

Short Thesis for the degree of doctor of philosophy (PhD)

**Functional Investigation of Voltage-Gated Ion Channels:
Implications for the Characterization of Human Arrhythmia
Mechanism**

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Introduction and literature review

1. Voltage-Gated Potassium Channels (Kv)

Voltage-gated potassium (Kv) channels are homotetrameric proteins composed of four α -subunits, which together form the two principal functional units of the channel: the voltage-sensing domain (VSD) and the pore domain (PD). Each subunit consists of six transmembrane helical segments (S1–S6) connected by intra- and extracellular amino acid loops. Segments S1–S4 constitute the voltage-sensing domain (VSD), whereas segments S5–S6 and the intervening extracellular loop form the pore domain (PD). This loop contains the highly conserved GYG D motif, which is responsible for K^+ selectivity termed as selectivity filter (SF). Sensing of the membrane potential is mediated by the VSD, while ion permeation is governed by the activation and inactivation gates located within the pore domain, together with the selectivity filter. The converging intracellular ends of the S6 segments form the activation gate. Upon membrane depolarization, the activation gate opens allowing the channel to enter a functionally conducting state. If depolarization is maintained, closure of the inactivation gate may lead to a non-conducting, inactivated state. Proper Kv channel function critically depends on the coordinated operation of the activation gate and the different inactivation mechanisms, namely N-type and C-type inactivation. In N-type inactivation, a ~40 amino acid-long sequence, located at the N-terminus of each channel subunit commonly referred to as the “inactivation ball”, occludes the pore via a diffusion-limited process, thereby directly blocking the permeation pathway. In contrast, C-type inactivation is structurally associated with conformational changes of the selectivity filter, involving closure of a C-type inactivation gate. The selectivity filter itself, its interactions with the pore-forming helices, and the so-called “inactivation water molecules” located in the cavity behind the selectivity filter are thought to play key roles in regulating the molecular rearrangements underlying C-type inactivation. C-type inactivation is generated

cooperatively by the four channel subunits, although the precise molecular sequence of events leading to this state remains incompletely understood.

The *Drosophila melanogaster* Shaker potassium channel shares a highly similar structural organization and gating behavior with human Kv channels. The mutant Shaker channels used in my experiments lack the N-terminal “ball” sequence; consequently, these channels are devoid of N-type inactivation (Shaker-IR). Shaker-IR channels are therefore widely accepted as model systems for the selective investigation of C-type inactivation in Kv channels. Residue T449 has particular importance for C-type inactivation. Accordingly, substitution of this residue with alanine accelerates inactivation, whereas replacement with aromatic amino acids, such as histidine, slows the process. In my experiments, I used Shaker mutants carrying the T449A and T449A/I470A substitutions, which exhibit faster inactivation kinetics than the wild-type Shaker-IR channel, albeit with different time courses, thereby facilitating detailed analysis of the process. In these channels, four principal gating states can be distinguished: closed (C), open (O), open-inactivated (OI), and closed-inactivated (CI). Upon depolarization, opening of the activation gate permits ion flow through the channel (O), while prolonged depolarization induces closure of the inactivation gate, leading to the development of C-type inactivation (OI) and a progressive reduction of the ionic current, culminating in a non-conducting state. Subsequent repolarization to negative membrane potentials first results in closure of the activation gate (CI), followed by reopening of the inactivation gate and restoration of the initial closed, excitable state (C).

2. Molecular Determinants Stabilizing C-Type Inactivation of Kv Channels

It is well established that the C-type inactivated state is stabilized by a network of hydrogen bonds formed behind the selectivity filter. This hydrogen-bonding network was first described in the bacterial pH-gated potassium channel KcsA, which exhibits a high degree of sequence

homology with Shaker-type potassium channels. In KcsA, the greatest contribution to inactivation stability is provided by the hydrogen bonds formed between Trp67 and Asp80, as well as between Asp80 and Glu71. A similar hydrogen-bonding network is present in the region behind the selectivity filter of Shaker voltage-gated potassium channels. In this context, the Trp67–Asp80 interaction in KcsA corresponds to the Trp434–Asp447 pair in Shaker channels, which represents the most critical interaction for stabilizing the inactivated state. Molecular dynamics simulations further indicate that water molecules located behind the selectivity filter play a key role in the formation and stabilization of the C-type inactivated state. These so-called “inactivation water molecules” constitute an integral part of the inactivated channel structure. Removal of these water molecules results in recovery of the channel from the inactivated to the resting state; accordingly, recovery from C-type inactivation requires the exit of these water molecules from the region behind the selectivity filter.

3. Voltage-Gated Sodium Channels (Na_v)

Voltage-gated sodium (Na_v) channels consist of a pore-forming α -subunit with a pseudotetrameric architecture and one or two auxiliary β -subunits with regulatory functions. The α -subunit is a single large polypeptide chain composed of four domains (DI–DIV), each containing six transmembrane helical segments (S1–S6) connected by intra- and extracellular loops. Within each domain, segments S1–S4 form the voltage-sensing domain (VSD), where positively charged residues of the S4 segment undergo depolarization-induced conformational changes that trigger channel opening and result in rapid Na⁺ influx. Segments S5–S6 of the four homologous domains, together with the connecting extracellular loops, form the pore domain (PD) and the selectivity filter (SF). The human genome encodes nine Na_v α -subunit isoforms, designated Na_v1.1–Na_v1.9. These channels exhibit tissue-specific expression patterns: Na_v1.1–Na_v1.3 are predominantly expressed in the brain, Na_v1.7–Na_v1.9 in the peripheral nervous system, Na_v1.6 in both the central and peripheral nervous systems, Na_v1.4 in skeletal muscle,

and Na_v1.5—of particular relevance to this work—exclusively in the myocardium. The cardiac fast voltage-gated sodium channel Na_v1.5, encoded by the SCN5A gene, has been the focus of extensive experimental research over recent decades, as mutations in this gene have been increasingly associated with primary electrical heart diseases. In our work, I studied a SCN5A mutation (R893C) identified in a patient with Brugada syndrome, providing its comprehensive clinical and biophysical characterization, demonstrating its pathogenic nature, and elucidating the molecular mechanisms underlying the associated loss of channel function.

4. Pathophysiology of Brugada Syndrome

Brugada syndrome (BrS) is a genetically determined ion channel disorder that predisposes affected individuals to potentially lethal cardiac arrhythmias and is considered a relatively rare disease entity. The prevalence of Brugada syndrome is low, affecting approximately 1–5 individuals per 10,000; however, it accounts for about 4% of all cases of sudden cardiac death. Among patients with structurally normal hearts who experience sudden cardiac death, the prevalence is markedly higher, approaching 20%, making BrS the most common cause of fatal arrhythmias in this subgroup. The mean age of patients who suffer sudden cardiac death due to BrS is approximately 41 years, and malignant arrhythmias occur more frequently during nighttime or sleep. Known precipitating factors include fever, the administration of sodium channel-blocking drugs, and conditions associated with increased vagal tone. Variants in more than ten different genes have been linked to Brugada syndrome, among which mutations in the SCN5A gene are the most common. SCN5A encodes the α -subunit of the cardiac fast voltage-gated sodium channel Na_v1.5 expressed in cardiomyocytes. In approximately 10–30% of genetically characterized BrS patients, a loss-of-function mutation in SCN5A can be identified, typically inherited in an autosomal dominant manner. Due to variable expressivity and incomplete penetrance, the same pathogenic mutation may result in heterogeneous clinical phenotypes with differing severity among affected individuals. To date, more than 300 distinct

SCN5A mutations have been identified in patients with Brugada syndrome, affecting virtually any structural domain of the Na_v1.5 protein. The vast majority of these variants are loss-of-function (LoF) mutations, which may reduce the number of Na_v1.5 channels available at the plasma membrane, decrease sodium current amplitude leading to channel dysfunction, or alter gating properties, including activation and inactivation kinetics and their voltage dependence. The precise mechanism of arrhythmogenesis in Brugada syndrome remains a subject of ongoing debate. According to the most widely accepted hypothesis, Na_v1.5 dysfunction leads to a reduction in the slope of the AP upstroke, causing slowed atrial and ventricular conduction, the development of endo–epicardial voltage gradients, and delayed activation of the right ventricle, thereby producing the characteristic electrocardiographic features of the disease. In contemporary arrhythmology, the development of sustained arrhythmias requires the presence of both a “trigger” and a susceptible “substrate.” In Brugada syndrome, the arrhythmogenic substrate is thought to consist of endo–epicardial voltage gradients and increased repolarization heterogeneity. Prolongation of the plateau phase (phase 2) of the action potential may lead to enhanced diastolic intracellular Ca²⁺ influx, sarcoplasmic reticulum Ca²⁺ overload, and the generation of early and delayed afterdepolarizations (EADs and DADs), which can serve as triggers for sustained arrhythmias.

5. Brugada Syndrome in Clinical Practice

Clinically, Brugada syndrome most commonly manifests as typical cardiac syncope or sudden cardiac death, predominantly resulting from rapid polymorphic ventricular tachycardia (VT) or ventricular fibrillation (VF). In most cases, sustained ventricular arrhythmias are triggered by short-coupled premature ventricular contractions (PVCs), with the first arrhythmic event typically occurring between 30 and 50 years of age. In addition to ventricular arrhythmias, atrial tachyarrhythmias are more frequent in the BrS population, with approximately 10% of patients developing supraventricular tachycardia during their lifetime. Given the key role of sodium

channels in cardiac excitation and conduction, it is not surprising that SCN5A-positive individuals and families may present with additional clinical entities beyond BrS, collectively referred to as “overlap syndromes,” including long QT syndrome, sick sinus syndrome, idiopathic ventricular fibrillation, atrial fibrillation, and various atrioventricular and intraventricular conduction disturbances. Three characteristic ECG patterns are described in Brugada syndrome, of which the type 1 Brugada pattern is diagnostic. This pattern is characterized by a coved-type ST-segment elevation of ≥ 2 mm followed by a negative T wave in the right precordial leads (V1–V2), representing the right ventricular outflow tract (RVOT). Because these ECG abnormalities may show dynamic fluctuation, pharmacological provocation testing with sodium channel–blocking agents plays an increasingly important role in diagnosis. In BrS patients, certain sodium channel blockers can unmask the characteristic ST–T changes; therefore, provocation testing is recommended in individuals with spontaneous type 2 or type 3 Brugada patterns or in those with a strong clinical suspicion based on symptoms and family history. The most commonly used agents worldwide include class Ia drugs such as ajmaline and procainamide, and class Ic drugs such as flecainide and pilsicainide, according to the Vaughan Williams classification. Aborted or survived sudden cardiac death is often the first clinical manifestation of Brugada syndrome; collectively, these events are referred to as severe arrhythmic events (SAEs). Risk stratification is crucial for appropriate patient management and aims to identify individuals at high annual risk of SAE. In addition to a history of sudden cardiac death, syncope and the presence of a spontaneous type 1 Brugada pattern on a standard 12-lead ECG represent the strongest predictors of future arrhythmic events. Increasing the availability of genetic testing and functional characterization of pathogenic variants may play an important role in future genetic risk stratification. Currently, four main therapeutic modalities are distinguished in the management of Brugada syndrome: lifestyle modification, pharmacological therapy, device-based therapy, catheter ablation, and combinations thereof.

Among these, the device-based therapy in this patient population primarily consists of implantation of an implantable cardioverter-defibrillator (ICD). The ICD is a specialized pacemaker capable of detecting life-threatening ventricular arrhythmias and terminating them via painless overdrive pacing or, if necessary, by delivering an electrical shock. ICD implantation may be indicated for secondary prevention in BrS patients who have experienced sustained ventricular arrhythmias or sudden cardiac death, or for primary prevention in patients without documented arrhythmic events but with a high estimated risk. Pharmacological treatment options are limited; the only agent with proven efficacy in arrhythmia prevention is quinidine, which blocks sodium channels and voltage-gated potassium channels, thereby reducing the slope of the action potential downstroke and exerting its antiarrhythmic effect through prolongation of the refractory period. Unfortunately, quinidine has a broad side-effect profile and limited availability, restricting its clinical use. In cases of therapy-refractory electrical storm, administration of the catecholamine isoproterenol may also be considered. In patients with recurrent ventricular arrhythmias requiring frequent ICD interventions despite optimal pharmacological and device-based therapy, catheter ablation may be indicated. This invasive procedure aims to eliminate the epicardial arrhythmogenic substrate located in the RVOT using radiofrequency energy. Additional endocardial radiofrequency ablation of short-coupled premature ventricular contractions serving as arrhythmic triggers may further improve long-term outcomes.

6. Brugada Syndrome – Case Report

The study focused on a 41-year-old male patient who was referred to our clinic by emergency medical services due to chest pain, with the aim of urgent coronary intervention. The patient's medical history was notable for recurrent episodes of syncope since childhood. A trans-telephonic ECG transmitted by the ambulance revealed a type 1 Brugada pattern; however, this finding was no longer present on the 12-lead ECG recorded upon hospital admission.

Transthoracic echocardiography demonstrated preserved left ventricular systolic function without regional wall motion abnormalities, while CT angiography excluded aortic dissection and pulmonary embolism. Based on the dynamic ECG changes, Brugada syndrome was suspected. During invasive electrophysiological testing, administration of 400 mg intravenous procainamide as well as programmed ventricular extrastimulation from the right ventricle both induced rapid, self-terminating polymorphic ventricular tachycardia. Following procainamide administration, a type 1 Brugada pattern appeared on the 12-lead ECG, thereby supporting the diagnosis of Brugada syndrome. In light of the patient's clinical history, it was considered likely that the recurrent syncopal episodes were in fact consequences of spontaneously terminating ventricular arrhythmias; therefore, implantation of an implantable cardioverter-defibrillator (ICD) was indicated. Following implantation, episodes of loss of consciousness continued to occur; however, the ICD did not record any arrhythmias during these events. In light of this, despite the presence of clinical features consistent with Brugada syndrome, the recurrent syncopal episodes over several decades may be attributable to a neurological disorder, such as epilepsy.

7. Open Questions and Controversies Regarding C-Type Inactivation and the Clinical Manifestations of Brugada Syndrome

To provide experimental evidence for the presence of the “inactivation water molecules” located behind the selectivity filter, as introduced above, our research group previously examined the effect of heavy water (D₂O) on channel inactivation. These studies demonstrated that heavy water slows inactivation of these channels, and that this effect is preserved across different channel clones, indicating a mutation-independent mechanism; however, the precise molecular basis of this phenomenon has not yet been fully elucidated. The clinical course and prognosis of Brugada syndrome show marked interindividual variability; therefore, identification and biophysical characterization of pathogenic SCN5A mutations are essential

for understanding the mechanisms underlying arrhythmia development in this patient population. Elucidating the relationship between newly identified pathogenic variants and clinical outcome provides valuable information for risk stratification and patient management, while detailed knowledge of the structural and functional alterations of mutant Na_v1.5 proteins may facilitate the development of novel and more effective therapeutic strategies for patients with SCN5A-related channelopathies.

Objectives

The specific effects of heavy water in slowing inactivation cannot be directly demonstrated by current recordings; therefore, we applied an indirect approach. By excluding nonspecific effects, we aimed to provide indirect evidence for the presence of specific mechanisms. The goal of my experiments on Shaker-IR channels was to demonstrate that the slowing effect of heavy water on inactivation is not due to its higher viscosity compared to normal water. To investigate the role of viscosity, cellular electrophysiological measurements were performed in the presence of glycerol and sucrose, during which I also acquired practical expertise in the patch-clamp technique.

Subsequently, I conducted translational studies aimed at identifying a novel SCN5A mutation (R893C) in a patient diagnosed with Brugada syndrome exhibiting unique clinical features, followed by a comprehensive structural and functional characterization of the mutant Nav1.5 channels. This approach enabled us to establish a link between the presumed pathogenic mutation and ion channel dysfunction. During the characterization of the R893C mutation, we extensively analyzed the clinical features of the affected patient and their family members, as well as the structural alterations and functional consequences of the mutant channel using electrophysiological and pharmacological investigations based on the patch-clamp technique.

Materials and Methods

1. Genetic Analysis

Genetic analysis was performed using whole-genome sequencing, which was carried out by Novogene (Cambridge, UK). Bioinformatic data processing and variant calling were conducted using the GATK pipeline. The identified putative pathogenic variant (SCN5A Arg893Cys) was confirmed by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's protocol. The primer sequences used for validation were 5'-TCCCCTCCTCTTCCTCCTTC-3' and 5'-GATGGATGGACGGATGGGTA-3'. The wild-type (WT) human SCN5A gene was first subcloned and N-terminally tagged with green fluorescent protein (GFP). This construct was kindly provided by H. Abriel (University of Bern, Switzerland). The Na_v1.5 R893C and R893H mutations investigated in this study were generated by site-directed mutagenesis using the overlap extension PCR method. Generation of the R893C mutant was performed by UD-GenoMed Ltd. (Debrecen, Hungary), and the mutant clone was verified by direct sequencing.

2. Cell Cultures and Transfection

Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells were cultured in high-glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin. Cells were maintained in 25 cm² culture flasks at 37 °C in a humidified atmosphere containing 5% CO₂ and were passaged twice weekly. Adherent cells were detached during passaging by incubation in PBS containing 0.2 g/L EDTA for 5 minutes. One day prior to transfection, cells were plated in 60 × 15 mm Petri dishes using a dilution series. For recordings involving Shaker-IR channels, cells were co-transfected with plasmids encoding the T449A or T449A/I470A mutant ion channels

(10 μ g) together with an EGFP plasmid (1 μ g) using the calcium phosphate precipitation method.

For Na_v1.5 measurements, cells were transfected as described above with plasmids encoding N-terminally GFP-tagged wild-type human Na_v1.5 (WT-Na_v1.5GFP), untagged wild-type human Na_v1.5 (WT-Nav1.5), or N-terminally GFP-tagged human Na_v1.5 carrying the R893C or R893H mutations (R893C-Na_v1.5GFP or R893H-Na_v1.5GFP). Transfections were performed using the Lipofectamine 2000 kit according to the manufacturer's instructions. As transient transfection was applied, all measurements were carried out within 24–36 hours after transfection. Immediately prior to electrophysiological recordings, cells were washed twice with 1 mL of external solution (see below), and subsequently incubated in 2 mL of the same external solution.

3. Cellular Electrophysiology

Electrophysiological recordings were performed using the patch-clamp technique, whole-cell configuration for Na_v1.5 channels and the outside-out configuration for Shaker-IR channels. An Axopatch 200B patch-clamp amplifier was used in voltage-clamp mode, together with a Burleigh PCS5000 micromanipulator. Voltage protocols and data acquisition were controlled by an IBM-compatible personal computer via an Axon Digidata 1550A interface using GraphPad Prism 8 and pCLAMP 10 software. Current traces were filtered with a three-pole boxcar filter. The holding potential was typically –120 mV, and 70–80% of series resistance errors were compensated electronically; recordings were accepted only if series resistance did not exceed 15 M Ω . Measurements were rejected when leak currents exceeded 10% of the maximal current amplitude; otherwise, online leak subtraction was performed using a P/4 protocol. Reported membrane potential (V_m) values were not corrected for junction potentials. Identical internal and external solutions were used throughout, yielding a junction potential of

−8.9 mV ($V_m = V_n - 8.9$ mV). Patch pipettes were pulled from GC150F-7.5 borosilicate glass capillaries using a Flaming–Brown programmable puller in four steps. Pipette tip diameters were 0.5–1 μm , with resistances of 2–8 $\text{M}\Omega$ in the applied solutions. All measurements were performed at room temperature (20–24 $^{\circ}\text{C}$).

For Shaker-IR recordings, the pipette solution (ICS) contained 105 mM KF, 35 mM KCl, and 10 mM HEPES. The sodium-based external solution (ECS) delivered via the perfusion system contained 150 mM NaCl, 2 mM KCl, 1.5 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES. In addition to the control solution, variants supplemented with 5% or 10% (w/w) glycerol or 10% sucrose were used. External solutions were prepared using either normal water or heavy water (D_2O). All solutions were adjusted to pH 7.38 with NaOH. Whole-cell recordings of $\text{Na}_v1.5$ channels were performed using an ECS containing 145 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, and 5.5 mM glucose (302–308 mOsm/L). To suppress endogenous K^+ currents, the ICS was Cs^+ -based containing 10 mM NaCl, 105 mM CsF, 10 mM HEPES, and 10 mM EGTA; pH 7.2 adjusted with CsOH; 295 mOsm/L. External solutions were applied using a gravity-driven perfusion system at 0.2–0.5 mL/min with continuous suction. Solutions containing tetrodotoxin (TTX) or dithiothreitol (DTT) were freshly prepared before experiments. The effect of the tested compounds at a given concentration was characterized as the normalized current calculated as I/I_0 , where I_0 and I are the peak currents in the absence and presence of the compound at the indicated concentration, respectively. A choline-based ECS (145 mM choline-Cl, 5 mM KCl, 10 mM HEPES, 5.5 mM glucose, 2.5 mM CaCl_2 , 1 mM MgCl_2) was used as a perfusion control.

For Shaker-IR channels, a two-step protocol was applied. Excised patches were held at −120 mV, followed by a 2 s depolarizing pulse to +50 mV, and then returned to −120 mV for 45 s. Currents recorded under control and test conditions were subsequently compared. All Shaker-

IR measurements were performed in the outside-out configuration, as test solutions were applied extracellularly, consistent with the extracellular site of action of heavy water.

Basic biophysical properties of $\text{Na}_v1.5$ variants were assessed in the whole-cell configuration. Activation and inactivation kinetics, recovery from inactivation, and voltage dependence of steady-state activation and inactivation were analyzed. For activation and inactivation measurements, cells were depolarized from -120 mV to 0 mV for 15 ms every 5 s (20 kHz sampling). To avoid the model dependence of the reported parameter characterizing Na_v channel activation kinetics, we assessed the time-to-peak variable. Time-to-peak was measured as the time between the initiation of the depolarizing pulse and the time point when I_{Na} reached the peak. The average of the time-to-peak for a particular cell was determined as the average of time-to-peak values obtained for 4-5 depolarizing pulses to 0 mV repeated at every 5 s. The current densities in pA/pF for a particular cell were defined as the ratio of the average of peak currents, obtained for at least three depolarizing pulses to the appropriate test potentials repeated at every 5 s in a sequence, to the cell capacitance. Inactivation time constants (τ_i) were obtained by fitting a single exponential function to the decay phase of the current traces: $I(t) = I_0 \cdot \exp(-t/\tau_i) + C$, where I_0 is the amplitude of the inactivating component and C is the steady-state current. Steady-state activation was determined by depolarizing cells from -120 mV to test potentials between -70 and $+70$ mV in 10 mV increments (50 kHz sampling). Conductance was calculated as $G(V) = I_{\text{peak}} / (V_m - V_{\text{Na}})$, where V_{Na} was -68.7 mV, and normalized to maximal conductance. Data were fitted with a Boltzmann function: $G/G_0 = 1 / [1 + \exp(-(V_m - V_{1/2})/s)]$. Steady-state inactivation (h_∞) was assessed by calculating the fraction of non-inactivated channels (I/I_{-120}) at each holding potential and fitting the data with a Boltzmann function. To evaluate the late I_{Na} , we analyzed the steady-state Na^+ current during the last 5 ms of the 15 -ms-long depolarizing pulses to 0 mV i.e., between 10 and 15 ms. Recovery from inactivation was examined using paired depolarizing pulses (100 ms to 0 mV) from a holding

potential of -120 mV with interpulse intervals ranging from 0.25 to 60 ms. Fractional recovery ($FR = I_2/I_1$) was plotted against interpulse interval and fitted with an exponential function: $FR(t) = 1 - \exp(-t/\tau_r)$. Nonlinear least-squares fitting was performed using the Levenberg–Marquardt algorithm.

4. Protein Structure Modeling

Structural modeling was performed based on the cryo–electron microscopy structure of the human $Na_v1.5$ channel. The conserved regions described above, including the site of the R893C mutation and its surrounding environment, were successfully resolved at a resolution of 3.30 Å. Based on the experimental structure, the arginine residue at position 893 was substituted with cysteine using UCSF Chimera X software, followed by protonation and energy minimization steps applying the GAFF force field. Putative ligand–protein interactions were visualized using the LigPlot program.

5. Data Analysis and Statistical Evaluation

Data analysis was performed using the pCLAMP 8/10 software package (Molecular Devices Inc., Sunnyvale, CA, USA). Current traces obtained during recordings were first corrected for the ohmic leak current determined at the -120 mV holding potential preceding each voltage pulse. The number of experiments on different cells (n) involved in the given analysis is shown in the text. The number of independent transfections/biological replicates (N) was at least 4 in most sets of experiments. Data are presented as means $\pm$ SEM, individual data points are indicated in the bar graphs. Statistical significance was estimated with GraphPad Prism 8 software (version 8.0.1, La Jolla, CA). The Shapiro-Wilk test was used to test for normality of the given set of data. For pairwise comparisons Student's t test or Mann–Whitney rank sum test (nonnormal distribution population) was applied. For multiple comparisons one-way ANOVA with post hoc Tukey's test was applied. Statistical significance is indicated in terms of P values.

Results

1. Results of Experiments Performed on Shaker-IR Channels

1.1 Effect of 10% (w/w) Sucrose on Slow Inactivation of Shaker-IR Potassium Channels

As previous studies have reported experimental removal of inactivation water molecules using highly viscous (2 M) sucrose solutions, these experiments are presented first. These experiments were performed on the T449A mutant Shaker-IR potassium channel. During the initial depolarizing pulse applied before solution exchange (i.e., before the start of the perfusion with sucrose-based ECS), the mean inactivation time constant derived from the current traces was 144 ms ($n = 6$). Following solution exchange, the mean inactivation time constant measured during the second depolarizing pulse was 159 ms ($n = 6$, $P = 0.05$). Thus, application of 10% (w/w) sucrose in the second solution did not produce a significant effect on the inactivation time constant, despite the fact that the viscosity of sucrose exceeds that of heavy water.

1.2. Effect of 5% (w/w) Glycerol on Slow Inactivation of Shaker-IR Potassium Channels

In the subsequent experiment, the effect of 5% (w/w) glycerol was examined. These experiments were performed on the more slowly inactivating T449A/I470A mutant, which accounts for the longer time constants observed compared with the previous measurements. Under control conditions, the mean inactivation time constant derived from the current traces was 485 ms ($n = 6$), whereas exposure to glycerol resulted in a mean value of 517 ms ($n = 6$, $P = 0.05$). Thus, increasing solution viscosity by the addition of 5% (w/w) glycerol did not lead to a significant change in the average inactivation time constant.

1.3 Effect of 10% (w/w) Glycerol on Slow Inactivation of Shaker-IR Potassium Channels

In these experiments, the second ECS solution contained 10% (w/w) glycerol, and recordings were performed on T449A mutant Shaker-IR channels. Under control conditions, the mean

inactivation time constant determined from current traces during the first depolarizing pulse to +50 mV was 157 ms ($n = 6$), whereas exposure to 10% (w/w) glycerol increased this value to 225 ms ($n = 6$, $P = 0.0011$). This significant difference was unexpected, as no such effect was observed with 5% (w/w) glycerol. Moreover, the viscosity of 10% (w/w) glycerol in aqueous solution (1.31 mPas) is slightly lower than that of 10% (w/w) sucrose (1.336 mPas), yet no significant slowing of inactivation was detected in the sucrose-containing solution. In light of this observation, an additional experiment was required to determine whether the increase in inactivation time constants observed with 10% (w/w) glycerol was due to viscosity or to a specific molecular interaction. To address this question, we examined whether the inactivation-slowing effects of heavy water and 10% (w/w) glycerol were additive. In this protocol, the control solution contained heavy water, while the second solution consisted of heavy water supplemented with 10% (w/w) glycerol. Experiments were again performed on T449A mutant channels. The mean inactivation time constant measured during the first depolarization before solution exchange was 370 ms ($n = 5$), whereas after solution exchange this value was 422 ms ($n = 5$, $P = 0.05$). The difference between these mean time constants was not statistically significant. These results indicate that the effects of heavy water and glycerol are not additive, supporting our hypothesis that the effect of 10% (w/w) glycerol on C-type inactivation is not attributable to increased solution viscosity. The mean values presented in this section were derived from multiple measurements, and in all cases, the current traces and corresponding inactivation time constants compared were obtained from experiments performed on the same excised patch.

2. Results of Experiments Performed on Na_v1.5 Channels

2.1. Identification of a Potentially Pathogenic Na_v1.5 Mutation

In the patient with Brugada syndrome described above, whole-genome sequencing revealed a heterozygous point mutation (C>T) at position 2677 of the SCN5A gene (c.2677C>T). This missense mutation results in substitution of arginine by cysteine at position 893 of the Na_v1.5 protein (R893C). In silico prediction tools assessing the impact of point mutations on protein structure and function (SIFT, PolyPhen-2, and Align-GVGD) consistently classified this variant as potentially pathogenic with a disruptive effect on Na_v1.5 channel function. According to available literature data, analysis of a large control cohort comprising approximately 6,500 healthy individuals did not identify the SCN5A R893C variant. Furthermore, this mutation is absent from major human genetic variation databases, including Ensembl, HapMap, and the 1000 Genomes Project. Taken together, these findings support a causal role of the R893C variant as a potentially pathogenic Na_v1.5 mutation in the development of the disease. Residue 893 is located in the extracellular loop connecting the transmembrane S5 and S6 segments of domain II (DII) of the Na_v1.5 protein, a region that forms part of the channel pore and lies in close proximity to the selectivity filter. Sequence alignment revealed a high degree of conservation of arginine at this position across different human Na_v isoforms and across species, including mouse, rat, and human, indicating strong evolutionary pressure to maintain this residue. We were unable to identify any pathogenic mutation explaining our patient's epileptic seizures.

2.2. Biophysical Characterization of Wild-Type Na_v1.5GFP

First, we examined the biophysical effects of N-terminal GFP tagging of the wild-type Na_v1.5 channel (WT-Na_v1.5GFP) by comparing its functional properties with those of the untagged wild-type channel (WT-Na_v1.5). Following a 15 ms depolarizing pulse to 0 mV, the mean

maximal current density in cells transfected with WT-Nav1.5 was 311.8 ± 31.8 pA/pF ($n = 15$), whereas WT-Nav1.5GFP exhibited a mean current density of 340.6 ± 36.2 pA/pF ($n = 31$), indicating no significant difference between the two conditions ($P = 0.6150$). Activation kinetics were also comparable, with time-to-peak values of 0.413 ± 0.019 ms ($n = 15$) for WT-Nav1.5 and 0.493 ± 0.018 ms ($n = 31$) for WT-Nav1.5GFP ($P = 0.008$). Similarly, inactivation time constants (τ_i) did not differ significantly between untagged and GFP-tagged channels, yielding values of 0.719 ± 0.065 ms ($n = 15$) and 0.869 ± 0.047 ms ($n = 26$) ($P=0.0658$), respectively. N-terminal GFP tagging did not significantly affect the voltage dependence of steady-state activation or the slope factor (s). For WT-Nav1.5, the half-activation voltage ($V_{1/2}$) was -30.1 ± 2.4 mV with $s = 5.8 \pm 0.4$ mV ($n = 6$), whereas for WT-Nav1.5GFP, $V_{1/2}$ was -34.2 ± 1.8 mV and $s = 6.1 \pm 0.3$ mV ($n = 23$) ($P = 0.2846$ and $P = 0.6591$ for $V_{1/2}$ and s , respectively). No significant differences were observed in the voltage dependence of steady-state inactivation, with $V_{1/2}$ values of -54.2 ± 2.9 mV and $s = -8.4 \pm 0.5$ mV for WT-Nav1.5 ($n = 10$), and -59.1 ± 2.1 mV with $s = -9.5 \pm 0.5$ mV for WT-Nav1.5GFP ($n = 12$) ($P = 0.1676$ and $P = 0.1461$). Finally, recovery from inactivation was assessed and revealed no significant effect of GFP tagging. The recovery time constant (τ_r) was 3.2 ± 0.40 ms ($n = 9$) for WT-Nav1.5 and 3.41 ± 0.20 ms ($n = 22$) for WT-Nav1.5GFP ($P = 0.6283$).

2.3. Biophysical Characterization of the SCN5A R893C Mutation

The pathogenic role of the heterozygous SCN5A mutation (R893C) identified in our patient was investigated by detailed biophysical characterization using the patch-clamp technique in the whole-cell configuration in both CHO and HEK cells. Our aim was to compare the gating properties of cells expressing wild-type (WT-Nav1.5GFP) and mutant (R893C-Nav1.5GFP) channels. In the first set of experiments, CHO cells expressing either wild-type or mutant channels were depolarized from a holding potential of -120 mV to 0 mV for 15 ms. Under these conditions, cells expressing the mutant channel exhibited a dramatic reduction in current

density. At 0 mV, the mean current density was 340.6 ± 36.2 pA/pF for WT-Nav1.5GFP ($n = 31$), whereas only 15.8 ± 2.2 pA/pF was measured in cells expressing R893C-Nav1.5GFP ($n = 19$) ($P < 0.0001$). Although only minimal sodium currents were recorded in non-transfected CHO cells (4.6 ± 1.2 pA/pF at 0 mV, $n = 15$), it was essential to exclude the possibility that the small currents observed in mutant-transfected cells originated from endogenous sodium channels expressed by CHO cells. To address this, we examined the effect of extracellular application of 0.5 mM tetrodotoxin (TTX). Endogenous sodium channels in CHO cells are sensitive to 0.5 mM TTX, whereas Nav1.5 is resistant. After at least 3 minutes of perfusion with TTX-containing external solution, depolarization to 0 mV did not result in a significant reduction in current density in either WT-Nav1.5GFP- or R893C-Nav1.5GFP-expressing cells. The normalized current (I/I_0), where I_0 is the peak current measured in the absence of TTX and I is the peak current measured at equilibrium block in the presence of 0.5 μ M TTX, was 0.927 ± 0.013 ($n = 4$) for WT-Nav1.5GFP and 0.913 ± 0.025 ($n = 6$) for R893C-Nav1.5GFP ($P = 0.7029$). Additional experiments performed in HEK cells yielded similar results: depolarization to 0 mV in cells expressing R893C-Nav1.5GFP produced an even smaller current density (5.4 ± 0.7 pA/pF, $n = 9$), indicating that the observed effects are not specific to the CHO expression system. In addition to reduced current density, significant alterations in gating properties were observed in cells expressing the mutant channel. Activation was markedly slowed, as reflected by a prolonged time-to-peak. The mean time-to-peak was 0.493 ± 0.018 ms for WT-Nav1.5GFP ($n = 31$) and 0.779 ± 0.079 ms for R893C-Nav1.5GFP ($n = 20$) ($P < 0.0001$). In contrast, no significant difference was detected in inactivation time constants (τ_i), which were 0.869 ± 0.05 ms for wild-type channels ($n = 26$) and 0.933 ± 0.08 ms for mutant channels ($n = 20$) ($P = 0.4892$). Analysis of steady-state activation and inactivation revealed pronounced shifts in voltage dependence in mutant channels. Compared with wild-type, both curves were shifted by approximately +10 mV toward more positive membrane potentials, accompanied by a

significant increase in the slope factor. For steady-state activation, the half-activation voltage ($V_{1/2}$) was -34.2 ± 1.8 mV with $s = 6.1 \pm 0.3$ mV for WT- $\text{Na}_v1.5\text{GFP}$ ($n = 23$), whereas R893C- $\text{Na}_v1.5\text{GFP}$ exhibited a $V_{1/2}$ of -24.9 ± 2.1 mV and $s = 8.9 \pm 0.7$ mV ($n = 12$) ($P = 0.0033$ and $P = 0.0002$, respectively). Similarly, for steady-state inactivation, $V_{1/2}$ values were -59.1 ± 2.1 mV with $s = -9.5 \pm 0.5$ mV for wild-type channels ($n = 12$) and -47.4 ± 3.0 mV with $s = -11.3 \pm 0.7$ mV for R893C mutant channels ($n = 10$) ($P = 0.0037$ and $P = 0.0455$, respectively). Finally, recovery from inactivation was assessed and revealed no significant difference between wild-type and mutant channels. The recovery time constant (τ_r) was 3.38 ± 0.19 ms for WT- $\text{Na}_v1.5\text{GFP}$ ($n = 22$) and 3.65 ± 0.36 ms for R893C- $\text{Na}_v1.5\text{GFP}$ ($n = 12$) ($P = 0.4798$).

2.5. Effect of R893C Mutant Channels on Wild-Type Channel Function

To model the heterozygous state of our patient and to assess a potential dominant-negative effect of the R893C mutant on wild-type channels, CHO cells were co-transfected with wild-type and R893C mutant $\text{Na}_v1.5$ channels at a 1:1 ratio (termed as “Mixed”). In this experimental series, 1 μg of WT and 1 μg of R893C plasmid DNA were co-transfected into CHO cells, resulting in a total DNA amount equivalent to that used in single-transfection experiments. Under these conditions, no dominant-negative effect of the R893C mutant on wild-type channel function was observed. Upon depolarization to 0 mV, the maximal current density was 340.6 ± 36.2 pA/pF ($n = 31$) in WT- $\text{Na}_v1.5\text{GFP}$ -expressing cells and 373.4 ± 33.9 pA/pF ($n = 47$) in co-transfected cells ($P = 0.7560$). Likewise, no significant differences were detected in the voltage dependence of steady-state activation or steady-state inactivation between wild-type and co-transfected cells. For steady-state activation, WT cells exhibited $V_{1/2} = -34.2 \pm 1.8$ mV with $s = 6.1 \pm 0.3$ mV ($n = 23$), whereas co-transfected cells showed $V_{1/2} = -29.4 \pm 1.5$ mV and $s = 7.6 \pm 0.3$ mV ($n = 44$) ($P_{V_{1/2}} = 0.0520$, $P_s = 0.047$). For steady-state inactivation, $V_{1/2}$ values were -59.1 ± 2.1 mV with $s = -9.5 \pm 0.5$ mV ($n = 12$) in WT cells and -52.1 ± 2.2 mV with $s = -9.7 \pm 0.5$ mV ($n = 27$) in co-transfected cells ($P_{V_{1/2}} = 0.0578$; $P_s = 0.7522$). Activation kinetics

were also comparable, with time-to-peak values of 0.493 ± 0.018 ms ($n = 31$) for WT cells and 0.532 ± 0.013 ms ($n = 46$) for co-transfected cells ($P = 0.0515$). In contrast, a modest but statistically significant increase in the inactivation time constant was observed in co-transfected cells compared with WT ($\tau_i = 0.869 \pm 0.05$ ms, $n = 26$ vs. 1.082 ± 0.05 ms, $n = 47$; $P = 0.0457$). No significant difference was detected in recovery from inactivation, with recovery time constants (τ_r) of 3.38 ± 0.20 ms ($n = 22$) for WT and 3.90 ± 0.19 ms ($n = 45$) for co-transfected cells ($P = 0.1020$).

2.6. Effect of Dithiothreitol (DTT) on the Function of R893C Mutant Channels

We hypothesized that substitution of arginine by cysteine at position 893 may promote the formation of aberrant Cys–Cys disulfide bonds within the pore region of the channel. To test this hypothesis, patch-clamp recordings were performed in the presence of the strong reducing agent dithiothreitol (DTT), which is capable of breaking disulfide bonds. Using the perfusion system, cells were exposed to 1 mM DTT and depolarized to 0 mV. Normalized sodium currents were calculated as $I(t)/I_0$, where I_0 denotes the peak current measured in the absence of DTT and $I(t)$ represents the peak current measured after a given duration (t) of DTT exposure. In CHO cells expressing WT- $\text{Na}_v1.5\text{GFP}$ channels, 2–3 minutes of DTT treatment did not result in a significant change in I_{Na} , with a normalized current of 1.02 ± 0.01 ($n = 8$). In contrast, cells expressing R893C- $\text{Na}_v1.5\text{GFP}$ exhibited a pronounced increase in sodium current following DTT application, with a normalized current of 1.39 ± 0.03 ($n = 8$) ($P < 0.0001$). These findings indicate that the cysteine introduced at position 893 forms disulfide bonds with native cysteine residues located in the pore region, thereby impairing channel opening. Reduction of these disulfide bonds by DTT restores channel function, providing direct mechanistic evidence for the molecular basis of the R893C-induced loss of $\text{Na}_v1.5$ function.

2.7. Role of Arg893 in Nav1.5 Channel Function

As described above, the arginine residue at position 893 (R893) is highly conserved and evolutionarily favored. To further demonstrate the functional importance of this residue, we generated a Nav1.5 construct carrying an R893H substitution. Patch-clamp recordings were performed in the whole-cell configuration on CHO cells expressing R893H mutant channels, and a pronounced loss of channel function was observed. Upon depolarization to 0 mV, current density was 340.6 ± 36.2 pA/pF ($n = 31$) for WT-Nav1.5GFP, whereas only 17.3 ± 3.5 pA/pF ($n = 28$) was measured for R893H-Nav1.5GFP ($P < 0.0001$). Similar reductions in current density were observed when R893H mutant channels were expressed in HEK cells. These results demonstrate that the presence of arginine at position 893 is essential for proper Nav1.5 channel function. To elucidate the structural basis of this requirement, we examined the cryo-electron microscopy structure of the human Nav1.5 channel. Structural analysis revealed that R893 plays a critical role in stabilizing the pore helix by forming three hydrogen bonds with the side chains of glutamate residues E898 and E901, thereby orienting their negatively charged side chains toward the pore lumen. This coordination likely contributes to the maintenance of a negatively charged electrostatic environment that facilitates Na⁺ permeation. In contrast, in the R893C mutant, the bulky arginine side chain required for this coordination is absent, leading to loss of these interactions. Consequently, the local negative charge density in this region is markedly reduced, which may hinder Na⁺ ion permeation through the channel pore.

Discussion

1. Evaluation of Results Obtained from the Investigation of Slow Inactivation in Shaker-IR K⁺ Channels

Although the precise mechanism of C-type inactivation has not yet been fully elucidated, increasing evidence suggests that a hydrogen-bond network formed in the region behind the selectivity filter plays a crucial role in stabilizing this process. Molecular dynamics simulations have proposed that the inactivated state is stabilized by so-called inactivation water molecules that enter the region behind the selectivity filter. To provide experimental evidence for the presence of these water molecules, our research group investigated the effects of heavy water (D₂O). However, the mechanism underlying the previously described slowing effect of heavy water on inactivation and recovery from inactivation remains unresolved. It has been suggested that this phenomenon may arise either from specific molecular interactions or from non-specific effects, such as the higher viscosity of heavy water compared with normal water or altered hydration energy of K⁺ ions. The latter non-specific effect has already been successfully excluded by our research group. Among the potential specific mechanisms, primary and secondary kinetic isotope effects have been proposed. Deuterium isotope effects on voltage-gated ion channels have been demonstrated previously, and subsequent studies have shown that the activation and inactivation kinetics of certain voltage-gated sodium channels are also slowed in the presence of heavy water compared with normal water. Kinetic isotope effects can influence protonation/deprotonation and/or H⁺ transfer reactions, particularly when proton transfer is directly involved in the rate-limiting step of the process. Hydrogen bonds formed in heavy water are stronger than those in normal water, as the difference in relative bond energies between hydrogen and deuterium leads to a reduction in zero-point vibrational energy (ZPVE). Zero-point vibrational energy, or molecular dissociation energy, is commonly used as a measure of bond strength. The presence of deuterium has a pronounced effect on O–H stretching

vibrations. According to Hooke's law, replacing hydrogen with the heavier deuterium results in a decrease in vibrational frequency and, consequently, a lower zero-point vibrational energy. Importantly, when an atom in a molecule is replaced by its isotope, the force constant (k) remains unchanged. Thus, in heavy water, hydrogen bonds between molecules become shorter and stronger. Because direct demonstration of these specific effects by electrophysiological current measurements is practically impossible, the experimental strategy of this work was to exclude the contribution of viscosity and thereby provide indirect evidence for specific molecular interactions. Based on our results, only 10% (w/w) glycerol in aqueous solution induced a significant change in inactivation time constants. No such effect was observed with the solution containing the highest viscosity, namely 10% (w/w) sucrose, nor with the moderately viscous 5% (w/w) glycerol solution. Furthermore, the effects of heavy water and 10% (w/w) glycerol were not additive, despite their different viscosities. Taken together, these findings support our previous hypothesis that heavy water stabilizes the inactivated state through direct molecular interactions after entering the region behind the selectivity filter. In this way, indirect experimental evidence was obtained for the presence of inactivation water molecules behind the selectivity filter. If heavy water is capable of accessing this region and forming direct molecular interactions, it is likely that normal water exerts a similar stabilizing effect on the inactivated state. This knowledge may contribute to a more detailed understanding of the molecular mechanisms underlying C-type inactivation in the future.

2. Evaluation of Results Obtained from the Biophysical Characterization of Na_v1.5-R893C

In the present study, genetic analysis of a 41-year-old male patient diagnosed with Brugada syndrome and displaying distinctive phenotypic features revealed a novel SCN5A mutation (c.2677C>T), followed by comprehensive biophysical characterization of the mutant protein. The c.2677C>T nucleotide substitution results in replacement of arginine by cysteine at position

893 (R893C). This residue is located in the extracellular loop between the S5 and S6 segments of domain II, a region contributing to formation of the channel pore and situated in close proximity to the selectivity filter (SF). The pathogenic nature of the R893C mutation was hypothesized based on several lines of evidence: 1) based on sequence comparison, the R893 side chain is highly conserved from an evolutionary perspective across different mammalian species as well as human Na_v isoforms, suggesting that arginine at this position is essential for normal channel function; 2) according to literature data and gene mutation databases, R893C has an extremely low allele frequency, consistent with its potentially pathogenic nature; 3) electrophysiological measurements revealed a dramatic reduction in current density in R893C- $\text{Na}_v1.5\text{GFP}$ channels compared with wild type, strongly supporting the pathogenic nature of the mutation. Numerous mutations associated with Brugada syndrome have been reported in the DII S5–S6 region of $\text{Na}_v1.5$, including position 893; however, this is the first study providing detailed functional information through full biophysical characterization of a mutation at this site. To exclude potential confounding effects of N-terminal GFP tagging, we first characterized the biophysical properties of WT- $\text{Na}_v1.5\text{GFP}$ and compared them with those of untagged WT- $\text{Na}_v1.5$. Only minor differences were observed, consistent with previous reports. In contrast, expression of R893C- $\text{Na}_v1.5\text{GFP}$ in CHO and HEK293 cells resulted in a marked reduction in current density accompanied by alterations in other biophysical parameters, demonstrating that this mutation causes a pronounced loss of $\text{Na}_v1.5$ channel function. In general, $\text{Na}_v1.5$ variants associated with Brugada syndrome are considered loss-of-function (LoF) mutations, most commonly impairing channel activation, opening, or recovery from inactivation through alterations in gating parameters. In heterozygous Brugada syndrome, a reduction in the number of functional $\text{Na}_v1.5$ channels leads to decreased upstroke velocity of the cardiac action potential, formation of an endo-epicardial voltage gradient, and delayed right ventricular activation, thereby generating the characteristic ECG abnormalities of the disease. This endo-

epicardial voltage gradient, together with consequent repolarization heterogeneity, constitutes the electrophysiological arrhythmia substrate. Reduced Na^+ conductance prolongs the action potential and increases vulnerability to early and delayed afterdepolarizations (EADs and DADs), which may act as triggers for sustained and potentially fatal arrhythmias. If such depolarizing events reach threshold, they can initiate new action potentials and, via the “R-on-T” phenomenon, give rise to polymorphic ventricular tachycardia (VT) or ventricular fibrillation (VF). In addition to the marked reduction in current density, R893C mutant channels exhibited slower activation kinetics and modest alterations in the voltage dependence of steady-state activation. Interestingly, the R893C mutation also caused an approximately 10 mV positive shift in the voltage dependence of steady-state inactivation. This shift toward more positive membrane potentials increases the fraction of available channels and may serve as a compensatory mechanism counteracting the reduced current density. In practical terms, at a given membrane potential, mutant channels display a lower probability of inactivation, which may enhance excitability. Thus, the loss of function induced by the R893C mutation, together with the resulting abnormal Na^+ homeostasis, contributes both to the arrhythmic trigger and substrate. CHO cells were used as an expression system in several experiments; these cells are known to express endogenous sodium channels that generate small inward background currents. Therefore, it was essential to exclude the possibility that the very low I_{Na} measured in R893C- $\text{Na}_v1.5\text{GFP}$ -expressing cells originated from endogenous channels rather than from the mutant $\text{Na}_v1.5$ channels themselves. In our experiments, background Na^+ currents were minimal, and expression of R893C mutant channels in HEK293 cells, which lack endogenous sodium channels, yielded I_{Na} values comparable to those recorded in CHO cells. Moreover, endogenous sodium channels are tetrodotoxin (TTX)-sensitive, whereas $\text{Na}_v1.5$ channels are TTX-resistant; application of 0.5 μM TTX had no significant effect on current density in R893C- $\text{Na}_v1.5\text{GFP}$ -expressing cells. These findings indicate that the reduced I_{Na} observed in

CHO cells reflects intrinsic functional impairment of the mutant Na_v1.5 channels rather than contamination by endogenous currents. To model the heterozygous state of the patient and to assess interactions between co-expressed WT-Na_v1.5GFP and R893C-Na_v1.5GFP channels, 1:1 co-transfection experiments were performed in CHO cells, followed by whole-cell patch-clamp analysis of current density and gating parameters. Because an identical amount of WT plasmid DNA (1 µg) was transfected in all experiments, similar current densities and gating properties were expected in WT and mixed conditions. Indeed, the presence of mutant channels did not exert a dominant-negative effect on WT channel function, as no significant differences were observed in current density or gating parameters upon depolarization to 0 mV. These results further demonstrate that R893C-Na_v1.5GFP channels remain nonfunctional even in the presence of WT-Na_v1.5GFP channels. Confocal microscopy revealed similarly preserved membrane expression of WT-Na_v1.5GFP and R893C-Na_v1.5GFP, indicating proper synthesis and trafficking of the mutant channels to the plasma membrane despite their functional impairment. The precise molecular mechanism underlying the loss of function of R893C-Na_v1.5GFP channels is not yet fully resolved. However, extracellular application of 1 mM dithiothreitol (DTT) partially restored channel function. Cryo-electron microscopy-based modeling suggests that the cysteine at position 893 may form aberrant disulfide bonds with nearby cysteines, such as C896 and C906, or with cysteines located in homologous regions of other domains (e.g., C373 in DI, C1272 in DIII, and C1703 in DIV). Such interactions could prevent proper formation of the native Na_v1.5 conformation or destabilize the active state of the channel. Recent evidence indicates that Na_v channel α-subunits may assemble and function as dimers, although the precise mechanisms governing this process remain incompletely understood. We propose that arginine at position 893 plays an important role in Na_v channel dimerization. Conserved arginine residues located behind the selectivity filter, including R367, R383, R878, and R893, form critical interactions with negatively charged amino acid side

chains. Mutations at these positions disrupt these interactions, leading to conformational changes of the selectivity filter and impaired Na^+ permeability. Specifically, R893 forms essential salt bridges with nearby glutamate residues E898 and E901. Structural modeling indicates that R893 orients these negatively charged residues toward the pore lumen, thereby maintaining a favorable electrostatic environment for Na^+ permeation. In contrast, the R893C mutation disrupts local negative charge density and abolishes these interactions, leading to altered selectivity filter conformation and defective Na^+ permeation. To further confirm the critical role of the conserved R893 residue, we examined the biophysical properties of another missense mutation detected in Brugada syndrome patients, R893H. Although histidine shares certain physicochemical properties with arginine and can carry a positive charge at physiological pH, expression of R893H- $\text{Na}_v1.5\text{GFP}$ in CHO and HEK293 cells failed to produce detectable macroscopic I_{Na} upon depolarization to 0 mV. These results further underscore the essential role of conserved arginine residues in $\text{Na}_v1.5$ channel function. Consistent with this notion, previously reported missense mutations at the similarly conserved R878 position (R878C and R878K) also resulted in nonfunctional channels despite the presence of positively charged side chains.

In summary, genetic analysis of a young male patient with Brugada syndrome identified a novel $\text{Na}_v1.5$ pore mutation (R893C). This mutation caused a pronounced loss of $\text{Na}_v1.5$ function, manifested by a dramatic reduction in I_{Na} and positive shifts in the voltage dependence of steady-state activation and inactivation, with only minor changes in recovery from inactivation. These biophysical alterations create an arrhythmogenic environment that plausibly underlies the patient's clinical phenotype. Although the functional impact of the R893C mutation is substantial, we cannot exclude the possibility that physiological regulatory mechanisms in native cardiomyocytes may modulate these effects differently from those observed in CHO and HEK293 expression systems. Our findings highlight the importance of the DII S5–S6 loop

region in Nav1.5 gating and further support the concept that elucidating correlations between ion channel structure, mutation-induced functional changes, and clinical phenotypes is essential for understanding the mechanisms of ion channel diseases. Moreover, improved insight into Nav1.5 structure–function relationships may provide valuable prognostic information for clinicians, aiding patient selection for ICD implantation and informing the development of more effective pharmacological therapies for SCN5A channelopathies.

Summary

In this work, we performed biophysical characterization of various wild-type and mutant voltage-gated potassium and sodium channels using cellular electrophysiological approaches. Our aims were to elucidate the molecular mechanisms underlying genetically determined arrhythmia entities associated with mutations of the SCN5A gene encoding the cardiac fast voltage-gated sodium channel (Nav1.5), and to improve understanding of C-type inactivation by identifying contributing factors using the patch-clamp technique.

In the first part of the study, we searched for experimental evidence supporting the presence of a hydrogen-bond network formed by so-called “inactivation water molecules” entering the region behind the selectivity filter and stabilizing C-type inactivation in voltage-gated potassium channels. As direct proof of specific molecular interactions is not feasible using electrophysiological current measurements, we aimed to provide indirect evidence by excluding non-specific effects. Measurements performed on Shaker-IR channels allowed us to rule out the contribution of increased viscosity, supporting the hypothesis that heavy water—and by extension normal water—can access the region behind the selectivity filter and stabilize the inactivated state.

In the second part, we carried out comprehensive biophysical characterization of a novel SCN5A mutation identified in a patient diagnosed with Brugada syndrome. Following clinical evaluation, family screening, and genetic analysis, the mutation was expressed in CHO and HEK cells, and its pathogenic nature was demonstrated using patch-clamp electrophysiology. Confocal microscopy and pharmacological experiments further revealed the molecular basis of the observed loss of function and highlighted the critical importance of the DII S5–S6 loop region and conserved arginine side chains in Nav1.5 channel function.

New scientific achievements

1. I successfully excluded the possibility that the retarded inactivation induced by heavy water on Shaker-IR channels is due to its higher viscosity, which further supports the hypothesis that heavy water and consequently lightwater as well, can access the region behind the selectivity filter, thereby stabilizing C-type inactivation.
2. In our patient, diagnosed with Brugada syndrome, a previously uncharacterized, potentially pathogenic Nav1.5 pore-region mutation (R893C) was identified by genetic analysis.
3. R893C mutant ion channels exhibited significantly reduced current density compared to wild-type channels, supporting the pathogenic nature of the mutation.
4. Confocal microscopy measurements demonstrated preserved membrane expression of the R893C mutant ion channels.
5. The presence of R893C mutant ion channels did not exert a dominant-negative effect on the function of wild-type channels.
6. Extracellular application of DTT partially restored the function of R893C mutant channels, suggesting that the formation of Cys–Cys disulfide bonds in the pore region underlies the observed loss of function.
7. The evolutionarily conserved arginine at position 893 is critical for Nav1.5 function, as supported by structural modeling of the channel and the loss of function observed in R893H mutant channels.

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