Integrative Studies on the Role of CaMKII in Cardiac Disease and Arrhythmias

DISSERTATION

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By

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Abstract

Heart disease accounts for one of every three deaths in the United States annually. The majority of these deaths are caused by disturbances in normal cardiac electrical activity, known as arrhythmias. While progress has been made towards addressing this concern, recent advances have been limited. Mounting data indicate that Calcium/Calmodulin Protein Kinase II (CaMKII) plays a critical role in promoting arrhythmogenesis and dysfunction in cardiac disease. Overactivation of CaMKII has a proarrhythmic effect and impacts the ability of cells to maintain proper function. Additionally, CaMKII has been shown to be elevated in end-stage human heart failure. Despite the apparent importance of CaMKII in disease, however, the specific mechanisms and phosphorylation targets that CaMKII uses to impact cellular function, particularly in vivo, remain unresolved.

In these studies, we utilized a combination of biochemistry, whole-animal, and mathematical simulation experiments to determine the role that CaMKII plays in cardiac arrhythmia and disease. Our studies have shown that CaMKII contributes to arrhythmias in a number of ways. Our major findings include: 1) CaMKII can induce structural changes that lead to heart disease, particularly in the sinus node; and 2) overactivation of CaMKII displays acute effects through its phosphorylation of ion channels, as we observed primarily in the sodium channel. Additionally, we developed new
mathematical tools to analyze sinus node cell stability that demonstrated how CaMKII dysregulation can contribute to arrhythmias by altering subcellular structures.

In summary, these experiments support an important role for CaMKII in the setting of heart disease. In the sinus node, we identified CaMKII as a contributor toward increased mortality in the setting of myocardial infarction in diabetes. Outside of the sinus node, our studies revealed CaMKII as a critical regulator of the late sodium current in disease, identified a site of singular control over this late current (S571 on Na\textsubscript{v}1.5), and clearly demonstrated that the late sodium current plays an important \textit{in vivo} role in cardiac dysfunction.
This document is dedicated to my family for their love and support throughout the years.
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Chapter 1: Introduction

1.1: Objective

Heart disease accounts for one of every three deaths in the United States annually.\(^3\) The majority of these deaths are caused by disturbances in normal cardiac electrical activity, known as arrhythmias. While progress has been made towards addressing this concern, recent advances have been limited. This is demonstrated by the fact that the major go-to drugs for heart failure remain angiotensin-converting-enzyme (ACE) inhibitors, which have remained largely unchanged since they were first approved in 1981.\(^{17}\) The Cardiac Arrhythmia Suppression Trial (CAST), a double-blind study conducted between 1986 and 1998 that examined the effectiveness of class I antiarrhythmic agents, exposed drugs that, while effective at reducing the amount of Premature Ventricular Contractions (PVCs), actually increased mortality by increasing arrhythmia susceptibility (e.g. flecainide, moracizine, and encainide).\(^{18}\) Because of the limited effectiveness of current therapies, there is a large need for additional research in this field. The overall goal of my research is to identify new potential heart disease therapy targets using a combination of advanced molecular science and mathematical modeling. In particular, my studies focus on the multifunctional Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII), due to mounting evidence that this signaling axis is critically involved in human cardiac disease and arrhythmias.\(^2,19-25\)
1.2: Overview of Cardiac Physiology and Cell Biology

The primary function of the heart is, of course, mechanical in nature and involves regular, robust circulation of blood throughout the body. Blood first enters the heart through the right atria, travels to the right ventricle, and then travels to the pulmonary system. It then re-enters the heart through the left atria, travels to the left ventricle, and circulates to the rest of the body. However, the mechanical work of the heart is impossible without coordinated electrical activation. In order for these chambers to effectively work together, the heart has an extensive conduction system that is responsible for coordinating electrical activation of cardiac muscle cells.

Cardiac muscle cells have an excitable membrane that allows for generation of action potentials. The cell membrane consists of a phospholipid bilayer that provides structure to the cell and regulates the entry and exit from the intracellular environment. An action potential is a temporary reversal of the electrical potential gradient across a cell membrane.

Because of its role in separating the extra- and intra-cellular compartments, the cell membrane can be thought of as a capacitor that regulates the separation of electrically-charged ions on either side of the membrane. In order to precisely control the separation and flow of ions, a number of proteins, including ion transporters, are
embedded in the membrane. Movement of charged ions across this barrier generates an electrical current. An example schematic can be seen in Figure 1.

There are three main classifications of ion transporters: channels, pumps, and exchangers. Ion channels may be gated by voltage, ligand binding, and/or mechanical stimulation allowing for specific ions to flow freely down their electrochemical gradient. Ion pumps, on the other hand, move ions against their electrochemical gradient by utilizing adenosine triphosphate (ATP). Finally, ion exchangers move ions against their electrochemical gradient by utilizing energy generated from moving a different ion down its electrochemical gradient.
When not perturbed, the electrical potential across a membrane tends toward a steady-state value known as the resting membrane potential. This value is approximately -90 mV for ventricular cardiomyocytes, and varies depending on cell type (e.g. -60 mV in SAN cells and -80 mV in atrial cells). Precise control of ion channels, transporters and pumps allows for the maintenance of the resting potential. However, when stimulated, the membrane is able to become depolarized and will experience a reversal in its electrical potential. This reversal is known as an action potential.

Figure 1: The cell membrane controls the flow of charged ions from the intracellular to the extracellular space through the use of ion transporters. These include, from left to right, $K^+$, $Ca^{2+}$, and $Na^+$ channels, the $Na^+/Ca^{2+}$ exchanger (NCX), and the $Na^+/K^+$ ATPase (NKA).
1.3: Cardiac Action Potential

The electrical state of the cardiac membrane is dependent upon precise regulation of a variety of ions, including sodium, calcium, potassium, and chloride. Voltage-gated ion channels are one major classification of ion channels that open and close in response to changes in the membrane potential. At rest, the membrane permeability to potassium is greater than that to other ions (e.g. sodium, calcium). Because of the fact that, at rest, the membrane is primarily permeable to potassium, the resting membrane potential is close to the value of the potassium Nernst potential. The Nernst potential is the equilibrium potential of a given ion such that there is no net movement of that ion across the cell membrane.\textsuperscript{26} It is described by the following equation:

$$E_x = \frac{61}{z} \log \frac{X_o}{X_i}$$

Equation 1: The Nernst Potential Equation

where $E_x$ is the equilibrium potential for ion $x$ (in mV), $[X]_o$ is the concentration of the ion outside of the cell, $[X]_i$ is the concentration of the ion inside the cell, and $z$ is the valence of the ion.

When an external, depolarizing stimulus is applied to a cell, voltage-gated sodium channels in the cell membrane open and allow sodium ions to rapidly enter the cell by
flowing down their electrochemical gradient. This sudden, massive influx of positively charged ions further depolarizes the membrane, causing more sodium channels to open, and further increasing the flow of sodium into the cell. The sodium channels then rapidly inactivate. This inactivation eliminates most of the sodium current, although a very small amount of sodium current persists through the rest of the action potential.

Additionally, as the sodium channels are closing, both calcium and potassium channels begin to open, causing an influx of calcium ions and an efflux of potassium ions. This opening occurs after the sodium channel activity due to slower channel kinetics. Because both calcium and potassium have positive charges, the simultaneous activity of these two channels leads to a “plateau” phase of the action potential. Shortly after reaching this plateau phase, however, calcium channel inactivation/deactivation together with the continued potassium channel activation shifts the balance towards a net repolarizing current. This pushes the cell membrane towards its resting potential. Ion pumps and exchanges work against ion concentration gradients to reestablish baseline concentration levels.
The action potential varies greatly across different heart regions. The sinoatrial node (SA node) is a critical heart region located in the right atrium that spontaneously generates action potentials. Action potentials from cells in this region lack a stable rest potential but have a maximum diastolic potential more positive than the resting potential for other
cell types (-60 mV). Other atrial cells do not generate their own action potentials, but instead receive stimulation from the electrical wave generated by the SA node. Atrial cells have a resting membrane potential of approximately -80 mV. From the atria, the electrical wave travels through the atrioventricular node (AV node) before conducting through the bundle of His and finally to the Purkinje fibers. These fibers are located in the ventricular wall, experience rapid depolarization followed by slow repolarization, and have a resting membrane potential of -90 mV. Finally, the Purkinje fibers conduct electrical signal to the ventricular myocytes, which also have a resting membrane potential of approximately -90 mV. An example of the ventricular action potential, and representative contributing currents, can be seen in Figure 2.

1.4: Sodium Channels

$Na_v$ activity is responsible for the rapid action potential upstroke in atrial and ventricular cardiomyocytes, and $Na_v$ dysfunction has been linked to abnormal cardiac cell excitability and congenital and acquired arrhythmia.$^{28}$

$Na_v1.5$ is the primary $Na_v$ alpha subunit expressed in heart. It is primarily localized to the cardiomyocyte intercalated disc to assist with AP propagation and tissue excitation and contraction.$^{29-32}$ This 220-kDa $\alpha$-subunit constitutes a functional channel by itself but associates with a family of 30-kDa accessory $\beta$-subunits that regulate the kinetics and voltage-dependence of the gating.$^{33,34}$ The importance of proper $Na_v1.5$
function is demonstrated by the variety of diseases associated with its malfunction, including cardiac conduction disease, sinus node disease, Brugada syndrome, and long QT type 3.\textsuperscript{33, 35, 36}

The Na\textsubscript{v} α-subunit consists of four repeating domains (labeled DI-DIV in Figure 3). Each of these domains is made up of six α-helical segments that span the membrane.\textsuperscript{34} The fourth segment is central to the voltage-sensing functions of the channel. The loop in between the 5\textsuperscript{th} and 6\textsuperscript{th} segments forms a narrow, ion-selective filter at the extracellular side of the pore, and the sixth segments combine to form the intracellular side of the pore.\textsuperscript{34} Inactivation is made possible by the highly conserved intracellular loop in between DIII-DIV that acts as an inactivation gate. This gate is frequently thought of as

![Figure 3: CaMKII phosphorylates Na\textsubscript{v}1.5. A variety of proteins play a role in targeting CaMKII to Na\textsubscript{v}1.5 for phosphorylation, including Ankyrin-G, Actin, βIV-spectrin, and α-spectrin.](image)
a “hinged lid,” as it swings closed to bind to the intracellular pore of the channel, causing inactivation.\textsuperscript{34}

Na, channels open to generate the action potential upstroke (Figure 4). This activation is followed by rapid inactivation (1-2ms).\textsuperscript{37} The conductance of these channels is gated by changes in the membrane potential.\textsuperscript{38} Less than 1\% of channels, however, remain active during the action potential plateau in a normal myocyte.\textsuperscript{39} The current generated by these channels is referred to as the “late” Na\textsuperscript{+} current (late I\textsubscript{Na}). There are four main methods by which this late Na\textsuperscript{+} current is thought to be generated: 1) Delayed inactivation of open channels, 2) Reopening of unstable inactivated channels, 3) fast recovery of channels from inactivation, and 4) Delayed opening of Na\textsuperscript{+} channels that did not open during the AP upstroke.\textsuperscript{40-42} While the amplitude of this current is small under normal physiological conditions (Estimates range from approximately 0.1\%-1\% of peak I\textsubscript{Na}), its susceptibility to increase when under pathological stress, and its association with a variety of diseases, makes it a potential target worth examining.\textsuperscript{43}

When increased above baseline levels, late I\textsubscript{Na} may promote elevation in intracellular Na\textsuperscript{+} concentration. This increased intracellular Na\textsuperscript{+} contributes to a variety of changes in these cells. One such change of particular physiological significance is an associated increase in internal Ca\textsuperscript{2+} via reverse mode Sodium-Calcium Exchanger (NCX) activity.\textsuperscript{44} NCX activity is regulated by the intra- and extracellular concentrations of Na\textsuperscript{+} and Ca\textsuperscript{2+} as well as the membrane potential. An increase in intracellular Na\textsuperscript{+} reduces
forward mode NCX activity (3 Na\(^+\) ions transported into the cell for every 1 Ca\(^{2+}\) ion removed), and increases reverse mode activity (3 Na\(^+\) out, 1 Ca\(^{2+}\) in). This causes an increased buildup of calcium in the cell.

An additional impact of enhancement of late I\(_{Na}\) is a prolongation of the AP plateau.\(^{45}\) This prolongation causes an increased susceptibility to afterdepolarizations.\(^{46}\) Afterdepolarizations are inappropriate secondary depolarizations of the membrane potential and are frequently split into two categories, Early and Delayed, depending upon the timing relative to the cardiac action potential phase. Early afterdepolarizations (EADs) occur during the plateau or late repolarization (phase 2 or phase 3), before repolarization is complete. Delayed Afterdepolarizations (DADs) occur during phase 4 of the action potential, after repolarization is complete, but before another action potential would normally occur.\(^{47}\)
EADs and DADs are commonly believed to differ by their triggering mechanism. EADs are thought to occur primarily due to the reactivation of L-type Ca\(^{2+}\) current due to prolongation of the action potential.\(^{48}\) DADs, on the other hand, are due to elevated cytosolic calcium levels. These elevated levels lead to sarcoplasmic reticulum overload, which causes spontaneous release of Ca\(^{2+}\) via RyR (RyR gating is regulated by both cytoplasmic and luminal calcium).\(^{19}\) This can then lead to reverse mode NCX activity, causing a net depolarizing current that may bring the membrane to threshold for activation of Na\(_v\).

The late sodium current is capable of increasing the likelihood of both EADs and DADs through different mechanisms. Late I\(_{\text{Na}}\) increases APD, which can lead to EADs.

![Diagram](image.png)

**Figure 4:** The impact of increased late sodium current on the ventricular action potential. Increased late current can prolong the action potential and potentially cause afterdepolarizations.
Additionally, as previously discussed, increased intracellular Na\(^+\) leads to an increase in intracellular Ca\(^{2+}\), and is thus also capable of inducing DADs.\(^{12}\) Regardless of the pathway, agents that selectively block late Na\(^+\) have shown antiarrhythmic potential in preclinical and clinical trials.\(^{49, 50}\) For example, in a study conducted by Scirica et al. referred to as “The Metabolic Efficiency With Ranolazine for Less Ischemia in Non–ST-Elevation Acute Coronary Syndrome (MERLIN)–Thrombolysis in Myocardial Infarction (TIMI) 36” (MERLIN-TIMI 36), a randomized group of 6560 patients hospitalized with a non–ST-elevation acute coronary syndrome were given either ranolazine or placebo in addition to standard therapy.\(^{50}\) The patients received continuous ECG monitoring for a week following admission. After analysis, the researchers found that patients who received ranolazine therapy experienced fewer episodes of ventricular tachycardia lasting more 8 or more beats, supraventricular tachycardia, or new-onset atrial fibrillation compared to placebo.\(^{50}\)

1.5: Causes of Increased Late I\(_{Na}\)

There are a variety of defects that can lead to an increase in late I\(_{Na}\). Generally, these can be broken down into three categories: Inherited mutations in SCN5A (the gene that encodes Na\(_v\)), Inherited mutations in proteins associated with Na\(_v\) (e.g. β-subunit, regulatory proteins, adapter/cytoskeletal proteins) and acquired channelopathies observed in myocardial ischemia and heart failure.\(^{38}\)
Inherited mutations in SCN5A, the cardiac sodium channel gene, have been shown to cause long QT (LQT) Syndrome 3. LQT3 Syndrome is defined as a disorder of the heart’s electrical system that causes a prolongation of the QT interval which can lead to a sudden, life-threatening ventricular tachycardia known as *torsades de pointes*. More than 80 different mutations in SCN5A have been identified that cause LQT3. The vast majority of these mutations are missense mutations (caused by a single nucleotide change that changes the coded amino acid), and they result in a gain-of-function by increasing the probability that Na\(_v\).1.5 will either fail to inactivate properly or will be more prone to reopening from a closed state. Many of these mutations are found in the DIII-DIV linker on Na\(_v\).1.5.

Inherited mutations in proteins that interact with Na\(_v\).1.5 can also cause an increase in late I\(_{Na}\). Primary examples of this are mutations in the Na\(^+\) channel β subunits. Mutations in these subunits have been shown to cause inherited LQT3 and Brugada Syndrome, as well as atrial fibrillation and conduction slowing. Four different β subunits have been shown to play an important role in localization and regulation of the Na\(_v\) α subunit. For example, loss of β\(_1\) expression in mice caused an increase in both peak I\(_{Na}\) and late I\(_{Na}\). Additionally, mutations in both β\(_3\) and β\(_4\) subunits have been identified in patients with inherited LQT3, and found to cause an increase in late I\(_{Na}\) when expressed with in HEK293 cells.
In addition to inherited mutations, myocardial ischemia and heart failure also lead to a number of changes that can impact late \( I_{Na} \). These changes include increased concentrations of \( Na^+ \) and \( Ca^{2+} \), reverse mode \( Na^+\)-\(Ca^{2+} \) exchange activity, and increased Calcium/Calmodulin Protein Kinase II (CaMKII) activity.\(^{56,57}\) These conditions can lead to hypoxia and an increased concentration of a number of potentially harmful products, such as nitric oxide (NO) and hydrogen peroxide (\( H_2O_2 \)). Many of these induced changes, including hypoxia, NO, and \( H_2O_2 \), have been shown to individually increase both late \( I_{Na} \) and internal sodium concentrations.\(^{38}\) Importantly for this discussion, this combination of increased intracellular calcium and other elevated intracellular ROS can lead to an increased activation of CaMKII. This increase in activated CaMKII can then cause an increase in the late sodium current.\(^{58}\) For example, studies using a \( Na_v1.5 \) pS571-specific antibody demonstrated an increased CaMKII-dependent phosphorylation of this site in disease.\(^2\) More specifically, these studies showed a relationship between regulation of the S571 site and \( Na_v1.5 \) in heart disease and monogenic arrhythmia. The relationship between CaMKII and \( Na_v1.5 \) will be further examined in section 1.7.

**1.6: Excitation-Contraction Coupling**

Excitation-contraction coupling (ECC) is a multistep process that is essential for linking electrical and mechanical activation of a cell. ECC begins when an action potential excites the cell membrane. Depolarization of specialized membrane
invaginations called t-tubules causes extracellular Ca\(^{2+}\) to enter the cell through voltage-gated L-type Ca\(^{2+}\) channels (LTCC, highly localized in t-tubular membrane). The increased inward Ca\(^{2+}\) current generated by these channels triggers ryanodine receptor 2 (RyR2) channels to open, in a process termed Calcium-induced Calcium release, leading to coordinated release of sarcoplasmic reticulum (SR) Ca\(^{2+}\) that contributes the major portion of the myofilament-activating increase in internal Ca\(^{2+}\). The Ca\(^{2+}\) released from the SR binds to troponin C, a part of the troponin-tropomyosin complex, on the actin filaments in sarcomeres. This facilitates formation of cross-bridges between actin and myosin and triggers myocardial contraction during systole. Voltage-gated K\(^{+}\) channels open to allow an outward current that leads to AP repolarization, allowing the relaxation process to begin. Relaxation occurs when Ca\(^{2+}\) is taken back up into the SR through the SR Ca\(^{2+}\) adenosine triphosphatase (SERCA2a) and is removed from the cell by the Na\(^{+}\) and Ca\(^{2+}\) exchanger (NCX).\(^ {59}\)

1.7: Regulation of Excitation-contraction Coupling in Response to Acute Stress

Adrenergic and cholinergic signals originating from the autonomic nervous system, in addition to neurotransmitters such as epinephrine and acetylcholine, play a major role in regulation of ECC.\(^ {60}\) Posttranslational modification of channels and other proteins involved in ECC by these compounds is an important way of maintaining intracellular homeostasis, especially in response to acute stress. One impact of these
signals is either the activation or inhibition of G-proteins, which promote the formation of cyclic AMP (cAMP).\textsuperscript{60} cAMP then activates PKA, which phosphorylates a number of proteins involved in Ca\textsuperscript{2+} handling. These proteins include phospholamban (PLB), L-Type Calcium Channels (LTCC), several substrates involved in controlling contractility (e.g. TnI and MyBP-C).\textsuperscript{61}

In addition to regulation by PKA, many of these targets, such as PLB, are also regulated by Calcium/Colmodulin Protein Kinase II (CaMKII).\textsuperscript{19} Members of the CaMKII family are multifunctional serine/threonine kinases that exist in a wide variety of cell types and show a high level of conservation across animal species.\textsuperscript{62} CaMKII regulates a variety of cellular functions including ion channel biophysics, cellular metabolism, and transcription and excitation-contraction coupling.\textsuperscript{63, 64} Additionally, CaMKII has been linked to human heart failure through a variety of mechanisms, including its role in ECC regulation.\textsuperscript{65}

There are four main CaMKII isoforms produced by four different genes: alpha, beta, delta, and gamma isoforms.\textsuperscript{66} In heart, the predominant CaMKII isoform is delta, but there is some secondary expression of gamma.\textsuperscript{66} CaMKII isoforms express high homology, with each CaMKII monomer containing an N-terminal catalytic domain, a regulatory domain, and a C-terminal association domain.\textsuperscript{67} The regulatory domain contains a Ca\textsuperscript{2+}/calmodulin binding pocket and a large number of regulatory sites, including autophosphorylation (Thr287), oxidation (Met281/282), and O-linked
glycosylation (Ser279). Following activation, CaMKII phosphorylates a large number of intracellular targets, such as ion channels, pumps, transporters, and a variety of other proteins. 

1.8: CaMKII-dependent regulation of Excitation-contraction coupling

There are three primary methods by which CaMKII plays a role in ECC. The first is through regulation of proteins directly involved in Ca$^{2+}$ handling, such as RyR2, PLB, and LTCC. The second is through regulation of channels that while do not participate directly in ECC coupling may alter Ca$^{2+}$ homeostasis by directly affecting other ion concentrations and/or the action potential duration (e.g. Na$^+$ and K$^+$ channels). The third way is by regulating Ca$^{2+}$ binding to troponin C, which plays a role in activation of myofilaments involved in contraction.

RyR2 is phosphorylated by CaMKII at serine 2814 (An additional serine, 2809, has been linked to Protein Kinase A phosphorylation). CaMKII-dependent RyR2 phosphorylation increases diastolic SR Ca$^{2+}$ release. Additionally, mice expressing a mutant S2814A site that prevents CaMKII phosphorylation have been shown to be resistant to MI-induced heart failure and arrhythmias. The importance of this site was studied further by the Wehrens group, where they created a knock-in mouse model expressing an RyR2-S2814D (phosphomimetic) mutation. Mice expressing this mutation experienced a significant predisposition to sudden arrhythmogenic death after
transverse aortic constriction surgery. In addition, a second population of mice with the site ablated (Ryr2-S2814A) experienced improved resistance to arrhythmias versus wild-type mice after transverse aortic constriction surgery. Taken together, these two findings indicate that the S2814 site may play a significant role in heart failure.

A couplon is made up of colocalized LTCC and RyR2 channels, and functions to release Ca\(^{2+}\) in bursts, or “sparks.” These sparks occur spontaneously in cardiac myocytes. Under normal conditions, Ca\(^{2+}\) spark frequency depends upon SR Ca\(^{2+}\) load. However, CaMKII has been demonstrated to increase Ca\(^{2+}\) spark frequency, causing what is called a SR Ca\(^{2+}\) “leak.”\(^89\) Additionally, studies have shown that CaMKII association with LTCC plays a role in Calcium-dependent facilitation of these channels, and additional exacerbates pathophysiology related to these channels’ function.\(^90,91\)

Phospholamban (PLB) is an inhibitor of SERCA2a in its unphosphorylated state. Once PLB is phosphorylated, SERCA2a activity and SR Ca\(^{2+}\) uptake are both increased. PLB can be phosphorylated by either PKA at Ser-16 or by CaMKII at Thr-17, with both phosphorylation sites producing a similar effect on SERCA2a activity.\(^78\) However, some positive effects of CaMKII phosphorylation of this site have also been reported, including improved recovery from acidosis\(^92\) and improving the recovery of Ca\(^{2+}\) transients and contractility in the “stunned” (fully reversible post-ischemic dysfunction) heart.\(^93\)
1.9: CaMKII-dependent regulation of Na\textsubscript{v}1.5

CaMKII has been shown to phosphorylate Na\textsubscript{v}1.5, and the amount of phosphorylation that occurs is elevated in disease states.\textsuperscript{94} CaMKII phosphorylation of Na\textsubscript{v}1.5 has three primary effects on I\textsubscript{Na} gating: 1) Increased steady-state Na\textsuperscript{+} channel inactivation, 2) Slowed open-state inactivation and 3) Increased late component of I\textsubscript{Na}.\textsuperscript{65}

As described above, increased late I\textsubscript{Na} has been linked to arrhythmias in disease. Even more telling, Na\textsubscript{v}1.5 phosphorylation by CaMKII appears similar to a combined disorder involving aspects associated with loss-of-function (as in Brugada syndrome),\textsuperscript{12} and gain-of-function (more associated with LQT3).\textsuperscript{95, 96}

A number of sites on Nav1.5 have been identified as potential CaMKII phosphorylation targets in the DI-DII linker region.\textsuperscript{2, 32} One of these sites, Ser571 was first identified by our group as a potential phosphorylation site for CaMKII through functional screening of a library of mutants in heterologous cells.\textsuperscript{32} Additional studies using a S571 phosphorylation-specific antibody demonstrated an increase in CaMKII-dependent phosphorylation of this site in disease.\textsuperscript{2} Another study used a phosphorylation assay and mass spectrometry to identify additional sites, such as Ser516 and Thr594\textsuperscript{97}. Thirdly, a mass spectrometry approach identified 11 potential sites as targets for CaMKII phosphorylation on Na\textsubscript{v}1.5 \textsuperscript{98}. The relative role that each of these sites play has not yet been determined, however, and additional experiments, particularly \textit{in vivo}, will need to be performed.
Despite the fact that CaMKII has been shown by multiple groups to phosphorylate $\text{Na}_v1.5$, there may actually be multiple $\text{Na}_v1.5$ channel populations throughout the cell that are targeted differently. In fact, different groups of $\text{Na}_v1.5$ channels have been shown to interact with different proteins for targeting and regulation. For example, most $\text{Na}_v1.5$ channels are localized to the intercalated disc. $\text{Na}_v1.5$ channels that localize to the intercalated disc interact with a complex involving ankyrin-G, $\beta_{IV}$-spectrin, and CaMKII (Figure 3).

The beginnings of this complex were first realized in 2004 when Mohler et al. identified a mutation on $\text{Na}_v1.5$ that blocks the binding of ankyrin-G. $^{30}$ They also noted that when this binding was blocked, it prevented proper localization of $\text{Na}_v1.5$ to the intercalated disc. Following up on this research, work in 2010 by Hund et al. identified a $\beta_{IV}$-spectrin-based complex that targets CaMKII to $\text{Na}_v1.5$ at the intercalated disc. $^{32}$ Disruption of $\beta_{IV}$-spectrin/CaMKII interaction disrupts CaMKII localization but not $\text{Na}_v1.5$ localization. Combined, this work indicated that $\text{Na}_v1.5$ forms a complex with several different proteins to regulate its localization and activity.

Additionally, a recent publication showed that mice lacking ankyrin-G expression in the heart have defects in the expression of $\text{Na}_v1.5$, $\beta_{IV}$ spectrin, and CaMKII at the intercalated disc. Additionally, these mice experienced defects in CaMKII regulation of the late sodium current $^{31}$. Notably, loss of ankyrin-G did not alter sarcolemmal membrane $\text{Na}_v1.5$ channels $^{31}$. 
A second population of Na\textsubscript{v}1.5 channels has been shown to localize to the lateral membrane of cardiomyocytes. This population of channels depends on a completely unrelated cellular pathway involving alpha\textsubscript{1}-syntrophin\textsuperscript{99,100}, a protein whose function has been linked with congenital long QT syndrome\textsuperscript{101}. Another recent publication showed that mice expressing a mutant alpha\textsubscript{1}-syntrophin that lacks the Na\textsubscript{v}1.5 binding domain had interrupted lateral membrane Na\textsubscript{v}1.5 targeting and reduced $I_{\text{Na}}$\textsuperscript{100}. However, Na\textsubscript{v}1.5 was still properly localized to the intercalated disc\textsuperscript{100}. As the variability between these two populations demonstrate, it is possible that CaMKII only plays a functionally significant role in a subpopulation of the total Na\textsubscript{v}1.5 channels.

Because most intracellular proteins regulate a variety of ion channel targets, it can sometimes be difficult to discern which channel mutations are actually playing a role in a disease state. Previous studies indicated that SAP97 played a role in proper Na\textsubscript{v}1.5 localization to the intercalated discs\textsuperscript{102}. However, recent data from a SAP97 knockout study showed no difference in Na\textsuperscript{+} current in these animals\textsuperscript{103}. Because of the multitude of potential regulatory targets for each of these proteins, knockout studies can be a very useful tool for determining the impact of a protein on individual ion channels.

1.10: Role of CaMKII in cardiac disease and arrhythmias

Mounting data indicate that CaMKII plays a critical role in promoting arrhythmogenesis and dysfunction in cardiac disease\textsuperscript{20}. For example, CaMKII
overexpression in the mouse leads to the development of heart failure while CaMKII removal prevents this development after transaortic constriction.\textsuperscript{104-106} In addition to heart failure, CaMKII has been shown to play a role in diabetes\textsuperscript{107-109} and CaMKII dysfunction has been indicated to play a role in both atrial fibrillation and sinus node disease\textsuperscript{110}.

Mechanistically, CaMKII contributes to cardiac disease by activating a variety of downstream effects. For example, CaMKII is able to activate AP24, which is a proapoptotic protease. This activation leads to DNA fragmentation.\textsuperscript{79} Additionally, CaMKII can increase expression of proapoptotic genes downstream of the protein kinase kinases TAK1 and ASK1.\textsuperscript{80, 82} Finally, CaMKII has recently been shown to be involved in mitochondria-dependent prodeath pathways. Increases in cytosolic Ca\textsuperscript{2+} and ROS are able to activate CaMKII and cause CaMKII to associate with mitochondria. If this occurs, CaMKII then promotes the release of cytochrome C, reduces mitochondrial membrane potential, and encourages mitochondrial-dependent apoptosis.\textsuperscript{81} A second pathway by which CaMKII is able trigger mitochondrial-dependent apoptosis is through CaMKII-dependent phosphorylation of PLN.\textsuperscript{83} Lastly, CaMKII has been shown to bind to HDAC4, a class IIa histone deacetylase, to play a role in gene transcription.\textsuperscript{111}
Overall, CaMKII plays a role in a wide variety of cellular activities. In addition to its effects on ion channels, it is able to interact with a number of intracellular proteins involved in Ca\(^{2+}\) signaling and cell death. Improper regulation of CaMKII is able to dramatically impact the ability of cells to maintain proper function. By and large, the impact that overactive CaMKII has on these cells appears to be proarrhythmic.

![Diagram of CaMKII Phosphorylation targets](image)

Figure 5: Example CaMKII Phosphorylation targets. CaMKII phosphorylates a wide variety of proteins in cardiac cells, including Phospholamban (PLB), the Ryanodine Receptor 2 (RyR2), L-Type Calcium Channels (LTCC), the voltage-gated sodium channel (Na\(_v\)1.5), and multiple potassium channels, such as the voltage-gated potassium channels K\(_v\)4.3 and K\(_v\)4.3.
Additionally, CaMKII has been shown to be elevated in end-stage human heart failure. Because of these reasons, a large amount of research is currently being conducted on whether or not impeding CaMKII activity is capable of providing protective properties against arrhythmias.

1.11: Overall Goal of My Studies

CaMKII is a complicated protein that plays a role in a variety of intracellular interactions important for proper heart function. In addition to interacting with a number of targets essential for ECC function, CaMKII also phosphorylates a variety of ion channels (Figure 5). Because of this multitude of phosphorylation targets, it can be difficult to determine the individual role that each target plays in normal heart function, as well as its contribution to a disease state.

The overall goal of my research is to add clarity to this plethora of phosphorylation activity, with a particular focus on CaMKII/Na,1.5 phosphorylation. To accomplish this, I will first spend the next chapter discussing one of the primary tools I used in my investigations, mathematical modeling, and the applications of this tool. After that, I will focus on the role of CaMKII in sinus node dysfunction, and discuss several examples of the importance of proper CaMKII regulation in this tissue. Next, I will step into a discussion of the role of CaMKII in regulation of ventricular arrhythmia,
beginning my focus on proper Na\textsubscript{v} function. Finally, I will discuss the mechanisms of CaMKII-dependent regulation of Na\textsubscript{v}1.5 \textit{in vivo}.
Chapter 2: Mathematical Modeling of Electrophysiology

2.1: Motivation

Mathematical models are invaluable tools for understanding the relationships between components of a complex system. In a biological context, mathematical models help us understand the complex relationships between various components (such as DNA, proteins, enzymes, and signaling molecules) of a biological system, gain a better understanding of the system as a whole, and predict its behavior in a disease state.

Mathematical modeling has enhanced our understanding of multiple complex biological processes like enzyme kinetics, metabolic networks, signal transduction pathways, gene regulatory networks, and electrophysiology. Because of recent advances in high throughput data generation methods, computational techniques and mathematical modeling have become even more important to the study of biological systems.

2.2: Quantitative Physiology Originations

Among the first and best known applications of mathematical modeling to a biological system was the pioneering work of Hodgkin and Huxley, who in their 1952 paper described a theory for action potential (AP) generation in the giant squid axon based on a set of coupled ordinary differential equations. In many ways, the work of...
Hodgkin and Huxley to understand the nerve AP remains a gold standard for how experiment and modeling should be used together in order to advance knowledge beyond what would be possible with either approach individually. Beginning with observations of the AP in a squid giant axon and a simple model of the nerve cell membrane as a parallel combination of capacitive and resistive elements, they conducted a series of experiments to separate and characterize transmembrane fluxes due to Na\(^+\) and K\(^+\). These were modeled as resistive elements in their model. Data from these experiments were used to determine rate constants for voltage- and time dependent gating variables (control Na\(^+\) and K\(^+\) fluxes) in their membrane model. Finally, they demonstrated that by solving the set of equations that described the time-dependent changes in transmembrane potential and gating variables (four coupled, ordinary differential equations), they were able to accurately reproduce the nerve AP (Figure 6).

The work of Hodgkin and Huxley had an immense impact on our understanding of electrophysiology not only because it created a robust theory for AP generation, but also because it created experimental and mathematical tools that remain in widespread use today.\(^{113-115}\) On the experimental side, they perfected the technique of the voltage clamp, which remains an essential method for measuring electrophysiological properties of excitable cells today. On the modeling side, their efforts laid the foundation for modern quantitative physiology.
Despite the age of this work, understanding the process followed by Hodgkin and Huxley to Huxley is an essential prerequisite to understanding the more advanced models being used today. The first important piece of information is the fact that their experiments began and ended with a model. In their particular case, their model was the parallel conductance model of the axon membrane. In addition to their model, they utilized a number of intelligent assumptions about how ions traversed the membrane (Fig. 6).

Figure 6: Hodgkin–Huxley theory for action potential generation. (A–B) Parallel conductance model of the giant squid axon cell membrane representing the membrane as an electrical circuit with a capacitor in parallel with time- and voltage-dependent Na$^+$ and K$^+$ conductances ($g_{\text{Na}}$ and $g_{\text{K}}$, respectively) and a constant leak conductance ($g_{\text{L}}$). $V_m$ = transmembrane potential; $E_{\text{Na}}$ = reversal potential for Na$^+$; $E_{\text{K}}$ = reversal potential for K$^+$; $E_{\text{L}}$ = reversal potential for leak. (C–D) Simulated action potential and Na$^+$ and K$^+$ conductances generated by numerically solving the Hodgkin–Huxley equations. Published in 10.
1). The model helped inform their experiments and, in turn, results from their experiments informed the model. The work of Hodgkin and Huxley laid the foundation for generations of researchers interested in understanding the activity of excitable cells from a wide range of systems.

Among the first researchers directly influenced by Hodgkin and Huxley were Richard FitzHugh and Wilfrid Rall. FitzHugh, while working at the NIH, generated important insight into the dynamics of excitability (especially threshold phenomena) by deriving a simplified two-variable system of equations based on the Hodgkin–Huxley equations.\textsuperscript{116, 117} Rall, commonly considered one of the founders of computational neuroscience, utilized mathematical approaches based on cable theory to show that dendritic branching of neurons affects processing of synaptic input, developed the discretized version of cable theory (compartmental modeling), and was one of the first to use digital computers in neuroscience.\textsuperscript{115, 118} Rall's work was among the first examples of how mathematical modeling can be applied to change the accepted views on a topic.

Before these studies, neurons were assumed to have uniform electrical potential and dendrites were not thought to have any real electrophysiological importance. Through the use of mathematical modeling, Rall demonstrated the need to take the flow of current in dendrites into account when interpreting data recorded in the soma. Although it was not until much later that these ideas were finally accepted by the community-at-large, ideas introduced by Rall, such as spatial summation and dendritic attenuation of synaptic input, are now considered integral to neuroscience.
2.3: Cardiac-Focused Quantitative Physiology

While Hodgkin and Huxley's influence spans all of biology, nowhere is it more apparent outside the nervous system than in the heart. Around the same time that Rall and FitzHugh were conducting their studies, the British biologist Denis Noble was attempting to use the Hodgkin–Huxley equations to understand the distinct morphology of the cardiac AP. This differed significantly from the neuronal AP, particularly because of its relatively long plateau phase.\textsuperscript{119, 120} These initial efforts generated predictions about ion channel differences between neurons and cardiac myocytes that predated by several years the first successful voltage clamp experiments in cardiac myocytes.\textsuperscript{121} Following these early efforts, cardiac researchers have generated a staggering number of cell models based in one way or another on the original model created by Hodgkin and Huxley.\textsuperscript{122} Today, experimentally-based mathematical models are available for the cardiac AP from virtually every region of the heart and across a wide variety of species, including human (based, in fact, on human data). Advanced models account for dynamic changes in intracellular ion concentrations (original work assumed these to be constant)\textsuperscript{123-126}, complicated ion channel gating kinetics\textsuperscript{127-129}, intricate spatial organization of membrane-embedded ion channels,\textsuperscript{130, 131} and intracellular signaling pathways.\textsuperscript{12, 132, 133} Furthermore, cell models have been incorporated into multi-dimensional models of cardiac tissue based on realistic myocardial geometry.\textsuperscript{134}
2.4: Current Methods and Implementation

Mathematical modeling studies are constructed similarly to the way that experimental studies are constructed: the formation of a hypothesis based on previous observations. In fact, the mathematical model can be thought of as a quantitative version of a central hypothesis. Because of this, one of the most difficult aspects of the modeling process is translation of a hypothesis into a set of mathematical equations. This is known as model formulation. During this step, the researcher will have to determine what components (e.g. pathways, reactions, reactants, etc.) to include in the model. Factors such as accuracy, availability of data, and computing resources will help determine the choices made at this step. Additionally, this step is often performed iteratively by first developing an initial model, comparing results of this model to experimental data, and then adjusting the model based on its level of agreement.

Once a model has been selected, the next step is model parameterization. During this step, model parameters (e.g. channel conductances, ion concentrations, reaction rates) will be selected based on available experimental data. This data can come from either previously published work or be original data. In some cases, a value for a model parameter may be assigned based on a direct experimental measurement. Often, it may be difficult to directly measure a parameter and instead the value must be estimated in a way that creates agreement between model and experiment with regard to an outcome that is able to be directly measured. This is known as parameter fitting. Following model parameterization, optimization may be performed. In this step, the values for parameters related to solving the governing equations (e.g. time step, grid size) may be determined.
Once model parameterization and optimization are completed, a validation step is important to evaluate accuracy of the model. In this step, model output is compared to a separate experimental dataset that was not used in the parameterization process.

As mentioned earlier, the steps outlined above will likely be repeated to arrive at a final model. Once the final model has been developed and validated, it is now ready for simulation and analysis to generate predictions regarding a process of interest. Ideally, the model prediction will lead to the design of a new experiment that may be used to test the prediction and lead to further advances in model development.

2.5: Applications

Mathematical modeling has been applied extensively to provide important insight into molecular and ionic mechanisms for both congenital and acquired disease. Essential to this effort has been work from the lab of Dr. Yoram Rudy. The Luo–Rudy dynamic model and its variants remain among the most cited cardiac action potential models and are widely used to study cardiac electrophysiology principles.\textsuperscript{135-138} Studies using these models have demonstrated the power of computational approaches in delivering new mechanistic insight into cardiac arrhythmias. An early example comes from studies that used the Luo–Rudy dynamic model to link channel defects resulting from a genetic mutation in the voltage-gated Na\textsuperscript{+} channel to lethal cardiac arrhythmias.\textsuperscript{127} Many studies following this original one have used a similar approach to examine the mechanisms responsible for a wide range of inherited arrhythmia syndromes.\textsuperscript{139}
In addition to inherited disorders, modeling has been applied to help understand mechanisms involved in the setting of acquired disease, such as myocardial ischemia and infarction, heart failure, and diabetes. For example, arrhythmia mechanisms in the setting of myocardial infarction have been studied extensively using a mathematical modeling approach. Specifically, mathematical models of the cardiac cell and tissue have been used to study the role of CaMKII in arrhythmias following myocardial infarction in the canine. Mathematical models have also been used to help determine the link between chronic CaMKII activation and sinus node dysfunction in the setting of cardiovascular disease.

While these studies have provided significant insight into the mechanisms involved in arrhythmia generation, going forward, it will be important to take into consideration the fact that cells have a complex web of regulatory networks between genes, proteins and other cellular molecules. These networks between various components in a cell control crucial processes which govern cell growth, differentiation, division and cell death. An in-depth understanding of these networks is important not only for appreciation of the underlying biology but also to develop efficient therapeutic interventions in diseased conditions. New, multi-scale models will likely play a central role in the discovery of new interactions among the existing cellular components and to predict interactions which may lead to diseases.
2.6: Future Tools and Directions

While mathematical modeling is a very useful tool for discovery, it is not readily utilized by most researchers. There are a variety of reasons as to why it is underutilized, including: 1) models are perceived as difficult to utilize, understand, or apply to a research project; 2) models are thought of as useful mostly to confirm experimental findings; 3) models are simply not thought of as a relevant or useful approach to a body of work; 4) models are time consuming to run. However, because a wide variety of research projects could greatly benefit by incorporating a mathematical model into their work, it is worth developing approaches to modeling that can be more readily used.

One approach to improve the utilization of mathematical models is through the use of machine learning. Machine learning is the construction and study of algorithms that can learn from data. More specifically, machine learning algorithms take a body of “input” and “output” data, and attempt to use that data to create an algorithm that, when fed future input data, can be used to predict an output. This differs significantly from using a set of predefined rules; the algorithm “learns” how each of the inputs maps to the desired output. Machine learning is already heavily used in image recognition, spam filtering, search engines, and a wide variety of other tasks. However, it has not yet seen widespread adoption into the biological sciences.

Applications of machine learning on biological mathematical models might have tremendous appeal. As an example, let’s say that a researcher has developed a novel mutant animal that contains a modification to a protein of interest, and has made some baseline electrical measurements on it (e.g. APD, [Ca$^{2+}$], and I$_{Na}$). They know that their
protein of interest targets a variety of intracellular targets (e.g. CaMKII) and wishes to narrow down future experiments. Normally, if a researcher wanted to utilize a mathematical model, they would have to plug their various measured values into a model, run the model, and try to create meaningful output from the results. However, if this researcher has previously developed a mathematical model (of the appropriate species) and created machine learning algorithms that mapped the behavior of that model from a wide variety of inputs (channel conductances, ion concentrations) to outputs (APD, currents), you could then use those algorithms to almost instantly create a list of likely research targets. Additionally, the algorithms can be applied to almost any other novel mutant animal where baseline work was performed, as long as the factors of interest were originally taken into account when creating the algorithm.

There are a variety of algorithms that can be used depending on the desired goal. Two of the most commonly used examples are classification and regression algorithms. Classification algorithms are designed to separate cases into 2 or more distinct categories. Regression algorithms, on the other hand, generate a continuous output. This output may be a score or ranking, but could also be used for a classification-type decision (e.g. generating a score ranging from 0-1, where 0 fall in one category, and 1 falls into another. A threshold can be set to separate the two categories).

While improving the power or accuracy of mathematical models is a great way to improve them, one of the biggest hurdles to mathematical modeling is in the resistance to their utilization. Because of this, I believe that implementing tools such as machine learning into existing mathematical models is a worthwhile use of resources.
Chapter 3: The Role of CaMKII and Related Proteins in Sinus Node Dysfunction

3.1: Background

As has been previously discussed, CaMKII is expressed throughout almost all cells in the human body. Because of its widespread expression, and the differences in function, channel expression, and electrical activity of different areas of the heart, a proper evaluation of the impact CaMKII can have on overall heart function must examine the role of CaMKII in various types of heart tissue. One of these distinct heart regions is known as the sinus node.

The sinus node, or sinoatrial node, is a specialized region of the heart located at the intersection of the right atrium and the superior vena cavae. The primary function of the sinus node is to spontaneously generate action potentials, which initiates the normal heart beat. A variety of ion channels are involved in the initiation of these action potentials, including two different types of Calcium channels: L-type (CaV1.2/1.3) and T-type (CaV3.1/3.2). Additionally, all of the previously mentioned players in excitation-contraction coupling play an important role in managing intracellular calcium levels in these cells (e.g. RYR2, PLB, and SERCA). Together, these systems ensure continuous pacemaker activity.
Activated CaMKII is able to phosphorylate multiple targets in SAN tissue, including both the L-type and T-type Ca\textsubscript{v}, and a variety of targets involved in excitation-contraction coupling, such as phospholamban (PLN) and ryanadine receptor 2 (RYR2). CaMKII-catalyzed phosphorylation increases Ca\textsuperscript{2+} entry through Ca\textsuperscript{2+} channels, increases SERCA2a uptake of cytoplasmic Ca\textsuperscript{2+} into the SR lumen through phosphorylating PLN, and increases Ca\textsuperscript{2+} release from RYR2 by phosphorylation of RYR2.\textsuperscript{110} The main impact of these changes is an increase in SAN cell action potential frequency.

3.2: Diabetes Increases Mortality after Myocardial Infarction by Oxidizing CaMKII

Background

Diabetes mellitus is a worldwide epidemic and a major public health problem that affects over 8% of the US population.\textsuperscript{149} Myocardial infarction (MI) is the most common cause of mortality in diabetic patients,\textsuperscript{150,151} and multiple studies have shown that these patients are twice as likely to die from MI compared to nondiabetic patients.\textsuperscript{152,153} However, the mechanisms as to how diabetes increases MI-related mortality in diabetic patients is unclear.\textsuperscript{154} Surprisingly, the excess mortality due to MI in diabetic patients has been shown to be independent of commonly recognized comorbid clinical conditions (e.g. extent of myocardial injury, left ventricular contractile dysfunction, coronary artery patency after reperfusion therapy).\textsuperscript{155} Improved understanding of the molecular mechanisms and pathways that promote death in diabetic patients after MI is a major goal of biomedical science.
Reactive Oxygen Species (ROS) are elevated after MI,\textsuperscript{156, 157} and increased ROS increase the disease complications of diabetes.\textsuperscript{158, 159} However, broad-spectrum antioxidant therapies have been unsuccessful in improving this situation.\textsuperscript{160} Because of this, detailed knowledge of oxidative injury mechanisms will be necessary in order to develop new and effective antioxidant therapies.

The multifunctional Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) is activated by oxidation (ox-CaMKII),\textsuperscript{161} and ox-CaMKII may increase the risk of sudden death after MI by promoting heart failure,\textsuperscript{69} cardiac rupture,\textsuperscript{162} and arrhythmias.\textsuperscript{163} Oxidation of methionines 281/282 in the CaMKII regulatory domain locks ox-CaMKII into a constitutively active conformation. The lab of Dr. Mark Anderson recently reported increased levels of ox-CaMKII in diabetic patients who had MI when compared to nondiabetic patients who had MI, suggesting that ox-CaMKII could be contributing to the increased mortality in these diabetic patients. Streptozotocin (STZ) is a pancreatic β cell toxin that induces a severe form of type I diabetes.\textsuperscript{164} The Anderson lab demonstrated that STZ-treated diabetic mice were twice as likely to die after MI surgery as vehicle-treated control mice, mimicking the increased mortality in diabetic patients compared with that in nondiabetic patients after MI. Additionally, The Anderson lab demonstrated that sinus node dysfunction (SND), either due to defects in SAN cells, autonomic activity, or both, contributes to increased mortality after MI in diabetic mice.\textsuperscript{8} SND occurs when SAN cell loss exceeds a threshold value, leading to a source-sink mismatch between SAN and surrounding atrial myocardium.\textsuperscript{163} The relatively small
number of cells in the SAN, compared with the larger number of contracting myocardial cells, makes physiological SAN function particularly vulnerable to SAN cell death.

**Results**

In order to more fully understand the contribution of diabetes, STZ, and SND to MI, we sought to compare the results of two different mathematical models: the first representing a tissue sample from a diabetic mouse, and the second representing a nondiabetic mouse. In order to quantify the contribution of SAN cell death to SND in our models, we incorporated measured values for SAN cell loss from nondiabetic and diabetic mice into a 2-dimensional computational model of SAN, based on histologically reconstructed right atrial geometry. Normal activation of the atrial myocardium (vehicle

![Mathematical model of intact sinoatrial node](image)

*Figure 7: SAN cell death and fibrosis lead to decreased conduction velocity and spontaneous beating. Published in 8.*
treated in Figure 7A) was triggered by a regular impulse that initiates in the central SAN. Our mathematical model demonstrated that increased cell loss in diabetic hearts greatly disrupted the normal capture of atrial myocardium by SAN impulses.

Specifically, the model with increased cell loss displayed a shift of the lead pacemaker site toward the SAN periphery, a delay in conduction time from the SAN to the atrial myocardium, and slowing of heart rate comparable to experimentally measured values (Figure 7). Thus, our mathematical model of the intact SAN predicts that CaMKII-induced cell loss alone can produce SND characterized by slowed heart rate. Measurements of spontaneous impulse formation in current-clamped SAN cells isolated from diabetic and vehicle-infused control mice confirmed model predictions. SAN cells from both groups showed similar spontaneous automaticity at baseline and in response to isoproterenol, suggesting that SAN cell loss in STZ-treated mice, rather than defects in the function of surviving SAN cells, was the primary cause of STZ-induced SND. Thus, computational and experimental analyses support a view that excessive SAN cell death is a fundamental event causing increased mortality after MI in the setting of diabetes.

Discussion

Elevated ROS is a consistent finding in cardiovascular diseases, but understanding of defined molecular targets and pathways that connect increased oxidation with disease phenotypes is limited. The experimental results of this study demonstrated that selective ablation of oxidative activation of CaMKIIδ was sufficient to eliminate the increased risk of sudden death in diabetic mice after MI.
In our mathematical model, SAN cell death and fibrosis lead to decreased conduction velocity and spontaneous beating, consistent with the experimental findings. As can be seen in Figure 7, we incorporated cell loss data from vehicle- and STZ-treated WT mice into a 2-dimensional histologically reconstructed mathematical model of the intact sinoatrial node. The STZ model with 19% cell loss in the SAN predicts a shift of lead pacemaker site (indicated by red asterisks on the action potential traces), slowing of conduction time, block (indicated by double red lines on action potential traces), and decreased heart rate similar to experimental measurements.

Figure 8: Mitochondrial ROS increases ox-CaMKII and SAN cell death. In the setting of hyperglycemia or myocardial infarction there are elevated amounts of mitochondrial ROS. The increased ROS increases ox-CaMKII, which leads to increased SAN apoptosis and fibrosis, and leads down a pathway to increased mortality. Modified from 8.
Conclusion

These studies identified SAN as a critical target of a novel and unanticipated oxidation-sensitive molecular pathway underlying increased mortality after MI in diabetic mice (Figure 8). This ox-CaMKII pathway may also increase mortality risk in diabetic patients, who show more severe heart rate defects consistent with SND and higher right atrial ox-CaMKII than nondiabetic patients after MI. Because multiple tissues are injured in diabetes, it will be important to learn if ox-CaMKII contributes to progression of diverse forms of diabetic injury, such as diabetic vasculopathy and neuropathy. It is intriguing that CaMKII inhibition was recently found to improve insulin sensitivity in diabetes, suggesting that CaMKII inhibition could benefit diabetic patients by multiple mechanisms and pathways.

3.3: Atrial Fibrillation and Sinus Node Dysfunction in Human Ankyrin-B Syndrome: a Computational Analysis

Background

Tight spatial and temporal control of both intra- and inter-cellular signaling is essential for normal heart function. When this control is disrupted, such as from defects in adapter/cytoskeletal proteins involved in control of local CaMKII signaling domains, arrhythmias and cardiac dysfunction may arise. One such example comes from the adapter protein ankyrin-B. Ankyrins are a family of multifunctional polypeptides
(ankyrin- R, ankyrin-G, and ankyrin-B are expressed in heart) required for proper membrane localization of ion channels, transporters, and signaling molecules in both excitable and nonexcitable cells.\textsuperscript{167} Importantly, ankyrin dysfunction has been closely linked to human disease. In heart, defects in both ankyrin-G- and ankyrin-B-dependent pathways have been associated with increased susceptibility to cardiac arrhythmia. In particular, loss of ankyrin-B function has been linked to both congenital and acquired arrhythmias.\textsuperscript{168-174} Several mutations in ANK2 (the gene that encodes ankyrin-B) have been identified as the cause of a complex cardiac phenotype in humans characterized by prolongation of the QT interval on the electrocardiogram and increased susceptibility to ventricular arrhythmias and sudden death.\textsuperscript{174-176} Initially described as long-QT type 4 based on the electrocardiographic and electrophysiology findings, the disease has more recently been reclassified as “ankyrin-B syndrome” because of the presence of additional supraventricular phenotypes not found in classic long-QT syndrome. Specifically, in addition to ventricular tachyarrhythmias, human patients with ankyrin-B syndrome show highly penetrant sinus node dysfunction coupled with increased susceptibility to spontaneous and inducible atrial fibrillation. Thus an important unanswered question is: how does loss of ankyrin-B give rise to such a broad spectrum of distinct arrhythmias?

Recent findings from our group and others have provided important clues into the molecular and cellular mechanisms for arrhythmia in the setting of ankyrin-B dysfunction. In ventricular myocytes, increased susceptibility to proarrhythmogenic calcium-dependent afterdepolarizations has been observed in conjunction with the loss of membrane localization of Na/K ATPase (NKA) and Na/Ca\textsuperscript{2+} exchanger (NCX) and
associated imbalance in intracellular ion homeostasis. In atrial and sinoatrial node cells, L-type Ca\(^{2+}\) current is uniquely affected because of loss of membrane targeting of Ca\(_v1.3\) (which is not expressed in ventricular tissue). In addition, ankyrin-B-deficient (ankyrin-B\(^{+/\text{-}}\)) mice show a decrease in atrial action potential (AP) duration (APD) together with slow and variable SAN cell spontaneous firing rate. Relevant for CaMKII, a recent study has demonstrated that ankyrin-B deficiency results in hyperphosphorylation of the ryanodine receptor via protein phosphatase 2A (PP2A), a protein phosphorylated by CaMKII. Additionally, utilizing AC3I mice, which overexpress a potent CaMKII inhibitory peptide, was sufficient to rescue these animals and prevent arrhythmias. Therefore, suppression of CaMKII activity may be a potential therapeutic target for patients suffering from ankyrin-B deficiency. While these studies provide important insight into the underlying cellular defects of ankyrin-B deficiency, the link between specific cell/tissue changes and arrhythmogenesis remains unclear.

Mathematical modeling of excitable cells has been applied extensively to analyze the molecular and cellular basis of human disease. In previous work, a computational approach was used to analyze the substrate for stress-induced ventricular arrhythmias in ankyrin-B syndrome. Wolf et al. found that loss of NCX and NKA membrane targeting resulted in calcium overload under stress conditions, promoting an inappropriate release of calcium from the sarcoplasmic reticulum and afterdepolarizations. Here, we take a similar approach to define the substrate for arrhythmias across the full spectrum of arrhythmias observed clinically in ankyrin-B syndrome. Detailed methods are located in Appendix A.
Results

*Increased fibrosis in ankyrin-B\(^{+/−}\) atria* - Ankyrin-B dysfunction alters membrane expression of select ion channels, pumps, and exchangers that may regulate the substrate for atrial fibrillation and/or sinus node dysfunction.\(^{168}\) Previous studies have defined the atrial and SAN cellular phenotype associated with ankyrin-B dysfunction.\(^{168,172}\) However, changes in atrial structure and/or dimension have been shown to increase susceptibility to arrhythmia.\(^{182,183}\) Therefore, we first evaluated whether loss of ankyrin-B function altered gross atrial structure through histological staining of normal and ankyrin-B\(^{+/−}\) mouse atrial sections. Interestingly, we found almost a threefold increase in the amount of fibrosis present in atria from ankyrin-B\(^{+/−}\) animals compared with age- and sex-matched WT mouse atria (Figure 9). These data indicate that in addition to cellular level changes in ion channel membrane targeting, ankyrin-B dysfunction results in structural remodeling that may influence the substrate for arrhythmias.
Altered cellular dynamics in ankyrin-B\(^{+/−}\) atrial cell - Altered cellular dynamics have been linked to increased susceptibility to formation of wave breaks and cardiac fibrillation.\(^{184}\) Therefore, we next wanted to determine whether ankyrin-B dysfunction alters the dynamic response of the atrial cell AP to pacing. Mathematical models of ankyrin-B\(^{+/−}\) and WT cells were paced to steady state over a CL range from 2,000 to 300 ms (Figure 10). Additionally, we determined the APD frequency response to isolated defects in NCX, NKA, and Ca\(_{\text{v}}\)1.3. To verify that findings were not model-dependent, simulations were performed using two different models of the human atrial AP: one from Courtemanche, Ramirez, and Nattel\(^{11}\) that was among the first developed (referred to as “CRN”) and a recent model from Grandi and colleagues (referred to as “Grandi”)\(^{185}\) that includes a more advanced formulation for Ca\(^{2+}\) handling (Figure 10). Importantly, the

![Figure 9: Increased fibrosis in ankyrin-B\(^{+/−}\) atria.](image)

(A) Masson trichrome staining of atrial sections from wild-type (WT) and ankyrin-B\(^{+/−}\) mice. Blue staining indicates collagen.

(B) Summary data on fibrosis as percentage of total tissue area in WT and ankyrin-B\(^{+/−}\) atrial sections. \(*P < 0.05, n = 5\). Scale bar =50 μm. Published in \(^{9}\).
CRN and Grandi models predicted a similar decrease in APD with ankyrin-B deficiency that agrees with experimental values (Figure 10). While data on maximum action potential upstroke velocity (dV/dt\(_{\text{max}}\)) in ankyrin-B-deficient cells are not available, studies in isolated human atrial myocytes show a value of 203 ± 11 ms with no difference between sinus rhythm and atrial fibrillation patients. For comparison, at the same CL (800 ms) we observe a dV/dt\(_{\text{max}}\) of 205 ms in sinus rhythm (for CRN) and 198 ms in ankyrin-B\(^{+/−}\) cells displayed a reduced capacity for APD adaptation with rate: APD decreases by 74 ms in CRN ankyrin- B\(^{+/−}\), as CL decreases from 2,000 to 400 ms, compared with 123 ms in WT, with a similar difference observed in the Grandi model (Figure 10). Loss of Ca\(_{\text{v}}\)1.3 is the primary determinant of altered APD adaption in both the CRN and Grandi models as Ca\(_{\text{v}}\)1.3 deficiency shows decreased APD at all CLs, similar to the ankyrin-B\(^{+/−}\) cell (Figure 10, A and B). Interestingly, loss of NCX had a greater impact on APD adaptation in the CRN model than in the Grandi model. Regardless of these minor differences between the models, in both models, the role of NCX was secondary to Ca\(_{\text{v}}\)1.3 with little to no role for NKA.
We also determined APD in response to a premature stimulus applied at a variable diastolic interval following steadystate pacing (APD restitution). The maximal slope of the restitution curve is shallower in the ankyrin-B\(^{+/−}\) cell compared with WT, indicating a decreased susceptibility to proarrhythmogenic AP alternans (Figure 11). While the shallow restitution curve in the ankyrin-B\(^{+/−}\) CRN model depended almost exclusively on loss of Ca\(_{\text{v}1.3}\), the Grandi model showed a more balanced contribution from Ca\(_{\text{v}1.3}\), NCX, and NKA. Thus these simulations demonstrate that loss of Ca\(_{\text{v}1.3}\) (and to a lesser extent NCX) contributes to abnormal APD adaptation and restitution in ankyrin-B\(^{+/−}\) atrial myocytes. While we expected APD shortening observed in ankyrin-B\(^{+/−}\) cells is proarrhythmic, the observed changes to restitution are likely antiarrhythmic,

Figure 10: Simulated action potentials (AP) in mathematical model of ankyrin-B\(^{+/−}\) (AnkB) atrial myocyte. 2 different models of the human atrial AP were used to simulate atrial APs from control (WT, black) and ankyrin-B\(^{+/−}\) (red) myocytes during slow [cycle length (CL) = 2,000 ms] and rapid (CL = 400 ms) pacing. Published in \(^9\).
as steep restitution has been linked to increased susceptibility to complex AP dynamics and fibrillation.  

**Two-dimensional model of atrial tissue** - Our experimental and computational results show complex electrical and structural remodeling in ankyrin-B<sup>+/-</sup> hearts. Based on these findings, we hypothesized that increased susceptibility to atrial fibrillation in the setting of ankyrin-B dysfunction is due to the presence of multiple defects at the cell and tissue level. Specifically, we hypothesized that decreased APD and slowed conduction...
(due to fibrosis) dramatically decrease ankyrin-B<sup>−/−</sup> AP wavelength. The AP wavelength is used as a measure of the amount of AP propagation (estimated as the product of conduction velocity and APD). To evaluate the role of cell and tissue level changes in atrial arrhythmias, we used a two-dimensional model of atrial tissue in which human WT or ankyrin<sup>−/−</sup> cell models (based on CRN model) are coupled together via gap junctions in a two-dimensional grid (Figure 12). Fibrosis was introduced by randomly dispersing inexcitable cells. Conduction velocities in longitudinal and transverse directions were determined using plane wave stimuli.
Reentry was then initiated using a cross-field stimulation protocol. The size of the two-dimensional grid was systematically reduced (while retaining an aspect ratio of 2.5) until it could no longer support a single reentry cycle. Duration of reentrant activation, average CL, and activation maps were determined at each grid size and compared for the WT and ankyrin-B+/− model with changes at both the cell and tissue level (3 and 9% fibrosis in WT and ankyrin-B+/−, respectively) (Figure 12). Reentry Figure 12: Increased susceptibility to sustained reentry in ankyrin-B+/− atrial tissue. Simulated duration of reentry as a function of tissue area in heterogeneous grids comprised of WT and ankyrin-B+/− cells [based on CRN model (7)] interspersed with poorly coupled electrically inexcitable cells (fibroblasts) at levels corresponding to experimental measurements (3% and 9% for WT and ankyrin-B+/−, respectively) (A-D) Duration of reentrant activation as a function of tissue area in WT and ankyrin-B+/− models with varying degrees of fibrosis. (E-H) Activation maps during one cycle of reentry for the ankyrin-B+/− and WT models with varying degrees of fibrosis. (I and J) Simulated APs from a single cell in WT and ankyrin-B+/− tissues. Ankyrin-B+/− tissue sustains longer periods of reentry at smaller tissue sizes compared to WT, due primarily to electrical remodeling with a secondary contribution from structural remodeling. Published in 9.
dynamics displayed a complex pattern in the ankyrin-B+/− model relative to WT. Specifically, duration of reentry tended to be shorter for Ankyrin-B+/− than for WT in larger tissue sizes (> 10 cm²), but longer in smaller tissue sizes (< 10 cm²). Importantly, while the WT model could not support more than one cycle of reentry in grids smaller than 9.6 cm², the ankyrin-B+/− model supported at least one cycle of reentry in a grid as small as 6 cm². Activation maps during one cycle of reentry reveal a shorter functional line of conduction block and earlier activation times in the ankyrin-B+/− model than in the WT model (Figure 12, E and F). Thus, unexpectedly, ankyrin-B dysfunction increases the likelihood of conduction block at larger tissue sizes but decreases the critical tissue mass that can support reentry.

To determine the relative contribution of cell and tissue level remodeling to the altered reentry dynamics observed in ankyrin-B+/− tissue, we next compared reentrant activation in homogeneous WT and ankyrin-B+/− grids (lacking fibrosis) (Figure 12B). Eliminating fibrosis eliminated the complex relationship between duration of reentry and grid size, suggesting that fibrosis promotes the complex reentry dynamics seen in ankyrin-B+/− tissue. Importantly, loss of ion channel membrane targeting alone is sufficient to produce a dramatic decrease in activation CL (compare values for 0% fibrosis in Figure 13A) and critical mass. Loss of Ca,1.3 is the primary determinant of reduced critical mass in ankyrin-B+/− tissue (Figure 12B), consistent with our findings that Ca,1.3-deficiency is largely responsible for decreased APD in the ankyrin-B+/− atrial cell (Figure 10).
To further clarify the role of fibrosis, we compared activation and reentry dynamics in ankyrin-B\textsuperscript{+/−} tissue with 0 (homogeneous), 3 (similar to WT), and 9% fibrosis (Figure 12). As the degree of fibrosis increases, the relationship between reentry duration and tissue size also became increasingly more complex. More specifically, there was an increased likelihood of block at larger tissue sizes but decreased likelihood of block at smaller sizes. The net result is a leftward (smaller) shift in the critical mass to support reentry with increasing fibrosis. Increasing fibrosis decreased conduction velocity to a similar degree in WT and ankyrin-B\textsuperscript{+/−} tissue (Figure 13B). We also examined the difference between activation and reentry dynamics in the ankyrin-B\textsuperscript{+/−} tissue with a diffuse or “stringy” pattern of fibrosis, where clusters of up to five cells were created (Figure 12D). We observed a similar effect of fibrosis on conduction velocity and reentry dynamics independent of fibrosis pattern. Thus structural remodeling in ankyrin-B\textsuperscript{+/−} contributes to the decrease in critical tissue mass to support reentry by decreasing conduction velocity. Fibrosis also introduces complex reentry dynamics by increasing the likelihood that the reentrant wave front will terminate by

Figure 13: Cycle Length and Conduction Velocity of WT and AnkB\textsuperscript{+/−} simulated tissue. (A): average CL during 2s of sustained reentry in WT and ankyrin-B\textsuperscript{+/−} grids with varying levels of fibrosis (0, 3, and 9%). (B) Longitudinal and (C) transverse conduction velocity (CV) of a plane wave propagating in WT and ankyrin-B\textsuperscript{+/−} grids with varying levels of fibrosis. Published in \textsuperscript{9}. 

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encountering an inexcitable barrier (fibrotic cluster or tissue edge). In summary, our simulations suggest that loss of ankyrin-B function decreases the critical tissue mass for sustained reentry through cellular changes in ion channel membrane expression (decreased APD) with a secondary contribution from altered tissue structure (decreased conduction velocity). Altered tissue structure also introduces complex reentry dynamics by increasing the likelihood for wave break.

**Mathematical model of SAN pacemaking** - Ankyrin-B dysfunction is associated with severe and highly penetrant SAN dysfunction in addition to atrial and ventricular arrhythmias. Similarly, ankyrin-B<sup>−/−</sup> mice show bradycardia coupled with increased heart rate variability. At the cellular level, loss of Ca<sub>v</sub>1.3 (normally highly expressed in SAN), NCX, and NKA is observed in SAN cells from ankyrin-B<sup>−/−</sup> mice. To determine the mechanism for SAN dysfunction in ankyrin-B syndrome, we first incorporated measured loss of Ca<sub>v</sub>1.3, NCX, and NKA into a recently published model of the murine SAN cell. Importantly, this model includes separate formulations for Ca<sub>v</sub>1.2 and Ca<sub>v</sub>1.3 channel populations and accurately simulates normal murine AP and calcium cycling.
In contrast to the WT SAN cell, which showed regular and rapid spontaneous activation, the ankyrin-B<sup>+/−</sup> SAN cell displayed highly irregular activity (Figure 14). Fourier analysis was performed to quantify observed changes in activation rate and pattern. Whereas the WT SAN cell shows a single peak at 4.7 Hz in agreement with experimental measurements from isolated SAN cells (4.8 ± 0.5 Hz<sup>172</sup>), the ankyrin-B<sup>+/−</sup> SAN cell shows multiple peaks at lower frequencies as well as a broadening of peaks.
reflecting an overall slowing of spontaneous activation coupled with increased variability (Figure 14), consistent with findings in the ankyrin-B\textsuperscript{+/-} mouse and human patients.\textsuperscript{172}

To determine the mechanism for SAN dysfunction in ankyrin-B\textsuperscript{+/-}, we next evaluated spontaneous activity under four different conditions: 1) loss of Ca\textsubscript{1.3} alone, 2) loss of NCX alone, 3) loss of NKA alone, and 4) coupled loss of NCX and NKA. Ca\textsubscript{1.3} loss was sufficient to produce a slowing of the spontaneous firing rate without having an impact on variability (single peak at 4.2 Hz). In comparison, loss of NCX or NKA together produced a slight increase in firing rate (Figure 14B). To investigate in detail the sensitivity of SAN pacemaking to changes in expression of Ca\textsubscript{1.3}, NCX, and NKA, we analyzed changes in SAN CL as we simultaneously varied Ca\textsubscript{1.3} and NKA expression or Ca\textsubscript{1.3} and NCX expression (Figure 15). While, in general, CL was most sensitive to changes in Ca\textsubscript{1.3}, a 30–40% reduction in NKA (depending on Ca\textsubscript{1.3} expression) induced a transition from regular periodic firing to a more irregular pattern (“chaotic”) characteristic of ankyrin-B\textsuperscript{+/-} (Figure 15A). A similar transition was observed as NCX was varied but only after a larger degree of loss (50–60% reduction, Figure 15B). Thus, whereas loss of Ca\textsubscript{1.3} is responsible for slowing of firing rate, loss of NKA (and to a lesser degree, NCX) provides a mechanism for the increase in variability observed in ankyrin-B\textsuperscript{+/-} SAN cells. The mechanism behind these complicated dynamics is discussed in greater detail in section 3.4.
Figure 15: Ionic mechanism for altered automaticity in ankyrin-B+/- sinoatrial node cell. Parametric analysis performed by simultaneously varying (A) maximal conductance of Ca$_{v}$1.3 current and maximal NKA pump rate or (B) maximal conductance of Ca$_{v}$1.3 current and maximal NCX scaling factor. Each parameter was varied from 0.3 to 1 times its control value in increments of 0.02 to produce 1,225 simulations for each panel. Cycle length following 200 seconds of spontaneous activity was plotted for each simulation. Three discrete patterns of activity were observed: Regular periodic, irregular “chaotic”, and failure. While cycle length was most sensitive to changes in Ca$_{v}$1.3, a 30-40% reduction in NKA induced a transition from regular firing to a “chaotic” pattern characteristic of ankyrin-B+/-.

Mathematical model of intact SAN - Regular SAN activity depends on the intricate structure of the distributed pacemaker complex in the context of the intact RA. Our experimental data indicate that ankyrin-B dysfunction results in both electrical and
structural remodeling of the RA (see Figure 9). To determine the interplay between cellular and tissue level changes on SAN dysfunction in ankyrin-B^{+/-} hearts, we used a two-dimensional physiological model of the intact SAN (Figure 16).\textsuperscript{142, 187, 188} The WT model displayed regular spontaneous activation that initiated from a point in the central node and spread through the peripheral node and into the surrounding atrial tissue with a conduction velocity that agrees with experimental measurements (Figure 16, B and D).\textsuperscript{187}

Interestingly, the ankyrin- B^{+/-} node showed a shift in the primary activation site toward the periphery as the system approached steady state (activation failure in central SAN region is indicated by the white area in Figure 16C). Furthermore, exit block occurred after only one beat, and eventually spontaneous activity terminated altogether at steady state (Figure 16C). While these simulations do not rule out the possibility that in three dimensions, pacemaking would be sustained at an alternative site, they do predict a dramatic shift in location of the primary pacemaking site consistent with experimental measurements in intact ankyrin-B^{+/-} SAN.\textsuperscript{189}
Figure 16: Sinus node dysfunction in model of ankyrin-B-/- intact sinus node. A: mathematical model of intact sinoatrial node (SAN) based on realistic geometry with cell types determined by immunohistochemistry (3, 13, 42). WT (B) and ankyrin-B-/- (C) activation maps corresponding to one complete cycle (top) and spontaneous APs (bottom) recorded from SAN (black lines) and right atrial free wall (red lines). For ankyrin-B-/- spontaneous SAN APs are shown for 2 sites (black and gray lines, locations indicated by asterisks) on either side of central block region (white area denoted by arrow). D: simulated and measured (3) conduction velocity from SAN into right atrium (RA). Ankyrin-B dysfunction results in shift of primary pacemaker site toward periphery (central SAN region fails to activate), sinus exit block, and ultimately sinoatrial node failure. E and F: comparison of activation of RA free wall and septum in WT and ankyrin-B-/- for different degrees of fibrosis and different values of cell-to-cell coupling. RA activation properties characterized by number of free wall activations (G), CL of RA activation (H), and latency to first activation (I) in WT, ankyrin-B-/-, Ca,1.3-deficient, NKA-deficient, NCX-deficient, and WT with 9% fibrosis. Rg, gap junction resistance. Published in 9.
To determine the mechanism for SAN dysfunction in the ankyrin-B/model, we evaluated sensitivity of RA activation to each component of ankyrin-B-deficiency in the model (Figure 16, G–I). Consistent with findings in the single SAN cell, loss of Ca,1.3 had the greatest impact on RA activation as determined by the number of successful beats (Figure 12G) and CL of RA activation (Figure 12H). Interestingly, whereas loss of NCX or NKA had little impact on RA activation, increasing the degree of fibrosis dramatically increased RA CL and latency likely because of increased loading of surviving SAN cells by surrounding atrial cells. Finally, we determined the effect of uniform uncoupling on SAN function in the ankyrin-B+/− model (Figure 16, E and F). Interestingly, in contrast to fibrosis that promotes SAN dysfunction, a moderate level of uniform uncoupling had protective effects on SAN function.

Discussion

Our studies identified ionic mechanisms underlying sustained atrial fibrillation and SAN dysfunction in cardiac disease. Specifically, we found that loss of Ca,1.3 leads to AP shortening which reduces the AP spatial wavelength and the critical tissue size needed to sustain reentry. In parallel, increased fibrosis also reduces spatial wavelength, but does so by slowing conduction. In the SAN, loss of Ca,1.3 contributes to a general slowing of activation, whereas defects in NKA, and NCX to a lesser degree, underlie increased variability. Simulations of pacemaking in the intact SAN reveal a shift in primary pacemaking site, episodes of SAN exit block, and failure in ankyrin-B+/−. In previous computational studies, we have shown that ventricular arrhythmias associated
with ankyrin-B deficiency are likely the cause of \( \text{Ca}^{2+} \) overload secondary to defects in NCX and NKA membrane targeting.\(^{179}\) In contrast, results from the current study predict that loss of \( \text{Ca}_{\text{v}}1.3 \) in ankyrin-B\(^{+/−}\) atrial and SAN cells compensates for loss of NCX and NKA, resulting in very little net change in intracellular \( \text{Ca}^{2+} \) cycling. Instead, defects in repolarization and/or spontaneous depolarization lead to distinct arrhythmia phenotypes in these specific cardiac regions.

Loss-of-function ankyrin-B defects have been observed in both congenital (ankyrin-B syndrome) and acquired disease.\(^{169-171, 174, 176}\) In the setting of ankyrin-B syndrome, it is clear that ankyrin-B dysfunction is responsible for changes in membrane expression of ion channels leading ultimately to increased susceptibility to arrhythmias. Although the situation in acquired disease is undoubtedly more complex, it is interesting to consider the possibility that ankyrin-B dysfunction underlies electrical and structural remodeling known to precipitate arrhythmias in heart disease (including heart failure). An important question going forward is: what leads to ankyrin-B defects in acquired disease?

One recent study that helps address this question has demonstrated a link between CaMKII and ankyrin-B deficiency. In this study, DeGrande et al. demonstrated that ankyrin-B deficiency results in hyperphosphorylation of the ryanodine receptor via PP2A.\(^{180}\) Previous studies have shown that PP2A is a phosphorylation target of CaMKII.\(^{190}\) Additionally, overexpression of AC3I, a potent CaMKII inhibitory peptide,\(^{181}\) was sufficient to rescue these animals and prevent arrhythmias. This strongly
suggests that CaMKII is providing a significant contribution to the disease-state associated with ankyrin-B dysfunction.

Our results indicate that loss of ankyrin-B regulates the arrhythmia substrate at the cellular level by altering membrane expression and at the tissue level by inducing fibrosis. We also predict that fibrosis has a very different impact on SAN function than uniform uncoupling. Specifically, whereas fibrosis compromises SAN activity, moderate uniform uncoupling actually preserves function. These findings are consistent with recent computational studies showing a similar relationship between electrical remodeling, structural remodeling, and AP wavelength.\textsuperscript{182}

Our findings also agree with previous work showing that fibrosis slows conduction and produces fragmented wave fronts conducive to multiple reentry circuits.\textsuperscript{191-193} An important limitation to consider when interpreting these results is that the exact degree of coupling between fibroblasts and myocytes \textit{in vivo} is unknown. Here, we assume poor coupling based on studies showing sparse gap junction formation between fibroblasts and myocytes \textit{in vivo}.\textsuperscript{182,194} Whether this coupling changes in disease or in different regions of the heart remains to be determined. Furthermore, while the molecular pathway for ankyrin-B-dependent regulation of ion channel membrane expression has been well characterized, the pathway linking ankyrin-B dysfunction to changes in fibrosis is unknown. However, fibrosis is a common finding in atrial fibrillation and has been shown to be regulated at least in part by reactive oxygen species-dependent activation of matrix metalloproteinases.\textsuperscript{195} Interestingly, calpain activation has also been identified as a potential inducer of fibrosis through TGF-Beta activation.\textsuperscript{196}
will be important for future studies to determine whether ankyrin-B dysfunction induces changes in tissue structure through similar or distinct pathways. It is also important to note that fibrosis measurements in this study come from age-matched WT and ankyrin-B\(^{+/-}\) mice. We anticipate that fibrosis in ankyrin-B-deficient atria will vary with age and species.

Our findings add to mounting evidence that a common mechanism may give rise to atrial arrhythmias and SAN dysfunction.\(^{197, 198}\) Fibrosis helps create the substrate for sustained reentry in the atria by slowing conduction while simultaneously contributing to SAN dysfunction (slowed activation, intermittent exit block) by altering the source-sink relationship between SAN and surrounding atrial tissue. At the same time, loss of Ca\(_v\)1.3 reduces the duration of atrial APs (favorable for sustained reentry) while slowing spontaneous SAN cell activation. Thus ankyrin-B dysfunction involves changes at both the cell and tissue level that favor the common manifestation of atrial arrhythmias and sinus node dysfunction. Interestingly, severe bradycardia has been reported in the Ca\(_v\)1.3 null (alpha1D\(^{-}\)) mouse.\(^{199}\) While spontaneous atrial tachyarrhythmias have not been observed, it will be important to determine if these animals are susceptible to induced arrhythmias. However, it is interesting to consider the possibility that atrial arrhythmias are more common in ankyrin-B\(^{+/-}\) due to the presence of multiple proarrhythmic defects at the cell and tissue level (e.g., loss of multiple ion channels combined with increased fibrosis).
Limitations

While our mathematical model of the ankyrin- B\textsuperscript{+/−} cardiomyocyte accounts for many known ankyrin-B targets, it has important limitations based on the available experimental data. Although the model accounts for the loss of NCX, NKA, and Ca\textsubscript{v}1.3, it does not consider the loss of the inositol 1,4,5-trisphosphate receptor in atrial cardiomyocytes. Furthermore, it is likely that dysregulation of kinase/phosphatase balance secondary to loss of protein phosphatase 2a targeting will play an important role in regulating the arrhythmia substrate and/or trigger\textsuperscript{180}. Our model will provide the framework into which new data on the function of these proteins in atrial cardiomyocytes may be incorporated. It is also important to note that these studies do not account for the detailed physiology of the three-dimensional atrium.

Nevertheless, there is good agreement between the fundamental behavior in our study and that observed using higher dimensional models\textsuperscript{182}. Finally, while simulation results are considerably robust across models, important distinctions exist, particularly with respect to AP restitution (Figure 10), that may have implications for behavior at the tissue level.
3.4: Cycle Length Restitution in Sinoatrial Node Cells: A Theory for Understanding Spontaneous Action Potential Dynamics

Background

As previously discussed, we observed an increase in complicated SAN cell firing in Ankyrin-B-deficient cells. Because of the described relationship between CaMKII, PP2A, and ankyrin-B, CaMKII phosphorylation levels may affect SAN dynamics. To help answer this question and understand the mechanism involved in the creation of these dynamics, we developed a theory to explain how the cycle length of sinoatrial node cells can be impacted by dysfunction of a variety of different channels.

Cardiac pacemaking depends on the tight temporal and spatial synchronization of a specialized population of spontaneously active cells in the sinoatrial node (SAN). Importantly, SAN dysfunction, characterized by asynchronous, irregular pacemaker activity and/or failure is linked to cardiovascular disease and associated with increased mortality in heart failure.\(^{200-202}\) Additionally, SAN disease is common in the aging population.\(^{197}\) While great progress has been made in understanding the role of specific ion channels, Ca\(^{2+}\) handling proteins, and regulatory molecules in controlling spontaneous SAN cell activity,\(^{197, 203-205}\) important questions remain about the nature of SAN dysfunction at the cell and tissue level. At the same time, unlike other cardiac cell types (e.g. atrial and ventricular myocytes), the method for SAN cell dynamic regulation is not known.
Previous work has defined a restitution relationship that can be used to describe myocyte action potential (AP) dynamics in response to pacing.\textsuperscript{184, 206-208} The restitution hypothesis states that AP duration (APD) depends on the duration of the preceding diastolic interval (DI), with a shorter DI resulting in shorter APD due to decreased recovery time for ion channel gates. The APD restitution curve is determined by pacing the cell to steady state, applying a premature stimulus with a progressively shorter coupling interval, and plotting the AP duration as a function of the preceding DI. A steep restitution curve (slope greater than or equal to one) is associated with increased susceptibility to beat-to-beat alternations in APD (APD alternans). APD alternans, in turn, are thought to be arrhythmogenic by introducing spatial gradients in APD that promote unidirectional conduction block and initiation of reentry.\textsuperscript{184}

In this study, we hypothesized that irregular SAN cell spontaneous activity reflects an inherent restitution property similar to that responsible for AP dynamics in paced atrial and ventricular myocytes. Here, we define a method for assessing CL restitution in SAN cells and demonstrate the utility of CL restitution in predicting SAN cell dynamics (e.g., the emergence of irregular spontaneous activity). We perform detailed analysis of ionic and structural factors that influence CL restitution in mathematical models of the SAN cell and tissue. Finally, we identify a link between CL restitution curve morphology and SAN behavior at the cell and tissue level. We anticipate that CL restitution will be a useful tool that may be implemented in both experimental and mathematical studies to predict susceptibility to dynamical instability and SAN dysfunction.
Results

Previous experimental and computational studies have identified distinct modes of termination in SAN cells in response to perturbation (e.g. decreased $[\text{Na}^+]_o$, L-type Ca$^{2+}$ channel block).$^{4-6, 209, 210}$ Similarly, our previous computational and experimental studies have demonstrated the emergence of complex irregular activity preceding termination of spontaneous activity in ankyrin-B-deficient mouse SAN cells.$^9, 172$ Furthermore, we demonstrated that irregular activity at the single cell level translated to a shift of the primary pacemaker site and even failure in a detailed two-dimensional 6 model of the intact sinoatrial node.$^9$ Based on these findings, we wanted to determine the mechanisms underlying emergence of irregular activity and termination of spontaneous activity in SAN cells. We first examined the response of SAN spontaneous activity to two perturbations previously shown to elicit very different dynamic behaviors: 1) decreased extracellular sodium concentration ($[\text{Na}^+]_o$)$^4, 6$, and 2) block of the rapid delayed rectifier K$^+$ current $I_{\text{Kr}}$ (Figure 17)$^4, 5$. Consistent with previous reports,$^4, 6$ we observed the emergence of irregular activity and skipped beats preceding termination of spontaneous activity as $[\text{Na}^+]_o$ was decreased in a stepwise fashion from 70 to 58 mM. In contrast, spontaneous action potentials gradually declined in amplitude until activity terminated as the maximal conductance of $I_{\text{Kr}}$ ($g_{\text{Kr}}$) was decreased from 0.37 to 0.31 times its control value (Figure 17). While previous studies have applied tools from nonlinear dynamics (e.g. bifurcation diagrams, Poincare plots) to describe these behaviors, the
underlying mechanism remains unclear. Furthermore, the field lacks an effective method for efficiently determining the inherent dynamical stability of a given cell.

We hypothesized that SAN cells possess an inherent CL restitution property that may be assessed using the newly defined perturbation protocol (described in Methods). Furthermore, we predicted that the slope of the CL restitution curve might provide important predictive information regarding the likely mode of termination of spontaneous activity (irregular activity vs. gradual decline) in response to perturbation. To test this
hypothesis, we determined CL restitution curves for the baseline model (control) and for two different conditions: low $[\text{Na}^+]_o$ and $I_{Kr}$ block, which promote termination through irregular activity and gradual decline, respectively (Figure 18). We specifically chose a value for $[\text{Na}^+]_o$ (100 mM) that was well above the threshold for irregular activity (threshold ~68 mM, see Figure 17) to test whether the restitution curve could be used as a predictor of susceptibility to irregular activity and mode of termination. Consistent with our hypothesis, the restitution curve was uniformly flat (maximal slope $> -1$) for control conditions and in the presence of $I_{Kr}$ block. In contrast, low $[\text{Na}^+]_o$ produced a curve with an abrupt transition from a relatively flat region to a very steep region (maximal slope $<< -1$, indicated by arrow on red curve in Figure 18). These simulations indicate that the CL restitution curve provides important information regarding the inherent dynamical stability of the cell and, potentially, the mode of termination.
To determine the robustness of the CL restitution curve as a predictor of the mode of termination, we performed a detailed parametric analysis in the model (Figure 19). Select parameters were incrementally changed from their control values until spontaneous activity was terminated or the parameter value was zero. Steady-state (200 seconds) spontaneous activation was determined for each parameter set. Resulting termination modes were compared to experimental reports in the literature, where available. In general, we found good agreement between model and experiment (agreement in 5 of 7 instances where comparison was possible), with irregular activity being the more common mode, accounting for 75% (6 of 8 cases) and 71% (5 of 7 cases) of termination in model and experiment, respectively (Figure 19A).

Figure 18: The cycle length restitution curve. (A–B) Simulated spontaneous action potentials during a protocol to determine the cycle length restitution curve. A 10-ms stimulus is applied with varying amplitude at the maximum diastolic potential to accelerate or delay the subsequent spontaneous action potential. Time between 2nd and 1st APs following perturbation (CL2) is then plotted as a function of time between 1st perturbed and steady-state APs (CL1). (C) CL restitution curves for control (black), low [Na\(^+\)]\(e\) (red) and I\(_{\text{Kr}}\) block (gray). Note that low [Na\(^+\)]\(e\) results in a curve with an abrupt transition from a relatively flat region to a very steep region (maximal slope >>−1 indicated by arrow; dashed line has slope of −1 for reference). Published in \(^{14}\).

Figure 19: The cycle length restitution curve.
We next used the model to determine the maximal slope of the CL restitution curve for each case, using a parameter value just above the threshold for termination (in the case of gradual decline to termination) or onset of irregular activity (Figure 19B). In every instance, parameters that promote irregular activity while progressing toward termination also produce steep CL restitution curve (maximal slope < -1, Figure 19). Conversely, parameters that fail to terminate (even at value of zero) or promote termination through gradual decline result in relatively flat return maps (maximal slope > -1).

Figure 19: Parametric analysis of termination modes and restitution curve slope. (A) Unless otherwise indicated, each model parameter was decreased stepwise from its control value until spontaneous activity terminated or the value reached zero. For ionic currents (e.g. I_{Kr}), the affected parameter was the maximum channel conductance. For the Na⁺/Ca²⁺ exchanger (NCX) and Na⁺/K⁺ ATPase (NKA), affected parameters were scaling factor (k_{NaCa}) and maximum current, respectively. Altogether data were collected from 500 independent simulations. Experimental data is provided for comparison where available (compiled from 4-6). (B) Maximal CL restitution curve slope in the model for each parameter just before onset of irregular activity, termination, or value reaches zero. Color of bar corresponds to mode of termination identified in A. Note that there is good agreement between parameters that produce skipped beats and result in steep restitution (indicated by green in A and B). Dashed line corresponds to a slope of −1. Published in 14.
It is also interesting to note that, in general, loss of repolarizing current tends toward shallow restitution and gradual decline of activity (e.g. $I_{Kr}$ block, elevated $[K^+]_o$) while loss of depolarizing current/gain of repolarizing current tends toward steep restitution and skipped beats/irregular activity (e.g. Cav1.3 block, NCX block, decrease in $[Na^+]_o$). These results support our central hypothesis that the slope of the CL restitution curve may be used as a predictor of likely termination mode. Furthermore, these findings suggest that by manipulating CL restitution it may be possible to alter SAN cell dynamics.

While our experiments have demonstrate the utility of restitution in analyzing SAN dynamics at the level of the single cell, cardiac pacemaking depends on coordinated activity of a well-organized collection of electrically coupled SAN cells. As a first step in extrapolating our analysis to the multi-cellular pacemaker complex, we sought to determine the effect of a constant (bias) repolarizing current (as an approximation of electrical loading) on restitution and spontaneous activity in the control model and with either $I_{Kr}$ block or low $[Na^+]_o$ (100 mM, above the threshold for spontaneous irregular activity) (Figure 20).

Interestingly, in all three cases, we identified a threshold bias current that led to irregular activity/skipped beats with the lowest threshold corresponding to low $[Na^+]_o$ and highest threshold for $I_{Kr}$ block (Figure 20). We next generated CL restitution curves at baseline and in the presence of the bias current just below the threshold for induction of irregular activity (Figure 20). In all cases, a repolarizing bias current led to a steeper curve when compared to baseline (Figure 20B-C). We next wanted to test whether or not a depolarizing bias current would have the opposite effect on the slope of the restitution
curve. It turns out that the depolarizing bias current (amplitude = -0.01 mA/mF) flattened the curve reducing the max slope from -0.27 to -0.15 in the control model and from -10.92 to -1 under low [Na⁺]₀ conditions (not shown; we were unable to examine the effects of Iₖₚ block as spontaneous activity terminated even for depolarizing bias current << -0.01).

Based on the previous findings, we hypothesized that conditions associated with steep restitution at the level of the single cell (e.g. low [Na⁺]₀) would demonstrate increased susceptibility to irregular activity and termination when examining coupled tissue where coupled cells experience electrotonic loading (analogous to repolarizing bias current). To test this hypothesis, we created a one-dimensional fiber of SAN cells coupled to atrial cells (Figure 21). We first systematically decreased [Na⁺]₀ or gₖp in the SAN region (similar to protocol outlined in Figure 17) to determine the termination mode and threshold in the coupled tissue (Figure 22).

Figure 20: Effect of bias current on cycle length dynamics. (A) Bias current threshold to induce irregular activity in control, Iₖₚ block (gₖp = 0.35*control), or low [Na⁺]₀ (100 mM). (B) CL restitution curves and (C) maximal slope for control, Iₖₚ block, and low [Na⁺]₀ at baseline and during bias current injection. Published in ¹⁴.
As expected from simulations with repolarizing bias current, both the onset of irregular activity and termination of spontaneous activity occurred earlier (higher [Na\textsuperscript{+}]\textsubscript{o}) in tissue compared to the single cell ([Na\textsuperscript{+}]\textsubscript{o} = 92 mM for termination in tissue compared to 58 mM for single cell). In contrast, the termination occurred later (more block) for g\textsubscript{Kr}.

Figure 21: One-dimensional sinoatrial node fiber model. (A) 50 mouse SAN cells are electrically coupled to 50 mouse atrial cells to create a one-dimensional fiber. (B) Spontaneous action potentials along the fiber during five consecutive cycles at steady-state. (C) Activation time and (D) dV/dtmax along the fiber during one cycle at steady-state. Published in\textsuperscript{14}. 

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in tissue compared to single cell ($g_{Kr} = 0.27 \times \text{control for termination in tissue compared to } 0.31 \times \text{control in single cell}$). We also sought to determine what effect uniform uncoupling would have on termination in the fiber. Increasing gap junction resistance two-fold in the SAN region delayed termination for low $[\text{Na}^+]_o$ (termination at 88 mM rather than 92 mM, Figure 22A) but slightly accelerated termination under $I_{Kr}$ block ($g_{Kr}=0.28 \times \text{control rather than } 0.27$, Figure 22B). Together, these findings suggest that the CL restitution curve is useful not only for predicting single cell dynamics but also for predicting the behavior at the level of the intact tissue. Namely, cells demonstrating steep restitution and irregular activity at the single cell level are likely more susceptible to activation failure in the context of the intact tissue.
In addition to the effect of coupling on the termination threshold, we observed an interesting impact on the overall activation pattern. While irregular activity in the single cell may display higher order periodic patterns and intermittent skipped beats (see Figure 17A), in the tissue we observed intervals of regular activity interspersed with long pauses (Figure 22A). To determine the mechanism for this distinct type of activity, we returned
to the single cell and applied a condition with low $[\text{Na}^+]_o$ (100 mM) and bias current stimulation (0.0077 $\mu$A/$\mu$F, just above the previously determined threshold for irregular activity). Consistent with the irregular pattern in the coupled fiber, we observe long skipped beat runs in the single cell model with low $[\text{Na}^+]_o$ and bias current injection (Figure 23).

We hypothesized that this behavior reflected the existence of system “memory” in addition to the beat-to-beat dynamics captured by the CL restitution (a first order approximation for restitution only uses the preceding CL). We further hypothesized that, similar to short-term memory effects observed in ventricular myocytes,\textsuperscript{212, 213} SAN

![Figure 23: Ionic mechanism for irregular SAN activity with long pauses. (A) Simulated spontaneous APs and (B) $[\text{Na}^+]$, from a single SAN cell subjected to low $[\text{Na}^+]_o$ (100 mM) and constant, low amplitude (0.0077 $\mu$A/$\mu$F) bias current stimulation. During AP firing, $[\text{Na}^+]$, rises until a threshold is reached (~6.98 mM) at which point spontaneous activation terminates and $[\text{Na}^+]$, slowly falls until a second threshold is reached (~5.91 mM) and the pattern repeats. Clamping $[\text{Na}^+]$, to the termination threshold (red line in A and B, clamp applied at time point labeled b) results in complete termination of activity, while clamping to the recovery threshold (gray line) eliminates the skipped beat runs resulting in regular periodic activity. (C) The CL restitution curve was determined for the model with low $[\text{Na}^+]_o$ and bias current stimulation just after onset of regular periodic activity (black line, determined at time point labeled a in panels A and B) or prior to onset of skipped beat run (red line, determined at point marked b). $[\text{Na}^+]$, was then reset to the low threshold value and restitution was determined again at the same time point (gray line). The restitution curve from the control model (normal $[\text{Na}^+]_o$, no bias current) is shown for reference (black line). Dashed line denotes a slope of $-1$. $[\text{Na}^+]$, alters the slope of the restitution curve with much steeper slope at higher $[\text{Na}^+]$, Published in \textsuperscript{14}.

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memory depends on intracellular ion concentration changes that typically occur with a slower time course than other AP processes (e.g. ion channel gating). Interestingly, we find that during the period of regular activity, intracellular [Na\(^+\)]\(_i\) ([Na\(^+\)]\(_i\)) slowly increases until activity terminates, allowing [Na\(^+\)]\(_i\) to gradually decrease until activity is restored and the pattern repeats itself (Figure 23B). [K\(^+\)]\(_i\) shows a similar, but inverted, pattern (increases when Na\(^+\) decreases and vice versa, not shown). The central role of [Na\(^+\)]\(_i\) in governing this behavior is demonstrated by clamping [Na\(^+\)]\(_i\) to either the recovery threshold (e.g. value at time point ‘a’ in Figure 23A-B) or the termination threshold (value at time point ‘b’) during spontaneous activation (in both cases, clamp applied at time point ‘b’). Clamping [Na\(^+\)]\(_i\) to the termination threshold (higher value) resulted in complete termination of spontaneous activity (red lines in Figure 23-B).

Conversely, clamping [Na\(^-\)]\(_i\) to the recovery threshold resulted in uninterrupted periodic activity (gray lines in Figure 23A-B). In contrast, implementation of a similar clamp with [K\(^+\)]\(_i\) had very little effect on activation pattern (not shown). We hypothesized that [Na\(^+\)]\(_i\) regulates SAN cell dynamics by altering CL restitution. To test this hypothesis, we determined restitution at two different points during the irregular periodic activity shown in Figure 23: 1) just after onset of regular periodic activity (time point ‘a’ in Figure 23A-B corresponding to low [Na\(^+\)]\(_i\)) and 2) just before onset of a long skipped beat run (time point ‘b’). We observed a dramatic change in the maximal slope of the restitution curve between the two time points. Specifically, at the onset of periodic activity, the curve is relatively flat (max slope \(>-1\)), but just before termination, the curve demonstrates an abrupt transition from a flat to a very steep region (max slope \(<-1\)).
We also measured restitution just before termination of activity but with $[\text{Na}^+]_{i}$ reset to the value it took just after onset of activity.

We found that lowering $[\text{Na}^+]_{i}$ in this manner both shifted and flattened (max slope $> -1$) the restitution curve. Together these simulations demonstrate that $[\text{Na}^+]_{i}$ affects CL restitution and SAN cell dynamics with $[\text{Na}^+]_{i}$ accumulation favoring a steep restitution and irregular SAN activity. Under conditions where the restitution curve shows an abrupt transition from a flat to very steep region, (see Figure 18C and Figure 23C), $[\text{Na}^+]_{i}$ promotes a distinct type of SAN activity characterized by regular activity periodically interrupted by long skipped beat runs.

**Discussion**

While it is well-established that SAN pacemaking depends on rhythmic activity of membrane ion channels and calcium cycling proteins at the level of the single cell, the governing dynamics responsible for regulating spatial and temporal control of SAN synchrony remain elusive. Research in this area has become increasingly reductionist and while this approach has yielded gains in understanding the underlying mechanism for monogenic disease, tremendous controversy remains regarding the factors that govern the regular heart rhythm. Approaches utilizing nonlinear dynamics are well-suited to the study of a complex system such as the sinus node. In fact, previous studies in this area have generated important insights into SAN cell dynamics and have laid the foundation for the SAN restitution theory outlined here. This previous research has described the different types of SAN spontaneous activity, associated phase space
diagrams, and role of major ion channels/cell factors (e.g. vagal stimulation) in regulating dynamics.

Here, we advance this work by using an analytical and computational approach to generate a number of testable predictions regarding the dynamic behavior of SAN cells. Our major findings include: 1) the CL restitution curve provides valuable information about the dynamical status of the SAN cell, is relatively straightforward to assess, and may be used to make predictions about likely termination mode and behavior in coupled tissue; 2) CL restitution gives rise to a wide range of irregular dynamical behavior from beat-to-beat alteration (CL alternans) to regular periodic activity interspersed with extended periods of quiescence (long skipped-beat 13 runs) (different classes of activity are illustrated in Figure 24); 3) Electrotonic loading of cells in the intact tissue (similar to repolarizing bias current stimulation in a single cell) alters SAN cell dynamics, in part, by increasing the slope of the CL restitution curve; 4) Single cell conditions favoring relatively steep CL restitution are associated with increased susceptibility to irregular activity and even termination in the coupled tissue; and 5) Similar to other cardiac cell types (e.g. ventricular myocytes)²¹³, SAN cell dynamics demonstrate “memory” as a result of intracellular ion changes (particularly Na+) that alter restitution.
Figure 24: A graphical method for understanding the relationship between spontaneous SAN activity and restitution. Simulated spontaneous APs are shown in top of each panel with schematic representation of corresponding restitution curve in bottom. The identity line may be superimposed on the restitution curve to create a return map for tracking CL dynamics in response to a perturbation (arrows demonstrate iterative response to perturbation from fixed point, defined as intersection of return map with identity line). (A) Regular periodic activity (control model) occurs when restitution slope is shallow (slope > −1). Perturbation from steady-state results in eventual return to stable fixed point. (B) 2:2 periodic behavior (APs correspond to control model with bias current = 0.0274 mA/mF) results from a monophasic curve with a slope equal to the critical value of −1 (in this example, CL stably alternates between 306 ms and 283 ms). (C) Higher dimensional periodic activity (e.g. 4:4) and skipped beats may result from a multiphasic curve with regions of steep slope (< −1) (APs correspond to [Na+]o = 63 mM). (D) Irregular activity with long skipped beat runs may occur in instances where the CL restitution curve experiences an abrupt transition from a shallow region (slope < −1) to a very steep region (slope >> −1) (APs are shown for [Na+]o = 100 mM, bias current = 0.0077 µA/µF). Schematic curves and corresponding return map trajectory are shown as spontaneous activity progresses from time point a (onset of activity) to b (just before termination). In this case, dynamic changes in [Na+]i may produce intermittent long skipped beat runs by shifting the curve and altering its slope. Published in 14.
Conclusions

SAN CL restitution serves as a clear parallel to APD restitution in quiescent myocytes (e.g. atrial and ventricular myocytes). Similar to APD restitution, CL restitution reflects a fundamental dependence of cell activity on preceding recovery time. APD and CL restitution both assume that activity depends only on events in the preceding cycle, a first order approximation that does not account for system memory due, for example, to relatively slow intracellular ion changes. Finally, in both cases, restitution is straightforward to assess and interpret. We anticipate that CL restitution will serve a valuable role in advancing our understanding of SAN dynamics and dysfunction, similar to the proven utility of APD restitution in analyzing AP dynamics and arrhythmias [27].

There are a number of implications from our findings. First, our analysis predicts that electrical coupling has a dramatic impact on cellular dynamics and that this effect depends, in part, on baseline restitution properties of the individual cell. This is a critical factor to consider in light of recent discussion regarding the safety and efficacy of anti-arrhythmic agents that target gap junctions to increase electrical coupling.²¹⁶,²¹⁷

Our findings suggest that care should be taken in how these drugs (e.g. rotigaptide) are administered as in certain cases such a drug would be predicted to exacerbate sinus node dysfunction. It is important to note the difference between uniform electrical uncoupling studied here and heterogeneous uncoupling/cell loss (e.g. due to apoptosis and/or fibrosis), which in previous studies we have shown to be, without
exception, detrimental to SAN function.\textsuperscript{8, 9, 142} Clearly, loss of spontaneously active cells within the SAN will disrupt the source-sink balance between the SAN and surrounding atrial myocardium, independent of restitution. Another important implication of our findings is that elevated $[\text{Na}^+]_i$ promotes steep restitution and irregular dynamics, providing a potential mechanistic explanation for SAN dysfunction closely associated with certain types of cardiac disease (e.g., atrial fibrillation, heart failure). It is worth mentioning that both decreased $g_{Kr}$\textsuperscript{218} and elevated $[\text{Na}^+]_i$\textsuperscript{97} are known side effects of CaMKII overactivity. Because of this, CaMKII-inhibition may be an interesting research target to attempt to mitigate the negative effects observed in either of these scenarios. Finally, in a general sense, our studies highlight the importance of considering SAN dynamics (rather than just steady-state CL, for example) when evaluating single cell behavior.

**Limitations**

In this study, we use a computational approach to develop and test a specific hypothesis regarding SAN restitution and dynamics. Going forward, it will be essential to experimentally validate model predictions. Specifically, important future experiments in SAN cells will determine: 1) whether the protocol outlined here may be efficiently implemented to assess restitution in vitro, in situ, and/or in vivo; 2) whether the restitution curve is indeed useful in predicting dynamics and termination mode at cell and tissue level; and 3) whether restitution may be used to better understand cardiac pacemaking mechanisms and dysfunction. It is also important to note that while our
study examines the effects of changes in electrical coupling on SAN dynamics in coupled tissue, these changes are likely less important than the inherent differences in coupling in the intact SAN where impedance changes dramatically from the central SAN to the right atrium. Going forward it will be important to consider the functional impact of electrical and structural heterogeneity within the intact SAN on restitution and overall dynamics.
Chapter 4: The Role of CaMKII in Regulation of Na, Function and Ventricular Arrhythmia

4.1: CaMKII Regulation of Na,

As has been addressed in previous chapters, Calcium/calmodulin-dependent kinase II (CaMKII) is a multifunctional serine/threonine kinase that has both a broad substrate specificity and wide tissue distribution. Several CaMKII targets in heart have been extensively studied, including L-type Ca\(^{2+}\) Channels, sarcoplasmic reticulum Ca\(^{2+}\) release channels (RyR), phospholamban, voltage-gated K\(^+\) channels, and voltage-gated Na\(^+\) channels.\(^65, 72, 218-220\)

During the cardiac action potential, Na\(^+\) channels open rapidly to generate the action potential upstroke. This opening is followed by an almost instantaneous inactivation of these channels. For the most part, a combination of voltage-gated Ca\(^{2+}\) channels and K\(^+\) currents shape the plateau and repolarization phases of the action potential. While the Na\(^+\) current rapidly turns off following the peak due to voltage-dependent inactivation of the majority of Na\(^+\) channels, a small persistent current remains throughout the remainder of the action potential for reasons outlined in Chapter 1. This current is frequently referred to as the “late” Na\(^+\) current and is present even under
normal physiological conditions but increases dramatically in disease (e.g. heart failure).\textsuperscript{221-223}

CaMKII phosphorylates the voltage-gated Na\textsuperscript{+} channel to regulate I\textsubscript{Na} and cell membrane excitability. More specifically, CaMKII-dependent phosphorylation has been shown to impact I\textsubscript{Na} steady-state inactivation, recovery from inactivation, and the levels of late current.\textsuperscript{32, 65, 73} While the molecular mechanism (especially in vivo) remains unclear, our group and others have identified several CaMKII phosphorylation sites in the DI-DII linker of Na\textsubscript{v}1.5 that may mediate CaMKII-dependent regulation of I\textsubscript{Na}.\textsuperscript{2, 32, 97, 98}

My studies have focused on the Na\textsubscript{v}1.5 residue Ser571. Ser571 first emerged as a potential CaMKII phosphorylation site from a functional screen in heterologous cells using a library of Na\textsubscript{v}1.5 mutants lacking putative CaMKII sites (Ser/Thr-to-Ala mutations) in the intracellular channel regions.\textsuperscript{32} After this initial screening, β\textsubscript{IV}-spectrin was shown to be required for proper targeting of CaMKII to Na\textsubscript{v}1.5 in excitable cells.\textsuperscript{32} Finally, these studies demonstrated that CaMKII regulates Na\textsubscript{v}1.5 by directly phosphorylating S571. In order to continue this research direction, studies discussed here sought to use an integrative approach to test the hypothesis that dysregulation of the CaMKII Ser571 phosphorylation motif contributes to arrhythmogenesis in both inherited and acquired cardiac disease.
4.2: Ca^{2+}/Calmodulin-Dependent Protein Kinase II-Based Regulation of Voltage-gated Na^{+} Channel in Cardiac Disease

Background

Gene mutations in ion channels have been mechanistically linked to human arrhythmia through their effects on ion channel biophysics.\textsuperscript{224} Discovery of these mutations not only has provided new insight into cellular mechanisms for human disease but also has advanced our fundamental understanding of ion channel structure and function. Deriving from this work, a second class of human arrhythmia mutations has been identified in genes encoding ion channel accessory proteins (eg, adapter and scaffolding proteins, channel subunits, chaperones) rather than ion channel subunits.\textsuperscript{169, 225-231} These mutations cause disease by altering channel targeting and/or biophysical activity and highlight the importance of proper channel localization in specific cellular domains for normal heart function.

Despite major research discoveries, the mechanistic link between specific molecular defects, channel dysfunction, and arrhythmias associated with many human arrhythmia variants remains elusive. Protein phosphorylation has been identified as an essential mechanism for regulating cell function in both heart and other systems. In heart, tight spatial and temporal control of local signaling domains ensures proper regulation of key ion channels, transporters, and receptors. Importantly, alterations in posttranslational modification of membrane proteins are associated with increased susceptibility to congenital arrhythmia and common causes of acquired arrhythmia,
including heart failure (HF).\textsuperscript{19, 101, 232, 233} In this work, we identify the mechanism for 2 human cardiac arrhythmia susceptibility variants in SCN5A (A572D and Q573E).

Voltage-gated Na\textsuperscript{+} channels (Na\textsubscript{v}) are critical for normal cell excitability and were among the first ion channels to be linked to a specific congenital cardiac arrhythmia.\textsuperscript{51} Over the past twenty years, hundreds of human gene variants have been identified in SCN5A linked to various forms of cardiac arrhythmia.\textsuperscript{28, 35} Na\textsubscript{v}1.5 dysfunction has also been identified in common forms of acquired heart disease (eg, HF and myocardial infarction) in which slow conduction and/or altered repolarization plays an important role in arrhythmia and sudden death.\textsuperscript{234} My group recently demonstrated that the multifunctional Ca\textsuperscript{2+}/calmodulin- dependent protein kinase II (CaMKII) directly phosphorylates Na\textsubscript{v}1.5 at residue S571 to decrease channel availability and to enhance persistent (late) current, leading to increased susceptibility to afterdepolarizations.\textsuperscript{32} A screen of identified human arrhythmia variants in SCN5A yielded 2 variants in the Na\textsubscript{v}1.5 DI-DII loop, the mechanism of which was unsolved (A572D and Q573E).

Here, we demonstrate that these variants are localized to the CaMKII phosphorylation motif of Na\textsubscript{v}1.5 and alter functional regulation of Na\textsubscript{v}1.5. We also evaluate the role of the CaMKII phosphorylation domain of Na\textsubscript{v}1.5 in a large-animal model of acquired heart disease and in failing human hearts and identify significant CaMKII-dependent changes in posttranslational modification of Na\textsubscript{v}1.5 at S571 in diseased hearts. From these findings, we propose that S571 in the Na\textsubscript{v}1.5 DI-DII cytoplasmic loop serves as a critical node for regulating channel function in diverse forms of cardiac disease associated with arrhythmias and sudden death.
**Previous Work**

*Human Na$_v$1.5 Arrhythmia Variants Proximate to CaMKII Phosphorylation*

**Site Disrupt Channel Regulation** - We recently identified Na$_v$1.5 S571 in the DI-DII loop as a target for CaMKII-mediated phosphorylation and a key regulatory point for multiple Na$_v$ properties, including channel availability, recovery from inactivation, and late current. Our findings suggested that variation in this regulatory region could potentially result in significant cardiac dysfunction in vivo. As a first step in evaluating a link between the CaMKII regulatory motif and disease, we analyzed this region for potential human cardiovascular disease variants and identified 2 variants proximate to the CaMKII regulatory motif: A572D and Q573E (Figure 25). Q573E was originally identified in a genetic screen of long-QT syndrome probands with autosomal-dominant Romano-Ward syndrome. The A572D variant was initially characterized in a proband with Romano-Ward long-QT syndrome, later found in larger arrhythmia cohorts, and likely has an allele frequency of ~0.5% in the general population. Both variants result in charge substitution consistent with the effects of phosphorylation (neutral to negative) at residues immediately adjacent to the CaMKII phosphorylation site S571. Thus, although they do not involve direct changes to S571, we hypothesized that these proximate variants alter CaMKII-dependent modulation of Na$_v$1.5, resulting in altered susceptibility to proarrhythmic phenotypes.

In previous studies, variants in this region were analyzed to assess basic gain- or loss-of-function status with mixed results. Therefore, to determine whether these
variants alter Na$_v$ function and/or CaMKII regulation, we measured Na$_v$ current (INa) from human full-length GFP-WT and variant channels (expressed with the h1 subunit) in HEK cells with or without constitutively active CaMKII-T287D (Figure 26).

A572D and Q573E variants showed increased late current (measured as current amplitude 50 milliseconds after peak) at baseline compared with WT, similar to S571E (phosphomimetic) and WT CaMKII-delta (Figure 26A and B). The Na$_v$ blocker ranolazine (10 umol/L; specific for late current) normalized late current for WT, A572D, and Q573E variants with CaMKII-delta(T287D) (Figure 26A; P=NS versus WT; n=5; not shown). A572D and Q573E variants also showed a leftward (hyperpolarizing) shift in Na$_v$1.5 steady-state inactivation and slowing of recovery from inactivation at baseline compared with WT, similar to S571E and WT CaMKII-delta(T287D) (Figure 26C–H). In contrast, S571A (phospho-resistant) I$_{Na}$ properties were not different from WT at baseline (Figure 26B, E, and H). Immunoblot analysis showed similar expression of WT and human arrhythmia variants expressed in HEK cells.

![Figure 25: Human arrhythmia variants proximate to Na$_v$1.5 Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) phosphorylation node. Human variants adjacent to the Na$_v$1.5 phosphorylation site (A572D and Q573E) have been linked to cardiac arrhythmia. Published in 2.](image)
(not shown). Changes in Na\textsubscript{v} properties observed in WT CaMKII-delta(T287D) were prevented by the CaMKII peptide inhibitor autocamtide-2-related inhibitory peptide (10 umol/L; not shown). These data indicate that A572D and Q573E human variants resulting in charge substitution immediately juxtaposed to the CaMKII phosphorylation site mimic CaMKII phosphorylation (A572D>Q573E) and suggest a proarrhythmic mechanism associated with these human variants.
Human Variants Alter Cardiomyocyte Membrane Excitability - We next
determined whether phosphomimetic A572D and Q573E mutant channels
produced abnormal cell excitability in primary cardiomyocytes. To express WT and variant
channels in cardiomyocytes and to distinguish them from endogenous channels, we
created tetrodotoxin-sensitive constructs containing a point mutation (C373Y) at a critical
aromatic residue in which tetrodotoxin binds. The C373Y mutation increases
tetrodotoxin sensitivity of Na, by almost 3 orders of magnitude. Ventricular myocytes

Figure 26: I, traces, late current, recovery, and inactivation kinetics of Human arrhythmia Na,1.5 variants
(A), Na, current I, traces measured from HEK cells expressing wild-type (WT), A572D, or Q573E
channels (coexpressed with hβ1 subunit) with or without 10 μmol/L ranolazine (Ran) to block late I,. Na,
function was also measured in S571A- and S571E-expressing cells as negative (phospho-resistant)
and positive (phosphomimetic) controls, respectively. Dashed line indicates measurement time of late
current (50 milliseconds after peak). (B), Summary data for late current measured at 50 milliseconds after
peak and expressed as percentage of peak (**P<0.005, ***P<0.001 vs WT at baseline; n = 5 except
Q573E + CaMKII [n = 6]). (E) steady-state inactivation V1/2 (**P<0.005, ***P<0.001 vs WT at baseline), (H)
recovery time constants (*P<0.05, **P<0.005, ***P<0.001 vs WT at baseline) in WT- and variant-expressing cells with or without CaMKII. S571E-, A572D-, and Q573E-expressing cells show
increased late I, and slowed recovery from inactivation similar to WT CaMKII. S571E, A572D, and
Q573E did not show further changes in Na, function with CaMKII. In contrast, the S571A
phosphoresistant variant (with or without CaMKII) resembles WT at baseline (without CaMKII).
Published in 2.
were transfected with GFP-expressing tetrodotoxin-sensitive WT and mutant constructs (Figure 27). $I_{Na}$ was measured from GFP-positive cells 24 hours after $Na_v$ expression (Figure 27).

Consistent with findings in HEK cells, A572- and Q573-expressing cardiomyocytes showed increased late current compared with WT that was blocked by 10 umol/L ranolazine (Figure 27A and B). Furthermore, a significant leftward shift in steady-state inactivation was measured in A572D and Q573E variants compared with WT (Figure 27C and D). Differences in endogenous $Na_v$ function were eliminated by low-dose (10 nmol/L) tetrodotoxin sufficient to block tetrodotoxinsensitive $Na_v1.5$ (Figure 27A, B, and D–F). Peak current was comparable in transfected cells at baseline and showed a similar decrease with 10 nmol/L tetrodotoxin (57.34.6%, 63.26.2%, and 61.74.6% in WT, A572D, and Q573E, respectively; P=NS for WT versus A572D or Q573E), indicating significant and comparable expression levels between WT and variants (Figure 27E and F). These data support findings in HEK cells that A572D and Q573E variants act in a phosphomimetic manner.
Figure 27: Expression of human arrhythmia variants in cardiomyocytes. $I_{Na}$ current ($I_{Na}$) was measured in 3-day-old neonatal mouse cardiomyocytes transfected with vehicle, wild-type (WT), A572D, or Q573E channels engineered to have increased sensitivity to tetrodotoxin (TTX; C373Y).41 (A), $I_{Na}$ traces, (B) late $I_{Na}$ amplitude with or without 10 $\mu$mol/L ranolazine (Ran; **P<0.005, ***P<0.001 vs control [no TTX/ranolazine]; n = 8 for control, n = 5 for ranolazine), (C) steady-state inactivation curves (*P<0.05 vehicle vs A572D or Q573E; n = 5 except for WT [n = 4] and A572D [n = 6]), and (D) steady-state inactivation $V_{1/2}$ (*P<0.05, ***P<0.001 vs vehicle control [no drug]) were measured with or without low-dose (10 nmol/L) TTX to block exogenous current. (E), Current-voltage relationships were also measured with or without low-dose TTX to verify similar expression levels for WT and human arrhythmia variants (*P<0.05, control vs 10 nmol/L TTX; n = 7 for control, n = 5 for TTX). (F), Summary data comparing peak TTX-sensitive (exogenous) current-voltage relationship in WT and variant-expressing myocytes (P = NS vs WT). Published in$^2$. 

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Having validated successful expression of tetrodotoxin-sensitive Na\textsubscript{v}, in primary cardiomyocytes, we measured APs in cardiomyocytes 24 hours after the expression of WT and variant constructs (Figure 28). A572D- and Q573E-expressing cardiomyocytes showed significant prolongation of AP duration at 90\% (APD\textsubscript{90}) compared with WT or vehicle, consistent with the clinical phenotype of QT prolongation (Figure 4A and 4B).\textsuperscript{236, 237} Furthermore, afterdepolarizations were observed in A572D-expressing myocytes (2 of 9 cells; Figure 28A, red arrow). Ranolazine (10 µmol/L) or low-dose (10 nmol/L) tetrodotoxin eliminated differences in late current and APD\textsubscript{90} between Q573E-, A572D-, and WT-expressing myocytes and eliminated afterdepolarizations in A572D-expressing cells (Figure 28A and B). These data indicate that the A572D and Q573E variants mimic channel phosphorylation and delay AP repolarization by increasing late I\textsubscript{Na} in cardiomyocytes.
Results

Computational Model of Phosphomimetic Na,1.5 Variants – Despite the strength of the in vitro findings, a number of limitations exist that would require further

Figure 28: Human arrhythmia variants adjacent to Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation site delay action potential (AP) repolarization when expressed in cardiomyocytes. Wild-type (WT), A572D, and Q573E channels engineered to have increased sensitivity to tetrodotoxin (TTX; C373Y) were expressed in neonatal mouse cardiomyocytes. (A), APs and (B) AP duration at 90\% (APD\textsubscript{90}) from vehicle-, WT-, A572D-, and Q573E-expressing cardiomyocytes with or without 10 nmol/L TTX (to block exogenous Na current; *P<0.05, **P<0.005, ***P<0.001 vs control [no TTX]; n = 12 for control, n = 5 for ranolazine and TTX). A572D and Q573E significantly increase APD\textsubscript{90} compared with WT and increase the likelihood of afterdepolarizations (red arrow in A). Low-dose TTX or 10 µmol/L ranolazine (RAN) eliminates differences in APD\textsubscript{90}. Myocytes were paced at 1Hz. Published in \textsuperscript{2}. 

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investigation. One of the primary limitations of this work is the fact that the experiments were conducted in an overexpression system. Because of this, any differences that we observe between populations could potentially be artifacts due to the inherent artificiality of the experiment. To help determine whether the measured changes in the late sodium current could indeed produce the observed changes in the action potential traces, we decided to perform computational modeling.

To determine whether measured differences in Na\textsubscript{v} function are responsible for AP prolongation in A572D- and Q573E-expressing myocytes and to predict electrophysiological consequences in humans, we performed computational modeling using detailed Markov models of WT and variants integrated into a computer model of the human ventricular AP (Figure 29 and Figure 30).\textsuperscript{244} State-rate transitions in a Markov model of Na\textsubscript{v}1.5\textsuperscript{1,12,245} were determined for WT, A572D, and Q573E channels (Figure 30A, Figure 30 A and D) on the basis of our electrophysiological measurements (parameters provided in Table 9 of the appendix). The Na\textsubscript{v}1.5 Markov model was then incorporated into a well-validated model of the human ventricular AP\textsuperscript{244} to determine the effect of variants on Na\textsubscript{v} function and cell excitability (Figure 29 and Figure 30B).
Consistent with experiments (Figure 26-Figure 28), phosphomimetic A572D and Q573E human variants were associated with increased late $I_{Na}$ and prolonged APD (Figure 30 B and C). Furthermore, slow pacing (cycle length $> 4000$ milliseconds) unmasked afterdepolarizations in variant-expressing cells (Figure 30 E and F). These results indicate that measured changes in $Na_v$ function are sufficient to produce APD prolongation and afterdepolarizations in A572D- and Q573E-expressing cells.

Finally, we simulated the effects of ranolazine (10 µmol/L) on APs from WT- and A572D-expressing cardiomyocytes. Ranolazine was assumed to preferentially block the $Na_v$1.5 open state with on- and off-rates of 8.2 umol/L$^{-1}$ * s$^{-1}$ and 22 seconds$^{-1}$, respectively (Figure 29A).$^{241,242}$ Consistent with experimental observations,$^{45}$ ranolazine preferentially blocked late $I_{Na}$ (Figure 30F). Moreover, in agreement with our myocyte experiments, ranolazine normalized APD in variant-expressing cells and eliminated afterdepolarizations at slow pacing (Figure 30E). These data support our
hypothesis that A572D and Q573E variants prolong APD by increasing late $I_{\text{Na}}$ and indicate that late $I_{\text{Na}}$ blockers (ranolazine) may be an effective therapy for human patients with Na_v variants that mimic CaMKII phosphorylation (A572D and Q573E).

**Discussion**

Cardiac tissue has evolved highly specialized pathways for regulating

![Image of Figure 30: Comparison of experimentally measured and simulated values for human arrhythmia variants. Parameter estimation results comparing experimentally measured (black) and simulated (red) values for (A) steady-state inactivation and (D) late current. Simulated (B) APs and (C) INa from wild-type– (WT; black), A572D– (red), and Q573E– (gray) expressing cells. Results are shown at steady state at a pacing cycle length of 1000 milliseconds. Simulated (E) APs and (F) INa in WT– (black) and A572D-expressing (red) cells at steady state during slow pacing (cycle length = 4000 milliseconds). Afterdepolarizations were observed in A572D and Q573E (not shown) variants. Simulating a block of late $I_{\text{Na}}$ with 10 µmol/L ranolazine (E and F) reduced late $I_{\text{Na}}$ and afterdepolarizations consistent with experiments. Published in 1^2.](image-url)
posttranslational modifications of ion channels, transporters, and membrane receptors. Importantly, defects in local signaling and regulation of specific ion channels have been associated with abnormal cell excitability and arrhythmia in heart disease, including human HF. Here, we use a variety of novel reagents and computer models to demonstrate a link between defects in CaMKII-dependent phosphorylation of Na\textsubscript{v}1.5 and both congenital and acquired human disease. We show that 2 previously identified human SCN5A arrhythmia variants are localized to the CaMKII phosphorylation motif of the Na\textsubscript{v}1.5 DI-DII loop. Experimental studies in heterologous cells demonstrate that these variants, resulting in negative charge substitution adjacent to the phosphorylation site, partially recapitulate CaMKII phosphorylation effects on channel availability, recovery, and late current. Furthermore, these defects in the CaMKII phosphorylation motif alter the response of the channel to active CaMKII. Expression of these variants in myocytes and computer simulations reveal delayed AP repolarization and afterdepolarizations resulting from increased late I\textsubscript{Na}. Computational modeling reveals that the isolated (13-mer) DI-DII domains corresponding to A572D and Q573E variants possess electrostatic and structural features very similar to the S571-phosphorylated channel, suggesting that they may have similar binding interactions with downstream partners, including regions of the channel itself (eg, channel pore). Although these simulations do not account for the true 3-dimensional structure of the intact DI-DII loop, they support our experimental data indicating that the human variants A572D and Q573E confer arrhythmia susceptibility by structurally and functionally mimicking the phosphorylated channel. Finally, we report dysregulation of Na\textsubscript{v}1.5 at S571 in common
forms of heart disease, including human HF, suggesting a common molecular link between known defects in CaMKII activity, Na, dysfunction, and arrhythmias. Future studies are needed to determine whether targeting this motif (genetically or pharmacologically) will be effective in preventing arrhythmia and/or progression of disease.

Remarkable progress has been made in understanding links between specific congenital molecular defects and human disease. Structure-function studies on ion channels, coupled with expression studies in heterologous cells, have greatly advanced our understanding of not only monogenic disease but also more common acquired disease. A classic example of our ability to link defects at the molecular level to a clinical phenotype comes from long-QT syndrome variants found in the DIII-DIV linker or C-terminal region that increase late current by interfering with rapid channel inactivation (eg, (capital delta)KPQ). Here, we propose that the A572D and Q573E human variants belong to an emerging class of atypical ion channel variants that produce abnormal cell excitability and arrhythmia by affecting posttranslational modification.101, 225, 246

Previous studies have yielded conflicting results on the functional status of the A572D variant.239, 240 Expression studies in oocytes report faster recovery from inactivation with no change in steady-state availability (late current was not assessed).239 Other studies have found decreased availability, altered recovery, and increased late current in A572D but only with the common polymorphism H558R.240 Importantly, previous studies did not analyze A572D function in the setting of CaMKII signaling and
therefore lack controls that would facilitate a more thorough comparison (eg, CaMKII
cotransfection/inhibition). It is very likely that A572D may have limited pathogenicity in
isolation and may depend on environmental cues/cofactors. In addition, it is interesting
to consider the possibility that A572D may be linked to diseases other than inherited
arrhythmia syndromes (cardiomyopathy, HF). Similarly, although our data establish a
role for S571 in CaMKII-dependent regulation of Na\textsubscript{v}1.5, the precise mechanism is likely
more complicated with potential contributions from multiple nearby sites.\textsuperscript{97} It will be
important going forward to determine exactly how phosphorylation/mutation events in
this DI-DII “hot spot” conspire to regulate channel activity.

Interestingly, despite the potentially lethal cellular phenotype associated with
delayed AP repolarization observed in our myocyte studies and computer simulations, the
allele frequency for at least one of the variants (A572D) is relatively high (~0.5%). This
raises the question, “Why are these variants not associated with a more severe clinical
phenotype?” One possibility suggested by our data is that although the variants mimic the
phosphorylated channel, they also make the channel resistant to further regulation by
endogenous CaMKII (see Figure 26), thereby preventing exacerbation of the phenotype
by multiple factors known to increase CaMKII activity (eg, beta-adrenergic stimulation).
Furthermore, the inherent nature of Na\textsubscript{v}1.5 regulation by CaMKII is complex with loss-
of-function (decreased availability) coupled with gain-of-function (increased late current)
effects. Thus, the net result of this compound regulation may be a cellular phenotype less
problematic than either one alone. Alternatively, allelic imbalance and variable
expression of variant Na\textsubscript{v}1.5 channels may produce heterogeneity in Nav function and
clinical phenotype.\textsuperscript{247} Finally, and perhaps most important, our findings predict that individuals harboring these variants will in part resemble a much larger population of HF patients (display S571 hyperphosphorylation), suggesting that although this modification may increase susceptibility to arrhythmia, it is not 100% penetrant.

Although our data support a critical role for CaMKII in dysregulation of Na\textsubscript{v}1.5, we cannot exclude contributions of other kinase pathways to this regulatory site in disease. In fact, we expect that although CaMKII is the primary regulatory kinase for this site, other kinases also contribute to phosphorylation at S571. Furthermore, phosphorylation of other sites, in addition to S571, likely contributes to the disease phenotype because Na\textsubscript{v}1.5 is directly regulated by both protein kinase C and A, the activities of which are altered in disease.\textsuperscript{234} This may be true in human HF, in particular, in which we measured a significant but relatively modest change in phosphorylation of S571. Although our data demonstrate elevated Na\textsubscript{v}1.5 pS571 in forms of canine and human disease, it is important to establish the relative contribution of these changes to dysfunction across disease pathologies. Finally, oxidation and other posttranslational modifications may regulate channel activity through direct (eg, lipid peroxidation) or indirect (eg, protein kinase C–dependent phosphorylation) pathways. Thus, future studies are important to determine the relative contribution of phosphorylation of Na\textsubscript{v}1.5 at S571 to dysfunction in diseased heart and upstream signals (eg, beta-adrenergic receptor stimulation, angiotensin II, reactive oxygen species, Ca\textsuperscript{2+}).
Conclusion

We identified the molecular mechanism for 2 human Na\textsubscript{v}1.5 variants localized to the CaMKII regulatory motif in the Na\textsubscript{v}1.5 DI-DII loop and provide data to support that a similar defect is present in a large-animal model of ischemic heart disease and in human HF. These studies add to mounting evidence that defects in local signaling and protein posttranslational modification help define the disease phenotype associated with a broad range of cardiac arrhythmia syndromes. It will be interesting in the future to determine whether therapies targeting CaMKII-dependent regulation of Na\textsubscript{v}1.5 at S571 will be effective in reducing arrhythmia burden in these patients.
Chapter 5: Mechanism of CaMKII-Dependent Regulation of Na\textsubscript{v}1.5 In Vivo

5.1: Background

While experimental and computational studies to date have demonstrated a potential role for phosphorylation at S571 in regulating I\textsubscript{Na} and cell excitability, all of these studies have been done in vitro. 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. In an effort to identify the molecular basis for CaMKII-dependent regulation of Na\textsubscript{v}1.5 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\textsubscript{Na,L}, cell excitability and heart function in response to stress (pressure-overload induced heart failure). Furthermore, we observe that ablation of the Ser571 site mitigates maladaptive electrical and mechanical remodeling observed in the failing heart. Based on these findings, we propose that
Ser571 serves as a molecular toggle for CaMKII-dependent activation of $I_{Na,L}$ (but not other channel properties) in vivo. We anticipate that this molecular pathway may yield new therapeutic avenues for reducing arrhythmia burden without disrupting normal physiology.
5.2: Results

Generation of S571E/A knock-in mice to test in vivo role of Ser571 - Previous in vitro functional studies have demonstrated a role for CaMKII-dependent phosphorylation at Ser571 in regulating Na\textsubscript{v}1.5 function (Figure 31A).\textsuperscript{2, 32, 97} To test the central hypothesis that phosphorylation of Na\textsubscript{v}1.5 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 31B). While

![Figure 32: Immunostaining of WT, S571E, and S571A ventricular cardiomyocytes.](image)

Permeabilized adult WT, S571E and S571A ventricular cardiomyocytes were immunostained for N-cadherin (green) and Na\textsubscript{v}1.5, βIV-spectrin, ankyrin-G, or CaMKII (red). DAPI staining (blue) indicates nuclei. Scale bar = 5 μm.
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phospho-Na\textsubscript{v}1.5(Ser571) was not detectable in S571A or S571E lysates (phospho-epitope eliminated by either mutation), protein expression levels and cellular localization of Na\textsubscript{v}1.5 were normal in S571E and S571A myocytes compared to WT at baseline. Furthermore, there were no differences in levels (Figure 31C-D) or localization (Figure 32A-C) of related associated proteins CaMKII, β\textsubscript{IV}-spectrin, or ankyrin-G\textsuperscript{2,31,32}

Expression/localization of the intercalated disc protein N-cadherin was also normal in S571E and S571A myocytes (Figure 32A-C). 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 a small but significant decrease in EF in both S571E and S571A animals without differences in other parameters (Table 1).

<table>
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<th>WT (n=19)</th>
<th>S571E (n=7)</th>
<th>S571A (n=10)</th>
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<tr>
<td>HR (bpm)</td>
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<td>477.4±18.2</td>
<td>483±13.1</td>
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<td>LVID\textsubscript{d} (mm)</td>
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<td>4.46±0.15*#</td>
<td>4.06±0.08</td>
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<td>EF (%)</td>
<td>63.3±0.8</td>
<td>51.5±1.4*#</td>
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<tr>
<td>FS (%)</td>
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<td>28.8±0.5*</td>
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<tr>
<td>LVAW\textsubscript{d} (mm)</td>
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<td>0.63±0.01</td>
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<tr>
<td>LVPW\textsubscript{d} (mm)</td>
<td>0.66±0.01</td>
<td>0.64±0.01</td>
<td>0.62±0.01</td>
</tr>
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Table 1: Baseline echocardiographic parameters in WT, S571E and S571A mice. HR = heart rate; LVID\textsubscript{d} = left ventricular inner chamber diameter in diastole; EF = ejection fraction; FS = fractional shortening; LVAW\textsubscript{d} = LV anterior wall thickness in diastole; LVPW\textsubscript{d} = LV posterior wall thickness in diastole *P<0.05 vs. WT; #P<0.05 vs. S571A

Phosphorylation at S571 selectively regulates the late sodium current and cell membrane excitability - Disruption of spectrin/CaMKII interaction reduces Na\textsubscript{v}1.5
phosphorylation at Ser571 and alters Na\textsubscript{v}1.5 activity and cell excitability.\textsuperscript{32} 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 33). 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. In contrast, S571A myocytes displayed a significant reduction in $I_{Na,L}$ (when normalized to peak) together with a small but significant increase in peak current compared to WT (Figure 33A-C). Surprisingly, no differences were observed in steady-state inactivation or recovery from inactivation in S571E or S571A compared to WT (Figure 33D-E), despite previous reports from our own group showing CaMKII-dependent changes in both properties in heterologous cells.\textsuperscript{2, 32, 73, 97, 248} These findings indicate that Ser571 regulates $I_{Na,L}$ without affecting other channel properties linked to CaMKII (e.g. steady-state inactivation, recovery).
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 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 34A-C). In contrast, S571A APD was not significantly different than WT at baseline. High dose isoproterenol produced frequent repolarization abnormalities, including afterdepolarizations and repolarization failure, in S571E but not WT or S571A myocytes [Numbers of cells with repolarization abnormalities (from two different preparations): 4/8 S571E cells, 0/8 WT cells, 0/8 S571A cells; P<0.05] (Figure 34D). Repolarization defects in S571E myocytes were normalized by treatment with ranolazine, supporting late $I_{Na}$ as a viable target for decreasing arrhythmia susceptibility (Figure 34E).
To determine whether Ser571 plays a role in regulation of cardiac excitability and/or arrhythmias in vivo, electrocardiograms were measured in awake, unanesthetized WT, S571E, and S571A mice. S571E mice demonstrated a significant prolongation of...
QTc interval compared to WT, in agreement with measured differences in $I_{\text{Na,L}}$ and APD (Figure 35A-B). S571E animals also showed a small but significant increase in QRS duration, while S571A animals displayed a small but significant increase in RR interval, although the underlying cause for these differences is unclear (Figure 35C-D). 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 35F-G). Treatment with Na$^+$ channel blockers flecainide or ranolazine eliminated differences in arrhythmia events between S571E, WT and S571A animals, consistent with involvement of $I_{\text{Na,L}}$ in the arrhythmia phenotype and supporting $I_{\text{Na,L}}$ as a viable anti-arrhythmia target in vivo (Figure 35G).
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 pressure-overload induced heart failure in the...
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 36 and Figure 37). Cardiac hypertrophy, as evidenced by increased LV wall thickness, was apparent in WT, S5571E and S571A animals following 6 weeks of TAC (Figure 37D-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 37G-K).

Specifically, while EF 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.

Figure 36: Representative echocardiograms from WT, S571E, and S571A animals at baseline and following 6 weeks of transaortic constriction (TAC).
Figure 37: Ser571 phosphorylation is necessary for maladaptive remodeling in response to pressure overload. (A-E) Summary data for changes in echocardiographic parameters 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). (F-G) 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.
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 33B), TAC resulted in a large $I_{Na,L}$ in WT comparable to that measured in S571E (Figure 38). In contrast, S571A myocytes were resistant to the increase in $I_{Na,L}$ observed in WT following 6 weeks of TAC (Figure 38). Peak $I_{Na}$ was not different between TAC groups (Figure 40). Previous reports have identified a potential positive feedback loop involving CaMKII and $I_{Na,L}$.\textsuperscript{95, 250, 251} To determine whether elimination of the Ser571 site impacted CaMKII activation via this feedback relationship, we assessed immunoreactive signal of phospho-CaMKII in permeabilized myocytes from TAC hearts. Consistent with previous reports,\textsuperscript{2, 221} phospho-CaMKII levels increased with TAC in WT
heart and were comparable to S571E sham or TAC levels (Figure 39). In contrast, phosho-CaMKII immunoreactive signal was low in sham and TAC S571A mice.

To determine whether phosphorylation at Ser571 contributed to electrical remodeling following TAC, APs were measured in WT, S571E and S571A TAC myocytes. Consistent with $I_{NaL}$ 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 41A,B). Telemetry was also performed to evaluate arrhythmia burden in vivo (Figure 41D-E). Frequent PVCs were observed in S571E but not S571A animals following 6 weeks of TAC, indicating an anti-arrhythmic benefit from eliminating CaMKII phosphorylation at Ser571 in vivo.
(Figure 41D,E). Taken together, these TAC data indicate that phosphorylation at Ser571 is required for increased $I_{Na,L}$, maladaptive remodeling (but not hypertrophy), and arrhythmias in response to pressure overload.

![Figure 40: Peak $I_{Na}$ for different TAC groups. $I_{Na}$ current-voltage relationship measured in WT, S571E and S571A myocytes following 6 weeks of TAC (P = NS; N = 8 from two different preparations for WT, N = 10 from two different preparations for S571E, N = 12 for S571A from three different preparations).]

5.3: 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, especially for cardiovascular disease patients with poor heart function. The apparent impasse may be partly attributed to failures of high profile trials designed to test ion channels as anti-arrhythmia targets. While $I_{Na,L}$ inhibition has emerged as a candidate with great 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 important new findings that suggest it may be possible to specifically target pathogenic component of $I_{Na}$ ($I_{Na,L}$) without altering a myriad of other properties (e.g. channel availability). Namely, using two novel mouse models (S571E and S571A) that we anticipate will serve as useful tools for the study of $in vivo$ molecular mechanism associated with a wide range of cardiovascular disorders, we report that Ser571 is critically important for CaMKII-dependent regulation of $I_{Na,L}$ but not other channel properties previously 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 while phosphorylation at Ser571 alone is not sufficient to induce gross cardiac dysfunction, it is required for maladaptive remodeling and arrhythmias 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 cardiovascular disease states, including heart failure, myocardial infarction, ischemia reperfusion, and diabetes. In heart failure, for example, elevated CaMKII expression and/or activity has been identified in animal models and in human patients. CaMKII dysregulation has also been shown to promote pathology in specific inherited arrhythmia syndromes, including catecholaminergic polymorphic ventricular tachycardia, long QT type 3, ankyrin-B syndrome (long QT type 4), and Timothy syndrome (long QT type 8). In light of this body of evidence,
it is logical to consider when or if a clinical CaMKII drug will be available. In fact, several commercial efforts are underway to develop therapeutic compounds that target CaMKII, in addition to the variety of CaMKII inhibitors already available for research purposes but not optimized for clinical application. Thus, it is not unreasonable to think that eventually a CaMKII inhibitor with cardiovascular indication will reach the market.

Figure 41: 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 (Vm,peak), and rest potential (Vm,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-E) Representative telemetry measurements following exercise and epinephrine injection (2 mg/kg) in S571E and S571A TAC mice. Frequent arrhythmia events (PVCs indicated by red arrows) were apparent in S571E but not S571A mice.
However, it would be short-sighted to not at the same time consider alternative nodes in the CaMKII signaling pathway as potential targets for therapy. $I_{Na,L}$ is one such target that has shown promise. For example, the large 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 (borderline significance, $P=0.07$, for patients with low EF).\textsuperscript{50} Ranolazine has also been evaluated for treatment of atrial fibrillation in both preclinical\textsuperscript{256, 257} and clinical\textsuperscript{258} settings with additional trials underway.\textsuperscript{260} Alternatively, rather than target $I_{Na,L}$ or CaMKII directly, it is interesting to consider targeting the protein(s) responsible for organizing the relevant signaling domain. For example, we have previously identified roles for both $\beta_{IV}$-spectrin and ankyrin-G in coordinating CaMKII and $I_{Na}$ localization at the membrane.\textsuperscript{31, 32} It is possible that “drugging” $\beta_{IV}$-spectrin, and specifically interaction with CaMKII, may have therapeutic benefit.

Previous studies have reported both “gain-of-function” (increased $I_{Na,L}$) and “loss-of-function” (decreased availability) effects of CaMKII phosphorylation on $I_{Na}$.\textsuperscript{2, 32, 73, 97} 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.\textsuperscript{261} A second possibility is that the overall phenotype is the result of multiple phosphorylation events, which have distinct effects on channel behavior. Our unexpected finding that Ser571 regulates $I_{Na,L}$ but not other properties is consistent with the latter scenario and supports involvement for more than
one site in determination of the overall phenotype. Possible candidates include other residues in the DI-DII linker (e.g. Ser516 and/or Thr594)\textsuperscript{97} that may coordinate effects on availability and/or recovery. Also, while our data support a requirement of Ser571 for CaMKII-dependent regulation of $I_{Na,L}$, they do not rule out that phosphorylation at additional sites is co-requisite. It is important to note that while our findings regarding the role of Ser571 in regulating $I_{Na,L}$ are consistent with previous \textit{in vitro} studies, results on availability and recovery are divergent.\textsuperscript{2,97} This discrepancy may arise from differences in protocol inherent in the use of \textit{in vitro} over-expression system (acute) compared to \textit{in vivo} knock-in (chronic) strategy. It will be important going forward to determine whether phosphorylation at other sites\textsuperscript{97,98} may explain other aspects of the phenotype.

Our observation that ablation of the Ser571 site prevents TAC-induced increases in $I_{Na,L}$, APD and arrhythmias is consistent with recent studies using ranolazine in pressure overload-induced murine heart failure.\textsuperscript{221} Specifically, it was shown that, similar to our S571A mouse, ranolazine treatment restored $I_{Na,L}$ and APD to baseline levels following 5 weeks of TAC. Our findings of the importance of phosphorylation of Ser571 for maladaptive remodeling and heart failure in response to pressure-overload are also consistent with previous studies in CaMKII knockout animals.\textsuperscript{105,106,249} These previous studies focused primarily on the role of CaMKII-dependent dysregulation of Ca\textsuperscript{2+} handling in pathogenesis. While our studies support a critical involvement of increased $I_{Na,L}$ in maladaptive remodeling and heart failure, perhaps upstream of Ca\textsuperscript{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\(_2\)).\(^{262, 263}\) Furthermore, direct effects of CaMKII on gene transcription, metabolism and/or cell survival likely play an equally important role in remodeling.\(^{20, 72}\)
Chapter 6: Discussion

6.1: Significance

Heart disease accounts for one of every three deaths in the United States annually.\(^3\) The majority of these deaths are caused by abnormal cardiac electrical activity, known as arrhythmias. The goal of my dissertation research has been to identify molecular pathways responsible for increased arrhythmia burden in the setting of disease. My studies focused on the multifunctional Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII), due to the fact that mounting evidence indicates it is critically involved in human cardiac disease and arrhythmias.\(^2, 19-25\) Overactivation of CaMKII has a proarrhythmic effect and impacts the ability of cells to maintain proper function. Additionally, CaMKII has been shown to be elevated in end-stage human heart failure. Because of these facts, my overall objective with this work is to identify new potential therapeutic targets to prevent arrhythmias in human cardiac disease patients.

6.2: Summary of Findings

My experimental and computational studies have generated new insight into the link between CaMKII dysfunction and a wide range of arrhythmias. Using detailed computational models of the intact sinus node, we were able to establish a link between CaMKII-dependent cell loss and sinus node dysfunction in both acquired (streptozocin-
induced diabetes)\textsuperscript{8} and congenital (ankyrin-B syndrome)\textsuperscript{9,180} disease. Our mathematical models also predicted an interesting relationship between complex activation dynamics at the single cell level and sinus node dysfunction.\textsuperscript{9} Using newly developed mathematical tools for analyzing dynamics of spontaneous AP generation,\textsuperscript{14} we hope to determine whether CaMKII also promotes sinus node dysfunction by promoting irregular SAN cell dynamics.

In the ventricles, previous work from our group established the existence of a complex involved in targeting CaMKII to Na\textsubscript{v}1.5 for phosphorylation (See Figure 3). We next identified the Na\textsubscript{v}1.5 S571 phosphorylation site from a number of other potential targets, and discovered the importance of this site as a CaMKII phosphorylation target \textit{in vitro} for regulation of the late sodium current. The final studies outlined in this text detail our discovery of the singular importance of this site in the context of the late sodium current, and also demonstrate its \textit{in vivo} significance.

\textbf{6.3: Relationship to Clinical Findings}

As a whole, my studies demonstrate the significant contribution that CaMKII makes to heart disease in both the sinus node and the ventricles, and how disruption of CaMKII activity can be cardioprotective in a variety of ways.

One point worth discussing is that in my whole-animal studies, I did not observe any evidence of increased levels of sinus node dysfunction in the Na\textsubscript{v}1.5 S571E animals compared to wildtype mice. This is likely because Na\textsubscript{v}1.5 is not present in all pacemaker cells, and the amount that is present may be blocked by the more positive membrane
potentials that these cells possess.\textsuperscript{264,265} However, sinus node dysfunction is present in human heart failure patients. De Luna et al. recorded Ambulatory monitoring in heart failure patients who died, revealing two patterns of cardiac rhythm immediately before the fatal arrhythmia: 1) decrease in cycle length, and 2) sinus pause. However, despite the lack of apparent sinus defects in these animals, CaMKII could be capable of producing both ventricular and supraventricular arrhythmias in the same disease. This is because of the multitude of intracellular proteins and ion channel targets of CaMKII. As was explored in section 3.2, myocardial infarction (or hyperglycemia) can increase CaMKII activity, leading to SAN dysfunction. Since the S571E animals were modeling increased phosphorylation of this Na\textsubscript{v}1.5 site, increased CaMKII activity could lead to the observed increase in late current.

Additionally, as has been extensively discussed, CaMKII phosphorylates a large number of different proteins, a number of which have been shown to play a role in arrhythmias and heart disease. For example, CaMKII-dependent RyR2 phosphorylation increases diastolic SR Ca\textsuperscript{2+} release, and mice expressing a mutant CaMKII phosphorylation site that prevents phosphorylation are resistant to MI-induced heart failure and arrhythmias.\textsuperscript{77} These two systems may actually be intrinsically related: increasing the late sodium current leads to an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (through reverse NCX activity), which then leads to an increase in CaMKII activation. Because of this, increased late sodium current may actually exacerbate arrhythmias seen through an increase in CaMKII-dependent RyR phosphorylation. Through this same mechanism
(increased CaMKII activity), increases in the late sodium current could actually be contributing to many of the pro-arrhythmic scenarios seen in ECC dysregulation.

6.4: Implications for Therapy

Current therapies for heart disease and arrhythmias are inadequate. This is demonstrated by the fact that the major go-to drugs for heart failure are still ACE inhibitors, which have remained largely unchanged since they were first approved in 1981.\textsuperscript{17} A recent focus of clinical research has been on the anti-anginal drug ranolazine because it has been shown to decrease arrhythmic events. However, despite its apparent promise, recent studies have demonstrated no increase in survival in patient groups given ranolazine when compared to placebo.\textsuperscript{50, 266, 267} This is extremely disappointing; The fact that ranolazine has become such a central research focus despite its apparent ineffectiveness on improving survival demonstrates how desperate we are for improvements in current therapy.

One of the main potential therapeutic elucidations from my work is the singular importance of the S571 site for regulation of the late sodium current. Because CaMKII-phosphorylation of this site appears to so specifically regulate the late sodium current, and the previously established role of the late sodium current in cardiac disease, drugs designed to block regulation of this site may have therapeutic benefit for the prevention of cardiac arrhythmia. While ranolazine does specifically regulate the late component of the sodium current, it is not a “clean” drug, as it also hits a myriad of other targets, including potassium channels and channels involved in calcium release.\textsuperscript{268} Because of
this, specific regulation of the S571 site may prove to be more therapeutic than current ranolazine treatment. However, because targeting a specific phosphorylation site may be difficult, it may make more sense to focus on interruption of targeting of CaMKII to Na\textsubscript{v}1.5 via the identified complex (Figure 3).

**6.5: Study Limitations**

Despite the strong evidence for the importance of the S571 site, there are a number of limitations of my study. One of the major limitations is the surprising fact that this site appeared to only impact the late sodium current. We expected this site to also play a role in regulation of the channel’s steady-state inactivation and recovery from inactivation, but we found no evidence that this was the case. Additionally, the S571E mice appeared to function normally in baseline conditions, and required a decent degree of agitation before demonstrating negative health consequences to this phosphomimetic mutation. This is likely because of compensatory effects going on within the cell. It can be very difficult to clarify all of the other changes occurring because of the increase in late current, but discovering what compensatory effects are involved with this upregulation could be very important before implementing any therapies specifically targeting this site.

An additional limitation of our studies was that they were performed in mice. Mice are a fantastic model for human disease, have a highly similar genome, and allow us to directly manipulate genetics so we can isolate the impacts of complex diseases on overall function. However, mice are, of course, not human, and so not all conclusions
drawn from mouse models are applicable for human models. This can be especially true in transgenic mice; the phenotypes in targeted mutant mice are not always a 1:1 parallel with observed human defects.  

6.6: Next Steps

Experimentally, there are a number of directions that this research could go. One of the most obvious approaches is to investigate the role of the other identified CaMKII phosphorylation sites on Na,1.5. Because of the combination of the singular importance of the S571 site on the late sodium current, but the previously identified role of CaMKII-based phosphorylation of Na,1.5 on steady-state inactivation and recovery from inactivation, it is likely that CaMKII targets some of these additional sites for regulation of those functions.

Additionally, a variety of other proteins have been shown to regulate Na,1.5 function. For example, Protein Kinase A (PKA) phosphorylates many common targets to CaMKII, including Na,1.5. However, it is currently unknown what role Na,1.5 phosphorylation by PKA plays in regulation of the late sodium current. Previous studies that linked overexpressed PKA to an increase in late current found that the late current returned to normal levels when CaMKII was suppressed. Because of this, the S571 transgenic mice may be a perfect model for determining the isolated impact of PKA on the late sodium current.

A third direction would be to look at proteins that are also dependant on βIV-Spectrin (the protein responsible for proper targeting of CaMKII to Na,1.5). One example of this is
the potassium channel TREK-1. Previous work from our group has shown that βIV-Spectrin regulates TREK-1 membrane targeting in the heart, and that interrupt of TREK-1/βIV-Spectrin is proarrhythmic. Because of this, it would be important to investigate any potential role that TREK-1 regulation plays in the setting of heart disease.

In addition, my research focused on studies examining the sinus node and ventricular cells. However, Ca\(^{2+}\) overload and CaMKII dysregulation have been shown to promote atrial arrhythmias as well. Because of this, the impact of regulation of the late sodium current on atrial tissue could be studied in the S571 animals.

Computationally, the next steps that I believe should be made are to try to apply the principles of machine learning to the output from current models. As described previously, machine learning algorithms “learn” from data in order to make predictions about the outputs of future data. Utilizing these tools could allow for non-modeling research laboratories to much more easily make use of mathematical models to enhance or direct their own research. Some of the primary uses for such a system could include identifying targets related to a disorder, clustering data into various groups (for example, grouping different ECG traces based on their apparent disorder), or predicting future research targets.

Within mathematical modeling, one future direction that could be taken includes creating a more complete model of CaMKII activity and regulation. However, not only are there a large number of identified phosphorylation targets already identified, but new targets of CaMKII are being realized each year, so this would be a significant undertaking. However, the creation of such a model could allow for significant insight
into how CamKII overexpression or increased activation can impact the overall health of a cell.

In conclusion, the integrative approach taken in the preceding work has developed new and important insight into the mechanisms of CaMKII regulation of the late sodium current and its contribution to other forms of cardiac disease. Our studies have shown that CaMKII contributes to arrhythmias in a number of ways. Our major findings include: 1) CaMKII can induce structural changes that lead to heart disease, particularly in the sinus node; and 2) overactivation of CaMKII displays acute effects through its phosphorylation of ion channels, as we observed primarily in the sodium channel. Additionally, we developed new mathematical tools to analyze sinus node cell stability that demonstrated how CaMKII dysregulation can contribute to arrhythmias by altering subcellular structures.
References


266. Zipes, D.P., ACC/AHA/ESC 2006 guidelines for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: a report of the American College of Cardiology/American Heart Association Task Force and the European Society of Cardiology Committee for Practice Guidelines (Writing Committee to Develop Guidelines for Management of Patients With Ventricular


Appendix A: Methods

Diabetes Increases Mortality after Myocardial Infarction by Oxidizing CaMKII

Model geometry was represented as a 2-dimensional 400 × 45 rectangular grid with a spatial resolution of 40 μm. Cell types in different regions of the model were determined by histologically reconstructed sections through the rabbit RA, as described previously.\textsuperscript{187,273} Cell loss in the SAN was simulated by randomly replacing a percentage of normal SAN cells (1% for vehicle treated and 19% for STZ treated) with poorly coupled inexcitable cells. Detailed mathematical models were used to simulate central SANC, peripheral SANC, and atrial action potentials (Table 2).\textsuperscript{11,13}

Regional differences in cell size, coupling, and ion channel expression within the node were taken into account by making cell properties a function of space, following the

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<th>R\textsubscript{gap, transverse}</th>
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<td>15.0 Ωcm\textsuperscript{2}</td>
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<td>Kurata et al. 2008\textsuperscript{†}</td>
<td>Equation 2</td>
<td>R\textsubscript{gap, long}</td>
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<td>Kurata et al. 2002\textsuperscript{§}</td>
<td>Equation 2</td>
<td>R\textsubscript{gap, long}</td>
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<tr>
<td>Block zone</td>
<td>Butters et al. 2010\textsuperscript{‡}</td>
<td>4000 Ωcm\textsuperscript{2}</td>
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<td>Inexcitable cell</td>
<td>Morita et al. 2009\textsuperscript{†}</td>
<td>4000 Ωcm\textsuperscript{2}</td>
<td>R\textsubscript{gap, long}</td>
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\textsuperscript{†Kurata et al. Biophys J. 2008;95:951-977 with sodium channel conductance = 1.8523x10\textsuperscript{-6} nS/pF.}
\textsuperscript{‡Butters et al. Circ Res. 2010;107:126-37. Block zone modeled as coupled region of inexcitable cells with passive leak conductance (Eleak = -70 mV).}

Table 2: Mathematical Models for different regions of intact SAN
previously described approach (Table 3).

\begin{align*}
\text{Capacitive membrane area (cm}^2) \\
\text{\small \hspace{1cm} \text{x is distance in cm.}} \\
A_{cap} &= 110 \times 10^{-6} - 45 \times 10^{-6} \left( \frac{1}{1 + \exp(-4(x-7.0))} + \frac{1}{1 + \exp(4(x-9.0))} \right) \tag{1}
\end{align*}

\begin{align*}
\text{Gap junction resistance (Ωcm}^2) \\
R_g &= 110 \left( \frac{1}{1 + \exp(-4(x-7.0))} + \frac{1}{1 + \exp(4(x-9.0))} \right) - 108.5 \tag{2}
\end{align*}

\begin{align*}
\text{Conductance of fast sodium current (mS/cm}^2) \\
\mathcal{g}_{Na} &= 3.7 \times 10^{-6} - 1.85 \times 10^{-6} \left( \frac{1}{1 + \exp(-5(x-6.5))} + \frac{1}{1 + \exp(5(x-9.5))} \right) \tag{3}
\end{align*}

Table 3: Equations for Mathematical Modeling

The 2-dimensional cable equation describing action potential propagation was solved using an alternating direction implicit method and a fixed time step of 0.005 ms.\textsuperscript{274} Initial conditions used for cells from different regions are provided in Supplemental Tables 5 and 6.

Eight seconds of spontaneous activity were simulated, and action potentials from different SAN regions were analyzed for activation pattern and rate. Computer code was written in C++, compiled using Intel Composer XE 2011 for Linux, and executed on a Dell PowerEdge R515 server (Dual 6 core, 32 GB RAM running CentOS-6.2). Data from simulation of activation were visualized in MATLAB (R2012a).
Atrial Fibrillation and Sinus Node Dysfunction in Human Ankyrin-B Syndrome: a Computational Analysis

Simulation of normal (wild-type) and ankyrin-B+/− atrial APs - Ion channel kinetics for the atrial cardiomyocyte were simulated using two different well-validated models of the human atrial cardiomyocyte. Modifications to the equations were made to account for experimentally measured changes in NCX, NKA, and L-type Ca2 channel membrane in ankyrin-B/cardiomyocytes. Specifically, NCX and NKA surface expressions were reduced by 40 and 25%, respectively, from their wild-type (WT) values, consistent with experimental measurements in ankyrin-B+/− cardiomyocytes. Furthermore, the surface expression of the L-type Ca2 channel was reduced to produce a 40% decrease in the L-type Ca2 current peak current at a test potential of -10 mV as measured experimentally in ankyrin-B/atrial myocytes. Single cell models were paced from rest to steady state (1,000 s of pacing to produce beat-to-beat change in APD < 0.1%) over a range of pacing cycle lengths (CLs from 2,000 to 300 ms; stimulus amplitude = -20uA/uF; stimulus duration = 2 ms) using a conservative stimulus. The steady-state values for all state variables were used as initial conditions for subsequent simulations. In the case of multicellular simulations, steady-state values from pacing the single cell at CL of 300 ms were used as initial conditions (Table A1).

Simulation of reentry in atrial tissue - Spiral wave reentry was induced in the two-dimensional tissue model by cross-field stimulation of two perpendicular rectilinear
waves. Fibrosis was simulated by replacing a percentage of normal cells (3% for WT, and 9% for ankyrin-B⁺⁻) with poorly coupled inexcitable cells. Inexcitable cells were modeled as passive elements with a leak conductance to produce a rest potential of 40 mV in agreement with experimental measurements from isolated adult atrial fibroblasts (21). Two different random fibroblast distributions were implemented [pseudo-random numbers generated using the rand() command in the stdlib.h C standard library]: 1) “diffuse,” where elements were distributed individually, and 2) “clustered,” where groups of up to five were designated at a time. The two-dimensional cable equation describing AP propagation was solved using an alternating direction implicit method, a mesh size of 200 m and a fixed time step of 0.005 ms.

Simulation of WT and ankyrin-B⁺⁻ SAN AP - Ion channel kinetics underlying the SAN AP were simulated using a recently published model of the murine SAN cell. Importantly, this model includes separate formulations for both L-type Ca2 channel subtypes found in the SAN (Caᵥ1.3 and Caᵥ1.2) and accurately reproduces spontaneous firing rate and AP morphology of the mouse SAN cell. Similar to the atrial cell model, NCX and NKA surface expressions were reduced by 40 and 25%, respectively. As ankyrin-B binds Caᵥ1.3 but not Caᵥ1.2, the surface expression of the Caᵥ1.3 (but not Caᵥ1.2) component of L-type Ca²⁺ channel was reduced by 40% as measured experimentally in ankyrin-B/SAN myocytes. Parametric analysis was performed by simultaneously varying Caᵥ1.3 maximal conductance and NKA pumping rate or NCX scaling factor.
Intact SAN - Detailed mathematical models were used to simulate SAN cell and atrial APs (Table 4). A model of the human atrial cell was modified to produce an AP waveform more similar to the murine atrial AP (equations provided in Appendix B). The goal was not to create a model of the mouse atrial myocyte (insufficient data currently available) but rather to generate a waveform with physiological morphology to couple with the murine SAN models. Importantly, measured and simulated APDs show agreement for both WT and ankyrin-B murine atrial cells. Geometry of the intact SAN was represented as a two-dimensional 400 45 rectangular grid. Cell types in different regions were determined by histologically reconstructed sections through the rabbit right atrium (RA), as described, scaled to a spatial resolution of 9 m to match dimensions of the mouse SAN. Regional differences in cell size, coupling, and ion channel expression within the SAN node were taken into account by making cell properties a function of space, following the previously described approach (equations provided in Appendix B). Initial conditions for cells from different regions were derived from steady-state values for individual cell models following either 200 s of

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<td>Morita et al. 2009†</td>
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Table 4: Mathematical Models for Different Regions of Intact SAN. Model equations for human atrial cell model are found in original publication. *Modified to produce shorter AP. #Conductances of select channels scaled from central to periphery according to Eqs. 2–4. †Eleak = -40 mV. Rgap, gap junction resistance; SAN, sinoatrial node.
spontaneous activity (SAN cell) or 1,000 s of rapid pacing (atrial cell) (values provided in Table 5 and Table 6). While these initial conditions do not represent true steady state for the coupled system, they greatly reduce transient initial fluctuations in CL and other properties. Ten seconds of spontaneous activity were simulated, and APs from different SAN regions were analyzed for activation pattern and rate.

All computer code was written in C++, compiled using Intel Composer XE 2011 for Linux and executed on a Dell PowerEdge R515 server (Dual 6 core, 32 GB RAM running CentOS-6.2). Simulation activation data were visualized in MATLAB (R2012a).

**Histology of atrium from WT and ankyrin-B^{+/-} mice** - Hearts from age- and sex-matched (2-mo-old males) WT and ankyrin-B^{+/-} mice were fixed in 4% paraformaldehyde. Tissues were sectioned and stained with Masson’s trichrome. Light microscopy images were analyzed using ImageJ. Hearts were obtained after animals were euthanized by acute CO2 asphyxiation followed by cervical dislocation in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and the protocols were approved by the Institutional Animal Care and Use Committee of Ohio State University.

**Cycle Length Restitution in Sinoatrial Node Cells: A Theory for Understanding**

**Spontaneous Action Potential Dynamics**

Ion channel kinetics underlying the SAN AP were simulated using a detailed model of the murine SAN cell. This particular model was selected based on its accurate
representation of important features of the murine SAN AP (e.g. morphology, firing rate), facilitating comparison to our previous experimental and modeling studies on SAN dynamics in the mouse.\(^8,9,142,172\) We expect that, similar to APD restitution, CL restitution will be broadly applicable for understanding SAN dynamics across species.

The model was allowed to reach steady-state (20 sec of spontaneous activity) before implementing a perturbation protocol to assess CL restitution. Briefly, a subthreshold conservative stimulus (10 ms duration)\(^{275}\) is applied at the maximum diastolic potential (MDP) between the last steady-state AP (APss) and the first perturbed AP (AP1). The stimulus amplitude is varied to advance or delay onset of AP1 until either phase 5 resetting occurs (for a depolarizing stimulus) or perturbation fails to cause any further change in onset of AP1 (for a repolarizing stimulus). The first perturbed CL (CL1) is defined as the time interval between AP1 and APss (time of peak used as reference), while CL2 is the time between the second AP following perturbation (AP2) and AP1. Finally, the CL restitution curve is created by plotting CL2 vs. CL1 over the entire range of applied stimuli.

Spontaneous activity and termination were also studied in a one-dimensional fiber comprised of 50 mouse SAN cells coupled to 50 mouse atrial cells. A gradient in electrophysiological properties and electrical coupling was introduced in the SAN section, as described,\(^9\) to allow for proper activation of the fiber. Computer code for mathematical models was written in C++ and compiled using Intel Composer XE 2011 for Linux. Computer simulations were performed on a Dell
PowerEdge R515 server (Dual 6 core, 32 GB RAM running CentOS-6.2), as described.\textsuperscript{2,8,142,179,275}

**Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II-Based Regulation of Voltage-Gated Na\textsuperscript{+} Channel in Cardiac Disease**

**Molecular Biology** - Na\textsubscript{v}1.5 alpha-subunit cDNA was engineered in frame into pIRES2–enhanced green fluorescent protein (GFP; Clontech). Na\textsubscript{v}1.5 arrhythmia variant and tetrodotoxin-sensitive (C373Y) constructs were generated by the Quikchange method with wild-type (WT) Na\textsubscript{v}1.5 as template. Vectors were completely sequenced. Na\textsubscript{v}1.5 constructs were cotransfected with murine pcDNA3.1 T287D constitutively active CaMKII Delta\textsuperscript{32} or empty vector into HEK and primary myocytes using X-tremeGENE 9 (Roche). For HEK experiments, the Na\textsubscript{v}1.5 beta subunit (pcDNA3.1 hbeta1) was cotransfected with the Na\textsubscript{v}1.5 alpha subunit. To verify successful cotransfection of CaMKII with channel constructs, Na current was measured with and without the competitive CaMKII inhibitor autocamtide-2-related inhibitory peptide (AnaSpec) in the pipette.

**Electrophysiology** - Electrophysiological recordings were obtained from GFP-positive cells. Whole-cell sodium currents were measured with standard protocols as described in detail.\textsuperscript{32,65,277} Action potentials (APs) were recorded with the perforated (amphotericin B) patch-clamp technique at physiological temperature with pacing frequency of 1 Hz.
Detailed electrophysiological protocols and conditions are provided in the online-only Data Supplement.

**Computational Model** - Transmembrane currents and ion concentration changes are described by a well-validated model of the human ventricular myocyte$^{244}$ with a Markov model of voltage-gated Na+ current (INa) assuming 1 normal and 1 variant allele (50% variant channels).$^{1,12,245}$ Transition rate expressions, model parameters, and initial conditions for WT and variant Nav1.5 can be found in Tables I through III in the online-only Data Supplement.

**Electrostatic Potential Molecular Modeling** - Nav1.5 loop motif (W565–S577) structures were built and energy minimized with Amber.$^{278}$ Electrostatic potentials were computed by the Delphi finite-difference Poisson-Boltzmann solver$^{279}$ and mapped to molecular surfaces of W565-S577 containing the CaMKII phosphorylation site.

**Experimental Model of Myocardial Infarction and Immunoblotting** - Myocardial infarction was produced in canines by total coronary artery occlusion as described previously.$^{170,280}$ Ventricular lysates were prepared and analyzed by SDS-PAGE as described.$^{32,170}$ Immunoblotting was performed with validated affinity-purified antibodies to phospho-Nav1.5(S571) or total Nav1.5.$^{32}$ Slight differences in protein loading were corrected by the use of an internal control standard (rabbit polyclonal antibody to actin; Santa Cruz Biotechnology, Inc). This investigation conforms to the
Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No. 85–23, 1996).

**Human Tissue Samples** - Left ventricular tissue was obtained from explanted hearts of patients undergoing orthotopic heart transplantation through the Cooperative Human Tissue Network: Midwestern Division at Ohio State University. Approval for use of human subjects was obtained from the Institutional Review Board of Ohio State University. Left ventricular tissue from healthy donor hearts not suitable for transplantation (subclinical atherosclerosis, age, no matching recipients) was obtained through the Iowa Donors Network and the National Disease Research Interchange. The investigation conforms to the principles outlined in the Declaration of Helsinki. Age and sex were the only identifying data acquired from tissue providers, and the Iowa Human Subjects Committee deemed that informed consent was not required.

**Adult Cell Isolation and Pacing** - Isolated ventricular myocytes from WT and AC3-I adult mice (age, 2–3 months) were plated onto a 6-well tray precoated with laminin (Invitrogen). Cells were pretreated for 30 minutes with the phosphatase inhibitor okadaic acid (2 umol/L) and paced for 10 minutes at 2 Hz with the C-pace multichannel stimulator (Ionoptix). Immediately before the onset of pacing, 100 nmol/L isoproterenol was added. A subset of cells were pretreated for 30 minutes before the onset of pacing with the CaMKII inhibitor KN-93 (10 umol/L). Cell lysates were analyzed by SDS-PAGE and immunoblotting. Slight differences in protein loading were corrected with monoclonal anti-GAPDH (Fitzgerald) used as an internal control standard.
Statistics - P values were determined with the unpaired Student t test (2 tailed) for single comparisons. Multiple comparisons were analyzed by use of 1-way ANOVA. The Bonferroni test was used for post hoc testing (SigmaPlot 12.0). If the data distribution failed normality tests with the Shapiro-Wilk test, rank-based ANOVA and the Dunn multiplecomparisons test were performed. The null hypothesis was rejected for P<0.05.

Voltage-Gated Sodium Channel Phosphorylation by CaMKII at Ser571 Regulates Late Current, Arrhythmia, and Heart Function In Vivo.

Animals - Scn5a S571E and S571A knock-in mice were generated (genOway) in C57/Bl6 background using a Flp-mediated strategy to remove neomycin selection cassette. Resulting animals expressed either the S571E or S571A point mutation (Figure 31). Experiments were performed in 2-month-old male mice. Animals were euthanized using CO2 and cervical dislocation followed by collection of tissue or cell isolation. Studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health following protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Electrophysiology - Ventricular myocytes were isolated from Langendorff-perfused adult mouse hearts, as described previously.2, 32, 271 $I_{Na}$ 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 calculated using two different methods: 1) average current over the interval 50-150 ms following the peak current, normalized to peak; 2) and 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. APs were recorded using the perforated (amphotericin B) patch-clamp technique at room temperature in Tyrode’s solution (bath). The pipette solution contained (in mM): potassium aspartate (130), NaCl (10), HEPES (10), CaCl2 (0.04), MgATP (2), phosphocreatine (7), NaGTP (0.1), and 240 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. AP duration (APD) was assessed as the time from the AP upstroke to 90% repolarization to baseline (APD90).

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.271 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.

**Mouse heart failure model with proximal aortic banding** - Transaortic constriction was performed to induce pressure overload conditions in adult male mice. 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 parameters. 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.

**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), phospho-Nav1.5(S571), CaMKIIδ (Badrilla), phospho-CaMKIIδ (Thermo Scientific), Ankyrin-G (Santa Cruz), N-Cadherin (Invitrogen), and GAPDH (Fitzgerald).
Statistics - Data are presented as mean±SEM. 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. 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. Contingency data were analyzed using Chi-Square test. The null hypothesis was rejected for $P<0.05$. 
Appendix B: Additional Modeling Parameters, Equations, and Tables

Diabetes increases mortality after myocardial infarction by oxidizing CaMKII

<table>
<thead>
<tr>
<th>State Variable</th>
<th>Definition</th>
<th>Initial Value</th>
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</thead>
<tbody>
<tr>
<td>$m$</td>
<td>Na$^+$ current activation gate</td>
<td>0.004147463955</td>
</tr>
<tr>
<td>$h$</td>
<td>Na$^+$ current inactivation gate</td>
<td>0.9421032891</td>
</tr>
<tr>
<td>$j$</td>
<td>Na$^+$ current slow inactivation gate</td>
<td>0.9319148046</td>
</tr>
<tr>
<td>$d$</td>
<td>L-type Ca$^{2+}$ current activation gate</td>
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<tr>
<td>$f$</td>
<td>L-type Ca$^{2+}$ voltage-dependent inactivation gate</td>
<td>0.8466729054</td>
</tr>
<tr>
<td>$f_{u}$</td>
<td>L-type Ca$^{2+}$ calcium-dependent inactivation gate</td>
<td>0.5727907784</td>
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<tr>
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<tr>
<td>$ui$</td>
<td>Ultrarapid rectifier K$^+$ current inactivation gate</td>
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<td>$[Ca^{2+}]_{NSR}$</td>
<td>Ca$^{2+}$ concentration in network SR (mM)</td>
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<tr>
<td>$V_m$</td>
<td>Transmembrane potential (mV)</td>
<td>-79.01495665</td>
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</table>

Table 5: Initial conditions for state variables in mathematical model of atrial action potential. Single cell was paced to steady-state at cycle length = 300 ms. Model equations for human atrial cell model are found in original publication. SR, sarcoplasmic reticulum.
Table 6: Initial conditions for state variables in mathematical models of central and peripheral SAN cells. Single cell underwent spontaneous activity for 10 seconds. Model equations for rabbit central and peripheral node cells are found in original publication.\textsuperscript{13} SR, sarcoplastic reticulum.

<table>
<thead>
<tr>
<th>State Variable</th>
<th>Definition</th>
<th>Initial Value (Central)</th>
<th>Initial Value (Peripheral)</th>
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<td>$m$</td>
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<td>$h$</td>
<td>Na$^+$ current inactivation gate</td>
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<td>$j$</td>
<td>Na$^+$ current slow inactivation gate</td>
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<td>0.2498661863</td>
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<td>$qa$</td>
<td>Sustained inward current activation gate</td>
<td>0.4591365202</td>
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<td>Sustained inward current inactivation gate</td>
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<td>$V_m$</td>
<td>Transmembrane potential (mV)</td>
<td>-57.88001647</td>
<td>-74.11420057</td>
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Capacitative membrane area (cm$^2$):

$A_{cap} = 110 \times 10^{-6} \left( \frac{1}{1+\exp \left(-4(x-7.0)\right)} + \frac{1}{1+\exp \left(4(x-9.0)\right)} \right)$  \hspace{1cm} [1]

Gap junction resistance (\Omega cm$^2$):

$R_g = 110 \left( \frac{1}{1+\exp \left(-4(x-7.0)\right)} + \frac{1}{1+\exp \left(4(x-9.0)\right)} \right) - 108.5$  \hspace{1cm} [2]

Conductance of fast sodium current (mS/cm$^2$):

$\tilde{g}_{Na} = 3.7 \times 10^{-6} - 1.85 \times 10^{-6} \left( \frac{1}{1+\exp \left(-5(x-6.5)\right)} + \frac{1}{1+\exp \left(5(x-9.5)\right)} \right)$  \hspace{1cm} [3]

Figure 42: Supplemental Equations for mathematical modeling.
**Atrial Fibrillation and Sinus Node Dysfunction in Human Ankyrin-B Syndrome: a Computational Analysis**

<table>
<thead>
<tr>
<th>State Variable</th>
<th>Definition</th>
<th>WT Initial Values</th>
<th>Ankyrin-B&lt;sup&gt;−&lt;/sup&gt; Initial Values</th>
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<tr>
<td>(h)</td>
<td>(\text{Na}^+) current inactivation gate</td>
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<tr>
<td>(j)</td>
<td>(\text{Na}^+) current slow inactivation gate</td>
<td>0.9319148046</td>
<td>0.9688717543</td>
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<td>(d)</td>
<td>L-type (\text{Ca}^{2+}) current activation gate</td>
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<td>(f)</td>
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<td>(X_S)</td>
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<tr>
<td>(v)</td>
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<td>(\text{Ca}^{2+}) concentration in myoplasm (mM)</td>
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<td>([\text{Ca}^{2+}]_{\text{Up}})</td>
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<td>133.6894531</td>
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\(V_m\) Transmembrane potential (mV)

\(-79.01495665\)

\(-80.62135663\)

Table 7: Initial conditions for state variables in mathematical models of wild-type and Ankyrin-B<sup>−</sup> atrial action potential. Single cell was paced to steady-state at cycle length = 300 ms. Model equations for human atrial cell model are found in original publication. SR, sarcoplasmic reticulum; AP, action potential; WT, wild-type.
Figure 43: Equations for heterogeneity in SAN region. 

\[ R_g = 110 \left( \frac{1}{1 + \exp(-310.8(x - 0.141))} + \frac{1}{1 + \exp[100.8(x - 0.22)]} \right) - 108.5 \]  

\[ \bar{g}_{Na} = 3.0 - \left( \frac{1}{1 + \exp[-310.8(x - 0.163)]} \frac{1}{1 + \exp[310.8(x - 0.186)]} \right) \]  

\[ \bar{g}_{Ca_{L2}} = 3.0 - \left( \frac{1}{1 + \exp[-310.8(x - 0.163)]} \frac{1}{1 + \exp[310.8(x - 0.186)]} \right) \]  

\[ \bar{g}_{K_{F}} = 5.0 - 2.0 \left( \frac{1}{1 + \exp[-310.8(x - 0.163)]} \frac{1}{1 + \exp[310.8(x - 0.186)]} \right) \]  

[4] Gap junction resistances (in Ωcm²), where x is distance (in cm).  
[5] Conductance of fast sodium current (in mS/cm²).  
[6] Conductance of Ca_L2 L-Type Ca²⁺ current (in ms/cm²).  
[7] Conductance of rapid delayed rectifier K⁺ current (in mS/cm²)
Figure 44: Equations and parameters for the mathematical model of murine atrial AP waveform.

Equations and parameters that differ from original publication are provided above.

Equations derived from model of mouse ventricular AP regarding:

1. L-Type Ca\(^{2+}\) Current
2. Ultrarapid delayed rectifier K\(^{+}\) current
3. Transient outward K\(^{+}\) current
4. Noninactivating steady-state K\(^{+}\) current
5. Ryanodine receptor Ca\(^{2+}\) release channel

All other equations and parameters may be found in original publication.

\[ g_{CaL} = 0.175 \text{ nS/pF} \]  [8]

\[ I_{Kur} = G_{Kur} a_{ur} i_{ur} (V_m - E_K) \]

\[ \tau_{ur} = 1200 - 170/[1 + \exp \left( \frac{V_m + 45.2}{5.7} \right) ] \]  [9]

\[ a_{ur,\infty} = \frac{1}{1 + \exp \left( \frac{-V_m - 22.5}{7.7} \right) } \]

\[ i_{ur,\infty} = \frac{1}{1 + \exp \left( \frac{-V_m - 45.2}{7.7} \right) } \]

\[ G_{Kur} = 0.14 \text{ nS/pF} \]

\[ I_{Kto,f} = G_{Kto} a_{tto} \alpha_{tto} (V_m - E_K) \]

\[ \alpha_{tto} = 0.18064 \exp \left( 0.03577(V_m + 30) \right) \]

\[ \beta_{tto} = 0.3956 \exp \left( -0.06237(V_m + 30) \right) \]

\[ \alpha_{tto} = \frac{2.2094 \times 10^{-5} \exp \left( -0.1429V_m \right) + 1}{5.6002 \times 10^{-4} \exp \left( -0.1429V_m \right) + 1} \]  [10]

\[ \beta_{tto} = \frac{0.1138 \exp \left( -0.1429V_m \right)}{6.14926 \exp \left( 0.1429V_m \right) + 1} \]

\[ G_{Kto,f} = 0.0798 \text{ nS/pF} \]

\[ I_{Kss} = G_{Kss} a_{Kss} (V_m - E_K) \]

\[ a_{Kss,\infty} = \frac{1}{1 + \exp \left( \frac{V_m + 22.5}{7.7} \right) } \]  [11]

\[ \tau_{aKss} = 39.3 \exp \left( -0.08628V_m \right) + 13.17 \]

\[ G_{Kss} = 0.035 \text{ nS/pF} \]

\[ \tau_{activation} = 4.0 \text{ ms} \]  [12]
Figure 45: Modified model of murine atrial action potential waveform. (A): simulated WT and ankyrin-B^{+/-} action potentials following steady-state pacing at CL = 1,000 ms. (B): simulated and measured (9) APD, APD90, APD at 90% repolarization.

Ca^{2+}/Calmodulin-Dependent Protein Kinase II-Based Regulation of Voltage-Gated Na+ Channel in Cardiac Disease
<table>
<thead>
<tr>
<th>Transition Rate (ms⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a_1 = \frac{p_{1a1}}{p_{2a1} \exp \left( -\frac{V_m + 2.5}{17} \right)} + 0.20 \exp \left( -\frac{V_m + 2.5}{150} \right) )</td>
<td>Bondarenko et al.¹</td>
</tr>
<tr>
<td>( a_2 = \frac{p_{1a1}}{p_{2a1} \exp \left( -\frac{V_m + 2.5}{15} \right)} + 0.23 \exp \left( -\frac{V_m + 2.5}{150} \right) )</td>
<td>“</td>
</tr>
<tr>
<td>( a_3 = \frac{p_{1a1}}{p_{2a1} \exp \left( -\frac{V_m + 2.5}{12} \right)} + 0.25 \exp \left( -\frac{V_m + 2.5}{150} \right) )</td>
<td>“</td>
</tr>
<tr>
<td>( a_4 = \frac{1}{p_{1a4} \exp \left( -\frac{V_m + 7}{16.6} \right)} + 0.393956 )</td>
<td>“</td>
</tr>
<tr>
<td>( a_5 = \frac{p_{1a5}}{p_{2a5}} \exp \left( -\frac{V_m}{p_{2a5}} \right) )</td>
<td>“</td>
</tr>
<tr>
<td>( a_6 = a_4 / p_{1a6} )</td>
<td>“</td>
</tr>
<tr>
<td>( a_7 = p_{1a7} a_4 )</td>
<td>“</td>
</tr>
<tr>
<td>( a_8 = p_{1a8} )</td>
<td>Grandi et al.¹²</td>
</tr>
<tr>
<td>( b_1 = \frac{p_{1b1} \exp \left( -(V_m + 2.5) / p_{2b1} \right)}{p_{2b1}} )</td>
<td>Bondarenko et al.¹</td>
</tr>
<tr>
<td>( b_2 = \frac{p_{1b2} \exp \left( -(V_m - p_{2b2}) / p_{2b1} \right)}{p_{2b1}} )</td>
<td>“</td>
</tr>
<tr>
<td>( b_3 = \frac{p_{1b3} \exp \left( -(V_m - p_{2b3}) / p_{2b1} \right)}{p_{2b1}} )</td>
<td>“</td>
</tr>
<tr>
<td>( b_4 = (a_3 a_4 a_5) / (b_3 b_5) )</td>
<td>“</td>
</tr>
<tr>
<td>( b_5 = p_{1b6} (p_{2b5}(V_m + 7.0)) )</td>
<td>“</td>
</tr>
<tr>
<td>( b_6 = p_{1b6} a_5 )</td>
<td>“</td>
</tr>
<tr>
<td>( b_7 = p_{1b7} a_5 )</td>
<td>“</td>
</tr>
<tr>
<td>( b_8 = p_{1b8} )</td>
<td>Grandi et al.¹²</td>
</tr>
<tr>
<td>( a_9 = [\text{Ranolazine}] p_{1a9} )</td>
<td>“</td>
</tr>
<tr>
<td>( b_9 = p_{1b9} )</td>
<td>“</td>
</tr>
</tbody>
</table>

Table 8: Transition rate expressions for mathematical model of Nav1.5.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wildtype</th>
<th>A572D</th>
<th>Q573E</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1a1</td>
<td>7.5207</td>
<td>6.8920</td>
<td>4.6305</td>
<td>Fit to IV curve.</td>
</tr>
<tr>
<td>P2a1</td>
<td>0.1027</td>
<td></td>
<td></td>
<td>Bondarenko et al.</td>
</tr>
<tr>
<td>P1a4</td>
<td>0.188495</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1a5</td>
<td>7.0e-7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2a5</td>
<td>7.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1b1</td>
<td>0.1917</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2b1</td>
<td>20.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1b2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2b2</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1b3</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2b3</td>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1b5</td>
<td>0.0108469</td>
<td>0.0604095</td>
<td>0.0453576</td>
<td>Fit to inactivation and IV curves.</td>
</tr>
<tr>
<td>P2b5</td>
<td>2e-5</td>
<td></td>
<td></td>
<td>Bondarenko et al.</td>
</tr>
<tr>
<td>P1a6</td>
<td>1000.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1b6</td>
<td>6.0448e-3</td>
<td>2.5121e-3</td>
<td>2.6199e-3</td>
<td>Fit to recovery.</td>
</tr>
<tr>
<td>P1a7</td>
<td>1.05263e-5</td>
<td></td>
<td></td>
<td>Bondarenko et al.</td>
</tr>
<tr>
<td>P1b7</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1a8</td>
<td>4.0933e-13</td>
<td>1.4458e-4</td>
<td>6.5226e-5</td>
<td>Fit to late current.</td>
</tr>
<tr>
<td>P1b8</td>
<td>9.5e-4</td>
<td></td>
<td></td>
<td>Grandi et al.</td>
</tr>
<tr>
<td>P1a9</td>
<td>8.2 mM⁻¹ms⁻¹</td>
<td></td>
<td></td>
<td>Wang et al.</td>
</tr>
<tr>
<td>P1b9</td>
<td>0.022</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum INa conductance</td>
<td>7.35</td>
<td>9.75</td>
<td>11.60</td>
<td>Fit to peak IV curve.</td>
</tr>
</tbody>
</table>

Table 9: Parameters for mathematical models of wildtype and variant Na,1.5
<table>
<thead>
<tr>
<th>State Variable</th>
<th>Definition</th>
<th>WT</th>
<th>A572D</th>
<th>Q573E</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁</td>
<td>$I_{Na}$ closed state</td>
<td>0.0003850597267</td>
<td>0.0003849053676</td>
<td>0.0003848309613</td>
</tr>
<tr>
<td>C₂</td>
<td>&quot;</td>
<td>0.02639207662</td>
<td>0.02638818278</td>
<td>0.02638630536</td>
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<tr>
<td>C₃</td>
<td>&quot;</td>
<td>0.7015088787</td>
<td>0.7015704583</td>
<td>0.7016001465</td>
</tr>
<tr>
<td>IC₂</td>
<td>$I_{Na}$ inactive state</td>
<td>0.009845083654</td>
<td>0.009840770794</td>
<td>0.009838691924</td>
</tr>
<tr>
<td>IC₃</td>
<td>&quot;</td>
<td>0.2616851145</td>
<td>0.2616320393</td>
<td>0.2616064508</td>
</tr>
<tr>
<td>IF</td>
<td>&quot;</td>
<td>0.0001436935221</td>
<td>0.0001435402196</td>
<td>0.0001434923636</td>
</tr>
<tr>
<td>IM₁</td>
<td>$I_{Na}$ intermediate inactivation state</td>
<td>3.913769904e-05</td>
<td>3.909441247e-05</td>
<td>3.907357776e-05</td>
</tr>
<tr>
<td>IM₂</td>
<td>&quot;</td>
<td>3.381242427e-08</td>
<td>3.376098643e-08</td>
<td>3.373623669e-08</td>
</tr>
<tr>
<td>LC₁</td>
<td>$I_{Na}$ burst mode closed state</td>
<td>1.659002962e-13</td>
<td>1.658337887e-13</td>
<td>1.65801729e-13</td>
</tr>
<tr>
<td>LC₂</td>
<td>&quot;</td>
<td>1.137084204e-11</td>
<td>1.136916421e-11</td>
<td>1.136835517e-11</td>
</tr>
<tr>
<td>LC₃</td>
<td>&quot;</td>
<td>3.02240205e-10</td>
<td>3.022667309e-10</td>
<td>3.022795176e-10</td>
</tr>
<tr>
<td>O</td>
<td>$I_{Na}$ open state</td>
<td>9.754706096e-07</td>
<td>9.747956198e-07</td>
<td>9.744703275e-07</td>
</tr>
<tr>
<td>LO</td>
<td>$I_{Na}$ burst mode open state</td>
<td>4.202747013e-16</td>
<td>4.199838793e-16</td>
<td>4.198437236e-16</td>
</tr>
<tr>
<td>C₁,mut</td>
<td>Mutant $I_{Na}$ closed state</td>
<td>-</td>
<td>0.000126208303</td>
<td>7.166307322e-05</td>
</tr>
<tr>
<td>C₂,mut</td>
<td>&quot;</td>
<td>-</td>
<td>0.009441839154</td>
<td>0.007980529607</td>
</tr>
<tr>
<td>C₃,mut</td>
<td>&quot;</td>
<td>-</td>
<td>0.2739249316</td>
<td>0.3446438288</td>
</tr>
<tr>
<td>IC₂,mut</td>
<td>Mutant $I_{Na}$ inactive state</td>
<td>-</td>
<td>0.02242017495</td>
<td>0.01409702075</td>
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<tr>
<td>IC₃,mut</td>
<td>&quot;</td>
<td>-</td>
<td>0.6504500644</td>
<td>0.6087860865</td>
</tr>
<tr>
<td>IF,mut</td>
<td>&quot;</td>
<td>-</td>
<td>0.000299688607</td>
<td>0.000126587513</td>
</tr>
<tr>
<td>IM₁,mut</td>
<td>Mutant $I_{Na}$ intermediate inactivation state</td>
<td>-</td>
<td>0.0001977778539</td>
<td>8.00620291e-05</td>
</tr>
<tr>
<td>IM₂,mut</td>
<td>&quot;</td>
<td>-</td>
<td>1.707703298e-07</td>
<td>6.911707407e-08</td>
</tr>
<tr>
<td>LC₁,mut</td>
<td>Mutant $I_{Na}$ burst mode closed</td>
<td>-</td>
<td>1.920497509e-05</td>
<td>4.919562139e-06</td>
</tr>
<tr>
<td>LC₂,mut</td>
<td>&quot;</td>
<td>-</td>
<td>0.001436754012</td>
<td>0.0005478513486</td>
</tr>
<tr>
<td>LC₃,mut</td>
<td>&quot;</td>
<td>-</td>
<td>0.04168284782</td>
<td>0.02365928023</td>
</tr>
</tbody>
</table>

Table 10: Initial conditions for state variables in mathematical model of human ventricular action potential
<table>
<thead>
<tr>
<th>State Variable</th>
<th>Definition</th>
<th>WT</th>
<th>A572D</th>
<th>Q573E</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O_{\text{mut}}$</td>
<td>Mutant $I_{\text{Na}}$ open state</td>
<td>-</td>
<td>2.929101154e-07</td>
<td>1.117291082e-07</td>
</tr>
<tr>
<td>$L\bar{O}_{\text{mut}}$</td>
<td>Mutant $I_{\text{Na}}$ burst mode open state</td>
<td>-</td>
<td>4.457180191e-08</td>
<td>7.670035154e-09</td>
</tr>
<tr>
<td>d</td>
<td>L-type $\text{Ca}^{2+}$ current activation gate</td>
<td>1.638090607e-05</td>
<td>1.637609944e-05</td>
<td>1.637378239e-05</td>
</tr>
<tr>
<td>f</td>
<td>L-type $\text{Ca}^{2+}$ voltage-dependent inactivation gate</td>
<td>0.9999364487</td>
<td>0.9999364687</td>
<td>0.9999364783</td>
</tr>
<tr>
<td>f_s</td>
<td>L-type $\text{Ca}^{2+}$ calcium-dependent inactivation gate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>$X_{r1}$</td>
<td>Rapidly activating $K^+$ current activation gate</td>
<td>0.0001497389543</td>
<td>0.0001496918853</td>
<td>0.000149669196</td>
</tr>
<tr>
<td>$X_{r2}$</td>
<td>Rapidly activating $K^+$ current activation gate</td>
<td>0.4963056182</td>
<td>0.4963285448</td>
<td>0.4963395991</td>
</tr>
<tr>
<td>$X_s$</td>
<td>Slowly activating $K^+$ current activation gate</td>
<td>0.002723153193</td>
<td>0.00272272626</td>
<td>0.002722520434</td>
</tr>
<tr>
<td>r</td>
<td>Transient outward $K^+$ current activation gate</td>
<td>1.61573943e-08</td>
<td>1.615146813e-08</td>
<td>1.614861155e-08</td>
</tr>
<tr>
<td>s</td>
<td>Transient outward $K^+$ current inactivation gate</td>
<td>0.9999986683</td>
<td>0.9999986689</td>
<td>0.9999986692</td>
</tr>
<tr>
<td>g</td>
<td>Ryanodine receptor $\text{Ca}^{2+}$ release activation gate</td>
<td>0.9999961213</td>
<td>0.9999961354</td>
<td>0.9999961421</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>$\text{Ca}^{2+}$ concentration in myoplasm (mM)</td>
<td>4.387145251e-05</td>
<td>4.384476863e-05</td>
<td>4.383218716e-05</td>
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<tr>
<td>$[\text{Ca}^{2+}]_{\text{SR}}$</td>
<td>$\text{Ca}^{2+}$ concentration in sarcoplasmic reticulum (mM)</td>
<td>0.1969056818</td>
<td>0.1967445959</td>
<td>0.196669525</td>
</tr>
<tr>
<td>$[\text{Na}^+]_i$</td>
<td>$\text{Na}^+$ concentration in myoplasm (mM)</td>
<td>8.355040031</td>
<td>8.349674873</td>
<td>8.3471155</td>
</tr>
<tr>
<td>$[\text{K}^+]_i$</td>
<td>$\text{K}^+$ concentration in myoplasm (mM)</td>
<td>145.439625</td>
<td>145.4478242</td>
<td>145.451705</td>
</tr>
<tr>
<td>$V_m$</td>
<td>Transmembrane potential (mV)</td>
<td>-87.64533404</td>
<td>-87.64753511</td>
<td>-87.64859638</td>
</tr>
</tbody>
</table>

Table 11: Additional Initial conditions for state variables in mathematical model of human ventricular action potential