The Voltage Sensor Module in Sodium Channels

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Contents

Abstract

The mechanism by which voltage-gated ion channels respond to changes in membrane polarization during action potential signaling in excitable cells has been the subject of research attention since the original description of voltage-dependent sodium and potassium flux in the squid giant axon. The cloning of ion channel genes and the identification of point mutations associated with channelopathy diseases in muscle and brain has facilitated an electrophysiological approach to the study of ion channels. Experimental approaches to the study of voltage gating have incorporated the use of thiosulfonate reagents to test accessibility, fluorescent probes, and toxins to define domain-specific roles of voltage-sensing S4 segments. Crystallography, structural and homology modeling, and molecular dynamics simulations have added computational

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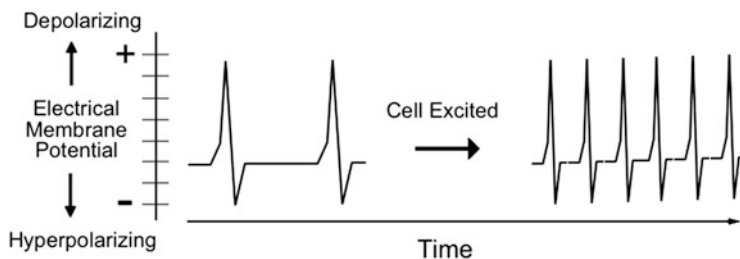
Fig. 1 Action potentials in excitable cells promoted by depolarization

approaches to study the relationship of channel structure to function. These approaches have tested models of voltage sensor translocation in response to membrane depolarization and incorporate the role of negative countercharges in the S1 to S3 segments to define our present understanding of the mechanism by which the voltage sensor module dictates gating particle permissiveness in excitable cells.

1   Sodium Channels and the Action Potential

Voltage-gated sodium channels initiate the action potential in excitable tissues such as neurons, cardiac, and skeletal muscle fibers (Armstrong and Hille 1998; Catterall 2012). These channels respond to membrane depolarization by opening, followed rapidly by an inactivating transition that limits the duration of action potentials. Their importance is underscored by the fact that action potential frequency codes for information flow in the nervous system and periphery (Fig. 1).

Cole and Curtis (1938, 1939) exploited preparations afforded by giant axons of *Nitella* and *Loligo* to measure alterations in membrane conductance during action potential generation. The subsequent development of an innovative voltage clamp technique allowed Hodgkin and Huxley (1952) to articulate a description of membrane excitability in which biological gating particles dictate permissiveness of ionic flux across the axonal membrane. Their work provided the requisite biophysical parameters for seminal equations describing voltage sensitivity and action potential propagation. The action potential was now described in mathematical terms, computational neuroscience was born, and the search for the basis of voltage gating in excitable cell membranes was on.
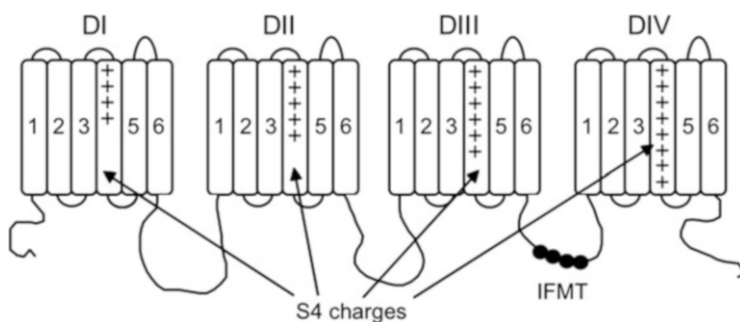
Fig. 2 Structure of the NaV channel, showing the asymmetric distribution of positive charges in the S4 segments in domains I to IV and the inactivation particle (IFMT)

2 S4 Segments as the Voltage Sensor

With the discovery of the genetic code and the subsequent elucidation of gene sequences for ion channels, a molecular approach to understanding bioelectric signaling was added to the growing arsenal of investigation. The first cloned gene for a voltage-gated sodium channel revealed a pattern of regularly spaced arginine and lysine residues in S4 segments (Noda et al. 1984), as for other members of the voltage-gated ion channel superfamily (for reviews see Yu et al. 2005; Bezanilla 2008; Catterall 2010). The hydropathic index of the sodium channel amino acid sequence predicts four domains of six transmembrane segments each. In these channels, S4 segments across the four domains are homologous but contain differing charge content (Fig. 2).

3 S4 Segments as Voltage Sensors: Experimental Approaches

3.1 Mutagenesis Studies

Several decades of research and the results from highly diverse experimental approaches support the premise that S4 segments move outward in response to membrane depolarization. As gene sequences for muscle and neuronal sodium channels were elucidated, classic mutagenesis strategy became possible. Here, the S4 segment was of immediate interest, and mutations of S4 arginine or lysine residues were employed to investigate the functions of these segments (Fig. 3). It was immediately apparent that several aspects of sodium channel gating are perturbed with mutation of positively charged residues, supporting the premise that S4 segments act as voltage sensors to promote activation and initiate fast inactivation (Stuhmer et al. 1989).

**Fig. 3** Mutational strategy for testing the role of charge in S4 segments
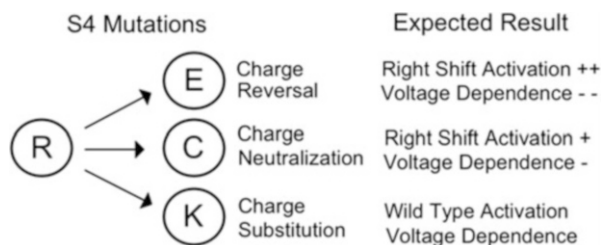
3.2 Domain-Specific Roles of S4 Segments in Sodium Channels

While mutations in S4 segments in each of the four domains of the sodium channels affect the probability or kinetics of channel activation and inactivation (Chen et al. 1996; Kontis and Goldin 1997; Kontis et al. 1997; Groome et al. 1999), homologous mutations across these domains are not equivalent. As investigations of sodium and other ion channels progressed, it has become apparent that sodium channel gating is not defined precisely by Hodgkin–Huxley parameters for which activation is due to biological structures that are independent of one another and contribute equally to sodium channel opening (gating particle permissiveness).

3.3 Channelopathy Voltage Sensor Mutations

The identification of point mutations in sodium channel genes sequenced from patients with skeletal muscle disorders has provided an area of research attention on structural determinants of voltage-dependent gating. For example, investigations of sodium channelopathies provide support for the hypothesis that the S4 segment in domain IV functions as the inactivation gate voltage sensor. Sodium channels enter into fast inactivation during the action potential (open-state) or during closed-state transitions (Armstrong 2006; and Fig. 4). Transition into, or recovery from, the fast-inactivated state is typically defective in channelopathy mutations.

For example, the outer arginine in DIVS4 is mutant in the skeletal muscle disorder paramyotonia congenita (PC, Jurkat-Rott et al. 2010). Sodium channel open-state fast inactivation is slowed by PC mutations at this locus including R1448P (Lerche et al. 1996), R1448C (Richmond et al. 1997), R1448H (Yang et al. 1994), and R1448S (Bendahhou et al. 1999). An example of the effect of a charge-neutralizing PC mutation is shown in Fig. 5 for sodium channels recorded with the macropatch configuration. Channel activation is unaffected, while entry into the fast-inactivated state is prolonged, and exhibits shallow voltage dependence. Other studies of paramyotonia mutations at this locus support the premise that DIVS4 couples the voltage-dependent process of channel activation to fast inactivation (Chahine et al. 1994). Structure–function analyses of the domain IV segment in other sodium channels support a prominent role of DIVS4 in fast inactivation (Chen et al. 1996; Kontis and Goldin 1997; Sheets et al. 1999).
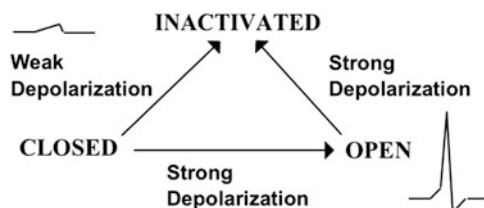
**Fig. 4** Sodium channel fast inactivation from closed or open states
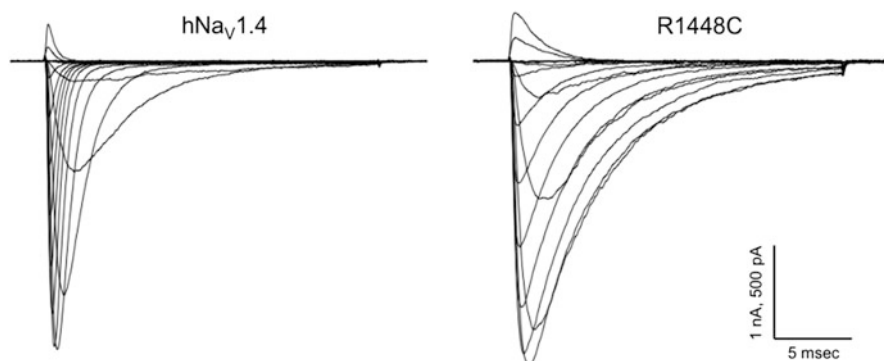
Fig. 5 PC mutant R1448C slows the entry of sodium channels into the fast-inactivated state. Groome and Ruben, unpublished

Channelopathy mutations in DIVS4 affect both routes into fast inactivation. For instance, PC mutations enhance closed-state transitions (Mohammadi et al. 2003, 2005). Mutation at the homologous locus in the cardiac sodium channel hNa$_V$1.5 (R1623Q) is associated with long QT syndrome 3 (Kambouris et al. 1998). Like PC mutations at DIVS4 R1, R1623Q slows channel inactivation from the open state but accelerates closed-state fast inactivation (Kambouris et al. 2000). These findings are consistent with those from cysteine-scanning mutagenesis of the DIV voltage sensor in cardiac channels (Sheets et al. 1999) indicating that the outermost arginine carries the predominant gating charge associated with fast inactivation.

In domains I to III, sodium channel voltage sensor mutations are often associated with periodic paralysis in skeletal muscle (for reviews see Cannon 2006; Jurkat-Rott et al. 2010). Gating defects for hypokalemic periodic paralysis (HypoPP) mutations in DIS4 (R222G; Holzherr et al. 2010), DIIS4 (Jurkat-Rott et al. 2000; Struyk et al. 2000; Kuzmenkin et al. 2002), and DIIIS4 (Carle et al. 2006) generally stabilize the fast-inactivated state, but do not explain the depolarization (and thus weakness) observed in patient muscle fibers in response to depressed serum potassium levels.

One interesting development in the search for the link between sodium channel mutations in HypoPP patients and the cellular phenotype has been the discovery of "omega" or gating pore currents associated with HypoPP mutations in S4 segments in the first three domains of Na$_V$1.4. First identified as a proton current in
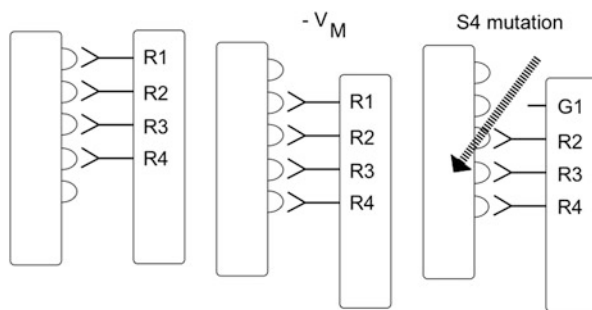
Fig. 6 Diagram of hyperpolarization-activated omega current produced by S4 mutation. In wild-type channels, side chains of S4 arginine residues maintain close contact with residues in the gating pore. With glycine substitution, ionic flow (*arrow*) through the gating pore is permitted as the voltage sensor moves downward

histidine-scanning mutagenesis of the *Shaker* K + channel (Starace and Bezanilla 2004), cationic omega currents are now hypothesized as an integral part of the pathogenesis of periodic paralysis for channelopathies of $Ca_V1.1$ and $Na_V1.4$ (for reviews see Cannon 2010; George 2012; Jurkat-Rott et al. 2012). The noncanonical currents that flow through the voltage sensor module with mutation of R1 or R2 in the voltage sensors of the first three domains are profoundly rectifying at hyperpolarized potentials (Fig. 6).

Channelopathy-associated omega currents identified in HypoPP include proton current observed with histidine mutation and cationic current observed in glycine, cysteine, or glutamine mutations (Struyk and Cannon 2007; Struyk et al. 2008; Sokolov et al. 2010; Francis et al. 2011). Omega currents have been detected in brain sodium channels (Sokolov et al. 2005) and recently in a cardiac sodium channelopathy mutation (Gosselin-Badaroudine et al. 2012a).

The pattern of voltage sensor mutations that produces the omega current has advanced our understanding of the structural basis of S4 translocation through the transmembrane electric field. Motivation for these studies, in part, was to test whether or not S4 traversed a lipid environment across the width of the membrane ("paddle hypothesis") or traversed a polar environment through a narrow gating pore comprising a focused electric field ("sliding helix or screw helical hypotheses"). Studies of *Shaker* K + channels demonstrating proton-selective (Starace and Bezanilla 2004) or cation-selective (Tombola et al. 2005) leak currents favored the sliding helix or screw helical models of S4 translocation. One of the interpretations of a voltage-dependent omega current is that in wild-type channels, positively charged residues traverse a short distance to comprise the gating charge moved in response to depolarization and channel opening (for review, see Chanda and Bezanilla 2008).

The highly conserved nature of the S1–S4 voltage sensor module in voltage-gated ion channels predicts that voltage-dependent gating charge transfer mechanisms are similar in these channels. In neuronal sodium channels, DIIS4 R1Q/R2Q mutations produce an inwardly directed current at hyperpolarized

potentials, whereas R2Q/R3Q mutations produce an outwardly directed current at depolarized potentials (Sokolov et al. 2005). The differential rectification for these mutations suggests the positioning of individual S4 arginine residues above (R1, R2) or below (R3) the gating pore for the resting state of the channel. Subsequent investigations of periodic paralysis mutations further clarified positions of R1 to R3 in resting and activated states of the channel. HypoPP mutations at R1 or R2 in domains I to III produce an inwardly rectifying cationic current (Struyk and Cannon 2007; Struyk et al. 2008; Sokolov et al. 2010; Francis et al. 2011), whereas normokalemic periodic paralysis mutations in DII (R3Q/G/W) produce an out-wardly rectifying cationic current (Sokolov et al. 2008a). These positions are supported by crystallographic evidence that arginines R1 to R3 are above the gating pore in the closed-activated state of the $Na_V Ab$ channel (Payandeh et al. 2011).

3.4 Thiosulfonate Experiments: Voltage Sensor Movement

From the initial amino acid sequence of the sodium channel, the S4 segment pattern of positive charges separated by hydrophobic residues immediately suggested a plausible biological substrate for the voltage sensor, if it could be demonstrated that this segment moved in response to membrane depolarization, and if that movement was the basis for pore opening. Early models predicted a screw-helical translocation of the S4 segment (Guy and Seetharamulu 1986) and investigations focused on identifying this putative movement of the voltage sensor to the extracellular space in response to a depolarizing change in membrane potential. Accessibility of residues to aqueous solutions on either side of the cell membrane has been tested using cysteine mutants of individual voltage sensor residues. Thiosulfonate reagents such as MTSEA or MTSET added to the bath solution test residue-specific access to the extracellular or intracellular compartments. A cysteine-substituted residue that gains access to the extracellular space would, with covalent linkage of reagent through the sulfhydryl group, cause a progressive loss of channel function with repetitive depolarization (Fig. 7). Thiosulfonate reagents may disrupt channel function if applied to the intracellular space when channels are in the resting state. Finally, residues with accessibility to the intracellular space at rest may become "buried" in response to depolarization, as shown by the lack of effect of thiosulfonate agents applied during depolarization.

Several findings from these studies are noteworthy. First, the outer three positive charges in the DIVS4 segment have accessibility to the extracellular space during depolarization, and only two of these charges actually traverse the transmembrane field to reach the extracellular space. These results suggest that a limited number of positively charged residues carry the gating charge during voltage sensor translo-cation prior to channel opening and fast inactivation (Yang and Horn 1995; Yang et al. 1996), consistent with estimates of 12–14 elemental gating charges crossing the field during activation (Aggarwal and MacKinnon 1996). In NachBac, MTS studies also suggest limited S4 movement, with only slight changes in accessibility
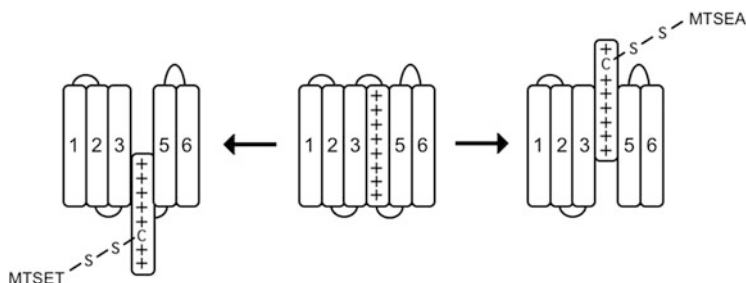
Fig. 7 Application of thiosulfonate to the intracellular compartment (*left*) or extracellular compartment (*right*) as probes for accessibility of voltage sensor charges

from the extracellular or intracellular space during depolarization (Blanchet and Chahine 2007).

3.5 Toxins: Site-Specific Actions on Voltage Sensors

Six pharmacologically distinct regional sites have been described for sodium channel toxins (Catterall 2010). The most widely used toxins in structure to function studies of the voltage sensors of sodium channels are from spiders, scorpions, and anemones (for reviews see Possani et al. 1999; Blumenthal and Seibert 2003; Zuo and Ji 2004; Catterall et al. 2007; Hanck and Sheets 2007; Moran et al. 2009; Bosmans and Swartz 2010). These toxins are differentially potent on insect or mammalian skeletal, cardiac, or neuronal sodium channels. Site-3 toxins include alpha scorpion toxins and anemone toxins, with their most studied actions at overlapping receptor sites in the S3–S4 extracellular loop of domain IV (Catterall and Beress 1978; Rogers et al. 1996). Site-4 toxins include beta scorpion toxins with a primary binding site in the domain II S3–S4 extracellular loop (Jover et al. 1980; Cestele et al. 1998). Site-specific toxins have proven invaluable probes for dissecting the roles of specific voltage sensors in activation and fast inactivation and are often referred to as gating modifier toxins.

Site-3, sea anemone toxins including ATXII and anthopleurin selectively target fast inactivation (El-Sharif et al. 1992; Hanck and Sheets 1995) and inhibit gating charge translocation (Neumcke et al. 1985; Sheets and Hanck 1995). Anthopleurin slows open-state fast inactivation, produces a "plateau" or persistent current, and accelerates recovery of neuronal, cardiac, and skeletal muscle channels with little effect on activation parameters (Hanck and Sheets 1995; Benzinger et al. 1998; Sheets et al. 1999; Groome et al. 2011). An example of the effect of anthopleurin A on brain type IIA sodium channels ($Na_V1.2$) is shown in Fig. 8.

The specific action of anthopleurin to inhibit translocation of DIVS4 (Sheets et al. 1999) has been used to dissect the contribution of individual voltage sensors and residues in gating charge movement during fast inactivation and its recovery. Mutation of the three outer arginine residues in DIVS4 of the cardiac sodium
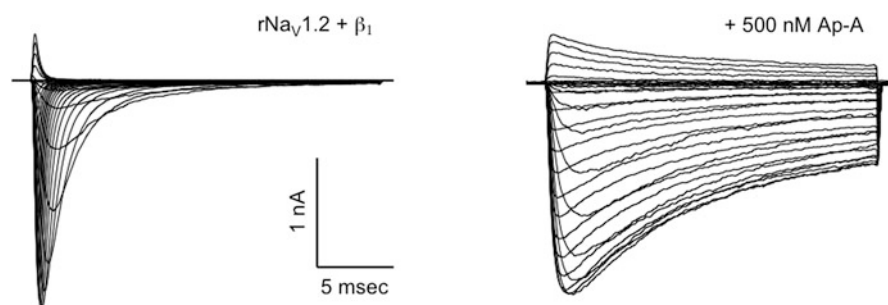
Fig. 8 Patch clamp recordings from *Xenopus* oocyte expressing rNa$_V$1.2 and beta subunit. Anthopleurin slows open-state fast inactivation. Groome, unpublished

channel results in loss of total gating charge (Q_{MAX}) by approximately 1/3, similar to that observed with exposure to anthopleurin. Contributions to gating charge of individual DIVS4 residues could be determined by the relative effect of toxin on Q_{MAX} observed in R to C constructs and show that R1 in DIVS4 carries the most gating charge during fast inactivation, with progressively less charge carried by R2 and R3 (Sheets et al. 1999). The effect of anthopleurin on charge neutralizing mutations in DIIIS4 shows that the outer positive charge there (K1) is outside the electric field (Sheets and Hanck 2002) and that gating charge translocated by DIIIS4 is carried by R2 > R3, with other residues not contributing.

During fast inactivation, a significant fraction of the gating charge becomes immobilized with voltage sensor movement in DIII and DIV (Cha et al. 1999). The respective roles of voltage sensor movement and fast inactivation per se on charge immobilization were determined in two studies (Sheets et al. 2000; Sheets and Hanck 2005). In the first, cysteine substitution in the inactivation particle (IFM–ICM) and exposure to the thiosulfonate reagent MTSET abolished fast inactivation, but did not abolish charge immobilization. In the second, the same ICM mutant was used along with R1C in DIVS4. Wild-type Q_{MAX} and the slow component of charge remobilization were restored by the thiosulfante reagent MTSEA by replacing lost positive charge in R1C, even with abolishment of fast inactivation by intracellular MTSET with the construct ICM/R1C. Thus, while DIVS4 movement is requisite for fast inactivation, the inactivation particle does not itself regulate the mobility of the voltage sensor during recovery.

Groome et al. (2011) found that anthopleurin accelerates charge immobilization during closed-state transitions in skeletal muscle sodium channels. Anthopleurin initially augments charge movement in the hyperpolarized voltage range (closed to closed transitions) with no effect on Q_{MAX}, and with full binding Q_{MAX} is depressed at voltages that drive channel opening. These results suggest that the site-3 toxin promotes DIVS4 toward an intermediate state, first augmenting closed state and then prohibiting open-state fast inactivation. DIVS4 movement promotes fast inactivation presumably by allowing access of the inactivation particle receptor in the distal portion of the DIV S4–S5 linker, as investigated in diverse sodium

channel isoforms (Mitrovic et al. 1996; Tang et al. 1996; Lerche et al. 1997; McPhee et al. 1998; Filatov et al. 1998).

Like anthopleurin, alpha scorpion toxins bind to site-3 and destabilize the fast-inactivated state without affecting activation. Studies with alpha scorpion toxins have uncovered additional features of the domain-specific role of DIVS4 to promote fast inactivation of sodium channels. For example, Ts3 scorpion toxin slows the entry of sodium channels into fast inactivation and accelerates their recovery, but is displaced by strong depolarization (Campos et al. 2004). The interpretation of the effects of toxin is that DIVS4 translocates in two steps; voltage sensor movement in domains I–III (O1) promotes activation, while latter stage DIVS4 translocation (O2) promotes fast inactivation. Subsequent experiments show that this toxin eliminates the voltage dependence of recovery (Campos and Beirao 2006) and decreases the charge immobilized during fast inactivation (Campos et al. 2008), an effect localized to the slow component of both ON and OFF gating currents. Taken together with the results of experiments with anthopleurin, these studies clarify the role of the gating charge comprised by DIVS4 to promote a rapid fast inactivation and to dictate the kinetics of recovery from that absorbing state.

The binding of alpha scorpion toxins from *Leiurus sp.* to the domain IV S3–S4 loop has been studied in some detail, with receptor site comparisons of *Leiuurus* and sea anemone site-3 toxins (Rogers et al. 1996) and structural queries of isoform specificity (Kahn et al. 2009). Extensive investigation using site-directed mutagenesis, chimeric swapping between sodium channels from mammalian and insect sources, and homology modeling have provided atomistic detail of the interaction of alpha scorpion toxin with sodium channels (for review, see Catterall et al. 2007) and that isoform specificity may be localized to sequence disparity in core and NC domains (Kahn et al. 2009). The *Leiurus* toxin core domain acts as a voltage sensor trapper with binding sites at the extracellular ends of domain IV S3 to S4 (part of the voltage sensor module) and the NC domain recognizes the extracellular ends of domain I S5 and S6 (part of the pore module, Gur et al. 2011; Wang et al. 2011). Like other alpha scorpion toxins, *Leiurus* LqhII toxin exhibits high affinity for the closed states of voltage-gated sodium channels. Exploiting this feature, homology models of the sodium channel in the resting state using structural data from $K_V1.2$ channels (Yarov-Yarovoy et al. 2006; Pathak et al. 2007) were subjected to toxin exposure in silico (Wang et al. 2011). The resulting interaction predicts specific interactions of positively charged S4 residues with putative negatively charged counterparts in S2 and S3 segments in the resting state of the channel.

Channel activation is enhanced by spider and beta scorpion site-4 toxins that trap DIIS4 in its activated position. The actions of beta scorpion toxins from *Centruroides sp.* such as CssIV have been studied in some detail. CssIV binds to a receptor complex including IIS1–S2 and IIS3–S4 loops (Zhang et al. 2011). The toxin binds to the channel in its resting state, and with depolarization, the IIS4 segment becomes "trapped" in its outward-favored position, enhancing activation (Cestele et al. 2006, 1998). An interesting finding from scanning mutagenesis of IIS4 is that mutations of hydrophobic residues enhance activation, and several of these residues also contribute to activation trapping. Additionally, mutations of

countercharges in S2 and S3 segments reiterate activation enhancement and toxin trapping phenotypes for mutations of IIS4 positive charge (Montegazza and Cestele 2005). Rescue of wild-type activation and trapping parameters in double charge swapping constructs suggest specific electrostatic interactions in the domain II voltage sensor module. In contrast, the tarantula toxin ProTx II suppresses activation and its gating charge movement, and mutation of the outermost arginines in DIIS4 abolishes the effect of the toxin (Sokolov et al. 2008b). The *Tityus* beta scorpion toxin Tz1 is capable of prohibiting (slowing) activation or deactivation (Leipold et al. 2012), suggesting that toxin binding itself is not dependent on the conformational state of the voltage sensor, but that voltage sensor position during binding dictates the effect of toxin on voltage sensor movement.

Voltage sensor trapping (by Lqh toxin) is also enhanced by domain III charge-reversing mutations (countercharge in S1 or mutations of central charges R4 and R5 in DIIS4; Song et al. 2011), possibly by allosteric modulation of the DIIS4 voltage sensor trapping effect. In domain III, beta scorpion toxin binding is explored by Zhang et al. (2011). Functional characterization of mutations and modeling of the receptor sites for CssIV in a Na$_V$Ab backbone reveal a third binding site, in the pore module of domain III. This site is shown to be in proximity with the "trappable" voltage sensor module of domain II, supporting the premise of an allosteric, beta scorpion toxin-binding site in domain III.

3.6   Fluorescent Probes of Domain-Specific S4 Functions

Gating charge movement during membrane depolarization has been studied using combined voltage clamp recordings and fluorescence signals, using cysteine residues covalently tagged with sulfhydryl reactive fluorescent dyes (Cha and Bezanilla 1997). For sodium channels, measurements of the combined gating charge transfer, concomitant with changes in fluorescence intensity during channel state transitions, have been utilized for the purposes of investigating the choreography of voltage sensor translocation and its immobilization, nature of elementary charge transfer across the electric field (Chanda and Bezanilla 2008), and domain-specific actions of toxins (Campos et al. 2007, 2008) and anesthetics (Muroi and Chanda 2008; Arcisio-Miranda et al. 2010), to name a few.

The nature of the gating particles that control voltage-dependent (sodium) ion permeability in the squid giant axon was experimentally confirmed by Armstrong and Bezanilla (1973, 1974), who determined that the gating charge moved with channel activation is not recovered instantaneously if channels enter into the fast-inactivated state (Armstrong and Bezanilla 1977). In other words, gating charge becomes "immobilized" during the process of fast inactivation. Cha et al. (1999) used simultaneous fluorescence and gating current measurements to define the roles of the four S4 segments in sodium channels, showing that movement of the voltage sensors in domains III and IV are simultaneous with fast inactivation and that these two segments contain the immobilizable fraction of the gating charge. Channel recovery and remobilization of the gating charge occur with the identical time

course (Armstrong and Bezanilla 1977). Thus, voltage sensor return in domains I and II is rapid, and the slow return of charge (remobilization) in domains III and IV is the primary determinant of sodium channel contribution to the refractory period of the action potential.

The Hodgkin–Huxley parameters describe a process in which three gating particle components m^3, h, and n^4 independently and randomly respond to membrane depolarization to dictate ionic permissiveness. Fluorescence measurements of voltage sensor movements in each domain during state transition have been crucial to our present understanding of the domain-specific and cooperative roles of voltage sensors in domains I to IV.

By correlating the time course of fluorescence intensity change of each S4 (labeled on the N terminal side of the outermost charge) with ionic flux and gating charge development, Chanda and Bezanilla (2002) observed a rapid and simultaneous movement of gating charge during activation in domains I to III, and a slower, delayed movement in domain IV. These results suggest a domain-specific action of voltage sensors in domains I to III for activation (without sequence specificity) and that charge movement in domain IV was not requisite for activation. It should be noted that these authors permitted the possibility of two separate translocations of domain IV, as suggested by the experiments of Horn et al. (2000) in which irradiation-induced immobilization of DIVS4 inhibited both activation and fast inactivation. The second Hodgkin–Huxley postulate of independent gating particles has been addressed by determining the effect of mutation of voltage sensors in one domain on the fluorescence tracking of a voltage sensor in a different domain (Chanda et al. 2004). Perturbation of any one voltage sensor produces a change in the gating charge movement of the other voltage sensors, demonstrating that S4 movement is cooperative. Coupling is strongest for DIS4 and DIVS4, suggesting that coupling of fast inactivation to the activation process has its basis in the cooperative interaction of these two voltage sensors.

4   X Ray Diffraction: Structural Modeling and Molecular Dynamics

The crystal structure of the prokaryotic potassium channel KcSA (Doyle et al. 1988) revealed for the first time interatomic distances within an ion channel that relate specific amino acid residues with the functions of ion permeation and selectivity. X-ray diffraction and electron microscopy data of prokaryotic voltage-gated potassium channel $K_V AP$ (Jiang et al. 2003, 2004; Cuello et al. 2004), and subsequent crystallization and characterization of mammalian *Shaker*-like $K_V 1.2$ (Long et al. 2005a, b; Lee et al. 2005) provided important structural detail into the voltage sensor module in ion channels and sparked a wave of experimental research emphasis to compare the voltage sensor paddle and screw-helical hypotheses. Importantly for this discussion, these efforts initiated a growing trend to incorporate structural detail of ion channels into investigation of their function.
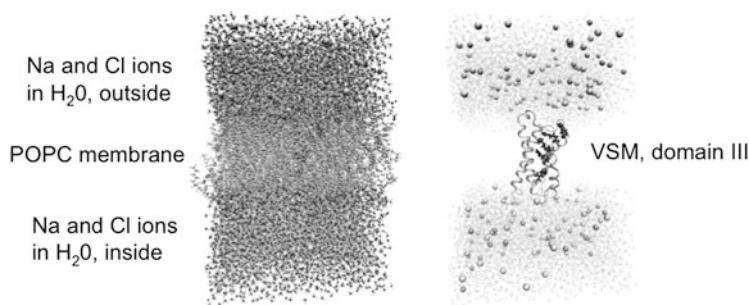
Fig. 9 Voltage sensor module of DIIIS4 of rNa$_V$1.4, embedded in POPC membrane. At *right*, lipid molecules have been removed with S4 charges highlighted. Groome and Winston, unpublished

One avenue of approach to studying the basis of voltage gating in sodium channels is to compare functional data for prokaryotic or eukaryotic channels to structural data inferred from X-ray diffraction data. Structural modeling often employs homology models of the channel of interest, based on the premise that voltage-gated ion channels share considerable sequence homology, especially for regions of voltage gating. Crystal structures are typically determined in a depolarizing environment, yielding data on the activated state in potassium (Long et al. 2005a, b, 2007) and sodium (Payandeh et al. 2011, 2012; Zhang et al. 2012) channels. Closed- or intermediate state models have been built from a comparison of these structures to experimental data on S4 movement using a combination of mutagenesis, fluorescence, spectroscopy, metal ion binding, and/or disulfide locking experiments to establish likely S4 charged residue positions or other interactions within the voltage sensor module (Silverman et al. 2003; Yarov-Yarovoy et al. 2006, 2012; Campos et al. 2007; Baker et al. 1998; Pathak et al. 2007; Broomand and Elinder 2008; Shafrir et al. 2008; DeCaen et al. 2008, 2009, 2011; Chakrapani et al. 2010; Horne et al. 2010; Paldi and Gurevitz 2010; Lin et al. 2011; Henrion et al. 2012). X-ray diffraction data of the S1–S4 region from the crystal structure of the bacterial cyclic nucleotide channel MIotiK1 resolved in the closed state (Clayton et al. 2008) has also proven useful in generating models of voltage-gated channels in the resting conformation. In general, these investigations support a model of voltage gating in which the S4 helix translocates a short distance across a focused electric field, with limited movement of the S1, S2, and S3 helices (studies reviewed in Delemotte et al. 2012; Vargas et al. 2012).

Structural or homology modeling of open and closed states of ion channels has been exploited in computer simulations of voltage sensor movement in molecular dynamics trajectory calculations (reviewed by Sigworth 2007; Dror et al. 2010; Roux 2010; Delemotte et al. 2012; Vargas et al. 2012). An example of a voltage sensor module embedded in a POPC membrane after equilibration and prior to simulation of an applied membrane potential is shown in Fig. 9.

All atom MD simulations for K$_V$ channels have been used to investigate or predict folding events (Gajewski et al. 2011), permeation events or intermediate

states (Jogini and Roux 2007; Treptow et al. 2009; Delemotte et al. 2011; Pan et al. 2011; Lacroix et al. 2012; Jensen et al. 2010, 2012, 2013), the 3–10 helix conformation proposed to provide energetic favoring of aligned S4 and countercharges in gating (Schwaiger et al. 2011), and the omega current defining the position of the gating pore (Delemotte et al. 2010; Jensen et al. 2012; Khalili-Araghi et al. 2012).

Molecular dynamics simulations of sodium channel gating have now been employed in several studies of the voltage sensor module. Incorporation of physical data into the model used for simulations is one method of constraining the protein in its predicted environment or evaluating the equilibration of that protein in the environment in the absence of constraints (Sompornpisut et al. 2008). The activated state of NachBac was predicted in this fashion by running MD simulations of the membrane-bound voltage sensor module after determining constraints for each of the residues in that module with respect to their accessibility to solvent using site-directed spin labeling (Chakrapani et al. 2010). The extensive physical characterization in this study provides a complementary support to MD simulation predictions of NachBac countercharge to S4 interactions. These and other MD simulations of bacterial sodium channels Na_VAb predict countercharge to S4 interactions during activation promoted by dynamic formation of S4 3–10 helical conformation (Amaral et al. 2012).

5 Counatercharges in the Sodium Channel VSM: Sliding Helix Model

With the demonstration of S4 segments as voltage sensors, an important goal has been to explain how voltage sensor movement is achieved in the energetically unfavorable environment dictated by a hydrophobic plasma membrane. Energetically favorable intermediate steps in translocation are an important consideration for efficient gating charge transfer across a hydrophobic barrier. Putative negative counatercharges might stabilize positively charged voltage sensor residues as they respond to membrane depolarization. This proposed mechanism has evolved from theoretical consideration to an experimentally derived, integral component of the sliding helix model. First studied in potassium channels, negatively charged amino acids in the voltage sensor module influence channel gating (*Shaker*, Papazian et al. 1995; Seoh et al. 1996; HCN2, Chen et al. 2000; BK, Ma et al. 2006; KCNQ, Eldstrom et al. 2010; Pless et al. 2011). Investigations based on potassium crystal structure data support a sliding helix or helical screw model (Yarov-Yarovoy et al. 2006; Pathak et al. 2007; Shafrir et al. 2008). Using this model as the basis for experimentation, recent studies have provided functional characterization of the role of counatercharges in sodium channels. It is interesting to note that the earliest models of sodium channel structure posit that negative charges in S1 and S3 provide salt bridge partners for positively charged S4 residues in the helical screw motion of voltage sensor translocation (Guy and Seetharamulu 1986).
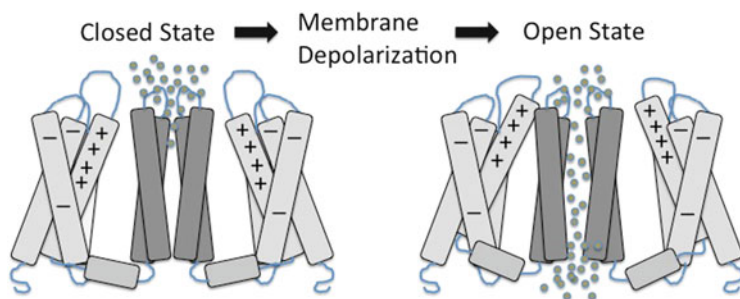
Fig. 10 Model of ion channel activation and pore opening. Countercharges identified in functional investigation of the NacBac channel are shown (−); these form sequential ion pairs with S4 (+) charge to drive that segment outward and via the S4–S5 linker pull open the ion permeation gate of the pore module

5.1 Countercharges in Prokaryotic Sodium Channels

Countercharge interactions with the S4 complement of positive charge have been investigated in prokaryotic sodium channels, with a focus on the bacterial sodium channel NacBac (DeCaen et al. 2008, 2009, 2011; Paldi and Gurevitz 2010; Yarov-Yarovoy et al. 2012). Specific ion pair interactions of negative countercharges in the S1 and S2 segments with S4 positive charges have been identified using disulfide locking experiments with cysteine mutants. The experimental design is based on the fact that the NachBac channel lacks native cysteine residues. By substituting S4 arginine and negative countercharges with cysteine, it is assumed that disulfide locking occurs with proximity of these two residues at 2–3 Å, well within the distance supported by an electrostatic interaction. If the residues lock, channel function is lost, as observed in electrophysiological recording. The sequential nature of these interactions is interpreted from the level of depolarization needed to induce disulfide locking and provide the choreography of salt bridge interactions during intermediate steps towards activation.

These electrophysiological data have been supported by computational methods and structural data to provide the current view of the activation process in sodium channels, in which S1–S4 voltage sensor module (VSM) dictates the opening or closing of S5–S6 segments comprising a pore module (Muroi et al. 2010; Yarov-Yarovoy et al. 2012 and reviewed by Vargas et al. 2012). The proposed mechanism for sodium ion channel activation is shown in Fig. 10. Briefly, sequential electrostatic interactions of countercharge ions with S4 residues are promoted by membrane depolarization. The favorable energetics of ion pairing define intermediate states that drive the S4 segment outwards toward the extracellular space. This movement is transferred through a rigid helix of the S4–S5 linker, providing a mechanical translation of the electrical force of membrane depolarization to open of the pore module gate at the base of S5–S6 segments, promoting ion permeation.

Crystal structures of the prokaryotic sodium channels Na$_V$Ab (Payandeh et al. 2011, 2012) and the NacBac orthologue Na$_V$Rh (Zhang et al. 2012) support
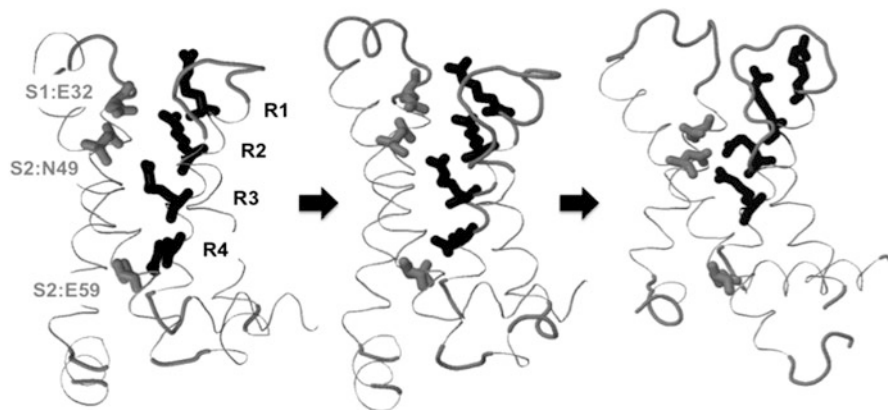
Fig. 11 Models of the voltage sensor module in action. *Left*: Closed-activated Na$_V$Ab (from Payandeh et al. 2011). Inactivated state models from Na$_V$Ab (*middle*, Payandeh et al. 2012) and Na$_V$Rh (*right*, Zhang et al. 2012) show the progressive outward movement of S4 and sequential ion pair formation

the results of functional characterization of the voltage sensor module in prokaryotic sodium channels. The Na$_V$Ab channel has been crystallized in two states of activation, and the Na$_V$Rh channel was crystallized in an even more depolarized state. A comparison of these structures show progressive movement of S4 charges through the gating pore towards potential interacting countercharge partners in the S1 and S2 segments (Fig. 11).

5.2 Countercharges in Eukaryotic Sodium Channels

Investigation of the voltage sensor module in eukaryotic sodium channels has been studied from a theoretical approach using homology models of voltage-gated potassium or sodium channels for which crystal structures have been determined and for several state transitions. Molecular dynamics simulations of the rNa$_V$1.4 (rat skeletal muscle) channel based on homology models of each domain predict a series of interactions of countercharges in S1–S3 segments with voltage sensor residues in each domain (Gosselin-Badaroudine et al. 2012b). Countercharge to S4 interactions define three intermediate states of voltage sensor translocation predicted in these simulations.

Functional characterization of S1–S3 countercharges in eukaryotic sodium channels with disulfide locking experiments has not yet been possible, since these channels contain native cysteine residues, precluding the interpretation of disulfide locking to a specific ion pair. Nevertheless, several studies have investigated the role of S1, S2, and S3 segments in eukaryotic sodium channels using a mutagenesis strategy. For example, charge-neutralizing mutations in S1 of insect channels slow
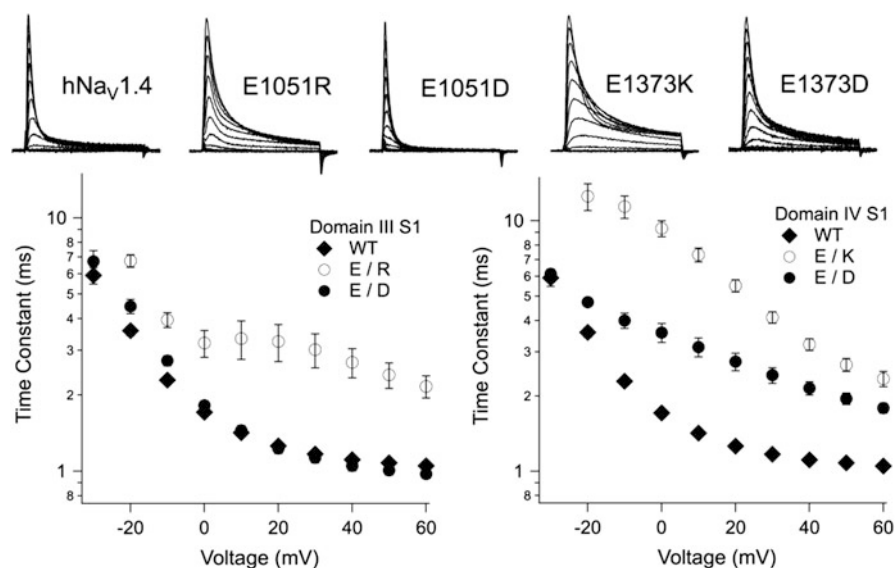
Fig. 12 Negative countercharges in the S1 segment of domains III (E1051) and IV (E1373) promote fast inactivation of the mammalian sodium channel. Plots show kinetics of entry into fast inactivation. Modified from Groome and Winston (2013)

activation and deactivation (Du et al. 2010). In mammalian sodium channels, mutations N1382C and E1392C (DIV S2) slow the entry of channels into fast inactivation ($rNa_V1.4$, Ma et al. 2009). In DIV S3, cysteine substitutions at D1420 or N1417 also slow fast inactivation ($hNa_V1.4$, Nguyen and Horn 2002). Effects of countercharge mutations across the four domains of $hNa_V1.4$ support the premise that domain-specific roles of S4 segments are facilitated by interactions of countercharges in these domains with S4 residues (Groome and Winston 2013). Figure 12 shows the effects of S1 countercharge mutations in domains III and IV on fast inactivation.

It is evident that charge content and structure must be considered to more completely explain the roles of countercharges in sodium channel function. For example, X-ray diffraction data and structural modeling predict networks of hydrogen bond interactions above and below the gating pore that may explain these diverse effects of mutations (Payandeh et al. 2011; Yarov-Yarovoy et al. 2012). Similarly S4 residue structure is important, as charge-conserving mutations of S4 residues often have dramatic effects (Kontis and Goldin 1997; Groome et al. 2002).

Conclusion

Voltage sensitivity of sodium channels is ascribed to movement of gating charge across the transmembrane electric field, as provided by positively charged residues in the S4 segments in each domain. Domain-specific functions of individual voltage sensing S4 segments have been investigated with mutations

including those found in channelopathies of nerve and muscle, chemical modification, and fluorescence. X-ray diffraction data, structural modeling, and molecular dynamics have added a new set of tools to study the interaction of S1 to S3 countercharges with the voltage sensing S4 segments to advance our understanding of the structural basis of channel functions effected by the voltage sensor module in sodium channels.

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