphosphine) were able to rescue channel function by breaking the disulfide linkage. For mutations in which partial loss of channel opening was observed, interactions were determined from results showing that oxidizing agents such as hydrogen peroxide more fully abolished sodium currents, and with subsequent treatment with reducing agents that restored channel function. Double-mutant

TABLE 2 | Specific countercharge/S4 residue interactions identified in NaChBac sodium channels.

| Channel | Countercharge locus | S4 locus | Experimental approach | Functional interaction | Reference |
|---------|---------------------|----------|-----------------------|------------------------|-----------|
| NaChBac | S2 ENC (D60) | R3 (R119) | Cysteine substitution disulfide locking | Activated state | DeCaen et al., 2008 |
|        | S2 ENC (D60) | R4 (R122) | Cysteine substitution disulfide locking | Intermediate | DeCaen et al., 2009 |
|        | S2 INC (D70) | T0 (T110) | Cysteine substitution disulfide locking | Activated states | DeCaen et al., 2011 |
|        | S1 ENC (E43) | T1 (T110) | Cysteine substitution disulfide locking | Resting state | DeCaen et al., 2011 |
|        | R1 (R113) | R2 (R116) | Cysteine substitution disulfide locking | Resting state | DeCaen et al., 2011 |
|        | R3 (R119) | R4 (R122) | Cysteine substitution disulfide locking | Intermediate | Yarov-Yarovoy et al., 2012 |
| S2 ENC (D60) | T0 (T110) | R1 (R113) | Mutant cycle analysis | Activated states | DeCaen et al., 2009 |
| S1 ENC (E43) | R2 (R116) | R3 (R119) | Mutant cycle analysis | Intermediate | DeCaen et al., 2011 |
| S1 ENC (E43) | R4 (R122) | R5 (R126) | Mutant cycle analysis | Activated states | DeCaen et al., 2011 |

Residues are identified in extracellular negatively charged (ENC) or intracellular negatively charged (INC) regions of S1–S3 segments, locus in S4 segment, and with interpreted role of their paired interaction.
cycle analysis of the free energy change required for activation was used in these experiments to corroborate interactions [for review see (Bosshard et al., 2004)].

These experiments showed that NaChBac S2 residues D60 (ENC) and D70 (INC) interact with R119 (R3) and/or R122 (R4) during activation (DeCaen et al., 2008; DeCaen et al., 2009). Subsequent studies employing disulfide locking (DeCaen et al., 2011), and/or mutant cycle analysis (Paldi and Gurevitz, 2010; Yarov-Yarovoy et al., 2012) revealed interactions between a highly conserved ENC S1 residue (E43) and S4 residues T110 (T0), R113 (R1) in the resting state, and with R116 (R2), R119 (R3), and R122 (R4) during intermediate to fully activated states of the channel. A combined set of results was compared in Rosetta models illustrating the pairwise interaction of countercharge/S4 residue interactions from resting to activated states (Yarov-Yarovoy et al., 2012), with an interpretation of VSD gating similar to that suggested by the aforementioned experiments in potassium channels.

**Scanning Mutagenesis of Countercharge Mutations in Eukaryotic Channels**

With the reports of crystal structures for prokaryotic sodium channels (Payandeh et al., 2011; Payandeh et al., 2012; Zhang et al., 2012) supporting the functional characterization of countercharge/S4 residue interactions in NaChBac, research attention was given to eukaryotic sodium channels. Here, both molecular dynamics simulations (Gosselin-Badaroudine et al., 2012) and scanning mutagenesis (Groome and Winston, 2013; Pless et al., 2014) probed each of the four domains of the skeletal muscle sodium channel (NaV1.4) for domain-specific roles of countercharges in activation and fast inactivation. Groome and Winston (2013) found that effects on activation probability were notable for mutations in domain II, while effects on fast inactivation kinetics were characteristic in domain IV (Figure 3). Using unnatural amino acid substitutions to precisely target the relative impact of charge content versus structure for gating contributions of countercharge residues, Pless et al. (2014) showed that mutations of ENC residues in domains I and II produce effects dependent on charge, whereas the effects of INC mutations are independent of charge alteration. These results were similar to those observed for Shaker channel mutations at ENC versus INC loci (Pless et al., 2011), and these authors presented the possibility that INC charges influence S4 movement through hydration of the inner vestibule of the VSD. Clearly, the significant differences observed in the functional effects of S1 to S3 mutations across domains, or at different loci (ENC versus INC) should be considered in the interpretation of the role(s) of countercharges in voltage-gating.

**Countercharges in Calcium and Proton Channels**

Domain specific roles for voltage sensors in calcium channels have also been identified (Pantazis et al., 2014; Tuluc et al., 2016a). One source of functional variability across domains in CaV channels may be related to an outer countercharge observed in DIIS3 and DIVS3 segments (Coste de Bagneaux et al., 2018). In CaV1.1, aspartate D1196, identified as D4 in the extracellular region of DIVS3, interacts with DIVS4 arginines R1 and R2, albeit differentially in distinct splice variants (Tuluc et al., 2016b). Specifically, the alternative splice variant CaV1.1e excluding 19 amino acids in the DIVS3-S4 linker enhances activation, suggesting that in CaV1.1a, the DIVS3-S4 linker disrupts D4 interaction with R1 and R2. Differential effects of DIVS3 D4 mutations in CaV1.2 and CaV1.3 are not solely dependent on the intact linker (Coste de Bagneaux et al., 2018) but also support a role for this countercharge in channel gating, and provide insight into domain-specific voltage-sensitivity in these channels.

VSD S1–S3 negative charges have roles in gating and selectivity in the voltage-gated proton channel Hv1. Countercharge/S4 residue interactions during Hv1 gating are suggested by mutant cycle

![Figure 3](https://example.com/figure3.png)

**FIGURE 3** | Scanning mutagenesis of putative countercharges in domains II and IV of the skeletal muscle sodium channel NaV1.4. Charge-reversing mutations in domain II decrease activation probability (indicated by shift in V0.5), while those in domain IV slow the entry of channels into a fast-inactivated state (indicated by the ratio of time constant [tau] of mutation with respect to that of wild type, at 20 mV). These effects are consistent with the hypothesis that countercharges facilitate certain domain-specific functions highlighted by green arrows (DI activation, DIV fast inactivation). Adapted from Groome and Winston (2013). Colors indicate countercharge loci as extracellular negatively charged (ENC) (green, red), or intracellular negatively charged (INC) (blue).
analysis of the effects of single and double cysteine mutations (Chamberlain et al., 2014), and molecular dynamics simulations have defined salt-bridge interactions between countercharge and S4 residues during H3:1 activation (Gianti et al., 2016). Interestingly, mutation of negative charge D112 in S1 results in channels that are non-functional (D112V), that display a shift in permeability to anions (i.e., D112H), or that maintain proton selectivity (D112E; Musset et al., 2012). The proton selectivity function of D112 might be based on its salt-bridge formation with S4 residue R211 (Chamberlain et al., 2015; Dudev et al., 2015). D112 and other countercharges in Hv1 S1–S3 appear to facilitate proton conduction through a mechanism in which proton binding sites are comprised of three pairs of acidic countercharges, with conformational change in the VSD promoting exchange of protons between sites (van Keulen et al., 2017). Proton-mediated disruption of the cation-pi interaction of S2 aromatic residue F150 with R211 has been proposed as a key determinant in the closed to open transition of the channel, facilitating the S2 E153 to S1 D112 proton exchange across the VSD permeation pathway.

**Structural Studies Illuminate Voltage Sensor Topology and Electrostatic Interactions**

Following the successful crystallization of the KvAP potassium channel (Doyle et al., 1998), efforts to determine the atomic structure of VGICs intensified, and led to alternative hypotheses about the mechanism through which S4 voltage-sensing segments promote channel activation. The first full length structure of a voltage-gated potassium channel (KvAP) was solved with crystallography at a resolution of 3.2 Å (jiang et al., 2003). In addition, an isolated VSD was solved at a resolution of 1.9 Å and whose structure revealed a salt-bridge between the S4 residue R133 (R5) with the S2 ENC countercharge D62. Importantly, the authors hypothesized that the second helical region in S3, along with the S4 helix, formed a voltage-sensor paddle that moved through the lipid membrane in response to membrane depolarization. Crystallization of the eukaryotic potassium channel Kv1.2 (Long et al., 2005a; Long et al., 2005b) and the chimera Kv1.2/2.1 (Long et al., 2007) partially reconciled the paddle hypothesis of charge displacement with functional data that supported vertical displacement of the S4 helix [reviewed by (Horn, 2002; Gandhi and Isacco, 2002; Elinder et al., 2007)]. As described later, structural data for Kv VSDs were utilized in molecular dynamics simulations (Delemotte et al., 2011; Jensen et al., 2012) that supported a mechanism of voltage-gating dependent on multiple salt-bridge interactions facilitating intermediate states of channel activation.

The crystal structure of the prokaryotic sodium channel NaVAb (Payandeh et al., 2011) revealed the proximity of ENC and INC countercharges with specific S4 residues. Importantly, Kv1.2/2.1 and NaVAb structures showed the S4 segment in nearly identical positions (Long et al., 2007; Payandeh et al., 2011), confirming the vertical position of the S4 segment in the activated state. Countercharge to S4 arginine residue interactions have been revealed with crystallographic data from NaVAb, NaVRh, and NaVMs (Payandeh et al., 2011; Payandeh et al., 2012; Zhang et al., 2012; Sula et al., 2017; Wisedchaisri et al., 2019). It has also been hypothesized that negatively charged residues tune the hydrophilicity of the inner and outer vestibules of the VSD (Palovcak et al., 2014), while the central, hydrophobic region separates these vestibules and focuses the electric field (Starace and Bezanilla, 2004; Ahern and Horn, 2005; Chanda and Bezanilla, 2008; Lacroix et al., 2014).

Recently, improvements in cryo-electron microscopy (cryo-EM) have allowed for rapid advances in structure determination of large membrane proteins, including eukaryotic sodium and calcium channels. Shen et al. (2017) used cryo-EM to determine the structure of NaVAP, the cockroach isoform of NaV1.4, at 3.8 Å. Shortly thereafter, the structure of EeNaV1.4, the electric eel construct of NaV1.4, was solved to 4.0 Å and in complex with the accessory β1 subunit (Yan et al., 2017). Many of the countercharge/S4 residue interactions described in the structures of prokaryotic sodium channels are reiterated in these eukaryotic structures, and in the cryo-EM structure of CaV1.1 (Wu et al., 2016). When the structure of the human NaV1.4 channel/β1 subunit was solved to 3.2 Å (Pan et al., 2018), an expanded set of interactions between S1 and S3 countercharges and S4 residues was presented, and suggested a role for polar residues in stabilizing VSDs across domains. Together, these cryo-EM structures have provided a structural framework to complement functional investigations of domain-specific functions of eukaryotic VSDs (Chanda and Bezanilla, 2002; Goldschen-Ohm et al., 2013; Tuluc et al., 2016a) and of domain-specific effects of S1–S3 countercharge mutations (Groome and Winston, 2013; Pless et al., 2014).

**Molecular Dynamics Simulations Bridge Structural and Functional Work**

Molecular dynamics simulations have been used extensively in computational approaches to investigate ion channel function, by providing a view of time-dependent interactions within the VSD in response to applied membrane potential on the system [for reviews see (Delemotte et al., 2009; Cournia et al., 2015; Howard et al., 2018)]. These mathematical simulations solve the equations for motion in three dimensions for atoms with a defined mass and charge. As parameters such as membrane potential and amino acid sequence can be easily manipulated, these simulations allow researchers to test specific hypotheses about countercharge interactions with S4 residues, as the S4 segment vertically transverses the electric field. However, these simulations are computationally limited to short timescales, making it difficult to simulate an entire conformational transition.

Molecular dynamics simulations of VSDs conducted following the publication of voltage-gated potassium channel structures (jiang et al., 2003; Long et al., 2005a; Long et al., 2005b; Long et al., 2007) probed the activated state of the voltage sensor. Several research groups were interested in hydration of the intracellular and extracellular vestibules, as well an interaction between the voltage sensor and surrounding lipid environment. Treptow and Tarek (2006) found that the outer S4 arginine residues are accessible to water in the activated state, and Jogini and Roux (2007) proposed that S4 arginine residues interact with both negatively charged lipid phosphate groups and S2
countercharges. While their intermittent electrostatic calculations showed that the membrane potential varies greatly across the membrane, the greatest membrane potential was focused on the center of the voltage sensor, a hypothesis that has been supported functionally (Starace and Bezanilla, 2004; Ahern and Horn, 2005). Simulations of S4 dynamics in response to an applied membrane potential suggested that the S4 segment transverses the membrane in a screw-like motion (Nishizawa and Nishizawa, 2008), with salt-bridges (Treptow et al., 2009) and hydrogen bonding networks (Bjelkmar et al., 2009) stabilizing intermediate states. These simulations supported a model of vertical translocation of the S4 helix through the membrane, and groups began focusing on defining the intermediate states of the voltage sensor and interactions that occurred within each state.

Due to computational limitations, simulating each intermediate state of the VSD required biasing simulations based on initial starting structure (Yarov-Yarovoy et al., 2006; Wood et al., 2012), a directed path of S4 helix motion through the membrane (Delemotte et al., 2011; Schwaiger et al., 2011) or enhanced sampling techniques (Delemotte et al., 2015). Resting state models have been built by multiple groups. Yarov-Yarovoy et al. (2006); Pathak et al. (2007), and Henrion et al. (2012) used a de novo and homology modeling approach, Delemotte et al. (2011) used a combination of harmonic restraints and steered molecular dynamics, Vargas et al. (2011) computed an average structure over multiple simulations, and Jensen et al. (2012) ran a long time-scale simulation to reach a resting state structure. Although the methodologies to produce them were unique, these models showed a high degree of similarity, with the S4 helix rotated and translated inwardly, and R1 located between the S2 ENC and INC countercharges, and near the conserved S2 aromatic residue of the GCTC. A consensus model of the resting state was then proposed (Vargas et al., 2012). While simulations have suggested that the S4 segment adopts a 3/10 helical secondary structure to favor the resting state and align S4 arginine side chains with those of negative countercharges (Villalba-Galea et al., 2008; Khalili-Araghi et al., 2010; Schow et al., 2010; Schwaiger et al., 2011), the functional contribution of 3/10 helical structure to VGIC gating remains to be clearly elucidated (Kubota et al., 2014; Bassetto et al., 2019).

Computational approaches to VSD activation have confirmed and refined the description of intermediate states. Nishizawa and Nishizawa (2008) initially hypothesized that the free energy landscape between intermediate states would be “ragged,” indicating multiple pathways of transition between states due to the large number of electrostatic interactions taking place. This hypothesis was confirmed by Delemotte et al. (2015), who characterized the transition between the resting and a partially activated state using well-tempered metadynamics. They showed that R1 must transit across the hydrophobic septum prior to the transition of R3 engaged with intracellular lipid phosphate groups, to R3 engaged with the INC S2 countercharge. Although this single most favorable pathway between a resting and intermediate state was found, their results did show a rough free energy landscape, as previously hypothesized. However, when the free energy was plotted as a function of gating charge, a smooth free energy landscape was produced, with two well-defined energy wells corresponding to the resting and intermediate states. It is important to note that the bulk of these simulations were completed using the Kv1.2 channel as a model. Thus, although many of these findings can be translated to other channel types, it is yet unknown how the differences between VSDs affect dynamics.

For example, the cryo-EM structure of hNav1.4 (Pan et al., 2018) suggests unique electrostatic interactions within each VSD, and differences in the extent of voltage sensor activation across domains. To further explore the differences in interactions between domains of hNav1.4, we have conducted a network analysis from short time-scale molecular dynamics simulations (Bayless-Edwards, 2019).

**Figure 4** shows each S4 segment translocated intracellularly from an initial activated structure (Pan et al., 2018) to produce both an intermediate state and a potential resting state structure, using methods similar to those of Wood et al. (2012). gRINN (get Residue Interaction eNergies and Networks) was used to complete the network analysis (Sercinoglu and Ozbek, 2018). Our results support the presence of unique electrostatic interactions in each domain over multiple conformations. The strongest interactions are between acidic countercharges with S4 residues, consistent with other simulations (Gosselin-Badaroudine et al., 2012) and functional experiments in sodium channels (Groome and Winston, 2013; Pless et al., 2014).

### Water Permeability Within and Across the Voltage Sensor Domain

Solvation of the VSD is an essential component in models of voltage-gating in which positively charged S4 arginine residues traverse the membrane through an aqueous environment (Freites and Tobias, 2015). Water permeation within the VSD is often measured by calculating either the solvent-accessible surface or the simulated average water occupancy along the z-axis of the membrane. As is expected by the hourglass shape of the VSD comprising inner and outer vestibules, water occupancy is highest at the edges of the membrane and decreases to the hydrophobic septum (Freites et al., 2006; Schow et al., 2010; Gosselin-Badaroudine et al., 2012). Chakrapani et al. (2010) used site-directed spin-labeling to quantify the hydrophilicity of outer and inner vestibules of the VSD and incorporated these environmental restraints into molecular dynamics simulations of NaChBac. Their results showed that S4 arginines R1 and R2 move through a hydrophilic environment toward their interaction with phospholipid heads of the bilayer as the S2 ENC (D60)/S4 R3 salt-bridge interaction is formed with activation of the channel. The significance of hydration in voltage-gating is further emphasized with the finding that S4 aspartate substitutions in the Shaker channel are remarkably tolerated with respect to gating function (Diaz-Franulic et al., 2018), and by the corollary observation that mutation of S1–S3 residues comprising the hydrophobic plug have dramatic effects on gating charge movement (Lacroix et al., 2014, and reviewed by Bezanilla, 2018).

In diseases such as hypokalemic periodic paralysis and dilated cardiomyopathy, mutation of S4 arginines can lead to...
permeation of protons or cations between the inner and outer vestibules of the VSD, a finding supported using both molecular dynamics simulations (Jensen et al., 2012; Monteleone et al., 2017) and cryo-EM (Jiang et al., 2018). This omega current is a characteristic effect of mutations in these diseases, and is a significant factor in pathogenesis [reviewed by Groome et al., 2018)]. Interestingly, the width of the hydrophobic region varies between sodium channel domains (Gosselin-Badaroudine et al., 2012), and the large hydrophobic septum in DIV may explain the lack of observed omega current associated with DIVS4 channelopathy mutations (Francis et al., 2011), even with periodic paralysis as a contributing element to the phenotype (Poulin et al., 2018).

It has been hypothesized that mutations near the hydrophobic septum disrupt not only steric inhibition of water molecules and ions (Jiang et al., 2018), but also hydrogen bonding (Monteleone et al., 2017; Poulin et al., 2018) and electrostatic interactions (Bayless-Edwards et al., 2018). This hypothesis is consistent with the premise that charged and polar residues play an important role in the hydration of ion channel vestibules in VSDs (Pless et al., 2011; Palovcak et al., 2014). An interesting set of questions remain as to the nature and extent of contributions of polar, countercharge residues to specific aspects of voltage-gating. For example, mutations of VSD polar residues do elicit substantial effects on channel activation, fast inactivation, and slow inactivation (Gamal El-Din et al., 2013; Groome and Winston, 2013; Pless et al., 2014; Groome et al., 2019). The domain-specific VSD conformations underlying these channel functions are an important target for investigation (Yarov-Yarovoy and DeCaen, 2019) and a
determination of the impact of hydration provided by polar countercharges in voltage-gating may provide some unique insight.

**Countercharge Mutations in Disease**

Inherited mutations in the genes for VGICs comprise a set of channelopathies including epilepsy and migraine syndromes, pain, cardiac arrhythmia syndromes, myotonia, periodic paralysis, and congenital myasthenia (for reviews see Catterall, 2012; Bezzina et al., 2015; Cannon, 2015). These mutations may result in a truncated protein, disrupt trafficking or assembly with accessory subunits, or produce gating defects. Channelopathy mutations have been studied in heterologous expression to access accessory subunits, or produce gating defects. Channelopathy mutations have been characterized, and are countercharge mutations identified in genetic screening of patients with intractable epilepsy syndromes. These countercharge mutations have yet to be functionally characterized in heterologous expression (Brunklaus et al., 2020). However, several countercharge mutations have yet to be functionally characterized in heterologous expression (Brunklaus et al., 2020). However, several countercharge mutations have yet to be functionally characterized in heterologous expression (Brunklaus et al., 2020).

A number of variants predicting VSD countercharge mutations associated with disease phenotypes have been identified, with missense mutations for these channelopathies given in Table 3. Noteworthy on this list are the prevalence of SCN1A mutations identified in genetic screening of patients with intractable epilepsy syndromes. These countercharge mutations have yet to be functionally characterized in heterologous expression (Brunklaus et al., 2020). However, several countercharge mutations identified in cardiac arrhythmia or skeletal muscle syndromes have been characterized, and are described here.

**Functional Characterization of Countercharge Channelopathy Mutations**

Channelopathy mutations identified in patients with Brugada syndrome and/or overlapping phenotypes such as dilated cardiomyopathy confer loss of function in the cardiac sodium channel hNa<sub>1.5</sub> (SCN5A; for reviews see Garcia-Elias and Benito, 2018; Asatryan, 2019). A few of these mutations are at countercharge loci, and for which biophysical characterization has been reported. Loss of function may be explained to some extent with observed reduction in current density, as in DIV2 ENC mutation E161Q (Meregalli et al., 2009). In contrast, N1541D (DIV1 ENC) elicits specific gating defects including enhanced probability of fast inactivation, and slowed recovery from open-state fast inactivation (Dharmawan et al., 2019). Interestingly, each of these gating defects are reiterated in the homologous Na<sub>v1.4</sub> mutation N1541D (Groome and Winston, 2013).

The SCN5A DIV3 INC mutation D1275N produces mild, loss of function gating defects including impaired activation and enhanced fast inactivation when studied in heterologous expression (Groenewegen et al., 2003; Hayano et al., 2017). However, D1275N promotes significant cardiac defects such as slowed conduction, heart block, atrial fibrillation, ventricular tachycardia, and dilated cardiomyopathy when inserted in transgenic mice (Watanabe et al., 2011) or zebrafish (Huttner et al., 2013). Two SCN5A channelopathy mutations have been characterized at D1595 (DIV3 INC). First, D1595N has been linked to atrioventricular block (Wang et al., 2002). The mutation reduces current density and enhances fast and slow inactivation consistent with the cardiac

### Table 3 | Voltage-gated ion channel (VGIC) countercharge S1–S3 mutations identified in human disease phenotypes.

| Gene | S1 mutation | S2 mutation | S3 mutation | Phenotype | Reference |
|------|-------------|-------------|-------------|-----------|-----------|
| SCN1A | D1: N191K/Y | D1: D194G/N | EIEE6       | Depienne et al., 2009, Depienne et al., 2010 | |
|      | D1: E1212K | D1: N186G   | EIEE6       | Depienne et al., 2009 | |
|      | D1: S1231R/T | D1: E1238D | EIEE6       | Depienne et al., 2009, Azmanov et al., 2010 | |
|      | D1: E1266A | EIEE6       | Depienne et al., 2009 | |
| SCN2A | D1: N132K | D1: E169G   | EIEE11      | Mataiol et al., 2014, Nakamura et al., 2013 | |
|      | D1: E1211K | D1: E169G   | EIEE11      | Pannini et al., 2017, Ogivara et al., 2009, | |
| SCN4A | D1: D1069N | D1: N1366S | EIEE6       | Wang et al., 2012, Marini et al., 2007 | |
| SCN5A | D1: E161K-Q | D1: D1069N | EIEE6       | Wang et al., 2012, Marini et al., 2007 | |

(Continued)
Countercharge Roles in Voltage-Gating: Concluding Remarks

Common sequence motifs in VSDs include the positively charged S4 region, and conserved aromatic, acidic, or polar amino acid residues in S1–S3 segments. Investigations of the mechanism of voltage-gating in VGICs have supported the hypothesis that VSD negatively charged residues act as countercharges to facilitate S4 translocation in an energetically unfavorable membrane environment. Functional experiments have revealed pairwise interactions between countercharge/S4 residues that play an important role in protein folding, the resting state of the channel, and in outward movement of the S4 segment in the steps leading to channel opening. Determinations of protein structure at atomic resolution supports such countercharge/S4 residue interactions, and have facilitated in-depth analyses of their choreography, using molecular dynamics simulations. These simulations have also been instrumental in present efforts to elucidate the mechanisms by which countercharge residues regulate VSD hydration and to explore the role of hydration in ion channel function. An expanding genetic database has revealed that countercharge mutations comprise a portion of published variants of ion channel genes in patients presenting with neural, cardiac, or skeletal muscle disorders. Genotype to phenotype correlation for countercharge mutations is an important aspect of continued research of the biophysical mechanisms of channelopathies.

AUTHOR CONTRIBUTIONS

JG and LB-E contributed equally to the writing of this review article.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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