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

Patric Glynn, PhD; Hassan Musa, PhD; Xiangqiong Wu, MD; Sathya D. Unudurthi, PhD; Sean Little, PhD; Lan Qian, MD; Patrick J. Wright, BS; Przemyslaw B. Radwanski, PhD; Sandor Gyorke, PhD; Peter J. Mohler, PhD; Thomas J. Hund, PhD

Background—Voltage-gated Na⁺ channels (Nav) are essential for myocyte membrane excitability and cardiac function. Nav current (INav) 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 INav (INavL) persists because of incomplete/failed inactivation of a subpopulation of channels. Notably, INavL is directly linked with both congenital and acquired disease states. The multifunctional Ca²⁺/calmodulin-dependent kinase II (CaMKII) has been identified as an important activator of INavL in disease. Several potential CaMKII phosphorylation sites have been discovered, including Ser571 in the Nav1.5 DI-DII linker, but the molecular mechanism underlying CaMKII-dependent regulation of INavL in vivo remains unknown.

Methods and Results—To determine the in vivo role of Ser571, 2 Scn5a knock-in mouse models were generated expressing either: (1) Nav1.5 with a phosphomimetic mutation at Ser571 (S571E), or (2) Nav1.5 with the phosphorylation site ablated (S571A). Electrophysiology studies revealed that Ser571 regulates INavL but not other channel properties previously linked to CaMKII. Ser571-mediated increases in INavL promote abnormal repolarization and intracellular Ca²⁺ 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 INavL. Relevant for improved rational design of potential therapies, our findings demonstrate that Ser571-dependent regulation of Nav1.5 specifically tunes INavL without altering critical physiological components of the current. (Circulation. 2015;132:567-577. DOI: 10.1161/CIRCULATIONAHA.114.015218.)

Key Words: action potential • arrhythmias, cardiac • calcium-calmodulin-dependent protein kinase type 2 • ion channels • Na⁺ current

Cardiac function depends on the tightly coordinated activity of voltage-gated Na⁺ channels (Nav) that are responsible for generating the action potential (AP) upstroke in response to an external stimulus. Importantly, Nav normally inactivate almost as quickly as they activate, a requirement for the cell membrane to repolarize in preparation for the next stimulus/heartbeat. Although voltage-dependent inactivation rapidly turns off most Nav current (INav), a small persistent (late) component (INavL) is apparent even under normal conditions. Aberrant INavL is directly linked with increased susceptibility to arrhythmia and dysfunction in cardiac disease. For example, increased INavL is present in congenital gain-of-function Nav channelopathies (eg, long QT 3), and in common forms of acquired disease (eg, heart failure), as well, and has been implicated in AP prolongation, abnormal ion homeostasis, and arrhythmia. Drugs that specifically target INavL are emerging as viable therapeutic agents to reduce arrhythmia burden in patients who have cardiac disease. A key feature of these agents is their selectivity for INavL over peak current. Thus, it is important to understand the molecular pathways for regulating INavL without altering other critical components of Nav1.5 current (availability, recovery kinetics, etc).

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From Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus (P.G., H.M., X.W., S.D.U., S.L., L.Q., P.J.W., P.B.R., S.G., P.J.M., T.J.H.); Department of Biomedical Engineering, College of Engineering, The Ohio State University, Columbus (P.G., X.W., S.D.U., L.Q., T.J.H.); Departments of Physiology & Cell Biology (H.M., S.L., P.J.W., P.B.R., S.G., P.J.M.) and Internal Medicine (P.J.M., T.J.H.), The Ohio State University Wexner Medical Center, Columbus; and Division of Pharmacy Practice and Administration, College of Pharmacy, The Ohio State University, Columbus (P.B.R.).

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Correspondence to Thomas J. Hund, PhD, The Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, 473 W 12th Ave, Columbus, OH 43210. E-mail Thomas.Hund@osumc.edu

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Although the precise mechanism for defective \( I_{\text{Na,L}} \) in cardiac disease remains unknown, dysregulation of the multifunctional Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII) has been linked to \( \text{Na}_\text{a} \) gating abnormalities in diverse settings, including heart failure, coronary artery disease, and diabetes mellitus.\(^{21-23}\) 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 \( \text{Na}_\text{a} \) to regulate the magnitude of \( I_{\text{Na,L}} \), and other properties, as well, including steady-state inactivation and recovery from inactivation.\(^{21,25-28}\) Although the molecular mechanism remains under investigation, several potential sites for CaMKII phosphorylation have been identified in the DI-DII linker of \( \text{Na}_\text{a,1.5} \), the predominant cardiac \( \text{Na}_\text{a} \) α-subunit.\(^{22,26,29,30}\) \( \text{Na}_\text{a,1.5} \) Ser571 first emerged as a potential CaMKII phosphorylation site from a functional screen in heterologous cells.\(^{26}\) Increased CaMKII-dependent phosphorylation of Ser571 was later observed under stress conditions in vitro and in samples from failing mouse, canine, and human hearts.\(^{22}\) Subsequent efforts have identified additional potential CaMKII sites in the DI-DII linker.\(^{29,30}\) Despite this important foundational work, there is a lack of evidence to support the physiological significance of any of the potential phosphorylation sites in vivo, because 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 \( I_{\text{Na,L}} \) has stalled because of the lack of relevant animal models.

In an effort to identify the molecular basis for CaMKII-dependent regulation of \( \text{Na}_\text{a,1.5} \) and cell excitability in vivo, we generated 2 novel \( \text{Scn5a} \) 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 owing to the replacement of the serine with an alanine. Using these new animal models, we generated 2 novel animal models. Resulting animals expressed either the S571E or S571A point mutation (Figure 1). Experiments were performed in 2-month-old male mice. Animals were euthanized with the use of CO\(_2\) 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.\(^{22,26,31}\) \( I_{\text{Na,L}} \) recordings were performed on freshly isolated (<1 hour in culture) myocytes at room temperature (20°C–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 by using 2 different methods: (1) average current over the interval 50 to 150 ms following the peak current, or (2) integral over the same time interval. Because 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 mmol/L): NaCl (5), CsCl (135), EGTA (10), MgATP (5), HEPES (5), pH 7.2. The extracellular solution contained (in mmol/L): NaCl (5), MgCl\(_2\) (1), CaCl\(_2\) (1.8), CdCl\(_2\) (0.1), glucose (11), CsCl (132.5), and HEPES (20); pH was maintained at 7.4 with CsOH. Only cells with membrane resistance >1 GΩ were used. Appropriate whole-cell capacitance and series resistance compensation (260%) was applied. Resulting currents were blocked with tetrodotoxin to verify isolation of \( I_{\text{Na,L}} \) (Figure I in the online-only Data Supplement). APs were recorded using the perforated (amphotericin B) patch-clamp technique at room temperature in Tyrode solution (bath). The pipette solution contained (in mmol/L): potassium aspartate (130), NaCl (10), HEPES (10), CaCl\(_2\) (0.04), MgATP (2), phosphocreatine (7), NaGTP (0.1), and 240 μg/mL amphotericin B. The pH was adjusted to 7.2 with KOH. APs were evoked by brief current pulses 1.5 to 4 pA, 0.5 to 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.\(^{32}\) Mice were anesthetized (isoflurane, 2.5%), intubated, and placed on a respirator (120 breaths/min, 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 6 weeks after 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 euthanized (2% Avertin, 20 μL/g intraperitoneally) via rapid thoracotomy at 6 weeks postsurgery (transaortic constriction [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 analysis of variance was used for multiple comparisons with the Bonferroni test for post hoc testing (data presented as mean±standard error of the mean). If the data distribution failed normality tests with the Shapiro-Wilk test, a Kruskal-Wallis 1-way analysis of variance 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 the \( \chi^2 \) test. The null hypothesis was rejected for \( P \leq 0.05 \).
Additional methods are provided in the 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 Naᵥ,1.5 (Figure 1A). To test the central hypothesis that phosphorylation of Naᵥ,1.5 at Ser571 is essential for CaMKII-dependent changes in Iₙa,L and channel kinetics in vivo, 2 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). Although phospho-Naᵥ,1.5(Ser571) was not detectable in S571A or S571E lysates (phosphoepitope
eliminated by either mutation), protein expression levels and cellular localization of Na\textsubscript{v}1.5 were normal in S571E and S571A myocytes in comparison with wild type (WT) at baseline. Furthermore, there were no differences in levels or localization of associated proteins CaMKII, β\textsubscript{IV}-spectrin, or ankyrin-G\textsuperscript{22,26,33} (Figure 1C through 1G). Expression/localization of the intercalated disc protein N-cadherin was also normal in S571E and S571A myocytes (Figure 1E through 1G). Histological examination of S571E and S571A hearts at baseline revealed no evidence of overt structural changes (eg, fibrosis) in comparison with WT at baseline (data not shown). Echocardiography revealed small but significant left ventricular 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).

### Table. Baseline Echocardiographic Features in WT, S571E, and S571A Mice

<table>
<thead>
<tr>
<th></th>
<th>WT (n=19)</th>
<th>S571E (n=7)</th>
<th>S571A (n=10)</th>
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<tbody>
<tr>
<td>HR, bpm</td>
<td>483 (471.495)</td>
<td>477 (466.524)</td>
<td>488 (465.504)</td>
</tr>
<tr>
<td>LVID,d, mm</td>
<td>4.08 (3.78,4.19)</td>
<td>4.34 (4.24,4.72)*</td>
<td>4.07 (3.82,4.29)</td>
</tr>
<tr>
<td>EF, %</td>
<td>63.1 (61.3,65.7)</td>
<td>53.1 (46.9,54.4)*</td>
<td>55.8 (54.3,57.2)*</td>
</tr>
<tr>
<td>FS, %</td>
<td>33.6 (32.2,35.7)</td>
<td>27.2 (23.5,28.0)*</td>
<td>28.6 (27.8,29.6)*</td>
</tr>
<tr>
<td>LVAW,d, mm</td>
<td>0.67 (0.61,0.69)</td>
<td>0.62 (0.59,0.68)</td>
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<td>LVPW,d, mm</td>
<td>0.67 (0.62,0.71)</td>
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<td>0.62 (0.60,0.64)</td>
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Data presented as median value with 25th and 75th percentiles; n=number of animals. EF indicates ejection fraction; FS, fractional shortening; HR, heart rate; LVID,d, left ventricular inner chamber diameter in diastole; LVAW,d, left ventricular anterior wall thickness in diastole; and WT, wild type.

*P<0.05 vs WT.

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\textsubscript{Na,L} measurements showed a significant increase (≈2-fold) in S571E I\textsubscript{Na,L} without any change in peak current in comparison with WT at baseline (Figure 2A and 2B, Figure I in the online-only Data Supplement). In contrast, S571A myocytes displayed a significant reduction in I\textsubscript{Na,L} despite an increase in peak current in comparison with WT (Figure 2A and 2B, Figure I in the online-only Data Supplement). Surprisingly, no differences were observed in steady-state inactivation or recovery from inactivation in S571E or S571A in comparison with WT (Figure 2C and 2D), 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\textsubscript{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 (Figures II and III in the online-only Data Supplement).

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### Table.

**Baseline Echocardiographic Features in WT, S571E, and S571A Mice**

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*P<0.05 vs WT.

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**Figure 2.** Ser571 controls late sodium current at baseline. **A,** Voltage-gated Na\textsuperscript{+} current (I\textsubscript{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\textsubscript{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\textsubscript{Na} steady-state inactivation and recovery from inactivation (D) were also measured in WT, S571E, and S571A myocytes (pulse protocols shown as insets; n=8 from 2 different preparations for WT, n=10 from 2 different preparations for S571E, n=12 for S571A from 3 different preparations). SEM indicates standard error of the mean; and WT, wild type.
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 $I_{Na,L}$, 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 in comparison with WT (Figure 3A through 3C). 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 $I_{Na,L}$. To determine whether increased $I_{Na,L}$ in S571E myocytes also affected Ca$^{2+}$ homeostasis, Ca$^{2+}$ sparks and waves were measured in isolated WT and S571E myocytes. Consistent with increased $I_{Na,L}$, S571E myocytes showed increases in Ca$^{2+}$ spark frequency, Ca$^{2+}$ wave frequency in response to isoproterenol, and sarcoplasmic reticulum Ca$^{2+}$ load in comparison with WT (Figure IV in the online-only Data Supplement). Isoproterenol-induced repolarization defects in S571E myocytes were normalized by treatment with ranolazine (Figure 3E and Figure V in the online-only Data Supplement), supporting $I_{Na,L}$ as a viable target for decreasing arrhythmia susceptibility.

To determine whether Ser571 plays a role in regulation of cardiac excitability and arrhythmias in vivo, electrocardiograms were measured in conscious WT, S571E, and S571A mice. S571E mice demonstrated significant prolongation of rate corrected QT interval (QTc) in comparison with WT or S571A mice. S571E mice demonstrated significant prolongation of rate corrected QT interval (QTc) in comparison with WT, in agreement with measured differences in $I_{Na,L}$ and APD (Figure 4A and 4B). S571A animals displayed a small but significant increase in RR interval (Figure 4D). Although no arrhythmias were observed in any group at baseline, S571E mice demonstrated frequent arrhythmia events, including premature ventricular contractions and ventricular tachycardia, in response to catecholaminergic stress in comparison with WT or S571A mice (Figure 4F and 4G). 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 antiarrhythmia target in vivo (Figure 4G).

**Phosphorylation of S571 Contributes to Myocardial Remodeling After Transaortic Constriction**

Several studies have implicated both CaMKII and $I_{Na,L}$ in maladaptive remodeling and arrhythmias in animal models of heart failure. Therefore, TAC was used 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 wall thickness, was apparent in WT, S571E, and S571A animals following 6 weeks of TAC (Figure 5A through 5E), indicating that hypertrophy occurs independent of phosphorylation at Ser571. However, ejection fraction, fractional shortening, and left ventricular chamber diameter following TAC showed improvement in S571A in comparison with WT or S571E animals (Figure 5F through 5J). For example, although the ejection fraction decreased by >40% in both WT and S571E animals following 6 weeks of TAC, the ejection fraction in S571A mice decreased by <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 electric remodeling. Although $I_{Na,L}$ was relatively small in WT myocytes at baseline (Figure 2B), TAC resulted in a large $I_{Na,L}$ in WT in comparison with 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 (Figure VI in the online-only Data Supplement). To determine whether phosphorylation at Ser571 contributed to electric 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 and 7B). Telemetry was also performed to evaluate arrhythmia
burden in vivo (Figure 7D and 7E and Figure VII in the online-only Data Supplement). Frequent premature ventricular contractions were observed with catecholaminergic stress in S571E and WT but not S571A animals following 6 weeks of TAC, indicating an antiarrhythmic benefit from eliminating CaMKII phosphorylation at Ser571 in vivo (Figure 7D and 7E). 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 (Figure VIII in the online-only Data Supplement). 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 3 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 \( I_{Na,L} \), 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 antiarrhythmia therapies.39 The apparent impasse may be partly attributed to the high-profile failure of trials designed to test ion channels as antiarrhythmia targets.2,40 Although \( I_{Na,L} \) 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 \( I_{Na,L} \) without altering other properties (eg, channel availability). Using 2 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 \( I_{Na,L} \) but not other channel properties linked to CaMKII (steady-state

![Figure 5. Ser571 phosphorylation is necessary for maladaptive remodeling in response to pressure overload. A through C, Representative echocardiograms from WT, S571E, and S571A animals at baseline and following 6 weeks of transaortic constriction (TAC). D through 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 and J, Hematoxylin and eosin staining of longitudinal heart cross-sections from WT and S571A sham or 6-week TAC animals (scale bar=1 mm). EF indicates ejection fraction; FS, fractional shortening; LVAW,d, left ventricular anterior wall thickness in diastole; LVID,d, left ventricular interior chamber diameter in diastole; LVPW,d, left ventricular posterior wall thickness in diastole; SA, S571A; SE, S571E; and WT, wild type.](http://circ.ahajournals.org/)

![Figure 6. Ser571 is required for increased late current in pressure overload conditions. A, 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 (means±SEM) for late \( I_{Na} \) (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 2 different preparations). CaMKII, Ca\(^{2+}\)/calmodulin-dependent kinase II; SEM, standard error of the mean; TAC, transaortic constriction; and WT, wild type.](http://circ.ahajournals.org/)
inactivation and recovery from inactivation). We also show that Ser571-mediated increases in $I_{Na,L}$ 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 $I_{Na,L}$ 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. Although 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 Metabolic Efficiency With Ranolazine for Less Ischemia in Non ST-Elevation Acute Coronary Syndrome Thrombolysis in Myocardial Infarction 36 (MERLIN-TIMI-36) trial tested arrhythmia therapy in patients with acute coronary syndrome and showed that ranolazine decreased the incidence of ventricular tachycardia without a significant effect on sudden death. Ranolazine has also been evaluated for the treatment of atrial fibrillation in both preclinical and clinical settings with additional trials underway. Although 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 (eg, 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 (eg, $\beta$-spectrin and ankyrin-G).

Previous studies have reported both gain-of-function (increased $I_{Na,L}$) and loss-of-function (decreased availability) effects of CaMKII phosphorylation on $I_{Na,L}$. 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 1 site in the 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.\(^{29}\) At the same time, proteomics-based approaches in mice\(^{30}\) and humans\(^{37}\) 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, although our findings regarding the role of Ser571 in regulating \( I_{Na,L} \) are consistent with previous in vitro studies, the results on availability and recovery are divergent.\(^{22,29}\) This discrepancy may arise from differences in protocol inherent in the use of in vitro overexpression system (acute) in comparison with in vivo knock-in (chronic) strategy. It will be necessary going forward to determine whether phosphorylation at other sites\(^{29,30}\) 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.\(^{6}\) 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 phosphorylation at other sites\(^{29,30}\) may explain other aspects of the phenotype.

Limitations

Although our findings provide insight into the mechanism linking Na\(_{1.5}\) phosphorylation, changes in \( I_{Na,L} \), remodeling, and arrhythmias, we acknowledge multiple study limitations. First, the mouse is distinct from human with regard to its electrophysiology and cardiac function.\(^{50}\) Although AP, ion homeostasis, and underlying ion currents vary greatly between mouse and larger animals, important electrophysiological features related to \( I_{Na,L} \) are conserved across species. Notably, the S571 site is conserved across species and \( I_{Na,L} \) is detectable at baseline and increases with disease in mouse and larger animals.\(^{3,6,21,28}\) It is also important to note that, whereas our studies indicate a role for S571 phosphorylation in controlling \( I_{Na,L} \), it remains unclear how S571 phosphorylation promotes increased \( I_{Na,L} \). 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. Although 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 \( I_{Na,L} \)\(^{34}\) may protect the cell from constitutive S571 phosphorylation making increased \( I_{Na,L} \) by itself insufficient for arrhythmia and maladaptive remodeling without additional factors (eg, catecholaminergic stress).

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Disclosures

None.

References


Normal activity of voltage-gated Na⁺ channels (Naᵥ) is critical for proper heart function. Although Naᵥ current normally activates and inactivates rapidly to generate the myocyte action potential upstroke, a small late component persists even under normal conditions. Increases in late current have been directly linked with arrhythmias in both congenital and acquired disease states. Previous studies have identified an important role for Naᵥ phosphorylation by Ca²⁺/calmodulin-dependent protein kinase II in the regulation of Naᵥ activity. Although in vitro studies and phosphoproteomic screens have identified Ser571 in the Naᵥ1.5 DI-DII linker as a potential Ca²⁺/calmodulin-dependent protein kinase II phosphorylation site, the relationship between Ser571 phosphorylation, late current, arrhythmias, and heart function in vivo remain unknown. Here, we use 2 novel Scn5a knock-in mouse models (S571A and S571E) to generate mechanistic insight into the link between phosphorylation at Ser571 and control of Naᵥ activity in vivo. Surprisingly, we find that Ser571 phosphorylation is highly specific for control of late current over other Naᵥ properties (eg, steady-state inactivation). Furthermore, we report that Ser571 phosphorylation alters action potential repolarization and intracellular Ca²⁺ homeostasis. Finally, we show that phosphorylation of Ser571 controls susceptibility to arrhythmias at baseline and maladaptive remodeling in response to pressure overload (transaortic constriction). Based on these findings and in light of recent preclinical studies and clinical trials demonstrating potential antiarrhythmia efficacy of drugs that block late current (eg, ranolazine), we propose Ser571 as an important locus for specific control of late current with therapeutic implications.
Voltage-Gated Sodium Channel Phosphorylation at Ser571 Regulates Late Current, Arrhythmia, and Cardiac Function In Vivo
Patric Glynn, Hassan Musa, Xiangqiong Wu, Sathya D. Unudurthi, Sean Little, Lan Qian, Patrick J. Wright, Przemyslaw B. Radwanski, Sandor Gyorke, Peter J. Mohler and Thomas J. Hund

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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\textsubscript{v}1.5 (Alomone or custom\textsuperscript{1, 5}), phospho-Na\textsubscript{v}1.5(S571), CaMKII\textsubscript{δ} (Badrilla), phospho-CaMKII\textsubscript{δ} (Thermo Scientific), Ankyrin-G (Santa Cruz), N-Cadherin (Invitrogen), and GAPDH (Fitzgerald).

Intracellular Ca\textsuperscript{2+} measurements: Intracellular Ca\textsuperscript{2+} cycling was monitored as described previously. Briefly, cardiomyocytes were loaded with cytosolic Ca\textsuperscript{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.
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 (1 μM) 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).
Supplemental Figure 6. (A) $I_{Na}$ current-voltage relationship measured in WT, S571E and S571A myocytes following 6 weeks of TAC ($P = \text{NS}; N = 8$ 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


