Functional interaction between S1 and S4 segments in voltage-gated sodium channels revealed by human channelopathies

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The p.I141V mutation of the voltage-gated sodium channel is associated with several clinical hyper-excitability phenotypes. To understand the structural bases of the p.I141V biophysical alterations, molecular dynamics simulations were performed. These simulations predicted that the p.I141V substitution induces the formation of a hydrogen bond between the Y168 residue of the S2 segment and the R225 residue of the S4 segment. We generated a p.I141V-Y168F double mutant for both the NaV1.4 and NaV1.5 channels. The double mutants demonstrated the abolition of the functional effects of the p.I141V mutation, consistent with the formation of a specific interaction between Y168-S2 and R225-S4. The single p.Y168F mutation, however, positively shifted the activation curve, suggesting a compensatory role of these residues on the stability of the voltage-sensing domain.

Introduction

In excitable tissues, the upstroke of the action potential is the result of the passage of a large and rapid influx of sodium ions (INa) through voltage-gated sodium channels (NaV). NaV dysfunction has been associated with an increasingly wide range of neurological, muscular and cardiac disorders. Mutations in the genes SCN1A, SCN4A and SCN5A, which encode NaV1.1, NaV1.4 and NaV1.5, respectively, lead to various epilepsy syndromes, muscular disorders and inherited cardiac arrhythmias.1-3

NaV channels are monomers with a tetrameric repeat of 6 transmembrane (TM) segments. The first 4 TM segments (S1-S4) comprise the voltage-sensing domain (VSD), and the last 2 TM segments (S5 and S6) form the pore of the channel when assembled in a tetrameric configuration. The S1 segment of domain I contains amino acids that are highly conserved between different species and different NaV isoforms. One of these amino acids is isoleucine 141. Its substitution to valine in NaV1.4, NaV1.5 and NaV1.7 channels has been linked to inherited channelopathies in patients with hyper-excitability phenotypes, i.e. myotonia, exercise-induced polymorphic ventricular arrhythmias and erythromelalgia.1-6

The p.I141V mutation in NaV1.4 and NaV1.5, as well as its homologous p.I136V mutation in NaV1.7, induce similar modifications in the biophysical properties of the voltage-gated sodium channels3-6 by shifting the voltage-dependence of steady state activation toward more negative potentials and hastening the activation and inactivation kinetics. In addition, the INa generated by these mutant channels have larger sodium window current peaks which are shifted toward more negative potentials. These observations highlight the important role of isoleucine 141 in the gating process of NaV channels.

The recently published crystal structure of the bacterial NaVAb channel demonstrates close proximity between the S1 and S4 segments of the VSD.7 Based on the fact that NaVAb can be used as a homolog for the mammalian NaV1.4, NaV1.5 and NaV1.7 channels, we generated a model of NaV1.4 and investigated the interplay between the p.I141V mutation and the stability of its VSDs. Through molecular dynamics (MD) modeling and biophysical characterization, we strived to determine if a modification of intra-segment interactions inside the VSD domain could stabilize...
the activated state of the channel that carries the p.I141V mutation.

**Results**

The p.I141V mutation stabilizes the activated state of Na,1.4 and Na,1.5

The functional effects of the p.I141V mutation on Na,1.4 and Na,1.5 were investigated using the whole-cell configuration of the patch-clamp technique. The initial step was to reproduce, under similar experimental conditions, previously published data regarding the effect of the p.I141V mutation on the voltage dependence of activation and inactivation of Na,1.4 and Na,1.5 found in patients with myotonia and cardiac arrhythmias (Fig. 1A-F).3,4 For both channels, the presence of the p.I141V mutation shifted the voltage dependence of activation toward negative potentials (Na,1.4-V1/2 act: \(-18 \pm 1.4 \text{ mV}, n = 9, \text{versus} -28 \pm 2.1 \text{ mV}, n = 5, ***P < 0.001; \text{slope}: 5.3 \pm 0.8 \text{ mV/nm})

![Figure 1](https://www.landesbioscience.com/Channels/415/)

Figure 1. (A and B) Representative current traces obtained with protocol in inset using HEK293 cells transfected with Na,1.4-WT and Na,1.4-I141V (A); Na,1.5-WT and Na,1.5-I141V (B). (C and D) Current-voltage (I/V) relationships curves of Na,1.4-WT and Na,1.4-I141V (C); Na,1.5-WT and Na,1.5-I141V (D). (E and F) Steady-state activation and inactivation curves for Na,1.4 (E) and Na,1.5 (F). Activation properties were determined from I/V relationships by normalizing peak INa to driving force and maximal INa. The protocol for the voltage-dependence of steady state of inactivation was 20-ms test pulse to 0 mV (Na,1.4) or \(-10 \text{ mV} (Na,1.5) \text{ after a 500 ms conditioning pre-pulse.}
The p.Y168F mutation destabilizes the VSD and abolishes the functional effect of the p.I141V mutation in Na\textsubscript{1,4} and Na\textsubscript{1,5}.

The MD simulations suggest that the p.I141V activated state is more stable than the WT. The stabilization of the DI VSD is proposed to be caused by the formation of a hydrogen bond between Y168 of the S2 segment (S2-DI) and R225 of the S4 segment (S4-DI) (Fig. 2C). To test this hypothesis, the Na\textsubscript{1,4} and Na\textsubscript{1,5} channels were mutated by substituting the Y168 residues for phenylalanines. The double mutant p.I141V-Y168F was also generated in order to prevent formation of the hypothetical hydrogen bond between Y168 and R225. Interestingly, the biophysical characterization of the p.I141V-Y168F double mutants showed abolition of the p.I141V effect on the Na\textsubscript{1,4} and Na\textsubscript{1,5} channels (Fig. 3A-F). The calculated half-potential values of the steady state of activation and inactivation of the WT channels and the p.I141V-Y168F mutants were not statistically different. The slope of the activation curve for Na\textsubscript{1,4} was not significantly modified. The voltage dependence of activation was shifted toward more positive potentials for the single mutant p. Y168F (Table 1, Fig. 3C-F), but the voltage dependencies of inactivation were not affected for the mutants nor the WT (Table 1, Fig. 3E and F).

**Discussion**

The substitution of the highly conserved isoleucine 141 to valine in the Na\textsubscript{1,4}, Na\textsubscript{1,5} and Nav1.7 channels has been associated with several inherited disorders of excitability, such as myotonia, cardiac arrhythmias, and erythromelalgia. This study investigated the biophysical effects of the p.I141V mutant on these channels and demonstrated that the p.I141V substitution stabilized the activated state of the DI VSD, manifested by a negative shift of the voltage dependence of the activation curve.

The crystal structure of the bacterial channel Na\textsubscript{Ab}, published by the Catterall group, shows close proximity between segments S1 and S4 of the VSD. Based on this observation, the hypothesis that the p.I141V substitution stabilizes the open conformation of the mammalian voltage-gated sodium channel Na\textsubscript{1,4} by modifying or creating new interactions between these specific segments was tested. An atomistic model of Na\textsubscript{1,4} was built using homology modeling and the structure of the Na\textsubscript{Ab} channel as a template. Unconstrained atomistic MD simulation data suggested that the p.I141V mutation influences the position of the Y168 residue in S2-DI. The Y168 residue was predicted to be spatially closer to R225 of the S4 segment in the mutant VSD, allowing for the formation of a hydrogen bond between the Y168 hydroxyl group and the R225 backbone. The observed hydrogen bond could be responsible for the stabilization of the activated state of the mutant channel in comparison to the WT. Therefore, to abolish the effect of the p.I141V mutation, the formation of a hydrogen bond in the mutant should be prevented.

Based on these predictions, a single (p.Y168F) and double mutant (p.I141V-Y168F) were generated in order to suppress...
the hydroxyl group of tyrosine, which was found to form a hydrogen bond with R225. The functional analyses of these mutant channels demonstrated the abolition of the p.I141V effect on the voltage dependency of activation for both the Nav1.4 and Nav1.5 channels. The recovery of the WT biophysical parameters in the double mutant (p.I141V-Y168F) is consistent with the MD simulation predictions. The functional experimental data of the single mutation (p.Y168F) showed a destabilization of the activated state of the Nav1.4 and Nav1.5 channels. This was reflected by a significant shift of the activation curves to more depolarized potentials. This effect was unexpected and raises the question of whether the neutralization of the biophysical effect of the p.I141V mutant is due to the abolition of a newly formed hydrogen bond or due to an additive effect of the p.I141V and p.Y168F mutants on sodium channel function. Given that the double mutation p.I141V-Y168F significantly modified the slope of the activation curve of Na\textsubscript{v}1.4, the hypothesis that the recovery of WT behavior
in the p.I141V-Y168F mutant is due to the functional changes of both channels induced by the given mutation seems to be more probable.

On the other hand, the functional effects of the p.I141V, p.Y168F, and p.I141V-Y168F mutation were slightly different between Nav1.4 and Nav1.5 channels. For example, in the presence of the p.I141V mutation, a difference of 10 mV and 8 mV in the $V_{1/2}$ of steady-state activation was observed for Nav1.4, and Nav1.5, respectively. This difference may be due to different intrinsic protein interactions that stabilize the open confirmation of the channels.
in these 2 channels. Several studies demonstrated that the substitution of one VSD residue is sufficient to affect its function.\textsuperscript{9,10} Although Nav1.4 and Nav1.5 are homologous channels with high sequence similarities, the environments of the S4 positive residues are not identical. This may result in different characteristics for the wild type channels and therefore may lead to different effects depending on the mutation that is introduced.

Altogether, these results highlight the functional role of 2 different segments (S1-S2) of the VSD in voltage-gated sodium channel gating, even though the exact interactions that influence the stability of the VSD are not yet fully understood.

### Materials and Methods

#### Site-directed mutagenesis

Site-directed mutagenesis was performed on pRC-CMV-hSCN4A or pCDN3.1-hSCN5A using the Quick-Change II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

#### Cell culture

Human Embryonic Kidney 293 cells (HEK293) were cultured at 37°C in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (Fetal bovine serum) FBS, 4 mM glutamine, and 20 mg/ml gentamicin in a humidified atmosphere of 5% CO2 and 95% air. All cell medium components, except glucose, and 20 mg/ml gentamicin in a humidified atmosphere of 5% CO2 and 95% air. All cell medium components, except glucose, were bathed with an extracellular solution containing (in mmol/L): NaCl 140, CaCl2 1, MgCl2 1, HEPES 10, EGTA 11 and Na2ATP 5. pH was adjusted to 7.4 with CsOH. Glass pipettes were filled with an intracellular medium containing (in mmol/L): CsCl 60, aspartic acid 50, CaCl2 1, MgCl2 1, HEPES 10, EGTA 11 and Na2ATP 5. pH was adjusted to 7.2 with CsOH.

#### Molecular modeling

The initial molecular model of Nav1.4 was built using homology modeling and the crystal structure of the bacterial Na+Ab channel (PDB code 4EKW) served as a template.\textsuperscript{7} Fifty different models of Nav1.4 were built using MODELLER.\textsuperscript{11} Ten were chosen based on their scoring functions (MOLDPF, DOPE and GA341). The quality of each model was analyzed using PROCHECK.\textsuperscript{12} The structure with the highest number of residues in the core regions of the Ramachandran plot and the lowest number of residues in the disallowed regions was selected for the current study. The selected model contained 94% of residues in the core regions and no residues in the disallowed regions.

The selected structure of Nav1.4 (both the native form and the I141V mutant) was embedded in a palmitoyl-oleyl-phosphatidylcholine (POPC) hydrated bilayer, surrounded by a KCl salt solution at a physiological concentration of 150 mM (total number of atoms: ~180,000). The CHARMM22 with the CMAP correction and the CHARMM36 force fields were considered for the protein and the lipids, respectively.\textsuperscript{13,14} Water was represented by the TIP3P model.\textsuperscript{15}

The molecular dynamics simulations were performed using NAMD.\textsuperscript{16} Langevin dynamics were applied to keep the temperature (300 K) and the pressure (1 atm) constant. The time-step of the simulations was 2.0 fs. Short- and long-range forces were calculated every 1 and 2 time-steps, respectively. Long-range electrostatics were calculated using Particle Mesh Ewald (PME). The cutoff distance of short-range electrostatics was taken to be 11 Å. A switching function was used between 8 and 11 Å to smoothly bring the vdW forces and energies to 0 at 11 Å. The equations of motion were integrated using a multiple time-step algorithm. During the calculations, chemical bonds between hydrogen and heavy atoms were constrained to their equilibrium values. Three d periodic boundary conditions were applied.

The following protocol of equilibration was used: 1) the entire protein was fixed for a 6 ns MD run in order to ensure the relaxation of

### Table 1. Electrophysiological characteristics of Nav1.4 and Nav1.5 (p.I141V-Y168F, and p.Y168F)

|                | Nav1.4 Activation | Nav1.4 Inactivation | Nav1.5 Activation | Nav1.5 Inactivation |
|----------------|-------------------|---------------------|-------------------|---------------------|
| V1/2 (mV)      | −18 ± 1.4         | −59 ± 1.9           | −32 ± 1.3         | −75 ± 1.6           |
| K (mM)         | 5.3 ± 0.4; n = 9  | 5.2 ± 0.2; n = 7    | 5.8 ± 0.2; n = 11 | 5.5 ± 0.3; n = 11   |
| n              |                   |                     |                   |                     |
|                | V1/2 (mV)         | −20 ± 1.8           | −31 ± 1.7         | −73 ± 2.6           |
| K (mM)         | 6.6 ± 0.3*; n = 7 | 6.3 ± 0.5; n = 9    | 6.1 ± 0.4; n = 8  | 5.7 ± 0.4; n = 9    |
| n              |                   |                     |                   |                     |
|                | V1/2 (mV)         | −13 ± 1.3*; K = 6.5 ± 0.3*; n = 7 | −21 ± 1.4***; K = 7.2 ± 0.3*; n = 11 | −73 ± 1.6; K = 5.7 ± 0.4; n = 9 |
| K (mM)         |                   |                     |                   |                     |
| n              |                   |                     |                   |                     |

*P < 0.05.
**P < 0.01.
***P < 0.001.
the lipids and the solution, 2) the backbone atoms were then constrained during an 8 ns MD run to enable subsequent reorganization of the side-chain groups, 3) the salt-bridges R225-E161, K228-E171, K228-D197, K175-E171 and K175-D197 in the DI VSD were also retrained, and 4) all constraints were released and the systems were further equilibrated for ~100 ns. The analysis of non-bonded energy was performed for the last 50 ns of the equilibration, for which the root mean square deviations (RMSD) from the initial structure of the channel calculated for the backbone atoms reached plateau.

Data analysis and statistical methods
The currents were analyzed with the Clampfit software (Axon Instruments, Inc.). The data were analyzed using a combination of pClamp10, Excel (Microsoft) and Prism (Graphpad). The comparisons between groups were performed with 2-tailed Student’s t test. All data shown are expressed as mean ± SD. A P-value < 0.05 was considered significant.

References
1. Abriel H, Zaklyazminskaya EV. Cardiac channelopathies: genetic and molecular mechanisms. Gene 2013; 517:1-11; PMID:23266818; http://dx.doi.org/10.1016/j.gene.2012.12.061
2. Meisler MH, O’Brien JE, Sharkey LM. Sodium channel gene family: epilepsy mutations, gene interactions and modifier effects. J Physiol 2010; 588:1841-8; PMID:20351042; http://dx.doi.org/10.1113/jphysiol.2010.188482
3. Petitprez S, Taib L, Chen L, Kappeler L, Rosler KM, Schorderet D, Abriel H, Burgunder JM. A novel dominant mutation of the Nav1.4 alpha-subunit domain I leading to sodium channel myotonia. Neurology 2008; 71:1669-75; PMID:19015483; http://dx.doi.org/10.1212/00002293-86248.55
4. Swan H, Amarouch MY, Leinonen J, Marjamaa A, Kucera JP, Laitinen-Forsblom PJ, Lahtinen AM, Palotie A, Kontula K, Toivonen L, et al. A Gain-of-Function Mutation of the SCN5A Gene Causes Exercise-induced Polymorphic Ventricular Arrhythmias 2014; http://dx.doi.org/10.1161/CIRCGENETICS.114.000703
5. Cheng X, Dib-Hajj SD, Tyrrell L, Waxman SD. A mutant sodium channel in a family with onset of erythromelalgia in the second decade. Mol Pain 2008; 4:1; PMID:18171466; http://dx.doi.org/10.1186/1744-8069-4-1
6. Lee MJ, Yu HS, Hsieh ST, Stephenson DA, Lu CJ, Yang CC. Characterization of a familial case with primary erythromelalgia from Taiwan. J Neurol 2007; 254:210-4; PMID:17294067; http://dx.doi.org/10.1007/s00415-006-0328-3
7. Payandeh J, Scheuer T, Zheng N, Catterall WA. The crystal structure of a voltage-gated sodium channel. Nature 2011; 475:353-8; PMID:21734777; http://dx.doi.org/10.1038/nature10238
8. Long SB, Tao X, Campbell EB, MacKinnon R. Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. Nature 2007; 455:376-82; PMID:18004576; http://dx.doi.org/10.1038/nature06265
9. Lacroix JJ, Benaimila F. Tuning the voltage-sensor motion with a single residue. Biophys J 2012; 103:23-5; PMID:22947880; http://dx.doi.org/10.1016/j.bpj.2012.06.030
10. Lacroix JJ, Campos FV, Frezza L, Benaimila F. Molecular bases for the asynchronous activation of sodium and potassium channels required for nerve impulse generation. Neuron 2013; 79:651-7; PMID:23972594; http://dx.doi.org/10.1016/j.neuron.2013.05.036
11. Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, Sali A. Comparative protein structure modeling using modeller. Curr Protoc Bioinforma 2006; Chapter 5:Unit 5 6; PMID:18429317; http://dx.doi.org/10.1002/0471140864.ps020950
12. Laskowski RA, MM. PROCHECK—a program to check the stereochemical quality of protein structures. J Appl Cryst 1993; 26:283-91; http://dx.doi.org/10.1107/S0021889892009944
13. Mackerell AD, Feig M, Brooks CL. Extending the treatment of backbone energetics in protein force fields: limitations of gas-phase quantum mechanics in reproducing protein conformational distributions in molecular dynamics simulations. J Comput Chem 2004; 25:1400-15; PMID:15185334; http://dx.doi.org/10.1002/jcc.20065
14. Kloda J, Venable RM, Freins JA, O’Conor JW, Tobias DJ, Mondragon-Ramirez C, Vorobyov I, Mackerrell AD, Pastor RW. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. J Phys Chem B 2010; 114:7830-43; PMID:20496934; http://dx.doi.org/10.1021/jp101759q
15. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. J Chem Phys 1983; 79:82; PMID:18004376; http://dx.doi.org/10.1063/1.445869
16. Phillips JC, Braun R, Wang W, Gumbart J,<Classification> J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kale L, Shulten K. Scalable molecular dynamics with NAMD. J Comput Chem 2005; 26:1781-802; PMID:16222654; http://dx.doi.org/10.1002/jcc.20289

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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