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Peter C. Ruben *Editor*

Voltage Gated Sodium Channels

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Voltage Gated Sodium Channels

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Preface

Just over 60 years ago, Hodgkin and Huxley first proposed the ionic basis of the action potential in neurons. Their insightful—and foresighted—work, based on experiments performed in squid giant axons, laid the groundwork for a new research field: ion channel biophysics. The elegance and importance of their work earned them a Nobel Prize 11 years after their groundbreaking work was first published and set the stage for other ion channel biophysicists to follow in their footsteps: Sakmann and Neher (1991) and MacKinnon (2003). Many others, however, substantively contributed to a rich research field, achievements, and advances which are celebrated each year at the Annual Meeting of the Biophysical Society. The study of ion channel biophysics has far-reaching implications in medicine, animal behavior, and evolution. Ion channels are targets for a vast and impressive array of naturally occurring and artificial compounds, including toxins and pharmaceutical agents. Their presence in the membrane of all cells makes them particularly vulnerable (in the case of toxins) and attractively accessible (in the case of drugs, some of which are modeled after toxins).

Voltage-gated sodium channels, the focus of this Handbook, are—in a sense—the first in a physiological lineage of ion channels, and the classic opening line of many publications about sodium channels exemplifies their importance; sodium channels are the basis of the rising phase of action potentials in nerve and muscle. That simple understatement characterizes the critical importance of sodium channels in the electrical activity of neurons and muscle cells. Sodium channels are responsible for the generation and propagation of action potentials along the cell membrane and are, therefore, the lynchpin in the processes of information transmission within the nervous system and muscle contraction.

Mutations in voltage-gated sodium channels impart changes in their structure and function. Some of these changes underlie diseases that span the spectrum from relatively benign to fatal. For clinical reasons, then, the study of mutations in sodium channels hold great potential for understanding the molecular basis of disease. Many mutations impart changes in the biophysical properties of sodium channels, particularly those associated with gating, and are thus of great interest in terms of understanding the structure/function relationship of channels. Indeed, site-directed mutagenesis has been, for nearly a quarter century, since Stuhmer et al. (1989),

the technique of choice to understand the structural basis of sodium channel function. The involvement of sodium channel mutations in disease is the subject of Chapters "The Voltage Sensor Module in Sodium Channels," "Slow Inactivation of Na^+ Channels," "The Role of Non-Pore-Forming β Subunits in Physiology and Pathophysiology of Voltage-Gated Sodium Channels," "Altered Sodium Channel Gating as Molecular Basis for Pain: Contribution of Activation, Inactivation, and Resurgent Currents," Regulation/Modulation of Sensory Neuron Sodium Channels," and "The Role of Late I_{Na} in Development of Cardiac Arrhythmias" of this Handbook.

By virtue of their critical importance in cellular excitability and their vulnerable position in the cell membrane, including an extensive exposure to the extracellular milieu, sodium channels are attractive targets for toxins, venoms, and drugs. In addition, sodium channel activity is modulated by a host of intracellular messengers. Studies of the interactions between sodium channels and agents that bind to them have revealed a wealth of information about their structure and function, and many biophysical properties of sodium channels have been revealed by these interactions. We have also learned much about evolution, predator–prey interactions, and discovered many agents to treat a wide range of diseases of excitability. The interaction between sodium channels and both naturally occurring and manufactured agents continues to be a vibrant and important line of research with implications for the treatment of diseases underlying the highest morbidity and mortality: cancer and heart disease. Chapters "Proton Modulation of Cardiac I_{Na}: A Potential Arrhythmogenic Trigger," "Probing Gating Mechanisms of Sodium Channels Using Pore Blockers," "Animal Toxins Influence Voltage-Gated Sodium Channel Function," "Ubiquitylation of Voltage-Gated Sodium Channels," and "Pharmacological Insights and Quirks of Bacterial Sodium Channels" in this Handbook deal with various aspects of sodium channel modulation by intrinsic and extrinsic agents.

Bacterial sodium channels have finally provided a way to visualize the structure of voltage-gated sodium channels through crystallography. This relatively recent breakthrough has allowed researchers in the field to "put it all together" through homology modeling. Soon, someone will crystallize a mammalian sodium channel and bring us one step closer to an even more complete understanding of how structure and function relate to one another, how drugs and toxins interact with the channel, and how changes in channel structure result in debilitating diseases. The authors and editor of this volume of the Handbook of Pharmacology hope that the chapters contained herein will inspire present and future sodium channel devotees to pursue the unanswered questions and resolve the structure and function of this complicated, fascinating, and physiologically pivotal protein.

September, 2013 Peter C. Ruben
Burnaby, BC, Canada

Contents

Introduction to Sodium Channels

Colin H. Peters and Peter C. Ruben

Contents

Abstract

Voltage-gated sodium channels (VGSCs) are present in many tissue types within the human body including both cardiac and neuronal tissues. Like other channels, VGSCs activate, deactivate, and inactivate in response to changes in membrane potential. VGSCs also have a similar structure to other channels: 24 transmembrane segments arranged into four domains that surround a central pore. The structure and electrical activity of these channels allows them to create and respond to electrical signals in the body. Because of their distribution throughout the body, VGSCs are implicated in a variety of diseases including epilepsy, cardiac arrhythmias, and neuropathic pain. As such the study of these channels is essential. This brief review will introduce sodium channel structure, physiology, and pathophysiology.

Keywords

Sodium • Channels • Structure • Physiology • Disease

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1 Overview

Voltage-gated ion channels are proteins that allow movement of ions across cellular membranes. As suggested by their name, the activity of these channels is regulated by the voltage difference across the membrane they span. The movement of charged ions, such as sodium, potassium, calcium, and chloride, as well as the voltage dependence of these channels allows them to produce and respond to electrical signals within the body.

One class of voltage-gated ion channels is the voltage-gated sodium (Na_V) channel. The kinetics of Na_V channels were studied by Hodgkin and Huxley in squid axon in 1952 and were part of their subsequent model (Hodgkin and Huxley 1952). Na_V channels have since been discovered to comprise a family consisting of $Na_V1.1$ through $Na_V1.9$ and a lesser studied group of Na_V2 channels (Catterall et al. 2005; Goldin 1999; Watanabe et al. 2000). Na_V channels are present in many electrically active tissues including neurons and both cardiac and skeletal muscle cells (Goldin 1999). In these cells voltage-gated sodium channels allow sodium to flow from the extracellular solution into the cytosol thus causing a depolarization of the cell. Different types of tissue express different sodium channel isoforms. $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, and $Na_V1.6$ are primarily expressed within the central nervous system; $Na_V1.4$ is found predominantly in skeletal muscle; $Na_V1.5$ is the primary variants in cardiac muscle; and $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ are found in the peripheral nervous system (Catterall et al. 2005; Goldin 1999).

2 Structure and Function

All the voltage-gated sodium channel variants share a common structure (Goldin 1999). The largest subunit of the sodium channel is the alpha-subunit. Smaller beta-subunits are associated with and modulate the channel, but the alpha-subunit alone is sufficient to conduct sodium (Egri et al. 2012). Similar to voltage-gated potassium (K_V) channels, the alpha-subunit of the voltage-gated sodium channel is made up of 24 alpha-helical transmembrane segments arranged into four domains (Fig. 1) (Catterall et al. 2005). Unlike K_V channels which are made of four identical subunits, in Na_V channels the four domains are not identical and are formed by a single protein, approximately 2,000 amino acids in length (Ahmed et al. 1992). Each of the four domains of the Na_V channel consists of a voltage-sensing domain formed by the first four transmembrane segments and a pore domain consisting transmembrane segments five and six as well as the extracellular linker between these two segments (the p-loop) (Payandeh et al. 2011). Recently multiple bacterial Na_V channel crystal structures were solved (McCusker et al. 2012; Payandeh et al. 2011, 2012). These structures show that the four voltage-sensing domains are arranged around a central aqueous channel formed by the pore domain (Fig. 2).

The selectivity filter for the channel is formed by residues in the p-loop segments. Sodium channels are highly selective for sodium entry into the cell with a potassium permeability to sodium permeability ratio of less than 0.10

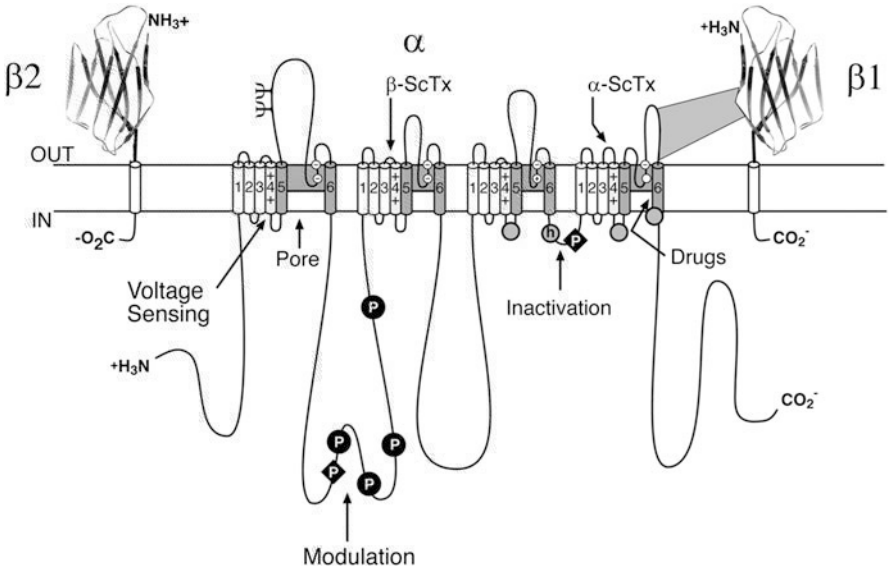

Fig. 1 The primary sequence of the voltage-gated sodium channel. The alpha-subunit is a single protein with 24 transmembrane segments arranged in four domains. The S5 and S6 segments of each domain as well as the S5–S6 linkers form the pore of the channel. The alpha-subunit is also associated with beta-subunits (Catterall et al. 2005)

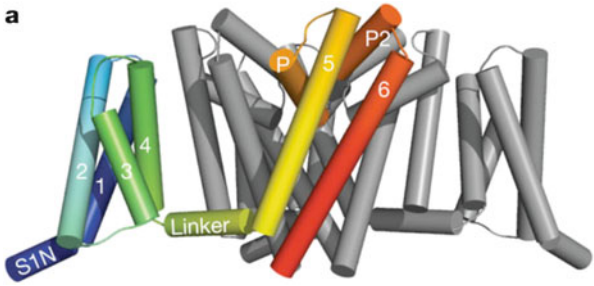

Fig. 2 Tertiary structure of a bacterial voltage-gated sodium channel. A single domain is highlighted in color. The voltage-sensing regions of each domain are arranged around the pore regions and are connected by the S4–S5 linkers (Payandeh et al. 2011)

(Favre et al. 1996; Lipkind and Fozzard 2008). The selectivity filter is formed by a ring of four residues: aspartate in domain I, glutamic acid in domain II, lysine in domain III, and alanine in domain IV (Heinemann et al. 1992; Lipkind and Fozzard 2008). Hydrated sodium passing through the selectivity filter interacts mainly with the two oxygen groups of the glutamate as it passes through the selectivity filter. In this process the DEKA residues shift to form both hydrogen bonds and van der waals interactions. Potassium does not pass through the channel easily as it forms a weaker interaction with the glutamate and is repelled more strongly by the lysine

then is sodium (Lipkind and Fozzard 2008). Previous researchers have shown that by replacing the lysine at position 1,237 the sodium selectivity of the channel is abolished and both potassium and calcium are allowed to move through the channel (Favre et al. 1996; Heinemann et al. 1992).

The voltage-sensing domain is connected to the pore-sensing domain by an intracellular linker between transmembrane segments four and five. Upon depolarization, the positively charged S4 transmembrane segments are believed to move toward the extracellular surface. This motion is transferred to the pore domain via the intracellular linker causing a conformational change resulting in the opening of the sodium channel. Upon repolarization the gate closes and the S4 segments return to their resting positions; this process is called deactivation.

Sodium channel currents in excitable tissues are often of large amplitude; however, the current through the channels lasts only a short period of time as the channel becomes impermeable to ions in a process called fast inactivation. Fast inactivation is a process whereby the channel ceases to conduct current while the voltage sensor is still in an active conformation. Fast inactivation is mediated by four residues in the linker between domains three and four of the sodium channel. This isoleucine, phenylalanine, methionine, and threonine (IFMT) motif is necessary for fast inactivation and mutation causes the abolishment of this process (West et al. 1992). When the cytosol is depolarized, this linker binds to residues on the intracellular side of domains III and IV. The particle, therefore, acts like a hinged lid to occlude the intracellular side of the sodium channel pore preventing sodium from moving through the channel (Goldin 2003). The movement of the fast inactivation gate is not independent from the activation of the channel. Recently Capes et al. showed that the movement of the DIVS4 transmembrane segment is the rate-limiting step in fast inactivation (Capes et al. 2013). After the cell repolarizes, the unbinding of the IFMT particle removes fast inactivation. The recovery from fast inactivation is also rate limited by the movement of the DIVS4 linker (Capes et al. 2013).

Fast inactivation is not the only process by which sodium channels cease to conduct. Sodium channels can also become inactivated in a process called slow inactivation (Richmond et al. 1998). While fast inactivation occurs in the millisecond time range, slow inactivation occurs on the time scale of seconds. Physiologically slow inactivation occurs during repetitive or prolonged depolarizations of sodium channels thereby limiting channel availability over longer time periods (Richmond et al. 1998). The exact process by which slow inactivation occurs is not fully known. Crystal structure data suggest that slow inactivation may be due to continued rearrangement of the channel during prolonged depolarization (Payandeh et al. 2012). This rearrangement can lead to collapse of both the pore and selectivity filter of the sodium channel, thus preventing sodium conduction. As with fast inactivation, the onset of slow inactivation occurs during depolarization and recovery occurs at repolarized potentials.

3 Physiology and Implications in Disease

The general physiological role of sodium channels is to depolarize excitable cells in the initial phase of an action potential. Upon opening, sodium channels pass a large inward sodium current. This influx of positively charged sodium makes the membrane potential more positive. In neuronal cells, depolarization of an axon segment allows the action potential to propagate to the next segment and trigger another action potential. In skeletal and cardiac muscle cells the action potential is used to trigger a contraction. The depolarization from sodium influx activates a calcium signaling cascade that causes cellular contraction. In skeletal muscle this contraction can cause movement of the body, while in cardiac muscle this contraction is used to pump blood.

Different tissues within the human body predominantly express different voltage-gated sodium channel subtypes. Thus mutations in neuronal channels cause neurological disorders such as epilepsy, while mutations in cardiac channels cause cardiac arrhythmias such as Brugada Syndrome or Long QT Syndrome (Meisler and Kearney 2005; Wang et al. 2009). The distribution pattern is also important as different variants show different voltage-dependence and drug-binding affinity. These differences can be exemplified by the difference between $Na_V1.2$ and $Na_V1.5$ which are found predominantly in neuronal and cardiac cells, respectively. Neuronal tissue has a more positive resting membrane potential than does cardiac tissue. Consequently the voltage dependence of activation and inactivation is shifted to more positive potentials in $Na_V1.2$ than in $Na_V1.5$ (Vilin et al. 2012). There are also differences in the degree of drug binding. For example, Tetrodotoxin (TTX), a potent neurotoxin, has an EC50 of approximately 12 nM in $Na_V1.2$ and a much higher EC50 of 1–2 mM in $Na_V1.5$ (Catterall et al. 2005). The differences in the distribution of these channels therefore can have large impacts in reference to the effects of mutations and pharmacological intervention.

Conclusion

From Hodgkin and Huxley's original paper in 1952 to modern crystallography and mutagenesis studies, many aspects of voltage-gated sodium channels have been studied (Capes et al. 2013; Hodgkin and Huxley 1952; Payandeh et al. 2011). This volume presents selected topics on the physiology, pathophysiology, and pharmacology of the voltage-gated sodium channel. Despite this vast body of research, new revelations about its critical structure, function, and role in disease are still being discovered.

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The Voltage Sensor Module in Sodium Channels

James R. Groome

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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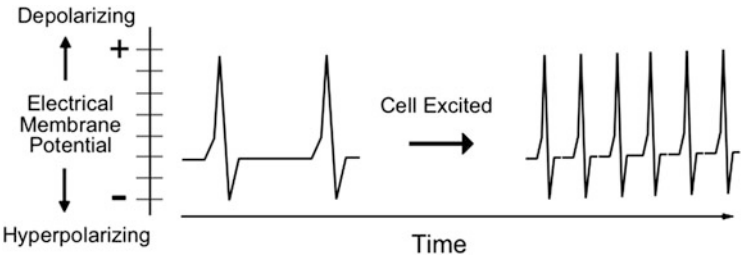

Fig. 1 Action potentials in excitable cells promoted by depolarization

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.

Keywords
Patch clamp electrophysiology • Segment four • Sodium channel • Ion channel • Voltage-gated • Voltage sensor module

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.

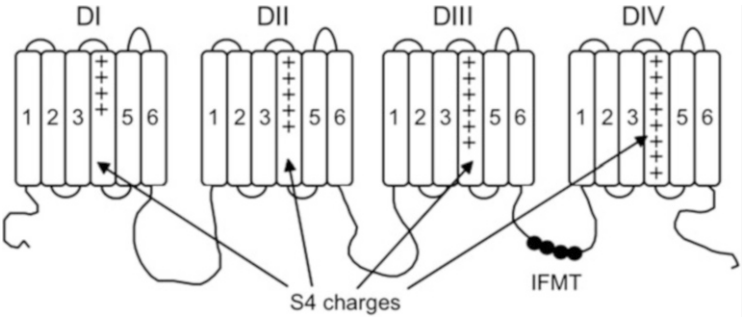

Fig. 2 Structure of the NaV channel, showing the asymmetric distribution of positive charges in the S4 segments in domains I to IV and the inactivation particle (IFMT)

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).

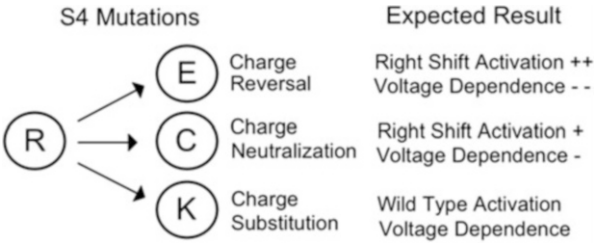

Fig. 3 Mutational strategy for testing the role of charge in S4 segments

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).

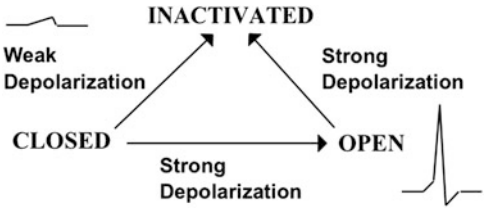

Fig. 4 Sodium channel fast inactivation from closed or open states

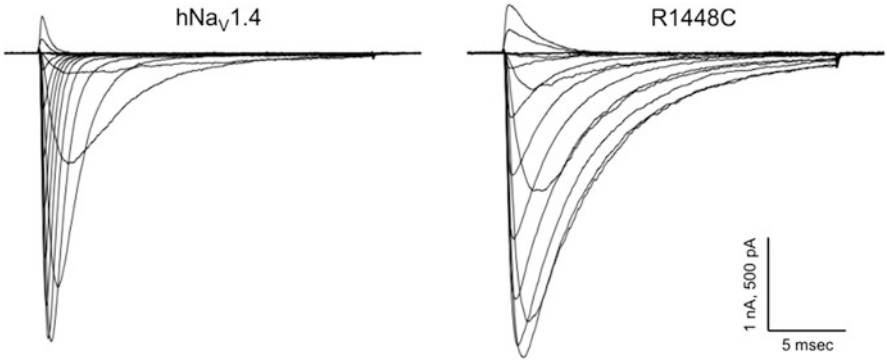

Fig. 5 PC mutant R1448C slows the entry of sodium channels into the fast-inactivated state. Groome and Ruben, unpublished

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

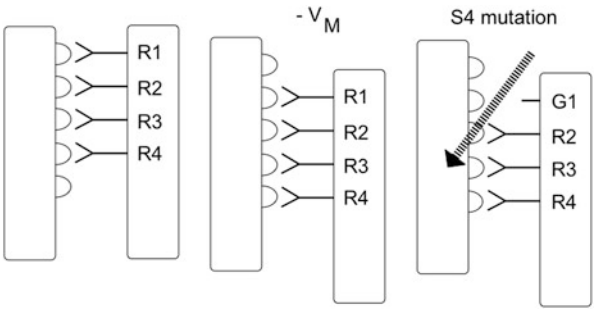

Fig. 6 Diagram of hyperpolarization-activated omega current produced by S4 mutation. In wild-type channels, side chains of S4 arginine residues maintain close contact with residues in the gating pore. With glycine substitution, ionic flow (*arrow*) through the gating pore is permitted as the voltage 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 outwardly 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_VAb 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 translocation 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

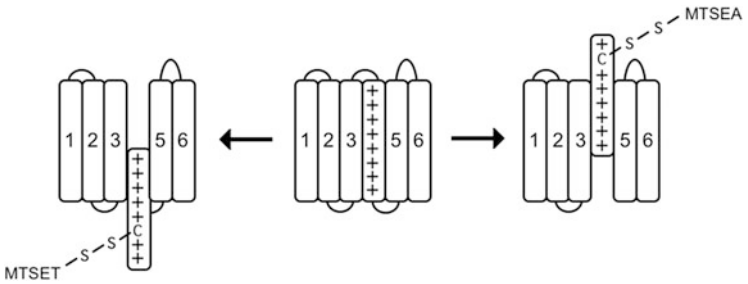

Fig. 7 Application of thiosulfonate to the intracellular compartment (*left*) or extracellular compartment (*right*) as probes for accessibility of voltage sensor charges

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

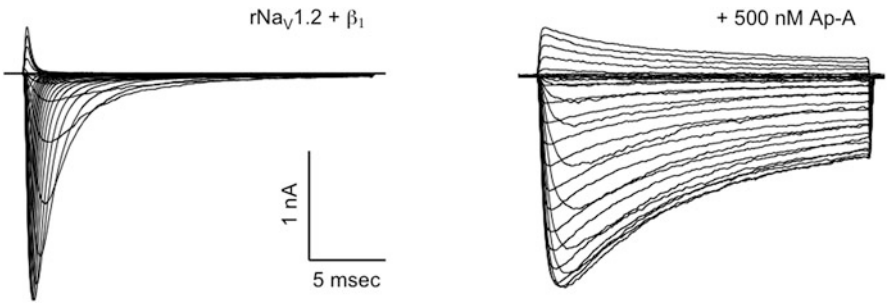

Fig. 8 Patch clamp recordings from *Xenopus* oocyte expressing rNa$_V$1.2 and beta subunit. Anthopleurin slows open-state fast inactivation. Groome, unpublished

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 DIIIS4; 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_VAb 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_VAP (Jiang et al. 2003, 2004; Cuello et al. 2004), and subsequent crystallization and characterization of mammalian *Shaker*-like K_V1.2 (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.

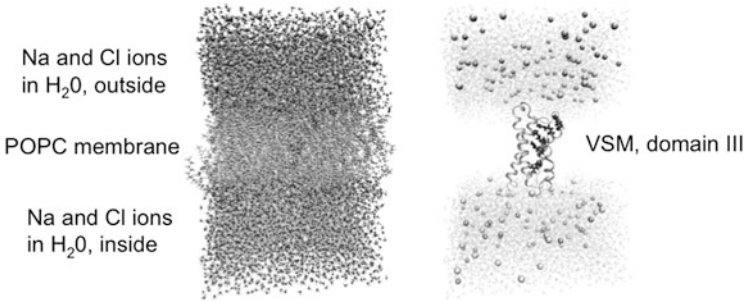

Fig. 9 Voltage sensor module of DIIIS4 of $rNa_V1.4$, embedded in POPC membrane. At *right*, lipid molecules have been removed with S4 charges highlighted. Groome and Winston, unpublished

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 Countercharges 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 countercharges 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 countercharges 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).

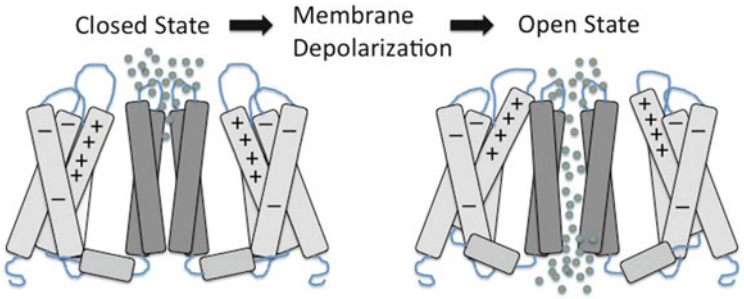

Fig. 10 Model of ion channel activation and pore opening. Countercharges identified in functional investigation of the NacBac channel are shown (−); these form sequential ion pairs with S4 (+) charge to drive that segment outward and via the S4–S5 linker pull open the ion permeation gate of the pore 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_VAb (Payandeh et al. 2011, 2012) and the NacBac orthologue Na_VRh (Zhang et al. 2012) support