Biophysical Investigation of Sodium Channel Interaction with β -Subunit Variants Associated with Arrhythmias

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Abstract

Background: Voltage-gated sodium (Na_V) channels help regulate electrical activity of the plasma membrane. Mutations in associated subunits can result in pathological outcomes. Here we examined the interaction of Na_V channels with cardiac arrhythmia-linked mutations in *SCN2B* and *SCN4B*, two genes that encode auxiliary β -subunits.

Materials and Methods: To investigate changes in *SCN2B*^{R137H} and *SCN4B*^{I80T} function, we combined three-dimensional X-ray crystallography with electrophysiological measurements on $\text{Na}_V1.5$, the dominant subtype in the heart.

Results: *SCN4B*^{I80T} alters channel activity, whereas *SCN2B*^{R137H} does not have an apparent effect. Structurally, the *SCN4B*^{I80T} perturbation alters hydrophobic packing of the subunit with major structural changes and causes a thermal destabilization of the folding. In contrast, *SCN2B*^{R137H} leads to structural changes but overall protein stability is unaffected.

Conclusion: *SCN4B*^{I80T} data suggest a functionally important region in the interaction between $\text{Na}_V1.5$ and $\beta4$ that, when disrupted, could lead to channel dysfunction. A lack of apparent functional effects of *SCN2B*^{R137H} on $\text{Na}_V1.5$ suggests an alternative working mechanism, possibly through other Na_V channel subtypes present in heart tissue. Indeed, mapping the structural variations of *SCN2B*^{R137H} onto neuronal Na_V channel structures suggests altered interaction patterns.

Keywords: $\text{Na}_V1.5$, arrhythmia, *SCN2B*, *SCN4B*

Introduction

VOLTAGE-GATED SODIUM (Na_V) channel mutations can cause dramatic alterations in cellular excitability leading to potentially life-threatening disorders.¹ In cardiac myocytes, $\text{Na}_V1.5$ is the main Na_V channel subtype responsible for heart muscle function.^{2,3} Consequently, mutations in this channel have indeed been associated with long QT syndrome (LQTS)⁴ and Brugada syndrome (BrS).⁵ However, it is now well established that Na_V channels do not function in isolation but form membrane-embedded macromolecular complexes that include auxiliary proteins such as β -subunits,^{1,6–8} fibroblast growth factor proteins, calmodulin, and others.^{9–16} β -subunits are known to modify gating properties, regulate trafficking, and influence plasma membrane recruitment of Na_V channels.⁶ Four main β -subunit isoforms ($\beta1$ – $\beta4$) have been identified and despite a shared, single transmembrane-segment protein topology, each interacts distinctly with and exerts disparate effects on specific Na_V channel subtypes ($\text{Na}_V1.1$ – $\text{Na}_V1.9$).^{6,17–19} All four β -subunits

have been identified in atrial and ventricular cardiomyocytes,² and mutations have been implicated in cardiac disorders.²⁰

Potential disease-associated mutations have also been found in *SCN4B* (p.Ile80Thr) and *SCN2B* (p.Arg137His), encoding the Na_V channel $\beta4$ - and $\beta2$ -subunit, respectively.^{1,21} These mutations were identified during genetic screening in two patients affected by familial atrial fibrillation and LQTS, respectively (www.ncbi.nlm.nih.gov/clinvar/). In this study, we investigated the functional and structural consequences of these mutations on Na_V channel complex function, providing initial evidence of altered interactions.

Methods

Electrophysiological recordings in *Xenopus laevis* oocytes

DNA sequencing of human (h) $\text{Na}_V1.2$, h $\text{Na}_V1.5$ C373Y, h $\beta2$, and h $\beta4$ (Origene, USA) was confirmed by automated Sanger sequencing.²² β -subunit mutants were generated

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TABLE 1. GATING PARAMETERS OF Na_V CHANNELS WITHOUT AND IN THE PRESENCE OF β -SUBUNITS AND MUTANTS

	Activation					Channel availability				
	N	$V_{1/2}$	SEM	d(x)	SEM	N	$V_{1/2}$	SEM	d(x)	SEM
$\text{Na}_V1.5$	7	-39.8	0.1	4.6	0.1	5	-73.8	0.5	8.0	0.3
$\beta 4$	6	-35.6 ^a	0.2	5.7 ^a	0.1	6	-74.4	0.5	9.6 ^a	0.3
I80T	5	-40.1 ^b	0.2	4.8 ^b	0.2	5	-78.5 ^{a,b}	0.5	8.9	0.2
$\beta 2$	5	-34.7 ^a	0.3	6.5 ^a	0.3	7	-67.2 ^a	0.4	7.9	0.2
R137H	5	-35.3 ^a	0.5	7.0 ^a	0.5	5	-68.7 ^a	0.5	7.8	0.3
$\text{Na}_V1.1$	6	-20.5	0.2	6.3	0.2	5	-32.3	0.4	7.6	0.3
$\beta 4$	5	-22.1	0.1	5.7	0.1	6	-31.4	0.2	7.3	0.3
I80T	4	-20.6	0.2	6.3	0.1	6	-31.8	0.2	7.7	0.1
$\text{Na}_V1.2$	5	-22.5	0.3	5.4	0.3	5	-37.7	0.4	9.00	0.4
$\beta 4$	5	-21.9	0.4	5.1	0.4	5	-34.9	0.3	7.1 ^a	0.3
I80T	5	-21.9	0.3	6.0	0.2	4	-35.6	0.3	9.1 ^b	0.2
$\beta 2$	5	-18.8 ^a	0.4	4.5	0.4	5	-34.9 ^a	0.4	9.3	0.4
R137H	5	-22.0 ^b	0.6	4.9	0.5	5	-36.0	0.6	9.4	0.6

N=number of experiments, d(x)=slope value.
^a $p < 0.01$ compared with WT channel by unpaired t -test.
^b $p < 0.01$ compared with WT β -subunit by unpaired t -test.
SEM, standard error of the mean; WT, Wild-Type.

using the Gibson assembly method. cRNA was synthesized using T7 polymerase (the mMessage mMachine Kit; Life Technologies, USA) after linearizing the DNA by restriction enzyme digest. h Na_V channel constructs were expressed in *Xenopus laevis* oocytes (sourced from Xenopus one[®]) with or without a β -subunit (1:5 molar ratio) and electrophysiological recordings were taken 2–4 days post cRNA injection. Oocytes were maintained at 17°C in Barth’s medium (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM MgCl₂, and 1.8 mM CaCl₂, 50 $\mu\text{g}/\text{mL}$ gentamicin, pH 7.6) and studied using the two-electrode voltage clamp recording technique (OC-725C; Warner Instruments, USA) with a 150 μL recording chamber. All data were filtered at 4 kHz and digitized at 20 kHz using pClamp 10 software (Molecular Devices, USA). The external recording solution used was ND100 (100 mM NaCl, 5 mM HEPES, 1 mM MgCl₂, and 1.8 mM CaCl₂, pH 7.6) and microelectrode resistances were 0.5–1.0 M Ω when filled with 3 M KCl. Recordings were performed at room temperature (~22°C) and leak and background conductance, identified by blocking $\text{Na}_V1.2$ and the tetrodotoxin (TTX)-sensitive $\text{Na}_V1.5$ C373Y channel with TTX (Alomone Labs, Israel), was subtracted for all channel currents. Off-line data analysis was performed using Clampfit 10 (Molecular Devices), Excel (Microsoft, USA), and Prism 7 (GraphPad, USA).

Qualitative surface biochemistry

X. laevis oocytes expressing h $\text{Na}_V1.5$, h $\beta 4$, h $\beta 4$ -I80T, h $\beta 2$, h $\beta 2$ -R137H were washed 2 $\times$ with ND100 and incubated with 0.5 mg/mL Sulfo-NHS-LC-biotin (Pierce, USA) for 1 h at 4°C. Oocytes were then washed 6 $\times$ in ND100 before lysis by sonication in 20 μL /oocyte Nonidet P-40 (NP-40, 1%) with protease inhibitor cocktail (Pierce). All subsequent steps were performed at 4°C. Lysates were gently shaken for 15 min and then centrifuged for 15 min at 14,000 rpm. The pellet was discarded and 40 μL of the supernatant was reserved as the total protein fraction. The surface fraction was generated by adding the remaining supernatant to 200 μL of MyOne[™] Streptavidin C1 magnetic beads (Invitrogen, USA), raising the volume to 1 mL with NP-40, and then

rotating overnight at 4°C. Beads were then washed 6 $\times$ with NP-40 and resuspended in 30 μL NP-40, after which the biotinylated protein was dissociated by addition of 1 $\times$ Bolt[™] Sample Buffer plus Reducing Agent (Thermo Fisher Scientific, USA) and boiling at 95°C for 5 min. Protein concentrations were measured by bicinchoninic acid assay (Pierce) and samples were run on a Bolt 4–12% Bis-Tris Plus gel (Thermo Fisher Scientific), then subsequently analyzed by western immunoblotting. Membrane images were cropped to show relevant bands only.

Statistical analyses

Mean data points for fast inactivation time constants (τ), persistent current, and recovery from fast inactivation (RFI) were analyzed using a one-way analysis of variance. For data on normalized conductance-voltage and steady-state inactivation relationships presented in Table 1, unpaired Student’s t -test against wild-type channel or wild-type β -subunit was used. Values in all cases reflect the mean and error bars reflect standard error of the mean. All analyses were carried out using Excel (Microsoft).

Recombinant protein production and crystallographic analysis

Human $\beta 4$ C58A (residues 32–157) and $\beta 2$ C55A (residues 30–153) were cloned into a modified pET28 vector.^{17,18} The $\beta 4$ -C58A/I80T and $\beta 2$ C55A/R137H mutations were introduced using the QuikChange Kit from Agilent Technologies (USA). Both mutants were expressed at 18°C in *Escherichia coli* Rosetta (DE3) pLacI strains (Novagen, USA), induced at an OD600 of ~0.6 with 0.4 mM IPTG, and grown overnight before harvesting. Cells were lysed through sonication in buffer A (250 mM KCl and 10 mM HEPES at pH 7.4), supplemented with 25 mg/mL DNaseI and 25 mg/mL lysozyme. After centrifugation, the supernatants were applied to a PorosMC column (Tosoh Biosep, USA), washed with buffer A plus 10 mM imidazole, and eluted with buffer B (250 mM KCl plus 500 mM imidazole pH 7.4).

The mutants were dialyzed overnight against buffer A and cleaved simultaneously with recombinant tobacco etch virus (TEV) protease. Next, the samples were run on another PorosMC column in buffer A, and the flowthrough was collected and dialyzed against buffer C (10 mM KCl plus 10 HEPES at pH 7.4), applied to a HiloLoadQ column (GE Healthcare, USA), and eluted with gradients from 0% to 30% buffer D (2 M KCl plus 10 mM HEPES at pH 7.4). Finally, samples were run on a Superdex75 (GE Healthcare) gel filtration column in buffer A. $\beta 4$ -C58A/I80T and $\beta 2$ -C55A/R137H were exchanged to 50 mM KCl plus 10 mM HEPES (pH 7.4), concentrated to 9.5 and 2.8 mg/mL, respectively using Amicon concentrator (3K MWCO; Millipore, USA), and stored at -80°C .

Crystallization, data collection, and structure solution

Crystals were grown using the hanging-drop method at 4°C . $\beta 4$ -C58A/I80T was crystallized in 0.1 M MES (pH 6.5), 15–17% (w/v) PEG 2000-MME and $\beta 2$ -C55A/R137H crystals were grown in 0.1 M HEPES (pH 7.0), 14% PEG 6000 using seeds from $\beta 2$ -C55A crystals at various dilutions. Crystals were flash frozen after transfer to the mother liquor supplemented with 30% glycerol. The datasets were collected at the beam line BL9-2 of the Stanford Synchrotron Radiation Lightsource and beam line 23-ID-D at the Advanced Photon Source (Chicago). Data were processed using XDS²³ and HKL3000.²⁴ Phases were obtained through molecular replacement through Phaser²⁵ using the available crystal structures of $\beta 4$ -C58A (4MZ2.pdb) and $\beta 2$ -C55A (5FEB.pdb). The structures were completed by manual model building in COOT²⁶ and refinement using Phenix.²⁷ Simulated annealing composite omit maps were calculated with CNS²⁸ software to verify the absence of residual model bias. All structure figures were prepared using PYMOL (DeLano Scientific, San Carlos, USA). Protein Data Bank (PDB) IDs for mutant β -subunits are 6VRR and 6VSV.

Circular dichroism spectroscopy

All proteins were diluted to $16\ \mu\text{M}$ in 50 mM KCl, 10 mM K-phosphate, pH 7.6 supplemented with 5 mM β -mercaptoethanol. Spectra were measured from 200–260 nm in a Jasco J-810. The initial thermal melt of $\beta 4$ -C58A from 25°C to 95°C in a Peltier controlled sample chamber connected to a water bath showed highest secondary structural changes at 213 nm. For all subsequent melts, the signal at 213 nm was monitored as a function of temperature.

Results

We examined the effects of *SCN4B*, *SCN4B*^{I80T} ($\beta 4$ ^{I80T}), *SCN2B*, and *SCN2B*^{R137H} ($\beta 2$ ^{R137H}) on Na_v channel gating. These mutations were chosen from the ClinVar database (www.ncbi.nlm.nih.gov/clinvar/), which contains many gene variants of unknown or unsubstantiated clinical significance. In this database, both variants are linked to LQTS 10, a cardiac electrophysiological disorder characterized by QT prolongation and T-wave abnormalities associated with tachyarrhythmias and syncope events that typically occur during exercise or emotional stress.^{29,30} Original data were submitted to ClinVar by Invitae, a genetic testing company based in California (USA). In this study, we exploit this in-

formation to investigate possible changes in *SCN2B* and *SCN4B* interaction with Na_v channels, which may lay the foundation for future experiments in which a link with the clinical phenotype is investigated.

Electrophysiological analysis

Since the clinical phenotype revolves around heart function, we used human (h) $\text{Na}_v 1.5$ as our model channel.³ Similar to wild-type $\text{h}\beta 4$ and $\text{h}\beta 2$, both $\text{h}\beta 4$ ^{I80T} and $\text{h}\beta 2$ ^{R137H} express in *Xenopus* oocytes and translocate to the membrane, indicating that protein trafficking is not affected, at least in a heterologous *Xenopus* oocyte expression system (Figs. 1 and 2). To discern potential subtle effects of $\text{h}\beta 4$ and $\text{h}\beta 2$ on Na_v channel function, we recorded and compared multiple gating parameters, such as the conductance-voltage (G-V) relationship, channel availability (I-V), RFI, and persistent current between h $\text{Na}_v 1.5$ alone or in the presence of $\text{h}\beta 4$ or $\text{h}\beta 4$ ^{I80T}. Channel opening kinetics were not studied due to inherent technical limitations associated with electrophysiological recordings in *Xenopus* oocytes.

We found that the G-V relationship of h $\text{Na}_v 1.5/\text{h}\beta 4$ ^{I80T} is shifted to more hyperpolarized potentials ($\pm 5\ \text{mV}$; $p < 0.01$) when compared with h $\text{Na}_v 1.5/\text{h}\beta 4$. A similar shift was seen in channel availability ($\pm 4\ \text{mV}$; $p < 0.01$). Another apparent effect of $\text{h}\beta 4$ ^{I80T} emerged when fitting the current decay phase with a single-exponential fit. Over a 45-mV range, $\text{h}\beta 4$ ^{I80T} caused $\text{Na}_v 1.5$ currents to inactivate significantly faster when compared with wild-type $\text{h}\beta 4$ ($p < 0.05$). Persistent current and RFI of h $\text{Na}_v 1.5$ were unaffected (Fig. 3). The G-V and I-V relationships of two neuronal Na_v channel subtypes, h $\text{Na}_v 1.1$ and h $\text{Na}_v 1.2$, were not disrupted by the $\text{h}\beta 4$ ^{I80T} mutation (Fig. 3). Markedly, $\text{h}\beta 2$ ^{R137H} did not have any apparent effect on the tested h $\text{Na}_v 1.5$ gating parameters as compared with wild-type $\text{h}\beta 2$ (Fig. 2).

Crystallographic analysis

Since $\text{h}\beta 2$ and $\text{h}\beta 4$ can be crystallized,^{17,18} we prepared crystals for the $\text{h}\beta 2$ ^{R137H} and $\text{h}\beta 4$ ^{I80T} variants (PDB IDs are 6VRR and 6VSV). Both resulted in high-resolution structures of 1.45 and 1.65 Å, respectively (Table 2). Figure 4 shows a comparison of wild-type and $\text{h}\beta 2$ ^{R137H}. The two structures can be superposed with an root-mean-square deviation (RMSD) of 0.79 Å for 90 C α atoms (with C α atoms of residues with double conformations not included). The R137H mutation results in multiple structural changes at distances $>12\ \text{\AA}$ away from the mutation site. In wild-type $\text{h}\beta 2$, the Arg¹³⁷ side chain is stacked against the side chain of Tyr¹²⁸, making cation- π interactions. It also forms two hydrogen bonds with a water molecule involved in a hydrogen-bond network, including Ser⁶⁴ and the Tyr¹²⁸ main chain. In the $\text{h}\beta 2$ ^{R137H}, these interactions are lost, resulting in a reorientation of the Ser⁶⁴ side chain. Instead, His¹³⁷ adopts a dual conformation and a 1.6 Å displacement of its main chain, which in turn propagates to several neighboring residues. This includes a near 10 Å shift in the side chain of Asn²⁸, which makes a hydrogen bond with one of the His¹³⁷ conformers, and large shifts of the Met¹³⁰ side chain, His¹³⁶, and Arg¹³⁵. Overall, the R137H mutation results in significant changes in the local area around the mutation site. Although this seems at odds with the lack of effect of this mutation on $\text{Na}_v 1.5$ function (Fig. 2), it suggests that the structural changes do not disrupt the interface

FIG. 1. (A–C) Representative traces of hNav1.5 alone or coexpressed with hβ4 (red) or hβ4^{I80T} (magenta). Oocytes were held at –120 mV and pulsed from –90 to 15 mV for 50 ms. (D) Western blot probing for the C-terminal myc-tag of hβ4. Signal is seen for wild-type hβ4 as well as hβ4^{I80T}, in both whole cell (closed circles) and surface (open circle) fractions. (E) Normalized conductance voltage (G-V, filled circles) and channel availability (I-V, open circles) relationships for hNav1.5 either without hβ4 (black), with hβ4 (red), or with hβ4 I80T (magenta). Summary data of G-V and I-V $V_{1/2}$ values, slope values, and p -values determined by unpaired t -test are reported in Table 1. (F) Rate (τ) of channel fast inactivation using the same color scheme described above. Time constant was obtained using a single-exponential fit of the current decay phase over a 45 mV range. Error bars in all cases reflect SEM with $n=5-7$; * $p<0.05$ or ** $p<0.01$ using one-way ANOVA. ANOVA, analysis of variance; hNav_v, human voltage-gated sodium; SEM, standard error of the mean.

FIG. 2. (A, B) Representative traces of hNav1.5 coexpressed with hβ2 (red) or hβ2^{R137H} (cyan). Oocytes were held at –120 mV and pulsed from –90 to 15 mV for 50 ms. (C) Normalized conductance voltage (G-V, filled circles) and channel availability (I-V, open circles) relationships for hNav1.5 without a β-subunit (black) or with either hβ2 (red) or with hβ2 R137H (cyan). Summary data of G-V and I-V $V_{1/2}$ values, slope values, and p -values determined by unpaired t -test are reported in Table 1. (D) Rate (τ) of channel fast inactivation using the same color scheme described above. Time constant was obtained using a single-exponential fit of the current decay phase over a 45 mV range. (E) Western blot probing for the C-terminal myc-tag of hβ2. Signal is seen for wild-type hβ2 as well as hβ2^{R137H}, in both whole cell (closed circles) and surface (open circle) fractions. Error bars in all cases reflect SEM, with $n=5-7$; * $p<0.05$ or ** $p<0.01$ using one-way ANOVA.

FIG. 3. (A) Normalized persistent current of hNav1.5 (black), hNav1.5 +h β 4 (red), or hNav1.5 +h β 4^{I80T} (magenta) measured 20 ms after depolarization over a 30 mV range. (B) Normalized recovery from fast inactivation measured over a 50 ms timeframe using a double-pulse protocol to the maximum current of the I-V in Figure 1. Recovery voltage used for this protocol was -10 mV. (C, D) Normalized conductance voltage (G-V, filled circles) and channel availability (I-V, open circles) relationships of h β 4 (red) or h β 4^{I80T} (magenta) coexpressed with either hNav1.1 (C) or hNav1.2 (D). Both wild-type channels without β -subunit coexpression are shown in black. Summary data of G-V and I-V $V_{1/2}$ values, slope values, and p -values determined by unpaired t -test are reported in Table 1.

between h β 2 and Nav1.5. However, this mutation may lead to altered interactions with other Nav channel subtypes.

The situation is very different for the h β 4^{I80T} mutation. Wild-type and mutant subunit superpose with an RMSD of 0.48 Å for 105 C α atoms (Fig. 5). In wild-type h β 4, Ile⁸⁰ is buried within the hydrophobic core of the Ig-like domain, which consists of Trp⁶⁸, Leu⁷⁹, Val⁹³, Leu¹⁰³, and Ile¹⁰¹. Substitution by a hydrophilic residue would thus be predicted to be destabilizing. In addition, as Thr is smaller than Ile, the packing is less optimal; resulting in a change in the side chain of Leu¹⁰³, which now adopts a dual side chain conformation. Importantly however, no structural changes are visible on the surface. As the surface of h β 4 would be interacting with Nav1.5, there is an apparent contradiction with the functional data, which clearly show an effect of the I80T mutation on the G-V relationship and steady-state inactivation (Fig. 1). However, as this protein crystallized at low temperature (4°C), and crystals were flash cooled with liquid N₂, a possible temperature-dependent effect on the structure may not be observed. We thus postulate that the suboptimal packing in the h β 4 core can result in changes on the surface at elevated temperature, such as used in electrophysiological experiments or present *in vivo*.

Protein stability

Amino acid substitutions may perturb protein folding. Although both β -subunit mutants still express to levels similar to wild type in *Xenopus* oocytes (Figs. 1 and 2), it is possible that these substitutions cause a thermal destabilization at higher temperatures. Such effect could explain how a mutant, which shows minimal structural changes in a crystallized form at low temperature, can still have a functional effect at physiologically relevant temperatures. We, therefore, prepared purified, recombinantly expressed extracellular subunits of h β 4, h β 2, and the two sequence variants, and utilized Circular Dichroism (CD) to measure thermal melting curves (Fig. 6). Mon-

itoring the CD signal at 213 nm, we increased the temperature from 25°C to 95°C in increments of 0.5°C. Wild-type h β 4 displays a melting temperature (T_M) of 61.1°C ± 0.3°C, whereas the I80T mutation results in a significant destabilization with a T_M of 51.4°C ± 0.1°C (Fig. 6).

Although the actual T_M in the context of a native cell, under oxidizing conditions and in the presence of a Nav channel is unknown, our observation that the mutation has an effect on stability in our assay hints at possible structural changes at elevated temperatures. Although the T_M is well above 37°C, a subtle degree of unfolding is already present at this temperature. Of note, the CD signal measures secondary structure, and it is thus conceivable that significant local structural perturbations, which do not affect secondary structure content, already occur at physiologically relevant temperatures, which would affect the interface between h β 4 and Nav1.5. Wild-type h β 2 is significantly less stable than h β 4, with a T_M of 47.4°C ± 0.1°C. However, in this case, no significant impact on protein stability was observed for the R137H mutation (T_M = 45.8°C ± 0.1°C).

Mapping β -subunit variants on cryoelectron microscopy structures

Next, we wondered whether the β -subunit mutations studied here are part of an interface with Nav1.5. A cryoelectron microscopy (EM) structure is available for Nav1.5 as well as crystal structures of cytosolic portions in complex with auxiliary proteins^{9,10,12–15,31} and also cryo-EM structures of the related Nav1.2, Nav1.4, and Nav1.7 channels, in complex with one or more auxiliary β -subunits.^{7,8,32} This includes structures of hNav1.2 in complex with β 2 (PDB 6J8E), hNav1.4 in complex with β 1 (PDB 6AGF), and hNav1.7 in complex with β 1 and β 2 (PDB 6J8G). In each of the β 2 complexes, a disulfide bond was observed with the pore-forming subunit. This corresponds to Cys⁹¹⁰ in Nav1.2, a residue we predicted to form a disulfide bond with Cys⁵⁵ in h β 2.¹⁷ Although no cryo-

TABLE 2. DATA COLLECTION AND REFINEMENT STATISTICS

Data collection	$\beta 4$ -C58A/I80T	$\beta 2$ -C55A/R137H
PDB code		
Space group	$P3_221$	$P2_12_12_1$
Cell dimensions		
a, b, c (Å)	43.1, 43.1, 107.5	40.3, 53.6, 59.3
α, β, γ (°)	90, 90, 120	90, 90, 90
Resolution (Å)	50–1.62 (1.68–1.65) ^{a,b}	40.3–1.45 (1.53–1.45) ^{a,b}
R_{meas}	7.9 (65.4)	4.3 (62.5)
$I/\sigma I$	33.5 (1.9)	24.4 (2.75)
Completeness (%)	99.8 (99.4)	99.9 (99.6)
Redundancy	5.1 (4.7)	6.9 (6.54)
CC (1/2) ^c	76.0	92.9
Refinement		
Resolution (Å)	37.3–1.62	40.3–1.45
No. of reflections	15397	43929
Molecules	1	1
$R_{\text{work}}/R_{\text{free}}$	18.47/20.81	14.8/18.35
No. of atoms		
Protein	990	2436
Ligand	—	28
Water	147	163
B -factors		
Protein	33.2	23.9
Ligand	—	44.2
Water	48.2	34.6
R.m.s. deviations		
Bond lengths (Å)	0.012	0.013
Bond angles (°)	1.229	1.623
Ramachandran plot ^d		
Favored (%)	96.3	97.6
Allowed (%)	3.7	2.4
Disallowed (%)	—	—

^aOne crystal for each structure was used for data collection and structure determination.

^bValues in parentheses are for highest-resolution shell.

^cValue is for highest-resolution shell.

^dUsing Procheck of CCP4 package.

EM structures have captured $\beta 4$ in complex with a Na_V channel, this subunit is also predicted to make a similar disulfide bond.^{17,18} However, as $\text{Na}_V1.5$ does not have a Cys residue at the corresponding position, the interaction between $\beta 2/\beta 4$ with $\text{Na}_V1.5$ cannot rely on this disulfide bridge.^{17,18,31}

Figure 7 shows the structure of the $\text{Na}_V1.2$ - $\beta 2$ complex (PDB 6J8E), illustrating that the interaction also involves several other residues that make extensive interactions with the Domain II pore-forming region of the channel. From these data, it is clear that the R137H variant is in a position to perturb this interaction. Although R137 is not directly located at the interface, the structural changes we observed for this mutation affect residues located at the $\beta 2$ - $\text{Na}_V1.2$ interface, including Arg,²⁸ Arg¹³⁵, and His¹³⁶. Thus, the h $\beta 2$ R137H variant could perturb the interface with $\text{Na}_V1.2$, and likely also closely related Na_V channel subtypes expressed in the heart (i.e., $\text{Na}_V1.1$ – 1.3).³³ Since h $\beta 2^{\text{R137H}}$ has no functional effect on $\text{Na}_V1.5$ compared with h $\beta 2$, whereas h $\beta 4^{\text{I80T}}$ does, it seems unlikely that $\beta 2$ and $\beta 4$ interact with $\text{Na}_V1.5$ similar to the $\text{Na}_V1.2$ - $\beta 2$ complex. Indeed, $\text{Na}_V1.5$ lacks the Cys equivalent to position 910 in $\text{Na}_V1.2$, suggesting that the manner in which these two β -subunits interact with $\text{Na}_V1.5$ is fundamentally different.^{17,31,34}

$\text{Na}_V1.2$ gating and $\beta 2^{\text{R137H}}$

Since neuronal Na_V channel subtypes expressed in the heart² contain the Cys equivalent to position 910 in $\text{Na}_V1.2$,^{7,17} the structural changes induced by the $\beta 2$ R137H mutation may disrupt channel function. To test this hypothesis with links to available structural data, we used h $\text{Na}_V1.2$ as our model channel, coexpressed with wild-type $\beta 2$ or $\beta 2^{\text{R137H}}$. While we observe no changes in channel availability of h $\text{Na}_V1.2$, the G-V relationship and fast inactivation in the presence of $\beta 2^{\text{R137H}}$ differs substantially from that observed with wild-type $\beta 2$ ($p < 0.001$) and is virtually identical to the condition when no subunit is present (Fig. 8).

FIG. 4. Crystallographic analysis of h $\beta 2^{\text{R137H}}$. Wild-type h $\beta 2$ (containing the C55A mutation to facilitate crystallization¹⁷) is shown in blue, and the R137H mutant in green. R137 is shown in black sticks and H137 in pink. **(A)** Overall superposition of both structures. **(B)** Details around the site of the mutation. Selected residues are shown in sticks and labeled. Hydrogen bonds are shown with dotted lines. In wild-type h $\beta 2$, the Arg137 side chain is involved in a hydrogen bond network as well as a cation- π interaction with Y128. Substitution by H137 results in multiple changes in side chain orientations, as well as the main chain conformation of the N-terminal region around N28.

FIG. 5. Crystallographic analysis of $h\beta 4^{I80T}$. Wild-type $h\beta 4$ (containing the C58A mutation to facilitate crystallization) is shown in blue, and the I80T mutant in orange. I80 is shown in black sticks, and T80 in pink. (A) Overall superposition of both structures. (B) Details around the site of the mutation. Select residues are shown in sticks and labeled. I80 is involved in packing of the hydrophobic core, making interactions with other hydrophobic residues. Substitution by Thr results in minor structural changes, including a rearrangement of the I101 side chain conformation.

Discussion

We investigated the functional and structural effects of two β -subunit variants, *SCN4B* (p.Ile80Thr) and *SCN2B* (p.Arg137His), found in patients with familial atrial fibrillation and LQTS 10. Our functional investigation showed a direct effect of the $h\beta 4^{I80T}$, but not the $h\beta 2^{R137H}$ variant, on the function of $hNa_v1.5$ (Figs. 1 and 2). However, structural investigation using X-ray crystallography revealed opposite results, with $h\beta 2^{R137H}$ causing significant structural changes and $h\beta 4^{I80T}$ resembling the wild-type protein under the crystallization conditions used (Figs. 4 and 5). A structural change does not necessarily have to cause a functional impact, especially if the perturbation is local and does not affect a protein–protein interface. The results presented here suggest that the region around Arg¹³⁷ in $h\beta 2$ may not be involved in the interface with $Na_v1.5$. However, the $h\beta 4^{I80T}$ results seem contradictory given that the absence of significant structural changes suggests that the interface with $Na_v1.5$ should be unaffected.

To better understand this conundrum, it is important to consider a possible effect of temperature. Proteins will unfold, both locally and globally, with rising temperatures, and a mutation could affect this thermal stability.^{35,36} If any unfolding of the mutant occurs at physiological temperatures, and this affects a relevant interface, then the mutant is likely to have a functional effect. In this regard, the $h\beta 4^{I80T}$ caused a significant reduction in thermal stability (Fig. 6). This is not

surprising, as our structural analysis showed that Ile⁸⁰ is buried in a hydrophobic core, and substitution by a hydrophilic residue should be destabilizing. One could thus postulate that the mutation causes local changes in structure at or near the interface between $h\beta 4$ and $Na_v1.5$ at physiologically relevant temperatures, leading to alterations in channel gating. Importantly, thermal melting curves obtained via CD only represent changes in secondary structure, so small structural perturbations may not be measured. Thus, although the melting temperature is above a physiologically relevant temperature, small local changes that do not affect secondary structure are still possible at physiological temperatures. In addition, the altered stability can affect the dynamics of $h\beta 4$, thus affecting its interactions with $Na_v1.5$ indirectly.

Temperature-dependent effects have previously been reported as a result of mutations in $Na_v1.5$. This includes amino acid substitutions in the EF-hand domain leading to thermal destabilization of this locus.^{37,38} Thermal destabilization induced by disease-causing mutations has also been described in other ion channel families.^{39,40} Since the $h\beta 4^{I80T}$ mutation was found in a LQTS patient, an increase in body temperature (e.g., fever or exercise) could elicit structural and functional changes in the cardiac Na_v channel complex, thereby causing an increase in QT interval time and subsequently an increased risk to develop torsade de pointes and polymorphic ventricular tachycardia. Indeed, fever-related ventricular arrhythmia and sudden death have been associated extensively with BrS⁴¹; however, few reports also describe fever-induced QT

FIG. 6. Thermal melting curves monitored through CD recorded at 213 nm. (A) Wild-type $h\beta 4$ (containing the C58 mutation—black) and the I80T mutant (red). (B) Wild-type $h\beta 2$ (containing the C58A mutation—black) and the R137H mutant (green). The T_M values were obtained from the midpoints of the transitions. CD, circular dichroism.

FIG. 7. Mapping the mutation sites on the Na_V1.2-β2 cryo-EM structure. **(A)** Overall cryo-EM structure of Na_V1.2 bound to hβ2 (PDB 6J8E). The pore-forming domain and VSDs are shown in gray and red, respectively, and hβ2 in blue. **(B)** Details of the interaction. Selected residues in hβ2 are labeled. Notice that several of the hβ2 residues, shown to be affected by the R137H mutation, are directly at the interface with Na_V1.2. EM, electron microscopy; VSDs, voltage-sensing domains.

prolongation and ventricular arrhythmias in individuals with type 2 congenital LQTS.⁴² As such, it is possible that the *SCN4B* p.Ile80Thr mutation could favor the appearance of an arrhythmic phenotype in concomitance with fever. We note that this remains a hypothesis, and it needs to be shown whether the mutation elicits small structural or dynamic changes at physiological temperatures.

Although the hβ2^{R137H} variant did not appear to affect any of the electrophysiological properties of Na_V1.5, mapping of the mutation on the Na_V1.2-hβ2 cryo-EM structure showed that this residue can affect channel-subunit interactions. Although not directly involved in the interface, we found that the structural changes we observed affect residues directly participating in their interaction, including R135 and H136 (Fig. 7). Subsequently, hβ2^{R137H} indeed restored hNa_V1.2 function as if no hβ2-subunit was present when considering channel closure (i.e., the fast inactivation process) (Fig. 8). Since several neuronal Na_V channel isoforms are expressed in the heart at functionally important locations,^{2,33} our result hints at a contribution of this hβ2 variant to arrhythmia,^{43,44} despite the absence of functional effects on the main cardiac isoform, Na_V1.5. From a clinical perspective, hβ2^{R137H} was found in a patient with familial atrial fibrillation and since

intrinsic and extrinsic cardiac innervations are pivotal in determining physiological modulations in cardiomyocytes,⁴⁵ it may be feasible that this mutation can modify neural activity in the heart.

Finally, it is worth noting that the extracellular Ig domain of β-subunits can participate in homo- and heterophilic cell adhesion processes, which may have implications for cardiomyocyte function.³⁴ Combined with previous work,^{17,18,30,31,34,46,47} it is unlikely that the Ig domains of hβ2 and hβ4 interact with Na_V1.5 in the same way that they do with other Na_V channel subtypes, since Na_V1.5 does not have a free Cys residue on the domain II extracellular loop.^{17,31} As such, the exact interface of hβ2 or hβ4 with Na_V1.5 remains to be described, but likely does not involve hβ2 residue R137 or the neighboring residues whose positions are affected by the R137H mutation. Ig domains of hβ4 (and presumably hβ2) can form both cis and trans cell adhesion contacts and some of the same amino acids involved in binding to the Na_V channel also contribute to these cis interactions.³⁴ Therefore, hβ2 and hβ4 Ig domains that associate with Na_V1.5 may be involved in crosslinking of associated channels suggesting the involvement of higher-order assemblies in potentially causing cardiac defects and illustrating the complexities in linking genetic data to patient phenotype.

FIG. 8. **(A)** Normalized conductance voltage (G - V , filled circles) and channel availability (I - V , open circles) relationships for hNa_V1.2 without a β-subunit (black) or with either hβ2 (red) or with hβ2 R137H (cyan). Summary data of G - V and I - V $V_{1/2}$ values, slope values, and p -values determined by unpaired t -test are reported in Table 1. **(B)** Rate (τ) of channel fast inactivation using the same color scheme described above. Time constant was obtained using a single-exponential fit of the current decay phase over a 45 mV range. Error bars in all cases reflect SEM. with $n=5$; * $p<0.05$ or **0.01 using one-way ANOVA.

Author Contributions

F.B and F.V.P. conceived the experiments, analyzed data, and wrote the article. J.D.W., L.C., and A.S. helped write the article and interpret (clinical) data. S.D. performed all crystallographic and thermal melt experiments. J.P.L. performed all electrophysiology and biochemical experiments. All co-authors have reviewed and approved of the article before submission.

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