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Gain-of-function mutation of $Na_v1.5$ in atrial fibrillation enhances cellular excitability and lowers the threshold for action potential firing

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ABSTRACT

Genetic mutations of the cardiac sodium channel (SCN5A) specific only to the phenotype of atrial fibrillation have recently been described. However, data on the biophysical properties of SCN5A variants associated with atrial fibrillation are scarce. In a mother and son with lone atrial fibrillation, we identified a novel SCN5A coding variant, K1493R, which altered a highly conserved residue in the DIII–IV linker and was located six amino acids downstream from the fast inactivation motif of sodium channels. Biophysical studies of K1493R in tsA201 cells demonstrated a significant positive shift in voltage-dependence of inactivation and a large ramp current near resting membrane potential, indicating a gain-of-function. Enhanced cellular excitability was observed in transfected HL-1 atrial cardiomyocytes, including spontaneous action potential depolarizations and a lower threshold for action potential firing. These novel biophysical observations provide molecular evidence linking cellular "hyperexcitability" as a mechanism inducing vulnerability to this common arrhythmia.

The cardiac sodium channel ($Na_v1.5$) plays a critical role in the generation of electrical activity in cardiac myocytes. Genetic mutations of the gene encoding the $Na_v1.5$ sodium channel (SCN5A) are associated with a variety of cardiac arrhythmia syndromes, including the Long QT syndrome type 3 (LQT3) [1], Brugada syndrome [2], and progressive cardiac conduction disease [3]. The biophysical abnormalities induced by disease-causing SCN5A mutations are diverse. In LQT3, SCN5A mutations result in a "gain-of-function" effect, invariably leading to a persistent late component of sodium current resulting in action potential prolongation [4]. In contrast, Brugada syndrome and conduction system disease are generally characterized by a primary loss of myocardial sodium current by various mechanisms, including impaired protein trafficking, more rapid inactivation kinetics, or altered voltage-dependence of activation [5,6].

A role of SCN5A mutations in the primary pathogenesis of atrial fibrillation (AF) is suggested by the known efficacy of sodium channel blocking agents in diminishing recurrences of AF. Further, genetic screening of SCN5A in a large cohort of patients with lone AF identified rare genetic variants of SCN5A in nearly 6% of AF cases [7]. Insight into the biophysical abnormalities of SCN5A mutations associated with lone AF is essential to understanding the molecular mechanisms of this common arrhythmia.

In this article, we describe unique electrophysiological characteristics of a novel SCN5A mutation associated with lone AF. The biophysical observations of this mutant $Na_v1.5$ channel are previously undescribed for the cardiac sodium channel, and include the demonstration of enhanced window current occurring near resting membrane potential, spontaneous action potential depolarizations and a lower threshold for action potential firing in transfected atrial cardiomyocytes. This study provides direct evidence of a novel molecular mechanism of cellular "hyperexcitability" inducing vulnerability to AF.

Methods

Molecular screening. Genomic DNA was extracted from whole blood from probands with "idiopathic" AF. Genetic analysis was

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performed by direct DNA sequencing of the coding region of the GJA5, KCNQ1, KCNA5, and SCN5A genes. Affected and control patients in this study were of Western European descent. All study participants provided written informed consent and the research protocol was approved by the Ethics Board of the University of Ottawa Heart Institute.

Expression of $Na_v1.5$ in tsA201 cells and HL-1 atrial myocytes. Mutant $Na_v1.5$ (K1493R) was introduced into a wild-type (wt) $Na_v1.5$ pcDNA1 clone using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) and sequences confirmed using an ABI 377 DNA sequencer (Perkin-Elmer/Applied Biosytems, Foster City, CA, USA). TsA201 cells were grown in high glucose DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (10 mg/ml) (Gibco BRL Life Technologies, Burlington, ON, Canada). The cells were transfected with 5 μg of cDNA coding for wt or mutant channel using the calcium phosphate method.

HL-1 cells, an atrial myocyte derived cell line, were a generous gift from Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). HL-1 cells were grown in 35 mm culture dishes to 50–70% confluency in Claycomb medium containing 10% FBS, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 0.1 mM norepinephrine. Cells were transfected in serum free and antibiotic free Claycomb medium containing 2 μl lipofectamine 2000 and 1 μg $Na_v1.5$/wt or $Na_v1.5$/K1493R mutant expression vectors mixed with 1 μg pECFP expression vector to denote cells that received the wild-type or mutant $Na_v1.5$. Only cells that exhibited cyano fluorescence were selected for further electrophysiological experiments.

Electrophysiological experiments. $Na_v1.5$ currents from tsA201 transfected cells were recorded using the whole-cell configuration of the patch clamp technique. Patch clamp recordings were made using low resistance electrodes (<1 MΩ), and a routine series resistance compensation by an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, USA) was performed to minimize voltage-clamp errors. The patch pipette contained 35 mM NaCl, 105 mM CsF, 10 mM EGTA, and 10 mM Cs-Hepes. The bath solution contained 150 mM NaCl, 2 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Na-Hepes. The pH of all solutions was adjusted to 7.4. Voltage-clamp command pulses were generated by microcomputer using pCLAMP software v8.0 (Axon Instruments). Experiments were carried out at room temperature.

In HL-1 atrial cardiomyocytes, recordings were carried out using the current-clamp analysis at room temperature. Cells were perfused with a solution of 140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose, pH 7.35. The recording pipette had a resistance of 2.5–3.5 MΩ when filled with an internal solution containing (in mM): 140 mM KCl, 5 mM Hepes, 0.5 mM EGTA, 20 mM glucose, 3 mM Na_2ATP, and 0.5 mM GTP, pH 7.35. Action potentials (APs) were induced by injecting a current of 800 pA for a duration of 4 ms. When required, current was applied to set a holding potential of −60 mV. AP traces were acquired and analyzed with pClamp software, filtered at 10 kHz and sampled at 20 kHz. To study the threshold for action potential elicitation, increasing amplitude of current was injected in 50 pA increments ranging from 100 to 800 pA until an AP was induced. The minimum current injected eliciting an AP was considered the threshold.

Data are presented as means ± standard error of the mean (SEM). When indicated, a *t*-test was performed using statistical software in SigmaPlot (Jandel Scientific Software, San Rafael, CA, USA).

Results

Mutation detection and clinical phenotype

DNA sequencing identified a novel, heterozygous mutation in exon 26 of the SCN5A gene in a single proband and his affected mother. This mutation leads to an amino acid substitution of lysine for arginine (K1493R) in the DIII–IV linker of the $Na_v1.5$ channel (Fig. 1A). K1493 is a highly conserved amino acid across species and is six amino acids downstream from the fast inactivation IFM (isoleucine–phenylalanine–methionine) motif of sodium channels (Fig. 1B) [8]. This genetic variant was absent from 200 alleles of an ethnically matched, healthy control cohort.

The affected proband developed AF at age 50. Coronary angiography documented normal coronary arteries. Echocardiography noted normal left atrial size, and normal left ventricular size and function. Twelve-lead electrocardiograms during normal sinus rhythm and AF consistently demonstrated a normal QTc interval (Fig. 1C). The proband's mother was diagnosed with AF at age 63 following many years of symptomatic palpitations. Her previous cardiac investigations documented a normal myocardial perfusion scan and normal parameters on echocardiography, aside from a mildly enlarged left atrium. The proband's only sibling, a brother, is healthy at age 60, and is not a carrier of the K1493R mutation.

Electrophysiological data

In mammalian tsA201 cells, activation and inactivation kinetics for wt and mutant $Na_v1.5$ showed no differences in peak current or current density (Fig. 2A and B). To compare the time course of inactivation for both channels, current decay was fitted with a double exponential function. Fig. 2C shows that time constants of both fast and slow inactivation were slower for mutant $Na_v1.5$/K1493R. For example, at +10 mv, $Na_v1.5$/wt: $\tau f = 0.45 \pm 0.02$ ms, $\tau s = 3.72 \pm 0.40$ ms; $Na_v1.5$/K1493R: $\tau f = 0.58 \pm 0.02$ ms, $\tau s = 5.05 \pm 0.54$ ms, $p < 0.01$.

To investigate the gating properties of $Na_v1.5$/K1493R and $Na_v1.5$/wt, we next analyzed the voltage-dependence of steady-state activation and inactivation. The potential for half-maximal activation and slope factor were similar for wt and mutant channels ($Na_v1.5$/wt: $V_{1/2} = -56.59 \pm 1.72$ mV, $k = -5.6$, $n = 6$; $Na_v1.5$/K1493R: $V_{1/2} = -58.17 \pm 0.79$ mV, $k = -5.3$, $n = 6$, p = NS). In contrast, $V_{1/2}$ for inactivation was significantly shifted to more positive potentials for mutant $Na_v1.5$ as compared to $Na_v1.5$/wt channels, without significant change in their respective slope factors ($Na_v1.5$/wt: $V_{1/2} = -104.33 \pm 1.58$ mV, $k = 4.79$, $n = 6$; $Na_v1.5$/K1493R: $V_{1/2} = -99.20 \pm 1.34$ mV, $k = 4.81$, $n = 6$, $p < 0.05$) (Fig. 2D). This depolarizing shift in steady-state inactivation produced a greater overlap of activation and inactivation curves resulting in a larger "window current" (Fig. 2E), predicting an increase in channel availability in the range of the resting membrane potential of atrial myocytes. To confirm a gain of frequency-dependent current for the mutant channel, currents were elicited by slow ramp depolarizations. Significantly larger ramp currents were elicited for mutant $Na_v1.5$/K1493R channels as compared to wt channels (Fig. 2F). The averaged peak ramp current occurred at a voltage of −67.11 ± 1.89 mV for the mutant channel, and was consistently larger than wt channels at all ramp slopes tested (data not shown). In view of the observed gain-of-function properties of the mutant $Na_v1.5$/K1493R, we evaluated whether this mutant imbues a late, persistent inward sodium current. No substantial persistent inward current at the end of a 400 ms depolarization was observed for either wt or mutant channel (Fig. 3A and B). Analyses of intermediate inactivation, time course of recovery from slow inactivation, and voltage-dependence of deactivation did not differ between wt and mutant $Na_v1.5$ channels (Fig. 3C–E).

Lastly, we assessed the effect of $Na_v1.5$/wt and $Na_v1.5$/K1493R mutant channels on the action potential (AP) duration and cellular excitability in HL-1 atrial cardiomyocytes. Induced AP duration did not differ in cells transiently expressing $Na_v1.5$/wt or $Na_v1.5$/K1493R channels, consistent with the absence of late, persistent sodium current (Fig. 4A and B). Next, we measured the threshold

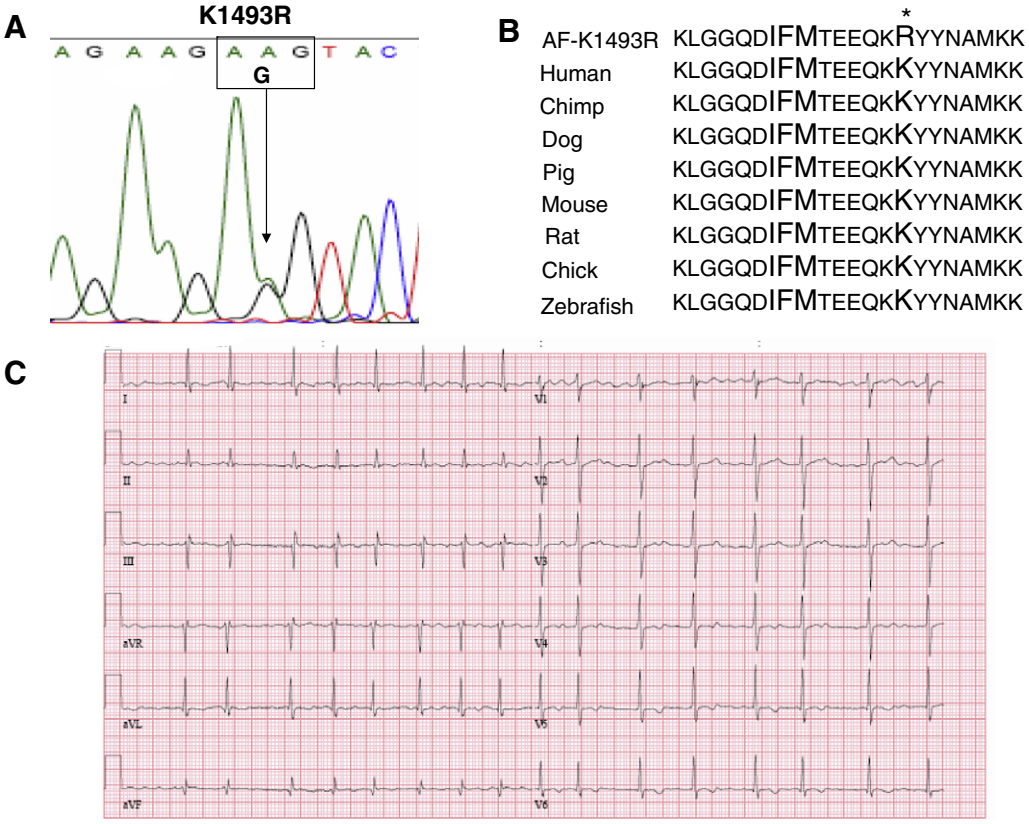

Fig. 1. Mutation and clinical data. (A) DNA sequence indicating the mutation in exon 26 of the SCN5A gene. (B) K1493 is in close proximity to the fast inactivation motif (IFM) within the $Na_v1.5$ channel and is highly conserved across species. (C) Representative ECG recording from the proband during atrial fibrillation. A normal QT interval is present.

of depolarization for elicitation of an all-or-none AP. HL-1 cells expressing mutant $Na_v1.5$/K1493R channels required 25% less depolarizing current for AP elicitation as compared to cells expressing wt channel ($Na_v1.5$/wt 693.3 ± 37.5 pA, $Na_v1.5$/K1493R 525 ± 30 pA, $p < 0.005$) (Fig. 4C). In addition, spontaneous cellular depolarizations were observed in 4 of 13 cells (31%) expressing mutant $Na_v1.5$/K1493R as compared to only 2 of 16 cells (12.5%) expressing $Na_v1.5$/wt (Fig. 4D), reaffirming a hyperexcitable phenotype induced by mutant $Na_v1.5$/K1493R. Mean amplitude of spontaneous depolarizations was 33.63 ± 17.97 mV in $Na_v1.5$/K1493 expressing cells versus 11.85 ± 1.36 mV in $Na_v1.5$/wt cells ($p < 0.05$). No difference was observed in resting membrane potential of HL-1 cardiomyocytes transfected with wt or mutant $Na_v1.5$ channels (data not shown).

Discussion

We describe the association of a novel genetic mutation in the voltage-gated cardiac sodium channel gene with lone atrial fibrillation. This missense K1493R mutation was present in a mother and son with early onset atrial fibrillation and no structural heart disease. The highly conserved, charged K1493 residue is located within the cytoplasmic loop domain which joins domains III and IV, and is six amino acids C-terminal to the fast inactivation IFM (isoleucine–phenylalanine–methionine) motif of the sodium channel. The IFM motif is invariable among sodium channels and is crucial to rapid channel inactivation following opening [8–10].

The unique gain-of-function properties exhibited by $Na_v1.5$/K1493R have not been previously described for the voltage-gated cardiac sodium channel. Consistent with the localization of the mutant to the fast inactivating region of the channel, mutant K1493R demonstrated a significant depolarizing shift in fast inactivation. These observations correlate with the site-directed mutagenesis studies of Miller et al. showing that deletion of charged lysines within the linker region of domains III and IV hasten the voltage dependence of sodium channel inactivation [10]. This alteration is expected to increase the fraction of channels available for activation close to resting potential due to an uncoupling of channel activation and inactivation. The increased overlap between activation and inactivation curves observed in our study resulted in an increased window current for the mutant channel. The window current represents a voltage region in which sodium channels continue to open, and therefore is predicted to lower the excitability threshold for excitable cells [11]. Indeed, expression of the wt and $Na_v1.5$/K1493R in HL-1 atrial cardiomyocytes confirmed a significantly lower depolarizing current threshold for single AP firing, and HL-1 cells expressing mutant $Na_v1.5$/K1493R demonstrated spontaneous depolarizations consistent with a hyperexcitable phenotype. AP duration in HL-1 cardiomyocytes were similar between cells expressing wt and mutant $Na_v1.5$ channels, consistent with the absence of late persistent current that is observed in $Na_v1.5$ gain-of-function mutations associated with LQT3.

Although not reported previously in the $Na_v1.5$ channel, non-inactivating sodium channel mutations resulting in larger window currents have been implicated in other inherited disorders of excitability [11–13]. In multiple animal models of spontaneous seizures, the voltage dependence of sodium current inactivation in neuronal cells was observed to be shifted toward depolarized potentials and resulted in increased window current, similar to our observations for the $Na_v1.5$/K1493R mutant [14–17]. The observed biophysical properties of sodium channel mutations responsible for epilepsy are consistent with the known efficacy of anti-epileptic drugs in functioning to shift the voltage dependence of inactivation in the

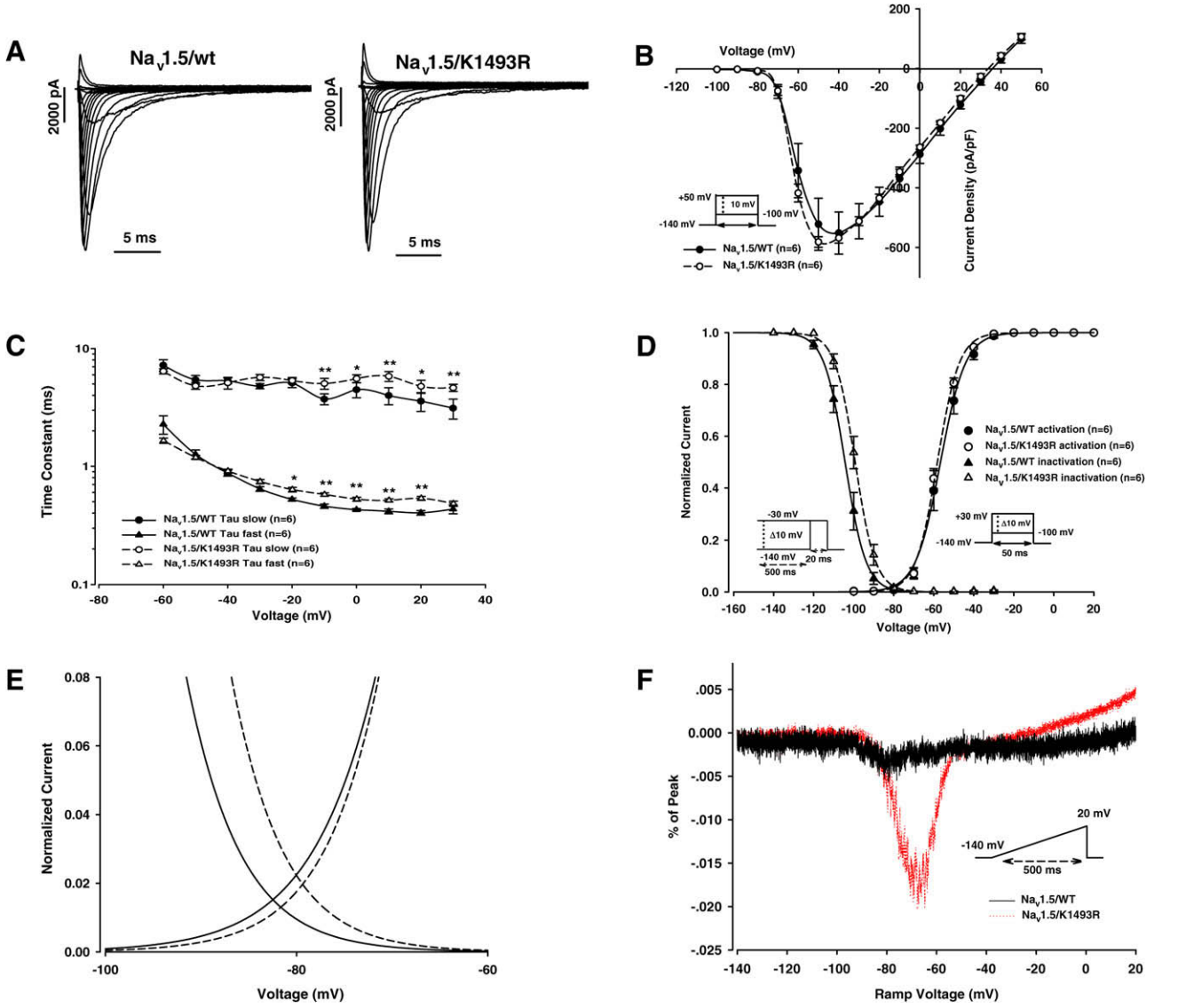

Fig. 2. Biophysical properties of Na$_v$1.5/wt and Na$_v$1.5/K1493R expressed in tsA201 cells. (A) Analysis of whole-cell currents recorded from cells expressing wt and mutant channels. Current was elicited by depolarizing pulses from −100 to +50 mV in 10 mV increments. (B) Current–voltage relationship of Na$_v$1.5/wt and Na$_v$1.5/K1493R. The current amplitude was normalized to the membrane capacitance. (C) The voltage-dependence time constants of fast inactivation in wt and K1493R expressing cells. Currents were fitted to a two-exponential function. Asterisks indicate significance ($^{*}p < 0.05$, $^{**}p < 0.01$). (D) Steady-state voltage-dependent properties of activation and inactivation for wt and K1493R sodium channels ($^{*}p < 0.05$, $^{**}p < 0.01$). Voltage-clamp protocols are shown as inset. (E) The view of the overlapping area between activation and steady-state inactivation was expanded from (D). (F) Window currents of wt and K1493R channels. Ramp currents of wt (solid line, $n = 6$) and K1493R (dash line, $n = 6$) sodium channels elicited with 500 ms ramp depolarizations from −140 to 20 mV.

negative direction [14,18–21]. Similar findings are seen in myotonic disorders associated with specific SCN4A mutations, whereby therapy with flecainide, a sodium channel blocker, provides clinical benefit [22].

Diverse molecular mechanisms of AF induced by ion channel genetic defects have been described. Gain-of-function mutations of potassium channels KCNQ1, KCNE2, and KCNJ2 are reported to result in a shortening of APD and are predicted to shorten atrial effective refractory period, inducing enhanced vulnerability to re-entry [23–25]. In contrast, a mutation in KCNA5 resulting in a loss-of-function is reported to increase APD and early-after-depolarizations in atrial myocytes promoting triggered activity [26]. Connexin40 mutations result in a loss of gap junction conductance and are proposed to enhance atrial conduction heterogeneity throughout myocardial tissue, facilitating a reentry substrate for AF [27]. Functional studies on two previously reported Na$_v$1.5 channel mutations associated with AF have shown contrasting functional differences [28,29]. Although both mutations were located in the C-terminus of the channel, a hyperpolarizing shift in steady-state inactivation suggesting a loss-of-function was noted in one study [30], while the other reported a depolarizing shift in steady-state inactivation and increased current density [31]. The gain-of-function findings of the latter study are similar to ours, although Na$_v$1.5/K1493R did not show an increased current density. The present study elaborates on the functional consequences of a depolarizing shift in steady-state inactivation, demonstrating for the first time that this biophysical abnormality in Na$_v$1.5, in the absence of other kinetic alterations, results in an enhanced window current, a lower threshold for action potential firing, and spontaneous depolarizations when studied in HL-1 atrial cardiomyocytes. These data suggest a distinct mechanism of AF vulnerability due to cellular hyperexcitability that results from a novel gain-of-function in the cardiac sodium channel.

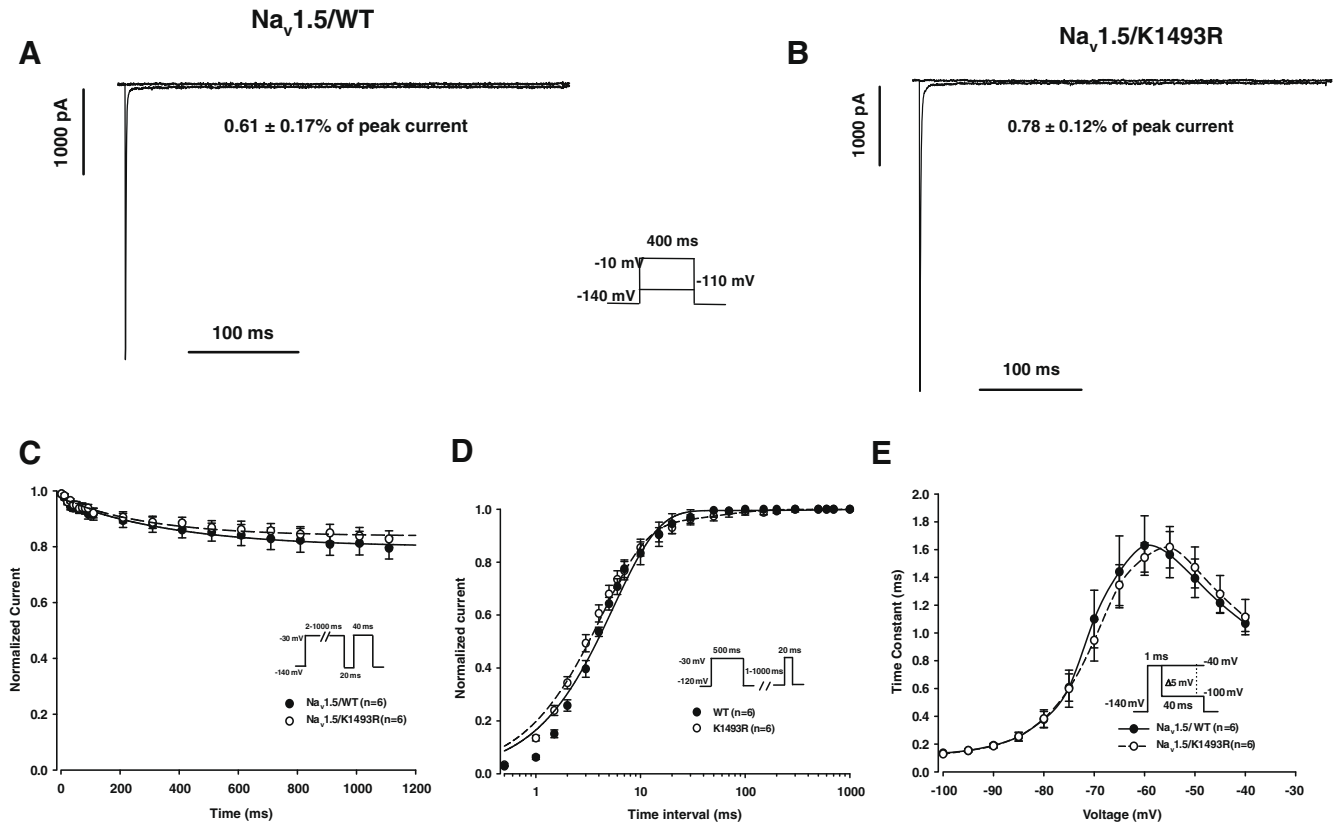

Fig. 3. The persistent sodium current from wt (A) and K1493R (B) $Na_v1.5$ channels. (C) Slow inactivation, (D) time courses of recovery from slow inactivation, and (E) deactivation from the open state from wt and K1493R $Na_v1.5$ channels. Protocols are indicated in inset.

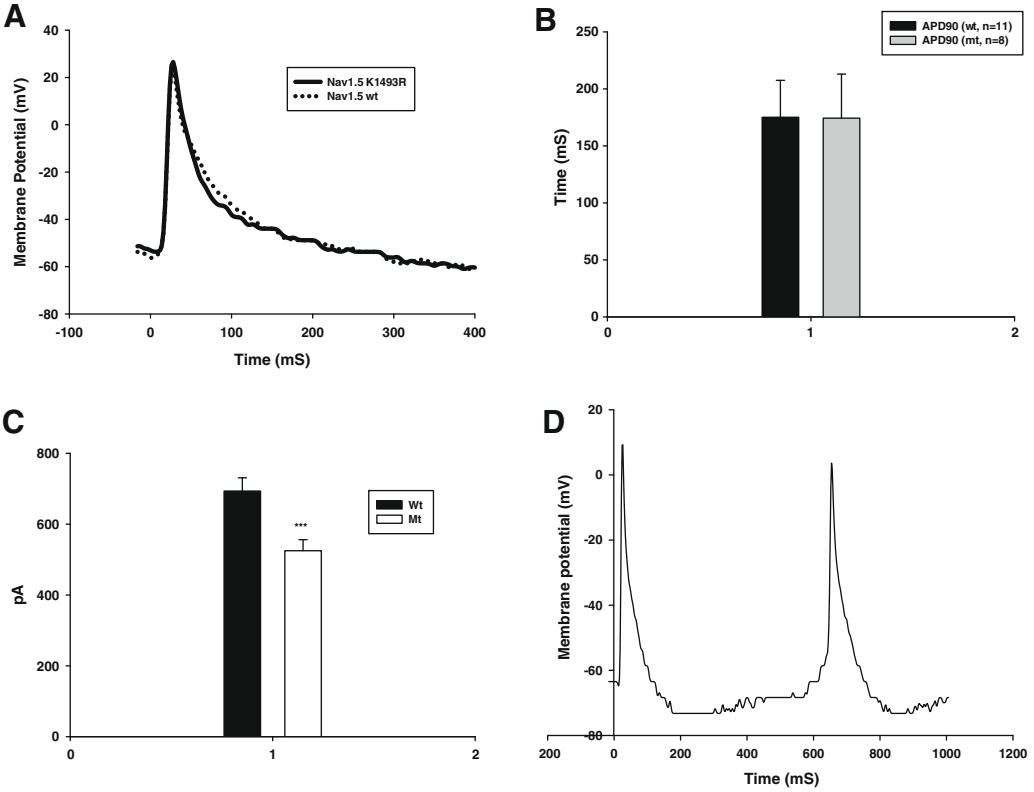

Fig. 4. Superimposed AP traces recorded from HL-1 cardiomyocytes transfected with $Na_v1.5$/wt or $Na_v1.5$/K1493R sodium channels (A). (B) The APD_{90} of APs recorded from HL-1 cells expressing wt or K1493 channels. (C) Depolarizing threshold current for elicitation of Aps from HL-1 cells expressing wt ($n = 6$) or K1493R ($n = 8$) sodium channels ($p < 0.005$). (D) Spontaneous all-or-none AP observed from HL-1 atrial cardiomyocytes expressing $Na_v1.5$/K1493, which was not observed from wt expressing cells.

The biophysical observations of $Na_v1.5$/K1493 are similar to those described in other sodium channelopathy diseases associated with hyperexcitability. In the context of atrial fibrillation, rapid focal atrial activity, commonly observed from pulmonary myocardial sleeves, may be the result of repetitive myocyte AP firing. Furthermore, a link between sodium channel activity and autonomic mediated AF has been suggested in a study by Scornik et al., which identified a high expression of $Na_v1.5$ channels in intracardiac ganglia [30]. These investigators demonstrated $Na_v1.5$-induced AP activity from these neurons and speculated that a gain-of-function of the $Na_v1.5$ channel might enhance AP activity of intracardiac neurons, augmenting acetylcholine release.

The data from this study provides a potential molecular link to the observations of focal triggers of AF [31], the efficacy of intracardiac ganglia denervation by catheter ablation [32], and the use of sodium channel blockers in reducing AF burden [33].

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