The role of CaMKII-dependent augmented $I_{Na,L}$ in arrhythmias during acute β-adrenergic stimulation

Undergraduate Research Thesis

By

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Abstract

The heart has evolved elaborate pathways for adapting function to acute stress stimuli [e.g. sympathetic stimulation of β-adrenergic receptors (β-ARs)]. This "fight-or-flight" cardiac response to acute stress involves rapid changes in heart rate and contractility. However, β-AR stimulation has been shown to enhance arrhythmogenesis due, in part, to induction of Ca^{2+} overload in cardiac myoctyes,[1,2,3] although the precise mechanism remains unknown. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is activated in response to β-adrenergic stimulation and phosphorylates a myriad of intracellular targets including the cardiac sodium channel ($Na_v1.5$) to alter myocyte excitability and Ca^{2+} handling. Our group and others have shown that CaMKII-dependent phosphorylation of $Na_v1.5$ at Ser571 selectively regulates late sodium current ($I_{Na,L}$) *in vivo*. CaMKII-dependent increases in $I_{Na,L}$ are linked to cardiac dysfunction.[4,5,18,19] We hypothesized that CaMKII-dependent phosphorylation of $Na_v1.5$, and subsequent increases in $I_{Na,L}$, are essential for Ca^{2+} handling defects and acute arrhythmic response to β-AR stimulation. Using optical mapping, we show that CaMKII-dependent phosphorylation of $Na_v1.5$ alters Ca^{2+} transients and arrhythmia susceptibility at baseline and in response to β-AR agonist isoproterenol. Our data demonstrate an important link between CaMKII-dependent phosphorylation of the $Na_v1.5$ Ser571 site and Ca^{2+} handling and arrhythmogenesis in response to acute β-AR stimulation.

Dedication

I would like to dedicate this work to my grandfather, Dr. Rodney F. Plimpton. Though you are not here to read it, thank you for being one of my biggest inspirations to eagerly invest in younger students, to persistently ask interesting questions, and to consistently build others up.

Acknowledgments

I would first like to acknowledge Dr. Amara Greer-Short, who spear-headed this project and who graciously invited me to take on a more active role in executing the experiments needed to explore the central questions of this study. I would also like to thank my research advisor, Dr. Thomas Hund, as well as my capstone mentor, Dr. Tanya Nocera, for serving on my thesis defense committee. Lastly, I would like to recognize the rest of the Hund Lab, including Dr. Sathya Dev Unudurthi, Dr. Drew Nassal, Dr. Birce (Ela) Onal, Nehal Patel, Taylor Howard, Dennison Min, Deborah Hong, Alyssa Dalic, and Tony Satroplus. You all have fostered an environment that nurtures collaboration, creativity, and community, and without that this project would not have been possible.

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- Unudurthi, SD, Greer-Short, A, Patel, N, Howard, T, Wu, X, Qian, L, Onal, B, Satroplus, T, Hong, D, Lane, C, Dalic, A, Musa, H, Smith, S, Mohler, PJ, Hund, TJ. Beta-IV-spectrin regulates STAT3 targeting and function to tune cardiac response to pressure overload. *Circulation*. 2017 (in review).

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Chapter 1. Introduction

Background and Motivation

The heart has inherent pacemaking abilities that enable it to establish its own rhythm, and it has developed elaborate pathways that enable it to alter this rhythm in response to changes in external stimuli. Specifically, sympathetic stimulation results in increased heart rate, contractility, conduction velocity, and relaxation rate, allowing the heart to adapt to the body's increased oxygen requirements during acute stress. Of particular interest in this sympathetic system are β-adrenergic receptors, a subfamily of G-protein coupled receptors that have been closely linked to the development of heart failure (HF).[6,7] β-adrenergic stimulation has been shown to magnify arrhythmogenesis during HF by inducing Ca^{2+} overload in cardiomyocytes.[1,2,3] However, the mechanism by which this stimulation contributes to arrhythmogenesis is not fully understood.

Previous studies have identified CaMKII as a serine/threonine-specific kinase that plays a critical role in cardiac excitation-contraction coupling and regulating ion homeostasis. Specifically, CaMKII has been shown to mediate "fight-or-flight" increases in heart rate by targeting regions of the heart responsible for Ca^{2+} homeostasis.[8] Prior research has found that the active Ca^{2+}/CaMKII complex phosphorylates the Ser571 site of the heart's primary voltage-gated sodium channel, $Na_v1.5$.[4] These channels are essential for cardiac

excitability and function, and their dysregulation can increase arrhythmia susceptibility and promote tissue remodeling.

The Ser571 site, specifically, is responsible for regulating the persistent late sodium current, $I_{Na,L}$, without altering other channel properties that contribute to vital physiological components of the sodium current.[5] This late component of the sodium current arises because at baseline conditions, a subset of sodium channels undergo failed or incomplete inactivation, allowing some sodium to continue to flow out of the cell, a phenomenon that has been directly linked with cardiac dysfunction and heightened arrhythmic susceptibility.[5] Furthermore, CaMKII is activated following β-adrenergic stimulation, and CaMKII-dependent increases in $I_{Na,L}$ have been linked to cardiac dysfunction in the form of Ca^{2+} mishandling and arrhythmogenesis.[4,5] In addition to this, $I_{Na,L}$ blockers like ranolazine have been shown to improve outcomes in patients with heart failure and atrial and ventricular arrhythmias, underscoring this action potential component's role in arrhythmogenesis.[18,19]

Although other groups have extensively examined the role of other channels—such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), ryanodine receptors (RyR2), and the sodium-calcium exchanger (NCX)—in arrhythmogenesis during β-adrenergic stimulation, much less is known about the role of $I_{Na,L}$.[10-16] Based on previous findings, it is likely that the interactions of CaMKII and $I_{Na,L}$ play an important role in Ca^{2+}-handling and arrhythmogenesis specifically in the context of β-adrenergic stimulation. Thus,

understanding the exact mechanism by which CaMKII contributes to arrhythmogenesis during β-adrenergic stimulation may lead to novel therapies for the treatment of heart failure. A proposed mechanism is described in Figure 1 with the novel mechanistic components indicated in dark blue. The roles of structures like SERCA, RyR2, and NCX are simplified in this representation in order to emphasize the novel components involving the CaMKII/$Na_v1.5$ axis acting simultaneously with isoproterenol-induced stimulation of β-adrenergic receptors.

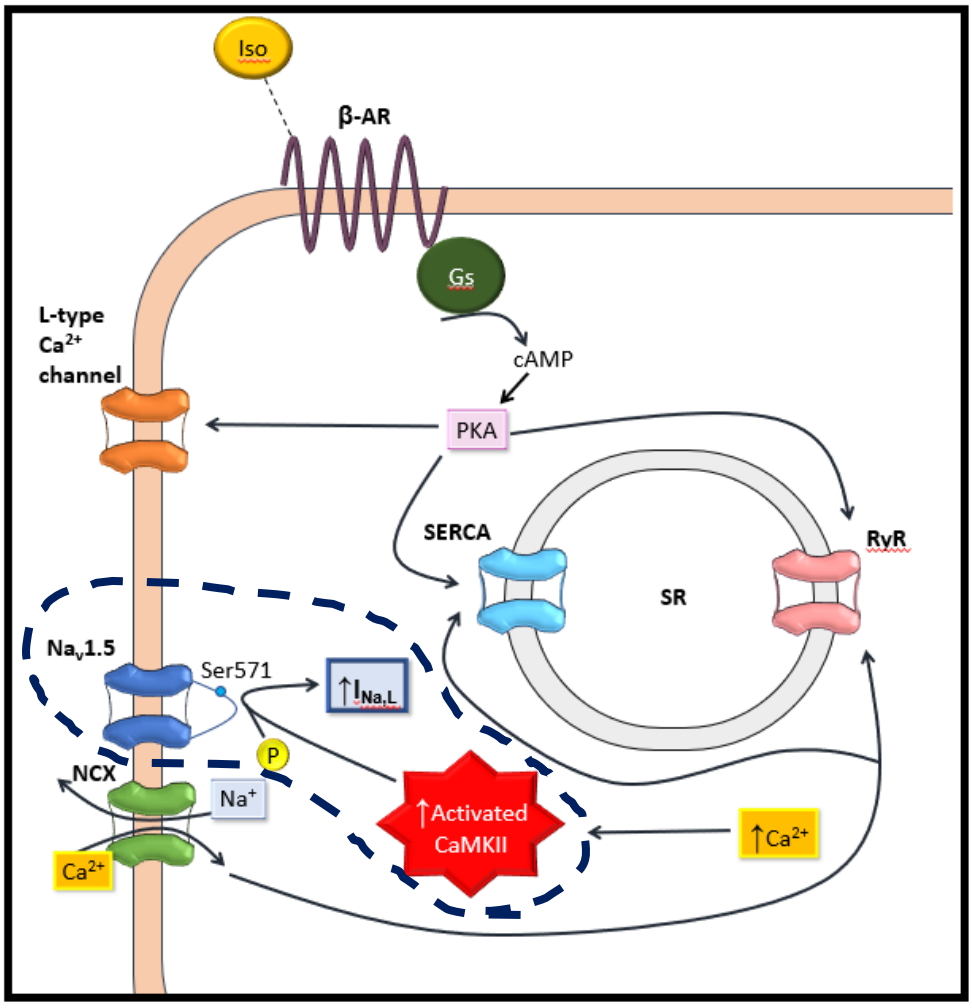

Figure 1: Hypothesized mechanism involved in cardiac Ca^{2+} handling and arrhythmogenesis during β-adrenergic stimulation.

Research Significance

Cardiac arrhythmias pose a significant health risk, associated with many life-threatening conditions like sudden cardiac arrest, heart failure, and stroke. Ventricular tachycardia and ventricular fibrillation are specifically implicated in cardiac arrest, and of the 360,000 out-of-hospital cases that occur each year in the United States, only 10-30% of patients survive to hospital discharge.[9] Understanding the underlying mechanisms that contribute to arrhythmias is therefore critical for improving patient outcomes. This research project is important because it deepens our understanding of the role of CaMKII as a regulatory protein, especially as it relates to acute arrhythmogenesis at cellular and whole-heart levels. Furthermore, it advances our knowledge of the pathway through which β-AR agonists induce arrhythmias

Based on the aforementioned findings, we hypothesized that CaMKII-dependent phosphorylation of the $Na_v1.5$ Ser571 site, and subsequent increases in $I_{Na,L}$, are essential for Ca^{2+} handling defects and acute arrhythmic response during acute β-adrenergic stimulation.. Through this study, I hoped to determine whether Ca^{2+} overload resulting from β-AR stimulation leads to heightened levels of activated CaMKII and subsequent phosphorylation of $Na_v1.5$, as well as to assess the extent to which this contributes to arrhythmogenesis at the whole-heart level.

Overview of Thesis

The next chapter, Methodology, details the protocols used for optical mapping and western blotting experiments in this study. Chapter 3: Results provides an analysis of the results obtained from these experiments, including CaD80, tau, occurrence of PVCs, and levels of protein expression. The final chapter presents the major conclusions of the thesis project, details the author's contributions to the study, projects future directions and additional applications of the techniques implemented in the study, and revisits the significance of the study's results.

Chapter 2. Methodology

Animal Models

Established strains of *Scn5a* knock-in mice that expressed either a phosphomimetic mutation at the Ser571 site of $Na_v1.5$ (S571E) or that had the phosphorylation site ablated (S571A) were used for these studies.[5] Experiments were performed in both male and female two- to six-month-old mice. Animals were anesthetized with isoflurane prior to tissue collection. All animal studies were performed in compliance 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.

Optical Mapping

Following isoflurane inhalation, hearts were rapidly excised and Langendorff-perfused with oxygenated modified Tyrode solution (140 mM NaCl, 1.0 mM $MgCl_2*6H_2O$, 1.2 mM NaH_2PO_4, 4.0 mM KCl, 1.8 mM $CaCl_2*H_2O$, 5.6 mM glucose, 10 mM HEPES) at 37°C and maintained at a pressure of 60-80 mmHg. Motion was reduced using 10 μM blebbistatin perfused for 15 minutes. Fluorescent rhod-2 (Biotium) was the Ca^{2+}-sensitive dye used to measure calcium transients.[1] The voltage-sensitive dye Rh-237 (Biotium) was also perfused in order to measure electrical activity, but it did not yield usable signals.

Following initial perfusion, hearts were transferred to a water bath maintained at 37°C and electrical activity was monitored using a continuously recorded volume-conducted bath electrocardiogram (ECG). A dual camera MICAM05 system was used to optically map the posterior epicardial surfaces of the ventricles during pacing with a unipolar electrode using an S1-S1 protocol with cycle lengths ranging from 40ms to 200ms. Slow pacing cycle lengths (i.e. 200 ms) were also used to induce early afterdepolarizations and associated ectopic activity in order to assess arrhythmogenesis. Hearts were mapped using the described protocol at baseline and after exposure to 150 nM isoproterenol (ISO), the drug used to stimulate the β-adrenergic receptors.

Calcium transients were manually assessed using a custom-made Zeng Analysis 2015b MATLAB program. Specifically, the calcium-transient duration at 80% relaxation (CaD80) and the decay constant (tau) were examined. Additionally, arrhythmogenesis was assessed by examining the occurrence of premature ventricular contractions (PVCs).

Western Blots

Equal quantities of ventricular lysates (determined by standard bicinchoninic acid protocols) were analyzed via western blot. Equal protein loading was verified through Ponceau stains of blots, and differences in protein loading were corrected using normalization to GAPDH levels with Image Lab software and Excel. The following antibodies were used for western blotting: total CaMKII (Badrilla), p-CamKII (Thermo),

Nav1.5[4], Serca2a (Thermo), ox-CaMKII (Millipore), p-PLB (Santa Cruz), PLB (Abcam), and GAPDH (Fitzgerald).

Chapter 3. Results and Discussion

When normalized to total protein content, ox-CaMKII expression was decreased in S571A mice relative to WT mice (Figure 2). This suggests that S571A hearts exhibit lower levels of activated CaMKII, which might contribute to their tendency to be less arrhythmogenic at baseline. Interestingly, there did not initially seem to be large differences in levels of p-CaMKII and p-PLB expression between the groups. This may be because the isoproterenol concentration used was causing such large Ca^{2+} overload that it obscured the differences between the groups. However, the number of replicates examined via western blot needs to be increased in order to draw any conclusions about statistical significance.

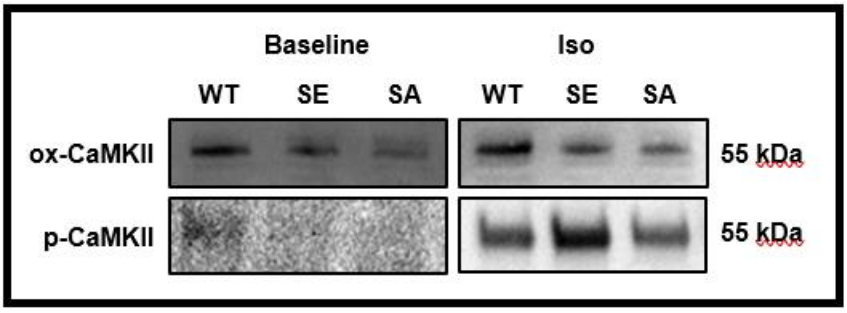

Figure 2: Representative western blots for expression of oxidized and phosphorylated CaMKII at baseline and with isoproterenol.

In addition to normalizing protein expression to total protein content, ox-CaMKII and p-CaMKII levels were normalized to total CaMKII levels, and p-PLB levels were normalized to total PLB. This was done in order to determine how the phosphorylation state of each protein changes with the introduction of isoproterenol. As expected, the amount of

phosphorylated CaMKII increased when exposed to isoproterenol, confirming that there was more activated CaMKII available to phosphorylate $Na_v1.5$ (Figure 2).

During optical mapping experiments, it became clear that CaMKII-dependent phosphorylation of $Na_v1.5$ alters CaD80 at baseline, as S571E mice exhibited significantly longer CaD80's than both WT and S571A. This is illustrated by representative calcium transient traces from WT, S571E, and S571A ventricles at baseline conditions, as shown in Figure 3a. Summaries of the average CaD80 for all mouse models further illustrate that under baseline conditions, S571E mice have significantly elongated calcium transients compared to WT or S571A mice (Figure 3b, *p<0.05 vs. WT, #p<0.05 vs. S571A).

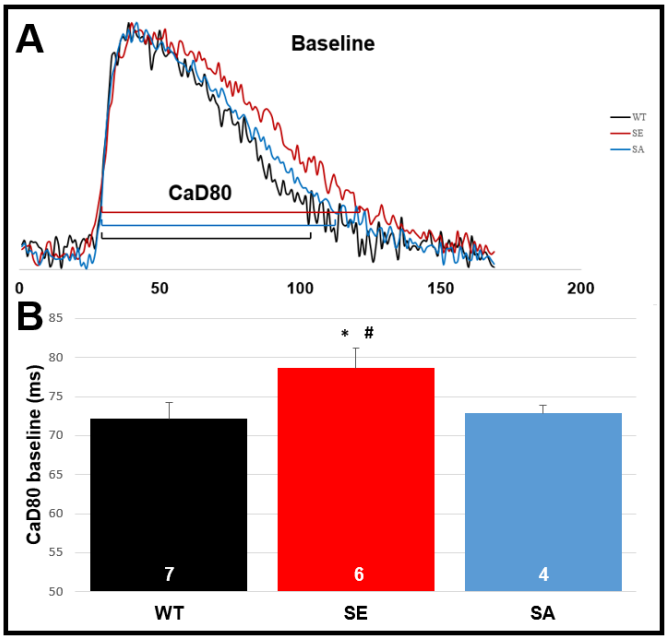

Figure 3. CaMKII-dependent phosphorylation of Nav1.5 alters CaD80 at baseline.

Interestingly, dosing these hearts with isoproterenol mitigated differences between S571E and WT, as the average CaD80 for mice in these two groups are virtually indistinguishable

(Figure 4). However, S571A mice appeared to exhibit some resistance to the effects of the drug. Although calcium transients experienced by these mice seemed to be slightly shortened as a result of isoproterenol, they were still significantly longer than transients from the other two groups (Figure 4, *p<0.05 vs. WT, #p<0.05 vs. S571E).

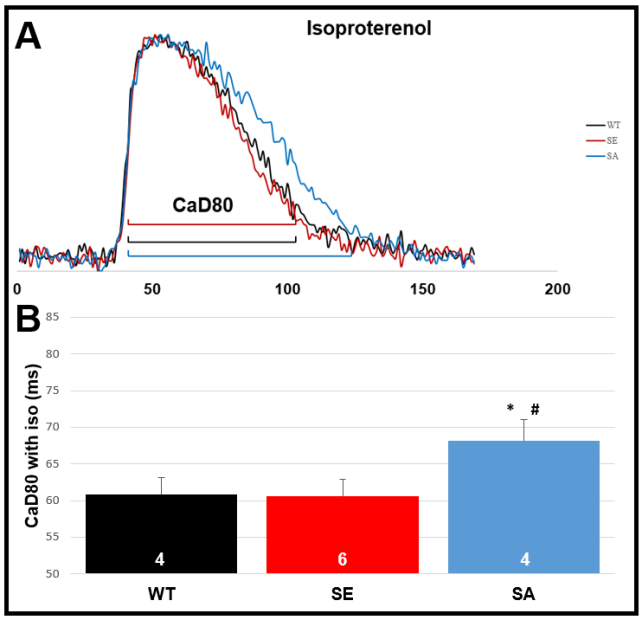

Figure 4. CaMKII-dependent phosphorylation of $Na_v1.5$ alters CaD80 with isoproterenol.

Mirroring the results observed with CaD80, S571E mice exhibited significantly longer decay constants (tau) than WT mice at baseline, suggesting that CaMKII-dependent phosphorylation of $Na_v1.5$ plays an important role in altering tau at baseline (Figure 5, *p<0.05 vs. WT). The differences in decay constant for S571E and S571A mice are not significant, though at baseline S571E mice tend to have longer decay constants than S571A mice. However, the number of replicates examined would need to be increased in order to confirm whether this difference is significant.

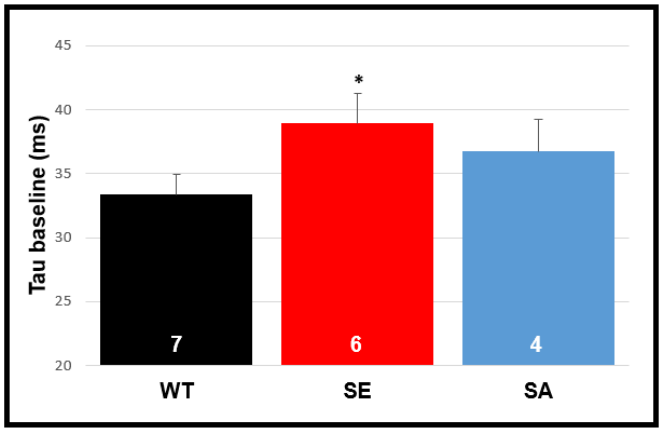

Figure 5. CaMKII-dependent phosphorylation of $Na_v1.5$ alters tau at baseline.

Further echoing the effects of CaMKII-dependent phosphorylation of $Na_v1.5$ on CaD80, isoproterenol reduced tau for all mouse models. Like with CaD80, S571A mice exhibited resistance to the effects of isoproterenol, and even though they experienced a reduction in tau, they were still significantly longer than tau for S571E mice (Figure 6, #$p<0.05$ vs. S571E). Though the decay constant with isoproterenol for S571A hearts seems to be slightly longer than the decay constant for WT hearts, the number of replicates need to be increased in order to draw conclusions about whether this difference is significant.

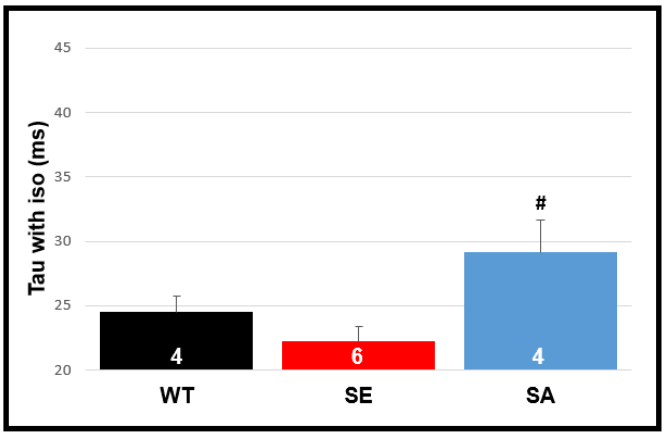

Figure 6. CaMKII-dependent phosphorylation of $Na_v1.5$ alters tau with isoproterenol.

Connecting the examined differences to arrhythmogenic phenotypes, the study also examined incidence of premature ventricular contractions, or PVCs, for all three mouse models at baseline. Representative ECGs from WT, S571E, and S571A hearts demonstrate that under baseline conditions, S571E mice exhibit increased induction of PVCs compared to the other mouse models (Figure 7a). This is further confirmed by examining the number of mice that experienced 3 or more PVCs in the 5 minutes prior to pacing under control conditions. As can be seen in Figure 6b, S571E mice were more arrhythmogenic than S571A mice.

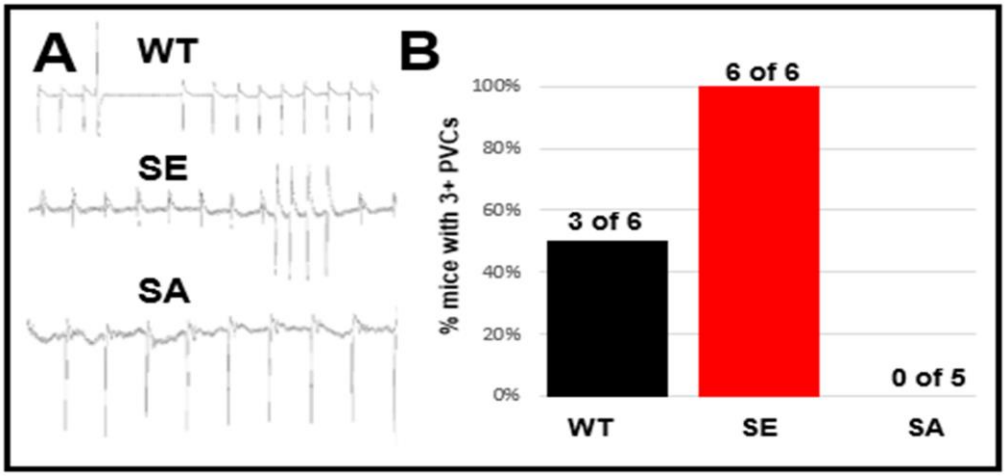

Figure 7. CaMKII-dependent phosphorylation of $Na_v1.5$ alters arrhythmogenesis at baseline.

Chapter 4. Conclusions

Contributions

Although CaD80 was prolonged in S571E mice relative to WT or S571A mice at baseline, isoproterenol eliminated these differences between S571E and WT mice. As expected, isoproterenol shortened CaD80 for all mouse models, although S571A hearts seemed to be resistant to CaD80 shortening in the presence of isoproterenol. As has been demonstrated previously, S571E hearts experienced more PVCs than WT or S571A mice before exposure to isoproterenol. Overall, these data demonstrate that CaMKII-dependent phosphorylation of the $Na_v1.5$ Ser571 site is integral in Ca^{2+} handling and arrhythmogenesis with and without β-adrenergic stimulation. Furthermore, this study suggests that the CaMKII/$Na_v1.5$ axis is a potential therapeutic target for patients that are vulnerable to sympathetically induced Ca^{2+} mishandling and arrhythmogenesis.

Additional Applications and Future Work

Although this study identified a key axis involved in arrhythmogenesis during β-adrenergic stimulation, much is still unknown about the precise mechanism involved. Future studies should thus explore the role of other potential players in causing Ca2+ mishandling and arrhythmias in these conditions. Specifically, a selection of ion channel blockers and/or inhibitors should be employed with and without β-adrenergic stimulation in order to

confirm actions of ion channels such as the L-type Ca^{2+} channel, NCX, SERCA, and RyR in the mouse models used in this study. Additionally, the number of replicates used for western blotting experiments should be increased in order to form a clearer picture of how various proteins are expressed. Patch-clamping experiments should be employed to determine whether the Ca^{2+}-handling trends observed on a whole-ventricle level are also achieved in single cardiomyocytes.

Summary

Despite recent therapeutic advances, heart disease remains the leading cause of death in the United States.[9] In the majority of cases, patients succumb to an electrical dysfunction, commonly referred to as an arrhythmia. Certain populations are especially vulnerable to arrhythmias during β-adrenergic stimulation, a type of signaling involved in acute stress. In order to develop more effective treatments for these patients, it is imperative that we understand the underlying cellular mechanisms that contribute to acute arrhythmogenesis. Through this study, we have identified CaMKII-dependent phosphorylation of the $Na_v1.5$ Ser571 site, as well as resulting increases in $I_{Na,L}$, as crucial contributors to Ca^{2+} handling and arrhythmogenesis both with and without β-adrenergic stimulation. As future studies seek to confirm how the late current affects other structures during these conditions, the CaMKII/$Na_v1.5$ axis provides a promising therapeutic target that can be harnessed to help patients with conditions that make them more susceptible to sympathetically induced cardiac dysfunction.

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