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PHOSPHATASE REGULATION IN CARDIOVASCULAR PHYSIOLOGY AND DISEASE

by

Sean Thomas DeGrande

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Molecular Physiology and Biophysics in the Graduate College of The University of Iowa

December 2012

Thesis Supervisors: Adjunct Professor Peter J. Mohler
Professor Mark E. Anderson

Reversible protein phosphorylation is an essential component of metazoan signaling and cardiovascular physiology. Protein kinase activity is required for regulation of cardiac ion channel and membrane receptor function, metabolism, and transcription, and aberrant kinase function is widely observed across disparate cardiac pathologies. In fact, multiple generations of cardiac therapies (eg. beta-adrenergic receptor blockers) have targeted cardiac kinase regulatory cascades. In contrast, essentially nothing is known regarding the mechanisms that regulate cardiac phosphatase activity at baseline or in cardiovascular disease.

Protein phosphatase 2A (PP2A) is a key phosphatase with multiple roles in cardiac physiology. Here we demonstrate the surprisingly complex regulatory platforms that control PP2A holoenzyme activity in heart. We present the first full characterization of the expression and regulation of the PP2A family of polypeptides in heart. We identify the expression of seventeen different PP2A genes in human heart and define their differential expression and distribution across species and in different cardiac chambers. We show unique subcellular distributions of PP2A regulatory subunits in myocytes, strongly implicating the regulatory subunit in conferring PP2A target specificity *in vivo*. We report striking differential regulation of PP2A scaffolding, regulatory, and catalytic subunit expression in multiple models of cardiovascular disease as well as in human heart failure samples. Importantly, we demonstrate that PP2A regulation in disease extends far beyond expression and subcellular location, by identifying and describing differential post-translational modifications of the PP2A holoenzyme in human heart failure. Furthermore, we go to characterize a mechanism for this method of post-translational modification that may represent a pathway capable of being therapeutically manipulated

in human heart failure. Lastly we provide evidence that dysregulation of phosphatase activity contributes to the cellular pathology associated with a previously described inheritable human arrhythmia syndrome, highlighting the importance of the PP2A in cardiovascular physiology and disease. Together, our findings provide new insight into the functional complexity of PP2A expression, activity, and regulation in heart and in human cardiovascular disease and identify potentially new and specific gene and subcellular targets for the treatment of human arrhythmia and heart failure.

Abstract Approved: _____
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Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Sean Thomas DeGrande

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Molecular Physiology and Biophysics at the December 2012 graduation.

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To the friends and family whose support and friendship over the years has made this work possible.

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My thesis Committee at the University of Iowa deserve special mention as their incredible efforts to facilitate the advancement of my thesis project proved valuable beyond words as I gathered this data in a research lab that was in the process of moving between institutions. Erwin Shibata, Dana Levasseur, Michael Anderson, Mark Anderson, and Scott-Moye Rowley provided endless intellectual and resource support that proved to be vital in making this project happen. This thesis would not have been possible without their tireless efforts to support this project and my career goals. Our collaborators in the lab of Mark Anderson and in the lab of Xander Wehrens, contributed significantly to several key experiments described in thesis and their expertise was greatly appreciated.

Most importantly, I owe a great deal of thanks to Peter Mohler, my thesis advisor, whose support and guidance have made this project possible. Not only have I learned many valuable lessons about the technical aspects of scientific research, but more importantly, I've learned how to communicate my ideas to the scientific community and how to formulate questions based on the patterns we observe around us. The lessons I have learned here I will build upon for the rest of my career and as I prepare to move on from this stage of my training, I find my interest in translational biomedical research at a new found peak.

Lastly, I wish to thank my parents, Tom and Bonnie, and my brother Christopher, whose unending support over the past 27 years is uniquely responsible for this achievement and whatever other achievements lie ahead.

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LIST OF ABBREVIATIONS

0 Na/0 Ca NT = Zero sodium, zero calcium normal Tyrode solution

β-AR = Beta-adrenergic Receptor

β-AR1 = Beta-adrenergic receptor subtype 1

β-AR2 = Beta-adrenergic receptor subtype 2

μM = Micromolar

A = Ampere

AC = Adenylyl cyclase

AKAP = A-kinase anchoring protein

AnkB= Ankyrin-B

AP = Action potential

APD = Action potential duration

ATP = Adenosine 5'-triphosphate

a.u. = Arbitrary units

bpm = Beats per minute

Ca2+ = Calcium

[Ca]i = Intracellular calcium concentration

[Ca]T = Total cellular calcium concentration

[Ca]SR = Free intra-sarcoplasmic reticulum calcium concentration

cAMP = Adenosine 3',5'-cyclic monophosphate

CaM = Calmodulin

CaMKII = Calcium/calmodulin-dependent Protein Kinase II

CHF = Congestive heart failure

CICR = Calcium-induced calcium release

Cl- = Chloride

CO = Cardiac output

CO2 = Carbon dioxide

CRU = Calcium-release unit

Ctrl = Control

DAD = Delayed Afterdepolarization

DHPR = Dihydropyridine receptor

DMSO = Dimethyl sulfoxideDNA = Deoxyribonucleic acid

EAD = Early Afterdepolarization

E-C coupling = Excitation-contraction coupling

EGTA = Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid

EM = Electron microscopy

Em = Membrane potential

Epi = Epinephrine

G-protein = Guanosine triphosphate binding regulatory protein

GDP = Guanosine 5'-triphosphate

GPCR = G-protein coupled receptor

Gs = Stimulatory G-protein subunit

Gi = Inhibitory G-protein subunit

HDAC = Histone deacetylases

HEPES = N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

HF = Heart failure

HR = Heart rate

Hz = Hertz

ICa = Calcium current

ICa, L = L-type Ca2+ channel current

IK1 = Outward potassium-mediated current

INa = Na channel-mediated inward current

*i*Na = Unitary sodium current

INCX = Sodium-calcium exchange-mediated current

Iti = Transient inward current

Ito = Transient outward current

ISO = Isoproterenol

jSR = junctional sarcoplasmic reticulum

K+ = Potassium

kDa = Kilodalton

KO = Knock out

[K]i = Intracellular potassium concentration

[K]o = Extracellular potassium concentration

L = Liter

LTCC = L-type calcium channel

M = Molar

MAPK = Mitogen activated protein kinase

mg = Milligram

min = Minute

mL = Milliliter

mM = Millimolar

ms = Millisecond

mV = Millivolt

Na+ = Sodium

[Na]i = Intracellular sodium concentration

[Na]o = Extracellular sodium concentration

NCX = Sodium-calcium exchanger

NF = Non-failing

NO = Nitric oxide

NOS = Nitric oxide synthase xxi

nm = Nanometer

nM = Nanomolar

NE = Norepinephrine

PKA = cAMP-dependent Protein Kinase

PKG = cGMP-dependent protein kinase

pL = Picoliter

PLB = Phospholamban

PLC = Polyphosphoinositide-specific phospholipase C

PMCA = Plasma membrane Ca2+ ATPase

Po = Open probability

PP = Protein phosphatase

RyR2 = Ryanodine receptor; sarcoplasmic calcium release channel

sec = Second

SA = Sinoatrial

Ser = Serine

SERCA = Sarcoplasmic-endoplasmic reticulum Ca2+-ATPase

SL = Sarcolemmal membrane SR = Sarcoplasmic reticulum

SV = Stroke volume

TAC = Trans-aortic constriction

Thr = Threonine

TnC = Troponin C

T-tubule = Transverse tubule

CHAPTER 1:

MECHANISMS FOR REGULATION OF CARDIAC SIGNALING

Introduction

The heart has evolved throughout the course of metazoan evolution to perform a host of critical physiological functions. Responsible for generating the pressure gradients that propel blood, oxygen, nutrients and metabolic waste products throughout the body, the heart is in essence no different from any other striated muscle with several important caveats. Unlike other striated muscles, the heart generates its own stimulus for contraction and then sends this signal through a specialized conducting system to ensure the heart operates in an effective and efficient manner during both diastole and systole. Furthermore, the heart can complete this process at a wide range of frequencies.

Like other muscles, the heart contracts when individual cardiomyocytes shorten in response to the movement of ions across the cardiomyocyte plasma membrane. In other muscles, this movement of charge particles, called an action potential, originates in the nervous system and terminates at a neuromuscular junction. In the heart, the action potential originates within specialized pacemaker regions of the heart itself that spontaneously depolarize and conduct action potentials to the rest of the contractile tissue in the heart. The pacemaker region located in the right atrium (RA), called the sinoatrial (SA) node, normally sets the pace of contraction in a healthy heart. From the SA node, this wave of electrical depolarization travels through the atria causing contraction of the atria and propelling blood into the ventricles (Fig.1). In normal conditions, there is only a single area of electrical connection between the upper and lower chambers of the heart. This area of cells, called the atrioventricular (AV) node, also contains cells that are capable of spontaneously depolarizing but at a slower frequency than the cells of the sinus node. The wave of electrical depolarization is slowed slightly in the AV node and is then conducted rapidly through the His-Purkinje system to the apex of the ventricles (Fig.1). From the apex, the wave of depolarization travels back upward through the

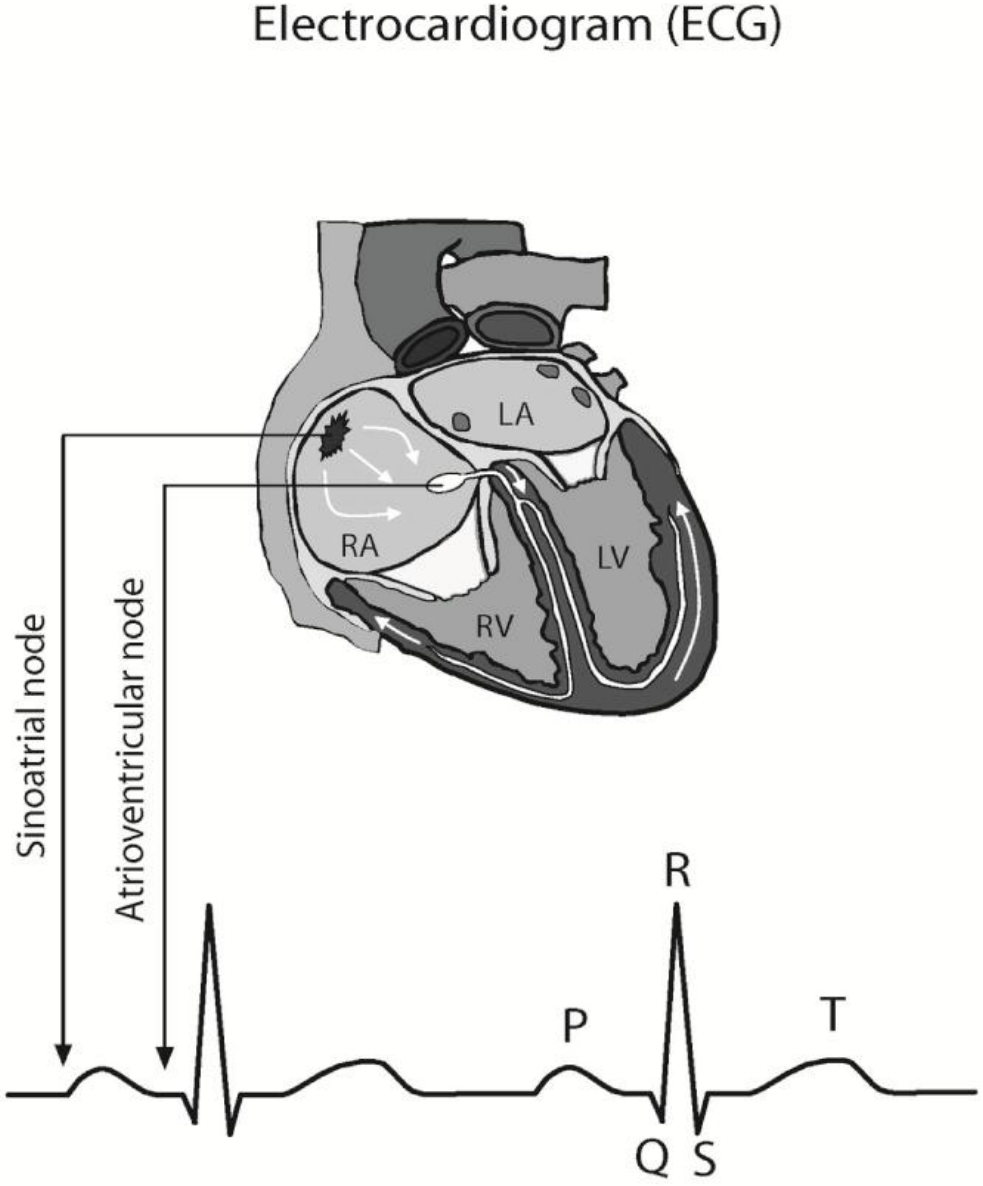

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Figure 1. Electrical Conduction in the Heart. White arrows indicate the paths of normal electrical conduction through a healthy heart. A sample ECG rhythm strip is seen at the bottom of this figure with the components of a complete contraction labeled. RA = right atrium, RV = right ventricle, LA = left atrium, LV = left ventricle.

muscular walls of the left and right ventricles towards the atria when the wave terminates at the atrial-ventricular border. This specialized conduction system ensures that the atria have moved blood downward in the heart into the ventricles before the ventricles contract in the opposite direction and send blood through either the pulmonary or systemic outflow tracks (Boron and Boulpaep 2005).

The Electrocardiogram

The electrocardiogram is an important devise used to monitor and evaluate the pattern of electrical conduction through the heart. This devise uses a series of six limb leads and six precordial leads to allow a physician interpreter or cardiovascular researcher to visualize the average wave of depolarization along 12 different axes in the heart. The data provided by an electrocardiogram can be used to diagnose a variety of cardiovascular diseases such as ventricular arrhythmia, atrial fibrillation, myocardial ischemia and heart failure.

Although a detailed understanding of ECG interpretation is not required to understand the work described in the rest of this thesis, a brief overview may prove useful. All of the components of a complete and effective heart beat described in the previous section can be detected by ECG analysis. The first electrical current produced during contraction is that of the atrial cells as the upper chambers of the heart contract to fill the ventricles. Atrial activity can be visualized on the ECG as the P wave. This first wave of depolarization is followed by a brief pause as the current passes from the atria to the AV node and into the Purkinje fibers. Following this brief pause is the QRS complex. This complex represents ventricular contraction and is normally many times larger than the atrial component due to the greater muscle mass of the ventricles when compared to the atria. Following the QRS complex is the T wave and this represents ventricular repolarization. Atrial repolarization is not visualized in an ECG due to fact that it is obscured by the QRS complex (Dubin 1970).

Conducting Cells of Heart

The heart, similar to every other organ in the body, is composed of multiple types of tissue and cells. For the sake of simplicity, this chapter will consider those cell types in the heart capable of conducting an electrical current and producing a contraction. There are several types of heart cells that have both of these properties. Ventricular cardiomyocytes produce what is referred to as a fast-response action potential. Normally, ventricular myocytes are at a resting equilibrium membrane potential of approximately -80 to -85 millivolts (mV). This resting potential is maintained in large part by the activity of the Na^+/K^+ ATPase and I_{k1} (Bers 2001). The Na^+/K^+ ATPase drives three Na^+ ions out of the cell while driving two K^+ ions into the cell, creating a net negative charge in the cell and relatively high concentration of Na^+ in the extracellular environment and a relatively high K^+ concentration in the cytosolic environment. The I_{k1} current is generated by open K^+ permeable channels (Kir2.1) in the plasma membrane that allow K^+ to diffuse down its electrochemical gradient (Ibarra, Morley et al. 1991; Dhamoon, Pandit et al. 2004). Since the cardiomyocyte at rest is many times more permeable to K^+ than to many other ions, such as Na^+, Ca^{2+} and Cl^-, the resting potential of the cell is much closer to the equilibrium potential of K^+ (about -89mV) than it is to the equilibrium potential of ions that are more concentrated in either the extracellular or intracellular environment.

Cardiac AP in Ventricular Cardiomyocytes

Phase 0 of the action potential begins when the cell is depolarized to its threshold potential and voltage gated Na^+ channels (predominately Nav1.5) are triggered to open. This allow an influx of Na^+ into the cell in the form of the I_{na} current. This sudden increase in membrane permeability to Na^+ allows the membrane potential to move quickly towards the Na^+ equilibrium potential of +70 mV. Simultaneously, several other channels open in response to rising membrane potential. The voltage gated L-type Ca^{2+} channel (predominately $Ca_v1.2$ in the ventricles and $Ca_v1.3$ in the atria) opens and

produces an inward Ca^{2+} current, I_{Ca}. Additionally, voltage gated K^+ channels (K_v4.2, K_v4.3 and K_v1.4) in the plasma membrane open and produce a transient outward K^+ current called I_{to}. The peak of the action potential (AP) occurs around +30 to +50 mV, or when the inward currents, I_{Na} and I_{Ca} are offset by the outward current I_{to} (Bers 2001; Boron and Boulpaep 2005).

Phase 1 of the AP is characterized by a very short but rapid partial repolarization of the cell. This repolarization is produced by the combined effect of the closure of the voltage gated Na^+ channels and I_{to} remaining active and effectively carrying positive charge, in the form of K^+ ions, out of the cell (Bers 2001; Boron and Boulpaep 2005).

Phase 2 of the action potential is a period of time when the membrane potential of the cell changes very little. During this phase, the inward I_{Ca} increases in intensity and although voltage gated K^+ channels responsible for producing I_{to} close during this period, other K^+ channels in the membrane open producing the outward delayed rectifier K^+ current. This delayed rectified current is composed of multiple currents that have different activation and inactivation kinetics. Also during phase 2, the Na^+/Ca^{2+} exchanger (NCX) becomes active and removes one Ca^{2+} ion from the cytosol and brings three Na^+ ions into the cell. This phase ends when the L-type Ca^{2+} channel closes (Bers 2001; Boron and Boulpaep 2005).

Phase 3 is characterized by repolarization of the cardiomyocyte to its resting membrane potential of near -80 mV. This phase is dominated by outward K^+ current. Phase 4 of the action potential consists of the ventricular cardiomyocyte at its resting membrane potential and able to generate another action potential (Bers 2001; Boron and Boulpaep 2005).

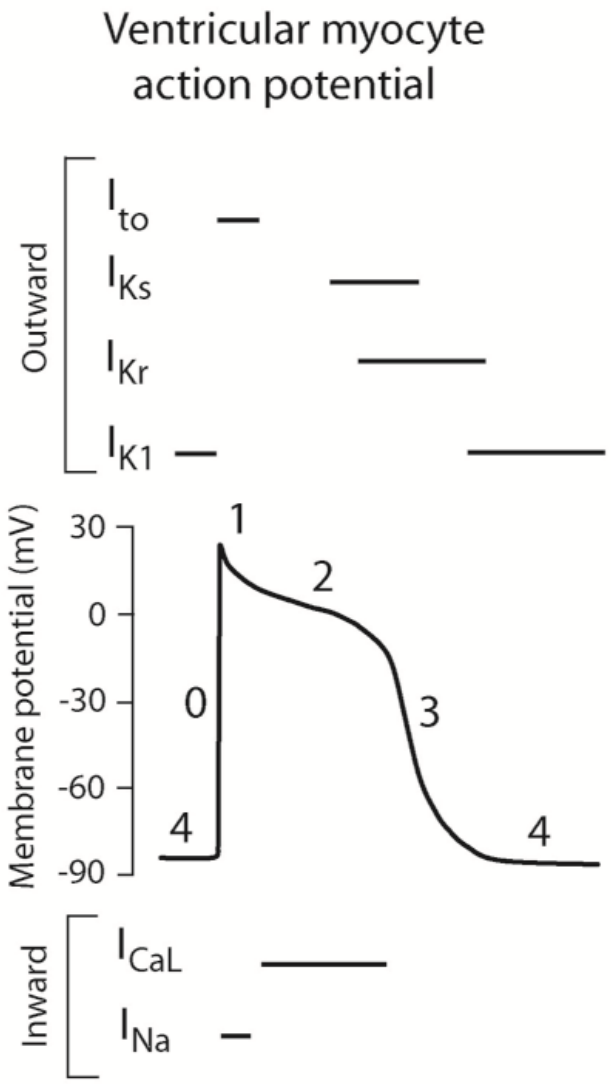

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Figure 2. The Ventricular Action Potential. The overall membrane potential of a ventricular cardiomyocyte is shown for the duration of one entire action potential. Individual outward currents are shown at the top and inward currents at the bottom. Black bars represent the portion of the action potential in which these currents are active

Cardiac AP in Other Conducting Cells of the Heart

As mentioned earlier, the different types of conducting cells present in the heart produce slightly different action potentials. The specialized conducting cells of the His-Purkinje fibers produce an action potential of longer duration than ventricular cells (APD_{90} is 321±5.6 ms in Purkinje cells vs. 242±5.7 ms in ventricular cardiomyocytes paced at the same frequency). Additionally, these cells produce a phase 0 rise in membrane potential of a greater maximal value and a greater rate of rise than ventricular cardiomyocytes (the peak membrane potential is 117±1.2 mV above resting potential in Purkinje cells and 106±1.1 mV in ventricular cardiomyocytes and the maximal rate of rise of phase 0 depolarization is 445±14.6 V/s in Purkinje cells vs. 230±9.5 V/s in ventricular cardiomyocytes) despite both cells having an equal average resting membrane potential (Dun and Boyden 2008).

Purkinje cells can also produce pacemaker currents that lead to spontaneous depolarization of the cell and the generation of an action potential that be conducted to neighboring cells. However, the pacemaker activity of Purkinje cells is over-driven by primary pacemaker cells found in both the sinoatrial (SA) and atrioventricular (AV) node under normal physiologic conditions. Pacemaker cells in these areas generally have a more positive resting potential and significantly less dominant I_{Na} in phase 0 of the action potential than do ventricular cardiomyocytes (Bers 2001). Spontaneous depolarization is achieved my numerous currents but the lack of a stabilizing I_{k1}, the decrease in outward delayed rectifier K^+ currents as repolarization proceeds and the slowly activating inward "funny" current, I_f, which is initiated by hyperpolarization at negative resting membrane potentials, are crucial to this process (DiFrancesco 1993; Mangoni and Nargeot 2008). I_f is produced by the hyperpolarization-activated cyclic nucleotide-gated channel (HCN1, 2,

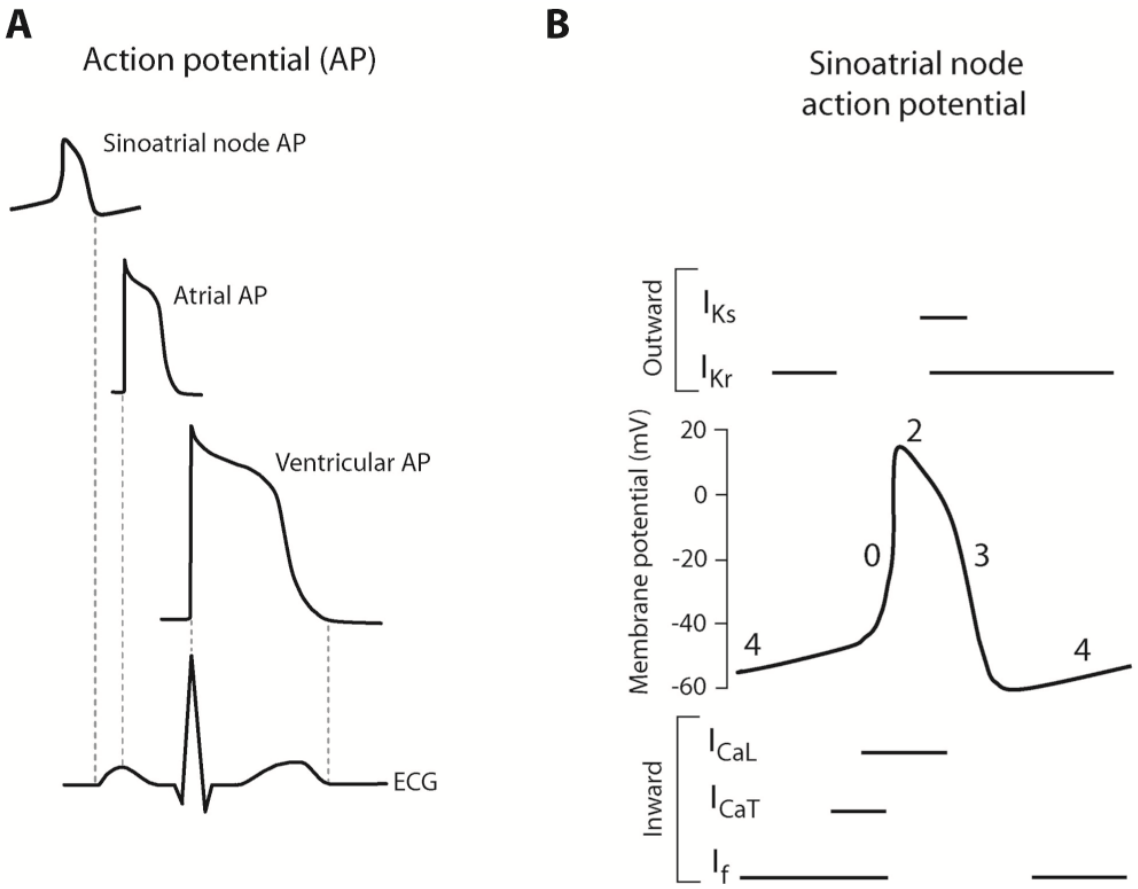

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Figure 3. Action Potentials in Other Cell Types. A) Representative action potentials are shown for three types of conducting cells in the heart along with the period of time in which they occur during one heart beat on an ECG rhythm strip. **B)** The phases of a typical action potential in a healthy sinoatrial node cell are shown along with individual outward and inward currents. Black bars indicate the time during which these currents are active during a SA nodal action potential.

the 4). The magnitude of this current is modified by stimulation from the autonomic nervous system (Brown, DiFrancesco et al. 1979) (reviewed in the next section).

Autonomic Innervation of the Heart

Although the afferent neural fibers of the central nervous system do not produce every action potential that reaches the heart like these neural fibers do in other striated muscle, afferent fibers from the autonomic nervous system do influence the frequency at which the intrinsic pacemaker regions of the heart spontaneously depolarize. The heart receives sympathetic stimulation directly from the cervical and cervico-thoracic stellate ganglion that innervate diverse regions in both the atria and ventricles and also indirectly from circulating catecholamines (Armour 1999). Parasympathetic innervation to the heart is supplied directly by the vagus nerve. These fibers converge in an area between the superior vena cava and the aorta as they continue on to innervate the SA and AV nodes of the heart (Chiou, Eble et al. 1997).

The effects of sympathetic stimulation on the contractile machinery of the heart will be discussed in more detail in later sections. In general the two arms of the autonomic nervous system can be conceptualized as working in an opposing fashion. However, there is a significant quantity of functional data suggesting both arms of the autonomic nervous system may act on the heart simultaneously, especially in pathologic processes (Kollai and Koizumi 1979; Shen, Choi et al. 2012).

The sympathetic and parasympathetic nervous systems have opposing effects on I_f. Sympathetic stimulation mediated by activation of the β-adrenergic receptor increases the inward current through the HCN class of channels at any given membrane potential, thereby increasing the frequency of spontaneous depolarization (i.e. heart rate). Parasympathetic activation mediated by the muscarinic receptors has the opposite effect. Furthermore, parasympathetic stimulation also activates an additional K^+ current, $I_{k(Ach)}$ mediated Kir 3.1 and Kir 3.4 channels, that functions to stabilize the resting potential of

the pacemaker cells closer to the much more negative equilibrium potential of K^+ (Corey, Krapivinsky et al. 1998). $I_{K(Ach)}$ thereby lowers the resting membrane potential of the cardiomyocytes in the pacemaker region to a more negative value and reduces the rate of spontaneous depolarization (Mark and Herlitze 2000).

Excitation Contraction Coupling

Overview

If the action potential is the means by which a cardiomyocyte is signaled to contract, then excitation coupling is the process by which a depolarizing action potential is translated into a lengthwise contraction of the cardiomyocyte. Ca^{2+}, a ubiquitous secondary messenger in a variety of cellular processes and signaling cascades, is a vital link in this process and, in essence, the process of excitation-contraction coupling serves to tightly regulate fluctuations in intracellular Ca^{2+} concentration $[Ca^{2+}]_I$ (Fig.4). Generally, activation of the voltage gated Ca^{2+} channel (L type Ca^{2+} channel) in the cardiomyocyte plasma membrane during the action potential produces and influx of Ca^{2+} into the cell, termed I_{Ca} in the previous section. This inward Ca^{2+} current in turn activates the sarcolemma Ca^{2+} channel, the cardiac ryanodine receptor (RyR2). This allows for Ca^{2+} to be released from its intracellular storage site, the sarcoplasmic reticulum, into the cytosol. This is called Ca^{2+} induced Ca^{2+} release. This cytoplasmic Ca^{2+} then interacts with the troponin complex, relieving troponin based inhibition of the contractile apparatus and allowing actin and myosin to interact and produce contraction along the long axis of the cardiomyocyte (Bers 2002) (Fig.4).

Ca^{2+} Release and Cardiomyocyte Contraction

In order for Ca^{2+} induced Ca^{2+} release to occur in an efficient manner, the human ventricular cardiomyocyte has several adaptations that bring the structures responsible for this process together in close proximity. This allows Ca^{2+} based signaling to occur in only a very small sub-cellular domain and does not require changing ion concentrations to

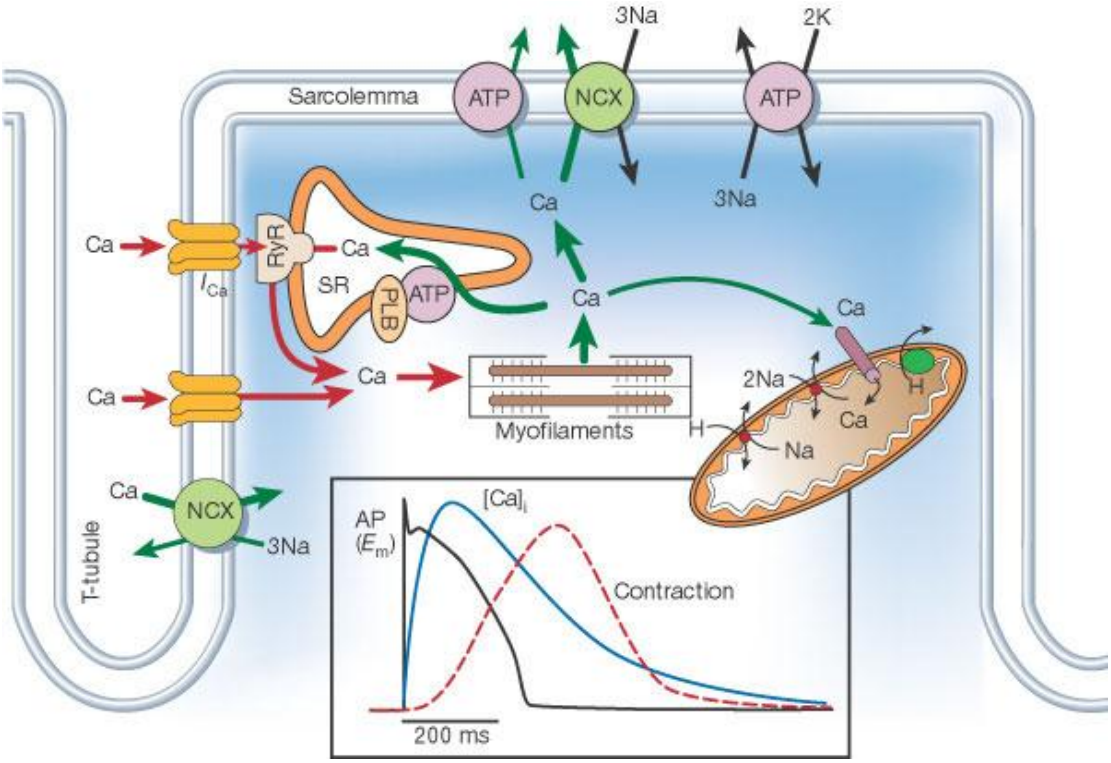

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Figure 4. Excitation Contraction Coupling. A diagram of the important Ca^{2+} cycling currents and events that occur during excitation contraction coupling in a cardiomyocyte.

equilibrate across the entire cytosol of the cardiomyocyte. Specialized invaginations of the plasma membrane, called transverse tubules, contain an increased concentration of the L-type Ca^{2+} channel and occur over the RyR2 bearing sarcoplasmic reticulum. This structure, where the junctional sarcoplasmicreticulum (containing RyR2) and the junctional plasma membrane (containing the L-type Ca^{2+} channel) meet is called the Ca^{2+} release unit (Scriven, Dan et al. 2000).

In this calcium release unit, RyR2 is more abundant than the L-type Ca^{2+} channel. While the exact ratio varies on the type of tissue in question and the species of origin, there are usually between 4-10 RyR2s for each surface L-type Ca^{2+} channel (Bers 2001). This means that activation of each L-type Ca^{2+} channel results in the activation of many more RyR2 channels and thus results in much larger amplitude of Ca^{2+} release from the sarcoplasmic reticulum. The Ca^{2+} release unit, also referred to as the couplon, can contain more than 100 RyR's and associated L-type Ca^{2+} channels in a relatively small area of space on the junctional SR about 200 nm in diameter (Franzini-Armstrong, Protasi et al. 1999). Due to the close proximity of neighboring RyR2 channels and the phenomenon of coupled gating between the all RyR2 channels in a couplon, the I_{ca} generated by a single L-type Ca^{2+} channel is sufficient to trigger the activation of the entire couplon. The inward I_{Ca} is rapidly inactivated in a Ca^{2+} dependent manner. Thus, the rapid rise in $[Ca^{2+}]_i$ in the local environment results in inactivation of the L-type Ca^{2+} channel, terminating the stimulus of Ca^{2+} induced Ca^{2+} release (Linz and Meyer 1998). Furthermore, the distance between couplons limits the effects of I_{Ca} at distant sites or even in neighboring couplons (Bers 2001).

Ca^{2+} Removal and Cardiomyocyte Relaxation

Although Ca^{2+} release has ceased, the Ca^{2+} now in the cytosol must be removed in order for relaxation of the cardiomyocyte to occur in preparation of the next depolarizing action potential. This process is accomplished predominately by four mechanisms. The SR Ca^{2+} - ATPase (SERCA) that returns cytosolic Ca^{2+} to the SR, the sarcolemmal

Na^+/Ca^{2+} exchanger (NCX) and Ca^{2+} - ATPase that moves Ca^{2+} from the cytosol into the extracellular environment, and the mitochondrial Ca^{2+} uniporter which moves cytosolic Ca^{2+} into the mitochondrial lumen (Bers 2002). In mammalian ventricles, the SERCA and the NCX are the predominate mechanisms for removing Ca^{2+} from the cytosol. Generally speaking, the action of SERCA removes most of the Ca^{2+} from the cytosol, but the exact proportion of Ca^{2+} removed by the NCX and SERCA varies according to species (Bers, Bassani et al. 1996). The activity of SERCA is regulated by the inhibitory actions of the phospholamban. Phosphorylation of phospholamban results in increased SERCA activity and facilitates more rapid Ca^{2+} cycling and a general increase in SR Ca^{2+} content (Frank, Bolck et al. 2003). Unlike the RyR2 which is located on the junctional SR, SERCA is located in the longitudinal portion of the SR away from the sites of near abutment between the t-tubule and SR (Fleischer and Inui 1989).

Another very important way in which Ca^{2+} is removed from the cytosolic compartment is through the activity of the NCX. This antiporter moves three Na^+ ions and 1 Ca^{2+} ion in opposite directions (Alberts 2002; Liao, Li et al. 2012). Aside from a very small portion of the action potential near the end of phase 0, this antiporter uses the energy contained in the electrochemical gradient across to the cell membrane to move three Na+ ions down their concentration gradient into the cell and move one Ca^{2+} out of the cell. This unbalanced movement of charged molecules across the cell membrane creates the inward I_{NCX} current that is important in maintaining the plateau phase of the ventricular myocyte action potential (Chiou, Eble et al. 1997; Bers 2001; Amin, Tan et al. 2010). Although the exact percentages vary depending on cell type and species, up to 30% of the total Ca^{2+} released from the SR during EC coupling can be removed from the cell via the actions of this antiporter.

The two other mechanisms for removing Ca^{2+} from the cytosol remove a significantly smaller portion of the Ca^{2+} released during EC coupling but are important nonetheless. A relatively small portion of the cytosolic Ca^{2+} is moved into the

mitochondria via a Ca^{2+} uniporter. Because the mitochondrial matrix carries a significantly more negative charge than the cytosol of the cardiomyocyte (-180mV and -80mV respectively), the mitochondrial uniporter (MCU) transports Ca^{2+} in a largely passive manner, allowing positively charged Ca^{2+} in the cytosol to diffuse down the electrical gradient (Dedkova and Blatter 2008). There is considerable debate as to whether mitochondrial Ca^{2+} content oscillates rapidly in response to every contraction of the cardiomyocyte or whether mitochondrial Ca^{2+} content changes more slowly in response to increased or decreased cytosolic [Ca^{2+}] at various heart rates (for a more comprehensive review see Dedkova & Blatter, 2008) however mitochondrial Ca^{2+} is important for the generation of nitric oxide (NO), regulation of apoptotic pathways, and in linking the metabolic energy demands of the cell with the energy produced by oxidative metabolism (Balaban, Bose et al. 2003; Orrenius, Zhivotovsky et al. 2003; Dedkova, Ji et al. 2004).

The plasma membrane Ca^{2+} ATPase (PMCA) is the last way in which Ca^{2+} released during EC coupling is removed from the cytosol to allow for relaxation of the cell. Like MCU, PMCA removes only a very small portion of the total Ca^{2+} released compared to the actions of SERCA and NCX. Whereas SERCA moves two Ca^{2+} ions for every ATP hydrolyzed, PMCA moves only a single Ca^{2+} ion per ATP hydrolyzed. Although PMCA probably makes little overall contribution to relaxation inducing Ca^{2+} removal, it does play an important role in organizing many intracellular signaling cascades that are beyond the scope of the work presented here. For a very complete review of the Ca^{2+} transporting ATPase proteins present in the heart, please see (Brini and Carafoli 2009).

Reversible Phosphorylation Regulation

Protein phosphorylation is a process by which a phosphate group from ATP is transferred to the hydroxyl residues in the side chain of the amino acids tyrosine, serine,

or threonine, thereby altering the baseline activity of the peptide. In metazoans, the delicate balance of protein phosphorylation status is tightly synchronized by the competing activities of protein kinases and phosphatases. Kinases catalyze the transfer of the phosphate group from ATP to the target amino acid residue in the peptide and more than 500 are encoded in the human genome (Anderson ME 2007). Phosphatases, on the other hand, while generally quite abundant, are more homogenous than the kinase repertoire present in any given cell type and only a handful of such proteins have been identified in the human genome (Wera and Hemmings 1995). These proteins catalyze the hydrolysis of the bond formed between the phosphate group and the hydroxyl group in the side chain of the previously mentioned amino acids and release free organic phosphate into the cytosolic environment. Reversible phosphorylation of the molecular components of EC coupling is an important way of regulating the rate and magnitude of cytosolic Ca^{2+} oscillations in cardiomyocytes.

G-Protein Coupled Receptors

In cardiomyocytes, G-protein-coupled receptors (GPCRs) play an important role in cardiac physiology and disease. These receptors are all composed of seven transmembrane spanning regions. However unlike the receptor tyrosine kinases, the GPCRs neither directly interact with nor directly activate downstream effector molecules. The GPCR recognize different ligands and effect cellular process in differing ways based on the specific composition of the heterotrimeric G-protein that is associated with the receptor. The heterotrimeric G protein is composed of 3 subunits, a Gα, a Gβ and Gγ, When inactive, the G protein subunits exist as a fully assembled heterotrimer with the α subunit bound to GDP and able to interact with the appropriate receptor. When the activating ligand binds to its G-protein-coupled receptor, it induces a conformational change allowing the receptor to interact with the corresponding G-protein and allow the GDP bound to the α subunit to be exchanged for GTP. This causes the Gα subunit to

dissociate from the Gβγ heterodimer and interact with its down-stream effectors. The inherent GTPase activity of the α subunit causes the GTP to be hydrolyzed to GDP and inactivates the subunit, allowing the heterotrimeric G-protein to reform.

The overall effects that the activation of the G-coupled receptor will have on cell signaling, metabolism, division, etc. is generally dependent on the α subunit associated with the receptor even though the β and γ subunits can also interact with downstream effector molecules as well (Clapham and Neer 1997). $G\alpha_s$ will activate adenylyl cyclase and increase expression of the secondary messenger cyclic AMP (cAMP), whereas $G\alpha_i$ has the opposite effect, decreasing the activity of adenylyl cyclase and reducing cAMP levels. $G\alpha_q$, when bound to GTP, will activate phospholipase C (PLC) (Wettschureck and Offermanns 2005).

There are several very important GPCRs in the heart. The β-adrenergic receptor is associated with $G\alpha_s$ and functions to increase the activity of kinases within the cardiomyocyte. The α adrenergic receptor is coupled to $G\alpha_q$, which activates PLC-β and the muscarinic receptor is coupled to $G\alpha_i$ and functions to decrease the availability of cAMP and reduce kinase activity (Rockman, Koch et al. 2002). True to form, the overall physiological effects of muscarinic and β-adrenergic receptor activation in the heart oppose one another. The muscarinic receptor is activated by parasympathetic (cholinergic) stimulation and is important in maintaining baseline resting cardiac output (the product of the heart's rate and force of contraction). On the contrary, the β-adrenergic receptor is activated by sympathetic (catecholaminergic) stimulation and functions to increase cardiac output. β-adrenergic receptor activation mediates the physiologic response to increased stress or systemic oxygen demand termed the 'fight or flight' response.

Fight or Flight Response

Overview

First described in 1920, the fight or flight response is the way in which the sympathetic nervous system alters an organism's energy and resource utilization in order to rapidly respond to a perceived threatening situation. The increased activity of the sympathetic nervous system and the resulting catecholaminergic stimulation of the heart that occurs as part of the physiologic fight or flight response increases the chronotrophy (rate of contraction) and inotrophy (force of contraction) of the heart (Salo, Campos et al. 2006). Although catecholamines released from the sympathetic nervous system will act on both the α and β adrenergic receptors, β adrenergic receptors are approximately ten times more numerous in the left ventricle than the α adrenergic receptors depending on the species.

As described previously, activation of the β adrenergic receptor causes $G\alpha_s$ to activate adenylyl cyclase which in turn results in an increase in cAMP production. This surge in cAMP production has several effects on the cardiomyocyte that contribute to the heart's overall response to catecholaminergic stimulation. One of these effects mediated by cAMP is to shift the activation voltage of the HCN channel to a less negative value. By directly binding to the HCN channel, cAMP causes the inward pacemaker current I_f to become active earlier in the repolarization phase of the SA nodal cardiomyocyte and increase the frequency of contraction (DiFrancesco, Ferroni et al. 1986; DiFrancesco and Tortora 1991). Perhaps more importantly, cAMP produced by adenylyl cyclase allows for the activation of cyclic AMP dependent kinases such as Protein Kinase A (PKA).

PKA Dependent Phosphorylation

PKA phosphorylation is a critical component of β-adrenergic stimulation in the heart. Composed of two regulatory and two catalytic subunits, cAMP binding to the regulatory subunits in this tetramer allow for the dissociation of the catalytic subunits

which then interact with an array of target molecules through interactions with various A-kinase anchoring proteins (AKAPS) (many very thorough reviews have been written discussing the role of specific AKAPS at key phosphorylation sites in the cell, for a recent review of AKAPS relevant to human cardiac physiology and disease please see (Diviani, Dodge-Kafka et al. 2011). The list of phosphorylation sites recognized by PKA is extensive however several PKA targets are well established critical components of EC coupling and their phosphorylation is important for the increase in heart rate and force of contraction associated with sympathetic stimulation of the heart.

CaMKII dependent phosphorylation

Beta-adrenergic signaling activates CaMKII activity in cardiomyocytes. Like PKA, CaMKII phosphorylation alters the properties and activity of many of the ion channels and signaling molecules important to the process of EC coupling. The CaMKII monomer contains three domains. Closest to the N-terminal side of the peptide is the catalytic domain that facilitates the transfer of a phosphate group from ATP to a serine of threonine residue within a CaMKII consensus motif (RXXS/T) within the target molecule. The c-terminal side of the peptide contains the association domain that allows for the interactions to occur between CaMKII monomers. Through interactions mediated by the association domain, multiple monomers come together to make up the dodecameric holoenzyme. Between these two domains is the regulatory domain that contains a pseudosubstrate sequence, and in the basal state, binds to and inhibits the catalytic domain.

As its name suggests, CaMKII activation is dependent on the cytosolic Ca^{2+} concentration. When Ca^{2+} concentrations are elevated, calmodulin takes up free Ca^{2+} and interacts with regulatory domain of CaMKII among a host of other proteins and enzymes, freeing the catalytic domain to interact with ATP and consensus sequences on target proteins. Additionally, CaMKII can be activated via Ca^{2+} independent mechanisms. Autophosphorylation of threonine residues in the regulatory domain is mediated by other

monomers in the activated holoenzyme and functions to allow for CaMKII to be activated without the need for CaM binding. Additionally, oxidation of specific methionine residues in the regulatory domain results in activation of the monomer. Due to the effects of rising Ca^{2+} concentrations, CaMKII activity is increased by an increased heart rate or a prolonged action potential duration (Anderson, Braun et al. 1998; Wehrens XH 2004).

Targets of phosphorylation dependent regulation

PKA and CaMKII share many important intracellular targets that are critical in EC coupling. Both kinases can exert effects on Ca^{2+} handling in cardiomyocytes by phosphorylating the L-type Ca^{2+} channel, RyR2 and PLN/SERCA (Vittone, Mundina et al. 1990; Yue, Herzig et al. 1990; Reiken, Lacampagne et al. 2003; Couchonnal and Anderson 2008). CaMKII phosphorylation of the LTCC results in an increase in peak I_{Ca} and frequent, extended openings of the channels in a process called facilitation (Dzhura, Wu et al. 2000). Phosphorylation of the cardiac ryanodine receptor results in an increase in the open probability of the receptor that causes an increase in Ca^{2+} release from the SR in both a triggered and spontaneous manner (Marx, Reiken et al. 2000). In the basal state, PLN is able to interact with SERCA to reduce the affinity of SERCA for cytoplasmic Ca^{2+}. When PLN is phosphorylated by either CaMKII or PKA, this inhibition is removed and Ca^{2+} removal from the cytoplasm is greatly accelerated and total SR Ca^{2+} load is increased (Vangheluwe, Sipido et al. 2006).

Phospho-Regulation in Cardiovascular Disease

Dysregulation of protein phosphorylation has been linked to mechanical dysfunction and arrhythmias in a host of cardiovascular disease states including atrial fibrillation, sinus node disease, heart failure, and myocardial infarction (Chelu MG 2009; Christensen, Dun et al. 2009; Shan, Betzenhauser et al. 2010; He, Joiner et al. 2011; Swaminathan, Purohit et al. 2011). In conditions like chronic heart failure, characterized

by the inability of the heart to provide enough blood flow to meet the body's metabolic demands, an increase in the phosphorylation levels of many of the targets described in the above section seems like it would be beneficial to survival in the face of decreasing pump efficiency and effectiveness. However, a wealth of research has shown that chronically increased β-adrenergic stimulation of the heart is associated with increased morbidity and mortality from this form of cardiovascular disease (Cohn, Levine et al. 1984). Consequently, great emphasis has been placed on defining levels and activity of protein kinases in the setting of cardiovascular disease states. Furthermore, pharmacological inhibitors of kinase activity have significantly enhanced our ability to treat cardiovascular disease phenotypes (Packer, Fowler et al. 2002; Reiken, Wehrens et al. 2003; Shelton, Clark et al. 2009). In fact, the success of beta-adrenergic receptor blockade in the treatment of arrhythmia, hypertension, and heart failure, likely depends on its ability to disrupt signaling cascades in several kinase pathways involved in disease. Nonetheless, kinase activity represents only one facet of the system responsible for regulating protein phosphorylation. Thus, while the past decade has illustrated the power of manipulating protein kinase activity for the treatment of cardiovascular disease, new therapeutic targets related to this pathway may offer additional avenues in the fight against heart disease. For example, while our understanding of kinase activity in cardiovascular health and disease continues to increase, our knowledge of phosphatase activity in cardiac pathophysiology is considerably less advanced. In fact, we lack even a basic fundamental understanding of expression, activity, and regulation of protein phosphatases in normal heart or in cardiovascular disease.

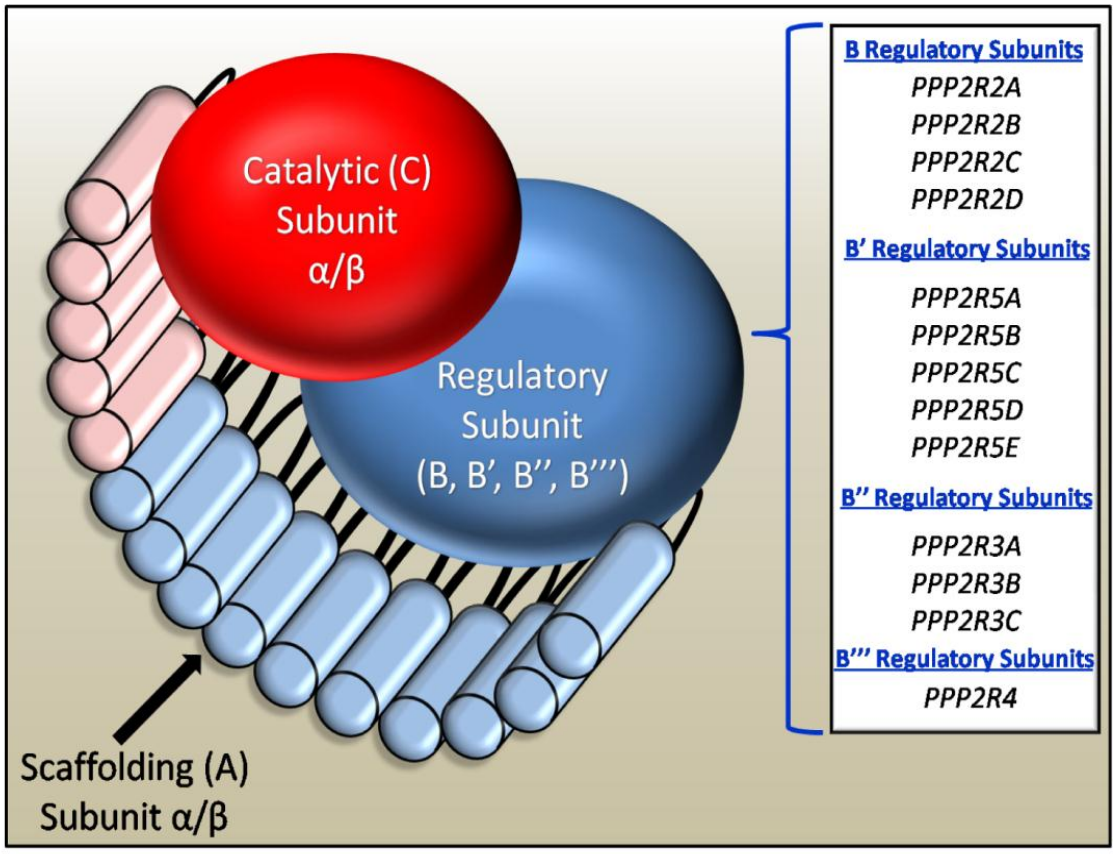

Figure 5. Protein Phosphatase 2A. A schematic drawing showing the relationship between the three different subunits that come together to form the PP2A holoenzyme. A list of the regulatory subunits as well as the families they belong to is shown on the right.

Protein Phosphatase 2A in Cardiomyocytes

Overview

Protein phosphatase 2A (PP2A) is a prominent serine/threonine phosphatase found across metazoan cell types. The canonical PP2A holoenzyme is comprised of three subunits that facilitate PP2A scaffolding (A subunits), regulatory (B subunits), and catalytic activity (C subunits) (Fig.5). Thus, diversity of PP2A function for cellular function arises from specific combinatorial holoenzyme products (2 A genes, 13 B genes, 2 C genes; 56 potential holoenzyme products) (Janssens and Goris 2001). PP2A function is critical for the regulation of a host of targets in both excitable and non-excitable cells ranging from ion channels and transporters, regulatory enzymes, transcription factors, and cytoskeletal proteins. Moreover, the role of PP2A for vertebrate physiology is clearly illustrated by its link to human disease. For example, PP2A dysfunction is liked with numerous forms of oncogenic regulation and Alzheimer's Disease (Eichhorn, Creyghton et al. 2009; Rudrabhatla and Pant 2011) and the design of PP2A-based inhibitors are a major area of research in cancer biology.

PP2A Targets in Cardiomyocytes

In cardiomyocytes, PP2A activity is linked with multiple target molecules important in membrane excitability and excitation-contraction coupling including ryanodine receptor (RyR2), connexin 43, Cav1.2, troponin I (cTnI), the Na^{+}/Ca^{+2} exchanger (NCX), and phospholamban (PLB)(Marx, Reiken et al. 2000; Schulze, Muqhal et al. 2003; Terentyev, Viatchenko-Karpinski et al. 2003; Ai and Pogwizd 2005; Deshmukh, Blunt et al. 2007; Kohr, Davis et al. 2009; Xu, Ginsburg et al. 2010; Wijnker, Boknik et al. 2011). Additionally, PP2A subunits directly associate with cardiac regulatory molecules including ankyrin-B (Bhasin, Cunha et al. 2007; Cunha and Mohler 2008). Notably, while manipulation of PP2A activity or expression in animal or cell models produce defects in myocyte physiology and cardiac phenotypes (Gergs, Boknik et

al. 2004; Deshmukh, Blunt et al. 2007; Yin, Cuello et al. 2010), the role and regulation of this critical enzyme family in cardiovascular disease is still largely elusive.

Possible Roles of PP2A in Cardiovascular Disease

Research conducted on PP2A activity in cardiac physiology and disease has been sporadic in recent years and has revealed a somewhat disjointed picture of how this important cellular enzyme functions to regulate cardiomyocyte signaling dynamics. Recent studies have indicated that PP2A activity is important in regulating oxidative stress induced apoptotic pathways (Liu and Hofmann 2004). Furthermore, we have learned that PP2A also plays an important role in the organization of sub-nuclear structures in cardiomyocytes (Gigena, Ito et al. 2005). A series of investigations also showed that expression of the catalytic subunit of PP2A is increased in the ventricles of mice exposed to hypoxic conditions and some have suggested the cytokine release related to hypoxia may alter PP2A expression or activity in cardiac tissue (Larsen, Lygren et al. 2008). Similarly, a different group investigating the mechanisms and cellular consequences of insulin resistance in the heart published findings suggesting that changes in calcineurin and PP2A activity are important in the pathologic processes associated with metabolic syndrome (Ni, Wang et al. 2007). Other work has also shown that *in vivo* exposure of mice to lipopolysaccharide (LPS) alters PP2A activity and expression in cardiac tissue and this work suggested PP2A may play an important role in cardiovascular related complications in sepsis. (Marshall, Anilkumar et al. 2009). All of these results are very interesting and strongly suggest that PP2A expression and activity is important in cardiovascular physiology and disease however we lack an all-important understanding of how PP2A activity, expression and localization is regulated *in vivo* and how this regulation is affected by human cardiovascular disease.

Thesis Overview

The data discussed in this dissertation was collected over the past several years and presents the first full characterization of the PP2A family of polypeptides in the heart. This work has identified seventeen different PP2A genes expressed by a healthy human heart and demonstrates unique subcellular distributions of PP2A regulatory subunits in myocytes, strongly implicating the regulatory subunit in conferring PP2A target specificity *in vivo*. This thesis describes the differential regulation of PP2A scaffolding, regulatory, and catalytic subunit expression in acquired human cardiovascular disease as well as in several well described animal models of acquired and inherited human disease. Finally, this work demonstrates more intricate regulation of PP2A activity by post-translational modification of the catalytic subunit and proposes a mechanism for how this level of regulation is altered in human disease. Together, these findings provide new insight into the functional complexity of PP2A expression, activity, and regulation in the heart and in human cardiovascular disease.

While the first section of this thesis describes a pattern of PP2A regulation in cardiovascular physiology and patterns of dysregulation in cardiovascular disease this work also explores the physiological consequences of PP2A subunit dysregulation. To do this, we investigated abnormalities in reversible phosphorylation in a previously characterized model of an inheritable human arrhythmia syndrome called Ankyrin-B Syndrome, or Long-QT Syndrome type IV (LQTS4). This condition is caused by point mutations in the ANK2 gene encoding ankyrin-B that result in a loss of ankyrin-B function, an important cytoskeletal scaffolding protein. It is widely speculated that this condition has direct link to catecholamine dependent abnormalities in reversible phosphorylation but this connection is currently unclear. Although ankyrin-B dysfunction results in abnormal localization of numerous ion channels and transporters in the heart, this protein was not known to interact with components regulating beta-adrenergic signaling in the heart until recently, when it was discovered that ankyrin-B is critical for

the proper localization of one of the PP2A regulatory subunits. The data presented in the latter half of this thesis will describe a mechanism for how dysregulation of a PP2A regulatory subunit causes unopposed kinase activity at specific intracellular targets in cardiomyocytes affected by this disease and contributes to the catecholamine induced arrhythmias seen in human patients carrying the ankyrin-B mutations.

This work provides new insight on PP2A regulation and how dysregulation of this enzyme occurs in human disease. This body of data also highlights new areas of investigation that require further study in order to more fully comprehend how phosphatase activity is affected by human disease and how such activity may be modulated to alleviate disease phenotypes.

CHAPTER 2:

PATTERNS OF PHOSPHATASE REGULATION IN CARDIOVASCULAR HEALTH AND DISEASE

Introduction

In metazoans, the delicate balance of protein phosphorylation is tightly synchronized by the competing activities of protein kinases and phosphatases. Dysregulation of protein phosphorylation has been linked to mechanical dysfunction and arrhythmias in a host of highly prevalent cardiovascular diseases including atrial fibrillation, sinus node disease, heart failure, and myocardial infarction (Reiken, Gaburjakova et al. 2001; Chelu, Sarma et al. 2009; Shan, Kushnir et al. 2010; Bers 2011; He, Joiner et al. 2011; Swaminathan, Purohit et al. 2011). Consequently, great emphasis has been placed on defining levels and activity of protein kinases in cardiovascular disease. Furthermore, pharmacological inhibitors of kinase activity have significantly enhanced our ability to treat cardiovascular disease phenotypes (Packer 1985; Reiken, Wehrens et al. 2003; Shelton, Clark et al. 2009). In fact, the success of beta-adrenergic receptor blockers in the treatment of arrhythmia, hypertension, and heart failure are attributed to the ability to dampen several kinase pathways involved in disease. Nonetheless, kinase activity represents only one facet of the system responsible for regulating protein phosphorylation levels. While the past decade has illustrated the power of manipulating kinase activity in cardiovascular disease, novel targets related to this pathway may offer additional therapeutic avenues. As our understanding of kinase activity in cardiovascular health and disease continues to increase, the conceptual framework for the role of phosphatase activity in cardiac homeostasis and pathology is considerably less advanced. In fact, we lack even a basic fundamental understanding of the expression, activity, and regulation of protein phosphatases in normal heart or in cardiovascular disease.

As discussed previously, PP2A in a prominent phosphatase present in the majority of cell types relevant to human physiology. Moreover, PP2A dysfunction is linked with numerous forms of human cancers and Alzheimer's disease (Eichhorn, Creyghton et al. 2009; Rudrabhatla and Pant 2011). Despite the established PP2A association with a variety of membrane bound ion channels and transporters in addition to key signaling molecules (see previous sections), little is known regarding the role of phosphatases in general or PP2A in specific in cardiovascular disease.

This section presents the first full characterization of the PP2A family of polypeptides in heart. We define the expression of seventeen different PP2A genes in human heart and demonstrate their differential expression and distribution across multiple species and in different cardiac chambers. We demonstrate unique subcellular distributions of PP2A regulatory subunits in myocytes, strongly implicating the regulatory subunit in conferring PP2A target specificity *in vivo*. We report striking differential regulation of PP2A scaffolding, regulatory, and catalytic subunit expression in multiple models of cardiovascular disease as well as in human heart failure samples. Finally, we demonstrate that PP2A regulation in disease extends far beyond expression and subcellular location, by identifying novel differential post-translational modifications of the PP2A holoenzyme in human heart failure. Collectively, our findings provide new insights into the functional complexity of PP2A expression, activity, and regulation in cardiac physiology and pathophysiology and identify potential therapeutic targets for heart failure and arrhythmias.

Materials and Methods

Human tissue samples

Left ventricular (LV) tissue was obtained from explanted hearts of patients undergoing heart transplantation through The Cooperative Human Tissue Network: Midwestern Division at The Ohio State University (OSU). Approval for use of human

subjects was obtained from the Institutional Review Board of OSU. LV tissue from healthy donor hearts not suitable for transplantation 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 information acquired from tissue providers.

Tissue preparation

Cardiac tissue from human, canine, and murine hearts was flash frozen with liquid N2 and ground into a fine powder using a chilled mortar and pestle. The resulting powder was then resuspended in homogenization buffer (1 mM NaHCO3, 5mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 1 mM NaF, 1 mM PMSF, and protease inhibitor cocktail [Sigma]) and further homogenized using a chilled Dounce homogenizer. Samples were flash frozen in liquid N_2 and stored at -80°C for immunoblots.

Immunoblots

Following quantification, tissue lysates were analyzed on Mini-PROTEAN tetra cell (BioRad) on a 4-15% precast TGX gel (BioRad) in Tris/Glycine/SDS Buffer (BioRad). Gels were transferred to a nitrocellulose membrane using the Mini-PROTEAN tetra cell (BioRad) in Tris/Glycine buffer with 10% methanol (v/v, BioRad). Membranes were blocked for 1 hour at room temperature using a 3% BSA solution and incubated with primary antibody overnight at 4°C. Antibodies included: anti-PP2A/A (Calbiochem 539509), anti-PP2A-C subunit (1:500, Millipore 05-421), Anti-PP2A subunit B isoform PR55α (1:500, Sigma SAB4200241), Anti-PPP2R5A (1:500, Abcam ab72028) or Anti-PP2A-B56-α (1:200, Santa Cruz sc-136045), anti-PPP2R5B (Abcam ab1366), Anti-PPP2R5C (1:500 Abcam ab94633), Anti-PP2A subunit B isoform B56-δ (Sigma SAB4200255), Anti-PPP2R5E (1:1000, Sigma HPA006034), anti-PPP2R3A (Sigma HPA035829), Anti-PP2A alpha phospho Y307 (1:500 Abcam, ab32104), anti-PP2A methyl L309 (1:500, Abcam ab66597). Secondary antibodies included donkey antimouse-HRP, donkey-anti-rabbit-HRP, and donkey-anti-goat-HRP (Jackson

Laboratories). Densitometry was performed using Adobe Photoshop software and all data was normalized to GAPDH levels present in each sample.

mRNA analysis

Touchdown PCR reactions were done in volume of 20µl using Platinum *Taq* Polymerase High Fidelity. Briefly, following an 5 minute denaturation step at 95°C the initial annealing temperature was set at 3°C above the maximum annealing temperature of the primer with the lowest annealing temperature in each primer set for ten rounds of PCR amplification. This was followed by ten rounds of PCR amplification in which the annealing temperature was set to the maximal annealing temperature of the primer with the lowest annealing temperature in the set, followed by ten rounds of PCR amplification in which the annealing temperature was set 3°C below the annealing temperature of the primer with the lowest annealing temperature. Following thirty rounds of PCR amplification, there was a final elongation step for 5 minutes at 72°C. The PCR products were then ran on a 1% agarose gel at 120V for 30 minutes in TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA). See Table 1 for a list of primer sequences used for these experiments.

Immunofluorescence

Isolated cardiomyocytes were blocked in PBS containing 0.075% Triton X-100 and 3% BSA for 1 hour at room temperature, and incubated in primary antibody overnight at 4 °C (same antibodies as listed in the immunoblots section of supplemental data). Following washes (PBS plus 0.1% Triton X-100), the slides were incubated in secondary antibody (Alexa 488, or 568) for 4 hours at 4 °C and mounted using Vectashield (Vector) and coverslips. Images were collected on Zeiss 510 Meta confocal microscope (40 power oil 1.40 NA (Zeiss), pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment.

Cardiomyocyte preparations

Neonatal and adult cardiomyocytes were prepared as previously described (Mohler PJ 2003; Hund, Wright et al. 2009). Murine hearts from adult wild type C57Bl/6, mice were obtained after animals were euthanized by acute CO_2 asphyxiation followed by cervical dislocation in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH and IACUC approved protocols. Hearts were removed from the animal immediately following euthanasia and canulated on a langendorff apparatus for perfusion. Hearts were perfused with modified Tyrodes solution (136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.5 mM HEPES, pH adjusted to 7.4 with NaOH) for 5 minutes. This was followed by 12 minute perfusion with modified Tyrode solution supplemented with collagenase type II (Worthington) and Protenase (Sigma) and 10 mM $CaCl_2$. The ventricles were then removed from the apparatus and mechanically agitated in a solution of Modified Tyrode Solution supplemented with 10mM $CaCl_2$ for 10-15 minutes to isolate ventricular cardiomyocytes.

Hearts were dissected from neonatal (1–3 day old) mice under sterile conditions by bathing the animal in 70% alcohol. Each heart was then placed in a 12-well flat-bottom tissue culture plate containing 2 ml of Ham's F10 nutrient media. The atrium and major blood vessels were removed and the ventricle was rinsed by squeezing with forceps to wash away the blood. The ventricles were minced finely in 1.5 ml of 0.05% trypsin, 0.2 mM NaEDTA, and placed in a humidified incubator (37°C, 95% air-5% CO2) for 15 min. Ventricles were then resuspended several times in a 1-ml Eppendorf pipette and incubated for another 15 min. After incubation, 0.2 ml of 2-mg/ml soybean trypsin inhibitor was added, followed by the addition of 0.2 ml of 0.2-mg/ml collagenase (type VII; Sigma). The homogenate was resuspended several times and incubated further for 30–50 min until dissociation of cells was complete. Then 2 ml of complete media (DME/Ham's F10, 10% FBS, and 10% HS) was added, and cells were pelleted by

centrifugation at 500 *g* for 5–10 min. Cells were resuspended in 2 ml complete media and transferred to a 35-mm petri dish to remove fibroblasts by differential adherence (2 h at 37°C). Cardiomyocytes in the supernatant were collected by centrifugation at 500 *g* for 5–10 min, and resuspended in 1 ml complete media. 0.25 ml of cell suspension was plated on a fibronectin-coated plate at a density of 1×10^6/ml and washed 24 h later with complete media to remove dead cells and debris. To minimize growth of nonmuscle cells, complete media was replaced with defined growth medium [DMEM/F10 with additions of insulin (1 μg/ml), transferrin (5 μg/ml), LiCl (1 nM), $NaSeO_4$ (1 nM), ascorbic acid (25 μg/ml), thyroxine (1 nm)], or with serum-free medium (DMEM/F10).

H_20_2 Treatment of cardiomyocytes and Fibroblasts

Neonatal murine fibroblasts and cardiomyocytes were separated by means of differential adherence during the isolation procedure as noted above. After 24 hours of culture at 37°C and 5% CO_2 in either defined growth media [DMEM/F10 supplemented with insulin (1 μg/ml), transferrin (5 μg/ml), LiCl (1 nM), $NaSeO_4$ (1 nM), ascorbic acid (25 μg/ml), thyroxine (1 nm)] for the cardiomyocytes or complete media (DMEM/Ham's F10, 10% FBS, and 10% HS) for fibroblasts, 75μM H_2O_2 was added to the cells for 60 minutes. Following the 60 incubation with H_2O_2, the media was removed and the cells were removed from the culture vessel and lysed via mechanical agitation in Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol).

Canine ischemic and non-ischemic heart failure models

Myocardial infarction was produced in healthy mongrel dogs by total coronary artery occlusion using the Harris method, as described previously (Harris 1950; Hund, Wright et al. 2009). This method involves a two stage ligature of the anterior descending coronary artery and was followed by cardiectomy one, five or ten days post total coronary artery occlusion procedure.

HF via chronic tachypacing was induced as previously described (Nishijima, Feldman et al. 2005; Gudmundsson, Curran et al. 2012). All dogs were initially verified

to have normal cardiac function by examination by a veterinarian. Adult hound-type dogs of either sex (2–3 years of age) received pre-anesthetics of butorphanol 0.2–0.4 mg/kg IM, and morphine (0.5–2 mg/kg IV) followed by anesthesia induction with isoflurane; anesthesia was maintained with isoflurane (1.5–2.5%). An RV pacemaker lead was implanted in the RV apex with fluoroscopic guidance followed by implantation of a modified Prevail 8086 pacemaker (Medtronic, Inc., Minneapolis, MN, USA). Following recovery from the pacemaker implant, the RV was paced at 180 bpm for 2 weeks; 200 bpm for the next 6 weeks, followed by 180 bpm for the duration of the protocol. Sequential echocardiograms were performed at baseline and during brief periods of sinus rhythm during butorphanol sedation (0.5 mg/kg, im) to confirm the presence of reproducible dilated cardiomyopathy. After 1 or 4 months of tachypacing, animals were administered butorphanol (0.2–0.4 mg/kg IM) followed by pentobarbital (25 mg/kg IV) or isoflurane (5% for induction, 2% for anesthesia). After achieving a deep level of anesthesia, the heart was rapidly removed. Cardiac tissues were snap frozen in liquid nitrogen and stored at − 80 °C until used. Control dogs were sacrificed in parallel.

All animal studies were performed in accordance with the American Physiological Society *Guiding Principles for Research Involving Animals and Human Beings*, and approved by the University of Iowa or The Ohio State University Institutional Animal Care and Use Committee. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996)

Co-Immunoprecipitation Experiments

Frozen wild type and canine heart samples (see *Canine ischemic and non-ischemic heart failure models* in the Materials and Methods section) were flash frozen in liquid nitrogen and ground into a fine powder. Samples were then resuspended in homogenization buffer (.32 M sucrose, 2.5 mM EGTA, 5 mM EDTA, 50 mM Tris, and 10 mM NaCl, pH 7.47) and further homogenized by mechanical agitation using a dounce

homogenizer. After douncing, 1% Triton-100 was added to each sample and the samples were then sonicated three times for 15 seconds with 5 minutes on ice between each round of sonification. Lysates were centrifuged for 15 minutes at 3000g and the supernatant was transferred to a new Eppendorf tube. The supernatant was then incubated with 40 µl TrueBlot anti-rabbit Ig beads (eBioscience) for 60 minutes at 4°C to remove as much background binding as possible. Samples were again centrifuged at 3000g for 2 minutes and 200 µL of sample was loaded into each of two tubes (two tubes per sample). One tube was incubated with 4µL of anti-PP2A/A antibody (Calbiochem 539509) at 4°C for 1 hour followed by the addition of 40µL TrueBlot anti-rabbit Ig beads overnight at 4°C. The other tube containing 200µL of sample lysate was just incubated with TrueBlot anti-rabbit IgG beads overnight at 4°C. 20µl and 10µL of each sample were set aside to be used as a 10% and 5% input loading control. Following overnight incubation with TrueBlot anti-rabbit IgG beads, the supernatant was removed from the beads and the beads were washed 3 times with wash buffer (1X PBS, .1% Triton, and 150 mM NaCl). Following the final wash, all samples were subjected to same western blot protocol as described in the *Immunoblots* subheading of this section.

Statistics

P values were determined with a paired Student's *t* test (2-tailed) or ANOVA, when appropriate, for continuous data. The Bonferroni test was used for post-hoc testing. The null hypothesis was rejected for $p<0.05$. Values are expressed as the mean ±SE.

Results

Defining the human cardiac PP2A 'phosphatome'

The human genome encodes at least seventeen different PP2A subunits (Fig.5; 2 A subunits, 13 B subunits, 2 C subunits, see Table 2 for detailed gene and protein nomenclature of subunit families). However, despite intense study of PP2A function in

metazoans, surprisingly little is known regarding the differential expression or activity of PP2A subunits across tissues or cell types. In fact, there are no studies that characterize the expression of the PP2A subunit family in human heart. We hypothesized that analysis of the expression profile of the different PP2A subunits in heart would provide important insight into how signaling specificity is achieved for phosphatase activity. We first identified PP2A subunits in healthy human heart using PP2A subunit gene-specific probes. We identified mRNA for both isoforms of the scaffolding subunit (A subunits α and β) in human LV (Fig.6). Additionally, message for both α and β isoforms of the PP2A catalytic subunit were present (Fig.6). Notably, while thirteen PP2A regulatory subunit genes are present in the human genome (Fig.6), we identified significant mRNA expression of only twelve of thirteen genes in human heart. Specifically, catalytic subunit (*PPP2AC),* two scaffolding subunits *(PPP2R1A*, *PPP2R1B),* and multiple regulatory subunits *(PPP2R2A, PPP2R2B, PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, PPP2R5E, PPP2R3A, PPP2R3B, PPP2R3C,* and *PPP2R4)* were detected in human heart. Message for *PPP2R2C,* not present in heart, was observed in human brain (Fig.6). All appropriate negative controls for PP2A subunit mRNAs were blank.

Expression of PP2A subunits in human heart

We next tested PP2A subunit gene product expression in human heart. Specifically, we tested the protein expression of the associated catalytic, regulatory, and scaffolding subunits with detected mRNA (Fig.7) in human LV using subtype-specific antibodies. Since we identified message for a large number of regulatory subunits, we hypothesized that unique intra-species and intra-chamber protein expression profiles might exist as a mechanism to confer signaling specificity. As predicted, we observed both PP2A catalytic and scaffolding subunits at the appropriate molecular weights in human LV (Fig.7). We observed nine different PP2A regulatory subunits (*PPP2R2A, PPP2R2B*, *PPP2R3A*, *PPP2R4*, *PPP2R5A*, *PPP2R5B*, *PPP2R5C*, *PPP2R5D*, and *PPP2R5E)* at their appropriate molecular weights by immunoblot (Fig. 7). Other

subunits were not detected. Notably, subsequent immunoblot tissue analyses across human heart chambers revealed differences in PP2A subunit expression. For example, both PP2A catalytic and scaffolding subunit expression were significantly higher in right atria (RA) and ventricle (RV) when compared with left atria (LA) and ventricle (LV) (Fig. 7). However, for regulatory subunits, we observed no statistical differences in relative protein expression between the four human chambers.

Finally, we tested whether subtype expression was conserved across species. Immunoblots of human, canine, rat, and mouse LV confirmed presence of both PP2A catalytic and scaffolding subunits (PP2A-A, PP2A-C) as well as regulatory subunits (*PPP2R2A*, *PPP2R2B*, *PPP2R3A*, *PPP2R4*, *PPP2R5A*, *PPP2R5B*, *PPP2R5C*, *PPP2R5D*, and *PPP2R5E*; Fig.8). While we did not observe differences in overall expression patterns of the catalytic or scaffolding subunits, several regulatory subunits had variable inter-species expression levels. *PPP2R5A* and *PPP2R5E* expression levels were significantly higher in rat and mouse than in human and dog (Fig.8) and *PPP2R2A and PPP2R5C* were significantly lower in rat and mouse than in human and dog (Fig.8). These new findings illustrate the complexity and diversity of PP2A subunit expression in mammalian hearts. Furthermore, they suggest that variable expression of the regulatory subunits may confer signaling specificity across regions, species, and disease states.

Differential subcellular expression of PP2A subunits

As illustrated above, diversity in PP2A holoenzyme activity may arise from differential organ or chamber mRNA and/or protein expression. However, a secondary level of target specificity may arise through unique subcellular distribution of specific PP2A holoenzymes. We tested our hypothesis by evaluating the subcellular localization of PP2A subunits in primary adult cardiomyocytes. We observed broad subcellular distribution of PP2A scaffolding and catalytic subunits throughout the cytosol and nucleus of ventricular cardiomyocytes. In fact, immunostaining for both A and C subunits was present within the entire myocyte (Fig.9). In contrast, we observed

remarkable specificity in the localization of PP2A regulatory subunits in ventricular myocytes. For example, *PPP2R5E* was localized specifically to the Z-line/T-tubule region as demonstrated by co-distribution with α-actinin (Fig.9I-J), while *PPP2R3A* was localized to both Z- and M-lines of the cardiomyocyte (Fig.9C-D). *PPP2R5C* was primarily localized to the myocyte nucleus, with a secondary population overlying the cardiac Z-line (Fig.9G-H). Finally, *PPP2R4* was concentrated in the cardiomyocyte nucleus, and in fact, localized with nuclear speckles (Fig.9E-F). Together, our findings illustrate an additional layer of complexity of PP2A holoenzyme regulation for specific subcellular targets. These findings will be critical in defining the target specificity and function of each PP2A holoenzyme *in vivo*.

PP2A subunit regulation in human heart failure

We next determined if expression levels of PP2A subunits were altered in human heart failure. In particular, our goal was to determine whether variability in expression of the regulatory subunits might serve as a unique molecular identifier for disease. We first investigated PP2A subunit expression in human ischemic heart failure samples. While PP2A scaffolding subunit levels were unaffected in LV tissue, we observed nearly a two fold increase in PP2A catalytic subunit expression when compared to control hearts (Fig.10, n=5 control and non-ischemic, $p<0.05$). We observed striking differential expression of regulatory subunits in ischemic heart failure, with elevated levels of *PPP2R5A, PPP2R5B, PPP2R5E, PPP2R3A,* and *PPP2R4* (Fig.10; n=5 control and non-ischemic, $p<0.05$). Other subunit levels were not statistically different between control and diseased hearts (Fig.10, n=5 control and non-ischemic, N.S.).

We examined PP2A subunit expression in LV samples from non-ischemic failing and control hearts. While we observed similar data for PP2A catalytic subunit (increased approximately two fold), PP2A scaffolding subunit levels were also elevated in non-ischemic LV (Fig.11, n=5 control and non-ischemic, $p<0.05$ for A, C subunits). Regulatory subunit overexpression was observed for *PPP2R5A, PPP2R5B, PPP2R5E,*

PPP2R3A, and *PPP2R4*, similar to ischemic heart failure data (Fig. 11; n=5 control and non-ischemic, $p<0.05$). Consistent with ischemic heart failure data, we observed no difference in the expression of *PPP2R2A*, *PPP2R2B*, *PPP2R5C*, or *PPP2R5D* gene products (Fig.11, n=5 control and non-ischemic, N.S.). These data provide the first insight into the complex regulation of PP2A subtype family proteins in human heart disease, and clearly demonstrate significant complexity in the regulation of specific PP2A holoenzymes in specific pathologies. These data are also congruent with differential target phospho-protein regulation in specific disease pathologies.

PP2A subunit regulation in canine models

We examined PP2A subunit expression in two different canine models of human heart disease. We first evaluated PP2A subunit expression following myocardial infarction (five days after total coronary artery occlusion), a leading cause of death worldwide (Roger, Go et al. 2012). Similar to both ischemic and non-ischemic human heart failure samples, we observed elevated levels of PP2A catalytic subunit in infarct border zone tissue compared to remote tissue of a well validated canine VT post myocardial infarction model (Baba, Dun et al. 2005) (Fig.12; n=5 control and post-occlusion, $p<0.05$). Additionally, PP2A scaffolding subunit levels were also significantly elevated, although only moderately at five days post-occlusion (Fig.12; n=5 control and post-occlusion, $p<0.05$). PP2A regulatory subunits with altered expression included *PPP2R5A*, *PPP2R5D*, and *PPP2R4* at five days post occlusion. At this early time point, we observed no difference in the expression of regulatory subunit products of *PPP2R2A*, *PPP2R2B*, *PPP2R2B*, *PPP2R5C*, *PPP2R5E*, or *PPP2R3A* (Fig.12; n=5 control and post-occlusion, N.S.).

In addition to the canine myocardial infarction model above, we also evaluated PP2A subunit expression in a well validated long-term tachy-pacing induced non-ischemic model of canine heart failure(Nishijima, Feldman et al. 2005). At four months of heart failure, we observed elevated levels of both scaffolding and catalytic subunits in

experimental samples compared with normal control tissue (Fig.13; n=5 control and HF, $p<0.05$). *PPP2R2B*, *PPP2R5B*, *PPP2R5D*, *PPP2R5E*, *PPP2R3A*, and *PPP2R4* gene products were significantly elevated in failing dog LV compared with control (Fig.13; n=5 control and HF, $p<0.05$). In contrast, levels of *PPP2R2A*, *PPP2R5A*, and *PPP2R5C* were unchanged between control and heart failure samples (Fig.13; n=5 control and HF, N.S.). Collectively, data in these canine ischemic and non-ischemic disease models demonstrate significant regulation of the PP2A enzyme through regulation of both PP2A catalytic and scaffolding subunits. However, these data also reveal that full holoenzyme regulation may be tightly regulated for target specificity in each disease through regulation of regulatory subunit expression. Our data further demonstrate that each specific disease pathology (ischemic versus non-ischemic, acute versus chronic, etc.) likely has its own PP2A subunit expression signature presumably corresponding with selective regulation of specific targets.

PP2A subunit regulation in mouse model of human CPVT

Dysregulation in cardiac sympathetic tone has been linked to both genetic and acquired forms of human ventricular arrhythmia (Marx, Reiken et al. 2000). In fact, mutations in select myocyte pathways that alter the phospho-protein axis have been identified as a primary cause of multiple forms of congenital catecholaminergic polymorphic ventricular tachycardia (CPVT) (Lehnart, Ackerman et al. 2007). One notable pathway is the ankyrin-B-pathway that coordinates the subcellular localization of membrane associated ion channels, transporters, and signaling molecule, which is altered in human CPVT as well as sinus node disease, heart rate variability, and atrial fibrillation (Mohler, Schott et al. 2003; Cunha, Bhasin et al. 2007; Le Scouarnec, Bhasin et al. 2008; Cunha, Hund et al. 2011). We hypothesized that PP2A subunit levels might be altered in a murine model of human ankyrin-B CPVT. Consistent with investigated forms of human and canine heart disease (Fig.10-13), we observed significant increases in both PP2A catalytic and scaffolding subunits in adult ankyrin-$B^{+/-}$ LV compared with

littermates (Fig.14; n=5 control and ankyrin-$B^{+/-}$, $p<0.05$). Additionally, we observed alterations in expression of PP2A regulatory subunits *PPP2R5A*, *PPP2R5B*, and *PPP2R5E* in ankyrin-B deficient hearts (Fig.14; n=5 control and ankyrin-$B^{+/-}$, $p<0.05$). While there were trends for differences in other PP2A regulatory subunits, we observed no significant difference in expression of *PPP2R2A*, *PPP2R2B*, *PPP2R3A*, *PPP2R4*, *PPP2R5C*, or *PPP2R5D* between WT and ankyrin-$B^{+/-}$ mice (Fig.14; n=5 control and ankyrin-$B^{+/-}$, $p<0.05$). Thus, consistent with a role for aberrant regulation of the kinase/phosphatase signaling axis in sympathetic-mediated ventricular tachycardia, our results indicate that multiple arms of the PP2A signaling cascade are differentially regulated in a mouse model of CPVT.

Post-translational regulation of cardiac PP2A

While tissue, cell, and subcellular subunit expression and localization play key roles in determining local holoenzyme function, an additional form of modulation, post-translational regulation of PP2A activity has been implicated in holoenzyme regulation in other organ systems and non-cardiac disease states (Janssens and Goris 2001; Longin, Zwaenepoel et al. 2007). Notably, the C-terminus of the PP2A catalytic subunit interacts with an interface of the A and B subunits. This C-terminal region is altered by both phosphorylation and methylation that serve to "switch" the active and inactive state of the full holoenzyme through altering the recruitment and docking of the regulatory subunit with the A and C subunits (Fig.15)(Longin, Zwaenepoel et al. 2007). We therefore examined whether these modifications were present in human heart and modified in human heart disease. We first examined the phosphorylation status of the catalytic subunit of PP2A at residue Y307, a site liked with inactivation of the phosphatase produced by $pp60^{vSrc}$, $pp56^{lck}$, epidermal growth factor, and insulin receptor signaling at baseline in heart, and in heart disease (Chen, Martin et al. 1992). Notably, we observed phosphorylated PP2A/C Y307 in control human heart (Fig.16). Moreover, we observed a significant increase in the expression level of the phosphorylated (inactivated) catalytic

subunit being expressed in heart failure samples compared to controls (Fig.16; $p<.05$, n=4). In contrast, we observed no difference in methylation of PP2A catalytic subunit at residue L309. This site is regulated by the opposing activities of leucine carboxymethyl-transferase 1 (LCMT-1) and phosphatase methyl-transferase 1 (PME1) to control recruitment of the *PPP2R2A* and *PPP2R2B* regulatory subunits (Janssens, Longin et al. 2008). Total PP2A catalytic subunit expression was increased in non-ischemic heart failure (Fig.5B,C). However, in parallel, levels of PP2A-phospho-Y307 (inactivated) were increased. Thus, we normalized expression of phosphorylated and methylated forms of the PP2A catalytic subunit to the expression level of the total amount of the PP2A/C subunit expressed in each sample. Notably, the ratio of PP2A subunit Y307 to total PP2A/C was increased in human heart failure samples compared to control (Fig.16; $p<.05$; n=4), favoring inactivation of the holoenzyme. Moreover, the ratio of methylated catalytic subunit to total catalytic subunit was significantly decreased in human heart failure compared to control samples (Fig.16; $p<0.05$), a modification further disabling the functional holoenzyme. We conducted the same experiments in lysates from human ischemic heart failure and canine pacing induced heart failure and found the same trends (Fig.17 and Fig.18) These data suggest that despite an increase in overall expression of the PP2A catalytic subunit in heart failure, the regulation of the subunit is altered dramatically and likely produces a net loss of phosphatase function. In summary, our findings provide the first data on PP2A subunit post-translational regulation in heart, and provide compelling data on the regulation of these critical subunits in human heart failure. Both post-translational pathways identified in heart failure appear to inactivate the holoenzyme.

Specific subunits are excluded from PP2A in HF

Data from other cell lines has shown that certain subunits, namely the B family of regulatory subunits (PPP2R2A and PPP2R2B) are more likely to be incorporated into the PP2A holoenzyme by the addition of a methyl group to the catalytic subunit. Based on

our data, we hypothesized that PPP2R2A, a member of the B family of regulatory subunits, is selectively excluded from functional PP2A holoenzymes in the cardiomyocytes of diseased hearts. To test this hypothesis we performed a co-immunoprecipitation assay in which an antibody for PPP2R2A was used to pull down the scaffolding subunit from heart lysates made from healthy control hearts and from hearts experiencing non-ischemic heart failure. In this experiment, the relative amount of scaffolding subunit pulled down by an equal quantity of anti-PPP2R2A antibody functions as a surrogate marker for the amount relative amounts of PPP2R2A incorporated into functional PP2A enzymes. Interestingly, we found a decrease in the amount of scaffolding subunit pulled down in the heart failure samples compared to control samples (Fig.19). This data strongly indicates that just like in other cell types previously studied by other groups, methylation of scaffolding subunit facilitates the incorporation of the B family of regulatory subunits into the PP2A holoenzyme.

Decreased LCMT-1 expression.

The process of catalytic subunit methylation is directly regulated by two enzymes, LCMT-1 that catalyzes the addition of the methyl group to the subunit and PME-1 that catalyzes the removal of this group (Fig.15). We next examined if differences in these two enzymes might mechanistically explain the observed differences in catalytic subunit methylation. Although we did not see a difference in PME-1 expression in whole heart lysates made from control samples and human heart failure samples (Fig.20; p=N.S, n=3), we did observe a statistically significant decrease in LCMT-1 expression in human heart failure samples (Fig.20; $p<.05$, n=3). Furthermore, the same trend was observed in the canine model of heart failure where we also did not detect a significantly different level of PME-1 expression between control and heart failure samples (Fig.20; p=N.S, n=3). However we were able to detect a significantly decreased level of LCMT-1 expression in heart failure samples when compared to control samples (Fig.20; $p<.05$, n=3). Based on these results, we believe the observed decreased methylation level of the

catalytic subunit in human heart failure is due to decreased expression of LMCT-1 in this disease process.

ROS treatment can induce post-translational modifications

Cardiovascular disease is frequently associated with extreme abnormalities in neurohormonal activity such as elevated catecholaminergic stimulation of target organs like the heart and vasculature, and alterations of the resting renin-angiotensin-aldosterone axis. Elevated levels of circulating aldosterone and norepinephrine have been found to be correlated with increased morbidity and mortality in heart failure and myocardial infarction (Cohn, Levine et al. 1984; Swedberg, Eneroth et al. 1990; Beygui, Collet et al. 2006). In addition to increasing Na^+ reabsorption by the distal convoluted tubule in the kidney, aldosterone increases the production of reactive oxygen species (ROS) in the myocardium (Rude, Duhaney et al. 2005). Furthermore, β-adrenergic stimulation of cardiomyocytes also causes an increase in ROS production (Srivastava, Chandrasekar et al. 2007). ROS production in cardiomyocytes alters a variety of receptor properties, signaling molecule activity, and ion channel dynamics that all lead to contractile dysfunction (Feldman, Elton et al. 2008).

In order to determine if ROS generation could alter post-translation regulation of the catalytic subunit, we exposed cardiomyocytes to 75 μM H_2O_2 for one hour and then performed immunoblots to detect the relative levels of the phosphorylated, methylated and total catalytic subunit. We found that although total expression of the catalytic subunit did not significantly change with H_2O_2 exposure, the level of methylated catalytic subunit was significantly decreased when compared to untreated cardiomyocytes (Fig.22, $p<.05$, $n=4$), and the level of phosphorylated catalytic subunit was significantly increased when compared to untreated cardiomyocytes (Fig.22, $p<.05$, $n=4$). In addition, we were also able to separate cardiomyocytes from fibroblast by taking advantage of their differential adherence to culture vessels during the isolation procedure (see Materials and Methods section for more details) and did not observe any significant change in either the

total expression or post-translational modification of the catalytic subunit in treated cells compared to untreated controls (Fig.21, p=N.S., n=4).

Based on these results, we believe that post-translational regulation of the catalytic subunit can be altered in a relatively quick manner by ROS generation within cardiomyocytes. In light of the fact that saw no change in catalytic subunit expression during this short time course, we hypothesize that this method of regulation may function to acutely regulate PP2A target selectivity in cardiomyocytes. Furthermore, these results provide additional confidence that the data reported in previous sections is representative regulatory mechanisms occurring in cardiomyocytes as opposed to any of the cells types that are present in healthy and disease cardiac tissue.

Discussion

Thousands of studies over the past decade have illustrated the complexity and dysregulation of the kinase/phosphatase axis in cardiovascular disease (Anderson, Brown et al. 2011). For example, increased activity of GSK-3Beta has been linked to cardiomyocyte cell death following ischemia and reperfusion (Miura and Miki 2009) and increased PKA, PKC and CaMKII activity have all been linked to a host of congenital and acquired cardiac pathologies (Braz, Gregory et al. 2004; Zhang, Khoo et al. 2005; Ling, Zhang et al. 2009). While our knowledge of how aberrant regulation of kinase activity contributes to human cardiovascular disease continues to expand and has seeded the development of new compounds for cardioprotection, phosphatase activity and its regulation in disease remains less well understood. Here, we provide the first study of the expression and regulation of the PP2A family of protein phosphatase polypeptides in heart. Our findings demonstrate that PP2A subunits show unanticipated complexity in their expression patterns in heart and heart cells with distinct expression profiles across cardiac chambers, species, and disease states. Steady state expression of the large family of regulatory subunits, in particular, is highly variable and may serve as a molecular

rheostat to tune signaling specificity. The regulation of specificity is further regulated by subunit specific expression within different subcellular domains, and ultimately local signaling is controlled by *multiple* post-translational modifications. In different cardiac disease pathologies, we demonstrate striking remodeling of not only subunit expression, but also subunit post-translational phosphorylation and methylation. Moreover, our data demonstrate that unique cardiac pathologies display specific combinations of subunit regulatory patterns associated with not only the severity of the phenotype, but also with the time course of the cardiac insult. Ultimately, these data provide a new compelling story of the complex nature of protein phosphatase regulation in health and disease, strongly supporting not only distinct, but spatially and temporally regulatory phospho-targets.

One unexpected finding from this study was the diversity in PP2A regulatory (B) subunit regulation between cardiac chambers, cell types, subcellular domains, and disease phenotypes. Based on our findings, we predict that the PP2A regulatory subunits provide the predominant regulatory step for PP2A function in heart. While PP2A scaffolding (A) and catalytic (C) subunits are expressed throughout all chambers and across cellular domains, we identified amazing diversity across the regulatory subunit family. For example, while *PPP2R5E* was expressed at the M-line, *PPP2R4* was enriched in the nucleus of the cell. These findings are even more compelling based on the high sequence conservation between subunits. Our past work has demonstrated that ankyrin proteins play a key role in the subcellular targeting of the *PPP2R5A* regulatory subunit through a unique C-terminal domain (Bhasin, Cunha et al. 2007). Based on our new findings, we predict that the C-terminal domain of each regulatory protein plays an important central role in holoenzyme targeting and specificity *in vivo*.

Post-translational regulation of PP2A has not been previously studied in heart or in heart disease. In human heart failure, the relative decrease in the methylated catalytic subunit, and increase in the phosphorylated catalytic subunit suggest that a specific subset

of regulatory subunits may be selectively excluded from the PP2A enzyme (Chen, Martin et al. 1992; Longin, Zwaenepoel et al. 2007; Janssens, Longin et al. 2008). There are competing interpretations to these data. The first is that pathological processes associated with heart failure alter the expression or enzymatic availability of LCMT-1 and PME-1 (enzymes that regulate catalytic subunit methylation) leading to exclusion of regulatory subunits from active holoenzyme formation. A second interpretation is that shifting PP2A enzymatic activity toward targets favored by the B' and B'' family of regulatory subunits is a physiologic response to heart failure. This study provides only an initial overview of what will ultimately contain multiple orders of magnitude of regulatory complexity. Additional studies on upstream regulatory molecules (e.g. LCMT1/PME1) will be necessary to further define regulation *in vivo,* and whether these alterations are adaptive or maladaptive.

One obvious limitation of this study is lack of data for local PP2A function in specific subcellular domains. For years, the literature has utilized "broad-stroke" functional kinase and phosphatase assays to assess the phosphor-substrate of a tissue or cell. Our new data suggest these global assays, while portraying the phospho-balance of an entire cell population, may have limited use in predicting the regulation of subcellular targets. In contrast, our findings suggest that monitoring the activity of specific regulatory subunits may offer a more sensitive, cell-specific and subcellular-specific assay for defining local holoenzyme activity.

In summary, our data provide new rationale and a new layer of complexity underlying the diversity in tissue, cellular, and subcellular target phospho-regulation in cardiac muscle and in heart disease. While our findings illustrate differential regulation of all PP2A subtype classes (scaffolding, catalytic, regulatory) in health and disease, we were struck by the complexity of the signature of expression and modification that controlled regulatory subunit organization and enzyme docking in each cardiac signature-whether at baseline or in disease. These data offer new insight into the complexity of

cardiac phospho-protein regulation, but also suggest new therapeutic targets to modify specific cellular and subcellular targets for cardioprotection. Moreover, our data suggest that other excitable organs (nervous system, skeletal muscle, beta cell) may also harbor similar regulatory networks for control of cell signaling and physiology.

Primer Name	Primer Sequence	Predicted PCR product length (BP)
PP2A/C_5'	5' - GTT CAG CAA CGA GCT GGA CCA GTG - 3'	610
PP2A/C_3'	5' - CCA CCA CGG TCA TCT GGA TCT GAC CA - 3'	
PP2A/Aα_5'	5' - ACC TCT CAG CTG ACT GTC GGG AGA ATG TGA TCA TGT CCC - 3'	505
PP2A/Aα_3'	5' - CTC CGG ACT GGC CAA GAC CTT GGG GAT GAT TGT GGA - 3'	
PP2A/Aβ_5'	5' - CGA TCG CGG TTT TAA TCG ACG AGC TCC GCA ATG AAG ACG TGC - 3'	658
PP2A/Aβ_3'	5' - ATA CTG ACA CAA GCT TCC ACA GCA AGG AGGG CGC ACT GAA TCC - 3'	
PPP2R2A_5'	5' - GCT GGA GGA GGG AAT GAT ATT CAG TGG TGT TTT TCT CAG GT - 3'	391
PPP2R2A_3'	5' GTA GGA TCT CTA TAC CTT CCA TCC TCC TCT TTC AAG TTA T - 3'	
PPP2R2B_5'	5' - GAT CCT GCC ACC ATC ACA ACC CTG CGG GTG CCT - 3'	747
PPP2R2B_3'	5' - GTC ACA CAG CCG GAT TGT CCC TTT GCT GCG GC - 3'	
PPP2R2C_5'	5' - CDC CDC CCA CTC ACT CCT GTC CAC CAA CGA T - 3'	305
PPP2R2C_3'	5' - GGA CGG CTT GAT GTC CAC GAT GTT GAA GCT CCT GTC GG - 3'	
PPP2R2D_5'	5' - TGG TGC TTC TCG CAG GTC AGG GGG GCC ATC GAC GA - 3'	389
PPP2R2D_3'	5' - GAC CCG TAG CGC CGT GAT CCT AAA TGG GTC TCG AAG TC - 3'	
PPP2R5A_5'	5' - GAG TAT GTT TCA ACT AAT CGT GGT GTA ATT GTT GAA TCA GCG - 3'	303
PPP2R5A_3'	5' - TCC CAT AAA TTC GGT GCA GAA CAG TCT TCA GG - 3'	
PPP2R5B_5'	5' - ATG GAG ACG AAG CTG CCC CCT GCA AGC ACC CCC ACT AGC CCC TCC TCC - 3'	375
PPP2R5B_3'	5' - GAC GGG CTC GAT GAG GAC ACC CCG GGT GCT CCC CAC ACT - 3'	
PPP2R5C_5'	5' - CAG TGA CAA CGC AGC GAA GAT TCT GCC CAT CAT - 3'	347
PPP2R5C_3'	5' - CTA GCG GCC GTC CTG GGA GGC CAG CTC ATC GGC CC - 3'	
PPP2R5D_5'	5' - GGC CCG GCT TAA TCC CCA GTA TCC CAT GTT CCG AGC CCC TCC - 3'	232
PPP2R5D_3'	5' - TCA GAG AGC CTC CTG GCT GGC AGT TAG GAA CTC TTC CGC CCG - 3'	
PPP2R5E_5'	5' - CCG GCT ATT GTG GCG TTG GTG TAC AAT GTG TTG AAG GC - 3'	203
PPP2R25E_3'	5' - TAA AGT TGG AAT TAT TCC ATC ACG TCT ACG TCT AAG ACC TCT CTT TAA - 3'	
PPP2R3A_5'	5' - GGA TGT GGT GGA TAC CCA CCC TGG TCT CAC GTT CCT - 3'	519
PPP2R3A_3'	5' - CTC ATA CAT GGA GAG TAC ACC GTC TCC ATC CAC ATC CAT - 3'	
PPP2R3B_5'	5' - ATG CCG CCC GGC AAA GTG CTG CAG CCG GTC CTG - 3'	280
PPP2R3B_3'	5' - CGT TCG TGG GGC TGG AGG CGG CGC CAA GGG - 3'	
PPP2R3C_5'	5' - TCG TCG GCG CCT AGC GAC GCC CAA CAC CTG - 3'	320
PPP2R3C_3'	5' - ATC GCT TCC TCT CCA ATC ATA GGT GGT GTC TGG TGT TTG TCC AGC - 3'	
PPP2R4_5'	5' - GCT GAG GGC GAG CGG CAG CCG CCG CCA - 3'	487
PPP2R4_3'	5' - GCC AGA TGG GTA GGG ACC ACT GTG GCC ACC - 3'	

Table 1. PP2A subunit primers.

Subunit type	Gene Name	Gene Product	Associated Names	Family (if applicable)
Catalytic (C) Subunits	*PPP2CA*	PP2A/Cα		
	PPP2CB	PP2A/Cβ		
Scaffolding (A) Subunits	*PPP2R1A*	PP2A/Aα		
	PPP2R1B	PP2A/Aβ		
Regulatory (B) Subunits	*PPP2R2A*	PPP2R2A	B55α	B Family of regulatory Subunits
	PPP2R2B	PPP2R2B	B55β	
	PPP2R2C	PPP2R2C	B55γ	
	PPP2R2D	PPP2R2D	B55δ	
	PPP2R5A	PPP2R5A	B56α	B' family of regulatory subunits
	PPP2R5B	PPP2R5B	B56β	
	PPP2R5C	PPP2R5C	B56γ	
	PPP2R5D	PPP2R5D	B56δ	
	PPP2R5E	PPP2R5E	B56ε	
	PPP2R3A	PPP2R3A		B" family of regulatory subunits
	PPP2R3B	PPP2R3B		
	PPP2R3C	PPP2R3C		
	PPP2R4	PPP2R4		B"' family of regulatory subunits

Table 2. PP2A subunit nomenclature.

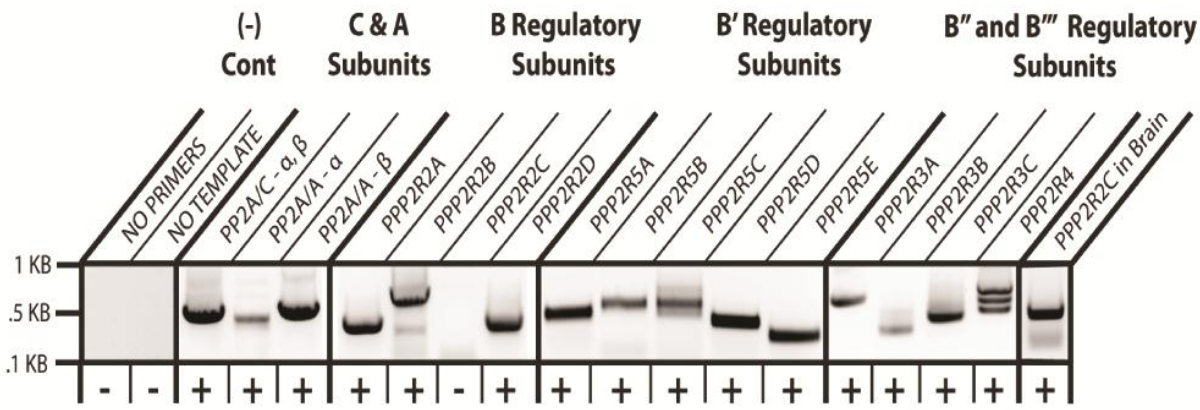

Figure 6. PP2A transcripts present in human heart. Primers designed to amplify the specific subunit noted above were used to determine the presence of PP2A subunit transcripts in normal human heart. PCR products are shown and a plus sign indicates the detection of a PCR product.

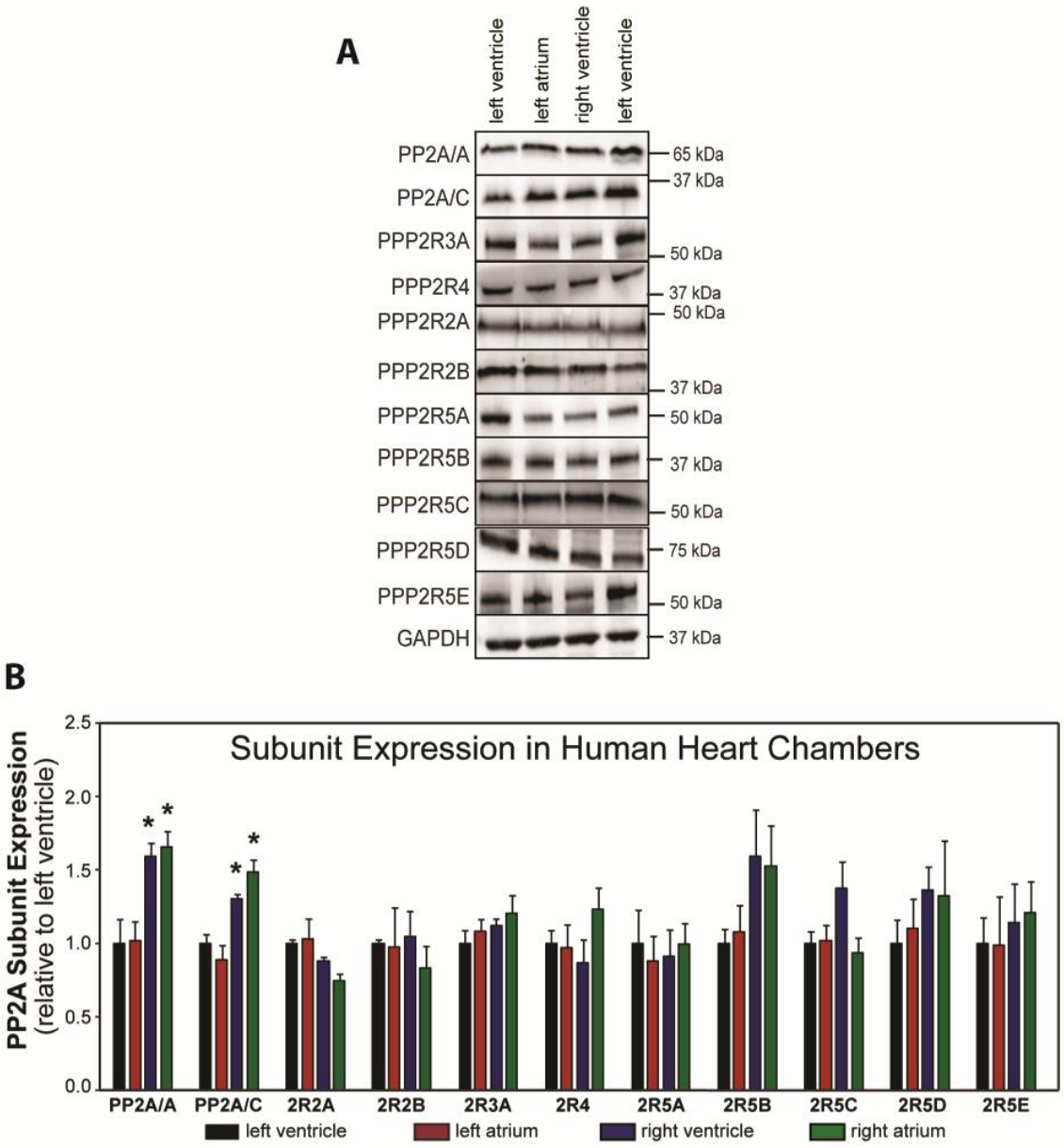

Figure 7. PP2A expression in healthy human heart chambers. A) A representative immunoblots showing expression of PP2A subunits across human heart chambers. B) Densitometry analysis indicating relative expression levels of the PP2A subunits across human heart chambers. Band densities were normalized to GAPDH and data is shown relative to expression levels in control human LV. N=3 for all experiments and asterisk denotes $p<0.05$.

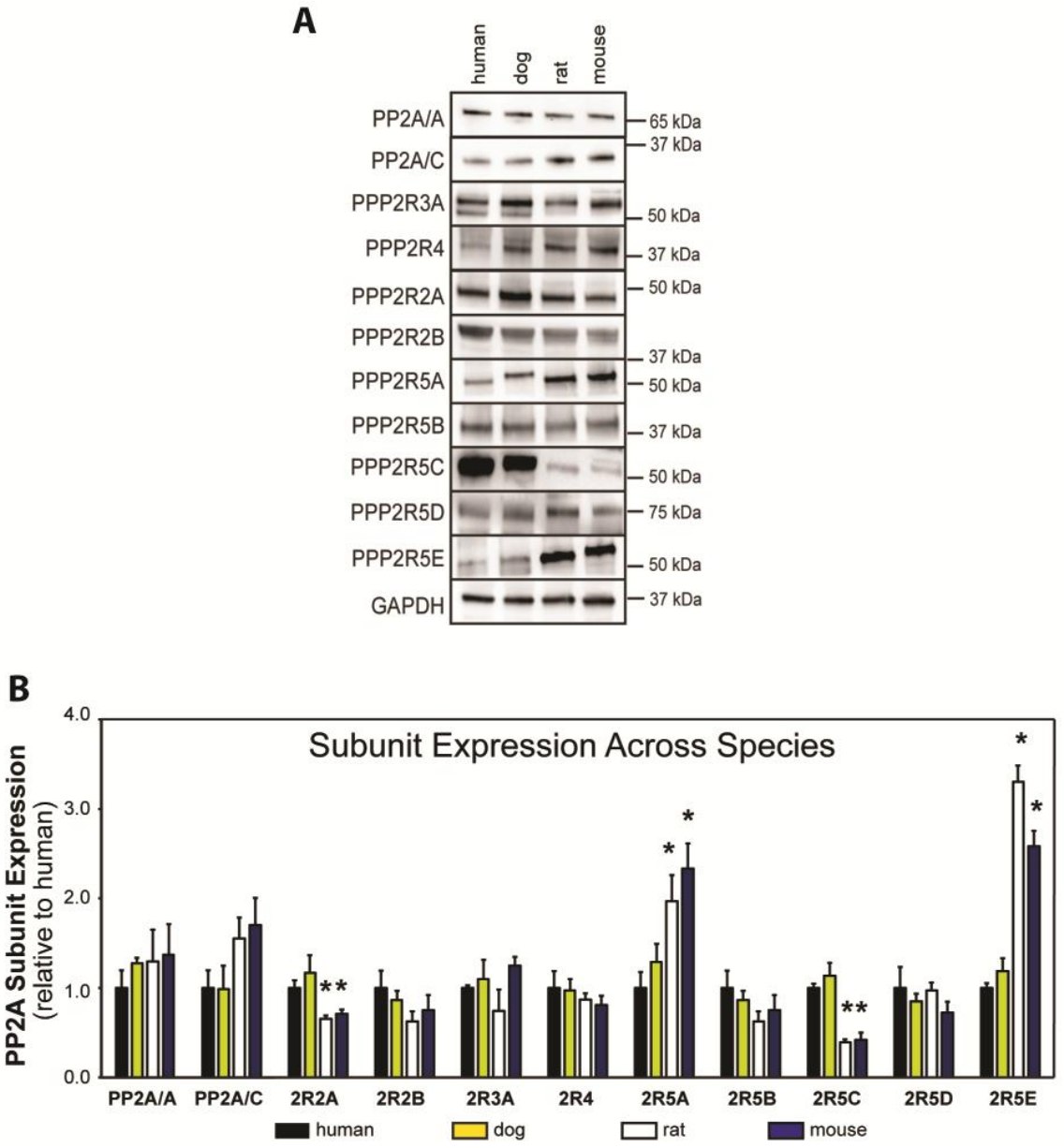

Figure 8. PP2A expression across species. A) A representative immunoblots showing expression of PP2A subunits in LV of four different species. B) Densitometry analysis indicating relative expression levels of the PP2A subunits in the LV of four different species. Band densities were normalized to GAPDH and data is shown relative to expression levels in control human LV. N=3 for all experiments and asterisk denotes $p<0.05$.

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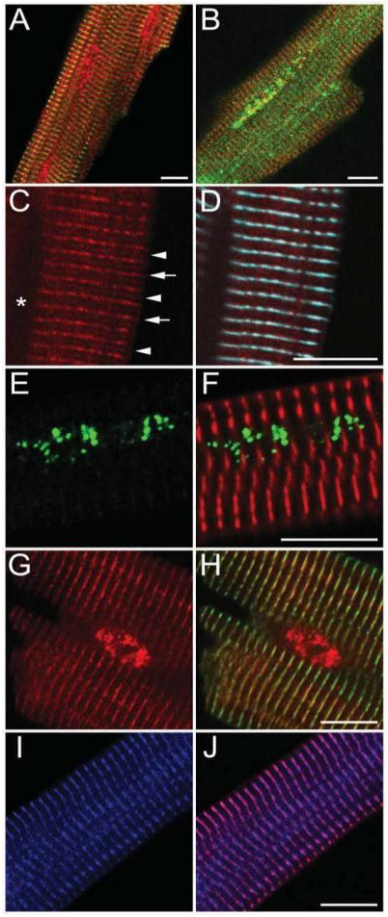

Figure 9. Differential subcellular localization of PP2A regulatory subunits in heart. Isolated adult mouse cardiomyocytes were analyzed by confocal microscopy for distribution of PP2A subunits. *A)* PP2A/A (red), α-actinin (green). *B)* PP2A/C (green), ankyrin-B (red). *C) PPP2R3A* (red), asterisk indicates nucleus, arrows indicate Z-line and arrowheads indicate M-line. *D) PPP2R3A* (red), α-actinin (blue). *E)* PPP2R4 (green). *F) PPP2R4* (green) and α-actinin (red). *G) PPP2R5C* (red). *H) PPP2R5C* (red) and α-actinin (green). *I) PPP2R5E* (blue). *J) PPP2R5E* (blue) and α-actinin (purple). Each experiment was done in triplicate using myocytes isolated from three different WT mice. The images shown above are representative of >100 cells analyzed/experiment. Bar= 10 μm

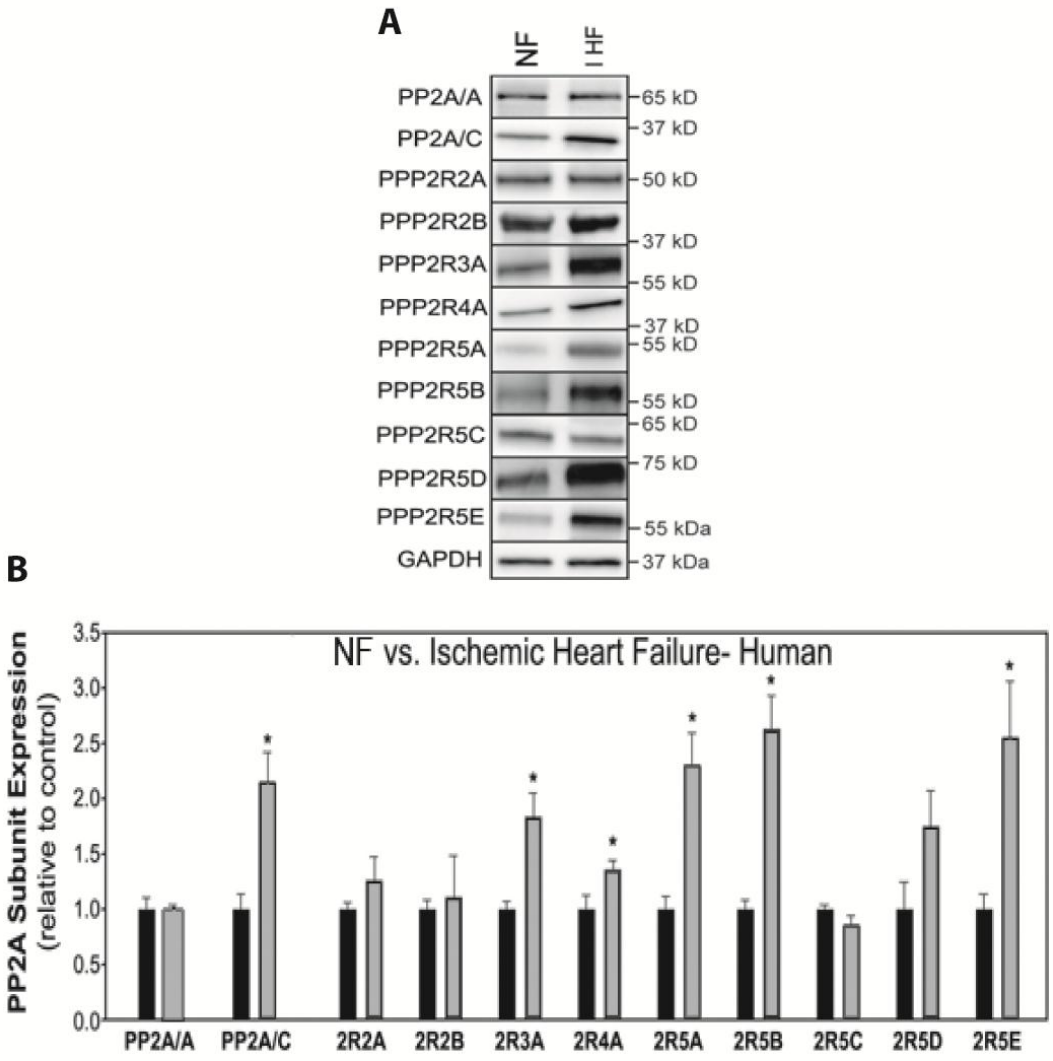

Figure 10. Differential PP2A subunit regulation in ischemic heart disease. A) PP2A subunit expression in control human LV and in LV of human hearts in end stage ischemic heart failure (IHF). B) Densitometry analysis describing PP2A subunit expression levels in control human LV and in the LV of human hearts in end stage ischemic heart failure. GAPDH was utilized as a loading control. N=5 for all experiments and asterisk denotes $p<0.05$ compared to control human LV.

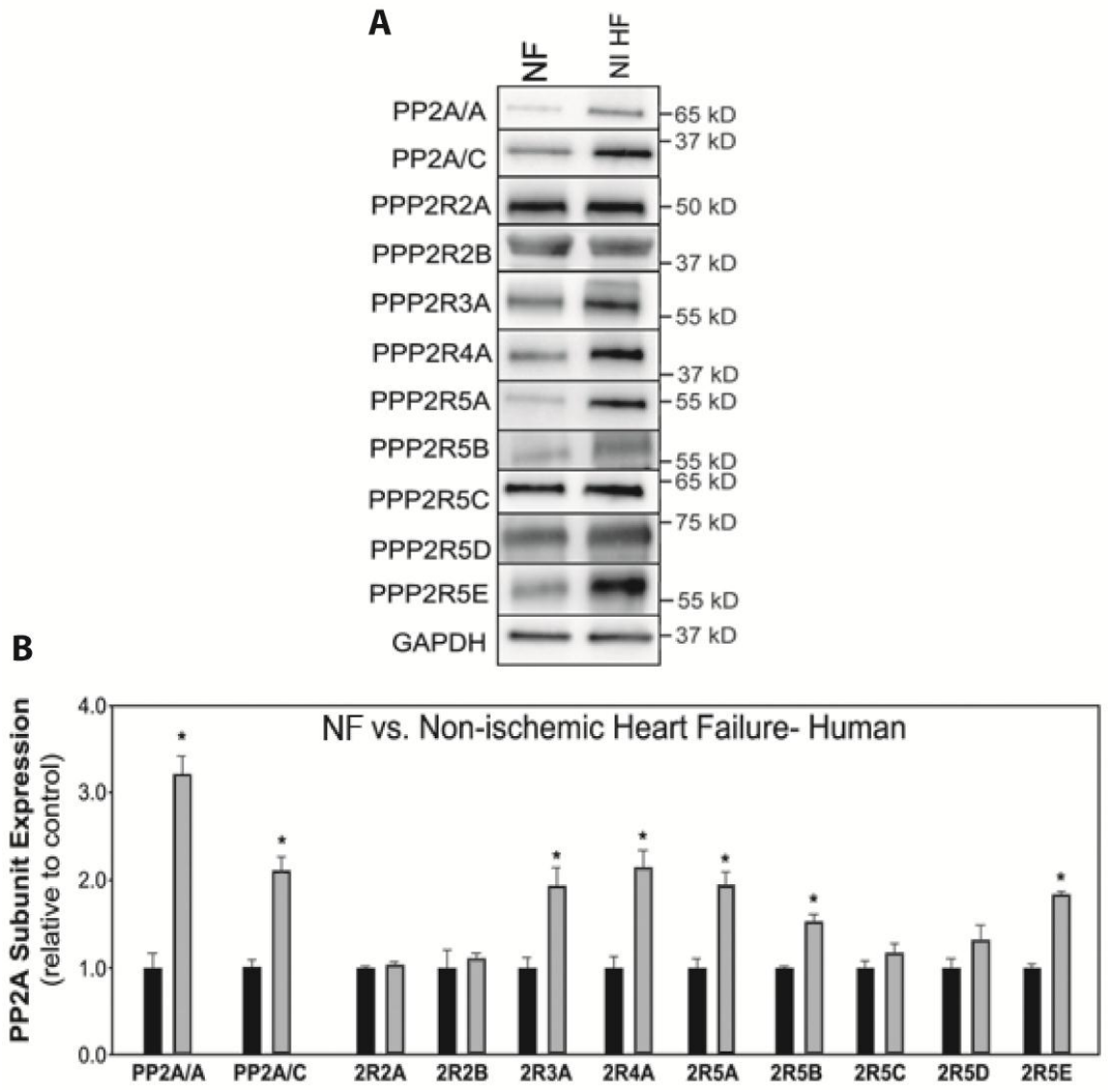

Figure 11. Differential PP2A subunit regulation in non-ischemic heart disease. A) PP2A subunit expression in control human LV and in LV of human hearts in end stage ischemic heart failure (IHF). B) Densitometry analysis describing PP2A subunit expression levels in control human LV and in the LV of human hearts in end stage ischemic heart failure. GAPDH was utilized as a loading control. N=5 for all experiments and asterisk denotes $p<0.05$ compared to control human LV.

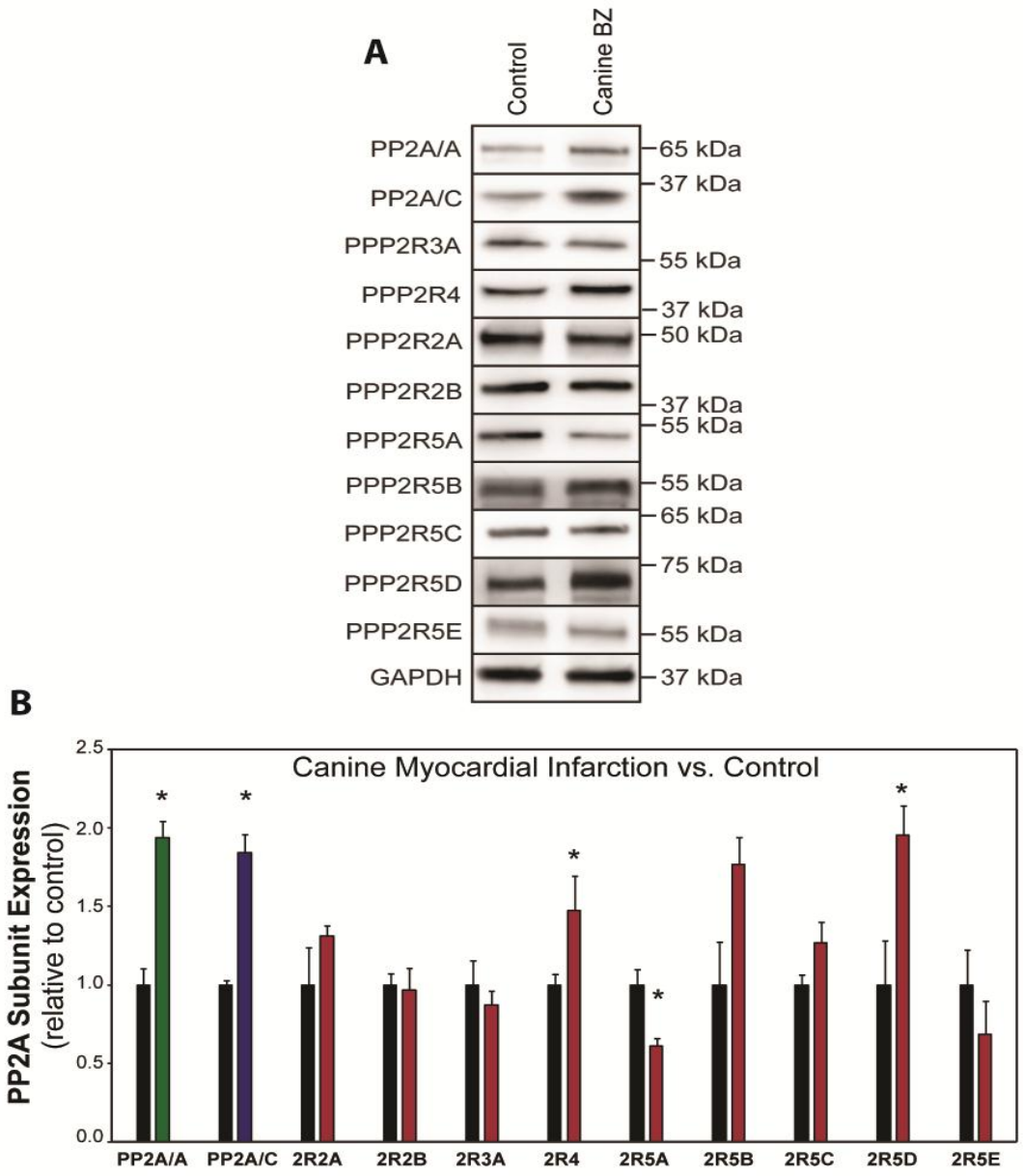

Figure 12. Differential PP2A subunit regulation in myocardial infarction. A) PP2A subunit expression in control canine LV and in canine LV from the border zone (BZ) region of a 5 day old infarct. B) Densitometry analysis describing PP2A subunit expression in control canine LV and in canine LV from the border zone (BZ) region of a 5 day old infarct. GAPDH was utilized as a loading control. N=5 for all experiments and asterisk denotes $p<0.05$ compared to control human LV.

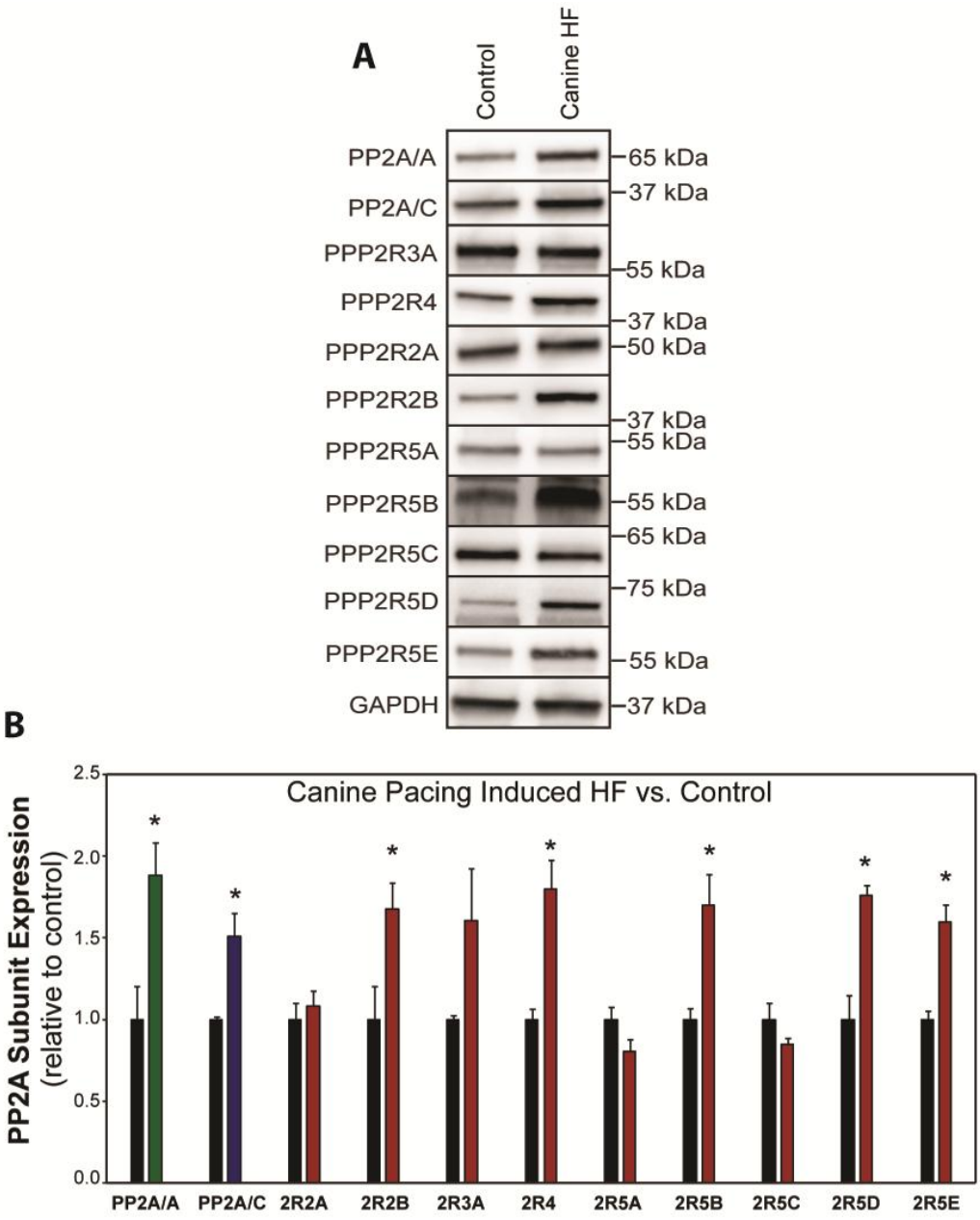

Figure 13. Differential PP2A subunit regulation in canine heart failure model. A) PP2A subunit expression in control canine LV and in LV of canine hearts following 4 months of pacing induced tachycardia (HF). B) Densitometry analysis describing PP2A subunit expression in control canine LV and in LV of canine hearts following 4 months of pacing induced tachycardia (HF) GAPDH was utilized as a loading control. N=5 for all experiments and asterisk denotes $p<0.05$ compared to control canine LV.

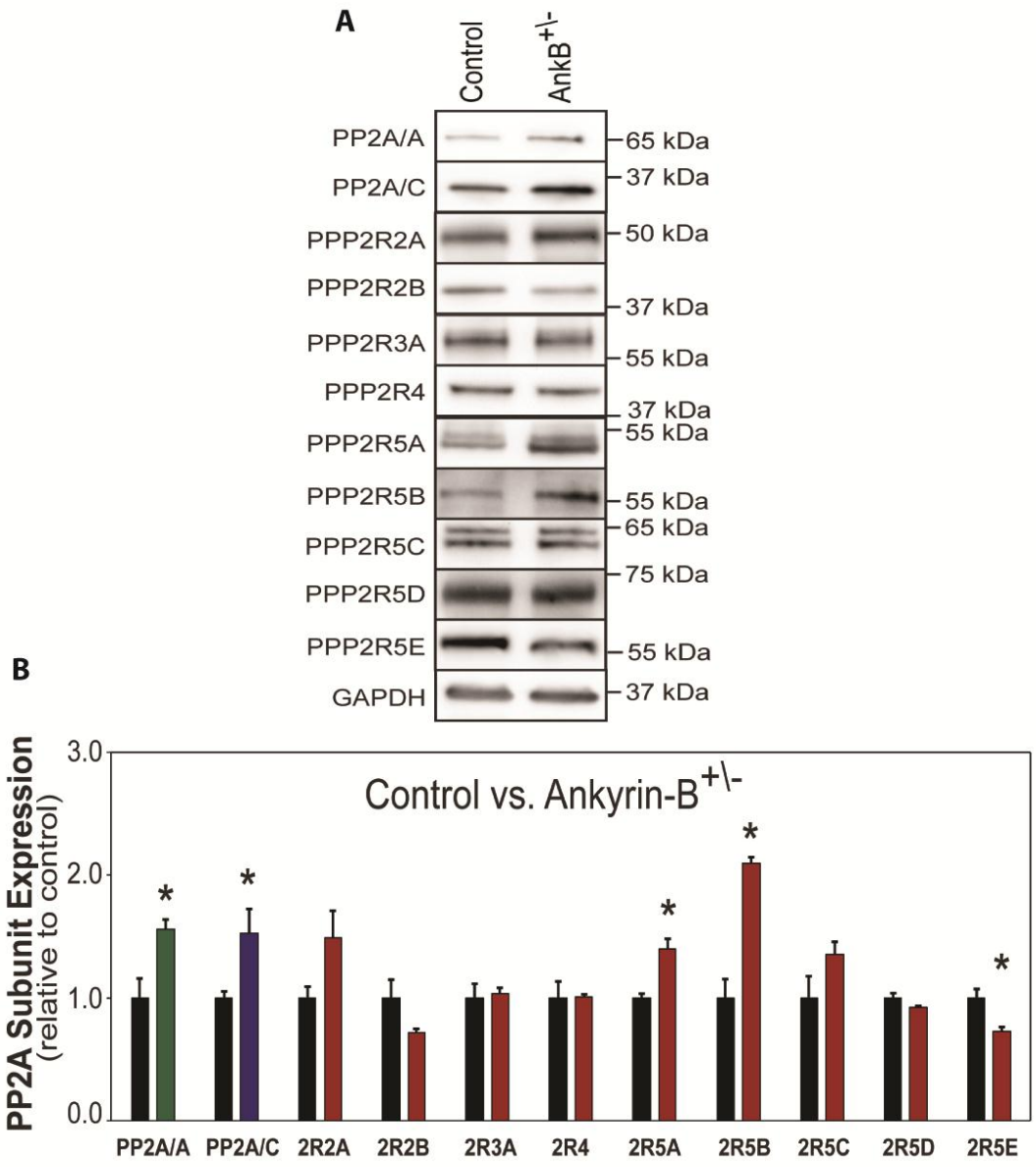

Figure 14. Differential PP2A subunit regulation in a murine model of ventricular arrhythmia syndrome. A) PP2A subunit expression in control mouse LV and in LV of ankyrin-B deficient hearts. B) Densitometry analysis describing relative PP2A subunit expression in control mouse LV and in LV of ankyrin-B deficient hearts. GAPDH was utilized as a loading control. N=5 for all experiments and asterisk denotes $p<0.05$ compared to control canine LV.

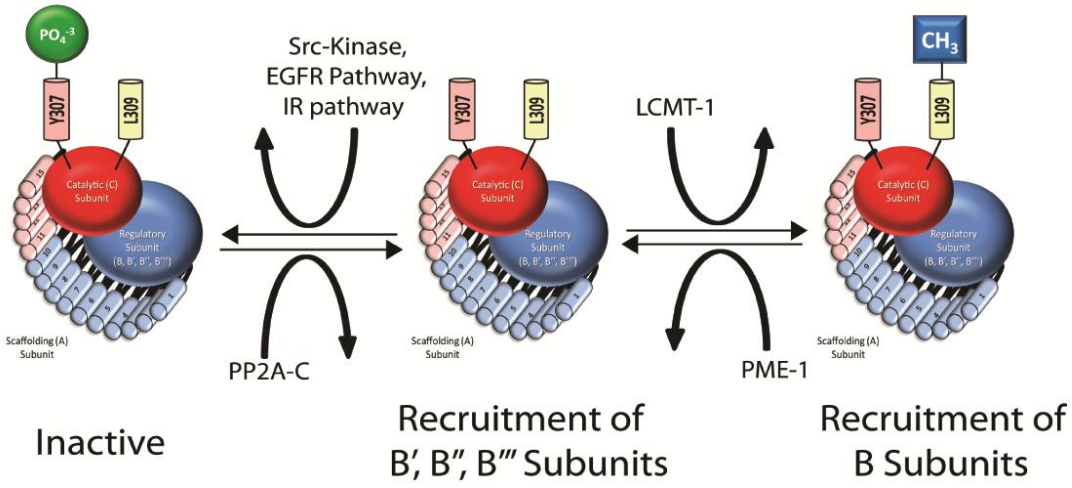

Figure 15. Post-translational modifications of the catalytic subunit. Model of post-translational modifications the PP2A catalytic subunit. Y307 phosphorylation of PP2A/C (regulated by EGF receptor, insulin receptor, and pp60 v-src) results in generalized inhibition of phosphatase activity; L309 methylation (regulated by LCMT1 and PME-1) results in the enhanced recruitment of the *PPP2R2A* and *PPP2R2B* into the holoenzyme.

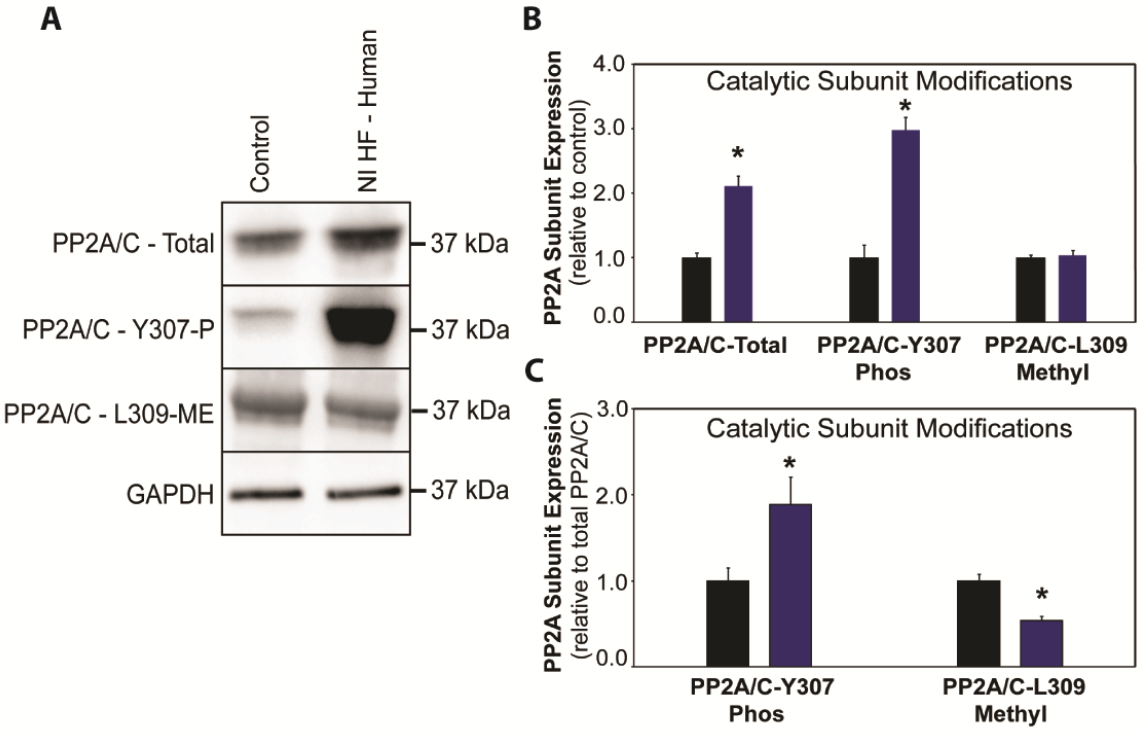

Figure 16. Post-translational modification of the catalytic subunit in human non-ischemic heart failure. A) Expression levels of total, phosphorylated (T307), and methylated (L309) PP2A catalytic subunit in LV samples of control and non-ischemic human heart failure. B) Densitometry analysis of total, phosphorylated (T307), and methylated (L309) PP2A catalytic subunit in LV samples of control and non-ischemic human heart failure (n=5; $p<0.05$). C*)* Adjusted phosphorylated and methylated catalytic subunit activities based on total PP2A catalytic subunit expression in control and non-ischemic heart failure samples. GAPDH was utilized as an internal loading control.

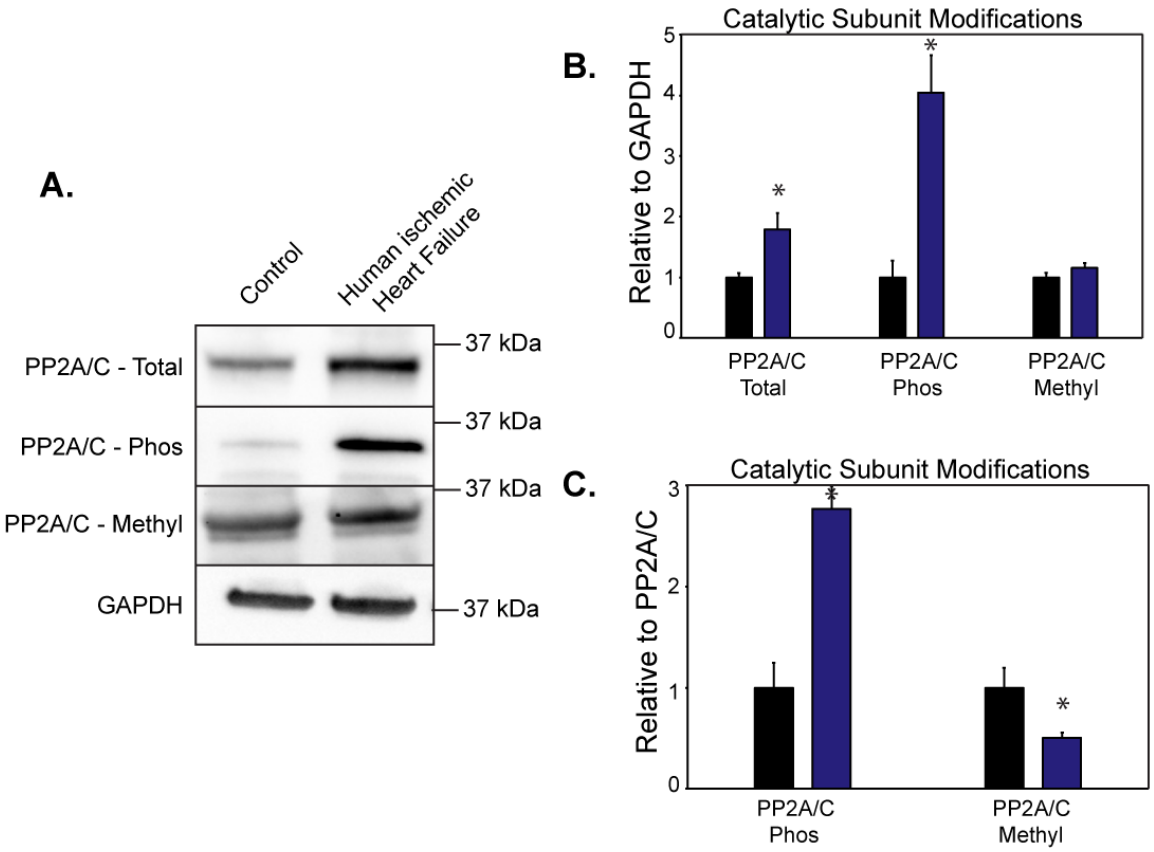

Figure 17. Post-translational modification of the catalytic subunit in human ischemic heart failure. A) Expression levels of total, phosphorylated (T307), and methylated (L309) PP2A catalytic subunit in LV samples of control and ischemic human heart failure. B) Densitometry analysis of total, phosphorylated (T307), and methylated (L309) PP2A catalytic subunit in LV samples of control and ischemic human heart failure (n=5; $p<0.05$). C*)* Adjusted phosphorylated and methylated catalytic subunit activities based on total PP2A catalytic subunit expression in control and ischemic heart failure samples. GAPDH was utilized as an internal loading control.

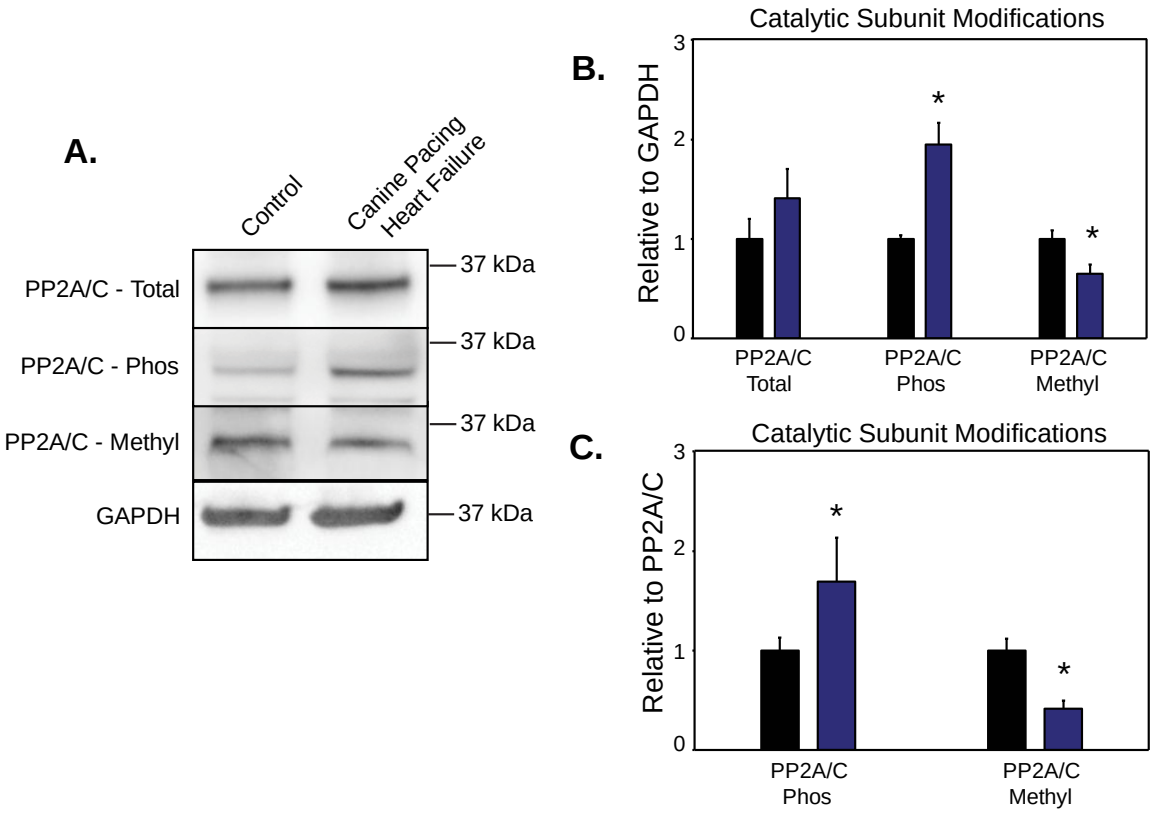

Figure 18. Post-translational modification of the catalytic subunit in canine pacing induced heart failure. A) Expression levels of total, phosphorylated (T307), and methylated (L309) PP2A catalytic subunit in LV samples of control and canine pacing induced heart failure. B) Densitometry analysis of total, phosphorylated (T307), and methylated (L309) PP2A catalytic subunit in LV samples of control and canine pacing induced heart failure (n=5; $p<0.05$). C*)* Adjusted phosphorylated and methylated catalytic subunit activities based on total PP2A catalytic subunit expression in control and canine pacing induced heart failure samples. GAPDH was utilized as an internal loading control.

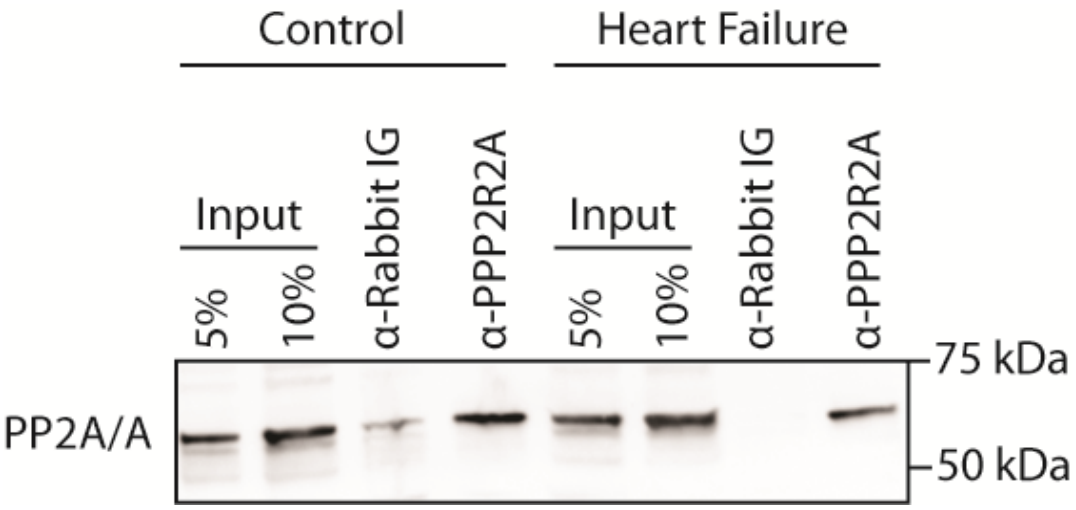

Figure 19. PPP2R2A is excluded from PP2A holoenzyme in heart failure. Co-immunoprecipitation experiments were done using an antibody against PPP2R2A followed by immunoblotting for the PP2A scaffolding subunit (PP2A/A). Detergent soluble samples from control and failing canine hearts were used in this experiment.

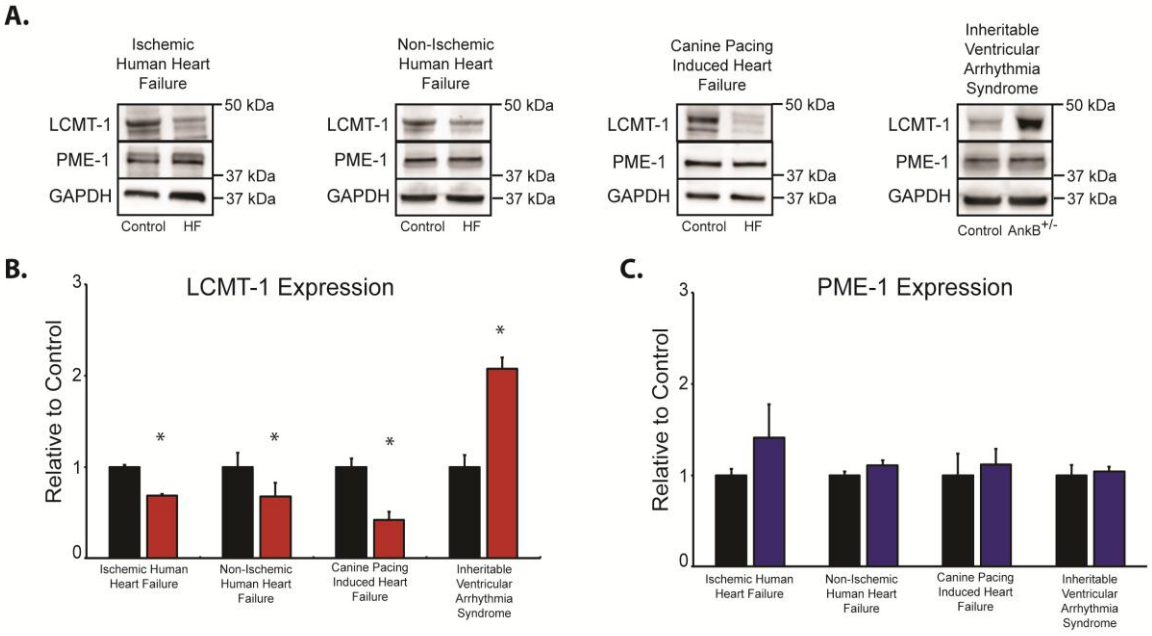

Figure 20. Decreased Catalytic Subunit Methylation is Due to Decreased LCMT-1 Expression. A) Representative immunoblots of LCMT-1, PME-1 expression in whole heart lysates from ischemic heart failure, non-ischemic human heart failure, canine pacing induced heart failure, and a mouse model of a human inheritable ventricular arrhythmia syndrome. B) Densitometry analysis indicating the expression level of LCMT-1 in ischemic heart failure, non-ischemic human heart failure, canine pacing induced heart failure, and a mouse model of a human inheritable ventricular arrhythmia syndrome relative to control. C) Densitometry analysis indicating the expression level of PME-1 in ischemic heart failure, non-ischemic human heart failure, canine pacing induced heart failure, and a mouse model of a human inheritable ventricular arrhythmia syndrome relative to control. In all experiments, GAPDH was used as a loading control. N=3 for all experiments and a p-value <.05 was considered statistically significant.

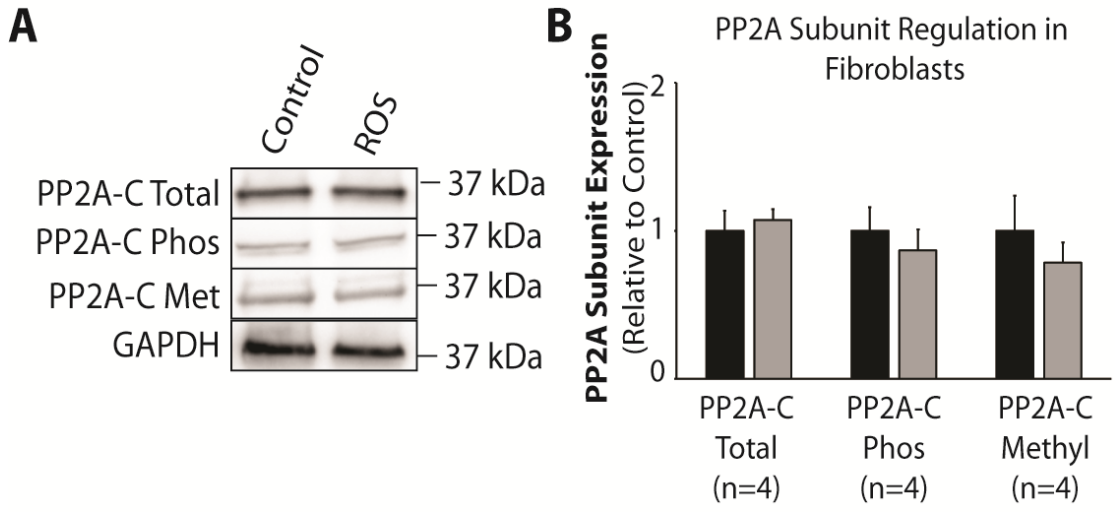

Figure 21. PP2A Subunit regulation in Fibroblasts. A) Representative immunoblots showing the expression of the PP2A catalytic subunit and its modified forms in control and ROS treated neonatal mouse fibroblasts. B) Densitometry analysis indicating relative expression levels of the catalytic subunit and its modified forms in H_2O_2 treated fibroblasts. No observed differences were statistically significant (n=4, p=N.S.). C) Representative immunoblots showing the expression of the PP2A catalytic subunit and its modified forms in control and ROS treated neonatal mouse cardiomyocytes. The phosphorylated form of the catalytic subunit was significantly increased in H_2o_2 treated samples when compared to controls (n=4, $p<.05$) whereas the methylated form of the catalytic subunit was significantly decreased H_2o_2 treated samples when compared to controls (n=4, $p<.05$).

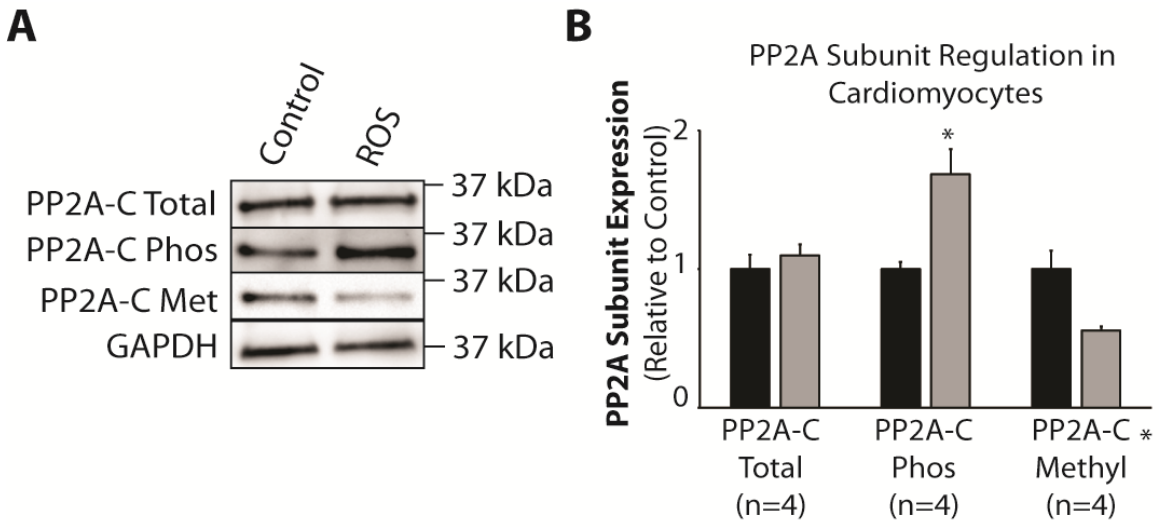

Figure 22. ROS generation induces changes in catalytic subunit regulation. A) Representative immunoblots showing the expression of the PP2A catalytic subunit and its modified forms in control and ROS treated neonatal mouse cardiomyocytes. B) Densitometry analysis indicating relative expression levels of the catalytic subunit and its modified forms in H_2O_2 treated cardiomyocytes. The phosphorylated form of the catalytic subunit was significantly increased in H_2O_2 treated samples when compared to controls (n=4, $p<.05$) whereas the methylated form of the catalytic subunit was significantly decreased H_2O_2 treated samples when compared to controls (n=4, $p<.05$).

CHAPTER 3:

REGULATORY SUBUNIT DYSREGULATION PRODUCES SITE SPECIFIC HYPERPHOSPHORYLATION

Introduction

Cardiovascular disease is the leading cause of death in the United States, resulting in more than a third of all annual deaths(Lloyd-Jones, Adams et al. 2010). Moreover, cardiovascular complications account for an estimated $40 billion in direct and indirect healthcare costs in the United States(Lloyd-Jones, Adams et al. 2010). Over half of heart disease fatalities are due to cardiac electrical and/or structural defects; therefore understanding the mechanisms underlying potentially fatal cardiac arrhythmias is paramount for uncovering new therapies.

Ankyrins play key roles in cardiac structural and electrical regulation. Three ankyrin genes encode ankyrin polypeptides (ankyrin-R, -B, and –G) with specific functions in cardiac physiology. Ankyrin-R isoforms are linked with organization and regulation of the sarcoplasmic reticulum (SR) and structural proteins obscurin and titin.(Zhou, Birkenmeier et al. 1997; Kontrogianni-Konstantopoulos, Jones et al. 2003) Ankyrin-G polypeptides target voltage-gated Na^+ channels to myocyte intercalated disc membranes and dysfunction in ankyrin-G pathways is linked with the Brugada Syndrome.(Mohler, Rivolta et al. 2004; Lowe, Palygin et al. 2008; Hund, Koval et al. 2010; Sato, Coombs et al. 2011) Finally, ankyrin-B is found at cardiac transverse-tubule/SR junctions and regulates the local organization of multiple structural and electrical proteins.(Smith, Curran et al. 2012) Ankyrin-B dysfunction is linked to acquired and congenital forms of human arrhythmia including sinus node disease, atrial fibrillation, ventricular tachycardia, and sudden cardiac death.(Mohler, Schott et al. 2003; Mohler, Splawski et al. 2004; Mohler, Le Scouarnec et al. 2007; Le Scouarnec, Bhasin et al. 2008; Cunha, Hund et al. 2011)

Individuals harboring ankyrin-B loss-of-function mutations and ankyrin-B deficient mice display catecholamine-induced ventricular arrhythmia (CPVT).(Mohler, Schott et al. 2003; Mohler, Splawski et al. 2004) However, the multifunctional nature of ankyrin-B complicates the process of linking ankyrin-B dysfunction to specific molecular targets in disease. Previously, we identified defects in ankyrin-B dependent targeting of Na/K ATPase and Na/Ca exchanger as important in providing a pro-arrhythmic substrate *in vivo*.(Mohler, Davis et al. 2005; Cunha, Bhasin et al. 2007; Camors, Mohler et al. 2012) However, strategies to define additional molecular events that support ankyrin-B-dependent arrhythmia in response to increased sympathetic tone have been unsuccessful. Despite the fact that recent work has shown a direct interaction between ankyrin-B and one of the regulatory units of PP2A, B56α or PPP2R5A, little evidence exists linking ankyrin-B to *in vivo* phosphorylation dependent regulation (Bhasin, Cunha et al. 2007; Cunha and Mohler 2008).

In this study, we utilized a combination of biochemical, electrophysiological, and *in vivo* approaches to identify a role for kinase/phosphatase imbalance in ankyrin-based arrhythmia at the levels of the myocyte and whole animal. We show that ankyrin-B deficiency results in ryanodine receptor hyperphosphorylation, and link this altered post-translational modification state with the calcium/calmodulin-dependent kinase (CaMKII). Utilizing AC3I mice that overexpress a potent CaMKII inhibitory peptide(Zhang, Khoo et al. 2005), we demonstrate that CaMKII inhibition is sufficient to rescue biochemical, cellular, and whole animal defects and is potent at preventing ankyrin-based cellular afterdepolarizations and fatal cardiac arrhythmias. In summary, these findings define new regulatory roles for ankyrin-B in the cardiac myocyte in the regulation of local CaMKII function in the cardiac dyad.

Methods

Electrophysiology

Current recordings were measured by conventional whole-cell patch-clamp technique with an Axon 200B patch-clamp amplifier controlled by a computer using a Digidata 1320A acquisition board driven by pClamp 8.0 software (Axon Instruments, Foster City, CA). Action potentials (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% (or 20 and 50%) repolarization to baseline (APD_{90}).(Koval, Guan et al. 2010) APs were recorded using the perforated (amphotericin B) patch-clamp technique in Tyrode's solution (bath) with the pipette filled with (mmol/L): 130 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 $CaCl_2$, 2.0 MgATP, 7.0 phosphocreatine, 0.1 NaGTP, and amphotericin B 240 μg⁄mL, with the pH adjusted to 7.2 with KOH.(Wu, Gao et al. 2009) APs were measured at physiological temperature ±1 μM isoproterenol.(Gao, Singh et al. 2011)

Statistics

Statistical significance was determined with a paired Student's *t* test (2-tailed) or ANOVA with the Bonferroni post-hoc test, when appropriate, for continuous data. The null hypothesis was rejected for $p<0.05$. For experiments in Figure 5B, contingency tables were generated and statistical significance was determined by Fisher's exact test. Data for Figure 5C were analyzed via the Mantel-Cox (logrank) test. Statistical analyses were conducted using GraphPad Prism V4 (GraphPad Software Inc., La Jolla, CA) or SigmaPlot.

Animal models

Mouse models included wild-type C57/Bl6 mice, ankyrin-$B^{+/-}$ mice, AC3I mice, and ankyrin-$B^{+/-}$ mice crossed with AC3I mice.

Single channel recordings

Single channel recordings were performed as described.(van Oort, McCauley et al. 2010) Cardiac SR membrane vesicles were prepared from mouse hearts and fused

with lipid bilayer comprised of a mixture of phosphatidylethanolamine and phosphatidylserine at a ratio of 3:1 (Avanti Polar Lipids, Alabaster, AL) dissolved in n-decane (25mg/ml). The trans chamber corresponding to the luminal side of the SR contained 250mM Hepes, 50mM KCl, and 53mM Ca(OH)2. The cis chamber corresponding to the cytosolic side of the SR contained 250mM HEPES, 125mM Tris-base, 50mM KCl, 1mM EGTA, 0.5mM CaCl2, pH 7.35. Addition of KN-93 was made to the cis chamber.

Immunoblotting

Immunoblots were done as described.(Mohler, Davis et al. 2005) Briefly, whole hearts were harvested from wild type, ankyrin-$B^{+/-}$, ankyrin-$B^{+/-}$ X AC3I and AC3I adult, age-matched, littermates and flash frozen in liquid nitrogen, ground into a fine powder and resuspended in bicarbonate buffer (1 mM $NaHCO_3$, 5 mM EDTA, 1 mM EGTA) supplemented with 2 mM Na_3VO_4 and 1 mM NaF. Following quantification, tissue lysates were analyzed on Mini-PROTEAN tetra cell (BioRad) on a 4-15% precast TGX gel (BioRad). Gels were transferred to a nitrocellulose membrane using the Mini-PROTEAN tetra cell (BioRad). Membranes were blocked for 1 hour at room temperature using a 3% BSA solution and incubated with primary antibody overnight at 4°C. Densitometry analysis was done using Adobe Photoshop software. For all experiments, protein values were normalized against an internal loading control (GAPDH).

Antibodies

The polyclonal anti-RyR_2 pS2808 (1:1000) and anti-RyR_2 pS2814 (1:1000) phosphorylated epitope-specific antibodies were custom generated using the peptide C-RTRRI-(pS)-QTSQV corresponding to the PKA phosphorylation site at RyR_2 S2808 and peptide CSQTSQV-(pS)-VD corresponding to CaMKII phosphorylation site at RyR_2 S2814, respectively. These custom antibodies have been used extensively and have been well characterized in previous work.(van Oort, McCauley et al. 2010) A monoclonal anti-

GAPDH (1:5000, Fitzgerald) and a polyclonal anti-RyR_2 antibody (1:1000, Millipore) were also used.

Conscious ECG experiments

ECG recordings of ambulatory animals were obtained using radio-telemetry (DSI) with transmitters implanted subcutaneously and superficial to the peritoneum seven days before recordings. Recording were obtained from mice both at resting conditions, post-exercise and epinephrine injection. For stress tests, animals were run on a treadmill for 1 hour and then injected IP with epinephrine (2 mg/kg). Non-sustained and sustained arrhythmias were identified using standard ECG analysis guidelines.(Mitchell, Jeron et al. 1998) Based on prior data linking significant arrhythmia and sudden cardiac death in the ankyrin-$B^{+/-}$ mouse mode l(Mohler, Schott et al. 2003), we limited experiments to a minimal number necessary to obtain statistical power. Arrhythmia was defined as sustained if >2 sec.(Mohler, Schott et al. 2003)

Adult cardiomyocyte preparations

Murine hearts from adult wild type C57Bl/6, mice were obtained after animals were euthanized by acute CO_2 asphyxiation followed by cervical dislocation in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH and IACUC approved protocols. Hearts were removed from the animal immediately following euthanasia and canulated on a langendorff apparatus for perfusion. Hearts were perfused with modified Tyrodes solution (136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5.5 mM HEPES, pH adjusted to 7.4 with NaOH) for 5 minutes. This was followed by 12 minute perfusion with modified Tyrode solution supplemented with collagenase type II.

Results

Ankyrin-B deficient mice display RyR_2 hyperphosphorylation.

Based on the role of ankyrin-B in regulation of protein phosphatase 2A targeting and regulation in the myocyte transverse-tubule/sarcoplasmic reticulum dyad, (Bhasin, Cunha et al. 2007; Cunha and Mohler 2008) we hypothesized that aberrant control of local phosphorylation events may contribute to ankyrin-B cardiac disease. The cardiac ryanodine receptor (RyR_2) is critical for sarcoplasmic reticulum (SR) calcium release, and RyR_2 dysfunction is linked with multiple forms of congenital and acquired cardiac pathologies including exercise- and stress-induced ventricular arrhythmias, atrial fibrillation, and heart failure (Go, Moschella et al. 1995; Chelu, Sarma et al. 2009; Dobrev and Wehrens 2010). Importantly, defects in RyR_2 phosphorylation status are linked with abnormal diastolic SR calcium release and arrhythmia.(Chelu, Sarma et al. 2009; Dobrev and Wehrens 2010; McCauley and Wehrens 2011) We first tested whether total RyR_2 expression levels were altered in ankyrin-$B^{+/-}$ (display ~50% reduction in ankyrin-B) mouse hearts by immunoblot. As shown in Figure 23, we observed no statistical difference in total RyR_2 expression in heart lysates prepared from age- and sex-matched wild-type and ankyrin-$B^{+/-}$ mice (Figure 23; 1.0 vs. 1.04; p=N.S.). We next evaluated the phosphorylation status of the cardiac RyR_2 by immunoblot using antibodies directed against two identified RyR_2 phosphorylation sites. RyR_2 S2808 has been previously linked with protein kinase A (PKA) regulation of RyR_2 open probability (Po) and function.(Wehrens, Lehnart et al. 2006) We did not observe statistical differences in RyR_2 S2808 phosphorylation between wild-type and ankyrin-$B^{+/-}$ cardiac lysates (Figure 23; 1.0 vs. 1.25; p=N.S.). In contrast, RyR_2 S2814, a regulatory site for the calcium/calmodulin-dependent kinase II CaMKII (Wehrens, Lehnart et al. 2004)) was hyperphosphorylated in ankyrin-$B^{+/-}$ cardiac lysates compared with wild-type mouse heart lysates (Figure 23; 1.0 vs. 1.79; $p<0.05$.). These data suggest that ankyrin-$B^{+/-}$ hearts display aberrant CaMKII-dependent regulation of cardiac RyR_2.

CaMKII and abnormal RyR_2 phospho-regulation.

CaMKII-dependent phosphorylation of RyR_2 is directly linked with catecholamine-triggered arrhythmias (McCauley and Wehrens 2011). Therefore, we evaluated whether RyR_2 hyperphosphorylation in ankyrin-$B^{+/-}$ hearts could be directly linked to CaMKII-dependent activity. We assessed the direct role of CaMKII by utilizing AC3-I mice, a transgenic mouse model that overexpresses a potent inhibitor of the CaMKII enzyme in cardiac myocytes (Zhang, Khoo et al. 2005). We compared the phosphorylation status of RyR_2 in wild-type mice, ankyrin-$B^{+/-}$ mice, and ankyrin-$B^{+/-}$ crossed with AC3I mice (ankyrin-$B^{+/-}$ X AC3I). We observed a striking reduction in RyR2 pS2814 phosphorylation in ankyrin-$B^{+/-}$ X AC3I mouse hearts compared with hearts from ankyrin-$B^{+/-}$ littermates (Figure 24, $p<0.05$). In fact, RyR_2 pS2814 levels in ankyrin-$B^{+/-}$ x AC3I mouse hearts were not significantly different from wild-type mouse hearts (Figure 24, p=N.S.). We observed no difference in total RyR_2 levels between the three genotypes (Figure 24, p=N.S.). These data support a direct link between RyR_2 S2814 hyperphosphorylation and CaMKII in ankyrin-$B^{+/-}$ mice.

CaMKII inhibition rescues cardiac electrical dysfunction

As noted above, ankyrin-$B^{+/-}$ mice display electrical dysfunction and patients with ankyrin-B loss-of-function mutations display QT_c abnormalities and arrhythmia susceptibility (Mohler, Davis et al. 2005; Cunha, Bhasin et al. 2007; Camors, Mohler et al. 2012). Moreover, ankyrin-$B^{+/-}$ myocytes display severe defects in myocyte electrical function and afterdepolarizations (Mohler, Schott et al. 2003; Camors, Mohler et al. 2012). Based on our biochemical data, we tested whether CaMKII inhibition was sufficient to prevent electrical dysfunction in primary ankyrin-$B^{+/-}$ adult ventricular cardiomyocytes. We observed mild prolongation of APD_{90} in ankyrin-$B^{+/-}$ myocytes compared with wild-type myocytes, consistent with QT_c interval changes in specific kindreds harboring *ANK2* loss-of-function variants (Mohler, Schott et al. 2003) (Figure 25; 0.5 Hz, $p<0.05$). Changes in APD_{90} in ankyrin-$B^{+/-}$ myocytes were exaggerated in

response to adrenergic stimulation (Figure 25, $p<0.05$) and as expected (Mohler, Schott et al. 2003) we observed significant afterdepolarizations in ankyrin-$B^{+/-}$ myocytes (Figure 25, $p<0.05$). In contrast, we observed normalized APD_{90} in ankyrin-$B^{+/-}$ x AC3I mouse myocytes at baseline compared with myocytes from ankyrin-$B^{+/-}$ littermates (Figure 3A-B, $p<0.05$). Similar trends were observed for APD_{20} and APD_{50} for ankyrin-$B^{+/-}$ X AC3I mice compared with ankyrin-$B^{+/-}$ mice (Figure 26). Furthermore, ankyrin-$B^{+/-}$ X AC3I mouse myocytes were resistant to isoproterenol-induced APD prolongation seen in wild-type or ankyrin-$B^{+/-}$ myocytes (Figure 25, $p<0.05$). Most notably, we observed complete inhibition of cellular afterdepolarizations in isoproterenol treated ankyrin-$B^{+/-}$ X AC3I mice compared with ankyrin-$B^{+/-}$ myocytes (Figure 25, $p<0.05$). All differences between genotypes reported above were consistent across multiple pacing frequencies (0.5, 1.0. 2.0, and 4.0 Hz, $p<0.05$). In summary, our findings demonstrate that CaMKII inhibition rescues both RyR_2 pS2814 hyperphosphorylation and pro-arrhythmic cellular phenotypes in ankyrin-B deficient mice.

RyR_2 open probability is rescued by KN-93 treatment.

We next tested whether ankyrin-$B^{+/-}$ mouse hearts displayed increased RyR_2 open probability compared to wild-type mouse heart. Consistent with our new findings, RyR_2 channels from ankyrin-$B^{+/-}$ cardiac SR membrane vesicles (van Oort, McCauley et al. 2010) displayed a significant increase in open probability (Po) compared with wild-type mice (Figure 27; 0.154 ± 0.043 vs. 0.007 ± 0.004, $p<0.05$). Notably, application of the CaMKII inhibitor KN-93 (10 μM) significantly reduced RyR_2 Po in ankyrin-$B^{+/-}$ preparations from 0.176 ± 0.039 to 0.099 ± 0.027 (Figure 4C, 4 channels/ 3 mice, $p<0.05$). In contrast, KN-93 did not alter RyR_2 Po in wild-type mice (Figure 27, 0.011 ± 0.008 vs. 0.006 ± 0.005, 3 channels/ 3 mice; p=N.S.). Collectively, these data support the role of CaMKII-dependent RyR_2 phosphorylation in ankyrin-$B^{+/-}$ electrical phenotypes.

CaMKII inhibition protects against AnkB arrhythmias

Individuals harboring ankyrin-B loss-of-function mutations display a host of cardiac phenotypes often linked with adrenergic dysregulation (Mohler, Schott et al. 2003; Mohler, Splawski et al. 2004; Mohler, Healy et al. 2007; Mohler, Le Scouarnec et al. 2007). Ankyrin-$B^{+/-}$ mice are haploinsufficient and display similar catecholamine-based arrhythmia (Mohler, Schott et al. 2003). Therefore, we tested whether CaMKII inhibition could protect the *in vivo* ankyrin-$B^{+/-}$ human disease model from life threatening arrhythmia. To assess arrhythmia susceptibility, we compared ECGs from conscious mice following surgical implantation of radio-telemetry devices. This protocol provides the ability to monitor cardiac activity at physiological heart rates (compared with anesthetized mice) as well as compare ECG parameters following physiological (exercise) or pharmacological intervention. Compared with wild-type mice, ankyrin-$B^{+/-}$ mice subjected to a catecholamine stress protocol (60 minutes exercise or fatigue as determined by three unsuccessful attempts to remain on the treadmill, whichever came first, plus intraperitoneal injection of an adrenergic receptor agonist) displayed consistent and severe polymorphic ventricular arrhythmia including ventricular tachycardia/torsade de pointes followed by severe bradycardia and ultimately death (Figure 28). In contrast, we observed no episodes of sustained arrhythmia (Figure 29, 0/6) and no death (Figure 29, 0/6;). Ankyrin-$B^{+/-}$ mice crossed with AC3I mice subjected to the identical protocol, while showing short intermittent (<2 sec) non-sustained arrhythmia events, lacked significant sustained arrhythmia phenotypes compared with ankyrin-$B^{+/-}$ mice (Figures 28 and 29). Similarly, *in vivo* CaMKII inhibition rescued survival following the cardiac stress protocol (Figure 29). In conclusion, our data link ankyrin-deficiency with cardiac RyR_2 hyperphosphorylation and abnormal electrical dysfunction at the level of the single cell and whole animal. Moreover, our studies demonstrate that CaMKII inhibition blocks RyR_2 hyperphosphorylation, and rescues single cell afterdepolarizations and arrhythmias.

Discussion

Protein kinase-dependent regulation of cardiac signaling is essential for normal physiology and excitation contraction coupling. Both congenital and acquired forms of human cardiovascular disease are now clearly linked with an imbalance in the kinase/phosphatase axis and cellular dysfunction. In fact, catecholaminergic polymorphic ventricular tachycardia, a disease directly linked with elevated sympathetic tone, exemplifies the striking requirement of precise adrenergic regulation for control of cardiac automaticity (Leenhardt, Lucet et al. 1995; Priori, Napolitano et al. 2002). Beta-adrenergic receptor blockers remain the mainstay for treatment of CPVT patients. However, emerging work from both human and animal models suggests that the protection provided by beta-blockers against potentially lethal arrhythmias is incomplete, demonstrating the need to identify new molecular therapeutic targets for this disease.(Priori, Napolitano et al. 2002; Cerrone, Colombi et al. 2005) Our data link human ankyrin-B-based arrhythmia phenotypes with alterations in the CaMKII signaling pathway. We identify aberrant RyR_2 pS2814 levels in animal models of cardiac ankyrin-B syndrome. Moreover, our data demonstrate that biochemical, cellular, and whole animal phenotypes that are linked with a pro-arrhythmic trigger are either reduced or eliminated in the presence of an *in vivo* cellular CaMKII inhibitory peptide. These studies not only provide important new data regarding the molecular components involved in human arrhythmia, but also define important new regulatory elements of the ankyrin-B pathway for normal physiology.

One interesting finding in this story is the apparent link between ankyrin-B and the kinase/phosphatase axis in the cardiac myocyte. Protein phosphatase 2A (PP2A) is an important negative regulatory factor for both PKA and CaMKII-dependent phosphorylation in heart. The PP2A holoenzyme contains three subunits (A, B, and C), that serve regulatory scaffolding, targeting and catalytic roles. Notably, we previously demonstrated that ankyrin-B directly associates with B56α, a PP2A regulatory subunit

that is highly expressed in heart at the cardiac transverse-tubule. (Bhasin, Cunha et al. 2007; Cunha and Mohler 2008) Moreover, ankyrin-B deficient myocytes display lack of normal B56α targeting presumably that is at least partially responsible for the alterations in RyR_2 phosphorylation observed in this study. Notably, work from Terentyev and colleagues previously showed that miR-1-targeted loss of B56α in myocytes results in CaMKII-dependent hyperphosphorylation of RyR_2 resulting in altered calcium handling and pro-arrhythmic electrical function.(Terentyev, Belevych et al. 2009) Together, these findings support a model in which ankyrin-B supports a key role in maintaining local kinase/phosphatase balance in the cardiac dyad by strategically positioning populations of PP2A near important targets including RyR_2 (Figure 6). Thus loss of ankyrin-B-dependent targeting of dyadic PP2A results in increased RyR_2 phosphorylation. It will be important in future experiments to define additional cardiac targets for ankyrin-B targeted PP2A.

CaMKII inhibition has been reported widely as a potential therapeutic strategy for the prevention of heart failure and arrhythmia.(Zhang, Khoo et al. 2005; Erickson, He et al. 2011; Swaminathan and Anderson 2011) In fact, relevant for this study, Priori and colleagues recently demonstrated the ability of CaMKII inhibition for protection against arrhythmia in an animal model of CPVT associated with human RyR_2 mutation.(Liu, Ruan et al. 2011) While our data link CaMKII inhibition to reduction in RyR_2 hyperphosphorylation, inhibition of afterdepolarizations, and protection from cardiac arrhythmia the direct mechanisms underlying these phenotypes is clearly complex and likely due to both direct and indirect effects. While CaMKII targets RyR_2 regulation, this multifunctional enzyme tunes the activity of a host of regulatory pathways including membrane proteins, transcriptional and metabolic pathways, as well as key SR proteins. (Kranias, Gupta et al. 1988; Hudmon, Schulman et al. 2005; Grueter, Abiria et al. 2006; Wagner, Dybkova et al. 2006; Hund, Koval et al. 2010; Erickson, He et al. 2011; Swaminathan and Anderson 2011) Thus, additional studies beyond the scope of this

study will be required to uncover the detailed calcium-based regulatory mechanisms altered in this incredibly complex human disease.

Limitations

There are a number of important limitations to our study. First, while our data implicate the CaMKII regulatory pathway in ankyrin-B cardiomyocyte function, we acknowledge that dysfunction in other key molecular components are involved in ankyrin-B cardiac phenotypes. For example, work from our group and others have demonstrated that ankyrin-B targets and regulates a number of key ion channels and transporters in the heart including the Na/Ca exchanger, Na/K ATPase, and Kir6.2- all contributing important roles in cardiac regulation at baseline and in disease.(Li, Burke et al. 1993; Mohler, Schott et al. 2003; Mohler, Davis et al. 2005; Kline, Kurata et al. 2009; Li, Kline et al. 2010) In fact, we predict that loss of specific transverse-tubule populations of Na/Ca exchanger and Na/K ATPase contribute to the pro-arrhythmic substrate by altering local Na^{+} and Ca^{2+} concentration gradients similar to the actions of cardiac glycosides. In fact, we predict that these cellular phenotypes, which ultimately raise SR calcium load, provide the substrate for the arrhythmias triggered by altered adrenergic and CaMKII balance observed in our current study and others.

To address this issue, we propose several future experiments that may help shed some light on the true importance of ankyrin-B dependent PPP2R5A targeting in cardiomyocytes. The first is to cross ankyrin-B deficient animals with a mutant RyR2 that cannot be phosphorylated at S2814. This model will effectively remove the effects of RyR hyperphosphorylation from the ankyrin-B deficient phenotype and allow us to evaluate how hyperphosphorylation at this one particular intracellular CaMKII targets contributes to the disease. The second experiment is to create a PPP2R5A knockout mouse. This mouse will allow us to determine how a lack of PPP2R5A expression in isolation contributes to an arrhythmogenic phenotype.

A second key limitation is the relative role of the cardiac RyR_2 for arrhythmia susceptibility related to CaMKII function in our study. As observed for the cardiac RyR_2 and voltage-gated Na^+ channel, multiple post-translational regulatory sites likely create an integrated "tuning" rheostat to control ion channel function and thus response to the immediate cellular environment. While our work has assessed RyR_2 S2808 and S2814 sites, these sites are not used to define the critical target for regulation, but instead provide a physiologically relevant surrogate of CaMKII function in heart. We predict that other CaMKII downstream proteins will also play critical roles in the ankyrin-B phenotype in response to altered sympathetic tone.

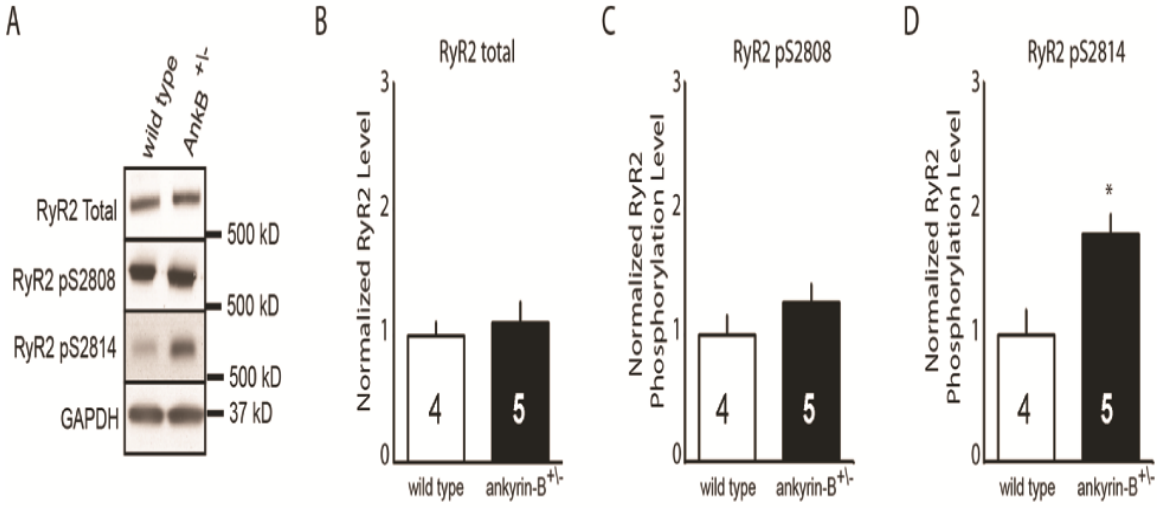

Figure 23. Altered RyR_2 phosphorylation in ankyrin-$B^{+/-}$ hearts. A) Representative immunoblot indicating expression of Total RyR_2, RyR_2 pS2808, and RyR_2 pS2814 in wild-type and ankyrin-$B^{+/-}$ hearts. For all experiments, protein values were normalized against internal loading control (GAPDH). B-D) Densitometry analysis indicating the relative levels of total RyR_2 (B), RyR_2 pS2808 (C), and RyR_2 pS2814 (D) expression in WT and ankyrin-$B^{+/-}$ hearts (N values are noted in each panel). In panel D, * denotes $p<0.05$.

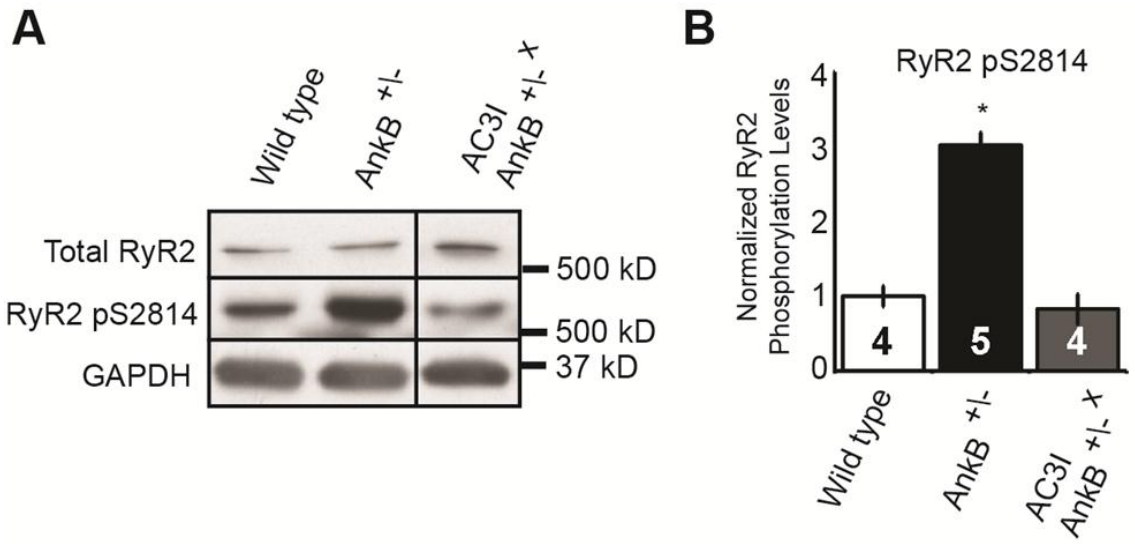

Figure 24. CaMKII inhibition normalizes RyR_2 hyperphosphorylation in ankyrin-$B^{+/-}$ hearts. A) Representative immunoblots showing levels of RyR_2 pS2814 in wild-type, ankyrin-$B^{+/-}$, and ankyrin-$B^{+/-}$ X AC3I hearts. Total RyR2 levels were unchanged between the three genotypes studies (p=N.S.). For all experiments, protein values were normalized against internal loading control (GAPDH). Line between lanes in A denotes that data collected from non-contiguous lanes of same gel. B) Densitometry analysis of RyR_2 pS2814 in wild-type, ankyrin-$B^{+/-}$, and AC3I x ankyrin-$B^{+/-}$ animals after treatment with isoproterenol. N values are listed in panel and * represents $p<0.05$ for wild-type vs. ankyrin-$B^{+/-}$ and ankyrin-$B^{+/-}$ vs. ankyrin-$B^{+/-}$ X AC3I.

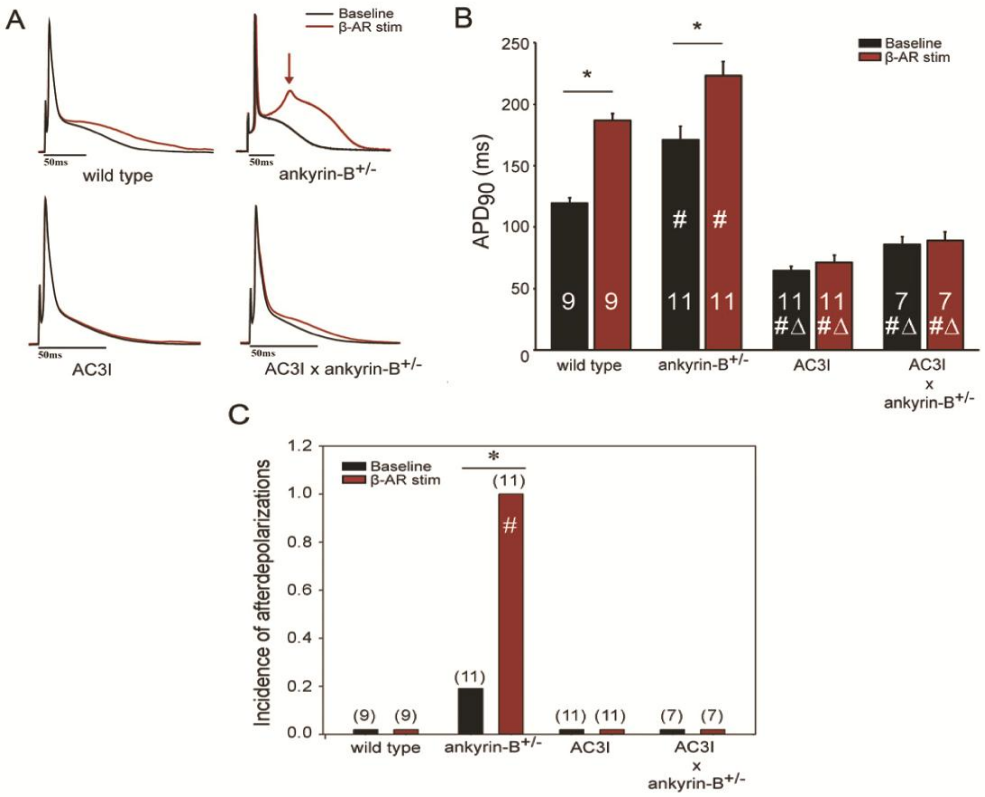

Figure 25. CaMKII inhibition normalizes ankyrin-B$^{+/-}$ myocyte electrical phenotypes. A) Action potential morphology in wild-type, ankyrin-B$^{+/-}$, AC3I, and ankyrin-B$^{+/-}$ X AC3I myocytes ± isoproterenol (1 μM). Red arrow indicates after-depolarization. B) Action potential duration (APD_{90}) of wild-type, ankyrin-B$^{+/-}$, AC3I, and ankyrin-B$^{+/-}$ X AC3I cardiomyocytes paced at 0.5 Hz. In B, * denotes $p<0.05$ for marked lanes; # denotes $p<0.05$ of ankyrin-B$^{+/-}$, AC3I, and ankyrin-B$^{+/-}$ X AC3I vs. wild-type myocytes ± β-AR-treatment; * denotes $p<0.05$ for AC3I and ankyrin-B$^{+/-}$ X AC3I myocytes vs. ankyrin-B$^{+/-}$ myocytes ± β-AR treatment. C) CaMKII inhibition normalizes ankyrin-B$^{+/-}$ myocyte afterdepolarizations. Bars denote incidence of myocyte afterdepolarizations ±isoproterenol treatment. In C, * denotes $p<0.05$ of ankyrin-B$^{+/-}$ myocytes ± isoproterenol; # denotes $p<0.05$ of ankyrin-B$^{+/-}$ isoproterenol treated myocytes vs. all other groups of myocytes ± isoproterenol. For B and C, N values are noted in each panel.

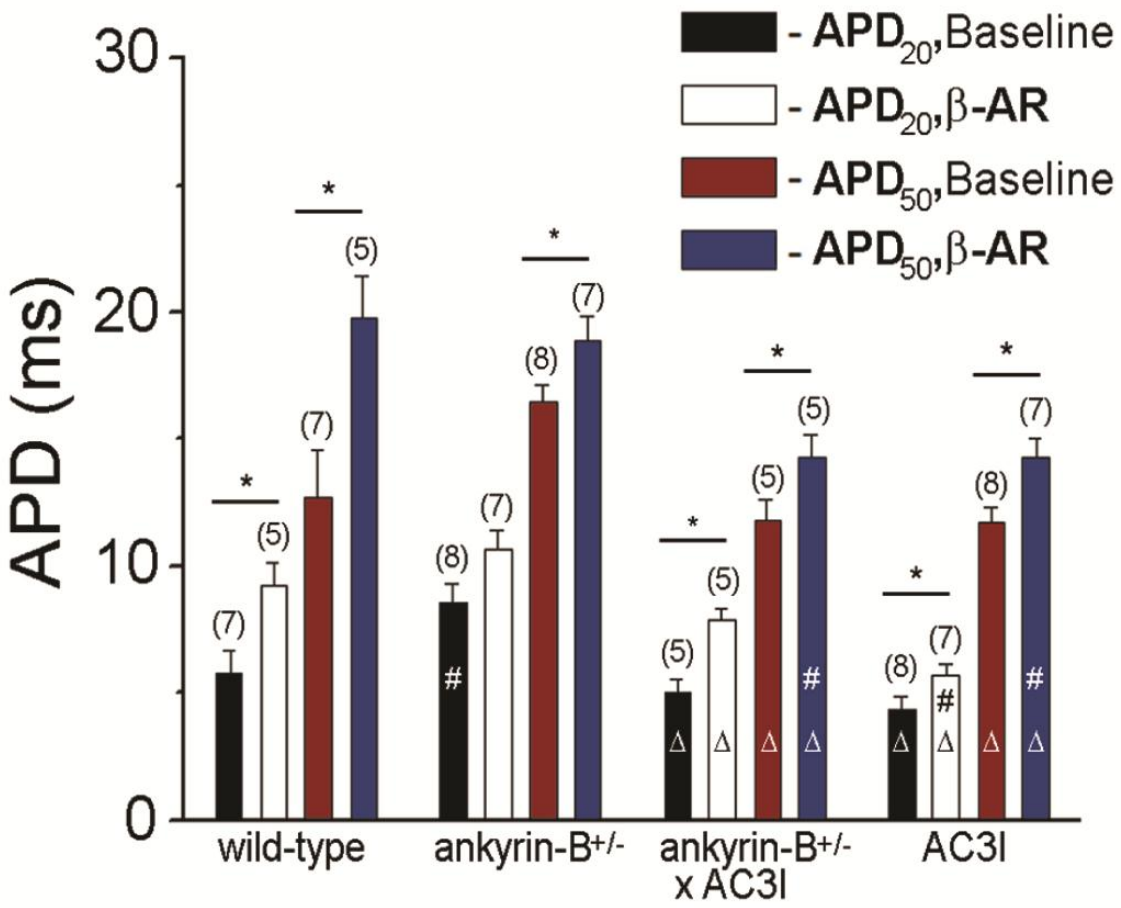

Figure 26. CaMKII inhibition normalizes ankyrin-B$^{+/-}$ myocyte electrical phenotypes. B) Action potential duration (APD_{20} and APD_{50}) of wild-type, ankyrin-B$^{+/-}$, AC3I, and ankyrin-B$^{+/-}$ X AC3I cardiomyocytes paced at 0.5 Hz. In Figure, * denotes $p<0.05$ for marked lanes; # denotes $p<0.05$ of ankyrin-B$^{+/-}$, AC3I, and ankyrin-B$^{+/-}$ X AC3I vs. wild-type myocytes ± β-AR-treatment; * denotes $p<0.05$ for AC3I and ankyrin-B$^{+/-}$ X AC3I myocytes vs. ankyrin-B$^{+/-}$ myocytes ± β-AR treatment. N values are noted for each measurement.

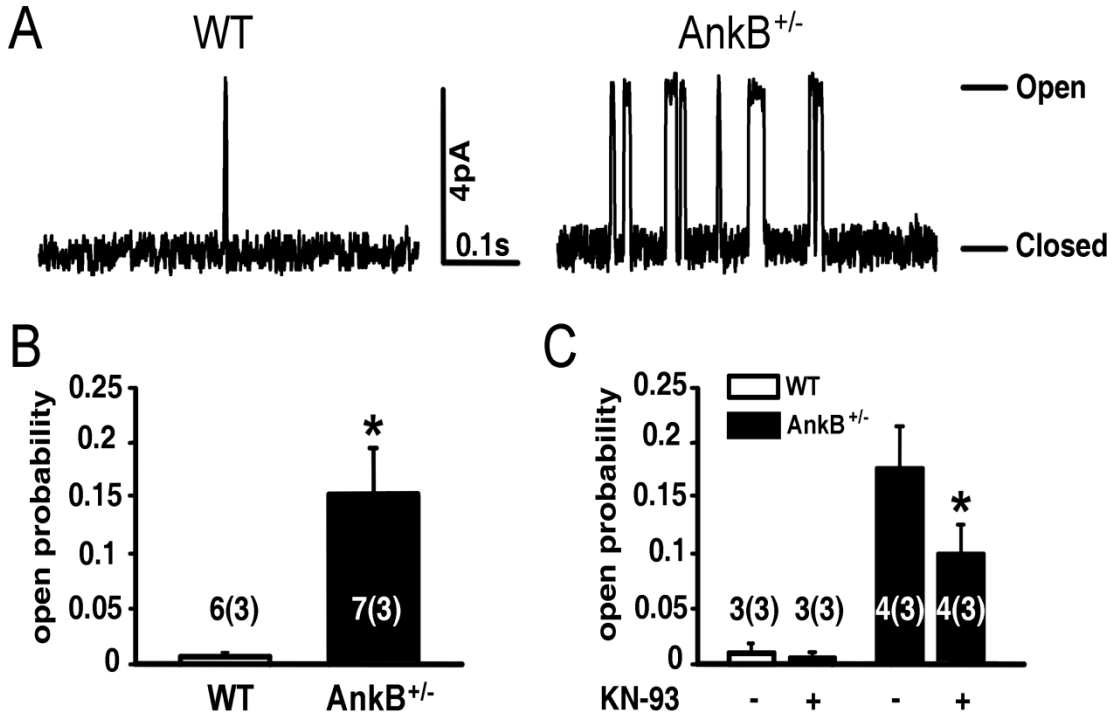

Figure 27. Ankyrin-B$^{+/-}$ mouse hearts display increased RyR$_2$ open probability (Po) A) Representative current recordings of RyR$_2$ from wild-type and ankyrin-B$^{+/-}$ mouse hearts. B) Average Po of RyR$_2$ from wild-type and ankyrin-B$^{+/-}$ mice. Po was increased in ankyrin-B$^{+/-}$ group (7 channels/3 mice) compared to wild-type group (6 channels/3 mice). C) Effect of KN-93 on Po of RyR$_2$ channels. Application of CaMKII inhibitor KN-93 reduced Po of RyR$_2$ from ankyrin-B$^{+/-}$ hearts, but did not alter Po of RyR$_2$ from WT mouse hearts. Numbers indicate channels/mice in the bar graph (* denotes $p<0.05$).

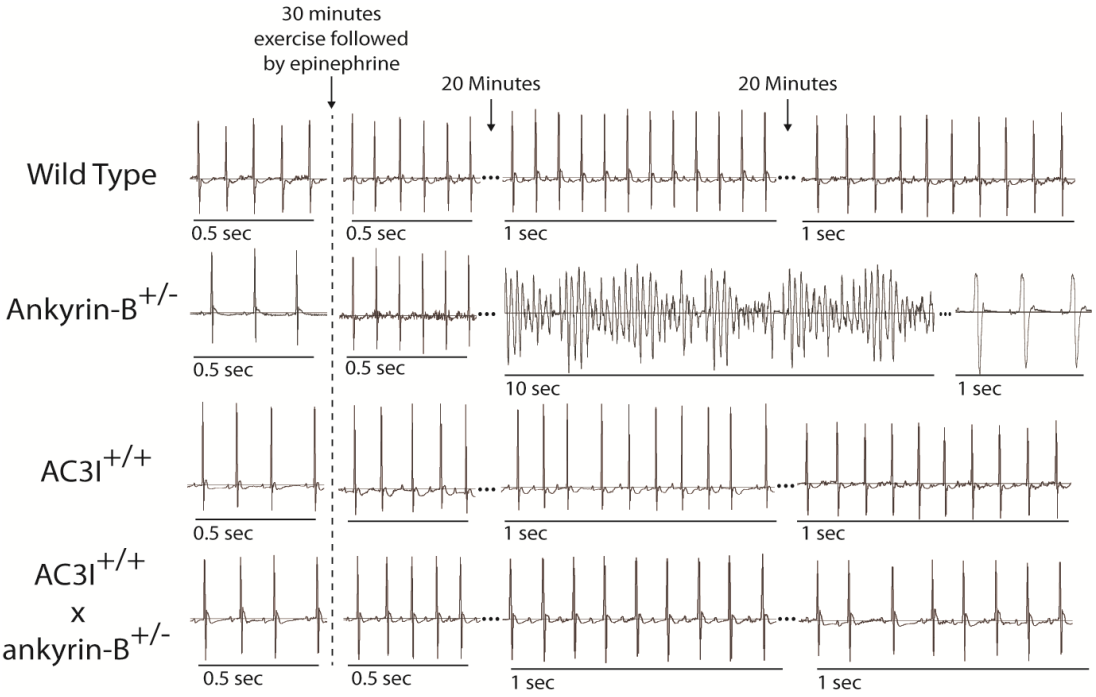

Figure 28. CaMKII inhibition normalizes ankyrin-B$^{+/-}$ arrhythmia phenotypes. Representative ECG recordings from wild-type, ankyrin-B$^{+/-}$, AC3I, and ankyrin-B$^{+/-}$ X AC3I mice at baseline and following exercise and treatment with adrenergic agonist. Center recording for ankyrin-B$^{+/-}$ mouse illustrates typical example of arrhythmia lasting minutes from this mouse model

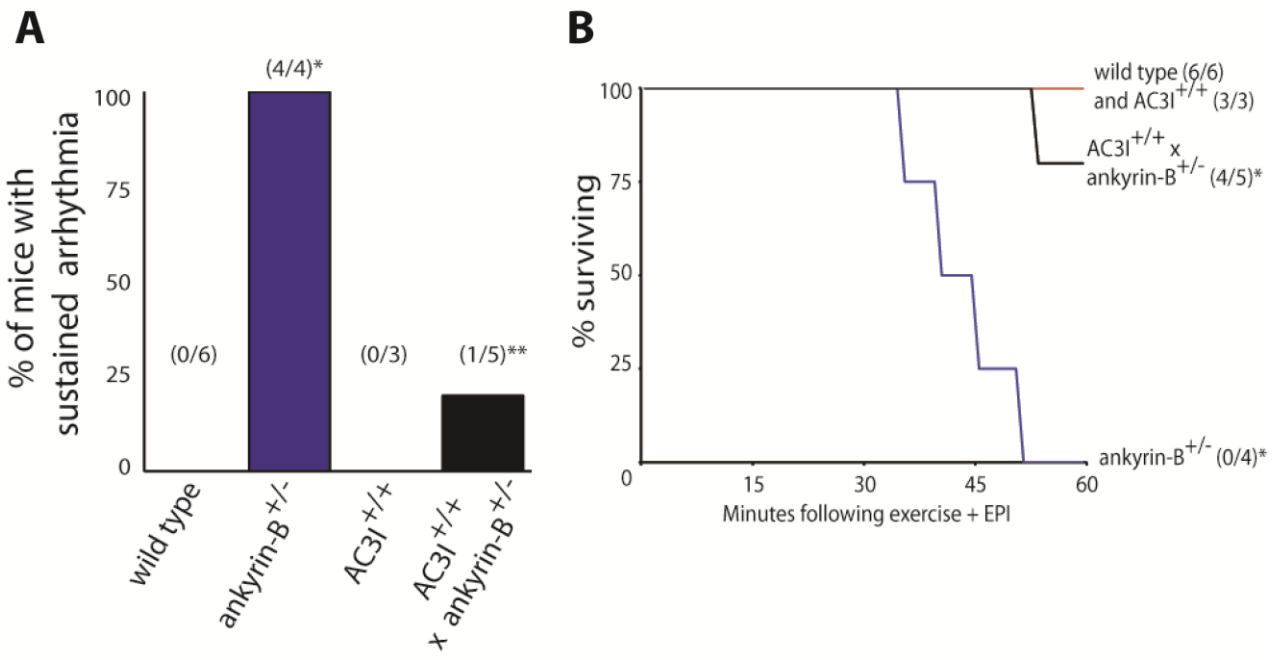

Figure 29. CaMKII inhibition reduces occurrence of lethal ventricular arrhythmia in Ankyrin-B Syndrome (LQTS type 4). A) Incidence of sustained ventricular arrhythmia in wild-type, ankyrin-$B^{+/-}$, AC3I, and ankyrin-$B^{+/-}$ X AC3I mice following exercise and treatment with β-adrenergic agonist., * denotes $p<0.05$ for ankyrin-$B^{+/-}$ mice vs. wild-type mice and ** denotes $p<0.05$ for ankyrin-$B^{+/-}$ mice vs. ankyrin-$B^{+/-}$ X AC3I mice. B) Survival of wild-type, ankyrin-$B^{+/-}$, AC3I, and ankyrin-$B^{+/-}$ X AC3I mice following exercise and treatment with adrenergic agonist. * denotes $p<0.05$ of ankyrin-$B^{+/-}$ vs. wild-type mice. For A and B, N values are listed in panels.

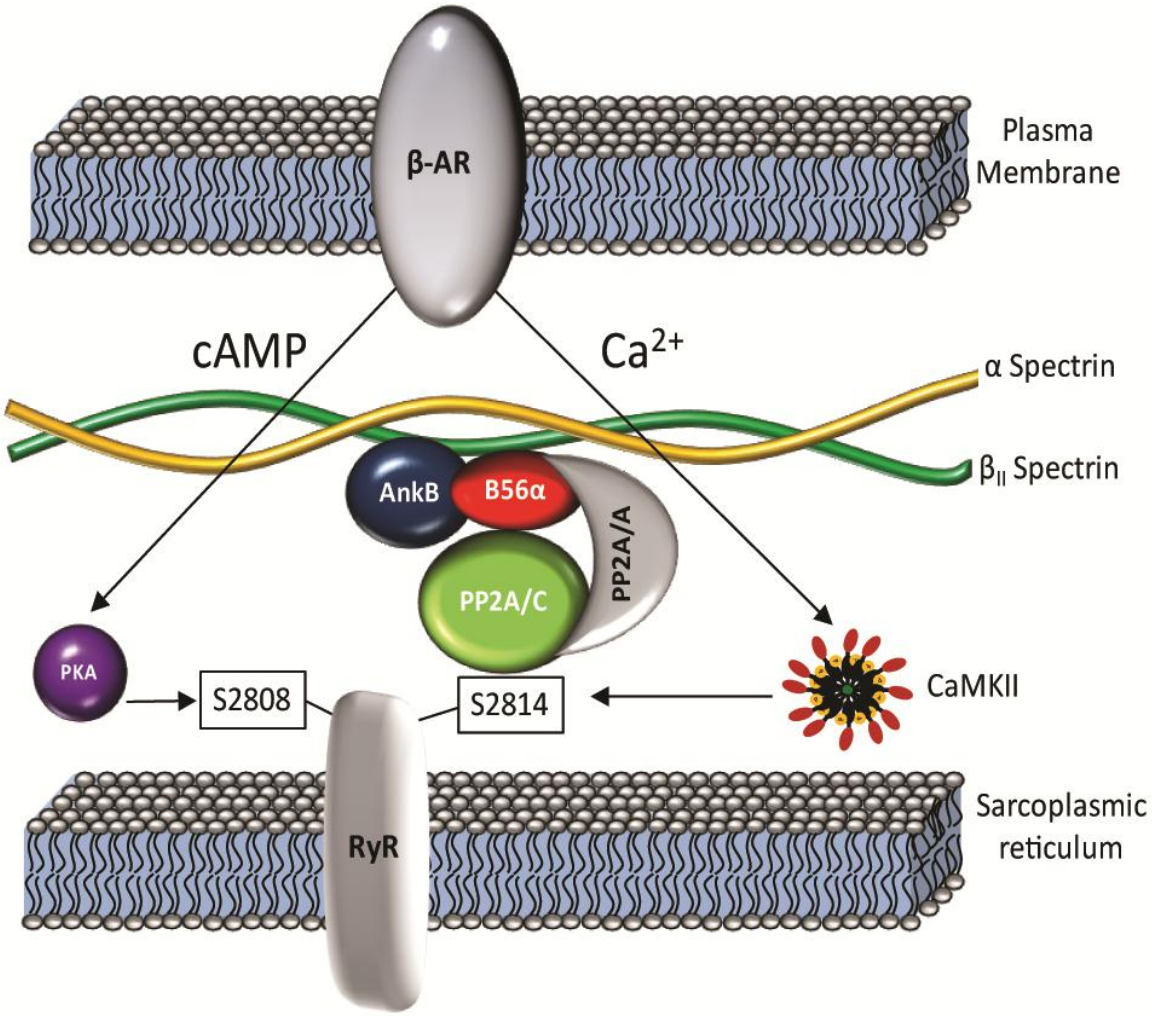

Figure 30. Model for link between ankyrin-B and local sympathetic regulation. In myocytes, ankyrin-B targets local PP2A activity through an interaction with B56α, one regulatory subunit of the PP2A holoenzyme. When ankyrin-B function is altered, this targeting mechanism is disrupted allowing CaMKII to function unopposed on myocyte targets (including RyR2) resulting in electrical dysfunction.

CHAPTER 4:

DISCUSSION AND FUTURE DIRECTIONS

These studies have established an important groundwork for understanding phosphatase regulation in cardiovascular health and disease. The data presented herein has established which of the PP2A subunits are expressed in all four chambers of the healthy heart and how the expression of these subunits changes in cardiovascular disease. One glaring questions that remains to be answered is whether or not the changes in subunit regulation reported here correlate to an increase or decrease in phosphatase activity in the disease heart.

Several studies have tried to answer this question using various treatments with phosphatase inhibitors (or conversely, treatments using exogenous PP2A over-expression) or *in vitro* phosphatase assays. Phosphatase inhibitors, while broad acting in their effects, have provided a useful starting point for exploring the role of phosphatases in cardiovascular disease. One study suggested that phosphatase inhibition maybe beneficial in the event of ischemic injury to the myocardium (Weinbrenner, Baines et al. 1998). In this study, fostriecin was used to inhibit enzymatic activity from PP2A and PP1. The results of this study showed that either pre-treatment of isolated rabbit hearts with fostriecin or treatment begun 10 minutes after the onset of ischemic conditions significantly reduced the infarct size compared to untreated infarcted hearts. Another study used a dominant negative form of the scaffolding subunit that was able to interact with the catalytic subunit but not the regulatory subunits to evaluate the role of PP2A in cardiomyopathy. This mutant had an increased level of activity against generic targets in an *in vitro* phosphatase assay that the authors assumed translated into an increase of *in vivo* phosphatase activity. The expression of this dominant negative mutant was linked to the development of dilated cardiomyopathy (Brewis, Ohst et al. 2000). Although these papers represent eloquent studies, their reliance on *in vitro* phosphatase assays to describe the *in vivo* activity of PP2A may not accurately portray phosphatase dynamics *in vivo*.

In vitro phosphatase assays essentially measure the activity of the catalytic subunit against a generic target. Based on the data I have presented here, it seems unlikely that activity against a generic target would accurately describe *in vivo* PP2A dynamics due to the fact that the targeting activity of the regulatory subunits is not accounted for in these assays. If we assume for the time being that the regulatory subunits define PP2A activity at specific targets, then the results of *in vitro* phosphatase assays likely only reflect changes in the relative expression level of the catalytic subunit in the tissues or cells these experiments are comparing, and not a true change in enzymatic activity of the holoenzyme against its *in vivo* targets. While these data suggest that phosphatase activity in cardiovascular physiology and disease is an interesting and worthwhile area of study, a true understanding of how phosphatase activity changes in cardiovascular disease will require a different system.

It is likely the best method to examine this question of overall phosphatase activity in cardiovascular disease is by stable genetic manipulation of the PP2A catalytic subunit activity or expression in transgenic mouse strains. While a pan PP2A catalytic subunit knockout would likely be a lethal genetic modification, a heart specific, conditional PP2A catalytic subunit knockout would likely produce a viable animal in which PP2A enzymatic activity could be selectively decreased. With these animals, a complete knockout of PP2A activity from birth would allow us to determine how the susceptibility of these animals to cardiovascular diseases such as heart failure, myocardial infarction, or arrhythmia compares to that of control animals. More interestingly, we would be able to compare the molecular, cellular, and whole animal phenotype of these animals in response to various treatments to control animals. For instance, we could more reliably test the hypothesis that inhibition of PP2A activity alleviates some of the cardiovascular damage associated with MI. Additionally, it would be interesting to see how these heart specific knockout animals respond to trans-aortic constriction (TAC) induced heart failure. An increase in morbidity and mortality of these mice following the

TAC procedure would provide strong evidence that phosphatase activity is needed in heart failure to promote survival. In this context it would be reasonable to hypothesize that new treatment modalities that enhance phosphatase activity may be beneficial to the treatment of human heart failure. On the other hand, an increase in survival and a decrease in morbidity following TAC procedure in these animals would provide strong evidence that phosphatase activity is detrimental to overall health and survival in heart failure and methods of inhibiting phosphatase activity may prove therapeutic. Morbidity could be evaluated in these animals by measuring several standard parameters routinely used to evaluate heart function including LV mass, ejection fraction, and systolic and diastolic volumes.

A knock-in mouse model in which PP2A activity is increased by over-expressing the PP2A catalytic subunit is a little bit more challenging. In this instance, simply creating a knock-in mouse model that over-expresses the PP2A catalytic subunit in the heart may create similar situation to that of using *in vitro* phosphatase assays. As such, the information gained from such a model would probably not be as useful as the information gained from the heart specific knock-out model discussed above. Regardless, this model will probably be more useful than other over-expression models based on transfection or viral transduction of isolated cells and thus could lend to support to any hypothesis derived from a knockout model.

Furthermore, and perhaps even more importantly, the data presented here sheds new light on potential mechanisms for rapidly altering PP2A activity through post-translational modification of the catalytic subunit. This regulatory mechanism for PP2A activity and target selectivity has been described in other diseases, and has been shown to be of particular importance in Alzheimer disease (Rudrabhatla and Pant 2011). Based on evidence reported here, we postulated that decreased LCMT-1 expression results in decreased methylation of the catalytic subunit, a modification of the subunit that is of critical importance in the recruitment a specific subset of regulatory subunits into the

functional holoenzyme. Interestingly, a different group reported similar evidence and proposed a very similar mechanism for PP2A dysregulation in Alzheimer's Disease. Although these disparate disease pathologies seem to have little in common at first glance, this may not be entirely accurate.

The incidence of both diseases, heart failure and Alzheimer's disease, increase with age (Jessup and Brozena 2003; Querfurth and LaFerla 2010). Aging has a wide variety of impacts on cellular activities and it is not difficult to postulate that perhaps aging has effects on phosphatase activity or on the availability of methyl transferase activity. Previous work has demonstrated that PP2A expression level decreases with age in mouse brain lysates but similar work has not yet been undertaken in the heart (Jiang, Tsien et al. 2001). Furthermore, additional studies need to be conducted to see if the expression and/or activity of the enzymes regulating post translational modification of the catalytic subunit, namely LCMT-1 and PME-1 changes with age, as no studies of this nature have been conducted in either cell type.

Another hypothesis linking the common observation in two seemingly disparate disease pathologies is a decreased availability of methylation intermediates. Leucine carboxy-methyltransferase-1 (LCMT-1) catalyzes the transfer of a methyl group from S-adenosyl-methionine (SAM) to a target molecule, or more specifically in this instance, to leucine 309 of the catalytic subunit of PP2A (Lee and Stock 1993). The immediate result of this action is the production of S-adenosylhomocysteine (SAH). This compound is then converted to homocysteine and adenosine by a different enzyme, SAH hydrolase (Selhub 1999; Vafai and Stock 2002). When homocysteine builds up to sufficiently high levels, this reaction is reversed and homocysteine and adenosine are converted back into SAH by SAH hydrolase. SAH in turn acts as competitive inhibitor of methyltransferase enzymes, including LCMT-1. Therefore high homocysteine levels back up the process of methylation of the catalytic subunit by increasing the concentration of SAH, a potent

inhibitor of methyltransferase activity (Yi, Melnyk et al. 2000; Caudill, Wang et al. 2001).

Homocystinuria is an autosomal recessive genetic condition caused by mutations in the genes encoding the enzymes responsible for homocysteine metabolism. This disease causes the concentration of circulating homocysteine to be significantly increased. Years of research have linked homocystinuria to an increased risk of cardiovascular disease (Clarke, Daly et al. 1991). Furthermore, studies conducted to examine the association between homocysteine and neurocognitive diseases like Alzheimer's disease have shown a positive correlation between elevated homocysteine levels and the occurrence of Alzheimer's disease (Seshadri, Beiser et al. 2002).

In light of the data presented here and of the data that exists in the literature, we hypothesize that elevated homocysteine levels can have a direct effect on the heart specifically in instances of relatively rare, severe genetic conditions affecting the enzymes involved in SAM dependent methylation and byproduct metabolism. Although no human disease is ever to small or to exceedingly rare to study, the big question remains, what role does homocysteine play in the development or in the pathology of cardiovascular disease or Alzheimer's Disease (although outside the scope of this cardiovascular biology based thesis) in instances of acquired disease in which no underlying genetic anomaly exists?

The data that currently exists relating to this question is confusing at best. It is well established that decreased methylation of the catalytic subunit is an important pathological process in Alzheimer's Disease; and the new data discussed above strongly suggests that a similar process may be involved in human heart failure, however it is unclear how homocysteine directly effects either of these pathological processes, if it does at all. It is well known that homocysteine is a remarkably reactive amino acid derivative and is extremely toxic to the vascular endothelium (Harker, Ross et al. 1976). Some studies have suggested that perhaps the connection between elevated homocysteine

and cognitive memory impairment related to Alzheimer's Disease is not due to any direct effects of homocysteine on diseased neurons but is related to an elevated risk of cerebral vasculature injury due to homocysteine mediated endothelial damage (Ford, Garrido et al. 2012). The best approach to determining the potential pathologic effects of homocysteine in the setting of human heart failure or other cardiovascular disease is to examine the expression level and activity of SAH hydrolase in disease vs. control tissue. A decrease in either the expression or activity of this enzyme would lend support to the hypothesis that a decrease in the availability of SAM to donate methyl groups for the methylation of the PP2A catalytic subunit contributes to observed changes in heart failure in combination with decreased LCMT-1 expression. Clinically, it would be interesting to know if elevated plasma homocysteine levels are correlated with worsening prognosis in the setting of adult onset heart failure. A prospective cohort study conducted on patients with newly diagnosed heart failure would be useful in helping us answer this question.

Lastly, the third and best supported potential mechanism linking this common observation of abnormal catalytic subunit regulation in such disparate disease pathologies is ROS generation. Pervious chapters have already discussed several pathways responsible for ROS generation in cardiovascular disease via aldosterone and β-adrenergic mediated signaling pathways. B-amyloid accumulation into large aggregates is the hallmark pathological feature of Alzheimer's disease (Querfurth and LaFerla 2010). This peptide is extremely toxic to mitochondria in general and to cytochrome C in particular (Caspersen, Wang et al. 2005). These damaged and dysfunctional mitochondria in diseased neurons release free radicals into the cytosol of the affected cells.

Free radicals create a variety of problems for the integrity of cellular structure and signaling in addition to the changes in PP2A catalytic subunit methylation and phosphorylation that have been described above. Based on the evidence presented here, the generation of free radicals is the most likely link between the observed decreased methylation of the catalytic subunit in both Alzheimer's disease and heart failure. In light

of the fact that we did not observe any changes in total expression levels of the catalytic subunit with short H_2O_2 exposure, we hypothesize that the short exposure to ROS used in this experimental protocol does not alter overall expression of PP2A subunit regulation. Nonetheless, I think it would be interesting to see if LCMT-1 expression or activity changes in cardiomyocytes with this treatment in a manner similar to what I observed in human heart failure lysates.

Although the data presented here clearly shows that exposure to ROS can induce changes in the post-translational modification of the catalytic subunit, we still don't know if this process is ROS dependent. The use of ROS scavengers might be useful in more completely defining the relationship between ROS generation and PP2A catalytic subunit modifications. To do this experiment, one could treat isolated adult cardiomyocytes obtained from canine models of heart failure with a ROS scavenger before conducting immunoblots to detect the methylated and phosphorylated form of the subunit. A decrease or elimination of the observed changes in catalytic subunit methylation and phosphorylation in diseased cells exposed to ROS scavengers compared to cells isolated from both untreated failing hearts and from healthy control hearts would support the hypothesis that the described changes in post translational modification are ROS dependent. Although these studies focused on the regulation of PP2A activity in the context of cardiovascular physiology and disease, the results described herein extend well beyond this very prominent form of human disease and contribute to a more complete picture of altered PP2A regulation globally in human disease by the generation of free radicals.

Despite the very interesting notion of possible connections between the pathological processes involved in heart failure and Alzheimer's disease, a more pressing issue that needs to be addressed is the importance of the observed post-translational regulation of PP2A in cardiovascular disease. A major impediment that currently stands in our way of answering this question is the fact that we lack any knowledge of what

macromolecular complexes these subunits are associated with. If we are able to determine which subunits direct PP2A activity to specific intracellular targets, we can form better hypotheses regarding the role of catalytic subunit post-translational modification in cardiovascular physiology and disease.

The data that currently exists linking PP2A to specific macromolecular complexes or even to specific phosphorylation sites on target peptides applies only the PP2A holoenzyme. Much of this data was assembled based on experiments that detected the presence of the catalytic or scaffolding subunit in association with a protein or phosphorylation site of interest (for a brief review of recent data published describing such interactions, please the section called *PP2A Targets in Cardiomyocytes* in the Background section of this thesis). In fact, a better understanding of the interactions between the regulatory subunits and their respective targets might help us explain some of the disjointed data that exists in the literature that at first glance seems contradictory. For example, one study published describing PP2A dynamics in skinned cardiomyocytes actually found decreased expression of the catalytic subunit associated with the contractile apparatus of diseased cardiomyocytes when compared to healthy controls (Wijnker, Boknik et al. 2011). These results are actually quite interesting in light of the new data discussed above. Realignment of PP2A activity from one set of targets in diseased cells vs. healthy cells, or at least an altered PP2A affinity for specific targets in diseased cells mediated by changes in subunit regulation, would explain how our results show an increase in overall catalytic subunit expression despite earlier results showing decreased expression (or more appropriately, decreased association) of the same subunit in the contractile apparatus. Again, all of this data highlights the importance of more precisely defining these interactions within cardiomyocytes. Such data will allow us to gain a better understanding of how these changes in PP2A regulation might be beneficial or pathologic to overall heart function in cardiovascular disease.

A yeast two hybrid experiment in which the subunits are used as bait constructs would be helpful to identify new complexes. This experiment would allow the ability to determine candidate targets for each of the PP2A regulatory subunits. Once a set of candidate molecular targets has been identified, these interactions could be further interrogated by direct *in vitro* binding assays, or by immunoprecipitation and pull-down experiments. These additional assays are necessary to not only confirm the findings of the initial yeast-2-hybrid experiment, but would also provide evidence to support or refute a direct interaction between the regulatory subunit and target molecule in question. An *in vitro* binding assay is an attractive option because it provides strong evidence for a direct interaction if positive results are obtained. A co-immunoprecipitation experiment, on the other hand, is also attractive option because it can provide useful information as to what other signaling components may also be complexed with the peptide, protein, or channel of interest and the regulatory subunit. These experiments would provide useful information as to the ability of the regulatory subunit to serve a scaffolding role, much like previously described AKAPs have been shown to do.

Furthermore, the striking similarity between the sequences of the regulatory subunits, especially when comparing regulatory subunits of the same family, has been noted previously in this dissertation. It seems that areas of variability between the subunits are confined to the near vicinity of the amino and carboxyl terminus of each regulatory subunit. We hypothesize that these areas will be responsible for much of the regulatory subunit specific interactions with target proteins found by the previously described experiments. Once the interactions are defined by the experiments above, further pull-down or direct *in vitro* binding assays can be completed using only fragments of the regulatory subunits as opposed to the whole subunit to determine which areas of the subunit facilitate the observed interactions.

While it is difficult to make any convincing hypotheses about which of the regulatory subunits might be more important to pathological or compensatory changes

associated with cardiovascular disease until we gain a better understanding of which regulatory subunits interact with which targets, the data we have presented here suggests some potentially useful places to start. The first is the regulatory subunit PPP2R5A.

CaMKII dependent phosphorylation of RyR2 has been linked to several pathological processes such as atrial fibrillation and heart failure (Chelu MG 2009; van Oort, McCauley et al. 2010; Li, Wang et al. 2012; Respress, van Oort et al. 2012). Based on data presented here and data in the literature, we hypothesize that dysregulation of the PP2A regulatory subunit PPP2R5A results in pathologic CaMKII dependent Ca^{2+} release from RyR2 and that an increase in PPP2R5A expression may reduce the severity of the pathogenic Ca^{2+} handling abnormalities seen in these diseases. The work presented in the latter half of this thesis supports previously published work that also suggested PPP2R5A was responsible for PP2A activity at the S2814 site on RyR2 (Terentyev D 2009). Although two independent lines of investigation have arrived at the same conclusion, that PPP2R5A targets PP2A activity to a specific CaMKII phosphorylation motif on RyR2, one significant drawback of both of these studies is that neither was very specific for isolating the effects of PPP2R5A dysregulation on RyR2. The first studied conducted by Terentyev et al studied PP2A targeting to RyR2 in the context of microRNA-1 (miR-1) expression and the current study examined PP2A targeting to RyR2 in the context of ankyrin-B deficiency. What is needed to decisively show a link between PP2A and RyR2 through the regulatory PPP2R5A subunit is a method of isolating and manipulating this interaction in an environment that is removed from the complicated intracellular pathology associated with either ankyrin-B deficiency or miR-1 expression.

A quick, preliminary way to conduct this study would be to knock down PPP2R5A in cardiomyocytes using siRNA followed by RyR2 characterization in these cells. Studies could be conducted to confirm the specificity of PPP2R5A for this particular site on RyR2 that would detect the phosphorylation level of RyR2 at both the PKA (S2808) and CaMKII (S2814) site. Additionally, a co-immunoprecipitation

experiment could be done to determine if RyR2 and the PP2A scaffolding subunit co-precipitate in the absence of PPP2R2A expression. Furthermore, functional data describing the open probability of the receptor in siRNA transduced cardiomyocytes with and without the addition of a β-adrenergic antagonist would be helpful in determining how the isolated occurrence of PPP2R5A dysregulation affects the function of this important receptor. However, the limited amount of time in which adult primary cardiomyocytes are viable in culture will limit the ability of this study to significantly reduce PPP2R5A expression.

While these studies would be helpful in isolated cells, siRNA technology has limited to no ability to create an environment for studying PPP2R5A dysregulation in whole hearts. To study the effects of this interaction *in vivo,* a heart specific knockout mouse would need to be created. With this mouse, we could the compare the susceptibility to arrhythmia or heart failure to that of wild type animals. In addition, such a mouse might be better suited for physiologic studies of RyR2 properties than would a siRNA transfection or transduction mediated knockdown due to the potential for low transfection or even transduction efficiency that is characteristic of primary cells.

Another interesting experiment that can be done to investigate the significance of an interaction between PPP2R5A and RyR2 is to overexpress PPP2R5A in cardiomyocytes. We hypothesize that PPP2R5A over expression would reduce the CaMKII mediated Ca^{2+} leak from RyR2 that has been described in heart failure, ventricular arrhythmia and atrial fibrillation. This can also be accomplished by a few different methods. The quickest method again relies on transfection of viral transduction of primary cardiomyocytes with PPP2R5A under the control of a highly active promoter. Again, a cardiomyocyte specific knock-in model would allow for whole animal physiology studies and would provide a more precise model for studying the molecular (i.e. RyR2 phosphorylation levels and RyR open probability) and cellular (i.e. action

potential, channel activity, and Ca^{2+} handling) consequences of PPP2R5A over expression in healthy and diseased hearts.

Lastly, and of the greatest interest to me, particular attention should be given to those regulatory subunits found to localize to the nucleus of cardiomyocytes. It is well established that the remodeling phenotype seen in cardiomyocytes of diseased hearts is associated with significant changes in gene expression mediated by several processes including phosphorylation of histone deacetylases (HDACs) and increased activity of several transcription factors such as nuclear factor of activated T-cells (NFAT), nuclear factor kappa light polypeptide gene enhancer in B cells (NFκB) and myocyte enhancing factor 2 (MEF2) among others (McKinsey and Kass 2007; Mudd and Kass 2008; Shah and Mann 2011). All of these changes result in a pro-hypertrophic remodeling phenotype.

Histone proteins regulate chromatin structure. When they are acetylated, they allow for relaxation of the chromatin structure and permit gene transcription to occur. However, when they are deacteylated, the histone is tightly bound to DNA and that area of DNA is not accessible by the DNA transcription machinery of the cell. Therefore HDACs function to repress gene transcription by deacteylating histone proteins. HDACs can be phosphorylated at two serine containing motifs resulting in their transport out of the nucleus and thus activation of gene transcription of many chromosomal areas that are normally repressed. Many of the kinases activated by heart failure signaling cascades, such as CaMKII, can phosphorylate these motifs (McKinsey and Kass 2007). Therefore kinase activity associated with cardiovascular disease results in diminished nuclear retention of HDAC protein compared to healthy control cardiomyocytes and contributes to abnormal gene expression diseased cells. Interestingly, PP2A has been found in association with several class II HDACs in the nucleus (Illi, Dello Russo et al. 2008). Furthermore, the results published by one study suggest that PP2A activity can reverse the process of nuclear export of class II HDACs thought to be important in inducing hypertrophic remodeling. In particular, this study found that HDAC4 required the actions

of the PP2A holoenzyme containing the PPP2R2A regulatory subunit for nuclear import (Paroni, Cernotta et al. 2008).

This report is of great interest as this is the same regulatory subunit that we described as being selectively excluded from the PP2A holoenzyme in heart failure due to a relative decrease in the methylated form of the PP2A catalytic subunit. We hypothesize that reduced incorporation of this particular regulatory subunit into the PP2A holoenzyme contributes to removal of the HDAC from the nucleus of diseased cardiomyocytes in heart failure. This hypothesis is relatively straight forward to test. Based on this experiment I would predict that overexpression of PPP2R2A might increase HDAC4 retention in the nucleus. Additionally, one might also predict that inhibition or knock down of PME-1 or over-expression of LCMT-1 might also have the same effect. This is of key interest to the process of drug discovery as attempts to alter the remodeling phenotype in cardiomyocytes have been the subject of scientific study for some time. While some of the experiments proposed in this section represent the only the formative beginnings of defining new therapeutic targets in the fight against heart disease, one might postulate that seeking ways of modifying catalytic subunit methylation in cardiovascular disease (or in Alzheimer's disease) might be a more reasonable goal to strive for in the near future.

However justified and seemingly well supported by the literature such an investigation may be, such a project will undoubtedly take time. The first step would be to evaluate the ability of PPP2R2A over-expression to alter the remodeling phenotype. An early investigation in this process would be transfect or virally transduce control neonatal cardiomyocytes with PPP2R2A and determine if more HDAC4 remains in the nucleus when compared to control cardiomyocytes. Experiments exploring the gene expression pattern of these cells compared to untreated controls would probably be of limited value as the cells over-expressing PPP2R2A are also healthy and therefore would not display the remodeling phenotype at baseline. The best way to test the ability of

PPP2R2A over-expression to modify the cardiomyocyte remodeling phenotype of heart failure is to create a genetically modified mouse that over-expresses PPP2R2A in a cardiomyocyte specific manner. With this mouse one could induce heart failure by means of trans-aortic constriction (TAC) and use immunofluorescent confocal microscopy to determine how nuclear HDAC4 localization in these cardiomyocytes compares to cells isolated from wild type animals subjected to the same heart failure inducing procedure. Additionally, comparing a gene expression profile of cardiomyocytes isolated from this model in heart failure vs. cardiomyocytes isolated from the wild type model in heart failure would also provide evidence for or against the hypothesis that PPP2R2A over-expression alters the remodeling phenotype. Assuming that changes were seen in the remodeling phenotype, one could compare the morbidity and mortality of the PPP2R2A over-expressing animals experiencing heart failure with that of the wild type animals experiencing heart failure to determine if changing the remodeling phenotype is harmful or beneficial in heart failure. This plan highlights a process for determining the ability of PPP2R2A over expression. One significant problem with this approach however is the fact that the decreased methylation of the catalytic subunit would not be reversed in this animal model over-expressing PPP2R2A and therefore PPP2R2A might still be selectively excluded from the holoenzyme despite increased expression of the subunit

An equally important course of study would be to evaluate the potential to alter PP2A catalytic subunit methylation by inhibiting or knocking down PME-1 expression, or conversely, by over-expressing or augmenting LCMT-1 activity; first in control cardiomyocytes and secondly in adult cardiomyocytes isolated from control and failing hearts. Despite the ease of use and little lead time required, notoriously low transfection and transduction efficiencies observed in experiments using primary cardiomyocytes may limit the usefulness of these types of studies. We would predict that either of these methods would increase methylation of the catalytic subunit especially in control neonatal cardiomyocytes that can be cultured for several days however isolated adult

cells from control and failing hearts may prove to be a bit more challenging. Although it does not seem that different physiologic properties should exist in neonatal vs. adult cardiomyocytes with regards to PP2A catalytic subunit methylation, the shorter period of time that adult cells are viable in culture might make changes in methylation more difficult to detect using these methods. However, if one could transfect or virally transduce cardiomyocytes isolated from healthy animals and from heart failure models in order to knock down PME-1 expression or over-express LCMT-1, one could then immuno-label the cardiomyocytes for HDAC4 to see if localization of this protein changes between the treated cardiomyocytes and control cardiomyocytes. Additionally, you could also repeat the co-immunoprecipitation experiments discussed in chapter 2 to determine if the relative amount of PPP2R2A found in association with the holoenzyme changes. This preliminary experiment might justify dedicating significant resources to further study of this system. Again, more in depth study of this potential method for altering class II HDAC localization will require stable knockout of PME-1 or stable overexpression of LCMT-1 using genetically modified animal models. Based on past literature indicating the lethality of pan PME-1 knockouts (Ortega-Gutierrez, Leung et al. 2008), such genetic modifications would have to be heart specific. With these animals, experiments would not only achieve a more complete inhibition of PME-1 activity or a more complete over-expression of LCMT-1 for molecular and cellular study, but one could characterize these mice to investigate the clinical usefulness of altering pro-hypertrophic remodeling in diseased hearts should the data indicate that altering the methylation of the PP2A catalytic subunit results in the nuclear retention of class II HDACs and changes the gene expression profile observed in diseased cardiomyocytes. If either of these animals showed reduced susceptibility to the development of heart failure, further work could be undertaken to explore pharmacologic methods of achieving the same effect.

In summary, the data presented here has confirmed the prominent role of PP2A activity in cardiac physiology and indicated that PP2A subunits are abnormally regulated in cardiovascular disease. Furthermore these studies highlight the importance of defining the specific intracellular targets of PP2A regulatory subunits as data obtained from a previously described model of a human arrhythmia syndrome indicate that dysregulation of one specific subunit can produce abnormalities in reversible phosphorylation that contribute to an arrhythmogenic phenotype. Perhaps most interestingly, this study identified an important means of PP2A regulation dependent of post-translational modification of the catalytic subunit. Although this project has identified many new areas for further investigation, identifying intracellular targets for each of the regulatory subunits and furthering our understanding of this method of post translational modification may shed light on new therapeutic targets in the fight against heart disease.

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