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

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Abstract

Background—Voltage-gated Na^+ channels (Na_v) are essential for myocyte membrane excitability and cardiac function. Na_v 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 $Na_v1.5$ 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) $Na_v1.5$ with a phosphomimetic mutation at Ser571 (S571E), or 2) $Na_v1.5$ 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+}

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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 $Na_v1.5$ specifically tunes $I_{Na,L}$ without altering critical physiological components of the current.

Keywords

arrhythmia (mechanisms); calmodulin dependent kinase II; Na^+ current; ion channels; 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 $Na_v1.5$ 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 mechanism remains under investigation, several potential sites for CaMKII phosphorylation have been identified in the DI-DII linker of $Na_v1.5$, the predominant cardiac Na_v alpha subunit.[22, 26, 29, 30] $Na_v1.5$ 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*, 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 $I_{Na,L}$ has stalled due to lack of relevant animal models.

In an effort to identify the molecular basis for CaMKII-dependent regulation of $Na_v1.5$ and cell excitability *in vivo*, we generated two novel *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 $I_{Na,L}$, 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 $I_{Na,L}$ *in vivo*. Importantly, Ser571-dependent regulation is selective for $I_{Na,L}$ 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 neomyocin 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 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_{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). $I_{Na,L}$ 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_{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), $CaCl_2$ (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 1.5–4 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.[32] Mice were anesthetized (isofluorane, 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 μL/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 25^{th} and 75^{th} percentiles (box) and 10^{th} and 90^{th} 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 $Na_v1.5$ (Figure 1A).[22, 26, 29] To test the central hypothesis that phosphorylation of $Na_v1.5$ at Ser571 is essential for CaMKII-dependent changes in $I_{Na,L}$ and channel kinetics *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-$Na_v1.5$(Ser571) was not detectable in S571A or S571E lysates (phospho-epitope eliminated by either mutation), protein expression levels and cellular localization of $Na_v1.5$ 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, β_{IV}-spectrin, or ankyrin-G[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 $Na_v1.5$ phosphorylation at Ser571 and alters $Na_v1.5$ activity and cell excitability.[26] To assess the specific role of Ser571 in CaMKII-dependent regulation of $Na_v1.5$ 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.[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^+ currents, or voltage-dependent Ca^{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 $I_{Na,L}$, 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 $I_{Na,L}$.[34] 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 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 $I_{Na,L}$ 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.[6, 35–38] 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 $I_{Na,L}$, 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 $I_{Na,L}$ 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} ($I_{Na,L}$) 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 $I_{Na,L}$ but not other channel properties linked to CaMKII (steady-state inactivation and recovery from inactivation). We also show that Ser571-mediated increases in $I_{Na,L}$ 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.[24] CaMKII dysregulation has also been shown to promote pathology in specific inherited arrhythmia syndromes.[22, 41–43] While efforts are underway to develop therapeutic compounds that target CaMKII,[44] 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.[11] Ranolazine has also been evaluated for treatment of atrial fibrillation in both preclinical[15, 16] and clinical[17, 18] settings with additional trials underway.[19] 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. β_{IV}-spectrin and ankyrin-).[26, 33]

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}.[22, 25–27, 29] 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.[45] A second possibility is that the overall phenotype is the result of multiple phosphorylation events, which have distinct effects on channel behavior.[29, 30, 46, 47] 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.[29] At the same time proteomics-based approaches in mouse[30] and human[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.[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[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 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.[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 (RyR_2).[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 $Na_v1.5$ phosphorylation, changes in $I_{Na,L}$, 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 $I_{Na,L}$ 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, 26] It is also important to note that while 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. 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 $I_{Na,L}$[34] may protect the cell from constitutive S571 phosphorylation making increased $I_{Na,L}$ by itself insufficient for arrhythmia and/or maladaptive remodeling without additional factors (e.g. catecholaminergic stress).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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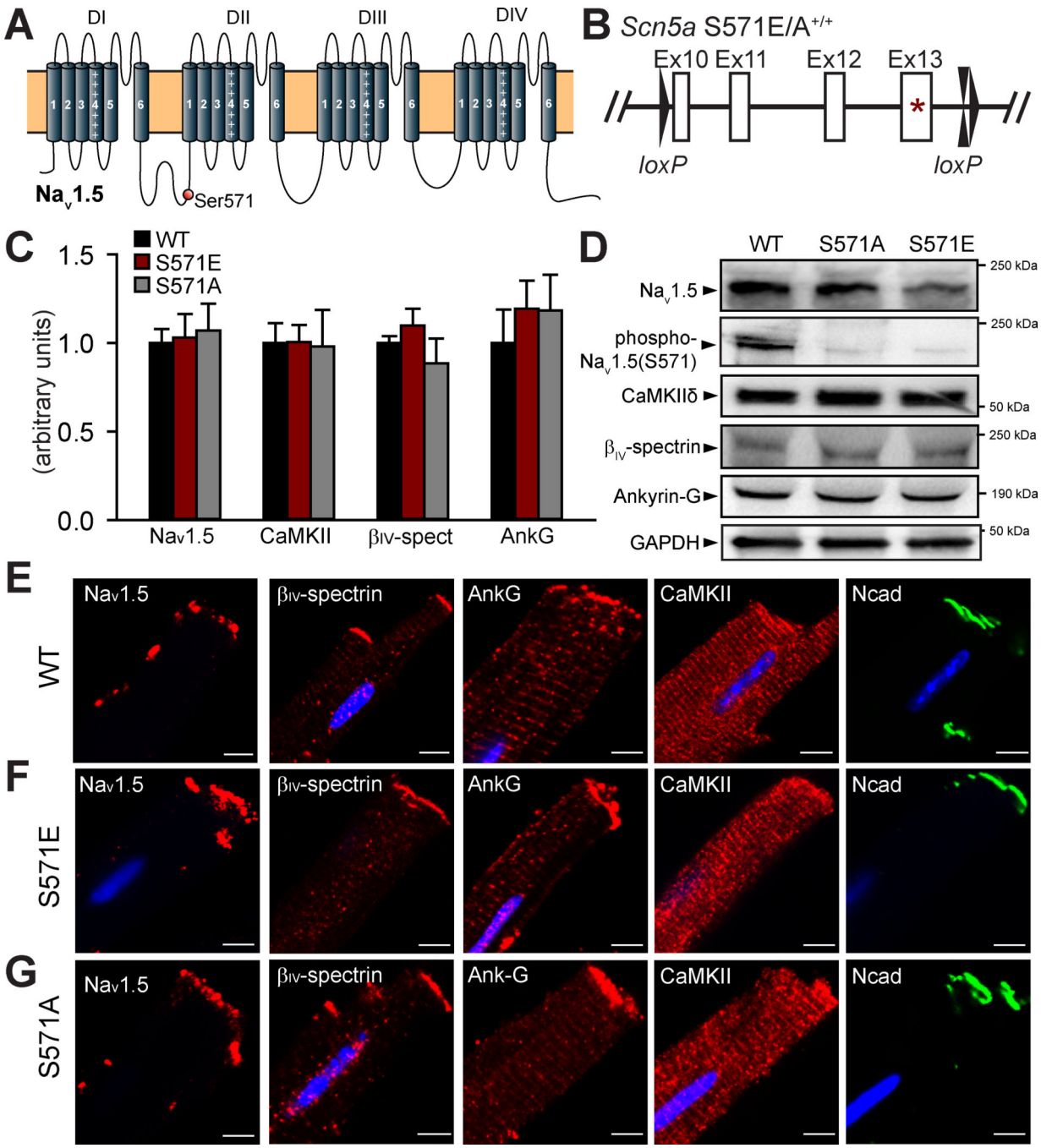

Figure 1.

Baseline characterization of *Scn5a* S571E/A knock-in mice. **(A)** Structure of voltage-gated Na^+ channel alpha subunit $Na_v1.5$. 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 $Na_v1.5$, phospho-$Na_v1.5$(S571), CaMKII, β_{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 $Na_v1.5$, β_{IV}-spectrin, ankyrin-G, or CaMKII (*red*). DAPI staining (*blue*) indicates nuclei. Scale bar = 5 μm.

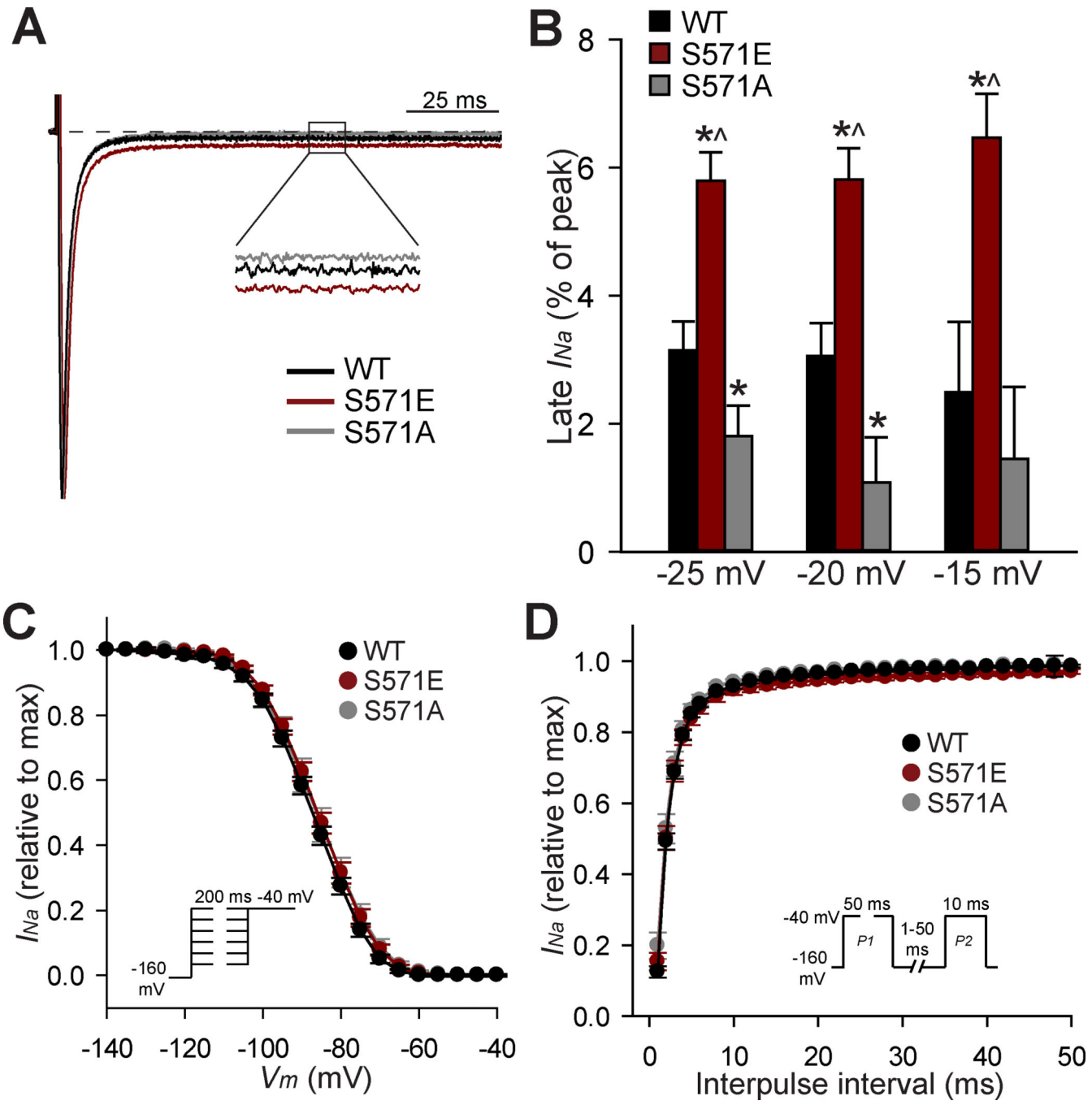

Figure 2.

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).

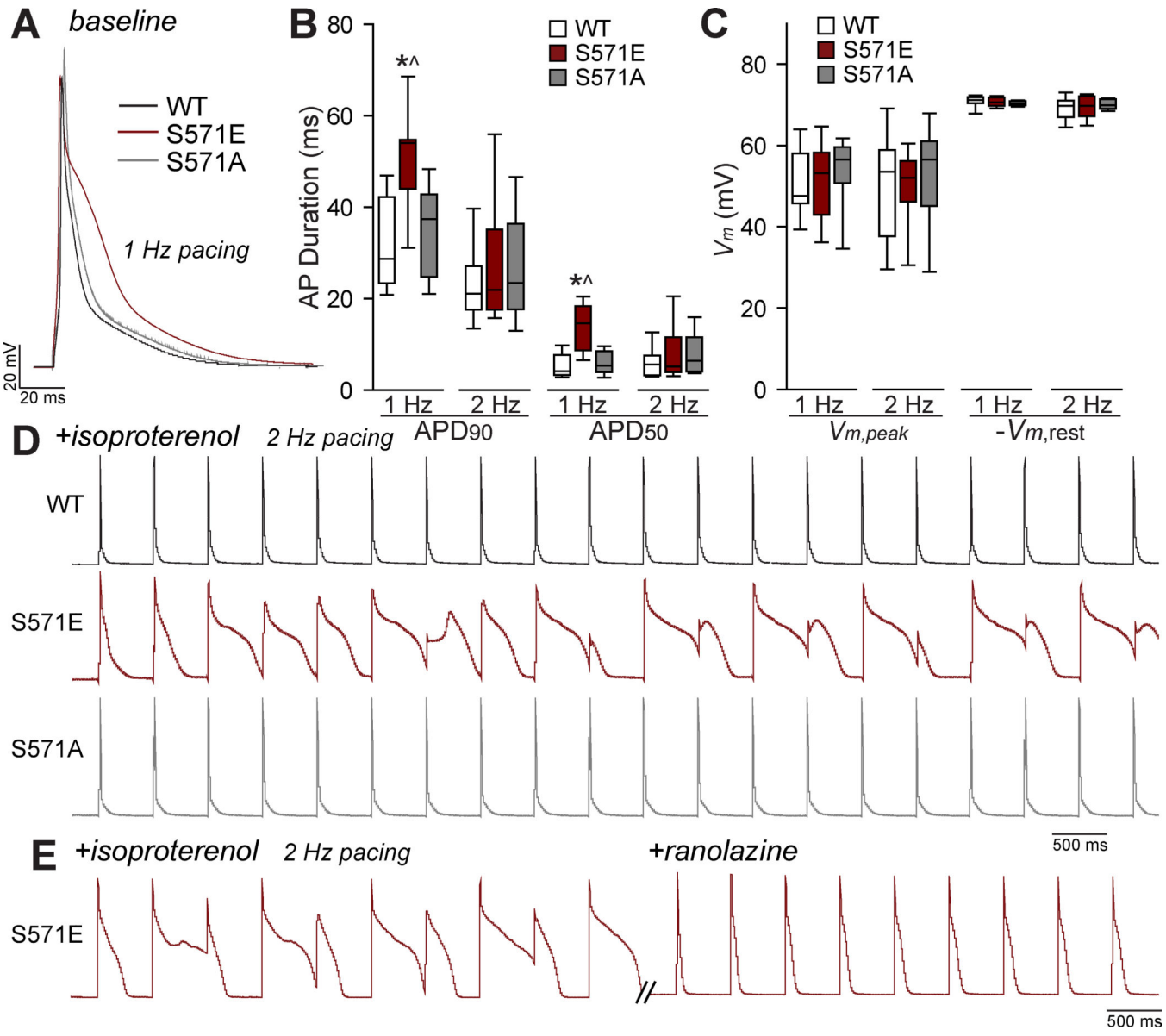

Figure 3.

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.

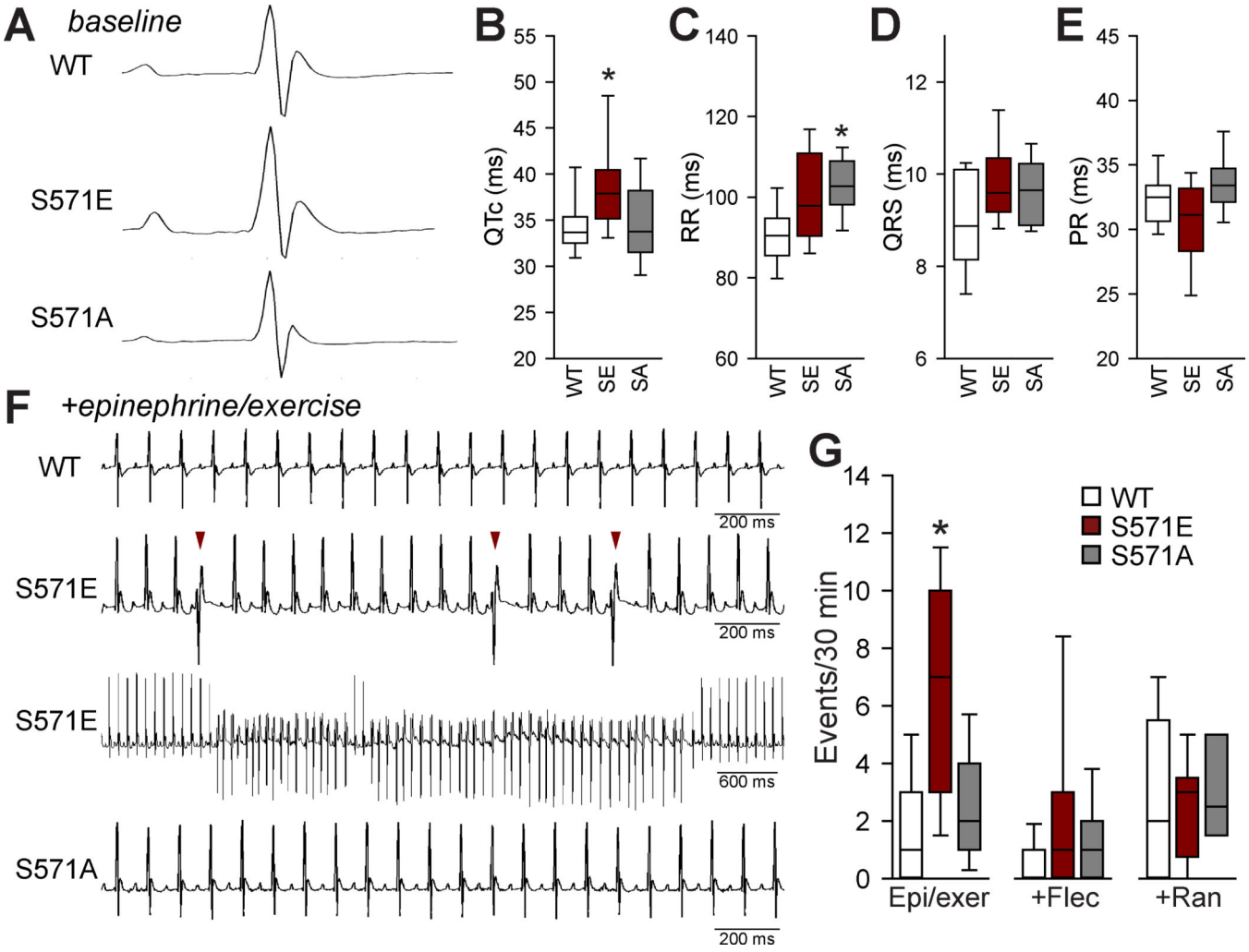

Figure 4.

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).

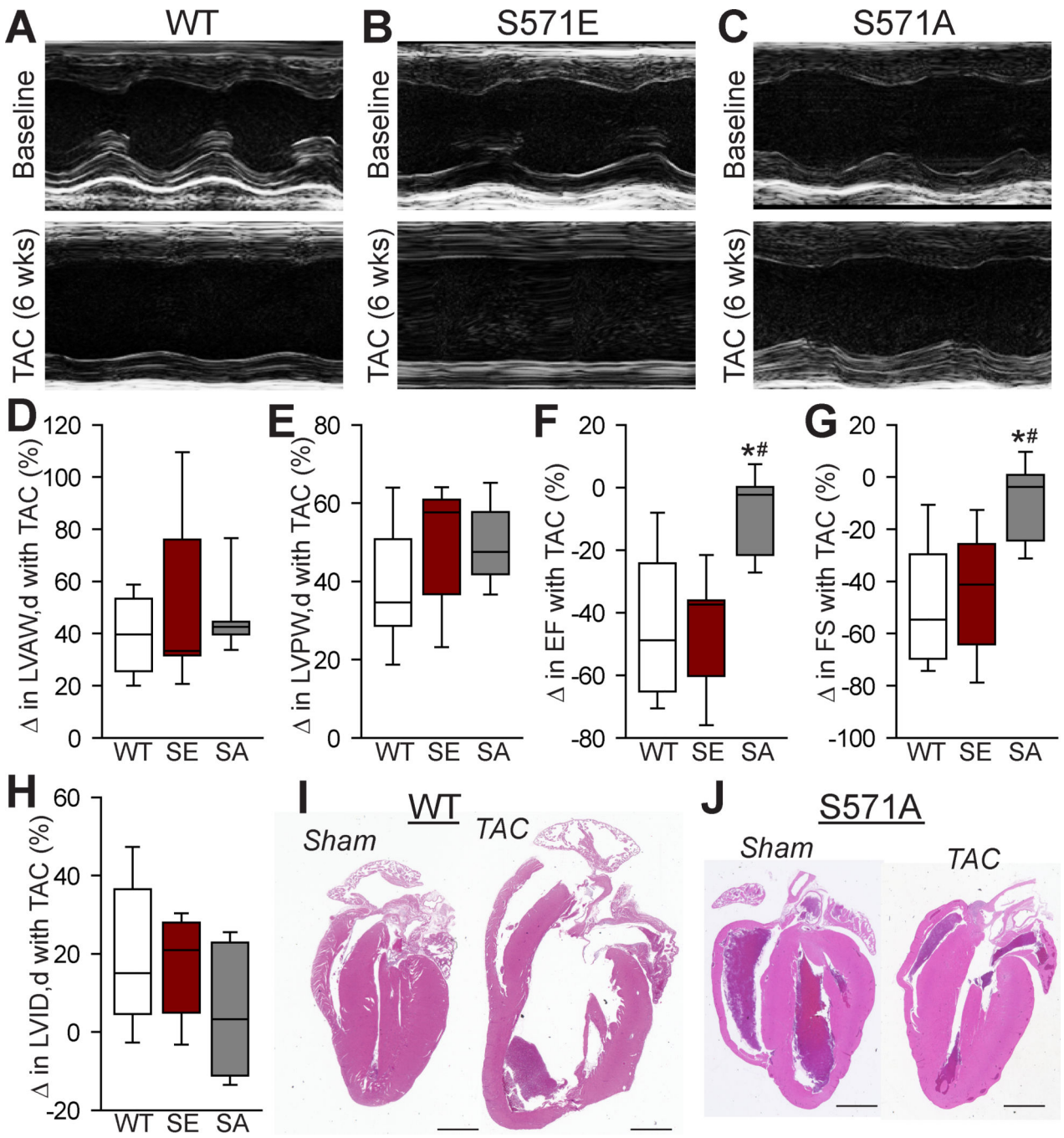

Figure 5.

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.

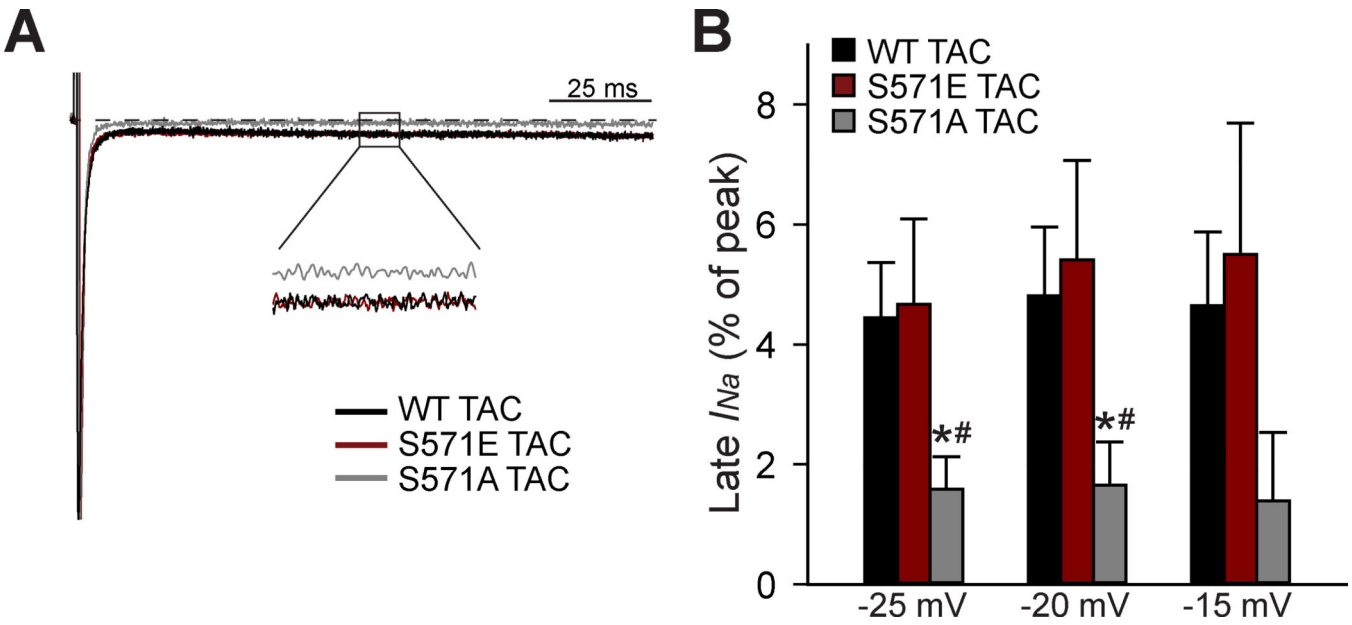

Figure 6.

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_{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).

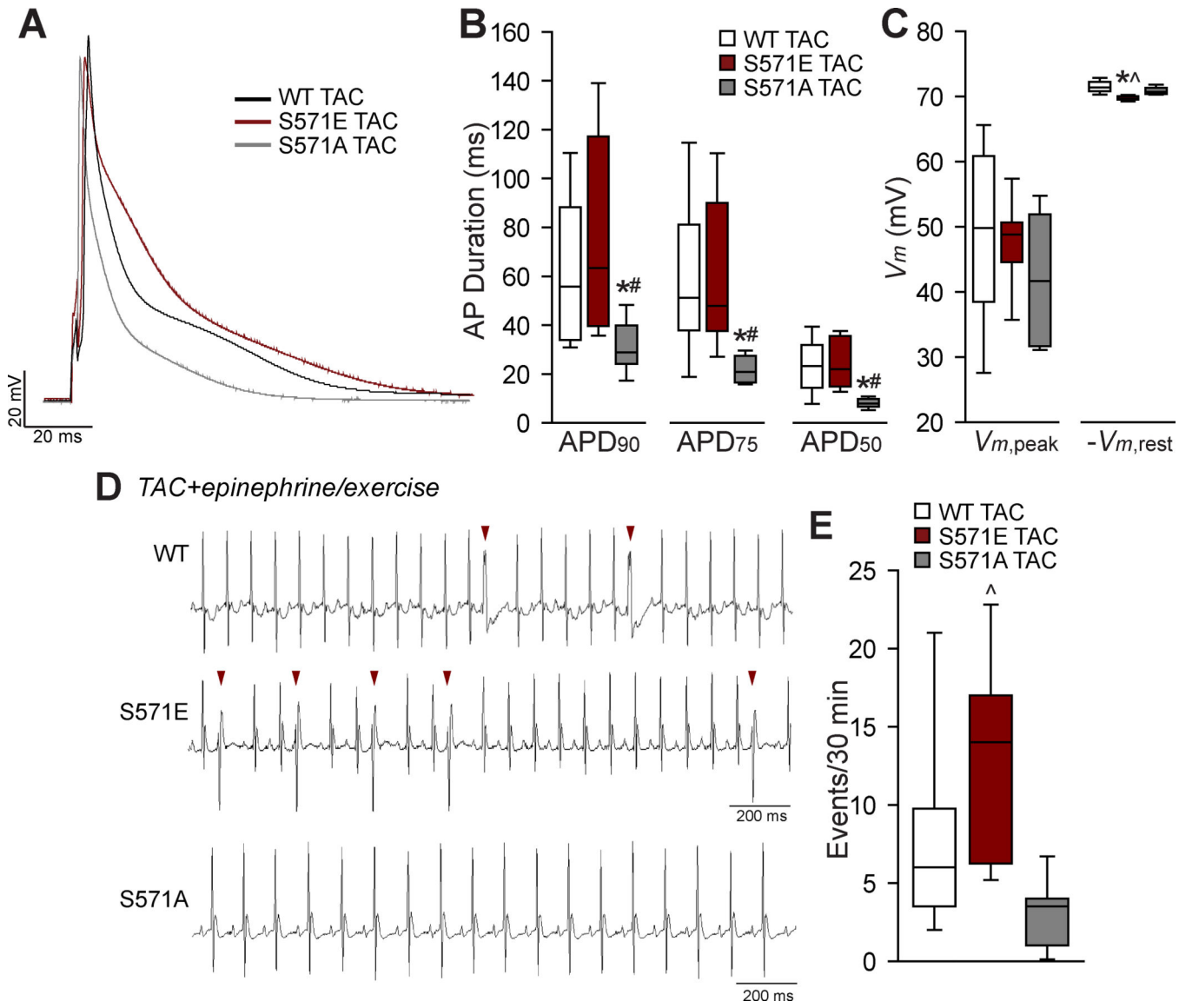

Figure 7.

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 ($V_{m,peak}$), and rest potential ($V_{m,rest}$) 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 25th and 75th percentile (box) and 10th and 90th percentile (whiskers).

Table 1

Baseline echocardiographic features in WT, S571E and S571A mice.

	WT (N=19)	S571E (N=7)	S571A (N=10)
HR, bpm	483 (471,495)	477 (446,524)	488 (465,504)
LVID,d, mm	4.08 (3.78,4.19)	4.34 (4.24,4.72)*	4.07 (3.82,4.29)
EF, %	63.1 (61.3,65.7)	53.1 (46.9,54,4)*	55.8 (54.3,57.2)*
FS, %	33.6 (32.2,35.7)	27.2 (23.5,28.0)*	28.6 (27.8,29.6)*
LVAW,d, mm	0.67 (0.61,0.69)	0.62 (0.59.0.68)	0.63 (0.60,0.65)
LVPW,d, mm	0.67 (0.62,0.71)	0.62 (0.61,0.67)	0.62 (0.60,0.64)

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