Potential Role of αKAP, a CaMKII Kinase Anchoring Protein in Myocardium

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ABSTRACT

The Sarco-endoplasmic Ca^{2+} ATPase (SERCA2a) plays a crucial role in sequestering cytosolic calcium into the sarco-endoplasmic reticulum (SR/ER) and is an important regulator of muscle contraction and relaxation. Recent findings suggest that a novel CAMKIIα splice variant, αKAP, that plays the role of a CAMKII anchoring protein in the myocardium, also directly interacts with SERCA2a. We examined the effects of αKAP on SERCA2a activity using transfection of HEK-293T cells as well as lentiviral infection of primary neonatal mouse cardiomyocytes (NMCM). Our data showed that αKAP reduced Ca^{2+} ATPase activity, and downregulated SERCA2a expression in both HEK-293T cells coexpressing αKAP and SERCA2a, as well as NMCM overexpressing αKAP. Interestingly in a rat model of myocardial infarction, αKAP expression was found to be elevated, alongside elevated CaMKIIδ, and depressed SERCA2a expression. These data suggest that αKAP may be a unique regulator of SERCA2a activity and cardiac function.

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AKAP	A-kinase anchoring proteins
αKAP	alpha-kinase anchoring protein
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ATPase	Adenosine triphosphatase
AChR	Acetylcholine receptor
AngII	Angiotensin II
BCA	Bicinchoninic acid assay
BSA	bovine serum albumin
β-AR	β-adrenergic receptor
Ca^{2+}	Calcium ion
cDNA	complementary DNA
CaMKII	$Ca^{2+/}$calmodulin-dependent protein kinase II
CASQ2	Calsquestrin
cAMP	Cyclic adenosine monophosphate
CaM	Calmodulin
CICaR	Calcium induced calcium release
DMEM	Dulbecco's Modified Eagle Medium
DCM	Dilated cardiomyopathy
ECL	Enhanced chemiluminescence
ECC	Excitation contraction coupling
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethyleneglcol-bis(B-aminoethylether)-N,N'-tetracetic acid
ER	Endoplasmic reticulum
FBS	fetal bovine serum

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione sepharose transferase
GRK2	G-protein receptor kinase 2
HBSS	Hank's buffered salt system
HF	Heart failure
HDAC	Histone deacetylases
KDa	Kilo Dalton
LDH	Lactate dehydrogenease
LTCC	L-type calcium channel
MOPS	3-(N-morpholino)propanesulfonic acid
MEF2	Myocyte enhancement factor 2
NADH	Nicotinamide adenine dinucleotide
NCX	Sodium calcium exchanger
NEAA	non-essential amino acids
NMCM	neonatal mouse cardiomycocyte
NP-40	Nonidet P-40
NLS	Nuclear localization domain
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEP	Phosphoenolpyruvate
Po	Open probability
PLN	Phospholamban
PVDF	Polyvinylidene fluoride
PK	Pyruvate kinase
PKA	Protein kinase A

PFA	Paraformaldehyde
PPI	Protein phosphatase inhibitor
PP1	Protein phosphatase 1
RIPA	Radioimmunoprecipitation buffer
RyR	Ryanodine receptor
SDS	Sodium dodecyl sulfate
SERCA2a	Sarco/endoplasmic reticulum Ca^{2+} - ATPase
SIKE	Suppressor of IKKepsilon
SLN	Sarcolipin
SUMO1	Small ubiquitin related modifier 1
SR	Sarcoplasmic reticulum
TRIS	Tris(hydroxymethyl)aminomethane
TBST	Tris buffered saline – tween
Wt	Wild type

CHAPTER 1: INTRODUCTION

1.1 Heart Failure

Heart failure is the disease state marked by the inability for the organ to adequately supply the body with the required blood flow based on the metabolic demands (Braunwald, 2000). In order to meet metabolic demands of the organs by supply of adequate blood flow, proper contraction and relaxation of the heart is imperative. Tight regulation of calcium homeostasis on a beat to beat basis is crucial in the regulation of contraction and relaxation of cardiac tissue and ultimately the proper function of the heart; disruptions in calcium homeostasis will ultimately lead to heart failure (HF). Heart failure is the progression of an underlying condition such as a myocardial infarct (damage caused by insufficient blood flow to an area), arrhythmias (irregular heart beat), dilated cardiomyopathy (DCM, an enlargement of the heart), or hypertrophic cardiomyopathy (HCM, a thickening of the heart muscle) (Gwathmey et al., 1987). Calcium handling proteins such as SERA2a and Ca^{2+}/calmodulin dependent kinase II (CaMKII) impact the beat-to-beat function of the heart and the studies here are focused on examining their regulation.

1.2 Excitation –Contraction Coupling (EC-C) in myocardium

Following membrane depolarization, the voltage gated L-type Ca^{2+} channels (LTCC), located on the transverse tubules/sarcolemmal membrane, open and enable an influx of Ca^{2+} into cardiomyocytes. This influx triggers calcium release through the ryanodine receptors (RyR), located on the membrane of the sarcoplasmic reticulum (SR). This is termed calcium induced calcium release (CICaR) and is necessary to initiate muscle contraction within cardiomyocytes

(See Figure 1) (Franzini-Armstrong, 1999). Muscle contraction is initiated when Troponin C, a protein bound to tropomyosin within actin filaments, binds calcium. Upon binding calcium, a conformational change in the troponin C-tropomyosin complex will allow cross bridging of actin and myosin, and thereby initiation of a contraction. For muscle relaxation to occur, cytosolic calcium must be actively sequestered back into the SR. This sequestration is achieved by the cardiac isoform of the calcium ATPase pump, SERCA2a (MacLennan & Kranias, 2003; MacLennan, Asahi, & Tupling, 2003; Vafiadaki, Papalouka, Arvanitis, Kranias, & Sanoudou, 2008). Down-regulation of SERCA2a expression in cardiac hypertrophy plays a critical role in the pathogenesis of the disease. Recent clinical studies using adenoviral mediated SERCA2a overexpression demonstrate an improvement in heart function and could potentially rescue the hypertrophic heart (Cutler, Wan, Plummer, Liu, Deschenes, Hajjar, & Rosenbaum, 2012).

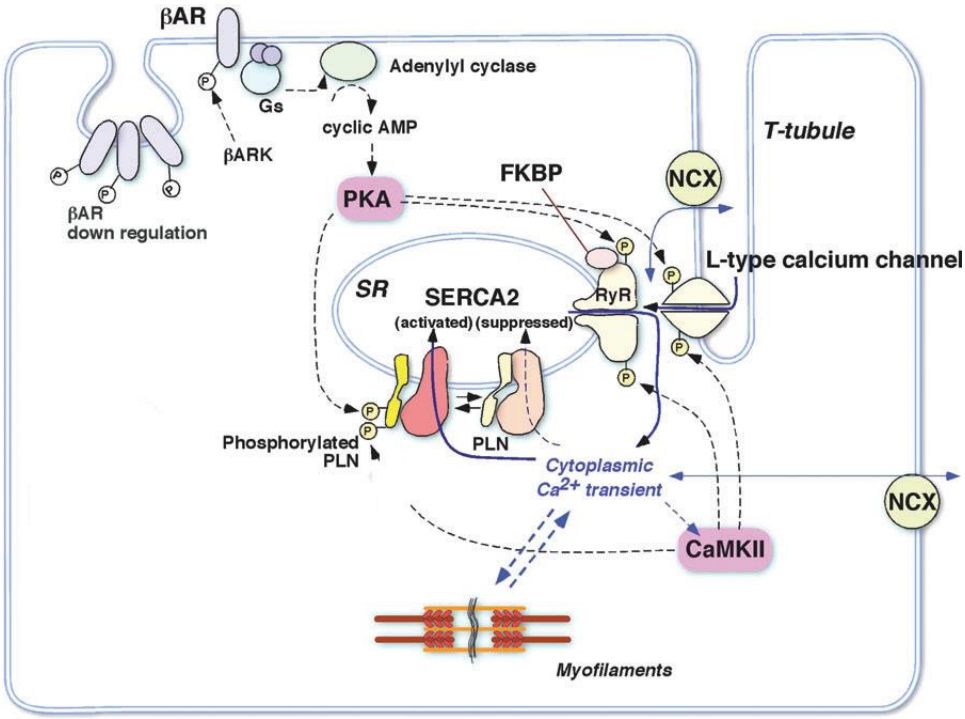


Figure 1: *Modeling EC-coupling in cardiac myocytes.* Following membrane depolarization, extracellular Ca^{2+} enters the cardiomyocyte via L-type Ca^{2+} channels, activating the calcium release channels (RyR) found on the SR and, causing a further influx of calcium into the cytosol. The increased intracellular calcium will then in turn initiate a muscle contraction. For muscle relaxation to occur, calcium is actively re-uptaken back into the SR via SERCA2a, whose function is modulated by phospholamban PLN, a natural SERCA2a inhibitor (Adapted from Kien et al, 2008).

1.3 Role of Sarcoplasmic Reticulum (SR) in cardiac function

Cardiac contraction relies on transient changes of cytoplasmic Ca^{2+} levels and therefore effective release and subsequent sequestration of Ca^{2+} back into the SR is imperative for proper contraction (McDonough, Yasui, Betto, Salviati, Glembotski, Palade, & Sabbadini , 2008). The SR is responsible for calcium release, uptake and storage and is a network of tubules and cisternae composed of two compartments: the longitudinal SR and junctional SR (Inui, Wang, Saito, & Fleischer, 1988). To initiate a muscle contraction, calcium is released from the SR into the cytosol via RyR, where the contractile force, and ultimately correct functioning of the heart, is highly dependent on the concentration of available calcium within the SR, also referred to as the 'calcium load' (MacLennan, 1970; Müller et al., 2002; Odermatt, Kurzydlowski, & MacLennan, 1996). Calcium re-uptake back into the SR via SERCA2a is required for muscle relaxation to occur and is crucial in regulating excitation-contraction coupling and ultimately heart function. Calsequestrin, CASQ2, is a high capacity, low affinity calcium binding protein in the SR, whose role is to bind and sequester calcium within the SR (Murphy, Larkins, Mollica, Beard, & Lamb, 2009). Calcium distribution within the SR is also of important consideration; uniform distribution will allow the released calcium to target regions equally within the myocyte and contribute to the synchronization of the EC- coupling mechanism. In heart failure, SERCA2a levels are decreased, whereby not only is the rate of calcium uptake reduced but the reduction in SERCA2a levels will also contribute to uneven calcium distribution within the SR (Picht, Zima, Shannon, Duncan, Blatter, & Bers 2011). Calcium leak, from the SR through the RyR, is a spontaneous release of calcium and plays an important in the role in the development of unwanted cardiac contractions and development of arrhythmias (Mackiewicz & Lewartowski, 2008). Under normal conditions, this diastolic leak of calcium is reuptaken back into the SR via

SERCA2a.

1.4 Sarco/endoplasmic Reticulum Ca^{2+} ATPase (SERCA2a): Regulation by Phospholamban (PLN)

Three isoforms of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) exist; a skeletal muscle isoform (SERCA1), the primary cardiac isoform (SERCA2a), and one that is ubiquitously expressed (SERCA2b). SERCA2a activity is triggered by intracellular calcium, more precisely, calcium binding to specific sites on SERCA2a. SERCA2a is further regulated by phospholamban (PLN), a 52 amino acid protein of ~5KDa (typically found in a inactive phosphorylated 25KDa pentameric form) which is a subunit of the pump. In its dephosphorylated, monomeric form, PLN binds to SERCA2a via its cytosolic domain and reduces the pump's affinity for Ca^{2+} (See figure 1) (Asahi, McKenna, Kurzydlowski, Tada, & MacLennan, 2000; Kimura, Kurzydlowski, Tada, & MacLennan, 1996; MacLennan et al., 2003). Phosphorylation of PLN occurs at two sites, Ser16 by protein kinase A (PKA) or at Thr17 by $Ca^{2+/}$calmodulin-dependent protein kinase II (CaMKII). This phosphorylation event causes PLN to undergo a conformational change, dissociating from SERCA2a, and thus relieving its inhibition on the pump and allowing Ca^{2+} re-uptake from the cytosol back into the SR lumen (Fujii, Ueno, Kitano, Tanaka, Kadoma, & Tada, 1987; Mortishire-Smith, Pitzenberger, Burke, Middaugh, Garsky, & Johnson, 1995; Simmerman & Jones, 1998). Sarcolipin (SLN), sharing sequence homology with PLN's transmembrane domain, was thought to be primarily the skeletal homologue of PLN (Asahi et al., 2003; MacLennan et al., 2003). However, it has been shown that cells in the cardiac atria express high levels of SLN (Minamisawa, 2003). Gene knockout of PLN in mice has been shown to enhance heart contractility and relaxation by increasing the affinity of SERCA2a to calcium. In contrast, overexpression of PLN decreased contractility

(Brittsan, 2000).

Although the mechanical aspects of cardiac muscle function are imperative to discuss, the nervous innervation has an equally influential role. The sympathetic innervation of the heart regulates cardiac function on a beat to beat basis, where β1 stimulation of the adrenergic receptors induces a G-protein coupled signaling pathway leading to an increase in cAMP levels (via adenylyl cyclase) and PKA levels. PKA will then phosphorylate its targets, among which are RyR and LTCC to cause increases in cytosolic calcium levels (See figure 1) (Bers, 2002; Harvey & Hell, 2013). For PKA to phosphorylate its target proteins, it needs to be in distinct subcellular location, within the vicinity of its target proteins. The co-localization and interaction of PKA with the RyR channel or LTCC is mediated by A-kinase anchoring proteins (AKAP). These proteins create complexes and act as scaffolding proteins (Fink, Zakhary, Mackey, Desnoyer, Apperson-Hansen, Damron, & Bond, 2001). AKAP79 targets PKA to the LTCC and enhances its response to β-adrenergic stimulation (increasing calcium release); thus enhancing the calcium induced calcium release crucial for EC coupling (Gao, Yatani, Dell'Acqua, Sako, Green, Dascal, 1997). On the other hand, mAKAP localizes at the junctional SR and complexes with RyR2 receptors and targets PKA at the channel (Ruehr, Russell, Ferguson, Bhat, Mal, Damron, Scott, & Bond, 2003). AKAP15/18δ mediate PKA phosphorylation of PLN at Ser16 and ultimately calcium uptake back into the SR. Cellular localization of kinases is crucial for them to be able to exert their effects. As mentioned earlier, PKA phosphorylation of RyR and LTCC will cause an increase in cytosolic calcium. The increase in cytosolic calcium is also followed by the activation of other kinases, notably CaMKII that will phosphorylate PLN at Thr-17 (Ji, 2005).

Phosphorylation, studies by Luo et al, where Ser16 was substituted by Ala, showed a decrease in Thr 17 phosphorylation. The substitution of Thr17 with Ala, however, did not inhibit

the phosphorylation of Ser16 (Luo et al., 1998) . The prerequisite of Ser16 phosphorylation prior to Thr17 phosphorylation has not been observed by all researchers as some have described these two events to be completely independent (Chu, 2000; Luo et al., 1998; Mattiazzi, Mundiña-Weilenmann, Guoxiang, Vittone, & Kranias, 2005). PKA is able to regulate the phosphorylation status of CaMKII via inhibition of protein phosphatase 1 (PP1). The role of PKA/PP1 regulation of CaMKII may explain the findings of Luo et al. Protein phosphatase inhibitors (PPI) are able to locally increase cellular calcium levels (by preventing dephosphorylation of RyR and LTCC) and activate Thr17 PLN phosphorylation independent of **β**1 adrenergic stimulation (Mundine-Weilmann, 1996).

In brief, the proper temporal and spacial localization of PKA near its target proteins is required for proper function, and is mediated via AKAPs. Like PKA, CaMKII has multiple cellular targets and is involved in various pathways regulating EC-C, therefore temporal and spacial localization of CaMKII is also crucial. A novel CaMKII splice variant of CaMKII has been elucidated in both skeletal muscle by Bayer et al, as well as in cardiac tissue by our lab. This novel protein, αKAP, is able to bind CaMKII via its association domain and is localized at the SR membrane via its N-terminal hydrophobic domain. It is thought that αKAP acts as a targeting molecule for CaMKII; however the exact mechanism still required further investigation (Bayer, Harbers, & Schulman, 1998; Nori et al., 2003; Oleary, Sui, Lin, Volpe, & Bayer, 2006; Singh, Salih, & Tuana, 2009).

1.5 Molecular Basis of Heart Failure: Role of Calcium Handling Proteins.

In patients suffering from heart failure, EC-C abnormalities and disrupted calcium homeostasis are observed due to the altered expression and function of calcium handling proteins. In order to meet cardiovascular demands, the failing heart will exhibit an increased β-adrenergic receptor (β-AR) activation. This chronic activation will nonetheless lead to the desensitization and downregulation of these receptors (Choi, Koch, Hunter, & Rockman, 1997). Stimulation of β-AR increases activation of cAMP that will in turn activate the cAMP dependent protein kinase, PKA. PKA will then phosphorylate its target proteins amongst which is G-protein receptor kinase 2 (GRK2) also known as βARK1. GRK2 will ultimately phosphorylate the β-AR, allowing β-arrestin to bind the β-AR and inactivate it. Desensitization of the β-AR has been noted in heart failure, and studies by Choi et al demonstrate that the elevated GRK2 levels contribute to the desensitization of β-AR (Lohse, 2003; Rengo, Lymperopoulos, Leosco, & Koch, 2011). The chronic stimulation of β-AR in heart failure leads to their abnormal desensitization and ultimately a disruption in their signaling. (Choi, Koch, Hunter, & Rockman, 1997). Furthermore, the disrupted β-AR stimulation will subsequently affect two key downstream effectors, PKA and CaMKII. Recent studies by Rengo et al describe the potential for GRK2 to be used as a novel target in the treatment of heart failure (Rengo, Lymperopoulous, Leosco, & Koch, 2011).

CaMKII plays an important role in the pathological and physiological development of HF altering both EC-C (short term) and gene transcription (Maier, 2002). In view of the increased CaMKII levels and the importance of CaMKII in the pathogenicity of the disease, it could potentially be a viable therapeutic target in the course of HF treatment (Anderson, 2009). Mice overexpressing AC3-I, a CaMKII inhibitor, showed normal cardiac function at basal levels and

when treated with the β-AR agonist, isoproterenol (ISO), demonstrated improved cardiac function, reduced hypertrophy and stress induced apoptosis, compared to Wt mice (Y. Yang et al., 2006, R. Zhang et al., 2005).

As described earlier, the failing myocardium exhibits elevated cytosolic Ca^{2+} levels. The origins of this increased cytosolic Ca^{2+} are numerous and can include an increased influx of extracellular calcium via the LTCC, or via either an increase of Ca^{2+} release from the RyR2 channels, or a decrease in SERCA2a mediated Ca^{2+} re-uptake during muscle relaxation.

In human heart failure, LTCC protein expression remained constant compared to the slight increase seen in the murine models; in both examples however, LTCC activity is significantly elevated via increase in LTCC phosphorylation levels (Schroder, Handrock, Beuckelmann, Hirt, Hullin, Preibe, Schwinge, Weil, & Herzig, 1998). CaMKII has been shown to directly bind and phosphorylate Thr-498 of the β2a-subunit, ultimately leading to an increase in extracellular calcium entering the cell, also known as inward calcium current (Ica) (Grueter, Abiria, Dzhura, Wu, Ham, Mohler, Anderson, & Colbran, 2006). Studies by Bünemann also demonstrate that PKA can phosphorylate Ser478 and Ser479 of the β_{2a} subunit as well as Ser1928 of the α_{1c} subunit, where AKAP79 and AKA15/18 are required for the targeting of PKA to the LTCC (Bünemann, Gerhardstein, Gao, & Hosey, 1999). Blocking the LTCC in murine models of heart failure inhibited cardiac remodeling following pressure overload, and prevented the development of cardiomyopathy; however clinical trials of LTCC blocking agents have shown no improvement in the patients (Mahé, Chassany, Grenard, Caulin, & Bergmann, 2003).

Another source for increased cytosolic calcium concentrations is calcium leak via the RyR channel. RyR is part of a large signaling complex, where calmodulin, CaMKII, PKA, PP1

and FKBP12.6 form a complex. It is thought that CaMKII phosphorylation dissociates FKBP12.6 from the RyR receptor complex, thus increasing the channel's sensitivity to Ca^{2+} and resulting in Ca^{2+} leak. The association of CaMKII and RyR within the complex, puts CaMKII near proximity to the RyR channel phosphorylating at Ser2814, chaperone protein mAKAP targets PKA near the complex to phosphorylate RyR at Ser2808 (Li, Wang, Wang, Cutler, Wang, Voigt, Rosenbaum, Dobrev, Wehrens, 2012; Marx, Reiken, Hisamtsu, Jayaraman, Burkhoff, Rosemblit, Marks, 2000). In cases of human and animal heart failure models, the phosphorylation of RyR is elevated, whereas RyR channel expression is unchanged. Protein phosphatase-1 (PP1) levels are reduced, resulting in increased levels of phosphorylated RyR. This increase in phosphorylated RyR will, as explained above, dissociate FKB12.6 from the complex and increase the channel's open probability (Po) and sensitivity to Ca^{2+} (Li, Wang, Wang, Cutler, Wang, Voigt, Rosenbaum, Dobrev, Wehrens, 2012; Marx, Reiken, Hisamtsu, Jayaraman, Burkhoff, Rosemblit, Marks, 2000; Wehrens, 2003). There has been some controversy as to how CaMKII and PKA mediated phosphorylation modulate RyR channel activity. Overexpression studies of CaMKII in adult ventricular cardiomyocyte culture by Kohlhass et al have demonstrated an increased Ca^{2+} leak, indicating increased RyR channel activity, whereas Zhang et al and Yang et al have found the opposite (Kohlhaas, 2006; Yang, Zhu, Xiao, Brochet, Chen, Lakatta, Xiao, Cheng, 2007; R. Zhang, Khoo, Wu, Yang, Greuter, Ni Price, Thiel, Guatimosim, Song, Madu, Shah, Vishnivetskaya, Atkinson, Gurevich , Salama, Lederer, Colbran, Anderson. 2005). Transgenic overexpression of CaMKII in mice by Maier et al also demonstrate increased Ca^{2+} leak (Maier, 2003). These opposing findings have been found in PKA overexpression studies by Hain et al and Valdivia et al (Hain, Onoue, Mayrleitner, Fleischer, & Schindler, 1995; Valdivia, 2005). What is clear though, is the role that the

phosphorylation event, and CaMKII play in the modulation and regulation of the RyR channel activity, either directly or through other proteins found in the complex (Kushnir, Shan, Betzenhauser, Reiken, & Marks, 2010; Li, Wang, Wang, Cutler, Wang, Voigt, Rosenbaum, Dobrev, & Wehrens, 2012).

Reduction of Ca^{2+} efflux, or reuptake, from the cytosol is also a contributor to the increased cytosolic calcium and the defective EC-Coupling observed in heart failure.

The main mechanism by which calcium is re-uptaken back into the SR is via SERCA2a ATPase pump accounting for 70-80%, while the NCX accounts for the remaining 20-30%. SERCA2a expression levels have been shown to be depressed by almost 50% in patients suffering from heart failure while PLN expression remains unchanged. The reduced SERCA2a expression coupled with the elevated PLN to SERCA2a ratio results in increased cytosolic Ca^{2+} concentrations and prolongation of Ca^{2+} transients during a diastole. Patients suffering from ischemia or dilated cardiomyopathy do in fact have a reduction in the amount of PLN expressed, but the ratio of SERCA2a to PLN is still decreased overall, when compared to the non diseased state. PLN a natural inhibitor of SERCA2a, is found bound to the Ca^{2+} ATPase. Phosphorylation of PLN, via CaMKII at Thr17 residue or PKA at Ser16, changes its conformation causing it to dissociate from SERCA2a, thus relieving the inhibition. Murine heart failure models are marked with reduced levels of both Thr17 and Ser16 phosphorylated PLN, whereas in human heart failure only Ser16 phosphorylated PLN is reduced. The reduction of PLN phosphorylation is indicative of increased SERCA2a inhibition, but phosphatases can also regulate the level of PLN phosphorylation. Heart failure patients have been shown to have an increased activity of PP1, protein phosphatase-1, increasing PLN dephosphorylation and allowing it to remain bound to SERCA2a, thus inhibiting Ca^{2+} reuptake. PP1 activity is indirectly regulated via PKA, where the

latter will phosphorylate and activate IPP-1, protein phosphatase inhibitor 1, inhibit PP1 and stop the PP1 mediated dephosphorylation of PLN. Patients with heart failure have decreased IPP-1, leading to an increased PP1 activity and ultimately increased PLN dephosphorylation and SERCA2a inhibition. It is clear that the regulation of PLN and SERCA2a is complex and requires a fine regulation between PLN phosphorylation and dephosphorylation. PKA and CaMKII both play a very important role in the modulation of PLN and SERCA2a either via direct PLN phosphorylation or regulation of phosphatases such as PP1 (Carr, Schmidt, Suzuki, del Monte, Sato, Lanner, Breeden, Jing, Allen, Greengard, Yatani, Hoit, Grupp, Hajjar, DePaoli-Roach, & Kranias 2002; Gupta, Mishra, Rastogi, Imai, Habib & Sabbah, 2003; Kho, Lee, & Hajjar, 2012; Steenaart, Ganim, Di Salvo, & Kranias, 1992) .

PLN-R9C mutation of PLN, where arginine is substituted for cysteine is found in human and causes dilated cardiomyopathy. Studies by Schmidt et al show that phosphorylation levels of PLN in these individuals is lower, and that a transgenic mouse overexpressing the R9C mutant had a decreased amount of PKA mediated phosphorylation of PLN (Schmidt, 2003). Cell culture and transgenic animal studies with the PLN-R9C mutated form demonstrated that it has a higher affinity for PKA, in turn this cause PKA to remain bound to PLN-R9C and would therefore be preventing the phosphorylation of the non mutated form of PLN (heterozygous mutation) (Haghigh, Kolokathis, Pater, Lynch, Asahi, Gramolini, Fan, Tsiapras, Hahn, Adamopoulos, Liggett, Dorn, MacLennan, Kremastinos, & Kranias. 2003). The effect of PKA "trapping" due to the mutation in PLN-R9C, it is localized only at the SR; and it has been proposed by MacLennan et al that this localized nature of the reduced PKA mediated PLN phosphorylation is due to AKAPs that target PKA at the SR (MacLennan, 2003). This localized effect further strengthens the importance of temporal and spatial localization of kinases in the modulation of cardiac

activity.

The sodium calcium exchanger, NCX, is also thought to contribute to the progression of heart failure (Studer, Reinecke, Bilger Eschenhagen, Bohm, Hasenfuss, Just, Holtz, & Drexler, 1994). NCX expression levels are shown to be elevated in heart failure and are thought to be a compensatory mechanism to the defective SERCA2a activity (Kho et al., 2012). The NCX extrudes calcium from the cytosol to the extracellular environment and in the process contributing to the decreased SR calcium load that is observed in heart failure. Other studies have shown the opposite, where the NCX will bring in extracellular calcium to be re-uptaken back into the SR in hopes of increasing SR calcium load. Due to the conflicting data, the exact role of the NCX in the modulation of heart failure remains unclear (Flesch, 1996; Anderson, 2001; Schillinger, 2003; Weber, 2003).

A newly identified protein, SUMO-1, or small ubiquitin related modifier 1, was shown to be involved in the increase of SERCA2a activity. SUMO-1 binds to its target proteins, a process termed SUMOylation, where it is involved in the regulation of subcellular localization, and protein stabilization. Kho et al have demonstrated that SUMO-1 levels are elevated in both human and animal models of heart failure, where it plays a role in the stability of SERCA2a, and is thought to be a contributor to the reduced SERCA2a expression and activity (Kho, Lee, Jeong D, Oh, Chaanine, Kizana, Park, & Hajjar, 2011; Schwartz & Yeh, 2012).

The SR's storage capacity is regulated via the SR protein calsequestrin CASQ2, and its ability to bind calcium; this ability allows the protein to buffer calcium concentrations within the SR and modulate its re-uptake and release. Furthermore, triadin and junctin form a complex with CASQ2 and localize it in proximity to the RyR2 channel where it is able to regulate calcium release via modulation of the RyR channel Po. Studies have shown that downregulation of

junctin in models of heart failure reduced calcium leak; and overexpression of triadin increased RyR2 activity increasing calcium leak and causing arrhythmias. Triadin and junctin levels are depressed in heart failure patients but it is unclear if the downregulation of protein expression is in response to the defective Ca^{2+} handling in order to improve cardiac function or whether the reduction in expression itself is a cause of heart failure. In heart failure, CASQ2 levels remain unchanged, but defective post-translational processing cause the mislocalization of the protein at the rough ER (Altschafl, Arvanitis, Fuentes, Yuan, Kranias, & Valdivia, 2011; Gyorke, Stevens, & Terentyev, 2009; Knollmann, 2009; Wehrens, 2003).

Increased Ca^{2+} levels activate Ca^{2+}/calmodulin dependent protein phosphatase 2B (calcineurin), which then in turn activates NFAT causing it to translocate from the cytosol into the nucleus. Nuclear NFAT then interacts with the GATA4 consequently regulating the expression of hypertrophic genes such as ANF. $CaMKII\delta_b$, the nuclear isoform, is able to alter gene regulation and signaling pathways. $CaMKII\delta_b$ is able to phosphorylate histone deacetylases (HDAC) that are bound to MEF2 (myocyte enhancement factor 2) (Akazawa, 2003; Molkentin, 2000; Molkentin et al., 1998). This phosphorylated state of HDAC allows for the activation of MEF2, as the two are no longer bound (Zhang, Kohlhaas, Backs, Mishra, Phillips, Dybkova, Chang, Ling, Bers, Maier, Olson, Brown, 2007). Interestingly enough, CaMKIIδc, the cytosolic isoform that lacks the NLS domain found in $CaMKII\delta_b$ can similarly phosphorylate MEF2, and studies by Zhang et al have demonstrated that transgenic mice overexpressing CaMKIIδc show an increased activation of MEF2. They propose that $CaMKII\delta_c$ is forming dimers with CaMKIIβ and the complex is then able to be targeted to the nucleus (Zhang, Kohlhaas, Backs, Mishra, Phillips, Dybkova, Chang, Ling, Bers, Maier, Olson, Brown, 2007).

1.6 Role for CAMKII in Cardiac Function

Free Ca^{2+} acts as a secondary messenger, whose function is mediated by the calcium receptor calmodulin (CaM). When Ca^{2+} binds CaM, it forms a complex, which is then able to activate kinases, mainly the calcium/calmodulin dependent protein II kinase (CAMKII). CAMKII has four different isoforms (α,β,γ,δ), where cardiac tissue predominantly expresses δ and β and gamma isoforms (δ being the most abundant). CAMKII δ_c is located in the cytosol and CAMKII δ_b in the nucleus (Zhang, Kohlhaas, Backs, Mishra, Phillips, Dybkova, Chang, Ling, Bers, Maier, Olson, Brown, 2007). Each CaMKII subunit contains a kinase catalytic domain on the N-terminus, a regulatory domain and a C-terminus association domain (figure 2).

Following calcium release from the SR, the increased intracellular calcium will bind calmodulin (CaM), the Ca^{2+}/CaM complex will then interact with the regulatory domain of CAMKII, changing the conformation of the protein, by disrupting the interaction of the autoinhibitory region and that of the catalytic region; thus, activating the enzyme. CaMKII isoforms have a C-terminal domain that allows six to twelve CAMKII molecules to associate and form a holoenzyme-like structure. When the Ca^{2+}/CaM complex binds to the regulatory domain of a CaMKII molecule and activates the enzyme, an inter-subunit phosphorylation event, also known as autophosphorylation, will phosphorylate neighboring CAMKII molecules at Thr287 (CaMKII β, δ, γ) or Thr 286 (CaMKII α), resulting in enzyme activation in a Ca^{2+}/CaM independent manner (Anderson, 2009; Hudmon & Schulman, 2002; Wu, 2002) (Figure 3).

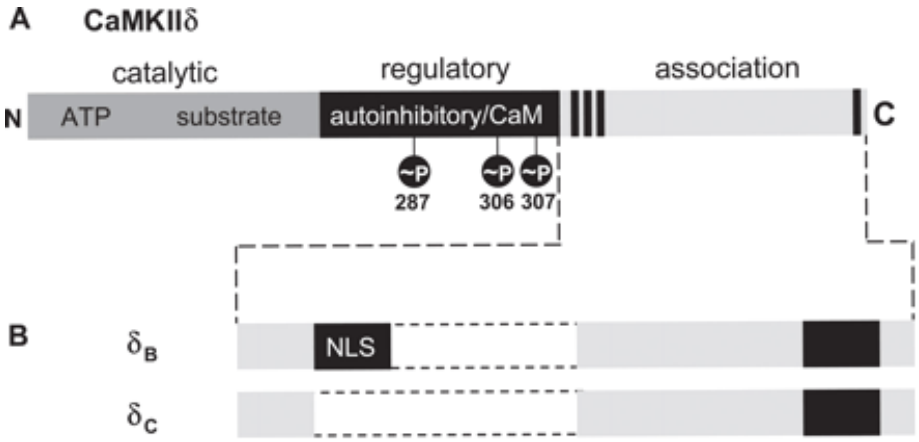


Figure 2: *Representation of CaMKIIδ-isoforms.* All CaMKII share a homologous catalytic domain at the N-terminus, and a regulatory domain but are differentiated via the C-terminus association domain. CaMKIIδ_B, a nuclear isoform differs from the cytosolic isoform CaMKIIδ_C by the presence of a nuclear localization domain, NLS.

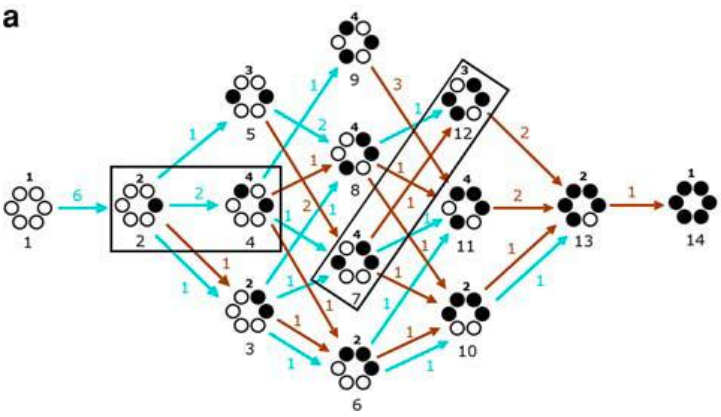


Figure 3: *A representation of CaMKII activation.* Brown arrows indicate autophosphorylation while green ones show Ca^{2+}/CaM mediated activation. Autophosphorylation of CaMKII only occurs on direct neighbouring subunits (Adapted from Lucic, 2008).

CaMKII plays a key role in both Ca^{2+} release and re-uptake; in a study by ME Anderson, it has been found to co-localize with the RyR as well as the LTCC (Anderson, 1994). Furthermore, CaMKII is also able to phosphorylate the LTCC and RyR causing a calcium influx into the cytosol (Bayer, Harbers, & Schulman, 1998; Kohlhaas, 2006). In brief, calcium levels in the cell regulate the activity of CaMKIIβ/δ, which will, in turn, regulate the phosphorylation level of PLN (and SERCA2a) and ultimately Ca^{2+} re-uptake into the SR.

The mechanism by which CaMKII localizes within the cells is not well elucidated. However recent studies by our lab have shown the presence of a non kinase anchoring protein known as αKAP in the heart (to be discussed later), specific to CaMKII, which may target CaMKII to subcellular compartments within cardiomyocytes for physiological control of the calcium signal.

PKA targeting and localization, as mentioned earlier, is crucial for the kinase to exert its effect on the target proteins. CaMKII targeting is not yet very well understood but we propose that αKAP may function as a targeting molecule for CaMKII.

1.6 CaMKII Kinase Anchoring Protein (αKAP).

Phosphorylation plays a critical part in the regulation of calcium dynamics and as described in the paragraphs above, heart failure is a manifestation of the disrupted calcium dynamics in which defective phosphorylation plays a key role. Also as mentioned above, AKAP mediated localization of PKA near its target proteins is understood to be of great importance, however, CaMKIIδ the other crucial calcium regulator has no known targeting molecule. CaMKII has been shown to directly bind the LTCC and NMDA receptors, but a targeting molecule is thought to be responsible for bringing CaMKII in close proximity to its target.

Recently, αKAP has been proposed to be a non-kinase, anchoring protein, specific to CaMKIIβ/δ. αKAP, a splice variant of CAMKIIα gene (a brain specific CaMKII isoform), encoding a 200 amino acid 22Kda protein was first identified by Bayer et al in skeletal muscle. αKAP's c-terminus is identical to that of CAMKII association domain and has an alternatively spliced hydrophobic N-terminal domain that targets the SR membrane. (Bayer, Harbers, & Schulman 1998).

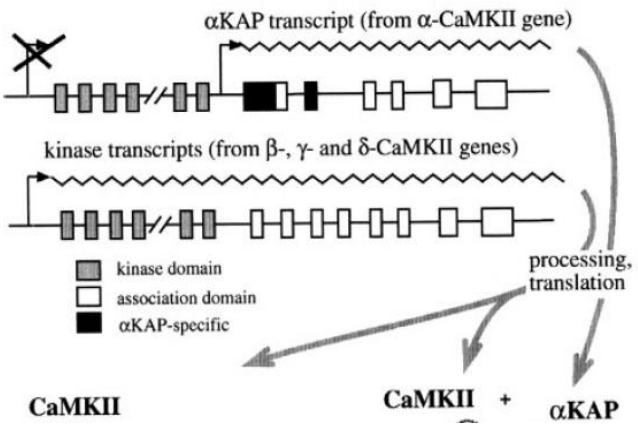


Figure 4: *Schematic of αKAP sequence.* αKAP was first characterized in skeletal muscle tissue and shown to physically interact with CAMKIIβ. It targets CAMKII to the SR and brings the kinase near the SERCA2a pump (Ca^{2+} re-entry site) to increase its concentration near its targets such as PLN and RyR. (Adapted from Bayer et al, 1998).

Recent studies from our lab show that αKAP is also found in the heart. Much like the skeletal form of αKAP, the hydrophobic N-terminal domain anchors to the SR membrane while the C-terminal domain binds to CaMKIIβ/δ. Cardiac αKAP is also able to bind SERCA2a (Singh, Salih, & Tuana 2009). The targeting of CaMKIIβ via αKAP to the SR also allows CaMKIIβ to interact with GAPDH and regulate the glycolytic pathway and control local ATP production (Singh, 2005). SERCA2a, an ATPase pump, requires ATP to actively re-uptake Ca^{2+} back into the SR and thus regulation of ATP levels also regulates Ca^{2+} re-uptake. Our lab has also demonstrated that αKAP enables localization of CAMKII to close proximity of SERCA2a, enabling CAMKIIβ/δ to phosphorylate PLN. Moreover, data from our lab shows that αKAP directly associates with SERCA2a. Figure 5 outlines the proposed model for αKAP's regulation of calcium dynamics within cardiomyocytes. The hydrophobic domain of αKAP, targeting the CaMKII to the SR in close proximity to SERCA2a/PLN could be a crucial in the phosphorylation of PLN and thus regulation of SERCA2a activity. Moreover, αKAP's direct interaction with SERCA2a may prove to be a novel method by which SERCA2a activity is regulated.

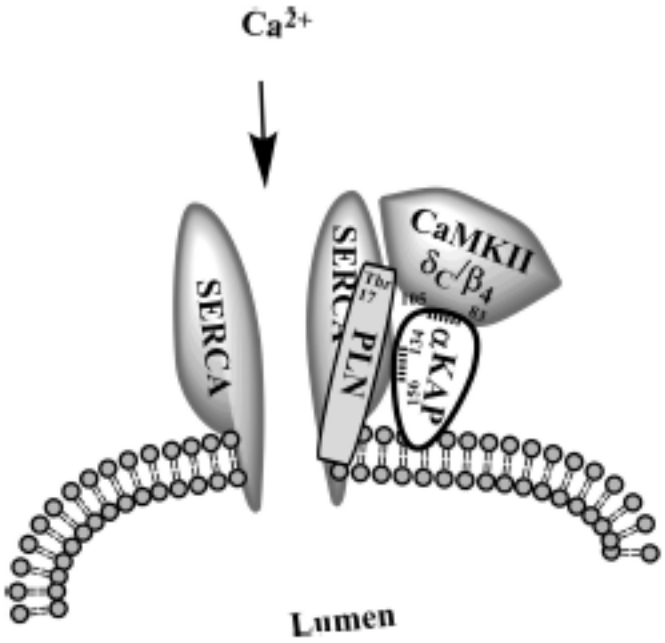


Figure 5: *Proposed model for SERCA2a, PLN, CaMKII and αKAP assembly at the cardiac SR.* αKAP, via its N-terminal domain, is directly targeted to the SR membrane directly interacting with SERCA2a and CaMKII (Singh et al, 2009).

In view of the importance of PLN phosphorylation in the regulation of SERCA2a in cardiomyocytes, we plan to investigate the role of αKAP in the modulation of PLN phosphorylation, as well as its role in Ca^{2+} uptake in the myocardium. In view of the proposed additional role for αKAP in proteosome targeting, we will investigate to the role of αKAP overexpression on SERCA2a activity and expression levels.

1.7 Statement of the Problem

SERCA2a downregulation and increased CaMKII activity have both been implicated in heart failure and the progression of the disease. SERCA2a and CaMKII both play an important role in the regulation of calcium dynamics within the myocardium and ultimately the proper functioning of the heart. The downregulated SERCA2a levels coupled with the increased CaMKII activity, both contribute in the disruption of calcium dynamics and are prime targets in the attempt to rectify and treat heart failure. CaMKII is known to regulate various calcium handling proteins, and as mentioned in the above, is crucial in the regulation of SERCA2a function via phosphorylation of PLN. How CaMKII is localized to its various targets within the myocardium is yet to be elucidated. PKA, like CaMKII is also a regulator of contractile proteins and cardiac function, it is targeted throughout the cells via anchoring proteins known as AKAP. Our lab has elucidated the existence of a novel CaMKII binding protein in myocardium known as αKAP. *Our hypothesis is that αKAP may serve as regulatory protein for SERCA2a and act as a targeting protein for CaMKII.* My objectives for this study are to examine the role of αKAP in, SERCA2a regulation by examining SERCA2a activity and Ca^{2+} uptake, CaMKII regulation and activity, and finally the expression of αKAP in an animal model of myocardial infarct. These objectives will be accomplished by using various cell culture models, subcellular fractionation,

enzymatic assays and radiolabelled ion transport assays to investigate the role of αKAP overexpression in the modulation of SERCA2a activity. Various other biochemical and molecular biological tools will be used to assay protein expression and activity.

CHAPTER 2: MATERIALS AND METHODS

2.1 Tissue Fractionation

a) Microsome fractionation from Heart Tissue: Hearts were collected from wild type CD-1 mice euthanized by CO2, rinse in ice cold 1x PBS, and homogenized, on ice, with a handheld Fisher Maximizer homogenizer in a buffer containing in mM: 20 Tris-HCl pH 7.8, 30 histidine and 600 KCl and protease inhibitor cocktail (Roche). The homogenate was centrifuged at 1600g for 10 minutes and the pellets was re-homogenized again in the above buffer and centrifuged again at 1600g for 10 minutes. The supernatants from both centrifugations were pooled together and again centrifuged at 14,000g for 15 minutes, pelleting the mitochondria and contractile proteins which were then subsequently discarded. The supernatant from the above spin was centrifuged at 45,000g for 50 minutes. The pellet was re-suspended in a buffer containing in mM 20 Tris-HCl pH 7.4, 30 histidine, 250 sucrose and centrifuged at 48,000g. the final pellet, containing cardiac SR fractions was resuspended in a buffer containing in mM: 20 Tris-HCl pH 7.2, 30 histidine, 250 sucrose, and aliquoted and stored at -80^{o}C.

b) Microsome fractionation from Cell Culture : Cells were scraped from the plates in ice cold PBS with protease inhibitor cocktail (Roche), and centrifuged at 500g for 5 min in a refrigerated centrifuge at 4^{o}C. The cells were then resuspended in the homogenization buffer described above and lysed with 20 passages through a 21 gauge syringe and the same centrifugation steps as described above were performed to fractionate the microsomes.

c) Protein Measurements: The protein concentration of the cardiac SR fractions was determined using the BCA protein assay kit (Thermo Scientific) as per the manufacturer's protocol

2.2 Immunoblotting

Whole heart tissue or cell culture tissue were homogenized with a Fisher handheld Maximzer homogenizer or 20 passages through a 21 gauge syringe respectively in a modified RIPA buffer (0.25% deoxycholate, 1 mM EDTA, 50 mM Tris-base, 1% NP-40, 100 mM NaCl) with the addition of a protease inhibitor cocktail (Roche). Protein concentrations were determined using BCA protein assay kit (Thermo Scientific) as per the manufacturer's recommendations. Isolated protein or fractions (see tissue fractionation) were run on a denaturing 5-15% gradient SDS-PAGE gel.

For Immunoblotting, gels were transferred overnight in a buffer containing, in mM, 25 Tris, 190 Glycine, 20% methanol to a PVDF membrane (Bio-Rad). All membranes were blocked at room temperature for 1hr in TBST (1 M Tris, 290 mM NaCl, 0.1% TWEEN20, pH7.2) with 5% milk. Primary antibodies (listed in Table 1) were incubated at room temperature for 4hrs or overnight at 4^{o}C in TBST with 5% milk. Membranes were washed 3 times for 10 min in TBST before adding the appropriate horseradish peroxidase labeled secondary antibody (Jackson) in TBST with 5% milk. Membranes were washed 3 times for 10 min in TBST and the conversion of ECL substrate (Roche) was detected on film. Membranes were stripped in a stripping buffer containing 25 mM Glycine, 1% SDS pH 2.2 for 10 minutes then washed in TBST 5 times for 10 minutes and reprocessed in the method described above. Bands were quantified by densitometry using ImageJ (National Institute of Health).

Table 1: *List of primary antibodies.* Primary antibodies used for western blots.

Target Protein	**Catalogue Number**	**Company/Source**
Calnexin	SPA-860	Stressgen
SERCA2a	MA3-919	Thermo Scientific
SERCA2a	A010-20	Badrilla
SERCA2a	SC-8095	Santa Cruz
CaMKIIδ	MAB4176	R&D Systems
CaMKII Thr286/287	A010-50AP	Badrilla
CaMKII Thr286/287	3361	Cell signalling
CaMKII Thr286-287	06-881	Uptstate
αKAP	610009	BD Transduction Labs
αKAP	SC-5390	Santa Cruz
αKAP	SC-13141	Santa Cruz
αKAP	3357	Cell Signaling
GFP	11814460001	Roche
GFP	A6455	Invitrogen
Myc Tag	A7470	Sigma
GAPDH	AM4300	Invitrogen
HA tag	H9658	Sigma

2.3 Ca^{45} Uptake:

Ca^{45} uptake in SR fractions was conducted as described by Swanson et al. in a buffer containing in mM: 20 MOPS pH7.0, 100 KCl, 5 MgCl, 5 Potassium Oxalate and varying amounts of EGTA and Ca^{2+}. A Ca^{2+}/EGTA buffer system was used to ensure constant calcium concentration throughout the experiment, and the program "Bound and Dissociated" was used to perform the calculations. To the above buffer, 0.5 mM EGTA and 0.4mM CaCl2 was added to yield a free concentration of 1uM calcium at 37^{o}C, pH 7.0. In 500 µl of pre-heated buffer (37^{o}C) containing 1µCi of Ca45, 20µg SR proteins were added and left to equilibrate for 5 minutes before the addition of 5mM ATP to initiate the reaction. To measure the effect of αKAP on Ca^{2+} uptake, the SR samples were incubated for 10 minutes on on ice with 1, 10, or 100 ng of GST-αKAP, or 100 ng of GST as a control, prior to the addition of the ATP. The reaction was then stopped via filtration on nitrocellulose filter discs and the immediately rinsed with ice cold water before being dried for 5 minutes prior to scintillation counting in 5ml of scintillation fluid.

2.4 NADH coupled ATPase Assay:

Protocol was adapted from Muinich G et al, and adaptation of the assay to a plate reader system was guided by work from Kiianista K et al (Kiianitsa, Solinger, & Heyer, 2003). The assay was performed on a SpectraMax M2 96 well plate reader. In brief, the ATPase assay was conducted at 37^{o}C in a buffer containing 21mM MOPS, 5mM NaN3, 100mM KCl, 0.06mM EGTA, 0.2mM NADH, 1mM PEP and 20U LDH/12U PK and 1mM ATP. The buffer was prepared freshly before conducting the assay and kept on ice. The reaction was started by the addition of 20ug/ml

of SR protein samples and 150 µl of the reaction mixture was added to each well in the 96 well plate. The rate of NADH oxidation, correlating directly to the ATPase velocity, was examined by measuring the decrease in OD of the samples at 340 nm. OD measurements were taken every minute for 45 minutes. The assay functions on the principle of one molecule of phospho-enol-pyruvate is converted to pyruvate using pyruvate kinase and hydrolyzing one ATP molecule to ADP via the SERCA2a ATPase. The pyruvate molecule is then converted to lactate via the lactate dehydrogenase enzyme in the process oxidizing one NADH molecule to NAD+. The assay measures decrease in absorbance of NADH (at 340 nm) as it is oxidized to NAD+. As with any microsomal fraction, contamination with mitochondria and other ATPases needs to be addressed. Thapsigargin, a specific SERCA2a inhibitor, was used at a concentration of 100nM, in order to quantify the SERCA2a independent activity and subtract it from the total measured activity in order to calculate SERCA2a specific activity.

2.5 Cell Culture

a) Cell maintenance: HEK293T cells were purchased from the American Type Culture Collection and grown at 37^{o}C in 5% CO2 and maintained in DMEM (Fisher) containing 10% FBS.

b) Cell Transfection: All transfections were performed using PEI at a concentration of 5ug of PEI per 4ug DNA. All pre-incubations of PEI with DNA were performed for 20 minutes at room temperature in serum free Opti-MEM (Gibco BRL). The cells were transfected with expression constructs of mouse αKAP or PLN cDNA ligated in frame with 6-myc tag (12KDa) and mouse SERCA2a cDNA ligated in frame with green fluorescent protein (GFP) tag (26KDa), in the pcDNA3 vector and driven by the CMV promoter.

2.6 Neonatal Mouse Cardiomyocyte Culture (NMCM):

a) NMCM Isolation: NMCM were cultured by collecting hearts from 1 day old mouse pups. The hearts were rinses in Hank's Buffer (HBSS) containing no calcium or Magnesium and incubated overnight in HBSS with 0.05% trypsin. The trypsinized hearts were then digested at 37^{o}C in 0.05% collagenase II dissolved in HBSS. The collagenase digestion steps were limited to 10 mins per treatment before the supernatant was collected and centrifuged at 500g for 5 minutes and pellet resuspended in DMEM containing 10% FBS, 20 mM HEPES, and NEAA and pen/strep. The above steps were repeated until all the hearts were completely digested (about 3 rounds). The resuspended cells were kept at 37^{o}C and 5% CO2 in falcon tubes until they were plated. Differential plating was used to separate fibroblast from cardiomyocytes; the cells were plate on uncoated dishes for 45 mins to allow the fibroblast to adhere (cardiomyocytes required gelatin coating). This step was performed again before a final plating of the cardiomyocytes on plates coated with 1% gelatin. The cells were transduced via a lentivirus viral transduction vector following 24 hours of plating with 8 μg/ml of polybrene to facilitate transduction.

b) NMCM Maintenance: NMCM were maintained in a DMEM growth medium containing 10% FBS, pen/strep, and 1% NEAA.

2.7 αKAP Lentivirus Production

The protocol and procedure to produce the lentivirus was taken from Campeau et al. Lenti-X 293T cells were purchased from Clonetech and grown in 37^{o}C in 5% CO2 and maintained in DMEM containing 10% FBS. The cells were then co-transfected with the pMD2G (envelope plasmid), psPAX2 (packaging plasmid) and either GFP, αKAP, or αKAPΔTM ligated into the pLenti overexpression vector. Briefly, the mouse αKAP cDNA was ligated into the Xba1 and

Bamh1 of the pLenti vector at the 5'end, in frame with the EGFP tag (enhanced green fluorescence protein) sequence found on the pLenti vector (Addgene plasmid 17448), driven by the CMV promoter. The media was changed 7 hours post transfection and the supernatant was collected 48 hours post transfection and centrifuged at 1500g for 10 minutes to remove cell debris. The cleared supernatant was centrifuged at 110,000g for 2 hours and the viral pellet was resuspended in PBS containing 1% BSA, aliquoted and frozen at -80^{o}C.

Table 2: *Primer Sequences Used.* Primer used for sequence cloning into lentiviral overexpression vector. (F) Forward primer, (R) Reverse primer

Primer	Targeted Gene	5'-3' Sequence
KAPV (F)	αKAP	GCTCTAGATGCTGCTCTTTCTCACGCTG
KAPV (R)	αKAP	CGGGATCCAGGACGGAGGGCGCCCCAG
KAPV23 (F)	αKAPΔTM	GCTCTAGATGCTGCTCTTTCTCACGCTG
KAPV23 (R)	αKAPΔTM	CGTCTAGATGGGAGGGAAGAGCGGAGGA

2.8 GST Fusion Protein Expression

E.coli BL21 cells expressing either GST alone or GST-αKAP was inoculated overnight in 5 ml of modified LB broth (20 g/L Peptone, 10 g/L yeast exgtract, 7 g/L NaCl) for 24hrs at 37^{o}C and then subsequently inoculated in 1 L of LB at 37^{o}C until *A*595 reached 0.5. The temperature was then reduced to 28^{o}C and the cells were induced with 0.1 mM of Isopropyl-β-D-thio-galactoside (IPTG) and maintained at the same temperature for 4 h. The cells were pelleted via centrifugation at 5000g for 15 mins at 4^{o}C and lysed via sonication in PBS containing 1% NP40. Cellular debris was again pelleted via centrifugation at 15,000g for 15mins at 4^{o}C. The supernatant was collected and the GST fusion proteins were isolated on glutathione-Sepharaose beads (GE Healthcare) by incubating the lysate for 60 mins at 4^{o}C and rotated end over end. The samples were finally centrifuged at 750g to collect the GST pellets and those were subsequently washed 4 times in ice cold PBS buffer containing protease inhibitors (Roche).

2.9 Statistical Analysis

Student's T-tests were performed using GraphPad Prism 5.0 (Graph Pad). P value < 0.05 was considered to be significant.

CHAPTER 3: RESULTS

3.1 Recombinant αKAP does not affect Ca^{2+} uptake in cardiac SR

SERCA2a, the SR membrane Ca^{2+} ATPase is a pump responsible for sequestration of cytosolic calcium (Ca^{2+} re-uptake) in cardiomyocytes and heart relaxation. The SERCA2a activity is sensitive to calcium concentration and is regulated by PLN. CaMKII and PKA mediated phosphorylation of PLN can further modulate its interaction with SERCA2a to modify the ATPase activity and calcium uptake into SR. Various studies show that addition of 10 ng of recombinant PLN can inhibit SERCA2a activity and calcium uptake in SR fractions isolated from myocardium (Sasaki, Inui, Kimura, Kuzuya, & Tada, 1992). Since αKAP can bind SERCA2a (Singh, Salih, & Tuana, 2009) we compared the effects of recombinant αKAP on SERCA2a activity/calcium uptake in the same manner as described for recombinant PLN (Sasaki, Inui, Kimura, Kuzuya, Tada, 1992). Cardiac SR enriched fractions were isolated from mouse hearts and the effect of αKAP on the rate of SERCA2a mediated Ca^{2+} uptake was examined. Cytosolic calcium concentrations in myocardium ranges from 0.1 μM (diastole) to 10 μM (systole). We first established a protocol to measure Ca^{45} uptake in a Ca^{2+}/EGTA buffered system in SR fractions isolated from mouse heart. A Ca^{2+}/EGTA buffered system maintains a constant, precise micromolar Ca^{2+} concentration ($[Ca^{2+}]_{free}$) throughout the time course of the assay.

The computer program "Bound and Determined" developed by Brook & Storey, takes into consideration pH, temperature, ion concentration and chelator concentration to calculate the concentration of free calcium that is unbound from the calcium chelator, EGTA (Brooks & Storey, 1992). In brief, whole heart lysate from mouse was fractionated to extract microsomes as

described in the materials and methods. We characterized the rate of SERCA2a uptake with $[Ca^{2+}]_{free}$ set within physiological limits, at 0.2 μM and 1 μM. Maintaining calcium levels within physiological limits is critical to prevent calcium from entering the SR via ionic gradient as opposed to active transport via SERCA2a. Since the accuracy of the Bound and Determined program is subject to pipetting and dilution errors, we limited the maximum free calcium concentration to 1μM and the minimum concentration to 0.2 μM. As predicted and in agreement with the studies by Odermatt, the rate of calcium uptake increases in the presence of higher calcium concentrations (Odermatt, Kurzydlowski, MacLennan, 1996). Ca^{2+} uptake was higher in the buffer system containing 1 μM $[Ca^{2+}]_{free}$ with a maximal rate of uptake of approximately 7000 nmol/mg/min (nmol of calcium per mg of SR protein per minute) compared to the experiment containing 0.2 μM $[Ca^{2+}]_{free}$ with a maximal rate of uptake of approximately 4000 nmol/mg/min (figure 6). In agreement with previously published works, careful examination of the uptake curve indicates that the linear range of Ca^{2+} uptake lies between 0-1000 s after which it eventually reduces to a plateau (figure 6).

To further demonstrate that the Ca^{2+} transport measured in the microsomal fractions was facilitated by SERCA2a, samples were treated with thapsigargin, a known inhibitor. Ca^{2+} transport was then measured in microsomal fractions treated with various amounts of the inhibitor thapsigargin. To measure the effect of thapsigargin on SERCA2a mediated Ca^{2+} transport, it is important to conduct the experiment within the linear range of uptake, as established above (0-1000s); our experiment was conducted at 10 min. Calcium transport without thapsigargin was measured at 400 nmol/mg/min and was gradually reduced with increasing concentrations of thapsigargin. The rate of calcium uptake at concentrations higher than 10 nM is not further reduced. This data is indicative of total SERCA2a inhibition with 10

nmol of thapsigargin; these findings are consistent with published data in the literature (Figure 7) (Lytton, Westlin, & Hanley, 1991).

Having established a robust protocol for measuring calcium uptake in mouse heart microsomal fractions, we wanted to examine the effect of αKAP on SERCA2a mediated calcium transport since αKAP is able to directly bind SERCA2a as demonstrated (Singh, Salih, & Tuana 2009). To specifically measure SERCA2a mediated Ca^{2+} transport, 100 nM of thapsigargin, was used as a control. By subtracting the measured calcium uptake in the presence of thapsigargin from the total measure calcium uptake, we are able to deduce SERCA2a mediated calcium transport activity exclusively. 1, 10 and 100 ng of recombinant αKAP tagged with GST were incubated with SR samples; Ca^{2+} uptake was then initiated with the addition of 5 mM of fresh ATP. The rate of calcium uptake in SR fractions incubated with 1, 10, and 10 ng of recombinant αKAP was 258 (+/- 33) nmol/mg/min, 292 (+/- 49) nmol/mg/min, 290 (+/- 43) nmol/mg/min respectively; compared to the control assay with recombinant GST alone which exhibited a calcium uptake rate of 200 (+/-33) nmol/mg/min. Recombinant αKAP did not have an effect on SERCA2a mediated Ca^{2+} uptake compared to the control experiment, $n>0.05$ (figure 8).

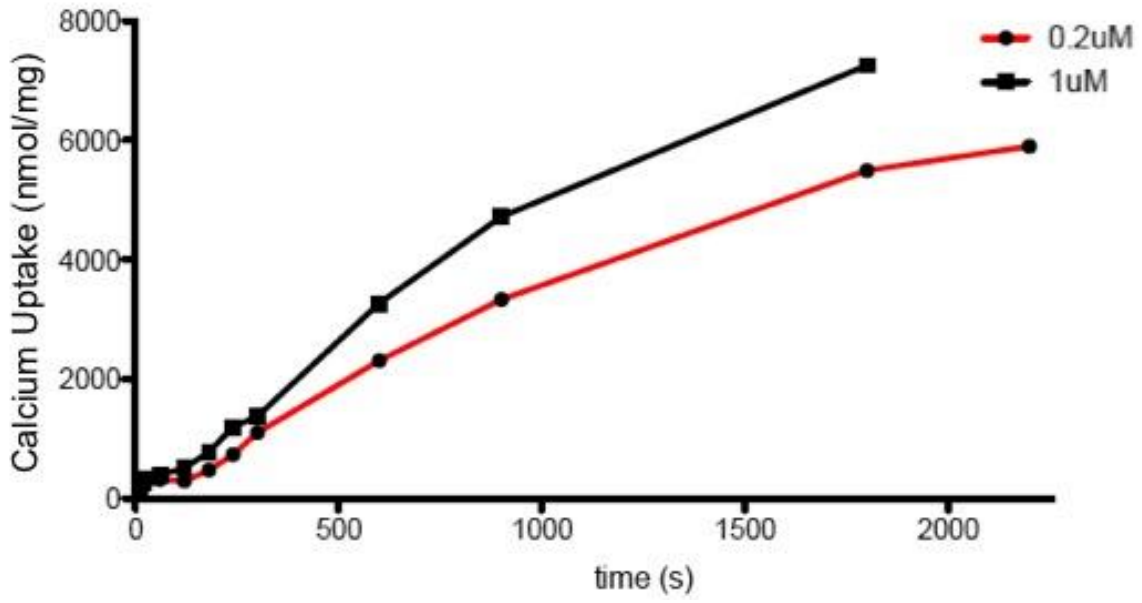


Figure 6: *Ca^{2+} uptake in cardiac SR.* Rate of cardiac SR Ca^{2+} uptake in a Ca^{2+}/EGTA buffered system with either 0.2 μM or 1.0 μM of free calcium, $[Ca^{2+}]_{free}$. Ca^{2+} uptake is expressed as nmol of calcium uptaken per mg of SR protein (nmol/mg). The reaction was terminated at the various time points using the filter disc method. Each time point represents the measured rate of calcium uptake in SR samples fractionated from 3 adult murine hearts, measured in triplicate, then averaged.

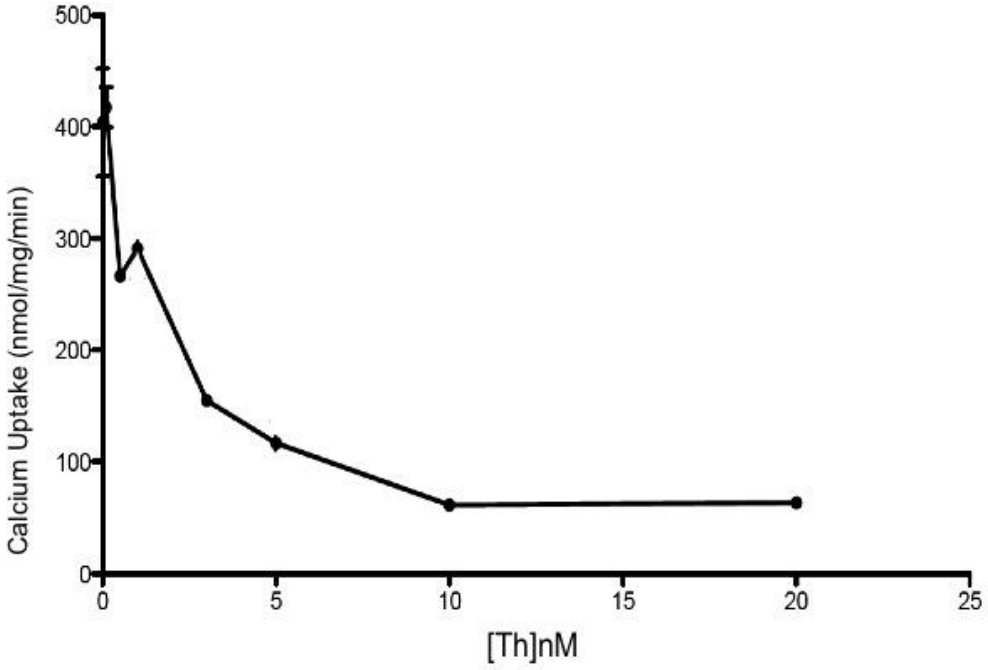


Figure 7: *Thapsigargin inhibits SERCA2a mediated* Ca^{2+} *Uptake.* Inhibitory effect of the known SERCA2a inhibitor, thapsigargin on Ca^{2+} uptake in cardiac SR fractions. Cardiac SR samples were pre-incubated on ice with various concentrations of thapsigargin and the Ca/EGTA $[Ca^{2+}]_{free}$ was set at 1uM. 5 mM ATP was added to start the reaction and the reaction was terminated after 10 minutes via the filter disc method and uptaken Ca^{45} was measured. The measured rate of calcium uptake was conducted in SR samples fractionated from 3 adult murine hearts, measured in triplicate, then averaged.

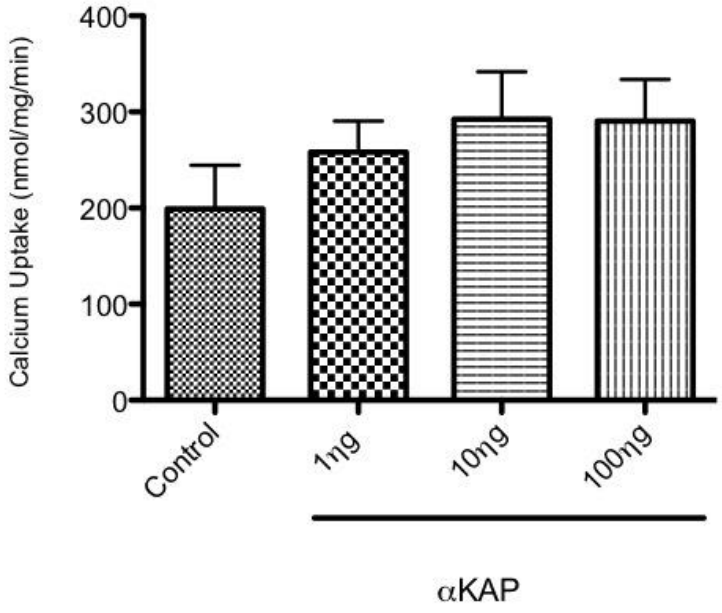


Figure 8: *Recombinant αKAP does not affect Ca^{2+} uptake in mouse cardiac SR.* Ca^{2+} uptake in mouse cardiac microsomal fractions pre-incubated with 1, 10 or 100 ng of recombinant αKAP-GST or 100 ng of GST as a control. The Ca^{2+}/EGTA buffer $[Ca^{2+}]_{free}$ was set at 1 μM. The reaction was initiated with the addition of 5 mM of fresh ATP and terminated at 10 minutes via the filter disc method. Bars represent the rate of calcium uptake from microsomal tissue fractionated from 3 adult hearts, the measurement was conducted in triplicate and the average was plotted (+/- SEM). P >0.05. n=3

3.2 Overexpression of αKAP reduces SERCA2a activity in Neonatal Mouse Cardiomyocyte Culture

Previous studies from our lab demonstrated that the N-terminal domain of αKAP, encodes a hydrophobic transmembrane sequence, which serves to localize αKAP at the SR. Furthermore, αKAP has been shown to directly interact with SERCA2a, through part of its association domain. The addition of recombinant αKAP, for the purpose of studying the direct SERCA2a/αKAP interaction did not have an effect on Ca^{2+} uptake in SR samples from cardiac tissue. Overexpressing αKAP in a neonatal mouse cardiomyocyte (NMCM) cell culture model would more closely resemble the *in-vivo* physiological system. Overexpressing αKAP, contrary to exogenous addition, will allow the expressed protein to be correctly localized at the SR membrane via its N-terminal transmembrane sequence. We overexpressed αKAP in NMCM by lentiviral delivery of an overexpression vector. In brief, HEK-293T cells were used to produce the lentivirus by transfecting the envelope, packaging and overexpression vector, and collecting the packaged virus 48 hours later by ultracentrifugation. The minimum amount of virus required for successful infection of >90% of the cultured cells was determined by adding increasing amounts of the virus to previously plated NMCM and expression was examined via fluorescence microscopy. Primary neonatal cardiomyocytes were extracted from 1 to 3 day old mouse pups and plated at a 50-60% confluency on 1% gelatin coated plates. The cells were then transduced with the lentiviral vector encoding pLenti-CMV- αKAPGFP and pLenti-CMV-GFP as a control and live cell fluorescence microscopy is performed to ensure expression of the overexpressed protein (figure 9). As expected, the expression of GFP protein was ubiquitous throughout the cells, whereas αKAP-GPP was expressed at reticular structures within the cell. The cells were then collected 96 hours post lentiviral transduction, and microsomal fractions isolated via subcellular fractionation in a slightly modified protocol from whole heart method, described in

detail in the methods section. The ATPase activity of SERCA2a is measured using the NADH coupled enzymatic assay, as described in the methods section. Thapsigargin inhibitable activity, defined as SERCA2a activity, was measured as described above in section 3.1. The overexpression of αKAP reduced SERCA2a activity from of 959.7 +/- 220.9 nmol/mg/min (nmol of phosphate released per mg of protein per min) in samples overexpressing GFP alone to 302.5 + 48.34 nmol/mg/min. This reduction in activity due to the overexpression of αKAP represented a 68.5% decrease in SERCA2a activity. The viral transduction had no notable effect on SERCA2a activity, evident by the ATPase activity of 1003 +/- 78.94 nmol/mg/min, in 3 samples that have not been infected with a lentivirus (figure 10).

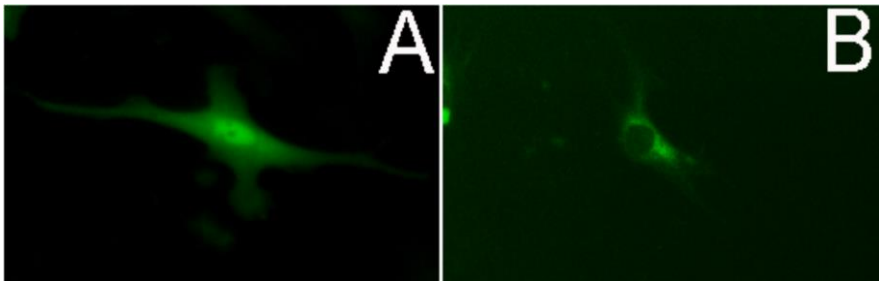


Figure 9: *GFP and αKAP overexpression in primary NMCM culture.* Live cell imaging of primary NMCM 96 hours post transduction of a lentiviral vector encoding pLenti-CMV-GFP (A) or pLenti-CMV-αKAP-GFP (B).

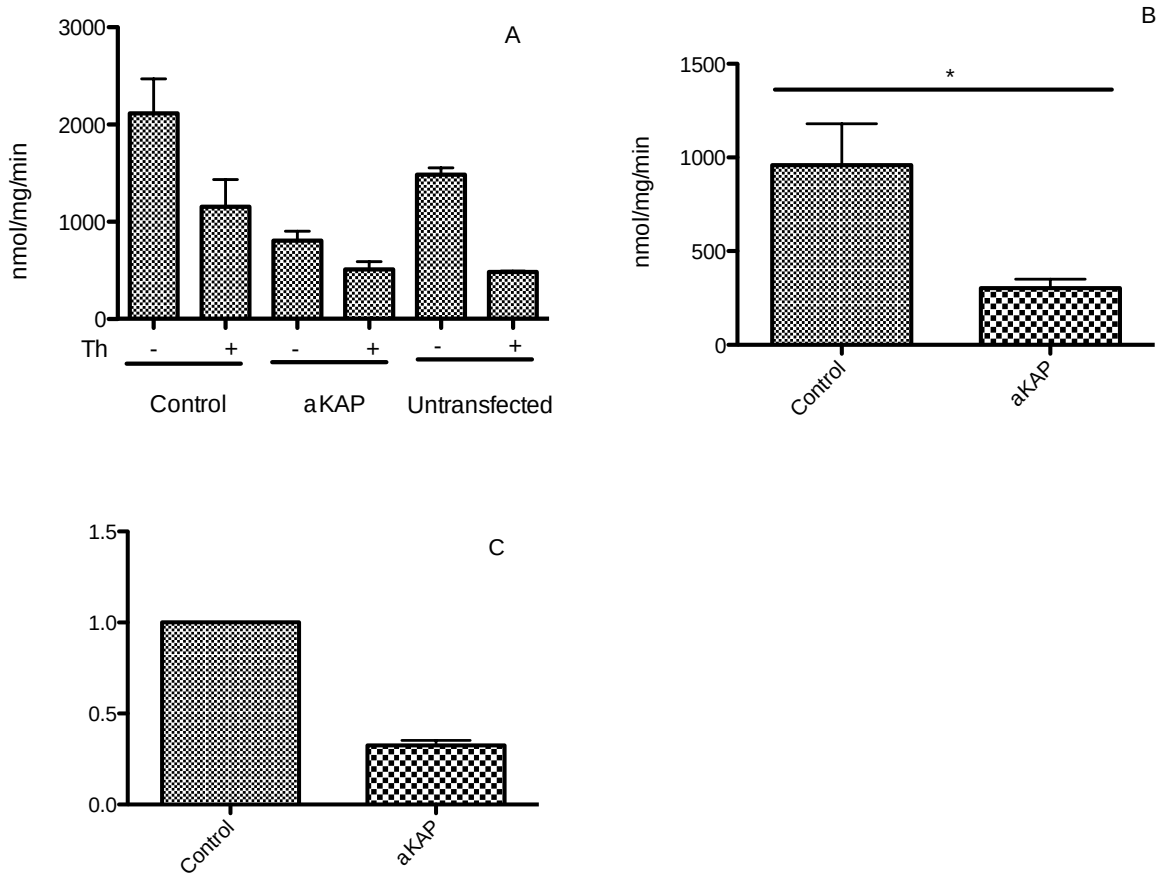


Figure 10: *αKAP decreases endogenous SERCA2a activity in primary NMCM.* Primary cultures of NMCM were transduced with pLenti-CMV-GFP or pLenti-CMV- αKAP -GFP. Microsomal fractions from these cells were collected and the endogenous SERCA2a activity was assayed using an NADH coupled enzymatic assay with thapsigargin as a control, to measure SERCA2a activity exclusively. Thapsigargin sensitive ATPase activity for each experiment was plotted (panel B). The data was normalized for easier representation (panel C). The bars represent the average rate of SERCA2a activity from 3 separate preparations (+/- SEM). Each sample was measured in triplicate, and then averaged. Student's t-test was performed, * $p<0.05$. N=3

3.3 Co-expression of αKAP and SERCA2a Does Not Affect SERCA2a activity in HEK-293T cells

From the data shown above, overexpression of αKAP in NMCM decreased SERCA2a activity in microsomal fractions. To study the effect of the direct interaction of αKAP with SERCA2a without the presence of other proteins that may interact with αKAP, HEK-293T cells were chosen as a model system. HEK-293T cells have the ability to be very efficiently and consistently transfected to a high level. After reaching 60-70% confluence, HEK-293T cells were co-transfected with expression vectors encoding for SERCA2a-GFP and 6Myc tag, SERCA2a-GFP and αKAP-6Myc, or SERCA2a-GFP and PLN-6Myc. The cells were collected 24 hours post transfection and microsomal fractions were collected in the same method as described for neonatal mouse cardiomyocytes. PLN is a known inhibitor of SERCA2a activity and is used as a control, in this experiment. As expected in our positive control, PLN coexpression decreased SERCA2a activity from a mean of 190 +/- 80 SEM nmol/mg/min (n=4) to 22.4 +/- 4 SEM nmol/mg/min (n=4). Co-expression of αKAP also reduced SERCA2a activity from 190 +/- 80 SEM nmol/mg/min (n=4) in cells expression SERCA2a alone, to 45.69 +/-20.31 SEM (n=4) (figure 11 A/B). Due to large variances in the SERCA2a activity of microsomal samples from control HEK-293T cells expressing SERCA2a alone, the data presented above was not statistically significant and failed the student's t-test. Because of the large variance in the measured SERCA2a activity of the control samples, normalizing the data gave a better representation of the observed effects of the co-expressed proteins. We see a 76% reduction in ATPase activity, in cells co-expressing αKAP and 75% in cells co-expressing PLN (figure 11C). Western blot analysis of the microsomal samples was used to confirm expression levels of SERCA2a and it was found that SERCA2a expression levels are reduced when co-expressed with αKAP compared to PLN or 6Myc tag control (figure 11D). Since plasmids encoding for

SERCA2a and αKAP share the same CMV promoter, we wanted to ensure that the observed reduction in SERCA2a expression was a direct result of αKAP and not promoter activity. We co-expressed SERCA2a with SIKE as a control protein, where SIKE gene expression is also controlled by the CMV promoter. SIKE is small 22KDa protein similar in size to αKAP, but has no known role or interaction with proteins involved in calcium regulation. In cells Co-expressing αKAP and SERCA2a, SERCA2a levels were reduced (figure 12A), compared to cells co-expressing SIKE and SERCA2a (Figure 12C). Interestingly, cells co-expressing αKAP and SIKE showed reduced expression of SIKE as well. From the date presented above, it seems that αKAP is playing a role in the modulation of SERCA2a expression stability and SERCA2a activity.

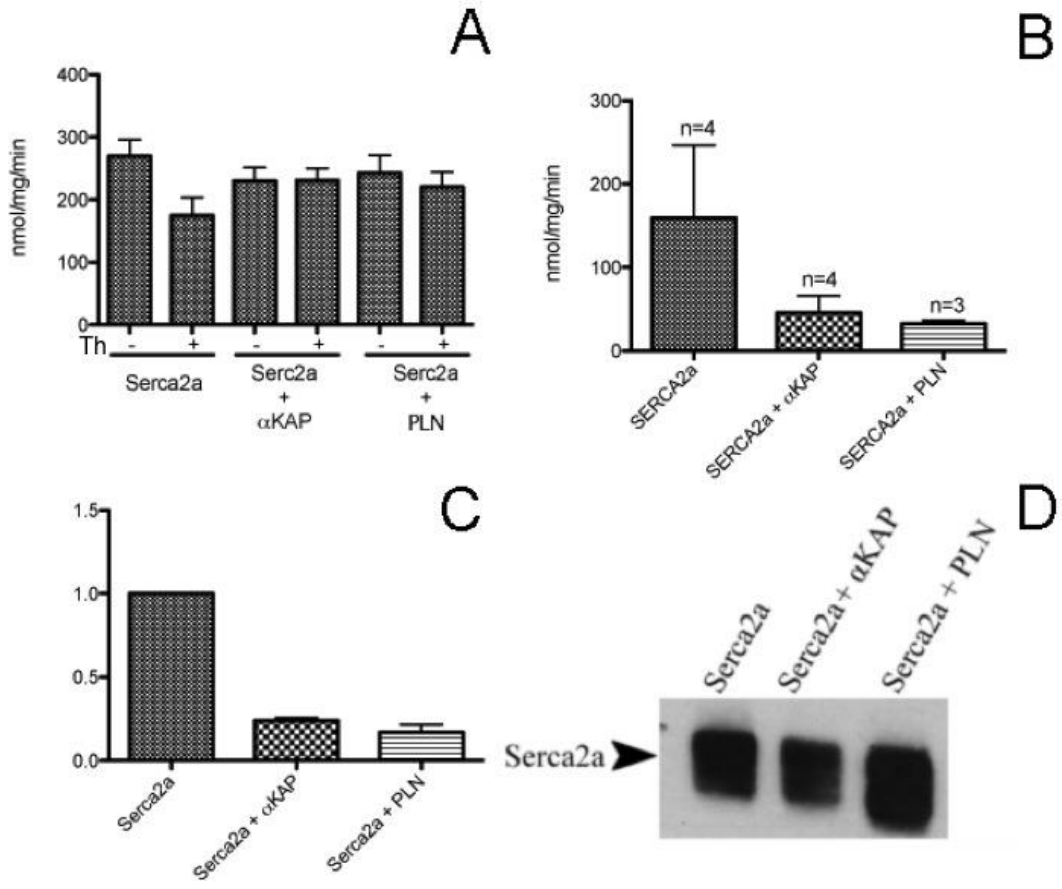


Figure 11: *αKAP reduces SERCA2a activity in HEK293T cells.* ATPase activity in microsomal fractions from HEK-293T cells co-expressing SERCA2a and αKAP or PLN was compared to cells expressing SERCA2a alone (A/B). The ATPase activity was normalized and plotted on a graph (C). Expression levels of SERCA2a, in the microsomal fractions, were examined by western blot (D) The bars represent the average rate of SERCA2a activity from 3 separate preparations. Each sample was measured in triplicate, and then averaged. n=4. $p>0.05$

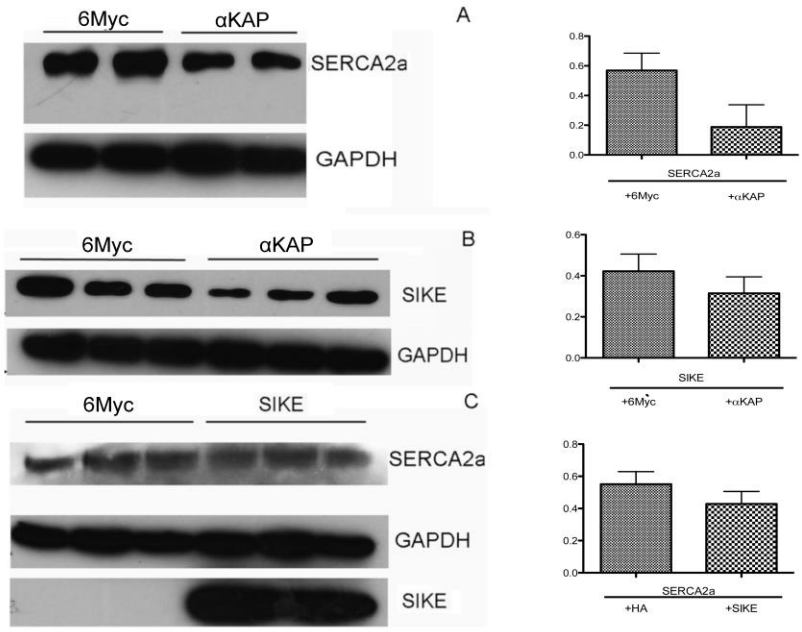


Figure 12: *αKAP reduces SERCA2a expression in HEK-293T cells.* HEK-293T cells were co-transfected with plasmids expressing αKAP-6Myc pCDNA3 and SIKE-HA pcDNA3 (C), SERCA2a-GFP pcDNA3 and SIKE-HA pcDNA3 (B), and lastly SERCA2a-GFP pcDNA3 and αKAP-6Myc pcDNA3 (A). Protein samples were run on a SDS 10% acrylamide gel and GAPDH was used as a loading control.

3.4 Immunohistochemical Studies of αKAP overexpression in HEK-293T cells.

From the data shown above, it is evident that αKAP reduces SERCA2a expression levels. αKAP did not have a role in targeting SERCA2a at the SR membrane. Previously published data from our lab has demonstrated that αKAP can bind SERCA2a directly and we hypothesized that it could target SERCA2a at the SR membrane via αKAP's N-terminal hydrophobic domain. The presence of SERCA2a in microsomal fractions from HEK-293T cells expressing SERCA2a without αKAP demonstrates that αKAP is not required for proper SERCA2a cellular localization (figure 11D).

Fluorescence microscopy was also used to further demonstrate that SERCA2a localization at the SR is not dependent upon αKAP. We coexpressed HEK-293T cells with SERCA2a-GFP and 6Myc tag or SERCA2a-GFP with αKAP -Myc. As expected, SERCA2a was properly localized at the ER membrane in HEK-293T cells expressing SERCA2a alone, as demonstrated by the colocalization of SERCA2a protein (green) with ER/SR marker calnexin (red) (figure 13). Co-expression of αKAP with SERCA2a does not affect the ER/SR localization of SERCA2a (figure 14), and as demonstrated previously by our lab, αKAP colocalizes with SERCA2a (figure 15). Co-expression of αKAP and SERCA2a is not required for proper cellular localization of SERCA2a and this finding correlates with the western blot data presented in section 3.3. SERCA2a was present in the microsomal fractions extracted from the HEK-293T cells expressing SERCA2a alone.

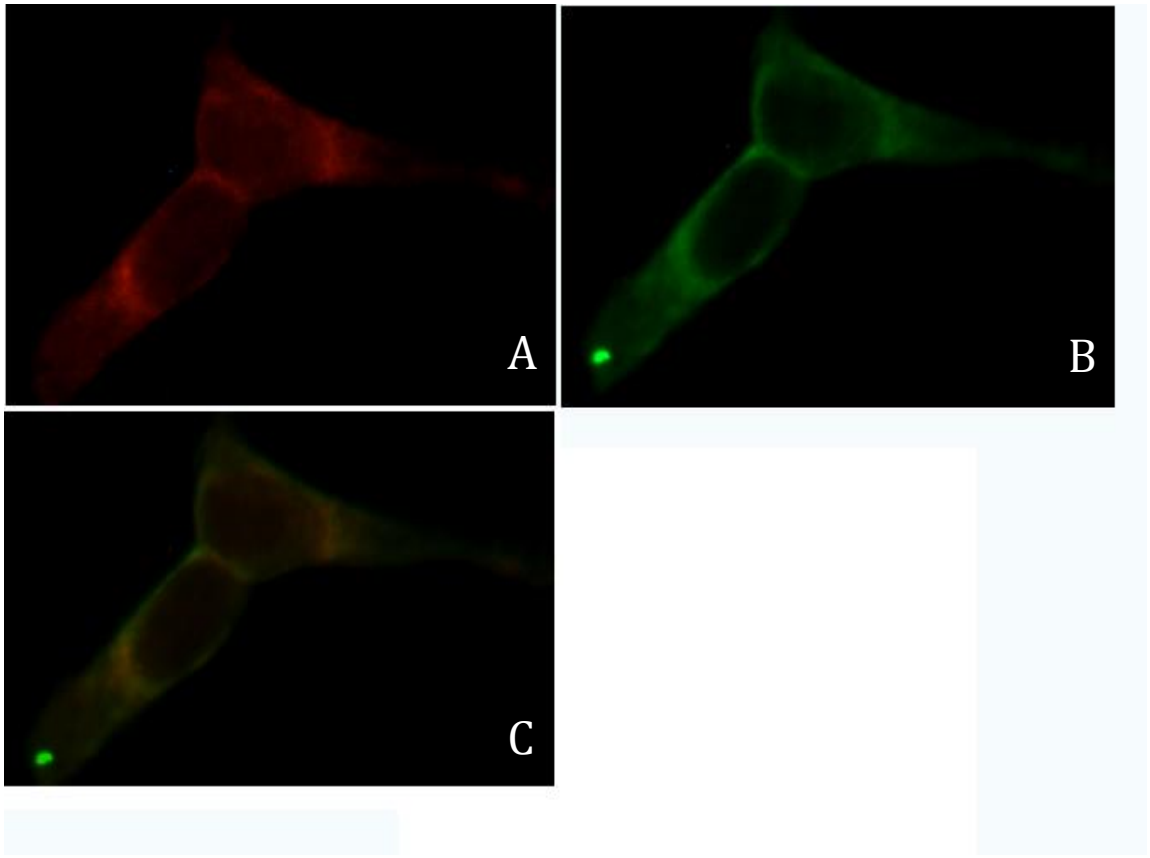


Figure 13: *SERCA2a localizes at the ER in HEK-293T cells.* HEK-293T cells were transfected with expression constructs encoding for SERCA2a-GFP. The cells were fixed 24 hours post transfection with 4% PFA. The cells were blocked in 1%BSA followed by immunostaining with ER/SR marker anti-calnexin (A) and Anti-GFP (B). SERCA2a-GFP proteins and calnexin protein are both co-localized at the ER

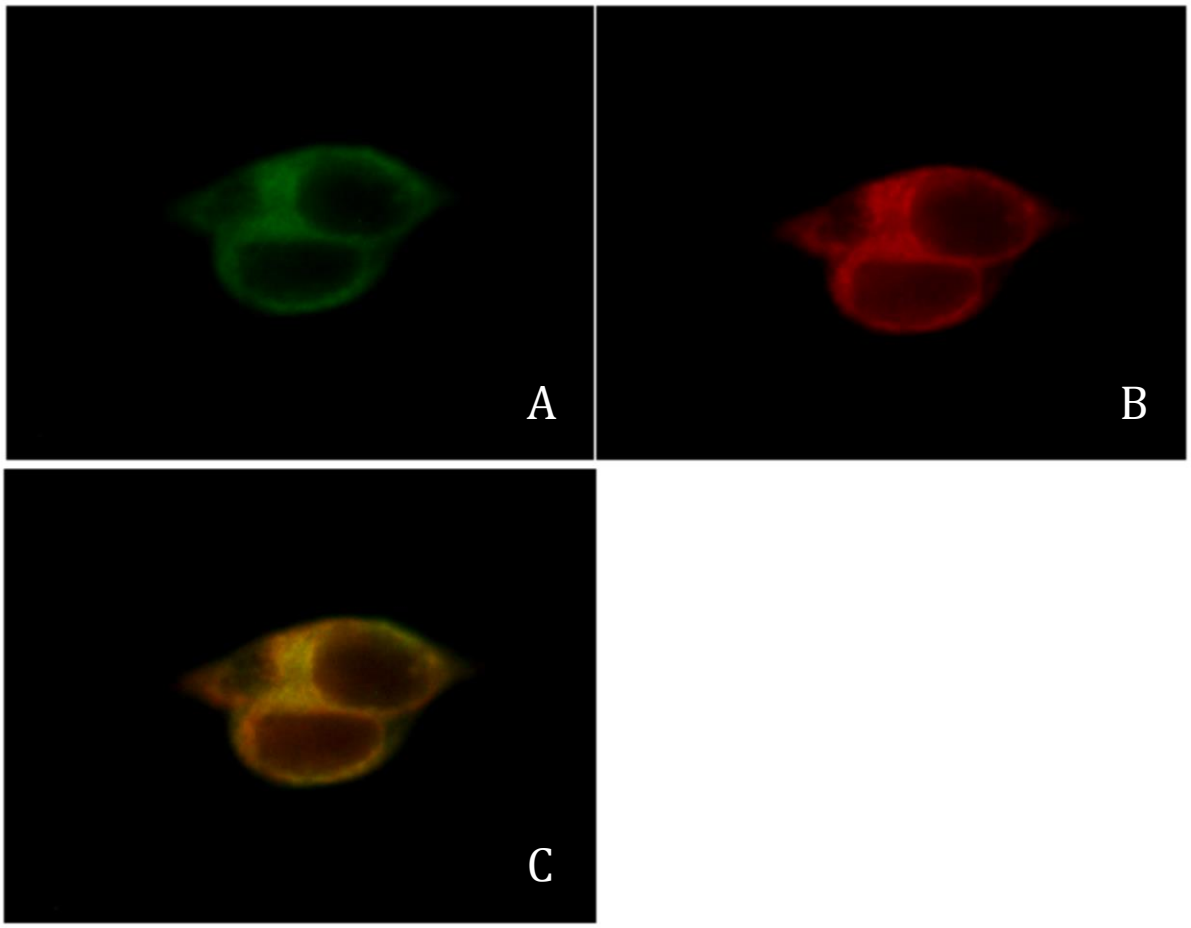


Figure 14: αKAP *does not alter SERCA2a localization in HEK-293T cells.* HEK-293T cells were co-transfected with expression constructs encoding for SERCA2a-GFP and αKAP -Myc. Cells were fixed 24 hours post transfection with 4% PFA. The cells were blocked in 1% BSA followed by immunostaining with ER/SR marker anti-calnexin (A) and Anti-GFP (B). SERCA2a-GFP proteins and calnexin protein are both co-localized at the ER and αKAP did not alter or affect SERCA2a's localization.

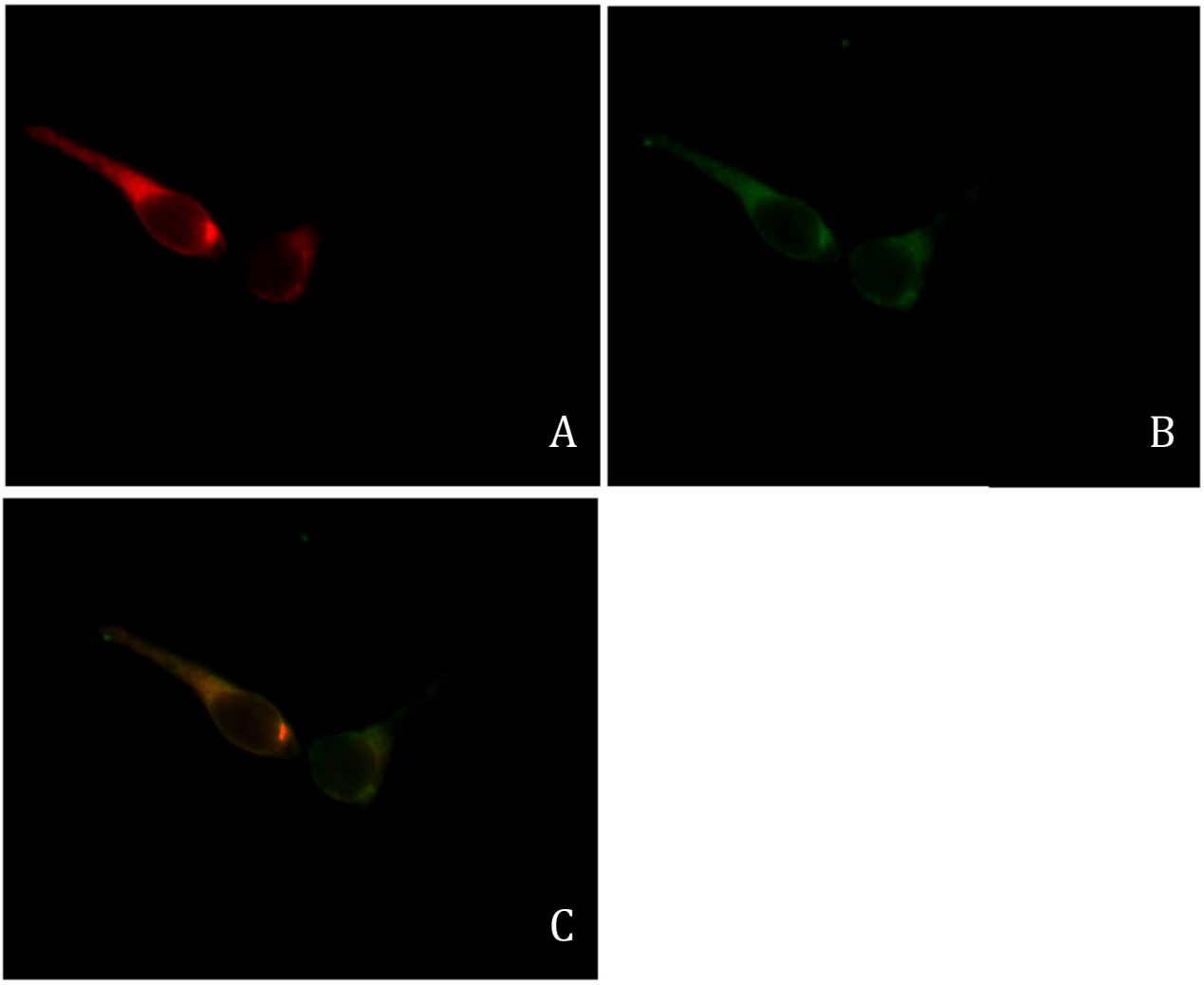


Figure 15: *SERCA2a and αKAP co-localize in HEK-293T cells.* HEK-293T cells were transfected with expression constructs encoding for SERCA2a-GFP and αKAP -Myc. The cells were fixed 24 hours post transfection with 4% PFA. The cells were blocked in 1% BSA followed by immunostaining with anti-6Myc tag (A) and Anti-GFP (B). SERCA2a-GFP proteins and αKAP -6Myc are both co-localized together.

3.4 αKAP Reduces SERCA2a Expression In NMCM.

The reduced SERCA2a expression levels seen in HEK-293T cells prompted the study of SERCA2a levels in NMCM. The SERCA2a activity in NMCM was reduced in cells overexpressing αKAP; thus, we overexpressed αKAP and analyzed SERCA2a levels via Western blot. To further strengthen the notion that αKAP is not affecting the targeting of SERCA2a; thus, reducing its presence in microsomal fraction, we also generated an expression construct of αKAP that is lacking the trans-membrane domain, αKAPΔTM. SERCA2a expression appears depressed in cells expressing αKAP and the mutated αKAPΔTM. To note, a greater reduction in SERCA2a expression was observed in NMCM overexpressing the un-truncated αKAP, compared to the truncation mutant, αKAPΔTM. The levels of αKAP protein overexpression were lower than αKAPΔTM, potentially indicating that proper localization of αKAP is required in order to exert its effect. The reduction in SERCA2a expression directly explains the depressed SERCA2a activity observed in NMCM experiments in section 3.2. Interestingly enough, CaMKIIδ expression levels are also reduced in the samples from NMCM overexpressing αKAP. As CaMKII can regulate PLN phosphorylation and ultimately SERCA2a activity, its reduced expression can help explain the decreased SERCA2a activity as well. A larger samples size is required to reach a statistically significant conclusion; however, with the ATPase activity data presented above, it is evident that αKAP overexpression has an effect on SERCA2a activity.

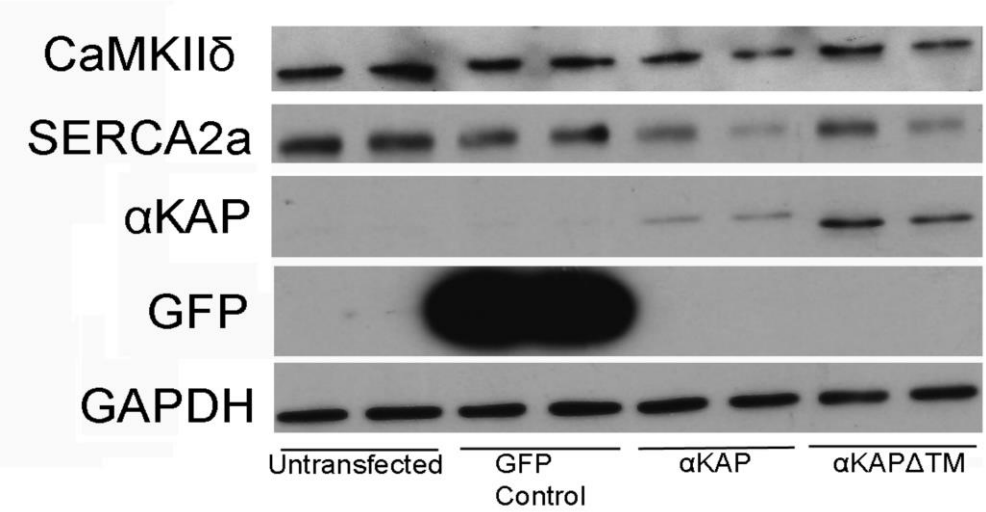
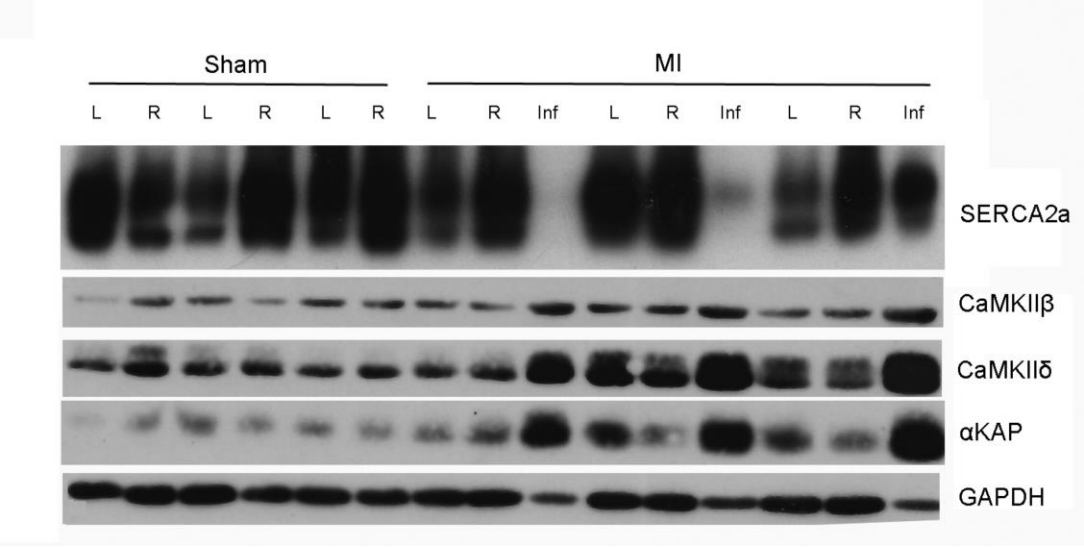


Figure 16: Effect of αKAP on SERCA2a expression in NMCM. Protein samples from NMCM infected with a lentiviral vector overexpressing pLenti-CMV αKAP-GFP (lanes 5,6) pLenti-CMV- αKAPΔTM-GFP(7,8), control, pLenti-CMV-GFP (3,4) or un-infected cells (1,2) were examined via western blot. The membrane was probed with antibodies against SERAC2a (Santa Cruz), CaMKIIδ (R&D Systems), αKAP (BD Transduction), GFP (Roche) and GAPDH (Invitrogen). Cells were collected and run on a 5-15% gradient SDS acrylamide gel 96 hours post transduction. GAPDH was used a control.

3.5 αKAP, CAMKIIδ and SERCA2a Expression Reduced in Myocardial Infarct Model.

In both human and animal models of heart failure, cardiac remodeling causes SERCA2a levels to be depressed, and CaMKIIδ to be elevated, both contributing to the greatly to the defective EC-coupling observed. In the data presented above, αKAP seems to play a role in SERCA2a activity and protein expression stability. The novel protein αKAP was never studied in heart failure and its role in the disease state of the heart is unknown. The CaMKII targeting protein αKAP could potentially play a role in the modulation of cardiac remodeling, particularly SERCA2a expression and activity. A Left Coronary Artery (LCA) occlusion experiment was conducted on adult rats in order to simulate a myocardial infarct (MI). Heart samples from the left ventricle, right ventricle and the infarct zone are compared to left and right ventricular samples from sham operated animals. The tissue samples were taken 4 weeks post-surgery, lysed and run on a 5-15% gradient gel. The gel was transferred on a PVDF membrane and probed with antibodies for CaMKIIδ, SERCA2a, and αKAP. Left ventricular remodeling is a crucial player in the development of heart failure and we will be focusing on comparing the expression levels of above mentioned proteins from the left ventricle (LV) of sham operated animals with samples taken from the infarct zone in the left ventricle from MI hearts. In agreement with the literature, samples from infarct zone demonstrate elevated levels of CaMKIIδ and decreased SERCA2a, both hallmarks of the diseased and failing heart. CaMKIIδ and SERCA2a's role in the development of heart failure have been reviewed extensively, most recently by Kho et Al (Kho et al., 2012) and it is accepted that CaMKIIδ activity and expression is elevated while SERCA2a is depressed. Our data shows a dramatic increase in CaMKIIδ levels in left ventricular tissue samples taken from the infarct zone of rats that have undergone a LCA occlusion operation, when compared to left ventricular tissue samples from sham operated animals (Figure 17A).

SERCA2a levels are also decreased in the above MI samples when compared to sham operated animals. Expression levels of the novel protein αKAP shows a dramatic increase in tissue samples from animals with MI (Figure 18A) compared to the samples from control sham operated animals. Western blot densitometry analysis of αKAP levels from the left ventricular tissue, and infarct zone tissue of rats with MI indicate a 3 fold and 10 fold increase in expression respectively, 2.99 +/-0.60, compared to LV tissue of sham operated animals , 0.27 +/-0.01; $p<0.005$ (Figure 18B).



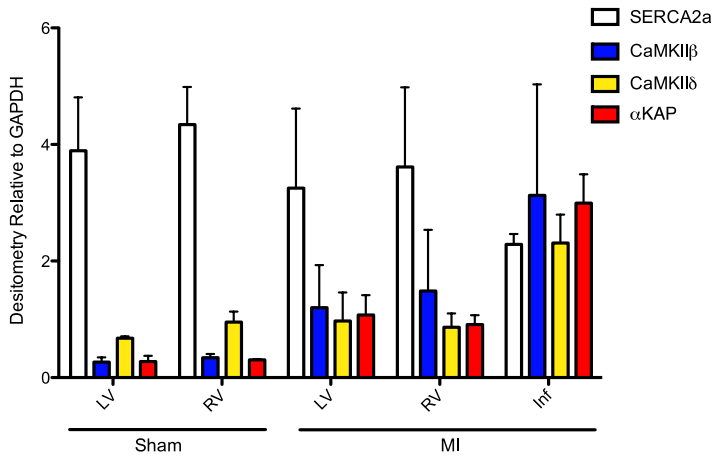


Figure 18: *Increased αKAP expression in adult rats with MI.* Left (L) ventricular samples and right (R) ventricular samples from 3 sham operated animals 4weeks post surgery (lanes 1,2 3, and 4,5,6 respectively) and samples from left ventricular, right ventricular and the infarct zone of 3 rats 4 weeks post LCA occlusion surgery (lanes 7,8,9; 10,11,12; and 13,14,15)(A). *The membrane was probed with antibodies against* SERAC2a (Santa Cruz), CaMKIIδ (R&D Systems), CaMKIIβ (Invitrogen), αKAP (BD Transduction), and GAPDH (Invitrogen). Densitometry analysis of protein expression relative to GAPDH was performed on the data plotted on a graph (B)

CHAPTER 4: DISCUSSION

Regulation of calcium signaling in the myocardium is of critical importance for the correct physiological function of the heart. CaMKII and PKA are critical players in the regulation of calcium handling proteins and calcium serves to exquisitely regulate cardiac contraction and relaxation (Sampson & Kass, 2010; Swaminathan, Purohit, Hund, & Anderson, 2012). Aberrant CaMKII and PKA activity has been directly implicated in the development of heart failure (Marx et al., 2000; Wu, 2002). PKA is targeted to appropriate cellular locale via A-Kinase Anchoring Proteins (AKAPs), and the importance of correct localization of PKA in the near vicinity of its targets has been demonstrated to be critical for the temporal and spatial control of cAMP signaling (Grimm & Brown, 2010; Lygren et al., 2007). The correct temporal and spatial organization of CaMKII for calcium signaling is also of critical importance but its understanding is underdeveloped and needs attention to detail (Bayer et al., 1998; Skelding & Rostas, 2009). Here, we propose the novel CaMKII splice variant, αKAP, serves as an adaptor protein, targeting CaMKII to the appropriate cellular locale. Previous studies have demonstrated that αKAP is able to bind CaMKII directly via the association domain, and target various subcellular membranes via its hydrophobic N-terminal domain; implying that CaMKII can be spatially organized within the cardiomyocytes (Nori et al., 2003; Singh et al., 2009). Data also indicated that αKAP can directly bind SERCA2a and whether it can modulate its function in terms of calcium transport is yet to be determined (Singh et al., 2009). Cellular localization studies demonstrate that SERCA2a and αKAP can colocalize at the ER membranes. We hypothesized that αKAP's direct interaction with SERCA2a could potentially modulate SERCA2a's activity. We had also hypothesized that αKAP may act to target CaMKII to SERCA2a and modulate calcium uptake to impact cardiac relaxation. Here we have examined

SERCA2a's activity in a HEK-293T cell culture model co-expressing SERCA2a and αKAP. Our results show a redduction in SERCA2a activity when coexpressed with αKAP. This reduction in SERCA2a activity was similar to that seen with the known SERCA2a inhibitor PLN. A neonatal mouse cardiomyocyte (NMCM) model was also used as a better representation of the physiological cardiac environment where both, SERCA2a and αKAP, would be expressed. Overexpression of αKAP in these NMCM, also inhibited SERCA2a's activity by ~60%, a reduction similar to that observed in the HEK-293T cells.

Protein expression studies indicated that overexpression of αKAP led to the reduction of SERCA2a expression, in both HEK-293T cells and NMCM. This reduction in SERC2a expression was not observed with PLN although PLN did inhibit SERCA2a activity in our assay system. Further analysis indicated that αKAP was also able to inhibit the expression of its other binding partner, CaMKIIδ, in NMCM. To further determine if αKAP was affecting SERCA2a expression exclusively, a protein of similar size (SIKE) but with no known interaction in the calcium handling pathways, was co-expressed with SERCA2a. Co-expressing SIKE with SERCA2a did not affect SERCA2a expression. However, co-expressing SIKE with αKAP reduced the level of SIKE expression. These data suggest that αKAP is somehow able to inhibit the level of co-expressed proteins as diverse as SERCA2a and SIKE.

While the mechanisms of how αKAP is exerting its effect on protein expression remains unknown, recent findings by Mouslim et Al, suggest that αKAP can serve roles in regulating the ubiquitination pathway and protein stability of the AChr. αKAP knockdown in skeletal muscle myotubes caused a decrease in AChR expression due to increased ubiquitination resulting in protein degradation (Mouslim, 2012). In our experiments, overexpressing αKAP caused a reduction in SERCA2a protein levels. This suggests that αKAP is able to target its binding

partner, may it be SERCA2a or CaMKIIδ for degradation. αKAP's role in protein degradation seems to be independent of its subcellular targeting. Overexpression of the truncated αKAPΔTM, lacking the transmembrane domain, also decreased protein expression, although it appears to be less potent. While experiments were attempted to explore the role of αKAP's regulation of the proteosome and protein degradation using MG132, the drug, even at the lowest effective concentrations cited in the literature, was toxic and the cells did not survive the treatment.

Recently SUMO-1, small ubiquitin related modifier 1, has been shown to play a role in the protein expression and stability of SERCA2a. SUMO-1 directly interacts with SERCA2a, a term called SUMOylation, and blocks lysine residue 480 and 585 preventing the subsequent 26S proteosomal mediated degradation. This was also linked to the decreased SERCA2a levels observed during heart failure progression (Kho et al., 2011; 2012; Schwartz & Yeh, 2012). Similarly to SUMO-1, αKAP can directly interact with SERCA2a, however the residues at which αKAP binds to have yet to be examined. It is possible that much like SUMO-1 prevents the ubiquitination of SERCA2a, αKAP may promote the ubiquitination of SERCA2a or even interact with SUMO-1 and prevent SUMOylation of SERCA2a, however this needs to be explored.

Reduced expression of SERCA2a may not be the only mechanism by which measured ATPase activity is decreased by αKAP. Previous studies demonstrate that αKAP downregulated PLN Thr17 phosphorylation in recombinant protein experiments (Singh et al., 2009). It is also possible that αKAP is affecting CaMKII activity, PLN-Thr17 phosphorylation and ultimately SERCA2a function, together with decreased protein expression as indicated above. CaMKII activity is dependent on the formation of the CaMKII holoenzyme (comprised of 6-12 CaMKII subunits). αKAP shares the CaMKII association domain allowing it to displace CaMKII subunits and disrupt the holoenzyme. The disruption of the holoenzyme by αKAP would lead to a

decrease in CaMKII autophosphorylation, impacting PLN-Thr17 phosphorylation. Studies on the effect of αKAP on CaMKII Thr286/287 autophosphorylation was not definitive, primarily due to the antibodies assayed. We have attempted to conduct phosphorylation assays to measure the CaMKII activity in the presence of αKAP using radiolabelled ATP (P^{32}), and again the results were not definitive.

The holoenzyme's physical arrangement is also of critical importance to its function as demonstrated recently by Chao et al, and αKAP's interaction with the holoenzyme could also disrupt its physical structure (Chao et al., 2011). In Chao et al's studied of CaMKIIδ, they demonstrate the presence of two physical arrangements of the holoenzyme, one that is extended and another that is compact. These two organizational states are differentiated by the distance between the association domain (forming the middle core of the holoenzyme) and the kinase domain of the enzyme (located on the outer portion of the holoenzyme ring). The compact holoenzyme, will have a shorter distance between the kinase and association domain, whereby the CaM binding sites are physically blocked and rendered inaccessible by the kinase domain, disrupting the regulation of the holoenzyme and its response and sensitivity to calcium, see figure 19 (Chao et al., 2011). This type of holoenzyme regulation relies on the large size of the subunits and the presence of the kinase domain. In light of these studies, αKAP's smaller size of 23Kda (versus 55-60Kda for CaMKII) and lack of kinase domain, could also impact the formation of the extended/compact arrangement of the holoenzyme and regulation of the CaM binding sites, ultimately affecting the holoenzyme's activity and function.

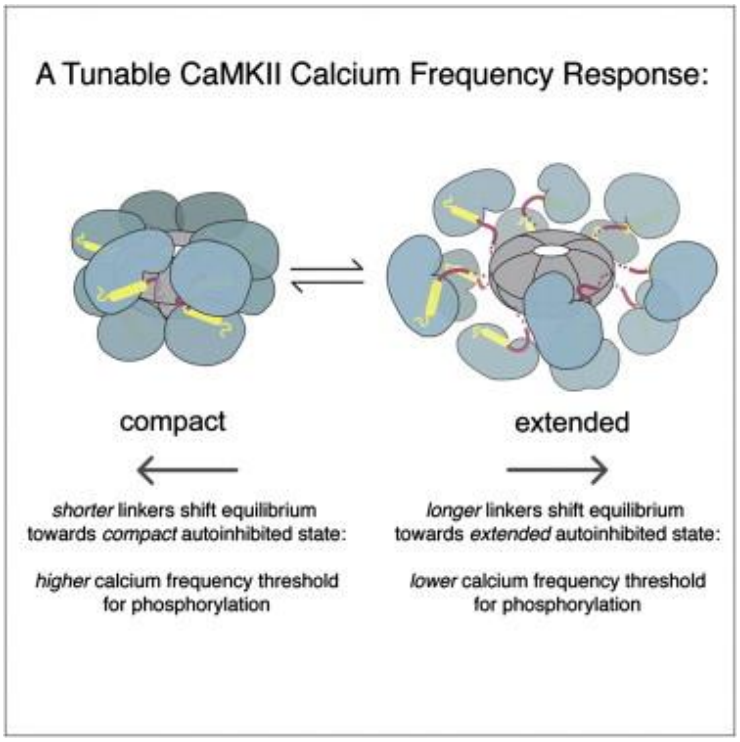


Figure 18: A schematic representation of the compact and extended physical arrangement of the CaMKII holoenzyme. The Extended arrangement of the holoenzyme has a larger distance between the core and kinase domains exposing the CaM binding sites. Adapted from Chao et al, 2011.

αKAP could also modulate SERCA2a activity via CaMKII targeting. The mechanism by which αKAP targets CaMKII is yet to be fully explored, but the interaction between these two proteins serves to target CaMKII at the SR membrane. Modulation of CaMKII targeting will then affect SERCA2a activity via PLN-Thr17 phosphorylation. Studies by Skelding and Rostas have demonstrated a new phosphorylation site on CaMKII, Thr253, whereby this phosphorylation is dependent on the localization of CaMKIIα, a brain isoform, at the post-synaptic density of neurons. This new phosphorylation site, however, has no known effect on CaMKII's activity but strengthens the notion that CaMKII targeting is imperative (Skelding & Rostas, 2009). The Thr253 phosphorylation site is conserved amongst all CaMKII isoforms, and could potentially be used as a tool to study αKAP's regulation of CaMKIIδ targeting in the myocardium.

The above statements regarding the αKAP holoenzyme regulation and CaMKII targeting could at first seem contradictory, whereby in one case αKAP is required for targeting of CaMKII and in another case αKAP is disrupting CaMKII activity. αKAP/CaMKII interaction seems to be a finely regulated event. αKAP could not only target CaMKII to its appropriate cellular locale but can be also used to effectively modulate CaMKII holoenzyme activity. The αKAP overexpression in our experiments could be disrupting this fine balance ultimately leading to a disruption in SERCA2a activity.

CaMKII and SERCA2a regulation are both very important targets in the treatment of heart failure. Both are known to play critical role in the pathogenicity of the disease, and in many cases of hypertrophy, arrhythmias and cardiac dysfunction, CaMKIIδ as well as the autophosphorylated CaMKIIδ Thr287 levels have been found to be elevated (Zhang T, 2003; Benji K, 2005; Anderson ME, 2005). Inhibition of CaMKII in animal models of heart failure has

been shown to slow down the progression of the disease, and with our finding eluding to the notion that αKAP is modulating CaMKII activity, this provides another avenue of studying the progression of disease.

In our myocardial infarct model, as expected CaMKIIδ levels were elevated and SERCA2a was depressed. Interestingly αKAP expression levels were found to be elevated by approximately 10 fold. αKAP can target CaMKII and our data shows that it can also regulate CaMKIIδ and SERCA2a expression as well as SERCA2a activity. The increased αKAP expression observed in our MI model could contribute to the decreased SERCA2a expression observed and explain the cardiac dysfunction present in MI. On the other hand αKAP could also target CaMKIIδ to SERCA2a, in a cardio-protective manner, in an attempt to improve SERCA2a function and muscle contractility. Understanding the fine regulation and balance between αKAP, SERCA2a and CaMKII could lead to a better understanding of heart failure and ultimately more effective treatments for the disease.

In conclusion, this study demonstrates that αKAP is regulating the protein expression of its two known binding partners, SERCA2a and CaMKII. Our data also indicated that αKAP is able to regulate SERCA2a activity, suggesting a potential role in αKAP's the modulation of cardiac contraction. In addition, our study has brought forth the novel observation of increased αKAP expression in a rat model of myocardial infarction. The data presented in this work suggest that αKAP may be a unique regulator of SERCA2a activity and cardiac function. These findings point to αKAP as a potential novel target in the treatment of heart disease.

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