Role of the voltage sensor module in Na\textsubscript{v} domain IV on fast inactivation in sodium channelopathies: The implication of closed-state inactivation

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ABSTRACT

The segment 4 (S4) voltage sensor in voltage-gated sodium channels (Na\textsubscript{v}s) have domain-specific functions, and the S4 segment in domain DIV (DIVS4) plays a key role in the activation and fast inactivation processes through the coupling of arginine residues in DIVS4 with residues of putative gating charge transfer center (pGCTC) in DIVS1-3. In addition, the first four arginine residues (R1-R4) in Na\textsubscript{v}s DIVS4 have position-specific functions in the fast inactivation process, and mutations in these residues are associated with diverse phenotypes of Na\textsubscript{v}-related diseases (sodium channelopathies). R1 and R2 mutations commonly display a delayed fast inactivation, causing a gain-of-function, whereas R3 and R4 mutations commonly display a delayed recovery from inactivation and profound use-dependent current attenuation, causing a severe loss-of-function. In contrast, mutations of residues of pGCTC in Na\textsubscript{v}s DIVS1-3 can also alter fast inactivation. Such alterations in fast inactivation may be caused by disrupted interactions of DIVS4 with DIVS1-3. Despite fast inactivation of Na\textsubscript{v}s occurs from both the open-state (open-state inactivation; OSI) and closed state (closed-state inactivation; CSI), changes in CSI have received considerably less attention than those in OSI in the pathophysiology of sodium channelopathies. CSI can be altered by mutations of arginine residues in DIVS4 and residues of pGCTC in Na\textsubscript{v}s, and altered CSI can be an underlying primary biophysical defect of sodium channelopathies. Therefore, CSI should receive focus in order to clarify the pathophysiology of sodium channelopathies.

Introduction

The family of voltage-gated sodium channels (Na\textsubscript{v}s), encoded by the sodium voltage-gated channel alpha subunit gene (SCN\textsubscript{nA}, n = number), consists of nine Na\textsubscript{v}s (Na\textsubscript{v}1.1-Na\textsubscript{v}1.9) [1]. Na\textsubscript{v}s are critical determinants for action potential initiation, formation and propagation in excitable cells, including nervous system and skeletal and cardiac muscles [1,2]. The sodium flow through Na\textsubscript{v}s is regulated by voltage-dependent transitions between resting (closed), activated (open) and inactivated-states [2]. Transition to inactivated-states (inactivation) of Na\textsubscript{v}s is divided into fast, intermediate and slow components, according to the time scale of the inactivation rate. Fast inactivation greatly regulates the sodium flow through Na\textsubscript{v}s and can occur from both an open-state (open-state inactivation; OSI) and a closed state (closed-state inactivation; CSI) [2-4].

The Na\textsubscript{v}s are composed of four homologous but non-identical domains (DIV-DIV), and each domain contains a segment 4 (S4) voltage sensor that consists of positively charged arginine and lysine repeats. S4 segments in Na\textsubscript{v}s have domain-specific functions, especially in the activation and fast inactivation processes [5,6]. For OSI during strong depolarization, the S4 segments in DI-DIV of Na\textsubscript{v}s move outward quickly and then the S4 segments in DIII and DIV become immobilized to promote the opening of the activation gate through a process known as electromechanical coupling, followed by the binding of the inactivation particle (conserved hydrophobic IFM motif in the cytoplasmic region linking DIII and DIV) to its binding sites [2,5-9]. The movement of the S4 segment in DIV (DIVS4) is particularly necessary for binding the inactivation particle to its binding sites [2,4]. On the other hand, for CSI during weak/subthreshold depolarization, only the S4 segments in DIII and DIV move outward without activation gate opening, but this is sufficient to allow access of the inactivation particle to its binding sites [2,4,8]. Conversely, for recovery from inactivation during repolarization, S4 segments return to their hyperpolarized/resting position, which allows the...
inactivation particle to unbind from its binding sites [2,4,8], and movement of DIVS4 is thought to be the rate-limiting step during repolarization [10]. Thus, DIVS4 of Na$_v$s plays a pivotal role in the activation and inactivation processes.

Mutations in Na$_v$s are responsible for multiple disorders (Na$_v$-related diseases: sodium channelopathies) depending on the expression of each of Na$_v$s on organs and its functional abnormalities [11]. Functional defects of mutations in Na$_v$s can be caused by multiple mechanisms, including defective membrane trafficking, production of non-functional channels, and altered channel gating properties. Among them, altered channel gating properties, especially altered fast inactivation, can cause both a gain- and loss-of-function (and the combination of both), which can be associated with diverse phenotypes. However, although alteration of OSI of Na$_v$s has received much attention, that of CSI has received considerably less attention in the pathophysiology of sodium channelopathies.

In this review, we describe the role of the voltage sensor module in Na$_v$ DIV on fast inactivation, including CSI, in sodium channelopathies.

**Voltage sensor module in Na$_v$ DIV**

Recent structural studies of Na$_v$s, such as prokaryotic Na$_v$Ab [12], cockroach Na$_v$PaS [13], eel Na$_v$1.4 [14] and human Na$_v$1.4 [9], have shown that arginine residues in the S4 are in proximity to residues that may facilitate gating charge transfer (putative gating charge transfer center; pGCTC) in the S1-3 segments (Figure 1), and have established the coupling of the S4 with S1-3 in the activation and inactivation processes [15]. For example, in prokaryotic Na$_v$Ab, R105 in S4 forms hydrogen bonds with N25 in S1 [12]. On the other hand, focusing on the most important domain, DIV, in the activation and inactivation processes, in Na$_v$1.4, R1457 in DIVS4 (homologous residue to R105 in Na$_v$Ab) forms hydrogen bonds with N1366 in DIVS1 (homologous residue to N25 in Na$_v$Ab), one of the residues of pGCTC [9,16]. Since homologous residues among Na$_v$s have common functional roles, the arginine residues of each DIVS4 and residues of pGCTC in DIVS1-3 play common pivotal roles in the activation and inactivation processes. Furthermore, it has become apparent that the first four arginine residues (R1-R4) in Na$_v$ s DIVS4 have position-specific functions in the activation and inactivation processes [9,15,17].

![Figure 1](image_url)

**Figure 1.** Upper panel: Predicted topology of Na$_v$. Arginine residues of DIVS4 are indicated by + (yellow), and putative gating charge transfer center (pGCTC) of DIVS1-3 are indicated by ★ (yellow). Lower panel: Positions of pGCTC of DIVS4 in Na$_v$1.4, Na$_v$1.5 and Na$_v$1.1 are listed. Asterisks indicate the positions at which disease-related mutations were functionally characterized.
Defective inactivation by mutations of arginine residues in SCN4A/Na\(_{\text{v}}\)1.4 DIVS4

SCN4A/Na\(_{\text{v}}\)1.4 is predominantly expressed in skeletal muscles, and mutations in Na\(_{\text{v}}\)1.4 are associated with multiple skeletal muscle disorders including myotonia, paramyotonia congenita (PC), hyper or hypokalemic periodic paralysis (hyperPP or hypoPP), and congenital myasthenic syndrome (CMS). Gain-of-function of Na\(_{\text{v}}\)1.4 is typically associated with myotonia, PC, hyperPP and hypoPP, and the clinical overlap of PC and hyperPP is extensive. In contrast, loss-of-function of Na\(_{\text{v}}\)1.4 is typically associated with CMS [11].

Several mutations in Na\(_{\text{v}}\)1.4 DIVS4 have been identified [11], and some of them have been functionally characterized (Table 1). Functional studies of R1 mutations, including R1448S, R1448H, R1448P and R1448C, associated with PC, commonly exhibited a delayed fast inactivation and hyperpolarizing shift in steady-state availability, with either a delayed or accelerated recovery from inactivation [18–24]. R2 mutations, including R1451C and R1451L, associated with PC (with or without hyperPP or hypoPP), also exhibited a delayed fast inactivation and hyperpolarizing shift in steady-state availability [25,26]. Contrary to the report of R1451L by Poulin et al. [25], Luo et al. reported that a homozygous R1451L carrier showed hypoPP and myotonia, whereas heterozygous R1451L carriers showed hyperPP and myotonia, and R1451L displayed an accelerated recovery from inactivation [26]. Intriguingly, they also reported that R1451L displayed an enhanced CSI. As described, mutations of R1 and R2 in Na\(_{\text{v}}\)1.4 commonly exhibited a delayed fast inactivation, resulting in a gain-of-function of Na\(_{\text{v}}\)1.4 and theoretically causing PC, although these mutations also exhibited a hyperpolarizing shift in steady-state availability that leads to a loss-of-function of Na\(_{\text{v}}\)1.4. In contrast, an R3 mutation R1454W and R4 mutation R1457H, associated with CMS, commonly exhibited a hyperpolarizing shift in steady-state availability, delayed recovery from inactivation and profound use-dependent current attenuation [27,28], resulting in a severe loss-of-function of Na\(_{\text{v}}\)1.4 and theoretically causing CMS, although these mutations also exhibited a delayed fast inactivation that leads to a gain-of-function of Na\(_{\text{v}}\)1.4.

Intriguingly, structural studies demonstrated that R1451 (R2) in Na\(_{\text{v}}\)1.4 DIVS4 coupled with E1373 (Figure 1), one of the residues of pGCTC, in DIVS1, and that R1451L disrupted its electrostatic interaction with E1373 [9,14,26]. Luo et al. hypothesized that the R1451L mutation may destabilize the S4 movement that should be immobilized and allow for a quicker recovery of the S4 to the hyperpolarized/resting state, which may lead to an accelerated recovery from inactivation [26]. This concept may be supported by the fact that neutralizing or charged-reversing mutations of E1373 also accelerate the recovery from inactivation [15]. Regarding CSI, the R1451L enhanced and accelerated entry into CSI. However, recovery from CSI has not been evaluated [26].

Defective inactivation by mutations of arginine residues in SCN5A/Na\(_{\text{v}}\)1.5 DIVS4

SCN5A/Na\(_{\text{v}}\)1.5 is predominantly expressed in cardiac muscles and the His-Purkinje conduction system. Gain-of-function of Na\(_{\text{v}}\)1.5 is typically associated with type-3 long QT syndrome (LQT3) [29]. In contrast, loss-of-function of Na\(_{\text{v}}\)1.5 can be associated with Brugada syndrome (BrS) [30], sick sinus syndrome (SSS) [31], atrioventricular conduction block [32] and supraventricular tachyarrhythmias (SVTs) [33,34], and these arrhythmic phenotypes can overlap with a single SCN5A mutation.

Several mutations in Na\(_{\text{v}}\)1.5 DIVS4 have been identified [11,35–37], and some of them have been functionally characterized (Table 1). Mutations of R1 and R2, R1623Q (R1) and R1626P (R2), commonly exhibited a delayed fast inactivation, which leads to a gain-of-function of Na\(_{\text{v}}\)1.5 and can be associated with LQT3 [38–41]. In contrast, mutations of R3 and R4, such as R1629Q (R3), R1632H (R4) and R1632C (R4), commonly exhibited a marked hyperpolarizing shift in steady-state availability, delayed recovery from inactivation and profound use-dependent current attenuation, resulting in a severe loss-of-function of Na\(_{\text{v}}\)1.5 [31,34,42,43]. These functional abnormalities can theoretically be associated with BrS with or without SSS and SVTs.

A comparison of the structural alignment of Na\(_{\text{v}}\)1.5 DIVS4 with Na\(_{\text{v}}\)1.4 DIVS4 revealed arginine residue-specific functional abnormalities. Of note, mutations of R1 and R2 in Na\(_{\text{v}}\)1.5 or Na\(_{\text{v}}\)1.4 displayed a common biophysical defect of a delayed fast
| Gene       | Protein | Arginine position | Mutation | Defective inactivation                                                                 | Phenotype                                      | References                                      |
|------------|---------|-------------------|----------|----------------------------------------------------------------------------------------|------------------------------------------------|------------------------------------------------|
| SCN4A      | Na,v,1.4| R1                | R1448S   | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation | PC                                             | Bendahhou et al. 1999 [18]                     |
| R1         | R1448H  | Delayed fast inactivation, hyperpolarizing shift in SSA, accelerated recovery from inactivation | PC       | Yang et al. 1994 [19]                                                                  |                                                 |                                                 |
| R1         | R1448P  | Delayed fast inactivation, hyperpolarizing shift in SSA | PC       | Mohammadi et al. 2003 [21]                                                             |                                                 |                                                 |
| R1         | R1448L  | N/A                | PC       | Matthews et al. 2008 [23]                                                               |                                                 |                                                 |
| R1         | R1448C  | Delayed fast inactivation, hyperpolarizing shift in SSA, accelerated recovery from inactivation | PC, hypoPP | Yang et al. 1994 [19]                                                                  |                                                 |                                                 |
| R2         | R1451C  | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from slow inactivation, enhanced CSI, accelerated entry into CSI | hypoPP    | Dice et al. 2004 [24]                                                                  |                                                 |                                                 |
| R2         | R1451L  | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from slow inactivation | PC, hyperPP, hypoPP | Poulin et al. 2018 [25]                                                               |                                                 |                                                 |
| R2         | R1451L  | Delayed fast inactivation, hyperpolarizing shift in SSA, accelerated recovery from inactivation | hypoPP and myotonia (homozygous), hyperPP and myotonia (heterozygous) | Luo et al. 2018 [26]                                |                                                 |                                                 |
| R3         | R1454W  | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation, delayed entry into slow inactivation, profound use-dependent current attenuation | CMS      | Habbout et al. 2016 [27]                                                               |                                                 |                                                 |
| R4         | R1457H  | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation, profound use-dependent current attenuation | CMS      | Arnold et al. 2015 [28]                                                                |                                                 |                                                 |
| Gene Protein | Arginine position | Mutation | Defective inactivation | Phenotype | References |
|--------------|-------------------|----------|-----------------------|-----------|------------|
| SCN5A Na\textsubscript{1.5} | R1 | R1623Q | Delayed fast inactivation | LQTS | Kambouris et al. 1998 [38] |
| | R1 | R1623L | N/A | LQTS | Makita et al. 1998 [39] |
| | R2 | R1626H | N/A | LQTS | Splawski et al. 2000 [35] |
| | R2 | R1626H | Delayed fast inactivation, hyperpolarizing shift in SSA | AF | Kapplinger et al. 2009 [36] |
| | R2 | R1626P | Delayed fast inactivation, hyperpolarizing shift in SSA | LQTS | Olesen et al. 2012 [41] |
| | R3 | R1629G | N/A | N/A | Ruan et al. 2007 [40] |
| | R3 | R1629Q | Accelerated fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation, enhanced intermediate inactivation | BrS | Amin et al. 2009 [37] |
| | R4 | R1632H | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation | BrS | Zeng et al. 2013 [42] |
| | R4 | R1632C | Hyperpolarizing shift in SSA, delayed recovery from inactivation, profound use-dependent current attenuation, enhanced CSI, accelerated entry intoCSI, delayed recovery from CSI | SSS, SVT | Benson et al. 2003 [31] |
| | | | | | Gui et al. 2010 [43] |
| | SCN1A Na\textsubscript{1.1} | R1 | R1636Q | N/A | LGS | Harkin et al. 2007 [47] |
| | R1 | R1636Q | N/A | DS | Heron et al. 2010 [48] |
| | R2 | R1639G | N/A | DS | Depienne et al. 2009 [49] |
| | R3 | R1642S | N/A | DS | Huang et al. 2017 [11] |
| | R3 | R1642M | N/A | DS | Lee et al. 2015 [50] |
| | R4 | R1645Q | N/A | DS | Heron et al. 2010 [48] |
| | R5 | R1648H | Delayed fast inactivation | GEFS+ | Lossin et al. 2002 [46] |
| | R5 | R1648M | Delayed fast inactivation | GEFS+2 | Spampanato et al. 2001 [44] |
| SCN2A Na\textsubscript{1.2} | R1 | R1626H | Accelerated recovery from inactivation, decreased use-dependent current attenuation | BrS3 | Soden et al. 2014 [51] |
| | R2 | R1629L | N/A | OS | Nakamura et al. 2013 [52] |
| SCN8A Na\textsubscript{1.6} | R1 | R1617Q | Delayed fast inactivation, depolarizing shift in SSA | EIEE13 | Wagnon et al. 2016 [53] |

AF, atrial fibrillation; BFIS3, benign familial infantile seizure 3; BrS, Brugada syndrome; CMS, congenital myasthenic syndrome; CSI, closed-state inactivation; DS, Dravet syndrome; EIEE13, early infantile epileptic encephalopathy type 13; GEFS+, generalized epilepsy with febrile seizures plus; GEFS+2, generalized epilepsy with febrile seizures plus type 2; HyperPP, hyperkalemic periodic paralysis; HypoPP, hypokalemic periodic paralysis; LGS, Lennox-Gastaut syndrome; LQTS, long QT syndrome; N/A, not available; OS, Ohtahara syndrome; PC, paramyotonia congenita; R1-R5, first five arginine residues (R1, R2, R3, R4, and R5) in domain IV-segment 4; SSA, steady-state availability; SSS, sick sinus syndrome; SVT, supraventricular tachyarrhythmia. Descriptions of closed-state inactivation are written in bold and italic.
inactivation, resulting in a gain-of-function of either Na\textsubscript{1.5} or Na\textsubscript{1.4}. In contrast, mutation of R3 and R4 in Na\textsubscript{1.5} or Na\textsubscript{1.4} displayed common biophysical defects of a hyperpolarizing shift in steady-state availability, delayed recovery from inactivation and profound use-dependent current attenuation, resulting in a severe loss-of-function of either Na\textsubscript{1.5} or Na\textsubscript{1.4}.

**Mutations of arginine residues in other Na\textsubscript{v} DIVS4**

SCN1A/Na\textsubscript{1.1} is predominantly expressed in neuronal cell bodies and axon initial segments in the central nervous system (CNS) [11,44]. Several mutations in Na\textsubscript{1.1} DIVS4 have been identified [11,45,46], including R1636Q (R1) [47,48], found in Lennox-Gastaut syndrome or Dravet syndrome (DS), R1639G (R2) [49], found in DS, R1642S (R3) [11], found in DS, R1642M (R3) [50], found in DS, and R1645Q (R4) [48], found in DS (Table 1). However, none of them have been functionally examined.

SCN2A/Na\textsubscript{1.2} is predominantly expressed in axons and dendrites in the CNS [11,44]. R1626Q (R1) in Na\textsubscript{1.2} [51], found in benign familial infantile seizure, and R1629L (R2) in Na\textsubscript{1.2} [52], found in Ohtahara syndrome, have been identified (Table 1). However, neither of them has been functionally examined. On the other hand, SCN8A/Na\textsubscript{1.6} is predominantly expressed in the neuronal cell bodies and proximal processes in the CNS and nodes of Ranvier in the peripheral nervous system [11,44]. R1617Q (R1) in Na\textsubscript{1.6} [53], found in early infantile epileptic encephalopathy type 13, displayed a delayed fast inactivation and depolarizing shift in steady-state availability, causing a gain-of-function of Na\textsubscript{1.6} (Table 1). Notably, the delayed fast inactivation of R1617Q (R1) in Na\textsubscript{1.6} is consistent with R1 mutations in Na\textsubscript{1.4} and Na\textsubscript{1.5} which also show a delayed fast inactivation. This fact further supports the notion that arginine residues in Na\textsubscript{v}s DIVS4 have position-specific functions in each of Na\textsubscript{v}s.

**Defective inactivation by mutations of pGCTC in Na\textsubscript{1.4} DIVS1-3**

In Na\textsubscript{1.4}, D1356, N1366, E1373, N1389, F1396, E1399 and D1420 in DIVS1-S3 are thought to be residues of pGCTC (Figure 1) and couple with arginine residues in DIVS4 in the activation and inactivation processes [9,15]. Among them, several substituted residues have been structurally and functionally examined (Table 2).

The functional effects of substitutions of these residues in Na\textsubscript{1.4} may vary depending on not only the position but also the charge of the substituted residues. Of note, structural studies showed that N1366 in DIVS1 forms hydrogen bonds with R1457 (R4) in DIVS4, and a functional study of N1366D (replaced with a negatively charged residue) displayed a hyperpolarizing shift in steady-state availability, altered voltage-dependence of fast inactivation and delayed recovery from inactivation, causing a loss-of-function of Na\textsubscript{1.4} [9,15] (Table 2). Conversely, N1366S (replaced with an uncharged residue), found in PC, displayed a cold-induced hyperpolarizing shift in activation, depolarizing shift in steady-state availability, delayed fast inactivation and accelerated recovery from inactivation, causing a gain-of-function of Na\textsubscript{1.4} that can be associated with PC [54] (Table 2). Intriguingly, a structural study of N1366S demonstrated disrupted interactions of N1366S and an arginine residue in DIVS4 at low temperature [54]. On the other hand, a structural study showed that D1420, one of the residues of pGCTC, in Na\textsubscript{1.4} DIVS3 is in close proximity to R1635 (R5) in DIVS4, and a functional study showed that D1420K (replaced with a positively charged residue) displayed a delayed fast inactivation and altered voltage-dependence of recovery from inactivation (Table 2) [15]. These findings reconfirm the importance of the coupling of arginine residues in DIVS4 with pGCTC in DIVS1-3 in the activation and inactivation processes. Regarding CSI, none of them have been examined.

**Defective inactivation by mutations of pGCTC in Na\textsubscript{1.5} DIVS1-3**

In Na\textsubscript{1.5}, D1531, N1541, E1548, N1564, F1571, E1574 and D1595 in DIVS1-S3 are thought to be residues of pGCTC (Figure 1) [9,15]. Among several disease-related mutations of these residues, D1595N (D1595 is the homologous residue to D1420 in Na\textsubscript{1.4}) in DIVS3, found in atrioventricular conduction block, and N1541D (N1541 is the homologous residue to N1366 in Na\textsubscript{1.4}) in DIVS1, found in BrS with SSS and SVTs, have been functionally examined (Table 2) [55-57].
D1595N (replaced with an uncharged residue) displayed a delayed fast inactivation, depolarizing shift in steady-state availability, delayed recovery from inactivation and enhanced slow inactivation (Table 2) [56]. In contrast, N1541D (replaced with a negatively charged residue) displayed a hyperpolarizing shift in steady-state availability, delayed fast inactivation and delayed recovery from inactivation (Table 2) [57]. It is noteworthy that these kinetic changes resemble those of N1366D in Na\textsubscript{v}1.4 [15]. Furthermore, N1541D displayed an enhanced CSI and accelerated entry into CSI without alteration of recovery from CSI (Tables 2 and 3) [57].

### Mutations of pGCTC in other Na\textsubscript{v} DIVS1-3

In Na\textsubscript{v}1.1, D1544, N1554, E1561, N1577, F1584, E1587 and D1608 are thought to be residues of pGCTC (Figure 1) [9,15]. Although several disease-related mutations, such as D1544A, D1544G, E1561K, D1608G and D1608Y, of residues of pGCTC in Na\textsubscript{v}1.1 have been identified, none have been functionally examined (Table 2) [11,58,59]. No disease-related mutations of residues of pGCTC in other Na\textsubscript{v}s have been reported.

### Coupling of S4 with S1-3 in Na\textsubscript{v} DIV may be implicated in the CSI process

Enhanced CSI by mutations of each Na\textsubscript{v} promotes the significant loss of channel availability, which can underlie the pathophysiology of sodium channelopathies. However, in functional analyses of disease-related mutations in Na\textsubscript{v}s, although alteration of OSI has been analyzed in detail, that of CSI has received less attention. The CSI of disease-related mutations in Na\textsubscript{v}1.4 has been the most studied among Na\textsubscript{v}s. Functional analyses of R1128C (R3) and R1128H (R3) mutations in rat Na\textsubscript{v}1.4 DIllS4, equivalent to R1135C (R3) and R1135H (R3) mutations in human Na\textsubscript{v}1.4 DIllS4 found in hypoPP, respectively, displayed an enhanced CSI, accelerated entry into CSI and delayed recovery from CSI (Table 3). It has been shown that,
during fast inactivation of Na\(_{1.4}\), DIII\(\text{S}4\) and DIV\(\text{S}4\) move outward, with immobilization of the DIII\(\text{S}4\) coupled to the binding of inactivation particle to its receptor, and return to its hyperpolarizing/resting position during repolarization [5,10]. Taken together with the results of structure-function studies, these CSI alterations of R1128C (R3) and R1128H (R3) mutations in rat Na\(_{1.4}\) DIII\(\text{S}4\) may be caused by an impaired DIII\(\text{S}4\) movement through disrupted native electrostatic interactions of DIII\(\text{S}4\) and pGCTC in DIII\(\text{S}2\) [60,61]. In contrast, R1451L (R2) in Na\(_{1.4}\) DIV\(\text{S}4\) displayed an enhanced and accelerated entry into CSI (Table 3), possibly by an impaired DIV\(\text{S}4\) movement through disrupted interactions of R1451L and a residue (E1373) of pGCTC in DIV\(\text{S}1\) [26], consistent with the notion that neutralization of arginines in Na\(_{1.4}\) DIV\(\text{S}4\) enhances CSI [62], although recovery from CSI of R1451L has not been examined. These findings suggested the involvement of coupling of DIII\(\text{S}4\) or DIV\(\text{S}4\) with each pGCTC in CSI.

Table 3. Disease-related mutations in Na\(_{1.4}\) and Na\(_{1.5}\) that enhance closed-state inactivation.

| Gene  | Protein Location | Mutation  | Defective inactivation | Phenotype | References |
|-------|------------------|-----------|------------------------|-----------|------------|
| SCN4A | DIII\(\text{S}4\) (R3) | R1135C* (rat R1128C) | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation, enhanced CSI, delayed recovery from CSI | hypoPP | Groome et al. 2014 [60] Groome et al. 2014 [61] |
|       | DIII\(\text{S}4\) (R3) | R1135H* (rat R1128H) | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation, enhanced CSI, delayed recovery from CSI | hypoPP | Groome et al. 2014 [60] |
|       | DIV\(\text{S}4\) (R2) | R1451L | Delayed fast inactivation, hyperpolarizing shift in SSA, accelerated recovery from inactivation, enhanced CSI, accelerated entry into CSI | hypoPP and myotonia (homozygous) hyperPP and myotonia (heterozygous) | Luo et al. 2018 [26] |
| SCN5A | DII-DIII linker | E1053K | Accelerated fast inactivation, delayed recovery from inactivation, enhanced intermediate inactivation, enhanced CSI | BrS | Mohler et al. 2004 [66] |
|       | DIII-DIV linker | Delta KPQ | Delayed fast inactivation, enhanced CSI, accelerated entry into CSI | LQTS | Viswanathan et al. 2001 [63] Chen et al. 2002 [64] |
|       | DIV\(\text{S}1\) | N1541D | Delayed fast inactivation, hyperpolarizing shift in SSA, delayed recovery from inactivation, enhanced CSI, accelerated entry into CSI | BrS, SSS, SVT | Dharmawan et al. 2019 [57] |
|       | DIV\(\text{S}4\) (R4) | R1632C | Hyperpolarizing shift in SSA, delayed recovery from inactivation, profound use-dependent current attenuation, enhanced CSI, accelerated entry into CSI, delayed recovery from CSI | BrS, SSS, SVT | Nakajima et al. 2005 [34] Dharmawan et al. 2019 [57] |
|       | C-terminus | 1795insD | Delayed fast inactivation, delayed recovery from inactivation, profound use-dependent current attenuation, hyperpolarizing shift in SSA, enhanced slow inactivation, enhanced CSI, accelerated entry into CSI, delayed recovery from CSI | LQTS, BrS | Veldkamp et al. 2000 [67] Viswanathan et al. 2001 [63] |
|       | C-terminus | L1825P | Delayed fast inactivation, hyperpolarizing shift in SSA enhanced CSI, accelerated entry into CSI | LQTS | Makita et al. 2002 [65] |
in native Na\textsubscript{\textalpha},1.4, and may support the notion that, during subthreshold depolarization, outward movement across the membrane and subsequent immobilization of DIII\textsubscript{4} and DIV\textsubscript{4} of native Na\textsubscript{\textalpha},1.4 drives entry into CSI, and its remobilization drives recovery from CSI during repolarization [2].

Regarding Na\textsubscript{\textalpha},1.5, enhanced CSI and accelerated entry into CSI of several mutations, such as ΔKPQ in DIII-DIV linker, L1825P in C-terminus, E1053K in DII-DIII linker and 1795insD in C-terminus, have been reported [63–67]. However, recovery from CSI has never been examined. We recently examined the CSI of N1541D (homologous residue to N1366 in Na\textsubscript{\textalpha},1.4), one of the residues of pGCTC, in DIV\textsubscript{1} and R1632C (R4) (homologous residue to R1457 in Na\textsubscript{\textalpha},1.4) in DIV\textsubscript{4} in Na\textsubscript{\textalpha},1.5, both of which were found in Br\textupsilon\textsubscript{S} with SSS and SVTs [34,57]. Both N1541D and R1632C displayed an enhanced CSI; however, the mechanisms underlying CSI were not uniform. N1541D displayed a marked acceleration of the entry into CSI without alteration of recovery from CSI, whereas R1632C displayed a slight acceleration of the entry into CSI and marked delay of recovery from CSI. These findings shed light on the mechanisms underlying CSI and the structure-function relationships of Na\textsubscript{\textalpha},1.5. The marked acceleration of the entry into CSI of N1541D may be caused by a voltage-dependent modification of coupling of N1541D in DIV\textsubscript{1} with R1632 in DIV\textsubscript{4} during subthreshold depolarization; N1541D markedly affects the coupling in the CSI process. In contrast, the marked delay of the recovery from CSI of R1632C may be caused by a slowed remobilization of DIV\textsubscript{4} to the hyperpolarized/resting position during repolarization through disrupted native electrostatic interactions of DIV\textsubscript{4} and DIV\textsubscript{1}, as with the R1128C (R3) and R1128H (R3) mutations in rat Na\textsubscript{\textalpha},1.4 DIII\textsubscript{4}.

Given the resemblance of functional abnormalities among homologous residues in Na\textsubscript{\textalpha}s, neutralizing or charge-reversing mutations of arginine residues (R1-R4) in DIV\textsubscript{4} or residues of pGCTC in DIV\textsubscript{1}-3 (such as mutations of R1457 and N1366 in Na\textsubscript{\textalpha},1.4, homologous residues to R1632 and N1541 in Na\textsubscript{\textalpha},1.5, respectively, and mutations of R1626 in Na\textsubscript{\textalpha},1.5, a homologous residue to R1451 in Na\textsubscript{\textalpha},1.4) may modify the native electrostatic interactions of DIV\textsubscript{4} and DIV\textsubscript{1}-3 in the activation and inactivation processes, including CSI. Further structure-function studies will be required to prove this hypothesis. Furthermore, in addition to the importance of coupling of DIV\textsubscript{4} with DIV\textsubscript{1}-3 in the CSI process, other mechanisms involved in CSI may be present and need to be elucidated. Clinically, enhanced CSI of R1632C and N1541D in Na\textsubscript{\textalpha},1.5 is the primary biophysical defect causing a severe loss-of-function of Na\textsubscript{\textalpha},1.5 and may underlie the pathophysiology of overlapping arrhythmogenic phenotypes [57]. In this manner, enhanced CSI of other mutations in other Na\textsubscript{\textalpha}s may also be an underlying factor as a primary biophysical defect in sodium channelopathies.

### Lack of gating pore currents by mutations of arginine residues in Na\textsubscript{\textalpha}, DIV\textsubscript{4}

Although the arginine residues of the S4 segments in DIII and DIV appear to have domain-specific important roles in the fast inactivation, those in DI and DII do not appear to have important roles [5,6]. In addition, the role of the S4 segments in Na\textsubscript{\textalpha}s differs with respect to gating pore currents (or ω-currents) [68–70].

Gating pore currents were originally observed in the Shaker channel by Starace and Bezanilla [71]. Substitutions of the R1 residue of S4 in the Shaker channel conduct protons or non-selective cations directly through the voltage sensor domains (gating pore or ω-pore), not through alpha (α)-pore, depending on substituted residues. It is thought that disrupted interaction of substituted arginine with pGCTC may create a water crevice spanning the membrane and open a continuous aqueous pathway [68–72]. On the other hand, in Na\textsubscript{\textalpha}s, gating pore currents were first observed in substitutions of R1 and R2 in Na\textsubscript{\textalpha},1.2 DII\textsubscript{4} [73]. Later, observations of gating pore currents spread to other Na\textsubscript{\textalpha}s. It is noteworthy that the gating pore currents in Na\textsubscript{\textalpha}s have small amplitudes (less than 1% of the ω-pore conductance) and are smaller than those in the voltage-gated potassium channels (K\textsubscript{s}s) because of the formation of α-pores: K\textsubscript{s}s have four identical gating pores but Na\textsubscript{\textalpha}s have only a single gating pore in each α-pore. Moreover, substitutions of the arginine residues of S4 in DIV are more resistant to the creation of gating pore currents than those in DI-DIII, possibly because the pGCTC in DIV is spread over a larger distance [70,74,75].

Evidence is accumulating on the pathophysiological roles of gating pore currents in sodium channelopathies, although there is no apparent evidence of
their pathophysiological roles in potassium channelopathies. Curiously, in Na,v, all hypoPP mutations that have been identified are missense substitutions at arginine (R1 or R2) residues of S4. More surprisingly, all mutations in DI-DIII that have been examined, except for those in DIV, create gating pore currents that flow either protons or non-selective cations, depending on the arginine positions and substituted residues [26,70,74,75]. For example, R669H (R1) and R672H (R2) in DIVS4 create gating pore currents that conduct protons. In contrast, R672G/S/C (R2) in DIVS4 create gating pore currents that conduct non-selective cations. In either case, these gating pore currents may account for the paradoxical depolarization in low serum potassium, resulting in paralysis, in spite of the loss-of-function of Na,v,1.4 [26,70,74,75]. Intriguingly, R1135C (R3) in Na,v,1.4 DIVS4, which is associated with hypoPP, showed enhanced CSI over a voltage range for which this mutation increases gating pore currents. This suggests that an increase of gating pore currents at subthreshold voltages may be one of the causes for the enhancement of CSI [61].

On the other hand, in Na,v,1.5, gating pore currents are thought to be associated with a particular clinical phenotype: dilated cardiomyopathy (DCM) with cardiac arrhythmias [69,70]. Most mutations in Na,v,1.5 associated with this phenotype are located at S4 in DI and DII, but are not present in DIV. R219H (R1) mutation of S4 in DI, which does not promote apparent alteration in Na,v,1.5, creates gating pore currents that conduct protons [68,70,76]. In contrast, R222Q (R2), R225P (R3) and R225W (R3) mutations in DI and R814W (R3) mutation in DII, which promote either gain- or loss-of-function of Na,v,1.5, create gating pore currents that conduct non-selective cations [69,70,77–79]. Notably, it appears that mutations of arginine residues in DIVS4 do not create gating pore currents and are not associated with DCM (Table 1). The mechanisms through which the gating pore currents of Na,v,1.4 and Na,v,1.5 contribute to the clinical phenotypes need to be clarified.

Conclusions

S4 segments in Na,v,s have domain-specific functions in terms of fast inactivation and gating pore currents. Regarding the fast inactivation, R1-R4 mutations in Na,v, DIVS4 have position-specific functions. R1 and R2 mutations in Na,v, DIVS4 commonly display a delayed fast inactivation, which causes a gain-of-function of Na,v. R3 and R4 mutations in Na,v, DIVS4 commonly display a marked hyperpolarizing shift in steady-state availability, delayed recovery from inactivation, and profound use-dependent current attenuation, which cause a severe loss-of-function of Na,v. Thus, R1-R4 mutations in Na,v, DIVS4 exhibit position-specific diverse phenotypes in sodium channelopathies. Regarding the gating pore currents, mutations of arginine residues of S4 in Na,v, DI-DIII, but not DIV, create those, which may underlie the pathophysiology of diverse clinical phenotypes. On the other hand, mutations of residues of pGCTC in Na,v, DIVS1-3 can also alter fast inactivation. These changes in fast inactivation of Na,v,s may be caused by disrupted interactions of DIVS4 and DIVS1-3. In addition, mutations of arginine residues in Na,v, DIVS4 and residues of pGCTC in Na,v, DIVS1-3 can alter not only OSI but also CSI through disrupted interactions of DIVS4 and DIVS1-3. Since altered CSI can be an underlying factor as a primary biophysical defect in sodium channelopathies, CSI should receive a greater degree of focus going forward in order to clarify the pathophysiology of sodium channelopathies.

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