Voltage-Gated Sodium Channel Phosphorylation at Ser571 Regulates Late Current, Arrhythmia, and Cardiac Function in vivo

Running title: Glynn et al.; Ser571 phosphorylation regulates late $I_{Na}$ in vivo

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Abstract

Background—Voltage-gated Na\(^+\) channels (Na\(_r\)) are essential for myocyte membrane excitability and cardiac function. Na\(_r\) current (I\(_{Na}\)) is a large amplitude, short duration “spike” generated by rapid channel activation followed immediately by inactivation. However, even under normal conditions, a small “late” component of I\(_{Na}\) (I\(_{Na,L}\)) persists due to incomplete/failed inactivation of a subpopulation of channels. Notably, I\(_{Na,L}\) is directly linked with both congenital and acquired disease states. The multifunctional Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) has been identified as an important activator of I\(_{Na,L}\) in disease. Several potential CaMKII phosphorylation sites have been discovered, including Ser571 in the Na\(_r\),1.5 DI-DII linker, but the molecular mechanism underlying CaMKII-dependent regulation of I\(_{Na,L}\) in vivo remains unknown.

Methods and Results—To determine the in vivo role of Ser571, two Scn5a knock-in mouse models were generated expressing either: 1) Na\(_r\),1.5 with a phosphomimetic mutation at Ser571 (S571E), or 2) Na\(_r\),1.5 with the phosphorylation site ablated (S571A). Electrophysiology studies revealed that Ser571 regulates I\(_{Na,L}\) but not other channel properties previously linked to CaMKII. Ser571-mediated increases in I\(_{Na,L}\) promote abnormal repolarization and intracellular Ca\(^{2+}\) handling, and increase susceptibility to arrhythmia at the cellular and animal level. Importantly, Ser571 is required for maladaptive remodeling and arrhythmias in response to pressure overload.

Conclusions—Our data provide the first in vivo evidence for the molecular mechanism underlying CaMKII activation of the pathogenic I\(_{Na,L}\). Relevant for improved rational design of potential therapies, our findings demonstrate that Ser571-dependent regulation of Na\(_r\),1.5 specifically tunes I\(_{Na,L}\) without altering critical physiological components of the current.

Key words: arrhythmia (mechanisms), calcium/calmodulin-dependent protein kinase II, Na\(^+\) current, ion channel, action potential remodeling
Introduction

Cardiac function depends on the tightly coordinated activity of voltage-gated Na\(^+\) channels (Na\(_v\)) that are responsible for generating the action potential (AP) upstroke in response to an external stimulus.\(^1,2\) Importantly, Na\(_v\) normally inactivate almost as quickly as they activate, a requirement for the cell membrane to repolarize in preparation for the next stimulus/heartbeat. While voltage-dependent inactivation rapidly turns off most Na\(_v\) current (I\(_{Na}\)), a small persistent (“late”) component (I\(_{Na,L}\)) is apparent even under normal conditions.\(^3\) Aberrant I\(_{Na,L}\) is directly linked with increased susceptibility to arrhythmia and dysfunction in cardiac disease.\(^4,5\) For example, increased I\(_{Na,L}\) is present in congenital gain-of-function Na\(_v\) channelopathies (e.g. long QT 3), as well as in common forms of acquired disease (e.g. heart failure), and has been implicated in AP prolongation, abnormal ion homeostasis and arrhythmia.\(^3,6-8\) Drugs that specifically target I\(_{Na,L}\) are emerging as viable therapeutic agents to reduce arrhythmia burden in cardiac disease patients.\(^9-20\) A key feature of these agents is their selectivity for I\(_{Na,L}\) over peak current.\(^20\) Thus, it is important to understand the molecular pathways for regulating I\(_{Na,L}\) without altering other critical components of Na\(_v\), I\(_{Na}\) current (availability, recovery kinetics, etc.).

While the precise mechanism for defective I\(_{Na,L}\) in cardiac disease remains unknown, dysregulation of the multifunctional Ca\(^{2+}/\)calmodulin-dependent protein kinase II (CaMKII) has been linked to Na\(_v\) gating abnormalities in diverse settings, including heart failure, coronary artery disease and diabetes.\(^21-23\) CaMKII is a central node in an expansive signaling network responsible for control of excitation contraction coupling, metabolism, cellular respiration, transcriptional regulation, and cytoskeletal dynamics.\(^24\) Among its numerous targets, CaMKII phosphorylates Na\(_v\) to regulate the magnitude of I\(_{Na,L}\), as well as other properties including steady-state inactivation and recovery from inactivation.\(^21,25-28\) Although the molecular pathways for regulating I\(_{Na,L}\) remain to be characterized, CaMKII is currently emerging as a therapeutic target for cardiac arrhythmias.
mechanism remains under investigation, several potential sites for CaMKII phosphorylation have been identified in the DI-DII linker of Nav1.5, the predominant cardiac Na\textsubscript{v} alpha subunit.\textsuperscript{22, 26, 29} Na\textsubscript{v}1.5 Ser571 first emerged as a potential CaMKII phosphorylation site from a functional screen in heterologous cells.\textsuperscript{26} Increased CaMKII-dependent phosphorylation of Ser571 was later observed under stress conditions \textit{in vitro} and in samples from failing mouse, canine, and human hearts.\textsuperscript{22} Subsequent efforts have identified additional potential CaMKII sites in the DI-DII linker.\textsuperscript{29, 30} Despite this important foundational work, there is a lack of evidence to support the physiological significance of any of the potential phosphorylation sites \textit{in vivo}, as functional studies to date have mostly involved overexpression of exogenous channels in heterologous cells or neonatal cardiomyocytes. Thus, this field while potentially fruitful in the search for new pathways to selectively regulate I\textsubscript{Na,L} has stalled due to lack of relevant animal models.

In an effort to identify the molecular basis for CaMKII-dependent regulation of Na\textsubscript{v}1.5 and cell excitability \textit{in vivo}, we generated two novel \textit{Scn5a} knock-in mouse models: 1) the S571E mouse that substitutes a phosphomimetic glutamic acid for the serine at position 571; and 2) the S571A mouse that lacks the phosphorylation site due to replacement of the serine with an alanine. Using these new animal models, we report that Ser571 is essential for targeted regulation of I\textsubscript{Na,L}, cell excitability and heart function in response to stress (pressure-overload induced heart failure). Furthermore, we observe that ablation of the Ser571 site mitigates maladaptive electrical and mechanical remodeling observed in the failing heart. Based on these findings, we propose that Ser571 serves as a binary molecular switch for CaMKII-dependent activation of I\textsubscript{Na,L} \textit{in vivo}. Importantly, Ser571-dependent regulation is selective for I\textsubscript{Na,L} without affecting other channel properties that may have deleterious consequences for cardiac function. We anticipate that this molecular pathway may yield new therapeutic avenues for reducing
arrhythmia burden without disrupting normal physiology.

Methods

Animals

Scn5a S571E and S571A knock-in mice were generated (genOway) in C57/Bl6 background using a Flp-mediated strategy to remove neomycin selection cassette. Resulting animals expressed either the S571E or S571A point mutation (Figure 1). Experiments were performed in 2-month-old male mice. Animals were euthanized using CO2 and cervical dislocation followed by collection of tissue or cell isolation. Studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health following protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Electrophysiology

Ventricular myocytes were isolated from Langendorff-perfused adult mouse hearts, as described previously.\textsuperscript{22, 16, 31} \(I_{\text{Na}}\) recordings were performed on freshly isolated (<1 h in culture) myocytes at room temperature (20-22 °C) by a conventional whole-cell patch-clamp technique with an Axon 200B patch-clamp amplifier controlled by a personal computer using a Digidata 1320A acquisition board and the pClamp 10.3 software (Axon Instruments). \(I_{\text{Na,L}}\) was measured using two different methods: 1) average current over the interval 50-150 ms following the peak current; or 2) integral over the same time interval. As results were not dependent on the specific method, all results are reported using the first method. Pipette resistance was < 2.8 MΩ when filled with solution containing (in mM): NaCl (5), CsF (135), EGTA (10), MgATP (5), HEPES (5), pH 7.2. The extracellular solution contained (in mM): NaCl (5), MgCl2 (1), CaCl2 (1.8),
CdCl₂ (0.1), glucose (11), CsCl (132.5) and Hepes (20); pH was maintained at 7.4 with CsOH. Only cells with membrane resistance greater than 1 GΩ were used. Appropriate whole cell capacitance and series resistance compensation (>60%) was applied. Resulting currents were blocked with tetrodotoxin (TTX) to verify isolation of \( I_{\text{Na}} \) (Supplemental Figure 1). APs were recorded using the perforated (amphotericin B) patch-clamp technique at room temperature in Tyrode’s solution (bath). The pipette solution contained (in mM): potassium aspartate (130), NaCl (10), HEPES (10), CaCl₂ (0.04), MgATP (2), phosphocreatine (7), NaGTP (0.1), and 240 \( \mu \)g/mL amphotericin B. The pH was adjusted to 7.2 with KOH. APs were evoked by brief current pulses 1.5-4 pA, 0.5-1 ms. In all cases, the electrophysiologist was blinded to animal genotype.

**Mouse heart failure model with proximal aortic banding**

Transaortic constriction was performed to induce pressure overload conditions in adult male mice. Mice were anesthetized (isoflurane, 2.5%), intubated, and placed on a respirator (120 breaths min⁻¹, 0.1 mL tidal volume). The aorta was exposed via a midline sternotomy and a 6.0 Prolene suture was placed around the aorta distal to the brachiocephalic artery. The suture was tightened around a blunted 27-gauge needle placed next to the aorta, the needle was removed, and the chest was closed. A group of sex- and age-matched sham mice underwent the same procedure with the suture step omitted as a control. Echocardiography was performed before surgery and at regular intervals for six weeks following surgery to assess cardiac function using the Vevo 2100 (Visualsonics). The MS-400 transducer was used in the short axis M-mode to assess heart function and contractile features. Mice were sacrificed (2% Avertin, 20 \( \mu \)L/g, I.P.) via rapid thoracotomy at 6 weeks post-surgery (TAC or sham). Hearts, lung tissue, and right tibias were gathered from each mouse for further analysis.
Statistics

SigmaPlot 12.0 was used for statistical analysis. The Wilcoxon-Mann-Whitney U-test was used to determine P values for single comparisons. One-way ANOVA was used for multiple comparisons with the Bonferroni test for post hoc testing (data presented as mean±SEM). If the data distribution failed normality tests with the Shapiro-Wilk test, a Kruskal-Wallis one-way ANOVA on ranks was applied with a Dunn multiple-comparisons test for significant P values [data presented as median with 25th and 75th percentiles (box) and 10th and 90th percentiles (whiskers)]. Contingency data were analyzed using Chi-Square test. The null hypothesis was rejected for P<0.05.

Additional methods are provided in online only Data Supplement.

Results

Generation of S571E/A knock-in mice to test in vivo role of Ser571

Functional in vitro studies have demonstrated a role for CaMKII-dependent phosphorylation at Ser571 in regulating Na\textsubscript{1.5} (Figure 1A).\textsuperscript{22, 26, 29} To test the central hypothesis that phosphorylation of Na\textsubscript{1.5} at Ser571 is essential for CaMKII-dependent changes in \textit{I}_{Na,L} and channel kinetics \textit{in vivo}, two novel Scn5a knock-in mouse models were generated: 1) an S571E mouse, where the serine at position 571 is replaced with a glutamic acid (phosphomimetic); and 2) an S571A mouse, where the serine is replaced with an alanine to eliminate the CaMKII phosphorylation site (Figure 1B). While phospho-Na\textsubscript{1.5}(Ser571) was not detectable in S571A or S571E lysates (phospho-epitope eliminated by either mutation), protein expression levels and cellular localization of Na\textsubscript{1.5} were normal in S571E and S571A myocytes compared to WT at baseline. Furthermore, there were no differences in levels or localization of related associated...
proteins CaMKII, βIV-spectrin, or ankyrin-G\textsuperscript{22,26,33} (Figure 1C-G). Expression/localization of
the intercalated disc protein N-cadherin was also normal in S571E and S571A myocytes (Figure
1E-G). Histological examination of S571E and S571A hearts at baseline revealed no evidence
of overt structural changes (e.g. fibrosis) compared to WT at baseline (data not shown).
Echocardiography revealed small but significant LV dilation in S571E animals at baseline and
small but significant decreases in ejection fraction and fraction shortening in both S571E and
S571A animals without differences in other features (Table 1).

**Phosphorylation at S571 selectively regulates $I_{Na,L}$ and cell membrane excitability**

Disruption of spectrin/CaMKII interaction reduces Na\textsubscript{v}1.5 phosphorylation at Ser571 and alters
Na\textsubscript{v}1.5 activity and cell excitability.\textsuperscript{26} To assess the specific role of Ser571 in CaMKII-
dependent regulation of Na\textsubscript{v}1.5 at baseline, electrophysiology experiments were performed on
isolated, adult ventricular WT, S571E, and S571A myocytes (Figure 2). Whole cell $I_{Na}$
measurements showed a significant increase (~two-fold) in S571E $I_{Na,L}$ without any change in
peak current compared to WT at baseline (Figure 2A-B, Supplemental Figure 1). In contrast,
S571A myocytes displayed a significant reduction in $I_{Na,L}$ despite an increase in peak current
compared to WT (Figure 2A-B, Supplemental Figure 1). Surprisingly, no differences were
observed in steady-state inactivation or recovery from inactivation in S571E or S571A compared
to WT (Figure 2C-D), despite previous reports from our own group showing CaMKII-dependent
changes in both properties in heterologous cells.\textsuperscript{22,25-27,29} These findings indicate that Ser571
regulates $I_{Na,L}$ without affecting other channel properties linked to CaMKII (steady-state
inactivation, recovery). No differences were observed in inward rectifier or transient outward K\textsuperscript{+}
currents, or voltage-dependent Ca\textsuperscript{2+} current (Supplemental Figures 2-3).

Action potentials (APs) were measured in isolated adult ventricular WT, S571E, and
S571A myocytes to assess the relationship between Ser571 and membrane excitability.

Consistent with observed differences in \( I_{\text{Na},L} \), S571E myocytes subjected to slow pacing (1 Hz) demonstrated an increase in AP duration (APD) at 50, 75 and 90% repolarization, without any change in resting or peak transmembrane potential compared to WT (Figure 3A-C). In contrast, S571A APD was not significantly different than WT at baseline. Differences in APD between S571E and WT or S571A myocytes were eliminated with rapid pacing (2 Hz, Figure 3B) consistent with the rate-dependent decrease of \( I_{\text{Na},L} \).\(^{34}\) To determine whether increased \( I_{\text{Na},L} \) in S571E myocytes also affected Ca\(^{2+}\) homeostasis, Ca\(^{2+}\) sparks and waves were measured in isolated WT and S571E myocytes. Consistent with increased \( I_{\text{Na},L} \), S571E myocytes showed increases in Ca\(^{2+}\) spark frequency, Ca\(^{2+}\) wave frequency in response to isoproterenol, and sarcoplasmic reticulum Ca\(^{2+}\) load compared to WT (Supplemental Figure 4). Based on observed AP prolongation and elevated Ca\(^{2+}\) at baseline, we hypothesized that S571E myocytes would be more susceptible to formation of arrhythmogenic AP afterdepolarizations in response to stress. While overdrive pacing (up to 5 Hz) followed by a pause failed to elicit afterdepolarizations even in S571E (data not shown), high dose isoproterenol elicited frequent repolarization abnormalities, including afterdepolarizations and repolarization failure, in S571E but not WT or S571A myocytes [Numbers of cells with repolarization abnormalities (from two preparations): 4/8 S571E cells, 0/8 WT cells, 0/8 S571A cells; \( P<0.05 \)] (Figure 3D and Supplemental Figure 5). Isoproterenol-induced repolarization defects in S571E myocytes were normalized by treatment with ranolazine (Figure 3E and Supplemental Figure 5), supporting \( I_{\text{Na},L} \) as a viable target for decreasing arrhythmia susceptibility.

To determine whether Ser571 plays a role in regulation of cardiac excitability and/or arrhythmias in vivo, electrocardiograms were measured in conscious WT, S571E, and S571A
mice. S571E mice demonstrated significant prolongation of rate corrected QT interval (QTc) compared to WT, in agreement with measured differences in $I_{Na,L}$ and APD (Figure 4A-B). S571A animals displayed a small but significant increase in RR interval (Figure 4D). While no arrhythmias were observed in any group at baseline, S571E demonstrated frequent arrhythmia events, including premature ventricular contractions and ventricular tachycardia, in response to catecholaminergic stress compared to WT or S571A mice (Figure 4F-G). Treatment with Na$^+$ channel blockers flecainide or ranolazine eliminated differences in arrhythmia events between S571E, WT and S571A animals, consistent with involvement of $I_{Na,L}$ in the arrhythmia phenotype and supporting $I_{Na,L}$ as a viable anti-arrhythmia target in vivo (Figure 4G).

**Phosphorylation of S571 contributes to myocardial remodeling following transaortic constriction**

Several studies have implicated both CaMKII and $I_{Na,L}$ in maladaptive remodeling and arrhythmias in animal models of heart failure. Therefore, transaortic constriction (TAC) was utilized to test the hypothesis that Ser571 serves as an important locus linking CaMKII dysregulation to increased $I_{Na,L}$ and maladaptive remodeling in response to pressure overload (Figure 5). Cardiac hypertrophy, as evidenced by increased left ventricular (LV) wall thickness, was apparent in WT, S571E and S571A animals following 6 weeks of TAC (Figure 5A-E), indicating that hypertrophy occurs independent of phosphorylation at Ser571. However, ejection fraction, fractional shortening and LV chamber diameter following TAC showed improvement in S571A compared to WT or S571E animals (Figure 5F-J). For example, while ejection fraction decreased by over 40% in both WT and S571E animals following 6 weeks of TAC, S571A mice decreased by less than 10% over the same period. These data indicate that phosphorylation at Ser571 contributes to development of heart failure but not hypertrophy in response to pressure overload.
In parallel, electrophysiology was performed to assess the role of Ser571 in TAC-induced electrical remodeling. While $I_{Na,L}$ was relatively small in WT myocytes at baseline (Figure 2B), TAC resulted in a large $I_{Na,L}$ in WT comparable to that measured in S571E (Figure 6). In contrast, S571A myocytes were resistant to the increase in $I_{Na,L}$ observed in WT following 6 weeks of TAC. $I_{Na,L}$ was large in S571E at baseline and did not change significantly with TAC, presumably reflecting its near maximal value at baseline. Peak $I_{Na}$ was not different between TAC groups (Supplemental Figure 6). To determine whether phosphorylation at Ser571 contributed to electrical remodeling following TAC, APs were measured in WT, S571E and S571A TAC myocytes. Consistent with $I_{Na,L}$ measurements, WT but not S571A APs showed significant prolongation following TAC such that WT TAC APD was not significantly different from S571E at 90%, 75% or 50% repolarization (Figure 7A,B). Telemetry was also performed to evaluate arrhythmia burden in vivo (Figure 7D-E and Supplemental Figure 7). Frequent PVCs were observed with catecholaminergic stress in S571E and WT but not S571A animals following 6 weeks of TAC, indicating an anti-arrhythmic benefit from eliminating CaMKII phosphorylation at Ser571 in vivo (Figure 7D,E). To determine whether the differential response to TAC could be linked with differences in CaMKII expression or activation, levels of total and phosphorylated (T287) CaMKII were measured by immunoblot at baseline and following TAC (Supplemental Figure 8). Total and phosphorylated CaMKII levels were not significantly different at baseline, although total CaMKII trended higher in S571A (P=0.07). Surprisingly, levels of phosphorylated CaMKII increased almost 6 fold with TAC in all three genotypes, including S571A, supporting the hypothesis that CaMKII activation occurs independently of S571 phosphorylation in response to pressure overload. Taken together, these
TAC data indicate that phosphorylation at Ser571 is required for increased $I_{\text{Na,L}}$ maladaptive remodeling, and arrhythmias, but not hypertrophy or CaMKII activation in response to pressure overload.

**Discussion**

As we consider progress in the field over the past 20 years, it is apparent that we have stagnated in our search for new anti-arrhythmia therapies.39 The apparent impasse may be partly attributed to high profile failure of trials designed to test ion channels as anti-arrhythmia targets.$^2, 40$ While $I_{\text{Na,L}}$ inhibition has emerged as a candidate with therapeutic potential, the question remains: How do we target pathogenic components of ion channels or other targets without affecting components that are essential for normal physiology? In this study, we present a number of new findings that suggest it may be possible to specifically target pathogenic component of $I_{\text{Na}} (I_{\text{Na,L}})$ without altering other properties (e.g. channel availability). Using two mouse models (S571E and S571A) that we anticipate will be useful to study the in vivo molecular mechanism for a wide range of cardiac disorders, we report that Ser571 is critically important for CaMKII-dependent regulation of $I_{\text{Na,L}}$ but not other channel properties linked to CaMKII (steady-state inactivation and recovery from inactivation). We also show that Ser571-mediated increases in $I_{\text{Na,L}}$ promote APD and QT prolongation and increase susceptibility to arrhythmia events at the cellular and organismal level. Finally, we demonstrate that Ser571 phosphorylation is required for maladaptive remodeling in response to pressure overload. Together, our results support CaMKII-targeted Ser571 as an important locus for specific control of $I_{\text{Na,L}}$ and identify a molecular pathway that may be manipulated for therapeutic advantage.

Growing evidence supports CaMKII as an important contributor to maladaptive
remodeling and arrhythmias in a variety of cardiac disease states. CaMKII dysregulation has also been shown to promote pathology in specific inherited arrhythmia syndromes. While efforts are underway to develop therapeutic compounds that target CaMKII, it would be short-sighted to not consider alternative branches in the CaMKII signaling pathway as potential targets for therapy. $I_{Na,L}$ is one such target that has shown promise. The MERLIN-TIMI-36 trial tested arrhythmia therapy in acute coronary syndrome patients and showed that ranolazine decreased incidence of ventricular tachycardia without a significant effect on sudden death. Ranolazine has also been evaluated for treatment of atrial fibrillation in both preclinical and clinical settings with additional trials underway. While our studies focused on ventricular arrhythmias and remodeling, it will be interesting to determine whether phosphorylation at S571 alters electrophysiology/function in other heart regions (e.g. sinoatrial node, conduction system, atrium). Furthermore, aside from $I_{Na,L}$ or CaMKII, it is interesting to consider targeting upstream protein(s) responsible for organizing the relevant signaling domain (e.g. $\beta$IV-spectrin and ankyrin-). Previous studies have reported both “gain-of-function” (increased $I_{Na,L}$) and “loss-of-function” (decreased availability) effects of CaMKII phosphorylation on $I_{Na}$. One possible explanation for this behavior is that a single molecular event (phosphorylation at a single residue) gives rise to complex changes in channel gating analogous to the 1795insD human arrhythmia mutation that produces long-QT at slow pacing but Brugada syndrome at fast pacing. A second possibility is that the overall phenotype is the result of multiple phosphorylation events, which have distinct effects on channel behavior. Our unexpected finding that Ser571 regulates $I_{Na,L}$ but not other properties is consistent with the latter scenario and supports involvement for more than one site in determination of the overall
phenotype. In fact, previous in vitro cell expression studies have identified a possible role for phosphorylation of residues Ser516 and Thr594 in mediating CaMKII-dependent regulation of $I_{Na}$ availability.\textsuperscript{29} At the same time proteomics-based approaches in mouse\textsuperscript{30} and human\textsuperscript{47} have identified these (Ser571 and Ser516, specifically) and other sites in the DI-DII loop as potential phosphorylation sites for CaMKII. It is important to note that while our findings regarding the role of Ser571 in regulating $I_{Na,L}$ are consistent with previous in vitro studies, results on availability and recovery are divergent.\textsuperscript{22,29} This discrepancy may arise from differences in protocol inherent in the use of in vitro over-expression system (acute) compared to in vivo knock-in (chronic) strategy. It will be necessary going forward to determine whether phosphorylation at other sites\textsuperscript{29,30} may explain other aspects of the phenotype.

Our observation that ablation of the Ser571 site prevents TAC-induced increases in $I_{Na,L}$, APD and arrhythmias is consistent with recent studies using ranolazine in pressure overload-induced murine heart failure.\textsuperscript{b} Specifically, it was shown that, similar to S571A, ranolazine treatment restored $I_{Na,L}$ and APD to baseline levels following TAC. Surprisingly, we did not find evidence that S571 phosphorylation had a significant effect on total or phosphorylated CaMKII in response to pressure overload. It will be important to determine whether a link between S571 and CaMKII expression/activation may be found in other animal models and/or disease states. Our findings of the importance of Ser571 phosphorylation for maladaptive remodeling and heart failure in response to pressure-overload are also consistent with previous studies in CaMKII knockout animals.\textsuperscript{35-37} These previous studies focused primarily on the role of CaMKII-dependent dysregulation of Ca$^{2+}$ handling in pathogenesis. While our studies support an involvement of increased $I_{Na,L}$ in heart failure, perhaps upstream of Ca$^{2+}$ handling defects, it is important to note that heart failure is a multifactorial disease that affects virtually every aspect of...
cell function. Among the notable molecular defects involved in the CaMKII-dependent remodeling process is dysregulation of sarcoplasmic reticulum Ca\(^{2+}\) release channels (RyR2).\(^48,49\) Furthermore, direct effects of CaMKII on gene transcription, metabolism and/or cell survival likely play an equally important role in remodeling.\(^24\)

**Limitations**

While our findings provide insight into the mechanism linking Na\(_{\text{v}}\)1.5 phosphorylation, changes in \(I_{\text{Na},L}\) remodeling and arrhythmias, we acknowledge multiple study limitations. First, the mouse is distinct from human with regards to its electrophysiology and cardiac function.\(^50\) While AP, ion homeostasis and underlying ion currents vary greatly between mouse and larger animals, important electrophysiological features related to \(I_{\text{Na},L}\) are conserved across species. Notably, the S571 site is conserved across species and \(I_{\text{Na},L}\) is detectable at baseline and increases with disease in mouse and larger animals.\(^3,6,21,26\) It is also important to note that while our studies indicate a role for S571 phosphorylation in controlling \(I_{\text{Na},L}\), it remains unclear how S571 phosphorylation promotes increased \(I_{\text{Na},L}\). Similarly, there is some question about why S571E animals with delayed AP repolarization and abnormal Ca\(^{2+}\) homeostasis do not show a greater propensity for arrhythmias and maladaptive remodeling. While we found no evidence for changes in other currents, it is possible that compensatory remodeling occurs in the S571E mouse to counteract deleterious effects of S571 phosphorylation. At the same time, rate-dependent decrease of \(I_{\text{Na},L}\)\(^34\) may protect the cell from constitutive S571 phosphorylation making increased \(I_{\text{Na},L}\) by itself insufficient for arrhythmia and/or maladaptive remodeling without additional factors (e.g. catecholaminergic stress).

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Conflict of Interest Disclosures: None.

References:


46. Marionneau C, Abriel H. Regulation of the cardiac Na channel Na,1.5 by post-translational modifications. J Mol Cell Cardiol. 2015;82:36-47.


Table 1. Baseline echocardiographic features in WT, S571E and S571A mice.

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<td>477 (446,524)</td>
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<td>28.6 (27.8,29.6)*</td>
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Data presented as median value with 25th and 75th percentiles;
N = number of animals; *P<0.05 vs. W;T
HR = heart rate;
LVID,d = left ventricular inner chamber diameter in diastole;
EF = ejection fraction;
FS = fractional shortening;
LVAW,d = LV anterior wall thickness in diastole;
LVPW,d = LV posterior wall thickness in diastole
Figure Legends:

Figure 1. Baseline characterization of Scn5a S571E/A knock-in mice. (A) Structure of voltage-gated Na\(^+\) channel alpha subunit Na\(_v\)1.5. CaMKII phosphorylates Ser571 in the DI-DII linker. (B) Schematic of the S571E and S571A Flp-mediated knock-in model containing point mutation at Ser571 and LoxP sites flanking exons 10 and 13. (C-D) Summary data (mean±SEM) and representative immunoblots for Na\(_v\)1.5, phospho-Na\(_v\)1.5(S571), CaMKII, \(\beta\)IV-spectrin and ankyrin-G in WT, S571E and S571A detergent-soluble heart lysates (N=3 from three separate hearts for all groups). (E-G) Permeabilized adult WT, S571E and S571A ventricular cardiomyocytes were immunostained for N-cadherin (green) and Na\(_v\)1.5, \(\beta\)IV-spectrin, ankyrin-G, or CaMKII (red). DAPI staining (blue) indicates nuclei. Scale bar = 5 \(\mu\)m.

Figure 2. Ser571 controls late sodium current at baseline. (A) Voltage-gated Na\(^+\) current (\(I_{Na}\)) traces from WT, S571E and S571A adult ventricular myocytes (expressed relative to peak value). Inset shows late component. (B) Summary data (mean±SEM) for late \(I_{Na}\) (as percentage of peak) in WT, S571E and S571A myocytes during test pulses to -25 mV, -20 mV or -15 mV (*P<0.05 vs. WT, ^P<0.05 vs. S571A, N = 8 for WT, N = 10 for S571E, N = 12 for S571A). (C) \(I_{Na}\) steady-state inactivation, and (D) recovery from inactivation were also measured in WT, S571E and S571A myocytes (pulse protocols shown as insets) (N = 8 from two different preparations for WT, N = 10 from two different preparations for S571E, N = 12 for S571A from three different preparations).

Figure 3. Ser571 regulates action potential duration and susceptibility to repolarization defects.
(A) Representative APs and (B-C) summary data for APD at 90%, 75%, and 50% repolarization, peak transmembrane potential ($V_{m,peak}$), and rest potential ($V_{m,rest}$) in WT, S571E and S571A myocytes at 1 Hz or 2 Hz pacing (*P<0.05 vs. WT, ^P<0.05 vs. S571A, N = 12 from three different preparations for WT, N = 17 from five preparations for S571E, N = 14 from three preparations for S571A). AP summary data were not normally distributed and are shown as median with 25th and 75th percentile (box) and 10th and 90th percentile (whiskers). (D) Representative AP traces from WT, S571E, and S571A ventricular myocytes following isoproterenol treatment (10 μM). Repolarization abnormalities (afterdepolarizations/repolarization failure) were apparent in S571E but not WT or S571A cells. (E) Representative AP trace from S571E myocyte treated with isoproterenol followed by ranolazine treatment (1 μM), which eliminated isoproterenol-induced repolarization defects.

Figure 4. Ser571 regulates susceptibility to arrhythmia events in vivo. (A) Representative signal-averaged telemetry recordings from awake, unanesthetized WT, S571E, and S571A mice at baseline. (B-E) Summary baseline ECG data showing corrected QT (QTc), RR, QRS, and PR intervals in WT, S571E, and S571A mice (*P<0.05 vs. WT, N=18 for WT, N=17 for S571E, N=8 for S571A, where N represents the number of animals from which ECGs were recorded). (F) Representative telemetry measurements during continuous recording following treadmill exercise and epinephrine (2 mg/kg) injection in WT, S571E and S571A mice. Frequent premature ventricular contractions (PVCs) (red arrows, upper S571E trace) and even ventricular tachycardia (lower S571E trace) were apparent in S571E mice. (G) Summary data showing number of arrhythmia events (PVCs, ventricular tachycardia) over 30 min of continuous recording in WT, S571E and S571A following exercise/epinephrine treatment. A subset of
animals were treated with flecainide (20 mg/kg, +flec) or ranolazine (20 mg/kg, +ran) following exercise/epinephrine. (*P<0.05 vs. WT; For epi/exercise: N=11 for WT, N=10 for S571E, N=8 for S571A; For +flec: N=6 for all groups; For +ran: N=5 for WT and S571E, N=4 for S571A, where N represents the number of animals from which ECGs were recorded). AP and arrhythmia summary data were not normally distributed and are shown as median with 25th and 75th percentile (box) and 10th and 90th percentile (whiskers).

**Figure 5.** Ser571 phosphorylation is necessary for maladaptive remodeling in response to pressure overload. (A-C) Representative echocardiograms from WT, S571E, and S571A animals at baseline and following 6 weeks of transaortic constriction (TAC). (D-H) Summary data for changes in echocardiographic features in WT, S571E and S571A animals following 6 weeks of TAC relative to baseline (*P<0.05 vs. WT, #P<0.05 vs. S571E; N = 19 for WT, N = 7 for S571E, N = 10 for S571A, where N represents the number of animals from which echocardiograms were recorded). Summary data were not normally distributed and are shown as median with 25th and 75th percentile (box) and 10th and 90th percentile (whiskers). (I-J) H&E staining of longitudinal heart cross-sections from WT and S571A sham or 6-week TAC animals (scale bar = 1 mm). Abbreviations: \( LVAW,d \) - left ventricular anterior wall thickness in diastole; \( LVPW,d \) - left ventricular posterior wall thickness in diastole; \( EF \) - ejection fraction; \( FS \) - fractional shortening – FS; \( LVID,d \) - LV interior chamber diameter in diastole.

**Figure 6.** Ser571 is required for increased late current and phospho-CaMKII in pressure overload conditions. Voltage-gated Na\(^+\) current (\( I_{Na} \)) traces from WT, S571E and S571A adult ventricular myocytes following 6 weeks of TAC (expressed relative to peak value). *Inset* shows...
late component. **(B)** Summary data (mean±SEM) for late \( I_{\text{Na}} \) (as percentage of peak) in WT, S571E and S571A TAC myocytes during test pulses to -25 mV, -20 mV or -15 mV (*P<0.05 vs. WT, ^\#P<0.05 vs. S571E; N = 4 for WT and S571E, N = 6 for S571A from two different preparations).

**Figure 7.** Ser571 is required for delayed repolarization and arrhythmias in pressure overload. **(A)** Representative APs and **(B-C)** summary data for APD at 90%, 75%, and 50% repolarization, peak transmembrane potential \( (V_{m,\text{peak}}) \), and rest potential \( (V_{m,\text{rest}}) \) in WT, S571E and S571A TAC myocytes (*P<0.05 vs. WT TAC, ^\#P<0.05 vs. S571E TAC, ^^P<0.05 vs. S571A TAC; N = 12 from three different preparations for WT, N = 8 from three preparations for S571E, N = 8 from two preparations for S571A). **(D)** Representative telemetry measurements following exercise and epinephrine injection (2 mg/kg) in WT, S571E and S571A TAC mice. Frequent arrhythmia events (PVCs indicated by red arrows) were apparent in S571E and WT but not S571A mice. **(E)** Summary data showing number of arrhythmia events (PVCs, ventricular tachycardia) over 30 min of continuous recording in WT, S571E and S571A TAC mice following exercise/epinephrine treatment. (^\#P<0.05 vs. S571A; N=5 for WT, N=7 for S571E, N=6 for S571A, where N represents the number of animals from which ECGs were recorded). AP and arrhythmia data were not normally distributed and are shown as median with 25\(^{\text{th}}\) and 75\(^{\text{th}}\) percentile (box) and 10\(^{\text{th}}\) and 90\(^{\text{th}}\) percentile (whiskers).
Figure 1
Figure 2

A. [Graph showing voltage-gated sodium current (I_Na) with different alleles: WT, S571E, and S571A.]

B. [Bar graph showing late I_Na (% of peak) at -25 mV, -20 mV, and -15 mV for WT, S571E, and S571A.]

C. [Plot showing voltage dependence of I_Na with voltage (Vm) on the x-axis and I_Na (relative to max) on the y-axis for WT, S571E, and S571A.]

D. [Plot showing time course of I_Na with interpulse interval (ms) on the x-axis and I_Na (relative to max) on the y-axis for WT, S571E, and S571A.]
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Voltage-Gated Sodium Channel Phosphorylation at Ser571 Regulates Late Current, Arrhythmia, and Cardiac Function in vivo
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SUPPLEMENTAL MATERIAL

Supplemental Methods

**Histology:** Hearts were fixed in neutral buffered 10% formalin, trimmed along the long axis to show both ventricles and both atria, processed routinely into paraffin, and then sectioned serially at 5 microns. Sections were stained using hematoxylin and eosin (H&E; to evaluate general organ structure and cell characteristics) and Masson's trichrome (to examine the amount of interstitial and perivascular fibrous connective tissue).

**Biochemistry and immunostaining:** Equal quantities of ventricular lysates (determined using standard BCA protocols and verified through Ponceau stain of blots) were analyzed by SDS-PAGE and immunoblot, as described. Any remaining small differences in loaded protein levels were corrected by normalizing protein levels to GAPDH. Adult cardiomyocytes were isolated, immunostained, and imaged as described previously. Briefly, cells were fixed in 100% ethanol and blocked in PBS containing 0.15% Triton X-100, 3% normal goat serum (Sigma) and 1% BSA (Sigma), and incubated in primary antibody overnight at 4 °C. Cells were then washed, incubated in secondary antibody (Alexa 488, 568) for 2 hours at room temperature, and mounted using Vectashield with DAPI (Vector) and #1 coverslips. Image collection was performed on a Zeiss 780 confocal microscope [Objective W Plan Apochromat 40x/1.0 DIC (Zeiss), pinhole of 1.0 Airy Disc] using Carl Zeiss Imaging software. The following antibodies were used for immunoblotting and immunostaining: βIV-spectrin (N-terminal), Na\(_v\)1.5 (Alomone or custom\(^{1,5}\)), phospho-Na\(_v\)1.5(S571), CaMKIIδ (Badrilla), phospho-CaMKIIδ (Thermo Scientific), Ankyrin-G (Santa Cruz), N-Cadherin (Invitrogen), and GAPDH (Fitzgerald).

**Intracellular Ca\(^{2+}\) measurements:** Intracellular Ca\(^{2+}\) cycling was monitored as described previously. Briefly, cardiomyocytes were loaded with cytosolic Ca\(^{2+}\)-sensitive indicators Fluo-4.
AM. The fluorescent probes were excited with the 488 nm line of an argon laser and emission was collected at 500–600 nm in the line scan mode of Nikon A1 laser scanning confocal microscope. The fluorescence emitted was expressed as F/F₀, where F is the fluorescence at time t and F₀ represents the background signal. Myocytes were paced at 0.3 Hz using extracellular platinum electrodes. Any Ca²⁺ release event (i.e. wave, wavelet) that increased cell-wide fluorescence intensity above 10% of the signal generated by the preceding stimulated Ca²⁺ transient was included in the analysis. Automated Ca²⁺ spark analysis was conducted with SparkMaster. To assess the SR Ca²⁺ load, 20 mM caffeine was applied at the end of the experiments.

Telemetry: Electrocardiogram (ECG) recordings were obtained in awake, unanaesthetized mice using implanted radiotelemeters (DSI, St. Paul, MN, USA) at baseline and following stress protocol, as described. Baseline heart rate analysis was performed by continuously collecting ECG data for 30 minutes on three separate days, and analyzed according to established protocol. For stress tests, mice were exercised to exhaustion on a treadmill and then were injected with epinephrine (2 mg/kg) followed by 90 minutes of continuous recording. A subset of animals was injected with flecainide (20 mg/kg) or ranolazine (20 mg/kg) prior to recording according to established protocol.⁷,⁸
Supplemental Figures

Supplemental Figure 1. (A) $I_{Na}$ current-voltage relationship measured in WT, S571E and S571A myocytes at baseline. (B) Summary data (mean±SEM) for late Na$^+$ current amplitude in WT, S571E and S571A myocytes during test pulses to -25 mV, -20 mV or -15 mV (*P<0.05 vs. WT, #P<0.05 vs. S571E, ^P<0.05 vs. S571A, N = 8 for WT, N = 10 for S571E, N = 12 for S571A). (C-D) Representative $I_{Na}$ traces from WT and S571E ventricular myocytes at baseline (control) and in the presence of 30 μM tetrodotoxin (TTX).
Supplemental Figure 2. K⁺ current measurements in WT, S571E and S571A ventricular myocytes. Representative current traces for WT (A) inward rectifier K⁺ current $I_{K1}$ and (B) transient outward K⁺ current (fast component) and remaining current after inactivation of $I_{to}$ ($I_{K_{slow}}$). (C-F) Summary data (mean±SEM) for K⁺ currents from WT, S571E and S571A (P=NS, N = 10 from two different preparations for WT, N = 11 from two different preparations for S571E, N = 13 from three different preparations for S571A).

Supplemental Figure 3. Voltage-dependent Ca²⁺ current-voltage relationship from WT, S571E and S571A ventricular myocytes. (P=NS, N = 6 for WT, N = 8 for S571E, N = 11 for S571A from two different preparations for each genotype).
Supplemental Figure 4 – Increased late Na⁺ current in S571E myocytes increases diastolic Ca²⁺ release. (A) Representative line-scan images of Ca²⁺ sparks and (B) Ca²⁺ waves from wild type (WT) and S571E ventricular cardiomyocytes. Cells were treated with isoproterenol (1μM) and paced at 0.3 Hz. Blue asterisks indicate Ca²⁺ waves. (C) Representative caffeine-induced (20 mM) Ca²⁺ transients to assess Ca²⁺ load. (D) Summary data (mean±SEM) showing increased Ca²⁺ spark frequency, Ca²⁺ wave frequency and SR Ca²⁺ load in S571E myocytes (*P<0.05 compared to WT).
Supplemental Figure 5. Summary data for (A) APD at 90% repolarization and (B) AP amplitude in WT, S571E and S571A myocytes at 2 Hz pacing at baseline (control), after addition of 10 μM isoproterenol (+iso), followed by 1 μM ranolazine (+ran). AP data were not normally distributed and are shown as median with 25th and 75th percentile (box) and 10th and 90th percentile (whiskers). (P=NS, N = 13 from three different preparations for WT, N = 17 from five preparations for S571E, N = 14 from three preparations for S571A).
Ser571 phosphorylation regulates late Na⁺ current in vivo

Supplemental Figure 6. (A) $I_{Na}$ current-voltage relationship measured in WT, S571E and S571A myocytes following 6 weeks of TAC (P = NS; N = 8 from two different preparations for WT, N = 10 from two different preparations for S571E, N = 12 for S571A from three different preparations). (B) Summary data (mean±SEM) for late $I_{Na}$ amplitude in WT, S571E and S571A TAC myocytes during test pulses to -25 mV, -20 mV or -15 mV (*P<0.05 vs. WT, #P<0.05 vs. S571E; N = 4 for WT and S571E, N = 6 for S571A from two different preparations).
Supplemental Figure 7. (A-D) Summary ECG data showing corrected QT (QTc), RR, QRS, and PR intervals in WT, S571E, and S571A TAC mice (*P<0.05 vs. WT N=6 for WT, N=7 for S571E, N=6 for S571A). Data were not normally distributed and are shown as median with 25th and 75th percentile (box) and 10th and 90th percentile (whiskers).
Supplemental Figure 8. (A) Summary densitometry data (mean±SEM) and (B) representative immunoblots for phospho- and total CaMKII in WT, S571E, and S571A detergent-soluble heart lysates at baseline and following 6-week TAC. (P=NS, N=3 for WT, S571E and S571A baseline, N = 4 for WT TAC and S571A TAC, N=5 for S571E TAC).
Supplemental References


