PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN CALCINEURIN AND THE CARDIAC L-TYPE CALCIUM CHANNEL

APPROVED BY SUPERVISORY COMMITTEE

_____
Joseph A. Hill, M.D. Ph.D.

_____
Marc C. Mumby, Ph.D.

_____
James T. Stull, Ph.D.

_____
Donald W. Hilgemann, Ph.D.

DEDICATION

For my family, who have endured a long history of heart disease.

PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN CALCINEURIN AND THE CARDIAC L-TYPE CALCIUM CHANNEL

by

SAMVIT TANDAN

DISSERTATION

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Finally, I am very grateful to my parents and immediate family for all their consistent love, support and interest in my educational endeavors over these years. It is for us, after all, that I toil in the hopes of better understanding the fascinating field of cardiovascular biology, and in turn, applying this knowledge for the benefit of those around me.

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SAMVIT TANDAN, Ph.D.

The University of Texas Southwestern Medical Center, 2008

JOSEPH A. HILL, M.D., Ph.D.

The L-type Ca^{2+} channel is the major mediator of Ca^{2+} influx in cardiomyocytes leading to both mechanical contraction and activation of signaling cascades. Among these cascades is calcineurin, a protein phosphatase that promotes hypertrophic growth of the heart. We previously reported from *in vivo* models of pressure-overload that calcineurin regulates Ca^{2+} channel function in the heart, such that, inhibition of calcineurin activity results in a decrease in channel function. Based on this, we hypothesize that calcineurin participates in the channel macromolecular complex. Initial immunohistochemical data demonstrated evidence for co-localization of calcineurin to $\alpha_1$1.2, the pore-forming

subunit of the L-type Ca^{2+} channel in mouse ventricular tissue. Additionally, co-immunoprecipitation biochemical experiments revealed evidence for binding between calcineurin and $\alpha_1$1.2 in native mouse and rat heart tissues. Pulldown assays using GST-fusion proteins of all intracellular $\alpha_1$1.2 regions provided evidence for direct binding of calcineurin at the N- and C-termini of $\alpha_1$1.2. At the C-terminus, calcineurin bound to aa 1909-2029 overlapping the well-characterized PKA/PKC site Ser-1928. *In vitro* kinase/phosphatase assays revealed Ser-1928 as a substrate for calcineurin dephosphorylation. Voltage-clamp recordings of L-type Ca^{2+} currents from cultured cardiomyocytes expressing constitutively-active calcineurin revealed significant up-regulation of channel function, similar to our previous observations from cardiac hypertrophy *in vivo*. Conversely, acute suppression of calcineurin, both pharmacologically or with specific peptide-inhibitors, induced a significant decrease in L-type channel function, while neither intervention had an effect on channel function in the absence of calcineurin activity. These data provide evidence for direct interaction between the L-type Ca^{2+} channel and calcineurin, and insights into the regulation of the channel by calcineurin. Furthermore, they highlight the specific role of calcineurin as a potential mediator of pathophysiological electrical remodeling in cardiac hypertrophy and failure.

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LIST OF ABBREVIATIONS

$\alpha_1$1.2	α subunit of L-type Ca^{2+} channel
α_{1C}	α subunit of cardiac L-type Ca^{2+} channel
$\alpha_2\delta$	α_2 and δ subunits of cardiac L-type Ca^{2+} channel
β_{2a}	β subunit of cardiac L-type Ca^{2+} channel
βAR	βeta adrenergic receptor
µg	microgram
µL	microliter
µM	micromolar
aa	amino acid
AdCnA*	adenovirus expressing constitutively-active calcineurin
AdGFP	adenovirus expressing GFP
AID	auto-inhibitory domain
AKAP	A-kinase anchoring protein
AP	action potential
APD90	action potential at 90% repolarization
ATP	adenosine-5'-triphosphate
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BW	body weight

C-terminal	carboxy terminal
CaM	calmodulin
CaMKII	Ca2+/calmodulin-dependent protein kinase II
$Ca_V1.2$	Voltage-gated Ca^{2+} channel 1.2
cDNA	complementary DNA
CMV	cytomegalovirus
CnA	calcineurin A
CnA*	constitutively-active calcineurin A
CnB	calcineurin B
CsA	cyclosporine A
CT	C-terminal (GST-fusion protein)
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-O, O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
FKBP	FK-506-binding protein
F.L.	full-length calcineurin A
GFP	green fluorescence protein
GST	glutathione-S-tranferase

GTP	guanosine-5'-triphosphate
HA	hemagglutinin
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-pressure liquid chromatography
hr	hour
HW	heart weight
Hz	hertz
$I_{Ca,L}$	L-type Ca^{2+} current
IB	immunoblot
IgG	immunoglobulin G
IP	immunoprecipitate
IP_3R	inositol 1,4,5-trisphosphate receptor
IPTG	isopropyl-β-D-thiogalactoside
IRES	internal ribosome entry site
Iso	isoproterenol
IV	current-voltage
kD	kilo-Dalton
MAP	mitogen-activated protein
MCIP	modulatory calcineurin interacting protein
MI	myocardial infarction

Mg	milligram
min	minutes
mL	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
ms	millisecond
mV	millivolt
N-terminal	amino terminal
NCX	sodium/calcium exchanger
NFAT	nuclear factor of activated T-cells
nM	nanomolar
NS	not significant
pA	pico Ampere
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phenylephrine
pF	pico Farad
pH	potential of hydrogen
pI	isoelectric point

PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B
PVDF	polyvinylidene fluoride
RCAN	regulator of calcineurin
rpm	revolutions per minute
RyR	ryanodine receptor
SDS	sodium dodecyl sulfate
SERCA2a	sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a
SR	sarcoplasmic reticulum
TAB	thoracic aortic banding
TEA	tetraethylammonium
Tg	transgenic
WT	wild-type

CHAPTER ONE: BACKGROUND AND SIGNIFICANCE

Epidemiologic burden of disease

Cardiac hypertrophy and failure are rapidly expanding public health concerns in the United States. Approximately 5 million patients in the country have heart failure, and over 550,000 new patients are diagnosed each year (Hunt SA et al., 2005). The disease is the primary reason for 15 million office visits and 6.5 million hospitalizations per annum (O'Connell JB & Bristow MR, 1994). One explanation for the increasing prevalence of disease comes with an increasing baby-boomer economy; a rise in heart failure is not unexpected as it is often considered a disease of the elderly (Kannel WB & Belanjer AJ, 1991). So much so, that the incidence approaches 1 per 100 population after the age of 65 (Hunt SA et al., 2005). Furthermore, another important reason for the increasing prevalence of heart failure comes with the advancements in 'rescue' and management of patients with acute myocardial infarctions (or heart attacks – MIs) earlier in life (Hunt SA et al., 2005). This particular subject is personally relevant and meaningful because of my family history of cardiovascular disease, including post-MI management and care.

As can be expected, the cost of management and treatment of these patients also adds considerable duress on the economy, and it was estimated that in 2005, the total direct and indirect cost of heart failure in the United States

equaled $27.9 billion (Hunt SA et al., 2005). It is of no coincidence, then, that at the time of writing this document, the American Heart Association (Dallas, TX) simultaneously announced and published the inaugural issue of Circulation: Heart Failure, a journal specifically aimed towards addressing this growing concern (Udelson JE, 2008).

Response to stress and pathophysiology

Heart failure, by definition, is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood (Braunwald E, 1997). Typically, left ventricular dysfunction begins with some injury to, or stress on, the myocardium and is generally a progressive process (**Figure 1.1**), even in the absence of a new identifiable insult. The principle manifestation of such progression is a change in the geometry and structure of the left ventricle, such that the chamber dilates and/or hypertrophies and becomes more spherical – a process referred to as cardiac remodeling. This change increases the hemodynamic stresses on the walls of the failing heart and depresses its mechanical performance. Cardiac remodeling generally precedes the development of symptoms (occasionally by months or even years), continues after the appearance of symptoms, and contributes substantially to worsening of symptoms despite treatment. The development of structural abnormalities can have one of three outcomes: 1) patients die before

developing symptoms, 2) patients develop symptoms controlled by treatment, or 3) patients die of progressive heart failure. Furthermore, sudden death, through arrhythmic events, can interrupt this course at any time (Hunt SA et al., 2005).

Along these lines, hypertrophic transformation of the myocardium is a milestone in the pathophysiologic progression to heart failure (**Figure 1.1**). At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis and heightened organization of the sarcomere. These changes in cellular phenotype are preceded and accompanied by re-induction of the so-called "fetal gene program", a pattern of gene expression mimicking that seen during embryonic development. Among the genes differentially expressed in cardiac hypertrophy and failure are those encoding sarcolemmal ion channels and electrogenic transporters. By altering the excitability and recovery responses of the heart – primarily by inducing action potential (AP) prolongation – these expression changes contribute importantly to the arrhythmias that are a prominent feature of these diseases.

Patients with echocardiographically documented hypertrophy are at significantly increased risk of developing malignant arrhythmias, which accounts for a substantial component of the mortality associated with cardiac hypertrophy (Brown DW et al., 2000). Mechanisms underlying these arrhythmias are multifactorial, but they stem, at least in part, from disordered electrical currents arising from prolongation of ventricular action potentials. The resulting delay in

the recovery of excitability, a consistent feature of ventricular hypertrophy (Hart G, 1994; Armoundas AA et al., 2001), predisposes to early and late afterdepolarizations. Hypertrophy is also associated with myocardial fibrosis, altered electrotonic coupling between cells, slowed conduction, and dispersion of refractoriness, all of which predispose to re-entrant mechanisms of arrhythmia. Together, these "electrical remodeling" responses underlie the propensity to arrhythmia, syncope, and sudden death. Further, there is evidence to suggest that alterations in transmembrane Ca^{2+} fluxes, and consequent perturbations in Ca^{2+} homeostasis, contribute to the pathogenesis of hypertrophy by abnormally activating Ca^{2+}-responsive signaling pathways (Hill JA, 2003).

Electrical remodeling and perturbations of Ca^{2+} dynamics

Perturbation of intracellular Ca^{2+} signaling (Wickenden AD et al., 1998; Frey N et al., 2000a) and prolongation of the action potential (Nabauer M & Kaab S, 1998; Tomaselli GF & Rose J, 2000; Tomaselli GF & Marban E, 1999; Wang Z et al., 2001a) contribute to the pathogenesis of cardiac hypertrophy and failure. Altered Ca^{2+} handling contributes to the activation of several kinase and phosphatase cascades, including those involving MAP kinases, protein kinase C, and calcineurin (recently reviewed by Vlahos CJ et al., 2003). Together, such abnormal profiles of signal transduction lead to disturbances of gene regulation, which may promote disease progression.

In heart failure, systolic increases in Ca^{2+} are blunted, whereas diastolic Ca^{2+} levels are increased, leading to depressed contractile function and ventricular stiffness, respectively (Balke CW & Shorofsky SR, 1998; Schwartz K & Mercadier JJ, 1996). Underlying mechanisms include a) defective regulation of the sarcoplasmic reticulum (SR) Ca^{2+}-release channel (RyR2), b) defective sequestration of Ca^{2+} secondary to decreased expression of the SR Ca^{2+} pump (SERCA2a) and calsequestrin, and c) alterations in the expression and phosphorylation of phospholamban, the major regulator of SERCA2a activity (Hasenfuss G & Pieske B, 2002). Less is known about hypertrophy, but there is evidence to suggest that Ca^{2+} signaling may be different from heart failure, with increased Ca^{2+} transients (Wickenden AD et al., 1998) and increased inward Ca^{2+} current through the L-type Ca^{2+} channel (Wang Z et al., 2001a; Mukherjee R & Spinale FG, 1998).

The voltage-gated L-type Ca^{2+} channel ($Ca_V1.2$) is the major mediator of Ca^{2+} influx into cardiomyocytes and an important determinant of action potential morphology. Indeed, the L-type channel transduces membrane potential changes into local intracellular calcium transients that initiate a host of physiological events. We and others have previously reported an increase in $Ca_V1.2$ activity in various models of cardiac hypertrophy (Wang Z et al., 2001a; Yatani A et al., 2001). However, molecular mechanisms responsible for the observed increase in channel function in hypertrophy are unknown, though evidence suggests that it

involves an increase in channel availability and open probability (Schroder F et al., 1998).

Action potential prolongation in hypertrophy often stems from increased inward Ca^{2+} current, particularly in models of modest hypertrophy. Indeed, some evidence suggests that L-type Ca^{2+} current density is inversely correlated with the severity of hypertrophy or ventricular dysfunction; in models of mild-to-moderate hypertrophy, L-type current is often increased, whereas in severe hypertrophy and failure, channel function is similar to control levels or sometimes decreased (see Armoundas AA et al., 2001; Tomaselli GF & Zipes DP, 2004 for discussion). Whereas the molecular basis for these observations is unknown, recent findings implicate Ca^{2+}-responsive signaling pathways (see below). These findings also suggest the logical hypothesis that increased Ca^{2+} channel activity contributes to the pathogenesis of the disease including progression from hypertrophy to failure.

Calcineurin signaling

One such Ca^{2+}-dependent signaling pathway involves calcineurin, or protein phosphatase 2B (PP2B), a Ca^{2+}/calmodulin dependent protein phosphatase that contributes to hypertrophic signaling in many models of cardiac hypertrophy including that induced in elevated afterload (Heineke J & Molkentin JD, 2006). Calcineurin links cytoplasmic Ca^{2+} to transcriptional regulation of multiple genes involved in the hypertrophic and failure programs (Molkentin JD et al, 1998).

Calcineurin is activated in heart failure in humans (Lim HW & Molkentin JD, 1999; Tsao L et al., 2000), participates in hypertrophic signal transduction in models of cardiac (Heineke J & Molkentin JD, 2006) and skeletal muscle (Dunn SE et al., 1999; Musaro A et al., 1999; Semsarian C et al., 1999) biomechanical stress, and in fiber type-specific gene expression in skeletal muscle (Chin ER et al., 1998). In transgenic mice, constitutively-active calcineurin is sufficient to drive robust hypertrophy and failure (Molkentin JD et al., 1998).

Indeed, the inhibition of calcineurin for therapeutic advantage remains an intensely studied paradigm (Leinwand LA, 2001). The use of and cytotoxicity associated with pharmacological inhibitors of calcineurin, such as cyclosporine A or tacrolimus (FK-506), are well documented (Schuler S et al., 1985; Laczkovics A et al., 1987). However, RCAN1, or regulator of calcineurin (formerly known as MCIP – modulatory calcineurin-interacting protein) (Davies KJ et al., 2007) is a recently described (Rothermel BA et al., 2000; Kingsbury TJ & Cunningham KW, 2000) protein that directly binds to and inhibits the catalytic subunit of the calcineurin holoenzyme and has rekindled interest in targeted calcineurin suppression for potential therapeutic gain. RCAN1 over-expression blunts the hypertrophic response to activated calcineurin *in vitro* (Rothermel BA et al., 2000) and in the setting of hormonal or exercise stress *in vivo* (Rothermel BA et al., 2001). Work from our laboratory, in fact, demonstrated that transgenic over-expression of RCAN1 blunted the hypertrophic response to thoracic aortic

banding (TAB) (Hill JA et al., 2002), an established surgical model of calcineurin-mediated hypertrophy induced by pressure-overload (**Figure 1.2**).

Central thesis

Thus, hypertrophy is a significant stepping stone in the progression to heart failure, and is characterized by critical alterations in Ca^{2+}-handling and electrical remodeling. While the role of calcineurin in pathophysiological remodeling is well established (as described above), the specific role of calcineurin in electrical remodeling, however, is unknown.

Previously, we reported an increase in the activity of the L-type Ca^{2+} channel and concomitant prolongation of the action potential in pressure-overload cardiac hypertrophy *in vivo* (Wang Z et al., 2001a). Intriguingly, these electrical remodeling events were significantly abolished with specific inhibition of calcineurin (**Figures 1.3 – 1.4**; Wang Z et al., 2001a), highlighting calcineurin as a potential modulator of pathological electrical remodeling in the heart.

Based on these findings, we **hypothesized** that calcineurin is a component of the L-type Ca^{2+} channel macromolecular complex in cardiac myocytes and participates in stress-dependent regulation of channel function. In this document, I report evidence of a direct physical interaction between calcineurin and the major pore-forming subunit ($\alpha_1 1.2/\alpha_{1C}$) of the cardiac L-type Ca^{2+} channel. I

map the interaction to specific intracellular domains of the channel protein. Finally, I provide evidence for an important role for calcineurin in regulating L-type Ca^{2+} channel function in heart.

Figures

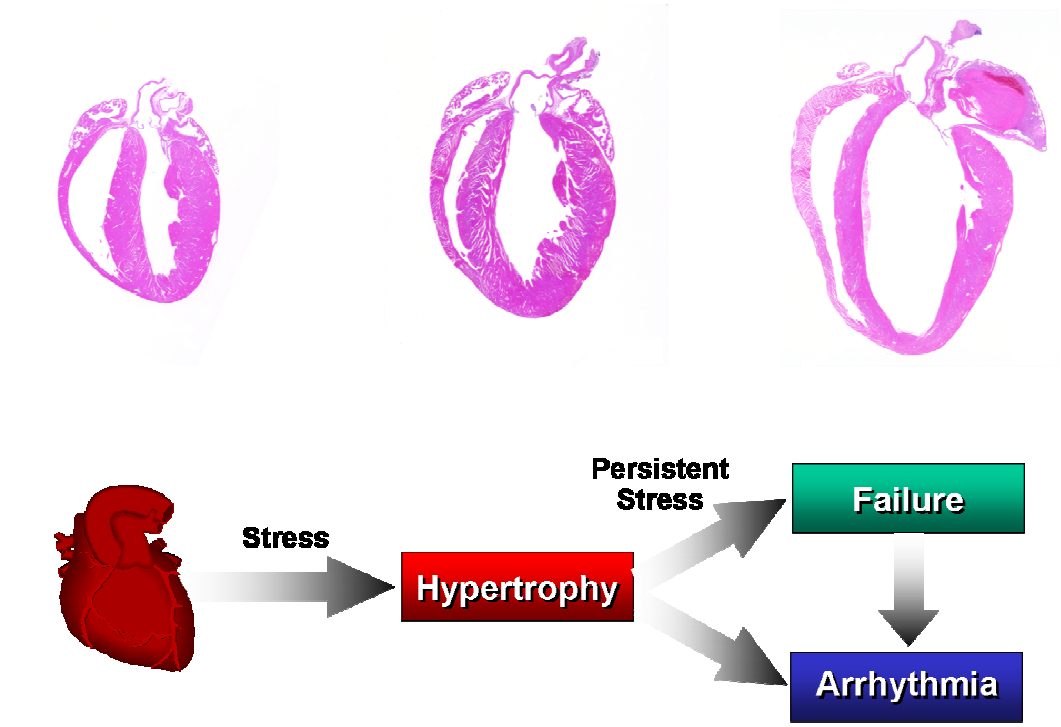


Figure 1.1: Progressive manifestation of disease pathophysiology. Left ventricular dysfunction begins with some stressful stimulus on the heart, and the concomitant remodeling events, in the face of persistent stress, render the heart prone to failure and arrhythmia. In some instances, severe stress can progress directly to heart failure.

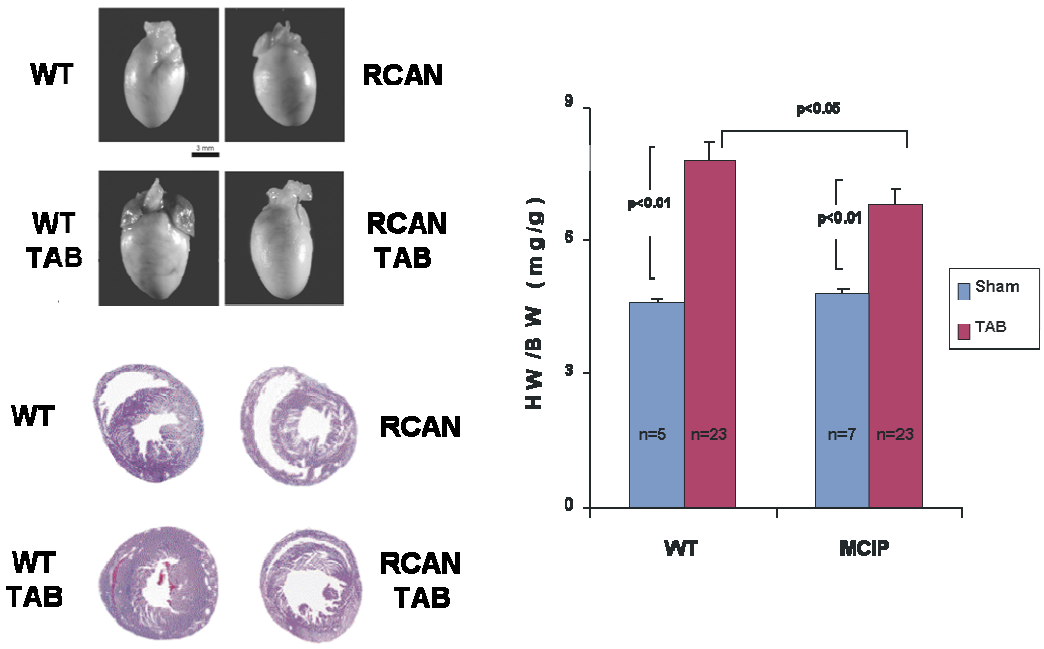


Figure 1.2: Genetic suppression of calcineurin by RCAN1 is cardioprotective. Cardiac-specific over-expression of RCAN1 (regulator of calcineurin) protects the heart from pressure-overload induced cardiac remodeling. WT = wild-type; TAB = thoracic aortic banding; HW/BW = heart-weight/body-weight.

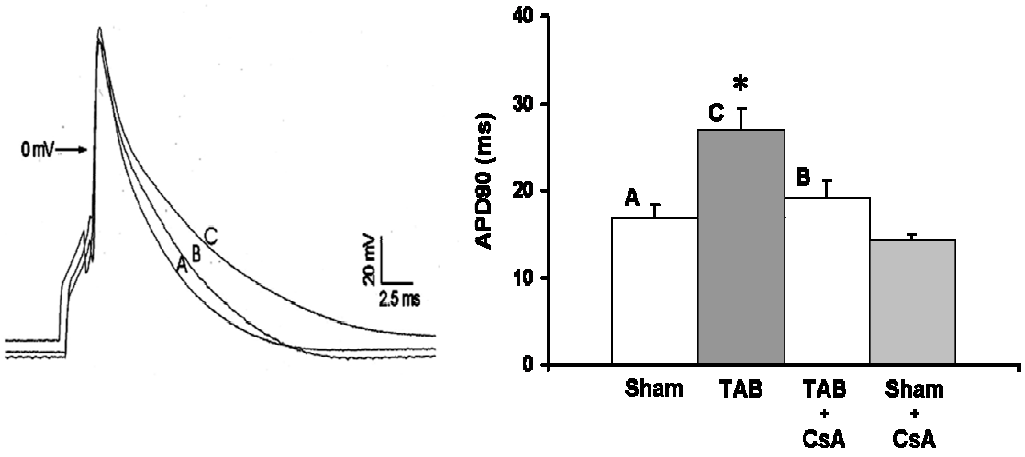


Figure 1.3: Action potential prolongation in calcineurin-dependent hypertrophy *in vivo*. Increase in phase 2 of the action potential suggests increased L-type Ca^{2+} channel activity. Specific inhibition of calcineurin, by cyclosporine A, virtually abolishes this increase in action potential duration. CsA = cyclosporine A; APD90 = action potential duration at 90% repolarization; TAB = thoracic aortic banding. (adapted from Wang Z et al., 2001a).

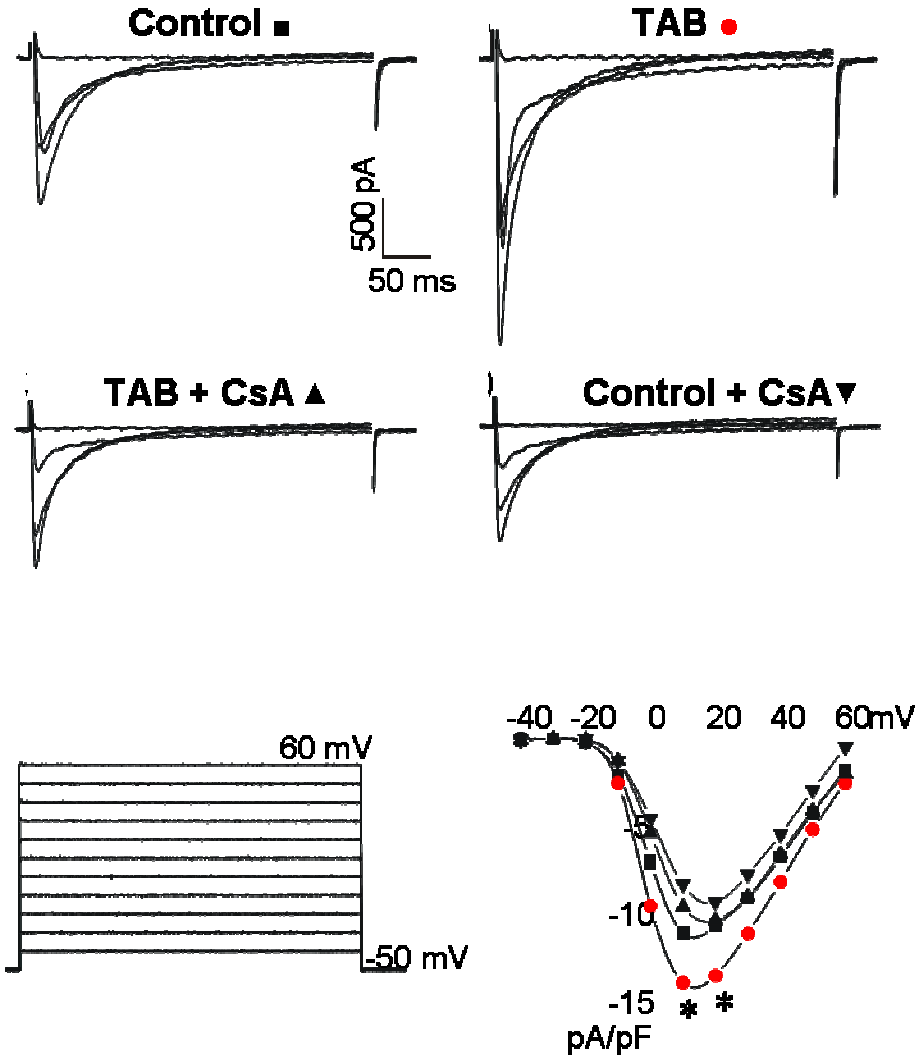


Figure 1.4: Increased L-type Ca^{2+} channel activity in calcienurin-dependent cardiac hypertrophy *in vivo.* Voltage clamp recordings reveal increased L-type channel function in pressure-overload induced cardiac hypertrophy. This increase in channel function is abolished with specific inhibition of calcineurin. Control =

sham-operated; TAB = thoracic aortic banding; CsA = cyclosporine A. (Wang Z et al., 2001a).

CHAPTER TWO: PHYSICAL INTERACTION BETWEEN CALCINEURIN AND THE L-TYPE Ca^{2+} CHANNEL

Introduction

Calcineurin has been shown to physically interact with several ion channels and transporters, especially those involved in Ca^{2+}-signaling and homeostasis. For example, most recently Katanosaka and colleagues demonstrated interaction between calcineurin and the cardiac sodium-calcium exchanger (NCX1) (Katanosaka Y et al., 2005). Using a yeast two-hybrid screening system, they further mapped the interaction sites to the C-terminus of calcineurin, containing the auto-inhibitory domain, and the β1 repeat of the central cytoplasmic loop of NCX1. Prior to these studies, Bandyopadhyay et al. had demonstrated physical association between calcineurin and the ryanodine receptor (RyR) in rat cardiac tissue (Bandyopadhyay A et al., 2000). In this instance, physical interaction was dependent upon Ca^{2+} (and presumably calcineurin activation state) as the interactions were disrupted in the presence of EGTA or FK-506 (a calcineurin inhibitor). This interaction between calcineurin and RyR may not be all too surprising, as many studies, including seminal work by Dr. Andrew Marks at Columbia University, NY, have established a direct and crucial regulatory interaction between the immunophilin FKBP12.6 and the RyR in the heart (Brillantes AB et al., 1994). Moreover, the interaction between

immunophilins, such as FKBPs, and calcineurin is well known (Milan D et al., 1994; Husi H et al., 1994). Therefore, calcineurin, through its association with FKBPs, could be recruited to the RyR. Such a possibility was further explored by Cameron et al. who used a yeast "three-hybrid" screening strategy to identify the association between calcineurin, FKBP12 and the inositol 1,4,5-trisphosphate receptor (IP_3R) (Cameron AM et al., 1995; Cameron AM et al., 1997).

More specifically, Lukyanetz et al reported evidence for co-localization of calcineurin and voltage-operated Ca^{2+} channels in dorsal root ganglion neurons (Lukyanetz EA, 1997). While Lukyanetz specifically investigated the distribution of the channel β-subunit, it can only be surmised that the N-type Ca^{2+} channel isoform ($Ca_V2.2$), an abundant isoform in the brain but not expressed in heart (Catterall WA et al., 2005), rather than the L-type channel ($Ca_V1.2$), was studied as a follow up to their earlier report (Lukyanetz EA et al., 1996). Nonetheless, the proximity of calcineurin and the L-type Ca^{2+} channel in neurons gained prominence with John D. Scott's continuous discoveries of A-kinase anchoring proteins (AKAPs) as seminal scaffolding elements responsible for the convergence of diverse signaling molecules. AKAP 79/150 was shown to be especially potent in combining PKA, calcineurin and PKC (Klauck TM et al., 1996) to the L-type Ca^{2+} channel as a macromolecular complex (Altier C et al., 2002). In fact, as recently as last year, the fundamental connection between calcineurin and the neuronal L-type Ca^{2+} channel through AKAP79 was

investigated in thorough detail, where calcineurin was shown to antagonize PKA-mediated regulation of the channel (Oliveria SF et al., 2007). In agreement, early on in my experiments, I also established an interaction between calcineurin and the L-type Ca^{2+} channel $\alpha_1$1.2 subunit in co-immunoprecipitation experiments from rat whole-brain lysates (**Figure A1**, see Appendices).

Here, using intensive biochemical and molecular-biology techniques, I report evidence that calcineurin directly binds to the cardiac L-type Ca^{2+} channel. Furthermore, I demonstrate that calcineurin, a protein phosphatase, recognizes the L-type channel $\alpha_1$1.2 subunit as an enzymatic substrate *in vitro*. As far as I know, this is the first and only report of such a physical interaction between these two Ca^{2+}-signaling proteins in the heart.

Results

Calcineurin co-localizes with the L-type Ca^{2+} channel

Calcineurin is a heterodimer of a catalytic A subunit (CnA) and a Ca^{2+}-binding regulatory B subunit (Klee CB et al., 1988). To determine whether calcineurin is a component of the L-type Ca^{2+} channel macromolecular complex, I immunoprecipitated $\alpha_1$1.2 from rat ventricular lysates and probed the resulting immunoblot with a CnA-specific antibody. A strong band migrating at ~61 kD was readily identified (**Figure 2.1A**). Directly immunoprecipitated CnA migrated at the same level, suggesting that this 61 kD band corresponds to CnA. In reciprocal experiments, anti-CnA co-immunoprecipitated two bands at 240 and 190 kD, which migrated at the same level as immunoprecipitated $\alpha_1$1.2. These bands are consistent with the full-length and processed $\alpha_1$1.2 isoforms prevalent in heart. A negative control using preimmune IgG did not immunoprecipitate either protein.

These data suggested that calcineurin co-localizes with the L-type Ca^{2+} channel complex in ventricular lysates. I also found an association between CnA and $\alpha_1$1.2 in immunoprecipitations from brain extracts (**Figure A1**, see Appendices), indicating that the association between these two endogenous proteins occurs in native tissues (i.e. not requiring protein over-expression).

To determine whether $\alpha_1$1.2 and CnA co-localize within cardiac myocytes, I performed immunohistochemistry on mouse heart tissue. Antibody against CnA (red) revealed localization of CnA to Z-bands (**Figure 2.1B**), consistent with other reports (Santana LF et al., 2002). Given this, I tested for co-localization by co-staining with antibodies against $\alpha_1$1.2 and CnA. Image overlay revealed co-localization (yellow; merge) of $\alpha_1$1.2 (green) and CnA (red) at Z-bands (**Figure 2.1B**). As a positive control, I co-stained for $\alpha_1$1.2 and α-actinin, an abundant protein found at Z-bands (**Figure A2**). $\alpha_1$1.2 localized at the Z-bands, as expected (Carl SL et al., 1995; Scriven DR et al., 2000). Together, these data provide support for co-localization of $\alpha_1$1.2 and calcineurin in ventricular cardiomyocytes.

Direct interaction between calcineurin and $\alpha_1$1.2

To map the calcineurin binding site within $\alpha_1$1.2, I engineered overlapping GST-fusion proteins spanning the intracellular domains of cardiac $\alpha_1$1.2. The lengthy C-terminus was further subdivided into numbered CT-fragments (**Figure 2.2A and Table 1**). Equal quantities of $\alpha_1$1.2 GST-fusion proteins were used to pull down CnA from tissue lysates. A consistent subset of GST-fusion proteins (CT-4, CT-8, CT-D) corresponding to overlapping regions of $\alpha_1$1.2 pulled down CnA from both cytosolic and membrane fractions (**Figure 2.2B**). Importantly,

CT-4, CT-8 and CT-D overlap considerably, and CT-8 (aa 1909-2029) is redundant to CT-4. Together, these findings suggested that calcineurin interacts with residues 1909-2029 of the cardiac $\alpha_1$1.2 protein. The N-terminus of $\alpha_1$1.2 also pulled down calcineurin, suggesting that calcineurin and $\alpha_1$1.2 may have multiple sites of interaction.

To test whether the interaction between calcineurin and $\alpha_1$1.2 is direct or mediated by intervening proteins, GST-$\alpha_1$1.2 fusion proteins were incubated with purified recombinant calcineurin (CnA/B, expressed in bacteria as a complex of catalytic A and regulatory B subunits). Immunoblots probed for CnA revealed an identical pattern of GST-fusion protein interactions as was seen with native lysates from heart or brain (CT-4, CT-8, CT-D and N-term) (**Figure 2.2C**). These data, then, demonstrate a specific and direct interaction between CnA and $\alpha_1$1.2 without requirement of intermediary scaffolding elements.

Search for a specific calcineurin-docking motif on $\alpha_1$1.2

Interestingly, the minimal interaction region (aa 1909-2029, encoded by CT-8) contained several potential PxIxIT motifs, a calcineurin-binding domain found in a variety of calcineurin-interacting partners (Feske S et al., 2003) (**Figure 2.3A, and Figure A3**). I tested whether one such potential motif was responsible for calcineurin binding to the $\alpha_1$1.2 C-terminus by mutating residues

PSI to AAA (aa 2018-2020) on CT-4. In GST pulldown experiments, CnA was able to bind wild-type CT-4 and CT-4 PsI-AAA with similar efficiency, suggesting this region is not the binding site (**Figure 2.3B**). As a second test, I performed competition binding assays utilizing overlapping peptides spanning amino acids 1976-2029 of CT-8, the region containing the potential PxIxIT motifs (**Figure A3**). Despite the presence of several-fold molar excess of peptide relative to both CnA and CT-8, CnA remained capable of avid binding to the channel (**Figure 2.3C**).

Chou-Fasman/Robson-Garnier algorithms (Garnier J et al., 1996) applied to the primary amino acid sequence of CT-8 (aa 1909-2029) suggested the presence of two distinct halves: an N-terminal domain (aa 1909-1968) containing an alpha-helix, and a C-terminal domain (aa 1969-2029) which contains the potential PxIxIT motifs and manifests no definitive structure (**Figure 2.3D**). As noted above, peptide competition assays suggested that the unstructured, C-terminal region is not responsible for binding to CnA. Thus, I analyzed the N-terminal alpha-helix for participation in $\alpha_1$1.2-CnA binding. To test this, I engineered both N- and C-terminal truncations of CT-8 (**Figure 2.3E**). GST pulldowns revealed binding of CnA to amino acids 1909-1971 and 1943-2029 within CT-8, implicating the alpha-helix (encapsulated by aa. 1943-1971) as sufficient to bind CnA (**Figure 2.3E**). To test this further, I substituted a proline residue for an alanine in the alpha-helix (CT-8 helix-Pro) to disrupt its structure.

Disruption of helical structure (**Figure 2.3F**) rendered the entire CT-8 fusion protein incapable of binding CnA (**Figure 2.3F**). Together, these data establish amino acids 1943-1971 of $\alpha_1$1.2 as the minimal region required for CnA-$\alpha_1$1.2 interaction.

The majority of the studies described above, while extensive, were performed with the use of GST-fusion proteins. As a caveat, the expression and purification of GST-fusion proteins in bacteria often represents a challenge due to early termination during synthesis or excessive degradation after rigorous detergent solubilization (**Figure 2.2B**). To by-pass these, I generated clones of the $\alpha_1$1.2 C-terminus fused to myc-epitope tags in plasmid constructs specific for expression in mammalian cells. In the experiments, I co-expressed calcineurin fused to GFP (~75 kD) for better identification and separation from heavy-chain bands (~50 kD), as well as, for a direct marker of transfection efficiency. Co-immunoprecipitation experiments from HEK 293 cell lysates using anti-myc agarose showed that calcineurin bound only $\alpha_1$1.2 portions containing aa 1909-2029 (**Figure 2.4**). These data are consistent with our GST pulldown experiments.

Calcineurin binds $\alpha_1$1.2 C-terminus with high affinity

To begin to quantify the affinity of interaction between $\alpha_1$1.2 and CnA, I performed GST pulldown experiments using varying amounts of CT-8 while

maintaining a constant, low concentration of recombinant calcineurin (15 nM) (**Figure 2.5**). Purified immobilized CT-8 was added in sequential approximate concentrations of 7.5, 22.5 and 45 nM, and supernatants were collected after each pulldown to assess bound (P; pellet) versus unbound (S; supernatant) calcineurin. Approximately equivalent amounts of CnA co-sedimented when CT-8 was present at half the concentration of CnA (CT-8:CnA = 0.5), whereas almost all the CnA was bound when more than three times as much CT-8 was present (CT-8:CnA $\geq$ 3). These data lend credence to the specificity of the pulldown assays, and they suggest that the interaction between $\alpha_1$1.2 and CnA occurs at high affinity.

Calcineurin regulatory region is required for $\alpha_1$1.2 binding

Experiments reported so far were performed in the presence of EGTA, which inactivates calcineurin by chelating Ca^{2+}, suggesting that $\alpha_1$1.2 binds CnA in its inactive conformation. Interestingly, I observed similar levels of binding when EGTA was absent in cardiac lysates (**Figure 2.6A**), a condition where free intracellular Ca^{2+} would allow for activation of enzymes. I also observed that CnA was able to bind CT-8 in the presence of cyclosporine A (CsA 10 μM) (**Figure 2.6B**), an inhibitor which blocks the calcineurin catalytic active-site (Hemenway CS & Heitman J, 1999). These data suggested that the calcineurin

catalytic site was not necessary for interaction with $\alpha_1$1.2. To test further the role of the catalytic domain, I expressed full-length CnA (F.L.) or a constitutively-active mutant (CnA*), which lacks the regulatory region that overlaps and obstructs the catalytic domain to inactivate the enzyme (**Figure 2.6C**). In six independent experiments, only F.L. (61 kD) bound CT-8, whereas no signal was detected with lysates of cells expressing CnA* (48 kD) (**Figure 2.6D**). Similar results were obtained using lysates from cells co-expressing both F.L. and CnA* (**Figure 2.6E**). Taken together, these findings suggest that the regulatory region is required for calcineurin A binding to the $\alpha_1$1.2 C-terminus.

Phospho-$\alpha_1$1.2 is a calcineurin substrate

As the Ca^{2+} channel is subject to regulation by several kinases and phosphatases, I set out to determine the functional significance of $\alpha_1$1.2-CnA binding. First, to determine whether $\alpha_1$1.2 is a potential substrate for calcineurin, GST-fusion proteins were phosphorylated *in vitro* by recombinant PKA and then tested for dephosphorylation by calcineurin. Initially, I studied overlapping fusion proteins CT-4 and CT-8, as both bind calcineurin and both contain the PKA/PKC/PKG site, Ser-1928 (**Figure 2.2A**). As expected, both CT-4 and CT-8 were readily phosphorylated by PKA (**Figure 2.7A**). The CT-B fragment of $\alpha_1$1.2, which is adjacent to CT-8 but does not bind CnA, was used as a control;

CT-B was phosphorylated to only a modest extent. As a further control, similar experiments were performed on the N-terminus of $\alpha_1$1.2, which binds CnA and can be phosphorylated by PKC (Kamp TJ & Hell JW, 2000) but does not contain consensus sequences for PKA phosphorylation (**Table A1**, see Appendices). This fragment was not phosphorylated by recombinant PKA (**Figure 2.7A**).

To determine whether these sites are dephosphorylated by calcineurin, I then incubated ^{32}P-phosphorylated CT-4, CT-8, and CT-B with purified recombinant calcineurin (CnA/B). Here, I observed marked decreases in the phosphorylation of $\alpha_1$1.2 fragments CT-4 and CT-8 (**Figure 2.7A**), consistent with their serving as substrates for calcineurin phosphatase. Phosphate incorporation in CT-B, a fragment which does not bind CnA, also appeared to be marginally reduced when treated with recombinant calcineurin, albeit not to the extent of CT-4 and CT-8, suggesting the presence of additional dephosphorylation sites of calcineurin contained within the $\alpha_1$1.2 C-terminus encoded by CT-B.

To test the specificity of CT-8 phosphorylation, an alanine was substituted for serine at position 1928 (S1928A), and the mutant CT-8 fusion protein was subjected to *in vitro* phosphorylation by PKA. PKA failed to induce phosphorylation of the mutated CT-8 (**Figure 2.7B**), indicating that the radioisotope incorporated on CT-8 is specific to Ser-1928. Together, these data, summarized in **Table 2**, demonstrate that calcineurin is capable of recognizing

$\alpha_1$1.2 as a substrate *in vitro*. Further, calcineurin dephosphorylates those $\alpha_1$1.2 fragments to which it binds, demonstrating specific targeting.

Figures

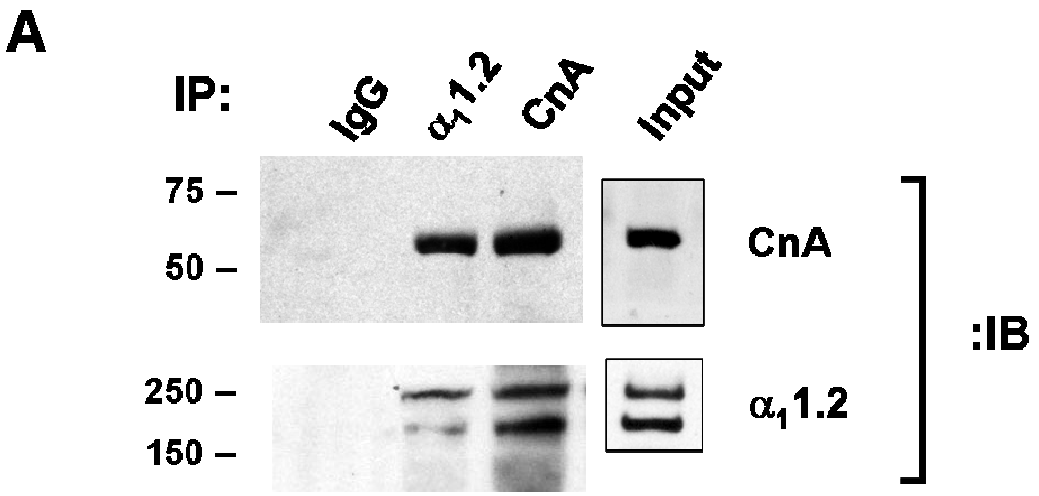


Figure 2.1: Calcineurin associates with L-type Ca^{2+} channel. A. Immunoprecipitations (IP) of $\alpha_1$1.2 and CnA from rat ventricular lysate, and immunoblots (IB) for both. Immunoprecipitation of $\alpha_1$1.2 and CnA reciprocally pulled down the other protein. Non-specific IgG was used as a negative control.

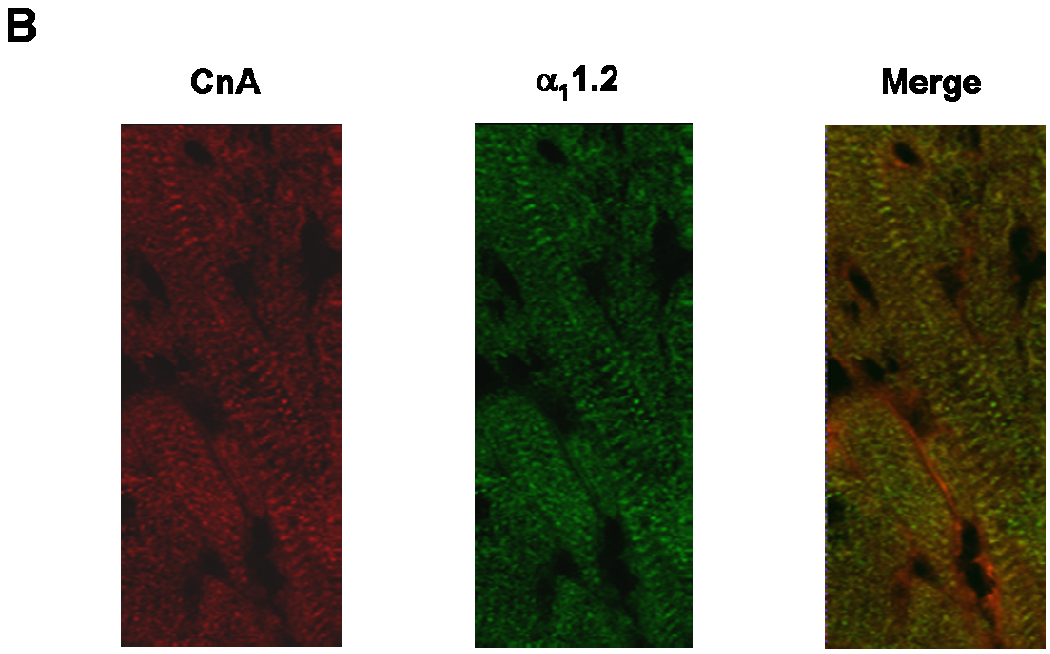


Figure 2.1: Calcineurin co-localizes with L-type Ca^{2+} channel. B. Confocal immunofluorescence images of adult mouse heart sections co-stained for CnA (red) and $\alpha_1$1.2 (green). Merged image demonstrates co-localization of both proteins (yellow).

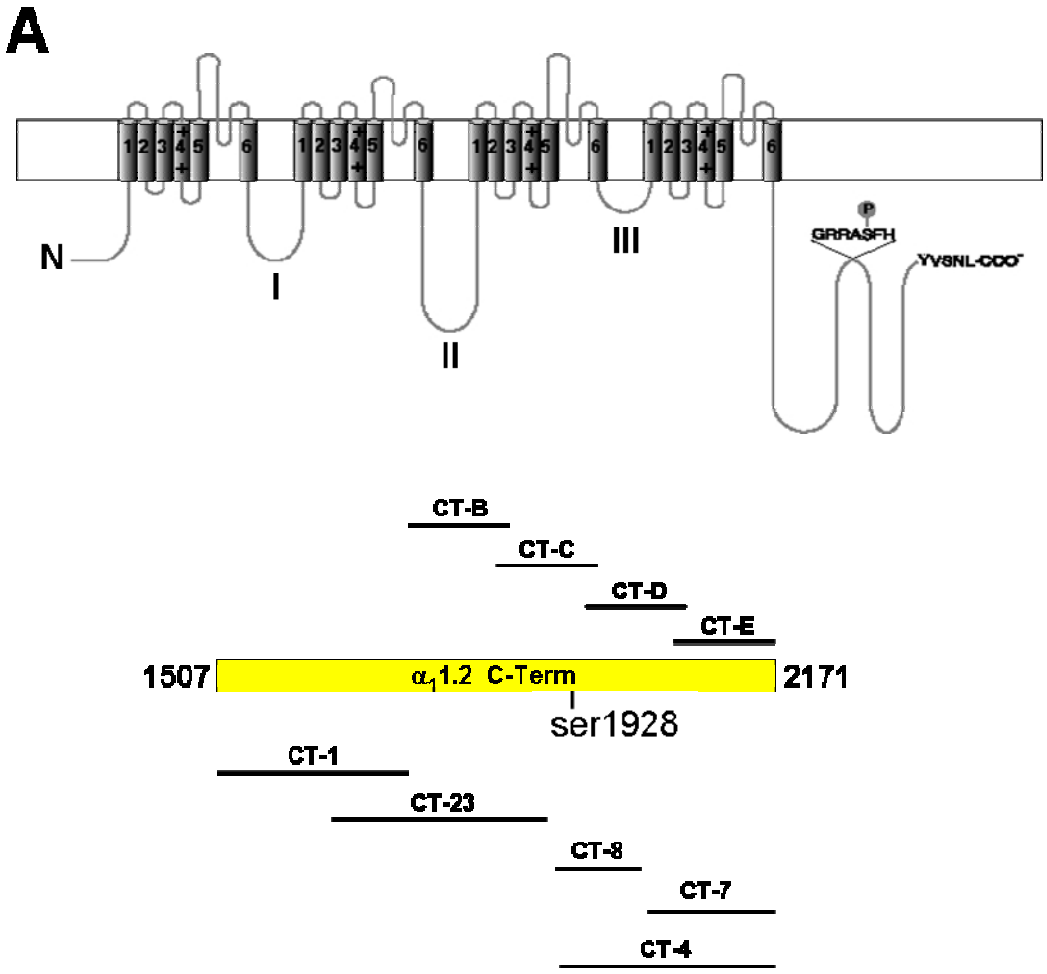


Figure 2.2: Schematic diagram of $\alpha_1$1.2. A. The C-terminus (CT) further subdivided into overlapping regions. GRRASFH contains Ser-1928; YVSNL-COO^- represents the C-terminal end of $\alpha_1$1.2.

Table 1. Amino acid residues in $\alpha_1$1.2 GST-fusion proteins.

Fusion	Rabbit cardiac $\alpha_1$1.2	Rat brain $\alpha_1$1.2
N-Term	1-154	**1-124**
Loop I	**437-554**	409-526
Loop II	**785-930**	754-901
Loop III	**1196-1248**	1165-1219
C-Term	1507-2171	1477-2140
CT-1	**1507-1733**	1477-1703
CT-4	**1909-2171**	1879-2140
CT-7	**2030-2171**	2000-2140
CT-8	**1909-2029**	1879-1999
CT-23	**1622-1905**	1592-1875
CT-B	1724-1847	**1694-1817**
CT-C	1834-1957	**1804-1927**
CT-D	1944-2067	**1914-2037**
CT-E	2054-2171	**2024-2140**
PKA/PKC/PKG site	**Ser 1928**	Ser 1898

Sequences listed in bold font were synthesized and studied here. Sequences not listed in bold are provided to facilitate comparison between rabbit cardiac and rat brain $\alpha_1$1.2.

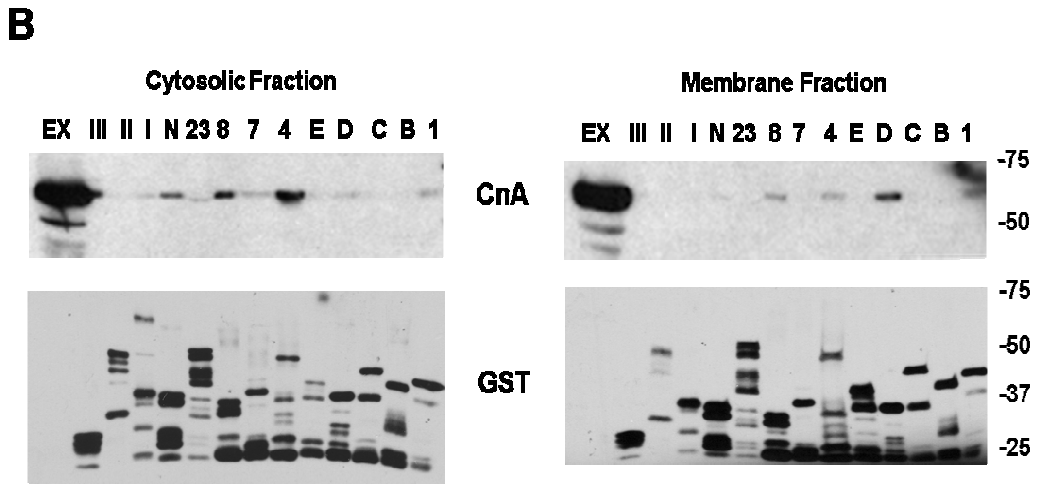


Figure 2.2: Calcineurin binds to C- and N-termini of $\alpha_1$1.2. B. Representative GST-pulldown experiments from rat ventricular lysate (cytosolic fraction, left; membrane fraction, right) probed for CnA reveals binding of CnA to CT-4 and CT-8, variable binding to CT-D, and N-terminus. Ex = tissue extract input. Lane numbers/alphabets represent GST-fusion protein, e.g. 8 = CT-8.

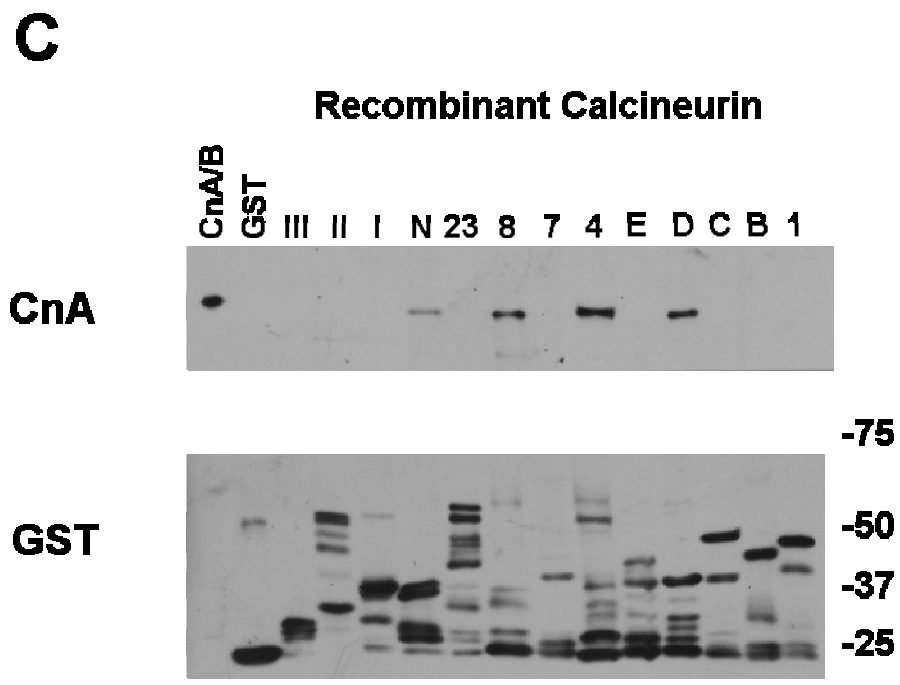


Figure 2.2: Calcineurin binds directly to C- and N-termini of $\alpha_1$1.2. C. Pulldown with recombinant calcineurin A and B subunits (CnA/B) shows direct binding of CnA to CT-8, CT-4, CT-D, and N-terminus. CnA/B = recombinant calcineurin A and B input. Lane numbers/alphabets represent GST-fusion protein, e.g. 8 = CT-8.

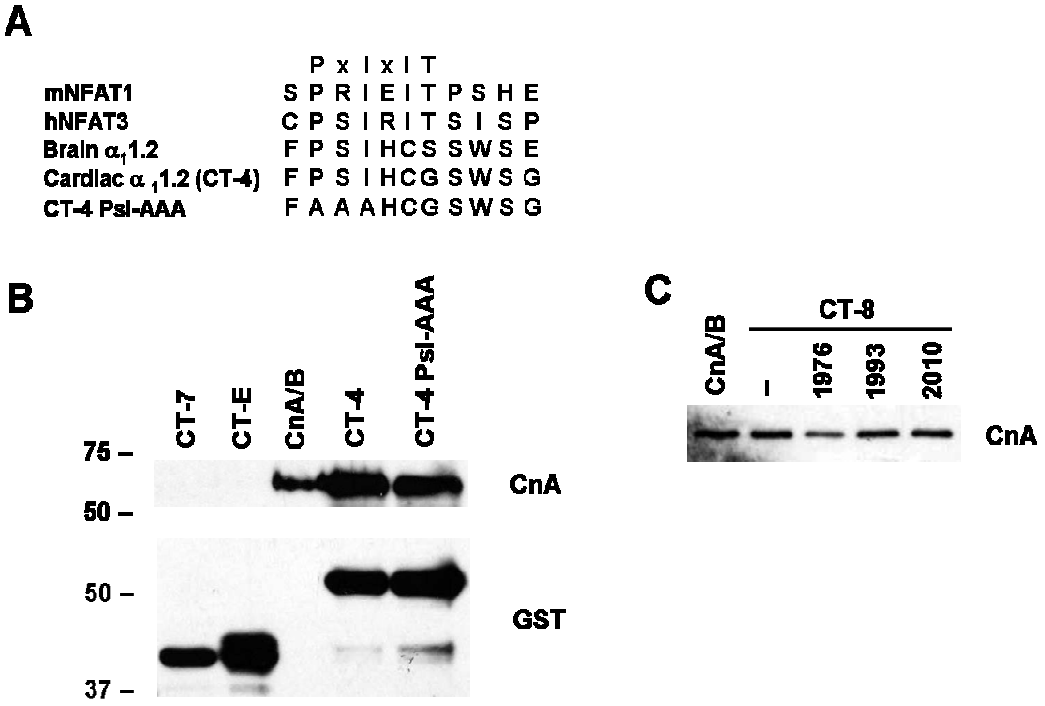


Figure 2.3: Calcineurin-$\alpha_1$1.2 interaction mapping. **A.** Potential PxIxIT motifs on $\alpha_1$1.2 CT-4. Alignment of primary amino acid sequences of murine NFAT1 (mNFAT1), human NFAT3 (hNFAT3), rat brain $\alpha_1$1.2, rabbit cardiac $\alpha_1$1.2, and the mutated CT-4 construct. **B.** GST-pulldowns of recombinant CnA using CT-4 or CT-4 PSI→AAA revealed comparable binding of CnA to each fusion protein. The membrane was re-blotted with anti-GST to confirm equivalence of fusion protein concentrations. **C.** Competition-binding assays were

performed with peptides spanning CT-8. Peptides were incorporated in molar excess (10 μM) relative to CnA/B (20 nM) and CT-8 (100 nM).

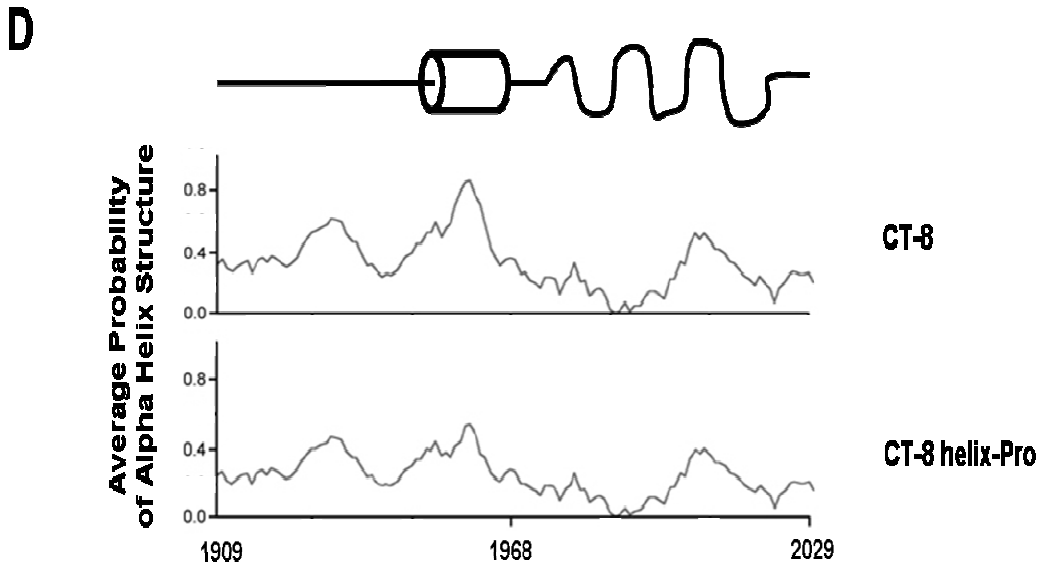


Figure 2.3: CT-8 (aa 1909-2029 of $\alpha_1$1.2) topology. **D.** Robson-Garnier prediction of CT-8 secondary structure. CT-8 contains one alpha-helix (within aa. 1943-1971) and an unstructured globular region (aa. 1969-2029).

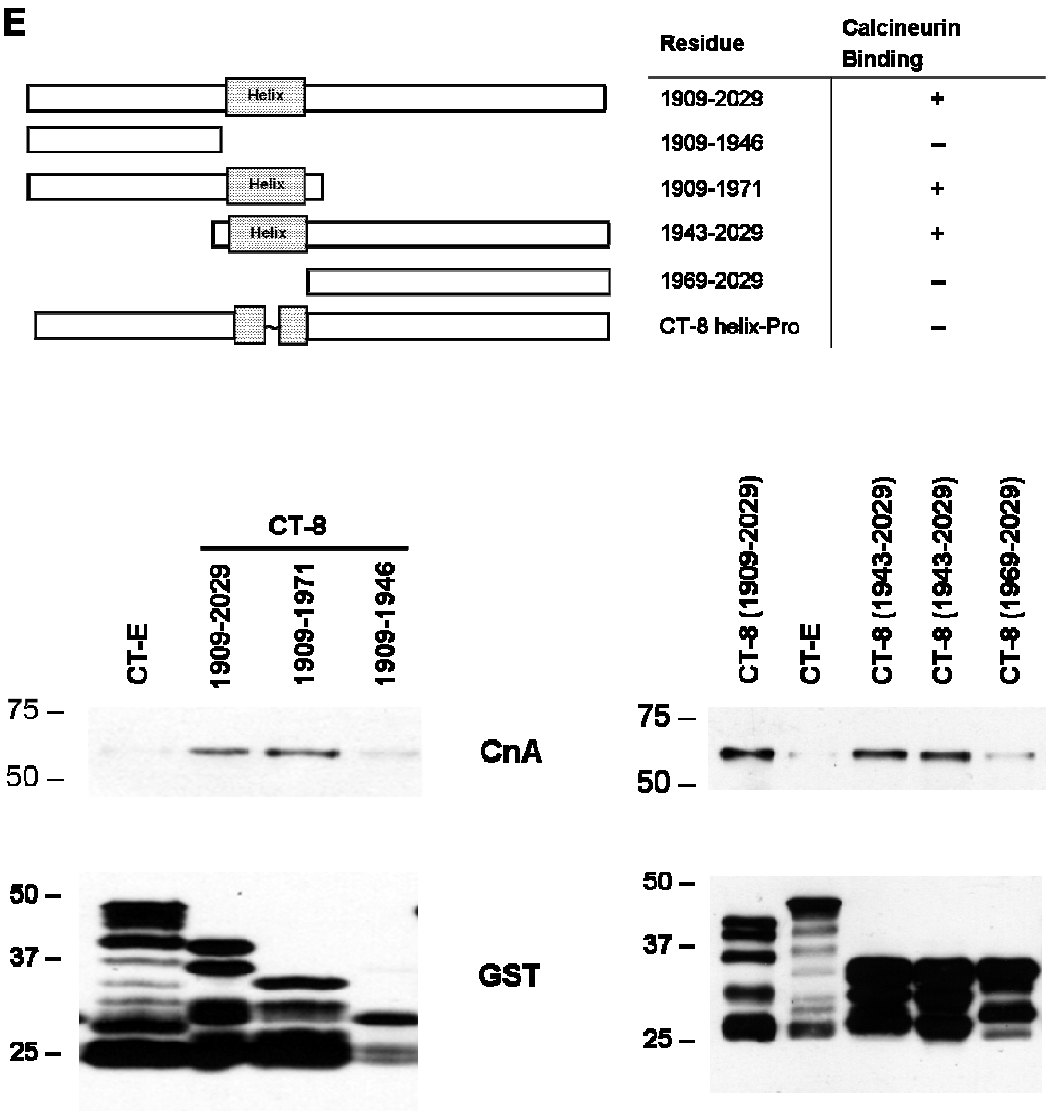


Figure 2.3: Calcineurin binds $\alpha_1$1.2 at aa 1943-1971. E. CT-8 truncations, depicted in tabular form with their respective CnA binding properties, were used in pulldowns from tissue extracts.

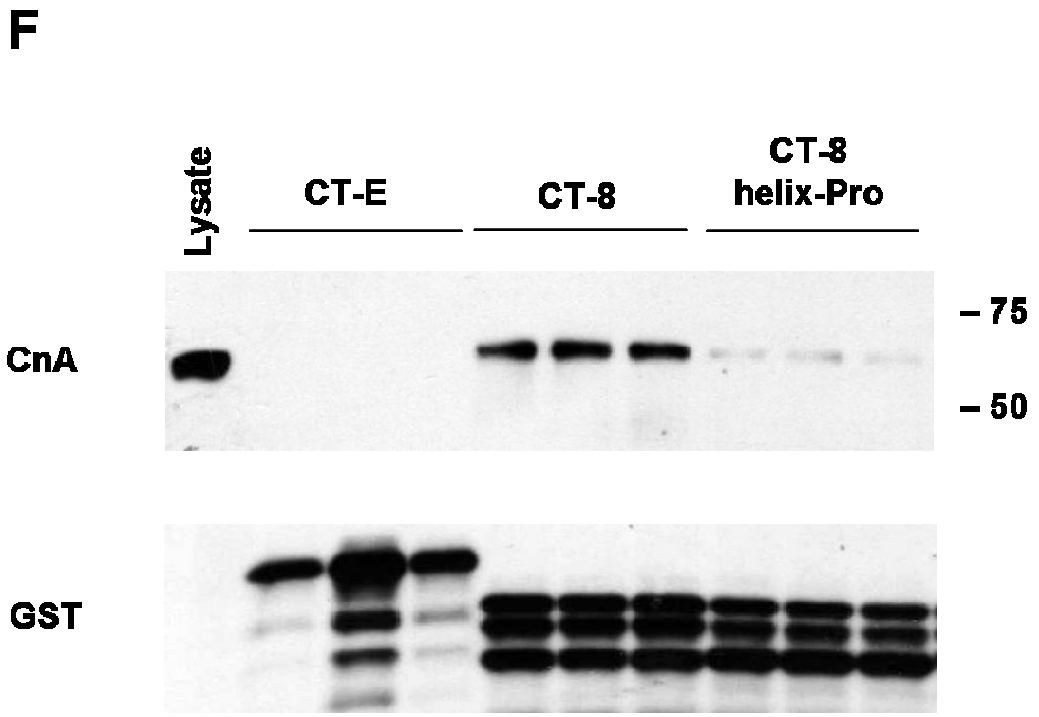


Figure 2.3: $\alpha_1$1.2 aa 1943-1971 is required for calcineurin-binding. F. Pulldown experiments with mutant CT-8 containing a single proline insertion in the CT-8 alpha-helix encompassed within aa 1943-1971 (CT-8 helix-Pro).

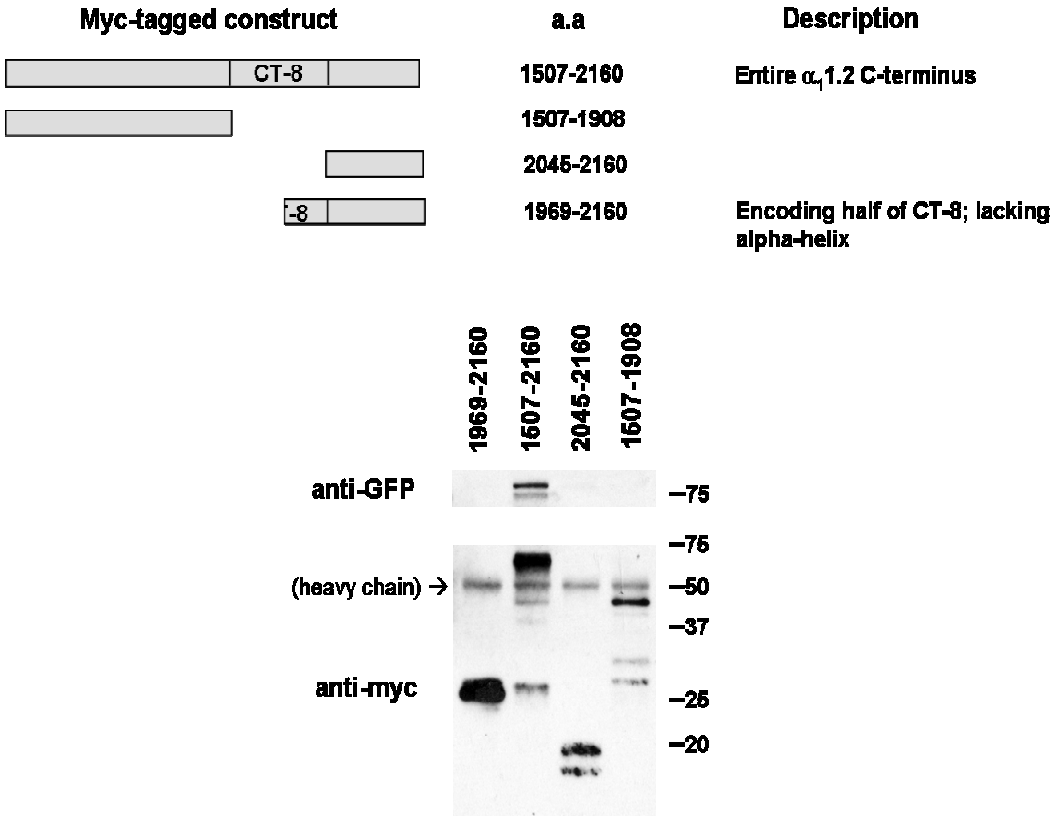


Figure 2.4: Calcineurin-$\alpha_1$1.2 interaction mapping. Immunoprecipitation experiments of myc-tagged $\alpha_1$1.2 C-terminus constructs co-expressed in HEK 293 cells with GFP-CnAα. Immunoblots for GFP show CnA binds $\alpha_1$1.2 containing aa. 1507-2160, but not the other fusion proteins.

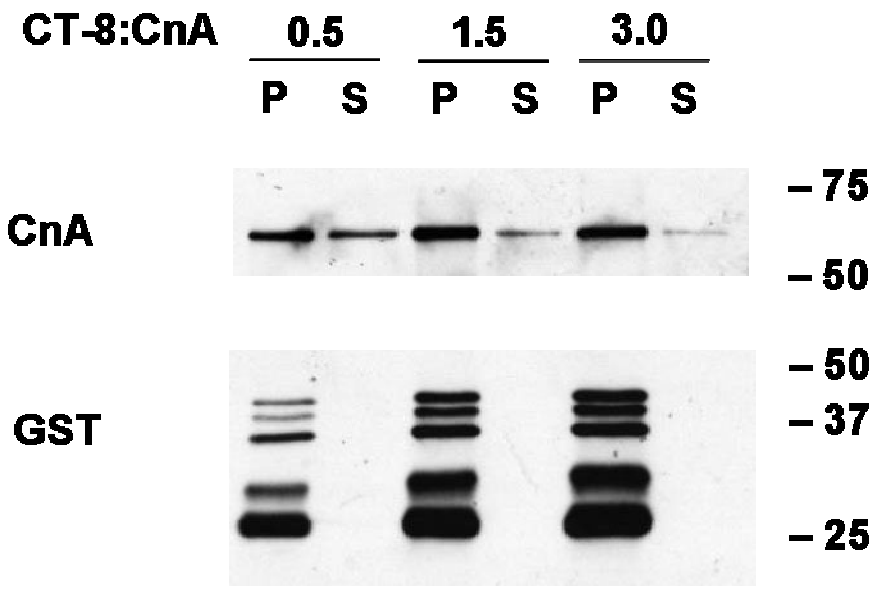


Figure 2.5: Calcineurin binds $\alpha_1$1.2 with high affinity. Representative GST-pulldown experiment with recombinant CnA/B (15 nM) and increasing concentrations (7.5, 22.5 and 45 nM) of CT-8. After each pulldown, supernatant (S) was saved to compare unbound to bound CnA co-sedimenting with bead-immobilized GST-CT-8 pellet (P). CT-8:CnA = molar ratio of CT-8 to CnA.

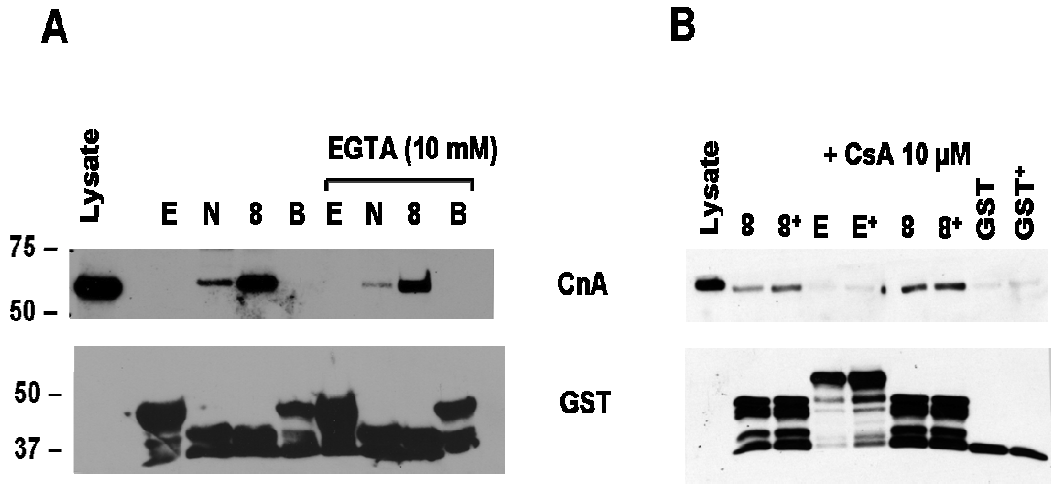


Figure 2.6: $\alpha_1$1.2 binds calcineurin regulatory region. Representative GST pulldown experiment using ventricular lysates prepared in the presence-vs-absence of 10 mM EGTA (**A**) or 10 µM Cyclosporine A (CsA) (**B**) revealing equivalent interaction of endogenous CnA. Lane numbers/alphabets represent GST-fusion protein, e.g. 8 = CT-8.

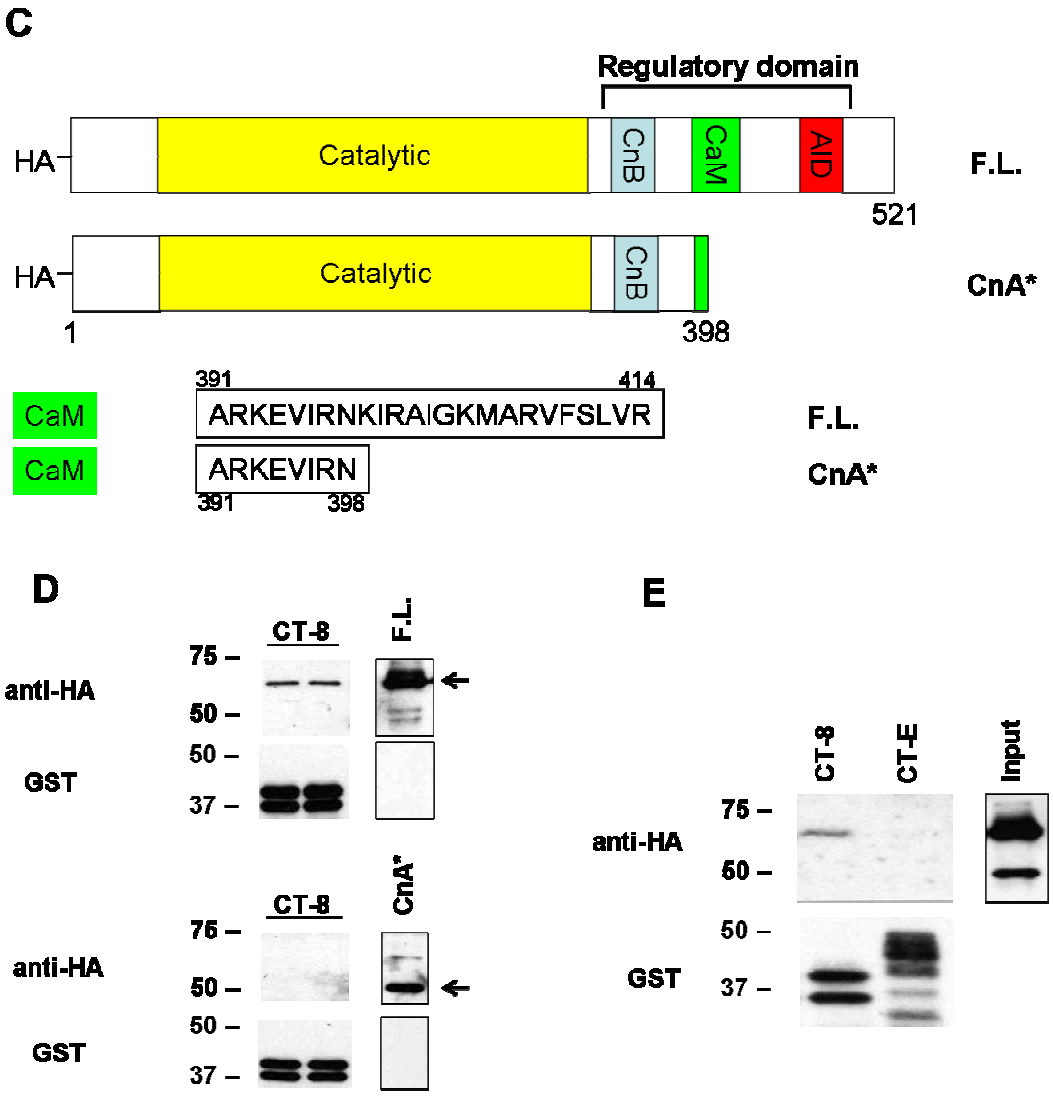


Figure 2.6: $\alpha_1$1.2 binds calcineurin regulatory region. **C.** Schematic representation of full-length (F.L.) and constitutively-active (CnA*) calcineurin constructs. Both proteins contain an N-terminal HA tag for identification from endogenous CnA. CnA* is truncated at aa. 398, and lacks the AID and most of

the CaM domain. CnB = calcineurin B binding domain, CaM = calmodulin binding domain, AID = auto-inhibitory domain. **D.** GST-pulldowns with lysates from HEK 293 cells expressing either F.L. or CnA*. Only F.L. (61 kD) bound CT-8 as detected by anti-HA immunoblot. **E.** Pulldowns were repeated with cells co-expressing F.L and CnA*.

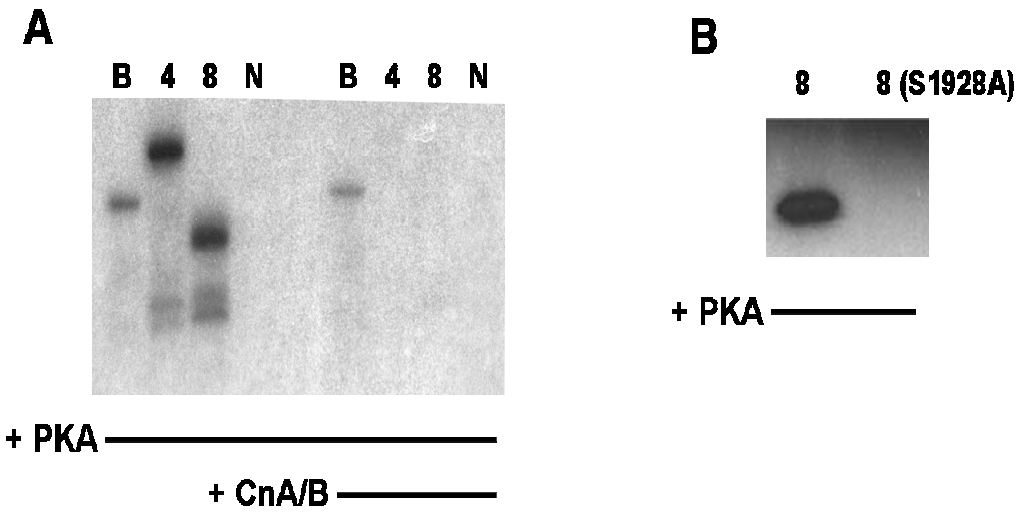


Figure 2.7: Calcineurin recognizes phospho-$\alpha_1$1.2 as a substrate. A. Representative autoradiogram of $\alpha_1$1.2 GST-fusion proteins phosphosphorylated with recombinant catalytic PKA and subsequently treated with recombinant calcineurin (CnA/B). PKA phosphorylates CT-8 and CT-4, which contain Ser-1928, as well as CT-B but not the N-terminus. Calcineurin dephosphorylates CT-8 and CT-4 completely, and CT-B to a lesser extent. **B.** Representative autoradiogram demonstrating that PKA does not phosphorylate mutant CT-8 containing a S1928A substitution. Lane numbers/alphabets represent GST-fusion protein, e.g. 8 = CT-8.

Table 2. ***In vitro*** **phosphorylation/dephosphorylation of $\alpha_1$1.2 GST-fusion proteins.**

fusion protein	PKA phosphorylated?	binds calcineurin?	calcineurin substrate?
CT-4	Yes	Yes	Yes
CT-8	Yes	Yes	Yes
CT-B	Yes	No	No
N-term	No	Yes	NA

Interpretation and Conclusions

Evidence for physical association

The results presented herein provide strong evidence for a direct physical interaction between calcineurin and L-type Ca^{2+} channel in cardiac tissue. Notably, co-immunoprecipitation studies from rat ventricular lysates show evidence for a physical association between calcineurin and the major pore-forming subunit of the L-type channel, $\alpha_1$1.2, regardless of the protein immunoprecipitated first (**Figure 2.1A**). The presence of this association between the two proteins from native lysates, without the need for over-expression in a heterologous setting, highlights the physiological specificity of this interaction. Immunohistochemistry on sections from mouse-heart further corroborate the findings by demonstrating co-localization of calcineurin and $\alpha_1$1.2 (**Figure 2.1B**). This particular experiment is very meaningful, especially given the paucity of successful immunolocalization of calcineurin from native tissues, whether cryofrozen or paraffin-embedded/fixed. Santana et al. reported localization of calcineurin in adult cardiomyocytes via immunocytochemistry, albeit they used an antibody against calcineurin B, the regulatory subunit of calcineurin (Santana LF et al., 2002), which at least in yeast has been purified from cell membrane fractions where it is believed to target possibly via myristoylation (Zhu D et al., 1995). If their data are true, then this raises the intriguing possibility that

calcineurin could associate with $\alpha_1$1.2 through its regulatory subunit, a possibility encompassing my results. Consistent with my data, others also report calcineurin localization to Z-disks in neonatal cardiac- and skeletal myocytes. (Frey N et al., 2000b; Torgan CE, 2006). Furthermore, my immunohistochemistry data suggest that not all calcineurin co-localizes with $\alpha_1$1.2 (presence of individual red/green signals in the merged image, **Figure 2.1B**), consistent with other reports of calcineurin localization with a host of other scaffolding proteins and cellular elements and suggesting the presence of specific calcineurin-signaling domains within the cardiomyocyte (Rusnak F & Mertz P, 2000).

Mapping the binding site within $\alpha_1$1.2

The subsequent extensive GST pulldown experiments further mapped the calcineurin-binding site within $\alpha_1$1.2, and demonstrated aa 1943-1971 within the C-terminus of $\alpha_1$1.2, as required for calcineurin interaction. The fact that a single residue substitution of a proline for an alanine at aa 1959 virtually abolished calcineurin–CT-8 interaction (**Figure 2.3F**) lends strong support for the requirement of that short region, encapsulating a putative alpha-helix, as the potential calcineurin-docking element. Interestingly, a GST-fusion construct expressing just the amino acids comprising the putative alpha-helix alone (aa. 1949-1965) was insufficient to pulldown calcineurin. A possible reason for this

negative result is that the expression of such a small fragment alone is insufficient for the amino acids to adopt an alpha-helical structure. Alternatively, surrounding elements encompassing a larger portion of $\alpha_1$1.2 (aa. 1943-1971) contain additional sites responsible for calcineurin interaction, especially in the context of appropriate protein folding and conformation of the $\alpha_1$1.2 C-terminus within the cardiomyocyte milieu. GST-fusion proteins, whilst ideal for such mapping studies, by virtue of their mass expression in bacteria often incompletely represent the true conformation of the protein being studied. To address this concern, I repeated the experiments by re-designing $\alpha_1$1.2 constructs in mammalian expression vectors (e.g. pCMV-Tag1 from Stratagene), a strategy that would allow me to overcome some of the problems associated with bacterial expression and rigorous protein purification. Moreover, this method would also allow for expression of the $\alpha_1$1.2 fragments in a more appropriate cell environment along with a shorter epitope tag (myc) replacing the bulky GST. **Figure 2.4** depicts results consistent with my observations from GST pulldown experiments, and once again highlighted aa 1909-2029, encompassed by GST CT-8, as the region within the $\alpha_1$1.2 C-terminus as required for calcineurin interaction since only the fusion protein that contained aa 1909-2029 (protein expressing aa 1507-2160) bound calcineurin while those fusion proteins lacking aa 1909-2029 (i.e. proteins expressing aa 1507-1908 and 2045-2160) did not bind calcineurin. In fact, a myc-$\alpha_1$1.2 construct expressing aa 1969-2160 showed further that in the absence of the

putative alpha-helix (contained within aa 1943-1971), calcineurin was unable to interact with the $\alpha_1$1.2 C-terminus. Furthermore, these results were especially significant since the proteins studied encoded the entire $\alpha_1$1.2 C-terminus (aa 1507-2160) vs. aa 1929-2029 of CT-8.

The binding site of calcineurin responsible for interaction with $\alpha_1$1.2

My primary objective was to identify where calcineurin anchored on the L-type Ca^{2+} channel $\alpha_1$1.2 subunit. Therefore, the majority of experiments focused on mapping the $\alpha_1$1.2 subunit, whether by means of GST-fusion proteins or myc-tagged versions. Nevertheless, additional pulldown experiments using cardiac lysates made with or without EGTA in the lysis buffer revealed equal interaction between calcineurin and CT-8 (**Figure 2.6A**). Given the fact that calcineurin is a Ca^{2+}-dependent molecule, these data suggested that the activation state of calcineurin, as it is activated by Ca^{2+} (and CaM), was not important for calcineurin to physically interact with $\alpha_1$1.2. Of course, this is assuming that lysis buffer without EGTA would allow for dispersion of free intracellular Ca^{2+} and therefore activation of a host of proteins including calcineurin in the eventual lysate. The response of calcineurin to Ca^{2+}/CaM is well established, with a classical unfolding of the protein, release of catalytic site by the auto-inhibitory segment, and therefore activation of the holoenzyme (Hemenway CS & Heitman

J, 1999 and references therein). Following this logic, I speculated that calcineurin likely bound $\alpha_1$1.2 through its C-terminal regulatory region (containing the auto-inhibitory segment) and not its catalytic site, because otherwise in the presence of EGTA, and consequent Ca^{2+}-chelation and calcineurin-inactivation, the masked catalytic site would not be exposed to interact with $\alpha_1$1.2. So the natural conclusion was that calcineurin bound through segments other than the catalytic site. I tested this conclusion by making use of two calcineurin constructs – one that expressed the full-length calcineurin isoform, and another mutant protein that expressed only calcineurin without a large proportion of its regulatory region (including auto-inhibitory domain and majority of CaM-binding domain) (**Figure 2.6C**). This mutation, by virtue of absence of regulatory elements would express a constitutively-active calcineurin (CnA*) and lack the regulatory region to mask the catalytic site. My pulldown experiments of $\alpha_1$1.2 GST-fusion proteins with either calcineurin type, or both when co-expressed, (**Figures 2.6D-E**) showed a remarkable bias towards $\alpha_1$1.2 interaction with only full-length calcineurin and not CnA*. These data suggested that the regulatory region was required for calcineurin A to bind to the channel, and as such, is reminiscent of the interaction of calcineurin with the NCX1 (Katanosake Y et al., 2005).

Affinity of interaction

To begin to test the affinity of interaction between calcineurin and $\alpha_1$1.2, I repeated the GST pulldown experiments with recombinant calcineurin and CT-8. Both recombinant proteins gave me the opportunity to control for relative concentrations, and pulldown experiments showed that at a 3:1 molar ratio of CT-8:calcineurin (i.e. 45:15 nM, respectively), virtually all the calcineurin was bound to CT-8, and co-sedimented with the GST-sepharose pellet (**Figure 2.5**). These data give crucial insights into relative affinity of interaction, and suggested that both proteins bind within nanomolar concentrations, which would be physiologically relevant. Furthermore, they also provided me the chance to test for efficacy of the pulldown assay itself, and demonstrated specificity of interaction because regardless of GST-sepharose content, buffer contents, or any other contaminating factors in the different reaction tubes, calcineurin bound proportionally to the amount of $\alpha_1$1.2 CT-8 fusion protein.

Nevertheless, immunoblot for GST revealed significant laddering of CT-8 (**Figure 2.5**) suggesting the presence of additional CT-8 fragments than just the full-length protein (estimated size ~40 kD; top-most band). In fact, CT-8 concentrations were calculated on the basis of only the full-length protein (see **Methods** for details), and therefore likely underestimated the actual concentration of CT-8 fragments that were binding CnA. In light of the data presented above

(**Figures 2.3-2.4**), it can be inferred that at least $\alpha_1$1.2 C-terminal aa 1909-1973 are sufficient to bind CnA. This length of protein would correspond to approximately 33 kD (including GST = ~27 kD). Therefore, assuming that the fragmentation of CT-8 represents early-termination of protein expression in bacterial cells, the top three bands are likely to contain aa 1909-1973 (prediction based on molecular weight), and therefore actual concentration of CT-8 that binds CnA would be higher than the calculated values. For example, the middle band appears to be of equal intensity relative to the top-most band, while the bottom band of the three, can be approximated to signify 3-4 times as much protein. Therefore, the initial concentration of 45 nM for the top-most band is more likely to be 45 + 45 + (3 x 45) = 225 nM total CT-8 species capable of binding CnA. Thus, a molar ratio of 3:1 CT-8:CnA would equate to 225:15 nM, wherein virtually all the CnA is bound to CT-8 with negligible amounts in the unbound fraction (**Figure 2.5**). These data suggest a high affinity interaction between CnA and the $\alpha_1$1.2 C-terminus.

Calcineurin recognizes $\alpha_1$1.2 as a substrate

The localization of the calcineurin binding site on $\alpha_1$1.2 to the proximity of Ser-1928 immediately raised the prospect that calcineurin may target this well-established phosphorylation site. Seminal work from Drs. Marlene Hosey and

William Catterall established Ser-1928 within the $\alpha_1$1.2 C-terminus as the bona fide target site of phosphorylation by protein kinase A (PKA) and consequent activation of the L-type Ca^{2+} channel (DeJongh KS et al., 1996; Gao T et al., 1997). These primary findings were performed by reconstitution of the cardiac L-type Ca^{2+} channel subunits in a heterologous setting such as HEK 293 cells. Mutation of Ser-1928 to an alanine essentially abolished the effects of PKA stimulation by isoproterenol or forskolin treatment of the cells. More recently, however, those earlier reports have come under question. Dr. Brian O'Rourke's group repeated the same experiments but in cultured cardiomyocytes (Ganesan AN et al., 2006). They engineered adenoviral constructs that expressed dihydropyridine-insensitive L-type Ca^{2+} channels allowing them to distinguish between Ca^{2+} currents from recombinant proteins versus endogenous channels. By mutating Ser-1928 to alanine, or through C-terminal truncation studies, they reported similar effects of PKA regulation on L-type Ca^{2+} channel compared to wild-type channels, suggesting that Ser-1928, while potentially sufficient, was not necessary for PKA-dependent regulation of the channel. Furthermore, their studies highlighted the differences between the use of heterologous systems such as HEK cells versus cardiomyocytes, and the potential role of additional cardiomyocyte-specific proteins and signaling pathways that are absent in HEK cells. Steve O'Marx's group more recently has shown both PKC (Yang L et al., 2005) and PKG (Yang L et al., 2007) to target Ser-1928, as well. Given the

apparent central role of this residue as a phospho-substrate, and the fact that calcineurin is a protein phosphatase, I tested whether calcineurin recognized Ser-1928 as a substrate *in vitro*. As shown in **Figure 2.7A**, calcineurin completely dephosphorylated $\alpha_1$1.2 GST-fusion proteins CT-8 and CT-4 after ^{32}P incorporation by PKA. Furthermore, this enzymatic event seemed to occur specifically at Ser-1928 (**Figure 2.7B**).

Conclusion

Taken together, these data demonstrate a novel direct and high affinity bimolecular physical interaction between calcineurin and the cardiac L-type Ca^{2+} channel $\alpha_1$1.2 subunit.

Materials and Methods

Co-immunoprecipitation and Western blot — Whole rat brain and cardiac left ventricle were homogenized on ice using a Dounce homogenizer. For cytosolic fractions, tissues were homogenized in buffer containing (mM) 10 Tris, pH 7.4, 10 EDTA, 10 EGTA, 1% sucrose, supplemented with Mini-Complete® protease inhibitor tablets (Roche). Following gentle centrifugation (5,000 rpm, 3 min) the supernatant was subjected to high-speed ultracentrifugation (200,000 g, 15 min) to collect the cytosolic fraction. The resulting pellet was re-homogenized in membrane extraction buffer containing (mM) 10 Tris-HCL, pH 7.4, 20 EDTA, 10 EGTA, 150 NaCl, 1% Triton X-100, and protease inhibitors and centrifuged as before to collect the membrane fraction (supernatant). Immunoprecipitates were recovered with protein-A Sepharose (Zymed) using antibody against $\alpha_1$1.2 (Alomone), PP2B (Santa Cruz) and AKAP79 (Santa Cruz), and separated by 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF or nitrocellulose membranes and immunoblotted with anti-calcineurin antibody (BD Transduction Laboratories).

Fusion proteins and pulldowns — Rat brain and rabbit cardiac $\alpha_1$1.2 cDNAs were used to engineer GST-fusion proteins spanning the intracellular domains of $\alpha_1$1.2. The C-terminus was further subdivided into shorter overlapping fragments

and designated alphabetically (CT-B through CT-E) for clones expressing the rat brain $\alpha_1$1.2 isoform (Genbank M67515) and numerically (CT-1 through CT-23) for clones expressing the rabbit cardiac isoform (Genbank CAA33546). GST-fusion constructs containing intracellular loops I, II, and III were expressed from rabbit cardiac $\alpha_1$1.2 (**Table 1**). The sequences were amplified by polymerase chain reaction (PCR) and cloned into pGEX-4T1 vector (Amersham Biosciences) in frame with GST, at the BamH I and Not I sites as described previously (Hall DD et al., 2006). Clones were transformed in *E. coli* BL-21 strain, and fusion-protein expression was induced by isopropyl-β-D-thiogalactoside (IPTG). The proteins were solubilized in buffer containing (mM) 15 Tris-HCl, pH 7.4, 0.1mg/mL lysozyme, 1.5% N-lauroyl-sarcosine, 150 NaCl, 15 DTT, 10 EDTA, and protease inhibitors, and purified on GST-Sepharose beads (Amersham Biosciences). The immobilized fusion-proteins were then incubated with extracts from rat ventricle in the presence of 10 mM EGTA or 10 μM Cyclosporine A, where indicated. Pulldowns were also performed from purified, recombinant calcineurin Aα and B subunits, prepared and purified as described (Mondragon A et al., 1997), or from HEK 293 cell lysates over-expressing constitutively-active (CnA*) or full-length (F.L.) calcineurin tagged with HA (see Cell transfections below). For peptide competition assays, recombinant calcineurin was pre-incubated with peptide for up to 1 hr before pulldowns were performed with the GST-fusion proteins. For affinity studies, 15 nM recombinant calcineurin was

mixed with 7.5, 22.5 and 45 nM of immobilized and purified CT-8 in a final volume of 100 μL. The mixtures were incubated at 4°C for 2 hours and centrifuged at 5,000 rpm for 2 min. The supernatant fractions were aliquoted and pellets washed twice in the same buffer. Pellet and supernatant fractions were resolved by SDS-PAGE and analyzed by western blot for bound and unbound calcineurin, respectively. Protein concentration was estimated using a standard curve of recombinant carbonic anhydrase and albumin (Sigma). After each GST protein purification, each of the proteins were loaded in 10, 20 and 30 μL amounts along with the protein standard of known concentration and resolved by SDS-PAGE. The gel was then stained by Coommasie dye, and estimations of fusion protein concentrations were made by comparing the intensity of the band representing the expected size of the full-length fusion protein versus that of the standard.

Myc-tagged fusion proteins encoding the rabbit cardiac $\alpha_1$1.2 C-terminus of various lengths were sub-cloned into pCMV-Tag1 (Stratagene) mammalian expression vector. Clones were confirmed by sequencing and expressed in HEK 293 cells (see Cell transfections). Cell lysates were incubated with anti-myc agarose conjugate (Sigma) for 2 hrs. at 4°C, washed in lysis buffer, and resolved by SDS-PAGE followed by western blot.

Cell transfections — HEK 293 cells were cultured (37°C) in standard DMEM supplemented with 10% FBS. Cells were transfected at 60-70% confluency using Lipofectamine Plus reagent (Invitrogen) with constructs encoding the murine calcineurin Aα subunit (full-length; tagged with HA or GFP) and constitutively-active mutant (CnA*; tagged with HA) truncated at amino acid 398 (**Figure 2.6C**). When using the HA-tagged constructs, cells were co-transfected with pEGFP-C1 (BD Biosciences Clontech) to localize and quantify transfection efficiency. Twenty-four hours after transfection, cells were scraped and lyzed in buffer containing (mM) 10 Tris, pH 7.4, 150 NaCl, 1 DTT, 20% glycerol, 0.1% Triton-X 100, protease inhibitors (Roche) and phosphatase inhibitor cocktails I and II (Sigma), and used for experiments.

Site-directed mutagenesis — PCR was performed on cloned plasmid encoding the GST fusion protein CT-8 ($\alpha_1$1.2 aa 1909-2029), using the forward primer 5'-CTGGGTCGAAGGGCTGCCTTCCACCTGGAG-3' and reverse primer 5'-CTCCAGGTGGAAGGCAGCCCTTCGACCCAG-3' to replace serine-1928 with alanine. The template vector was digested with Dpn I, and the remaining mutant plasmid was directly transformed into *E. coli* XL1-Blue Supercompetent cells (Stratagene). Mutant plasmid was purified and sequenced to confirm mutation. A similar PCR approach was also used to mutate the putative PxIxIT motif in GST

fusion protein CT-4 (PsI → AAA; $\alpha_1$1.2 aa 2018-2020), using the forward primer 5'-CAACAGCAGCTTCGCGGCCGCCCACTGCGGC-3' and reverse primer 5'-GCCGCAGTGGGCGGCCGCGAAGCTGCTGTTG-3'. Two C-terminal truncations of CT-8 (aa 1909-1946 and 1909-1971) were made via substitution of Gln1946 and Arg1971, respectively, into stop codons. The N-terminal truncations of CT-8 (aa 1943-2029 and 1969-2029) were made via PCR using primers 5'-CGAGGATCCGACATCTCTCAGAAGACAGTCC-3' and 5'-TTGGATCCCTGCAGAGAAGCCATTCCC-3' along with the same reverse primer 5'-TCCGGGAGCTGCATGTGTCAGAGG-3', respectively. Finally, an alanine to proline mutation was introduced at residue 1959 within the alpha-helix region ($\alpha_1$1.2 aa 1952-1966) using the mutagenic oligonucleotide forward primer 5'- GTCCACCACCAGCCATTGGCAGTGGCG-3' and reverse primer 5'-CGCCACTGCCAATGGCTGGTGGTGGAC-3' to generate "CT-8 helix-Pro".

In vitro kinase/phosphatase assay — In vitro phosphorylation and dephosphorylation assays on select GST fusion proteins were performed using recombinant PKA and calcineurin. Briefly, immobilized GST fusion proteins were phosphorylated with the recombinant murine PKA catalytic subunit (α isoform; Calbiochem) in the presence of $^{32}\gamma$P-ATP (30 min, 30°C) in kinase buffer containing (mM) 50 Tris, pH 7.2, 10 $MgCl_2$, 0.5 DTT and protease

inhibitors. The kinase and unincorporated radiolabeled ATP were removed by thorough washing, and the proteins were divided into 2 aliquots, one of which was put aside for subsequent gel analysis. The other aliquot was treated with a recombinant heterodimer of human calcineurin Aα + calcineurin B subunits (60 min, 30°C) in calcineurin buffer containing (mM) 100 Tris, pH 7.5, 200 NaCl, 12 $MgCl_2$, 1 DTT, 1 $CaCl_2$, 0.05% NP-40, and 2 μM calmodulin. Reactions were stopped in SDS buffer followed by PAGE and autoradiography.

Immunohistochemistry – Mice were anesthetized with intraperitoneal injection of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) and sacrificed by cervical dislocation in accordance with institutional and NIH animal use guidelines. A midline thoracotomy was performed and hearts excised while still beating. Hearts were grossly trimmed in the coronal plane and blotted free of excess blood prior to cryoembedding. Dorsal and ventral halves of heart specimens were placed in tissue freezing medium (TFM, Triangle BioScience, Raleigh, NC) and oriented for coronal sectioning in peelaway cryomolds (Polysciences, Warrington, PA). Cryoembedments were flash frozen by partial immersion in liquid-nitrogen-supercooled-isopentane and stored at -80°C until time of sectioning. Subsequently, samples were equilibrated to -22°C and eight-micron frozen sections were made on a Leica CM3000 cryostat (Wetzlar,

Germany). Sections were air-dried and returned to frozen storage at -80°C until time of immunohistochemical staining.

For immunostaining, slides were thawed and sections fixed for 10 minutes in ice-cold methanol. Sections were rinsed in phosphate-buffered saline, pH 7.3 (PBS) and permeabilized in 0.1% Triton X-100/PBS. Residual triton surfactant was removed with a PBS rinse and sections were blocked with Mouse IgG Blocking Reagent according to kit manufacturer's instructions (Mouse on Mouse Kit, Vector Laboratories, Burlingame, CA) Sections were incubated with primary antibody (diluted in PBS) against $\alpha_1$1.2, calcineurin and α-actinin (overnight, 4°C). After rinsing with PBS, bound primary antibody was detected with Alexa 488 or 555-labeled secondary antibody (1 hr, RT). Sections were rinsed in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). A Nikon (Eclipse TE2000-U) confocal microscope was used to scan the tissue sections viewed at 60X objective-lens magnification, and the images were obtained with the EZ-C1 version 2.0 software (Nikon Instruments).

Peptides – All but one peptide used were synthesized (>70% purity) and HPLC analyzed by the Protein Chemistry Technology Center at UT Southwestern Medical Center. The peptides were designed based on the primary amino acid sequence of the C-terminus of the cardiac L-type Ca^{2+} channel $\alpha_1$1.2 subunit (and

corresponding to CT-8 GST fusion protein). The peptides overlapped by three residues as follows:

peptide 1976: TSLPRPCATPPATPGSRGWP (aa 1976-1995 of $\alpha_1$1.2)

peptide 1993: GWPPPQPIPTLRLEGADSSEK (aa 1993-2012 of $\alpha_1$1.2)

peptide 2010: SEKLNSSFPSIHCGSWSGEN (aa 2010-2029 of $\alpha_1$1.2)

Calcineurin-inhibitory peptide was purchased from Sigma (#C-3937) and Santa Cruz (#sc-3055).

peptide AID: ITSFEEAKGLDRINERMPPRR (aa 457-482 derived from autoinhibitory domain of rat brain calcineurin Aα)

Reagents — Antibodies were purchased from Alomone Labs ($\alpha_1$1.2 #ACC003), BD Biosciences (CnA #610259), Santa Cruz (PP2B-A #9070, AKAP79 #10764), Roche (HA #12CA5), Sigma (GST #A7340, α-actinin #A7811), Invitrogen Molecular Probes (GFP #A11122), and Cell Signaling Technology (myc #2276).

CHAPTER THREE: FUNCTIONAL INTERACTION BETWEEN CALCINEURIN AND L-TYPE Ca^{2+} CHANNEL

Introduction

My studies in Chapter Two described specific binding between calcineurin and a C-terminal region of $\alpha_1$1.2, and therefore evidence for a physical interaction between the two proteins in the heart. My second hypothesis, in light of our previous findings (Wang Z et al., 2001a), was to test whether calcineurin regulates L-type Ca^{2+} channel function (for discussion see Central Thesis, Chapter One).

Calcineurin has been shown to regulate the function of a variety of ion channels, transporters and receptors involved in Ca^{2+}-signaling (Rusnak F & Mertz P, 2000). As mentioned in the previous chapter, the physical interaction between calcineurin and various channels has been extensively studied and characterized. Here, I will focus on some of the functional aspects associated with those interactions.

Katanosaka et al. previously reported calcineurin inhibition of sodium/calcium exchanger (NCX1) function in the setting of phenylephrine (PE)-induced cardiac hypertrophy (Katanosaka Y et al., 2005). Their findings were in

good agreement with ours, where using a pressure-overload model of cardiac hypertrophy, we also demonstrated regulation of NCX1 function by activated calcineurin (Wang Z et al., 2001b). Similar inhibitory effects of calcineurin were also implicated in studies of calcineurin-mediated regulation of the cardiac ryanodine receptor (Bandyopadhyay A et al., 2000).

Whilst the above two examples are sparse, calcineurin regulation of the L-type Ca^{2+} channel has been studied extensively, although the direction of effect on channel activity remains intensely debated (**Table 3**, see Chapter Four). Early studies performed by Chad & Eckert suggested calcineurin-dependent down-regulation of L-type Ca^{2+} channel function, whereas more recently, studies have shown both an absence of or up-regulation of channel activity via calcineurin (**Table 3**).

In this report, I present evidence to suggest that calcineurin activates the cardiac L-type Ca^{2+} channel, such that overexpression of a constituvely-active isoform of calcineurin is associated with a robust increase in channel function, while acute inhibition of calcineurin activity induces a significant decrease in channel function in a calcineurin-dependent manner.

Results

Calcineurin activation increases L-type Ca^{2+} current

To test whether calcienurin regulates L-type Ca^{2+} channel function I infected cultured neonatal rat cardiomyocytes with adenovirus expressing constitutively-active calcineurin (AdCnA*). Control cells were infected with adenovirus expressing GFP (AdGFP). As we reported before, over-expression of activated calcineurin provoked typical hypertrophic cell growth (Richardson KE et al., 2005), and the concomitant increase in two-dimensional cell surface area (by 84%±9%, $p<0.05$; n=100 AdCnA*, n=100 AdGFP) (**Figure 3.1A**). Recordings of L-type channel function in AdCnA*-infected myocytes revealed a statistically significant increase of 26% (±4, $p<0.05$; n=10 AdCnA*, n=6 AdGFP) current density with no significant shift in the steady-state current-voltage (IV) relation (**Figure 3.1B**). Hypertrophy-associated increases in channel function exceeded that expected from simple increases in cell size, as current density was significantly increased. These data are consistent with our *in vivo* findings of calcineurin-dependent up-regulation of L-type channel activity in pressure-overload hypertrophy (Wang Z et al, 2001a) and suggest that calcineurin is capable of regulating cardiac Ca^{2+} channel function.

Inhibition of calcineurin diminishes L-type Ca^{2+} current

Calcineurin-dependent increases in channel function do not exclude the possibility that changes in channel activity are secondary to the hypertrophic phenotype. To address this possibility, working in collaboration with Dr. Yanggan Wang, we acutely inhibited calcineurin with cyclosporine A (CsA) and evaluated changes in Ca^{2+} currents from dissociated ventricular myocytes of normal mice. To minimize current run-down or dialysis of intracellular contents, we used an amphotericin B perforated patch technique. The bath solution did not contain Na^{+} or K^{+} ions, and the cells were held at -80 mV, stepped transiently to -50 mV to inactivate sodium currents, and then L-type Ca^{2+} currents were measured (0.1 Hz) at test potentials between -30 and +50 mV in 20 mV increments. Under these conditions, addition of 10 μM CsA significantly inhibited L-type channel activity at all potentials without altering the potential at which peak current was observed (**Figure 3.2A**). Similar results were observed using tacrolimus (FK-506), a structurally distinct calcineurin-inhibitor (**Figure 3.2B**).

Suppression of peak (+10 mV) Ca^{2+} currents occurred over a time-course of several minutes suggesting underlying molecular post-translational events, and was partially reversible following drug wash-out (**Figure 3.2C**). Together, these findings suggest that the effects of CsA or FK-506 on L-type channel function are

mediated by inhibition of calcineurin activity rather than by non-specific actions of either compound or by current rundown.

Furthermore, CsA inhibited Ca^{2+} current without inducing a shift in the steady-state IV relation (**Figure 3.2D**), and the kinetics of channel inactivation were not altered (**Figure 3.2E**). Phosphorylation of the channel by PKA has been shown to induce a hyperpolarizing shift in the IV relation (McDonald TF et al., 1994), and therefore the results with CsA and FK-506, discussed above, suggested that calcineurin-mediated regulation of the channel does not involve antagonism of PKA-dependent phosphorylation of the channel. To test this further, I investigated the effects of isoproterenol in the presence of calcineurin activation and inactivation (**Figure 3.3**). Isoproterenol (Iso; 1 μM) induced similar alterations in L-type Ca^{2+} currents and IV relation from cells infected with AdGFP (51.9%±3%, n=3), AdCnA* (54.1%±7.4%, n=5, p=NS vs. control), or AdRCAN1 (regulator of calcineurin) an endogenous inhibitor of calcineurin (Rothermel BA et al., 2000) (46.1%±7%, n=3, p=NS vs. control) (**Figure 3.3**). Thus, these data indicate that PKA and calcineurin likely activate L-type Ca^{2+} channel through independent pathways.

Inhibition of L-type Ca^{2+} current is calcineurin-dependent

To determine whether the inhibitory effects of CsA on L-type Ca^{2+} channel function are calcineurin-dependent, as opposed to non-specific effects of the compound, we studied L-type currents in the context of negligible calcineurin activity. Calcineurin is inactive in the absence of Ca^{2+}, so we hypothesized that pharmacological suppression of calcineurin should have no effect on Ca^{2+} currents in the presence of BAPTA, a chelator that binds Ca^{2+} nearly two orders of magnitude faster than EGTA (Smith PD et al., 1984). These kinetics allow BAPTA, but not EGTA, to buffer Ca^{2+} effectively at the mouths of open Ca^{2+} channels, up to the limits imposed by the diffusivity of the chelator (Bauer PJ, 2001).

L-type currents were recorded in dissociated ventricular myocytes using standard whole-cell methods, where the pipette filling solution included 10 mM BAPTA. Under these conditions, where intracellular Ca^{2+} is irreversibly chelated, addition of 10 μM CsA did not diminish channel activity (**Figure 3.4A**); Ca^{2+} current declined by only 2.5% (±0.8%, n=7, p=NS) in the presence of intracellular BAPTA as compared with 44% (±5%, n=5, $p<0.05$) without BAPTA (**Figure 3.4D**). To determine whether the loss of CsA-induced inhibition in the presence of BAPTA was simply an artifact of the disrupted-membrane recording method, we studied channel function under conditions where intracellular Ca^{2+} was only slightly buffered. To accomplish this, the pipette was filled with 5 mM EGTA.

Under these conditions, CsA suppressed L-type Ca^{2+} currents to an intermediate extent (21±5%, n=9, $p<0.05$) (**Figures 3.4B, D**).

As another test, we took a genetic approach to inhibiting calcineurin by transgenic over-expression of RCAN1 (Rothermel BA et al., 2000). If our hypothesis is correct, we would predict that the addition of CsA should have no effect on L-type Ca^{2+} currents in cells over-expressing RCAN1, as calcineurin would already be inactive. We evaluated the effects of CsA on Ca^{2+} currents in myocytes dissociated from transgenic (Tg) mouse hearts over-expressing RCAN1 and consistent with our hypothesis, CsA had no effect on L-type currents (3.0%±0.8% decline, n=6, p=NS) in RCAN1-Tg myocytes (**Figure 3.4C, D**).

Targeted suppression of calcineurin inhibits L-type Ca^{2+} current

The above experiments were carried out with pharmacological inhibitors of calcineurin. I tested the effects of non-pharmacologic suppression of calcineurin by intracellular dialysis of the calcineurin-inhibitory peptide "AID", derived from the autoinhibitory domain of calcineurin. Intracellular dialysis of 10 μM AID through the patch pipette induced a 36% (±2.7%, n=5, $p<0.05$ vs. control peptide) decrease in L-type channel activity (**Figure 3.5A, B, D**). By contrast, no declines in Ca^{2+} currents were seen with intracellular dialysis of a control peptide (peptide 2010; 1.7%±4.3%, n=4, p=NS vs. no peptide) or no peptide at all

(6.1%±2.5%, n=6) (**Figure 3.5A, B, D**). Peptide 2010 was chosen as a control, because it did not interfere with calcineurin-binding (**Figure 2.3C**, Chapter Two) and was of a similar molecular mass and pI to AID. Consistent with our findings when calcineurin was suppressed pharmacologically, AID induced inhibition of L-type Ca^{2+} channel function to a similar extent (36% vs. 44%) and was not associated with shifts in the steady-state IV relation (**Figure 3.5C**). Furthermore, AID did not elicit reduction in Ca^{2+} currents recorded from RCAN1-Tg myocytes (2.6%±1.6%, n=5, p=NS vs. control) or intracellular BAPTA (7.4%±0.8%, n=4, p=NS vs. control) (**Figure 3.5D**), suggesting again that the observed inhibition of L-type channel was due to specific inhibition of calcineurin and not due to non-specific actions of peptide dialysis.

Figures

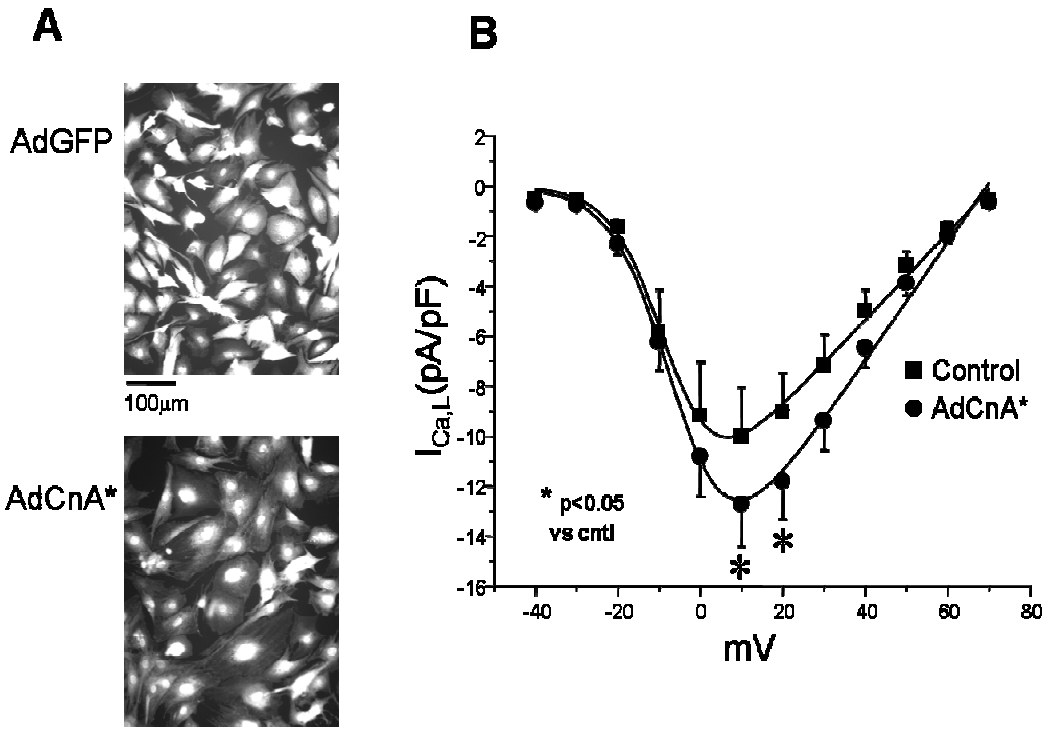


Figure 3.1: Activated calcineurin increases L-type Ca^{2+} current. **A.** Neonatal rat cardiomyocytes were infected with adenovirus expressing constitutively-active calcineurin (AdCnA*) or GFP (AdGFP) and visualized with fluorescent cell tracer (CellTracker^{TM}). AdCnA* led to the expected increase in cell size and associated myocyte hypertrophy. **B.** Voltage clamp recordings of L-type Ca^{2+} current ($I_{Ca,L}$) at increasing membrane-potentials. AdCnA* was associated with an increase in peak $I_{Ca,L}$ density (• AdCnA*; 12 pA/pF at +10 mV, n=10 vs. ▪

control; 10 pA/pF at +10 mV, n=6; * $p<0.05$), with no shift in current-voltage (IV) relation.

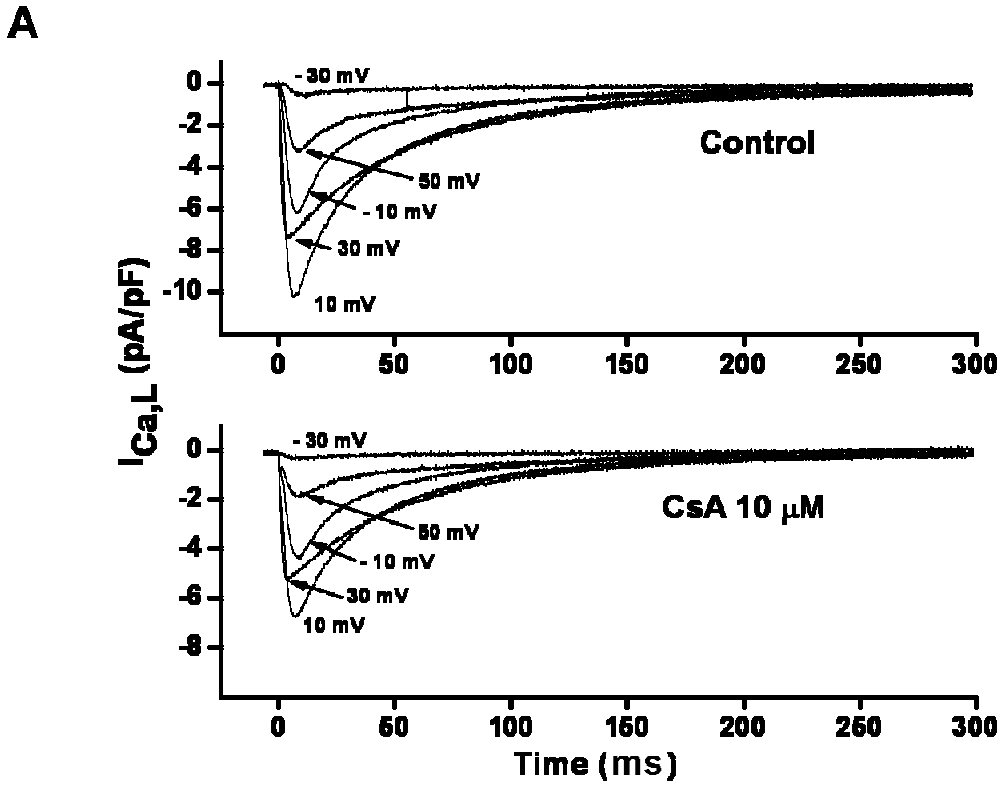
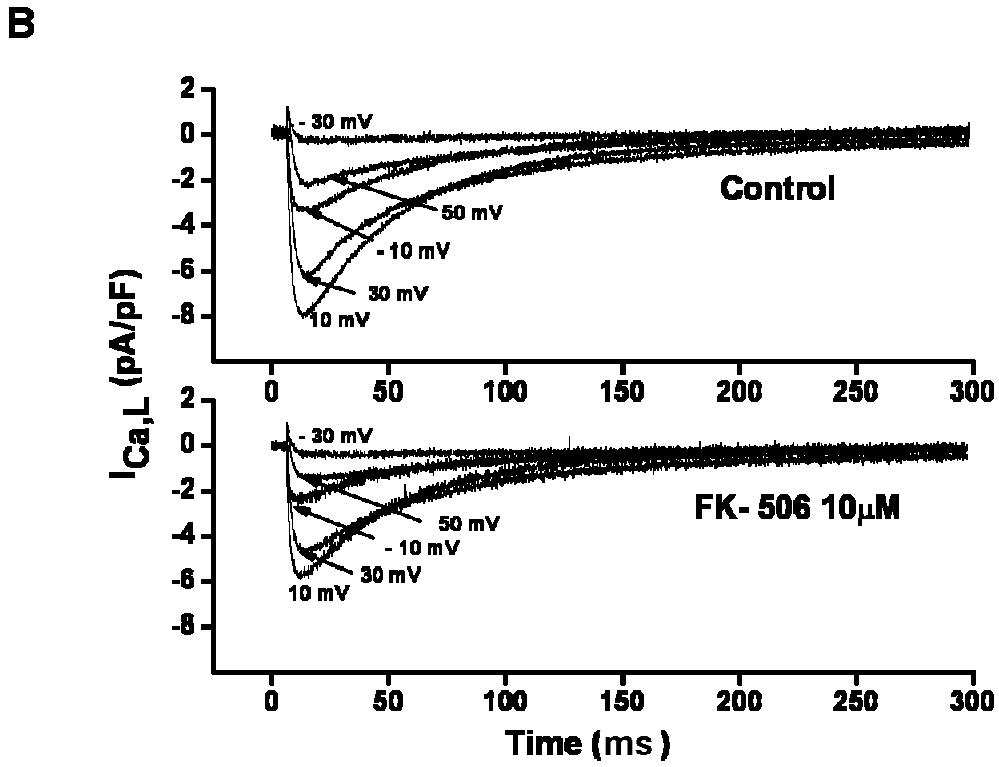


Figure 3.2: Acute suppression of calcineurin diminishes L-type Ca^{2+} current. Representative whole-cell voltage clamp measurements of $I_{Ca,L}$ density in dissociated ventricular myocytes before and after calcineurin suppression by two structurally distinct calcineurin-inhibitors, (**A**) CsA (10 μM) and (**B**) tacrolimus (FK-506; 10 μM) (next page).



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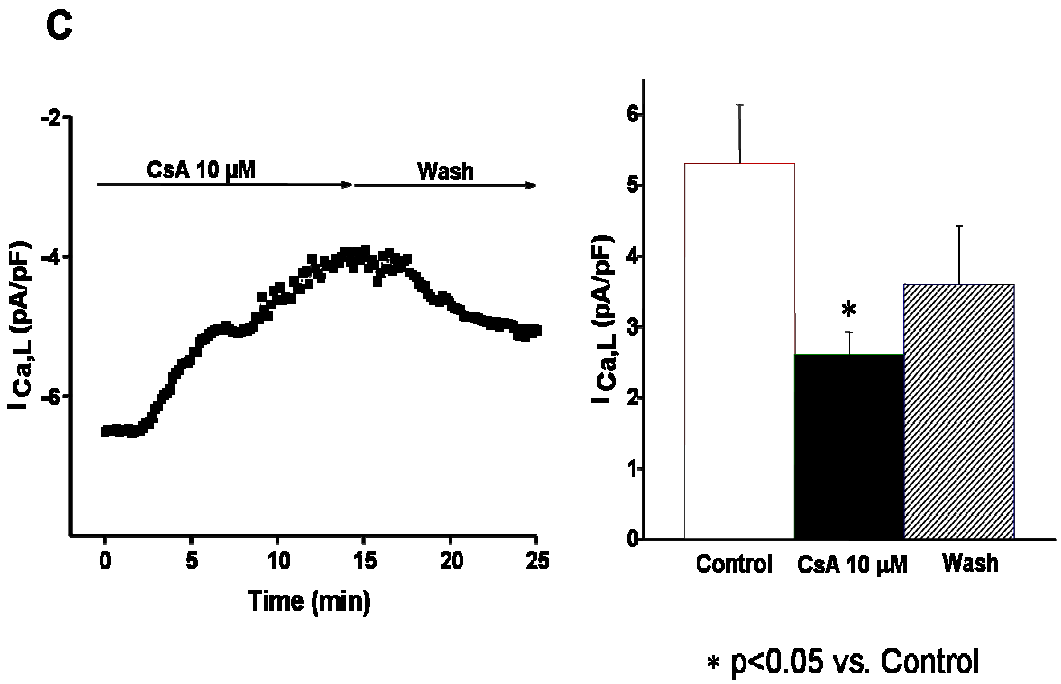
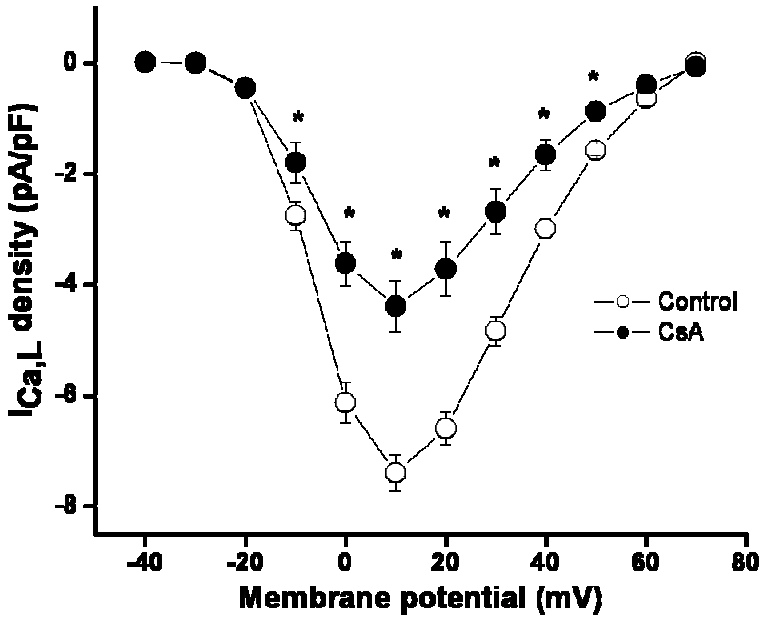


Figure 3.2: Acute suppression of calcineurin diminishes L-type Ca^{2+} current. **C.** Representative recording depicting time-course of CsA-induced $I_{Ca,L}$ inhibition, and partial reversibility on drug washout. Mean data from 4 experiments are shown.

D


0
-2
-4
-6
-8
$I_{Ca,L}$ density (pA/pF)
-40
-20
0
20
40
60
80
Membrane potential (mV)
Control
CsA
*

E

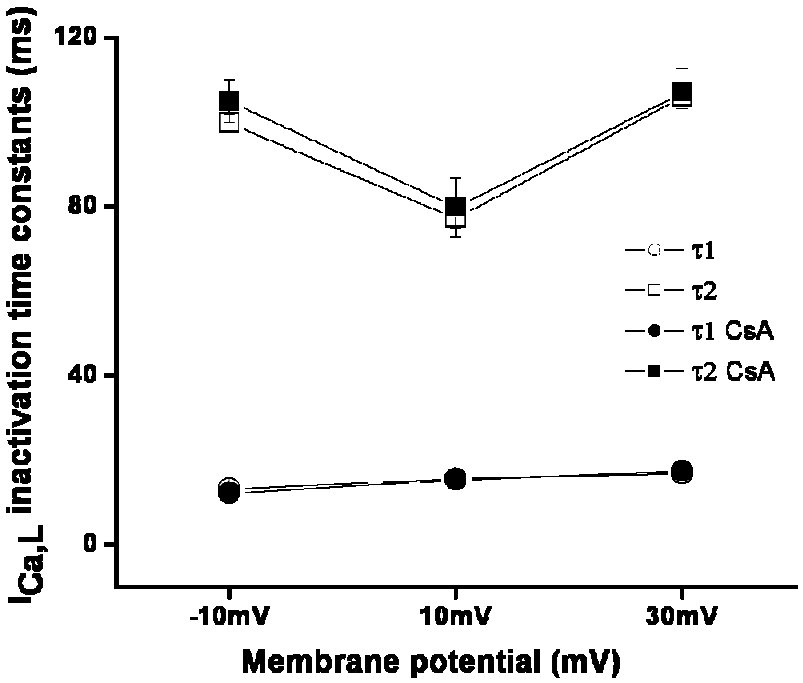

120
80
40
0
$I_{Ca,L}$ inactivation time constants (ms)
-10mV
10mV
30mV
Membrane potential (mV)
τ1
τ2
τ1 CsA
τ2 CsA

Figure 3.2: Acute suppression of calcineurin diminishes L-type Ca^{2+} current. **D.** Current-voltage relation depicting mean $I_{Ca,L}$ in the presence-vs-absence of CsA. CsA-induced suppression of $I_{Ca,L}$ elicited no shift in the steady-state IV relation (n=8 myocytes from 3 hearts in each treatment group). **E.** Mean data demonstrating that CsA-induced suppression of $I_{Ca,L}$ did not elicit changes in fast ($\tau 1$) or slow ($\tau 2$) time-constants of $I_{Ca,L}$ inactivation.

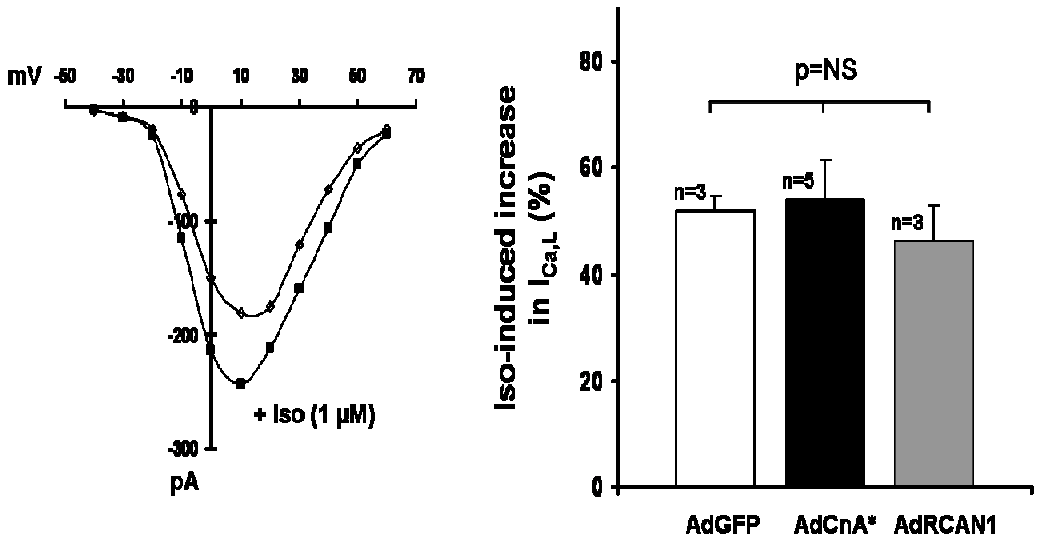


Figure 3.3: PKA activates L-type Ca^{2+} current independent of calcineurin. Representative IV curve and cumulative data (bar graph) showing equivalent increases in $I_{Ca,L}$ after exposure to isoproterenol (Iso 1 μM) under control, activated calcineurin (AdCnA*), or inactivated calcineurin (AdRCAN1) conditions.

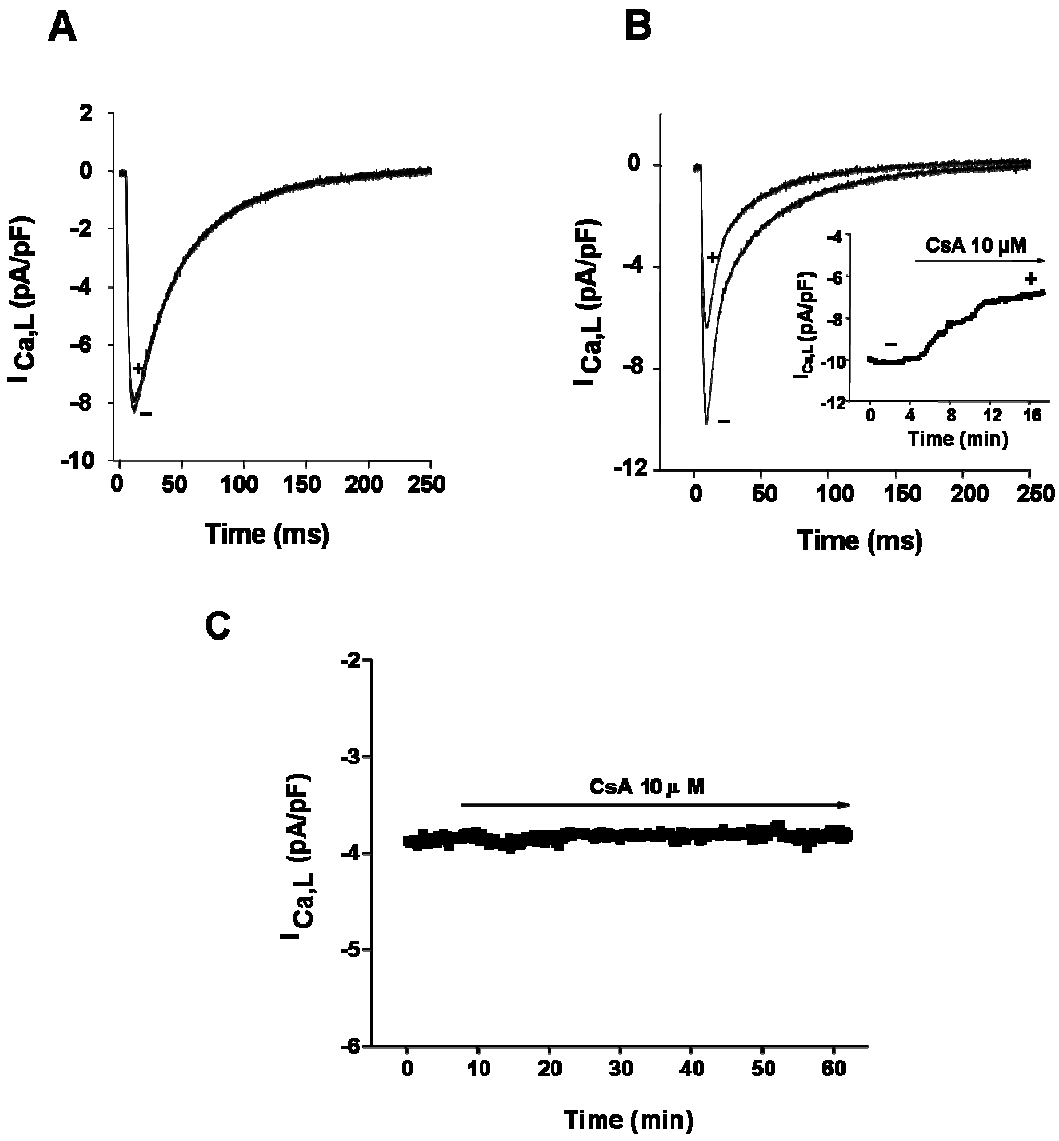


Figure 3.4: CsA inhibits L-type Ca^{2+} channel in a calcineurin-dependent manner. **A.** Whole-cell voltage clamp recordings of $I_{Ca,L}$ density in dissociated ventricular myocytes treated with 10 mM BAPTA. In the presence of Ca^{2+} chelation, and consequent calcineurin inactivation, 10 µM CsA had no effect on

$I_{Ca,L.}$ (+, 10 mM BAPTA; -, control). **B.** With incomplete Ca^{2+} buffering, 10 μM CsA induced a partial reduction in $I_{Ca,L}$ (+, 5 mM EGTA; -, control). **C.** Representative time-course of $I_{Ca,L}$ density recorded from a ventricular myocyte dissociated from a transgenic mouse over-expressing RCAN1 in cardiac myocytes. When endogenous calcineurin is chronically inactivated by RCAN1 over-expression, CsA-induced suppression of $I_{Ca,L}$ is abolished.

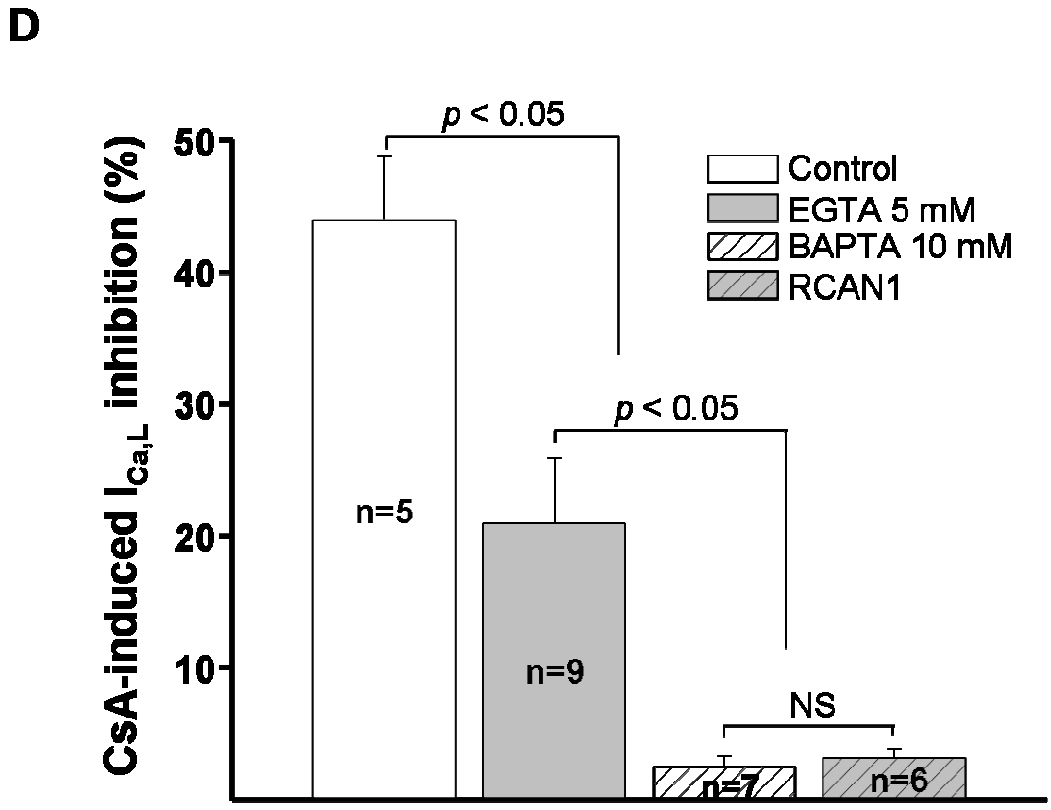


Figure 3.4: CsA inhibits L-type Ca^{2+} channel in a calcineurin-dependent manner. D. Mean data of percent-inhibition of $I_{Ca,L}$ induced by 10 μM CsA vs. control (perforated patch, 44% ±5%, n=5, $p<0.05$), when 5 mM EGTA or 10 mM BAPTA is dialyzed through the patch pipette (21% ±5%, n=9, $p<0.05$, and 2.5% ±0.8%, n=7, $p<0.05$, respectively), or recordings from ventricular myocytes isolated from RCAN1 transgenic mouse hearts (perforated patch, 3% ±0.8%, n=6, $p<0.05$).

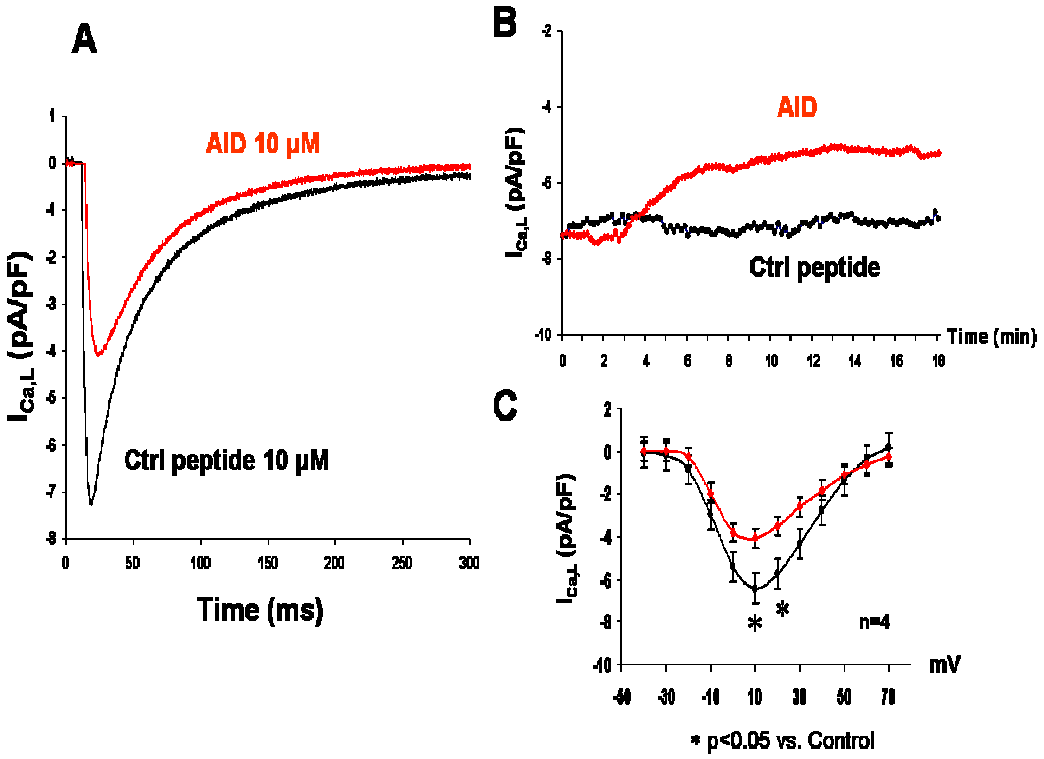


Figure 3.5: Targeted suppression of calcineurin inhibits L-type channel activity. **A.** Representative peak $I_{Ca,L}$ densities (at +10 mV) and (**B**) time-course recorded from ventricular myocytes after peptide-dialysis of 10 µM control peptide (2010, black line) or calcineurin auto-inhibitory domain (AID, red line) peptide. **C.** Current-voltage (IV) relation demonstrating that calcineurin inhibition by AID induced a 36% decrease in peak inward $I_{Ca,L}$ density (4.08 ± 1 pA/pF at +10 mV, n=4; $p<0.05$) vs. control peptide (6.42 ± 0.32 pA/pF at +10 mV, n=4), with no shift in IV relation.

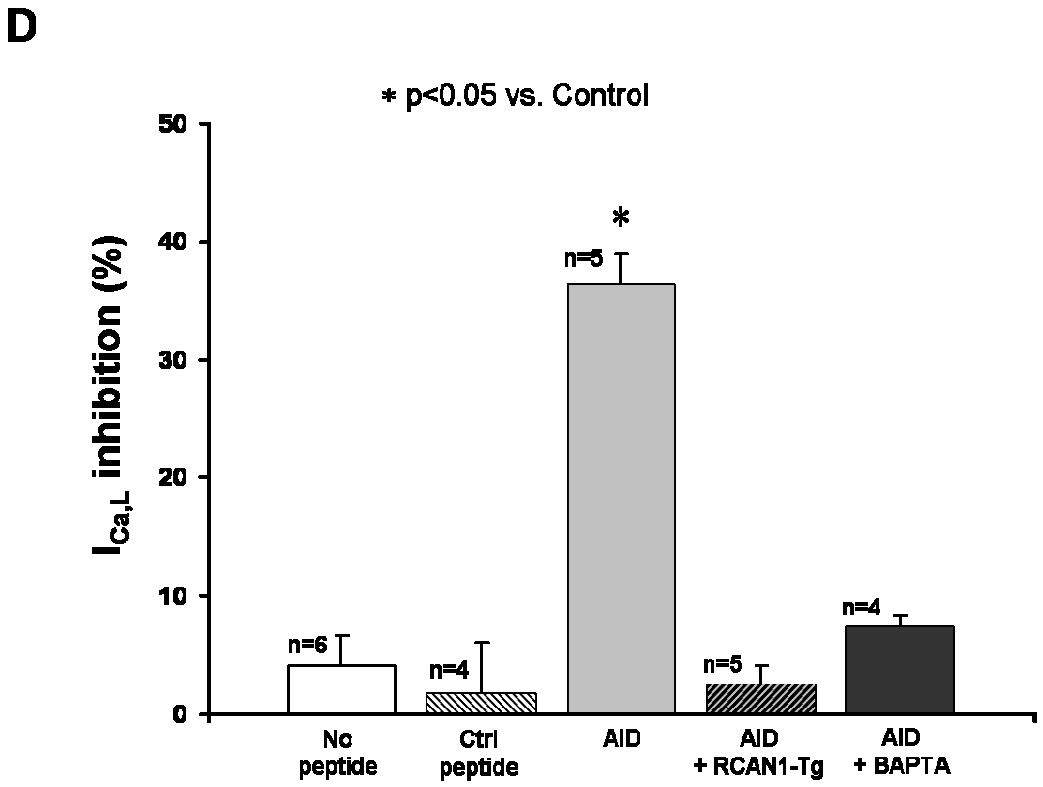


Figure 3.5: Targeted suppression of calcineurin inhibits L-type channel activity. D. Mean data demonstrating that AID induced a 36% (± 2.7%, n=5, $p<0.05$ vs. control peptide) decrease in $I_{Ca,L}$ density vs. control peptide (1.7%±4.3%, n=4, p=NS) or no peptide (4.1%±2.5%, n=6). AID had no effect on $I_{Ca,L}$ density in the absence of calcineurin activity in RCAN1-Tg myocytes (2.6%±1.6%, n=5, p=NS vs. control) or in the presence of BAPTA (7.4%±0.8%, n=4, p=NS vs. control). All experiments were performed on myocytes isolated from at least 3 different mice of the same strain.

Interpretation and Conclusions

Calcineurin activation is associated with increased L-type Ca^{2+} channel function

The results presented herein provide strong evidence that calcineurin regulates the function of the cardiac L-type Ca^{2+} channel. Using an established *in vitro* model of cardiac hypertrophy, I show that over-expression of constitutively-active calcineurin in cultured cardiomyocytes is associated with a significant increase in endogenous L-type Ca^{2+} channel activity. These findings are in keeping with our previously published data of increased channel function in pressure-overload induced cardiac hypertrophy *in vivo* (Wang Z et al., 2001a). Furthermore, it is noteworthy that the increase of 26% in L-type Ca^{2+} currents I observed in my *in vitro* model was very similar to the 30% increase we had observed previously *in vivo* (Wang Z et al., 2001a). Calcineurin-induced activation of L-type Ca^{2+} channel function has also been reported by Dr. Jeff Molkentin's group, where using a transgenic model of cardiac-specific calcineurin over-expression, they reported a significant increase in L-type Ca^{2+} currents (Yatani A et al., 2001).

An important caveat inherent to all these results, however, is that the activation of L-type channel function can be construed as either directly downstream of calcineurin regulation or as a by-product of the hypertrophic

phenotype induced by calcineurin activation in the first place. Therefore, while my results were at least consistent with previous observations *in vivo*, it was imperative to extend these investigations in the setting of normal (i.e. non-stressed) cardiomyocytes, as discussed below.

Inhibition of calcineurin activity decreases L-type channel function

Given the associative nature of the above findings, I repeated the electrophysiology experiments in the setting of normal or non-stressed adult cardiomyocytes. In other words, what was the effect of endogenous calcineurin on L-type Ca^{2+} current, without over-activation and expression of the enzyme? I investigated this question thoroughly, first in collaboration with assistant professor Dr. Yanggan Wang, an expert electrophysiologist in our laboratory, with the use of specific pharmacological inhibitors of calcineurin (**Figure 3.2**), and then independently, using a specific peptide-inhibitor of calcineurin expressing the auto-inhibitory domain of the enzyme (**Figure 3.5**). Remarkably, both diverse means of calcineurin inhibition, either small-molecule blockers (CsA, FK-506) or by a specific peptide (AID), induced similar reductions in L-type Ca^{2+} channel function (44% and 36%, respectively). These data suggest that calcineurin maintains baseline L-type Ca^{2+} channel activity in the heart, and lend credence to the fact that the observed effects of the calcineurin-inhibitors on

channel function were likely due to inhibition of calcineurin activity rather than non-specific effects of any inhibitor on the channel.

In addition, the time course of inhibition of L-type channel activity with CsA occurred over several minutes (**Figure 3.2C**), suggesting underlying molecular post-translational events and not simple channel blockade or current rundown. Furthermore, the partial recovery of current after drug washout indicated that the inhibitory effects of the drug were specifically acting through calcineurin and not non-specific to channel blockade. Indeed, complete recovery of current would require time if the underlying molecular events were to be reversed (e.g. hyperphosphorylation of the channel), which was also consistent with the partial recovery we observed within the time-frame of our current recordings (**Figure 3.2C**).

Specific regulation of L-type Ca^{2+} channel by calcineurin

Our findings with CsA, FK-506 and AID gave us strong evidence that specific inhibition of calcineurin induced a significant (and consistent) decrease in baseline L-type Ca^{2+} channel function. Due to the substantial structural differences between CsA and FK-506 (Leinwand LA, 2001), as well as the peptide-inhibitor, AID, the likelihood that they non-specifically inhibited the L-type Ca^{2+} channel by similar amounts is very low. Yet, their effects on channel function have been reported with a diversity of outcomes (**Table 3**), most notably

by Matthes et al. who describe non-specific inhibition of L-type channel activity with CsA (Matthes J et al., 2004). In their studies, CsA (10 μM) inhibited both ventricular and atrial L-type Ca^{2+} channel activity when only Ba^{2+} was used as the charge carrier. In contrast, channel activity remained constant when Ca^{2+} permeation was provided and increased in the presence of thapsigargin and BayK 8644, a channel agonist. They therefore concluded that CsA induced a dual modulation of the L-type channel (Matthes J et al., 2004 and discussion therein).

To ascertain the specificity of our approach, we utilized a variety of different strategies to test our inhibitors in the absence of calcineurin activity. In other words, could CsA, for example, inhibit the L-type channel when calcineurin was absent, or inactivated *a priori*?

First, we tested the effects of CsA in the presence of BAPTA, a powerful Ca^{2+} chelator (**Figure 3.4A**). In the presence of BAPTA, CsA had no effect on L-type Ca^{2+} currents. This was in stark contrast to the approximate 44% decrease in channel function under control conditions (**Figure 3.4D**). This observation allowed us two interpretations: First, that calcineurin was inactivated due to chelation of intracellular Ca^{2+}, and therefore CsA lacked any additional effect. Or second, that due to the differences in experimental design (i.e. perforated- vs. standard whole-cell patch clamp), CsA was now somehow rendered ineffective. The perforated patch clamp technique is a powerful means of limiting diffusion of intracellular contents with those of the patch pipette (Sakmann B & Neher E,

1995). Antibiotics such as amphotericin B intermittently puncture small pores in the surface of the membrane, at the interface between the cell and the pipette (Rae J et al., 1991). In fact, unlike in the standard whole-cell setting, the perforated patch experiment is performed by plain attachment of the pipette onto the cell surface without membrane disruption. This allows the diffusion of only small molecules and ions across the membrane. Under these conditions, the effects of calcineurin inhibitors, added extraneously, can be specifically tested in the context of preserved intracellular contents and therefore appropriate cardiomyocyte milieu. In this setting, CsA induced a ~44% decrease in L-type Ca^{2+} channel function, while in the standard whole-cell setting using BAPTA and thereby allowing free diffusion of BAPTA and cellular contents in and out of the cells, the effects of CsA were abolished.

Furthermore, to test whether the disrupted membrane patch due to standard whole-cell configuration was causing a loss of CsA efficacy, we repeated the experiments with 5 mM EGTA (vs. 10 mM BAPTA), where we expected less buffering of intracellular Ca^{2+}. BAPTA and EGTA are known to share similar affinities for Ca^{2+} ions, however BAPTA binds Ca^{2+} two orders of magnitude faster than EGTA (Naraghi M & Neher E, 1997). In fact, working on the foundation of the "rapid buffer approximation," Naraghi and Neher demonstrated a decade ago that BAPTA which contained a length-constant of 30 nm, but not EGTA (larger length-constant: ~400 nm), was able to buffer Ca^{2+} ions closer to

the mouths of ion channels (Naraghi M & Neher E, 1997). More recently, David T. Yue's laboratory extended those observations through a host of mathematical algorithms and experimental techniques by demonstrating that 10 mM BAPTA was sufficient to completely buffer intracellular 'global' Ca^{2+} concentrations within a cardiomyocyte (Tadross MR et al., 2008). Consistent with these reports, under conditions where EGTA was used as the Ca^{2+}-chelating agent, CsA had only a partial inhibitory effect on the L-type channel activity (**Figure 3.4B, D**). This interesting result led us to interpret that the partial buffering of intracellular Ca^{2+} induced partial inactivation of calcineurin and therefore partial inhibitory effects of CsA, as compared to complete inactivation of calcineurin and abolition of the effects of CsA with BAPTA.

An important constraint of the above protocol is the use of Ca^{2+}-chelators as a means of calcineurin suppression. Not only do EGTA and BAPTA (used at high concentrations) act globally within the cardiomyocyte, but also the lack of a specific means to test calcineurin activity (Leinwand LA, 2001), especially in real-time during electrophysiological experiments, represents a limitation of the experimental design. Both EGTA, and especially BAPTA, would be expected to inactivate calcineurin, but also a host of other Ca^{2+}-signaling molecules, including those responsible for regulation of L-type Ca^{2+} channel itself, such as the Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), for example (Anderson ME, 2004; Pitt GS, 2007; Wang Y and Tandan S, 2008).

Therefore, to preclude the potential confounding factors described above, we utilized a second approach to test the specificity of CsA-mediated inhibition of L-type channel function. This time, using a genetic means of calcineurin-suppression, we recorded L-type Ca^{2+} currents from RCAN1-Tg cardiomyocytes. RCAN1-Tg mice over-express RCAN1 (regulator of calcineurin) protein, an endogenous biological inhibitor of calcineurin (Rothermel BA et al, 2001) specifically in the heart. This method allowed us to test the effects of CsA particularly in the absence of calcineurin activity already established (Rothermel BA et al., 2001). Consistent with our observations of Ca^{2+}-chelation, CsA had no effect on L-type channel function in RCAN1-Tg cardiomyocytes (**Figure 3.4C, D**).

Taken together, these data lend support to the hypothesis that calcineurin activity increases L-type Ca^{2+} channel function in cardiac myocytes, and that inhibition of endogenous calcineurin induces a decrease in channel activity. Under conditions where calcineurin cannot be activated (e.g. chelation of intracellular Ca^{2+} or RCAN1-over-expression), addition of CsA, FK-506, or AID does not decrease current, confirming a direct effect through calcineurin.

Antagonism of PKA as a mechanism?

Our *in vitro* phosphorylation/dephosphorylation experiments suggested that at the very least, calcineurin was able to recognize an $\alpha_1$1.2 GST-fusion

protein phosphorylated by PKA *a priori* (**Figure 2.7** and discussion therein, Chapter Two), suggesting possible antagonism of PKA as a mechanism of action on the channel. PKA-dependent phosphorylation of the channel has been well studied and characterized, and is known to increase channel function along with a hyperpolarizing (or, left-ward) shift in IV relation (Catterall WA, 2000). Albeit a variety of calcineurin-inhibitors (both small molecules of distinct molecular structure, as well as a peptide-blocker) decreased L-type Ca^{2+} currents by similar amounts, another consistent theme within my results was that this reduction in current was not associated with any shift in steady-state current-voltage relation (**Figures 3.2D and 3.5C**). In fact, by corollary, calcineurin-associated increase in L-type Ca^{2+} channel function also did not induce a shift in IV relation (**Figure 3.1B**). Furthermore, the CsA-induced inhibition of L-type Ca^{2+} channel activity was not associated with any change in time-constants of channel inactivation (**Figure 3.2E**). All of these traits are characteristic of PKA-mediated regulation of the channel (McDonald TF et al., 1994; Catterall WA, 2000), and therefore my results suggested that calcineurin, a protein phosphatase, regulates the L-type channel via a pathway distinct from PKA.

I tested this hypothesis in further detail by examining the effects of isoproterenol (Iso), and consequent PKA activation, on L-type Ca^{2+} channel function in the setting of both calcineurin activation (by over-expression of constituvely-active calcineurin; AdCnA*) as well as inactivation (by over-

expression of RCAN1; AdRCAN1) (**Figure 3.3**). As expected, Iso induced equivalent increases in L-type Ca^{2+} current regardless of the activation state of calcineurin, further suggesting that calcineurin-mediated regulation of the channel was independent of PKA. Furthermore, PKA induced a similar increase in channel activity in the control setting of over-expressed GFP (AdGFP) thereby ruling out any specific masking effects of my adenoviral over-expression system. In addition, PKA is known to increase L-type channel function, an outcome similar to that observed with calcineurin activation. Therefore, it is unlikely that calcineurin-mediated regulation of the channel occurs through a pathway involving PKA antagonism as both have the same effect. Furthermore, calcineurin also caused a partial dephosphorylation of the $\alpha_1$1.2 subunit C-terminus encoded by CT-B (**Figure 2.7A**) suggesting the presence of additional PKA phosphorylation sites within the $\alpha_1$1.2 subunit C-terminus that calcineurin recognizes as substrate. Whether these sites are targeted by calcineurin *in vivo*, and their downstream effects of channel regulation remain to be resolved. In addtion, it is intriguing that Ser-1928 is also a target for PKC (Yang L et al., 2005) and PKG (Yang L et al., 2007), both of which have been shown to decrease channel function (McHugh D et al., 2000; Fischmeister R et al., 2005). These observations would be consistent with my findings, whereby it can be envisaged that calcineurin antagonizes these particular kinases and therefore activates the L-type Ca^{2+} channel.

Conclusion

In conclusion, my data strongly support a model of calcineurin-mediated regulation of native cardiac L-type Ca^{2+} channel. Calcineurin activation is associated with a significant increase in channel function, while specific suppression of calcineurin induces a significant decrease in channel activity.

Materials and Methods

Adenoviral infection and primary culture of neonatal rat ventricular myocytes — Cardiomyocytes were isolated from the ventricles of 1-2 day old rat pups (Sprague Dawley) and plated (1250 cells/mm^2) in medium containing 10% fetal calf serum as described (Ni YG et al., 2006). Myocyte cultures obtained from this differential plating method contained less than 5% non-cardiomyocytes as determined microscopically using a myocyte-specific α-actinin antibody (data not shown). Cells were transferred to serum-free medium containing the serum supplement Nutridoma (Roche) at 0.5x concentration 24 hours before treatment or infection with adenovirus. For expression of constitutively-active calcineurin and RCAN1, cells were infected with either AdCMVCnA*iresGFP or AdCMVMCIP, respectively, which contained the cDNAs for human calcineurin or RCAN1. Each gene was expressed from the CMV promoter, and AdCMVCnA*iresGFP co-expressed GFP from the bicistronic IRES transcription unit to ascertain infection efficiency and localize infected cells for voltage-clamp experiments. Experiments were performed at a multiplicity-of-infection (MOI) of 100 (≥ 95% infection efficiency), and voltage-clamp experiments (see *Electrophysiology*) were performed on plated cells 48 hours after infection.

Cardiomyocyte Isolation — Adult mouse cardiomyocytes were isolated after enzymatic dissociation as described (Wang Z et al., 2001a) with slight modification. Briefly, after retrograde perfusion with Krebs-Ringer solution at (2 mL/min, 5 min), the heart was perfused with fresh solution containing 0.8 mg/mL collagenase (Worthington type II) for another 12-15 min. The left ventricle was removed and cut into small pieces in "KB" solution [(mM) taurine 10, glutamic acid 70, KCl 25, KH_2PO_4 10, Glucose 22, EGTA 0.5, pH 7.2]. After trituration, cells were studied within 4 to 6 hours. All isolation steps were carried out at 36°C with continuous gassing with 95% O_2 + 5% CO_2. Only Ca^{2+}-tolerant, quiescent and rod-shaped cells, showing clear cross striations were used.

Electrophysiology — Cultured neonatal rat cardiomyocytes or acutely isolated adult myocytes were studied in a continuously superfused (1.5 mL/min) recording chamber fixed to an inverted microscope. For recordings of L-type Ca^{2+} current ($I_{Ca,L}$), the whole-cell voltage clamp configuration was used on cells bathed in (mM) 135 tetraethylammonium (TEA) Cl, 0.53 $MgCl_2$, 1.8 $CaCl_2$, 20 CsCl, 5 HEPES. The pipette solution for $I_{Ca,L}$ recording contained (mM): 110 CsOH, 90 aspartic acid, 20 CsCl, 10 TEACl, 10 HEPES, 10 EGTA, 5 Mg-ATP, 5 Na_2 creatine phosphate, 0.4 GTP (Tris), 0.1 leupeptin, pH 7.2 with CsOH. 5 mM EGTA or 10 mM BAPTA were substituted for 10 mM EGTA in this internal

solution where indicated. For certain studies, the amphotericin patch clamp technique was used. Here, patch clamp pipettes were prepared and the tips filled by dipping into internal solution containing (mM): 120 K-aspartate, 20 KCl, 5 $MgCl_2$, 10 HEPES, 1.8 $CaCl_2$, pH adjusted to 7.2 with KOH. The remainder of the pipette was back-filled with the same solution plus amphotericin B (200 μg/mL). Where indicated, bath solution was complemented with 10 μM CsA or FK-506. For peptide inhibitory studies, peptides were dissolved in pipette solution and dialyzed into the cell during whole-cell configuration.

$I_{Ca,L}$ run-down, when present, typically occurs within the first 5 minutes of recording. In order to minimize the impact of run-down, we added 5 mmol/L Mg-ATP to the pipette solution (see above) and commenced data acquisition after 5-10 minutes of equilibration between pipette solution and intracellular contents (Wang Y et al., 2000). Cells showing continuous current run-down (≈ 5%) were excluded from the analysis.

Peptides – All but one peptide used were synthesized (>70% purity) and HPLC analyzed by the Protein Chemistry Technology Center at UT Southwestern Medical Center. The peptides were designed based on the primary amino acid sequence of the C-terminus of the cardiac L-type Ca^{2+} channel $\alpha_1$1.2 subunit (and

corresponding to CT-8 GST fusion protein). The peptides overlapped by three residues as follows:

peptide 1976: TSLPRPCATPPATPGSRGWP (aa 1976-1995 of $\alpha_1$1.2)

peptide 1993: GWPPPQPIPTLRLEGADSSEK (aa 1993-2012 of $\alpha_1$1.2)

peptide 2010: SEKLNSSFPSIHCGSWSGEN (aa 2010-2029 of $\alpha_1$1.2)

Calcineurin-inhibitory peptide was purchased from Sigma (#C-3937) and Santa Cruz (#sc-3055).

peptide AID: ITSFEEAKGLDRINERMPPRR (aa 457-482 derived from autoinhibitory domain of rat brain calcineurin Aα)

CHAPTER FOUR: DISCUSSION AND PERSPECTIVE

Summary

The L-type Ca^{2+} channel is an element central to multiple processes in the heart, including action potential repolarization, excitation-contraction coupling, and activation of Ca^{2+}-responsive signaling pathways. The L-type channel is also a prominent target of disease-related remodeling, being up- or down-regulated in a variety of disease states. Having previously demonstrated an important role for calcineurin signaling in Ca^{2+} channel regulation and hypertrophy-related action potential repolarization (Wang Z et al., 2001a), we hypothesized that calcineurin is a component of the Ca^{2+} channel complex in heart and regulates channel function. In this study, I demonstrate that a) calcineurin co-purifies with the L-type Ca^{2+} channel in lysates from ventricle (and brain), b) calcineurin interacts directly with N- and C-terminal intracellular domains of the major pore-forming subunit $\alpha_1$1.2, c) calcineurin is capable of recognizing $\alpha_1$1.2 as an enzymatic substrate *in vitro*, and d) calcineurin up-regulates L-type channel activity, as acute inhibition of calcineurin decreases channel function in a calcineurin phosphatase-dependent manner. Together, these data uncover a previously uncharacterized regulatory mechanism governing the activity of the cardiac L-type channel with potential implications in a variety of disease processes.

Calcineurin participates in the L-type Ca^{2+} channel macromolecular complex

Specificity and kinetics of reversible protein phosphorylation are governed by subcellular targeting of distinct pools of kinases and phosphatases. Consistent with this, an emerging theme in cardiovascular biology emphasizes the importance of specific localization of signaling molecules in proximity to their targets (Marx S, 2003). The L-type Ca^{2+} channel comprises a macromolecular complex of various enzymes and anchoring proteins, most of which associate with the long intracellular C-terminus of the $\alpha_1$1.2 subunit (Catterall WA, 2000; Felix R, 2005; Pitt GS et al., 2006). A recent study in neurons implicated calcineurin in antagonizing PKA-mediated activation of the L-type Ca^{2+} channel by anchoring to the scaffolding protein AKAP79/150 (Oliveria S et al, 2007). In heart, however, it is unclear whether AKAP79/150 is expressed (Ruehr M et al., 2004), and here, I report that calcineurin binds directly to both the N- and C-termini of cardiac $\alpha_1$1.2 without the need for an intermediary protein. I have identified residues 1943-1971 on $\alpha_1$1.2 as the minimal region required for specific calcineurin binding. That this interaction occurs with at least low micromolar affinity is consistent with its being physiologically relevant.

Phosphorylation-dependent regulation of Ca^{2+} channel function

The Ca^{2+} channel is subject to regulation by multiple hormones and neurotransmitters largely through activation of kinases and phosphatases (Kamp TJ & Hell JW, 2000; Pitt GS et al., 2006). Prominent among these is a transient increase in channel activity triggered by PKA phosphorylation. Interestingly, recent work by Ganesan et al suggests that much of PKA-dependent activation of L-type channels in ventricular myocytes does not involve Ser-1928 (Ganesan AN et al., 2006). PKC-dependent mechanisms are similarly complex, acting on two threonine residues at the N-terminus of $\alpha_1$1.2, as well as at Ser-1928 (Kamp TJ & Hell JW, 2000). In the case of N-terminal phosphorylation, an initial transient activation of the channel is followed by sustained inhibition (McHugh D et al., 2000). It is noteworthy that my results demonstrate calcineurin anchoring at the $\alpha_1$1.2 N-terminus, although the functional implications of this interaction are beyond the scope of the current study. Further, a recent report mapped PKG-mediated inhibition of heterologously expressed L-type channel activity to Ser-496 on the β_{2a} subunit, as well as phosphorylation of $\alpha_1$1.2 Ser-1928 *in vitro* (Yang L et al., 2007). Thus, there is considerable variance in the literature regarding the effects and mechanisms of Ca^{2+} channel phosphorylation in health and disease, as important differences have been reported depending on the site of phosphorylation and the kinase involved.

Whereas a great deal of work has focused on channel regulation by kinases, including PKA (De Jongh KS et al., 1996; Kamp TJ & Hell JW, 2000), PKC (Kamp TJ & Hell JW, 2000; McHugh D et al., 2000), PKG (Fischmeister R et al., 2005; Yang L et al., 2007), CaMKII (Hudmon A et al., 2005; Anderson ME, 2007), and src (Dubuis E et al., 2006), less is known about the functional effects of countervailing actions by protein phosphatases. PP2A (Davare MA et al., 2000; Hall DD et al., 2006) and PP1 (duBell WH & Rogers TB, 2004) have been reported to dephosphorylate the channel, but the functional significance of these actions remains poorly characterized. Indeed, I have localized calcineurin binding to a region of $\alpha_1$1.2 close to a site where PP2A binds (Hall DD et al., 2006). Some studies have suggested that PP2A inhibits L-type channel function (Chen X et al., 2002), possibly by antagonizing the phosphorylation of Ser-1928. Here, I show that calcineurin elicits robust up-regulation of channel activity. While still unresolved, one possible mechanism may involve interference with PP2A binding and consequent antagonism of PP2A-dependent dephosphorylation.

Calcineurin-mediated regulation of L-type Ca^{2+} channel

We have previously shown that targeted inhibition of calcineurin, either pharmacologically with CsA (Wang Z et al., 2001a) or genetically via RCAN1 over-expression (Rothermel BA et al., 2001), attenuated action potential

prolongation and eliminated the increase in heart mass associated with pressure-overload hypertrophy. Similarly, calcineurin suppression prevented the hypertrophy-associated up-regulation of L-type Ca^{2+} currents (Wang Z et al., 2001a). Here, I extend these observations to demonstrate direct effects of calcineurin on the L-type channel. These findings, which are consistent with our overall hypothesis, cannot be explained by simple channel blockade, as two entirely divergent means of calcineurin inhibition – pharmacological inhibition with small molecules (CsA, FK-506) of distinct molecular structure, and intracellular peptide (AID) exposure – manifested the same response. As the effect of calcineurin inhibitors was partially reversible, current run-down cannot account for these findings either. I posit that calcineurin suppression induces progressive increases in channel phosphorylation as the cognate kinase is no longer antagonized by calcineurin. Accordingly, I am not surprised to observe only partial wash-out of the inhibitory effects, as a return to steady-state levels of $\alpha_1$1.2 phosphorylation would be expected to take time. Intriguingly, CsA and AID induced similar degrees of Ca^{2+} channel inhibition (44% and 36%, respectively).

There are reports of potentially non-specific actions of CsA on the L-type Ca^{2+} channel function (Matthes J et al., 2004). Here, I report evidence against this. Under conditions where calcineurin is inactive by complete chelation of intracellular Ca^{2+} (intracellular dialysis with 10 mM BAPTA), CsA had no effect on channel function. Also, electrophysiological recordings in ventricular

myocytes isolated from RCAN1 transgenic mice demonstrated no effect of acute CsA exposure or peptide-inhibitor dialysis in the context of negligible calcineurin activity. These data, then, indicate that the effects of CsA we observe are mediated by direct inhibition of calcineurin enzymatic activity.

Precedents in other systems

In a wide variety of cell types, calcineurin, activated by intracellular Ca^{2+}, feeds back to regulate Ca^{2+} homeostasis through its interaction with other Ca^{2+}-regulatory proteins. In yeast, calcineurin is involved in the regulation of Ca^{2+} pumps and exchangers responsible for Ca^{2+} homeostasis (Rusnak F & Mertz P, 2000). In mammalian tissues, calcineurin directly regulates the function of several proteins involved in Ca^{2+} homeostasis including the inositol triphosphate and ryanodine receptors in heart and the neuronal sodium/calcium exchanger (Carafoli E et al., 1999). Calcineurin regulates expression of the cardiac sodium/calcium exchanger (NCX1) and regulates NCX1 function by direct protein-protein interaction (Katanosaka Y et al., 2005; Wang Z et al., 2001b).

Calcineurin regulates voltage-gated Ca^{2+} channels in a variety of systems, although there is disagreement regarding the nature and extent of its regulatory function; some studies suggest an inhibitory role, whereas others suggest activation (**Table 3**). Early studies in molluscan and mammalian neurons

implicated calcineurin as an inhibitor of voltage-gated Ca^{2+} channels; more recently, studies in rodent neuroblastoma/glioma hybrid cell lines (NG108-15) (Lukyanetz EA et al., 1998) and vascular smooth muscle (Schuhmann K et al., 1997) demonstrated calcineurin-mediated negative feedback, possibly involving dephosphorylation of the channel complex itself. Conversely, other studies using electrophysiological and biochemical assays in rat pituitary tumor (GH_3) cells present evidence against calcineurin-mediated dephosphorylation and inactivation of L-type Ca^{2+} channels (Victor RG et al., 1997; Zeilhofer HU et al., 2000). Work performed in cultured hippocampal neurons provided the first evidence that calcineurin selectively enhances L-type Ca^{2+} channel activity in cultured neurons (Norris C et al., 2002). Thus, differences in species, cell type, expression system, and experimental conditions all may contribute to these discrepancies (**Table 3**). Indeed, the preponderance of different L-type Ca^{2+} channel genes, as well as alternatively spliced isoforms, expressed in diverse organ systems may contribute to these disparities (Liao P et al., 2005; Catterall WA, 2000; Catterall WA et al., 2005).

In rodent heart, the majority of studies have shown either no effect or an activation of L-type Ca^{2+} channel function by calcineurin, in some instances despite the use of similar experimental strategies (e.g. 25 μM FK-506, duBell WH et al., 1997 vs. Fauconnier J et al., 2005) (**Table 3**). Using 10 μM FK-506 (similar to my studies) Su Z et al. further report diverse effects of pharmacological

suppression of calcineurin on intracellular Ca^{2+}-handling between rat, mouse and rabbit ventricular myocytes (Su Z et al., 2003). Alternatively, using a genetic model of calcineurin activation by cardiac-specific over-expression of constitutively-active calcineurin in transgenic mice, Yatani et al. document increased L-type Ca^{2+} channel activity but a lack of effect of CsA on channel function. My findings of CsA and FK-506-mediated inhibition of L-type Ca^{2+} channel in heart are in agreement with those of Fauconnier J et al. and Mijares A et al., the latter demonstrating a dose-dependent inhibition of L-type Ca^{2+} channel function by CsA (Fauconnier J et al., 2005; Mijares A et al., 1997) (**Table 3**).

Ca^{2+} channel remodeling and significance in cardiac hypertrophy and failure

Cardiac hypertrophy is associated with significantly increased risk of both heart failure and arrhythmia and poses a major public health problem (Hill JA & Olson EN, 2008). There is evidence to suggest that alterations in transmembrane Ca^{2+} fluxes, and consequent perturbations of Ca^{2+} homeostasis, contribute to the pathogenesis of hypertrophy by abnormally activating Ca^{2+}-responsive signaling pathways. In several forms of heart disease, increases in L-type Ca^{2+} channel activity are a proximal trigger that activate pathological signaling cascades. Indeed, various transgenic models of L-type channel over-expression reveal the development of hypertrophy and severe cardiomyopathy associated with

ventricular fibrosis, myocyte necrosis and remodeling, often in an age- and time-dependent manner (Muth JN et al., 2001; Nakayama H et al., 2007).

Entry of a small amount of Ca^{2+} via L-type channels triggers the release of much larger amounts of Ca^{2+} from intracellular stores (Bers DM, 2002). As a result, modest changes in channel–mediated Ca^{2+} flux are amplified within the cell. In many species, membrane impedance is relatively high during phase 2 of the action potential, so changes in channel activity have important effects on action potential morphology and duration.

The L-type channel in heart is comprised of a large pore-forming subunit $\alpha_1$1.2 (also known as α_{1C}) plus auxiliary subunits β_{2a} and $\alpha_2\delta$ (Catterall WA, 2000). In failing human ventricular myocytes, Ca^{2+} channel activity often exceeds that predicted from constant levels of $\alpha_1$1.2 transcript and protein, suggesting post-translational regulation of the channel complex, possibly via phosphorylation (Schroder F et al., 1998). Several kinases have been shown to phosphorylate the channel, including protein kinases A and C, and Ca^{2+}-calmodulin-dependent kinase (Kamp TJ & Hell JW, 2000). These reactions have been shown to regulate numerous aspects of channel function, including protein abundance, voltage-dependent activation and inactivation, and recovery from inactivation. These alterations in channel function, in turn, are important in controlling heart rate, inotropy and lusitropy.

As noted above, the cellular and molecular phenotype of hypertrophy is dependent upon the degree of hypertrophy and the primary lesion that induces hypertrophy. Working with a model of compensated, pressure-overload hypertrophy in mice, our lab has found that outward K^+ currents (I_{to}, I_{sus}, I_{K1}) do not change, whereas inward L-type Ca^{2+} current increases, inducing greater AP prolongation. Other models of moderate hypertrophy also manifest increased L-type channel activity (Armoundas AA et al., 2001).

In our model of pressure-overload hypertrophy, the kinetics of channel inactivation are unchanged, suggesting little involvement of CaMK- (Mukherjee R & Spinale FG, 1998) or PKA- (Kamp TJ & Hell JW, 2000) dependent phosphorylation. Similarly, steady-state activation and inactivation are unchanged. In contrast, recovery from Ca^{2+} current inactivation is accelerated in hypertrophied myocytes, an effect blocked by CsA (Wang Z et al., 2001a). Together, these data implicate calcineurin-dependent signaling in the hypertrophy-associated electrical remodeling of L-type Ca^{2+} channel, and they point to possible post-translational changes in the channel protein itself.

Limitations and Future Directions

I report strong evidence here for a direct physical and functional interaction between calcineurin and the cardiac L-type Ca^{2+} channel. However,

the specific mechanisms underlying the functional regulation remain to be elucidated. One possible mechanism of regulation may involve direct protein-protein interaction and conformational changes in the $\alpha_1$1.2 subunit through calcineurin binding. My pulldown experiments revealed equivalent binding of calcineurin to $\alpha_1$1.2 in the presence of CsA, suggesting that the inhibition of L-type Ca^{2+} current by CsA is presumably not due to disruption of protein-protein interaction, but rather enzymatic action of calcineurin. Alternatively, I report that calcineurin is capable of recognizing $\alpha_1$1.2 as a substrate, although the specific site of action remains unresolved. Ser-1928 is an unlikely target as PKA-dependent phosphorylation of this site is associated with increased channel activity and a hyperpolarizing shift in the steady-state current-voltage relation (Catterall WA, 2000). That said, both PKC (Yang L et al., 2005) and PKG (Yang L et al., 2007) have been shown to target Ser-1928, and both kinases are known to have an inhibitory effect on L-type Ca^{2+} current (McHugh D et al., 2000; Fischmeister R et al., 2005). Finally, actions mediated indirectly by downstream targets of calcineurin and/or components of the channel complex may exist (Khoo MS et al., 2006).

Future studies can focus on exploring some of the above-mentioned possible mechanisms of calcineurin-mediated regulation of the L-type Ca^{2+} channel. A specific challenge I encountered during these studies was the ability to consistently reconstitute the L-type channel complex in a heterologous system

despite the large-scale use of this technique by others in the literature. Reconstitution of channel-calcineurin interaction would allow for immediate insights into mechanistic underpinnings, and also the direct investigation of the potential connection between the physical and functional aspects of interaction between the two proteins. Such further examinations could include, for example, the question whether calcineurin activation of the channel is preserved when the calcineurin docking motif within $\alpha_1$1.2 (aa 1943-1971) is mutated to disrupt binding.

In addition, the potential antagonistic effects of calcineurin on PKC and PKG inhibition of the channel should also be directly tested, including the elucidation of phosphorylation sites responsible for this regulation. Ser-1928 on $\alpha_1$1.2, along with those within the other channel subunits, could be mutated and tested in the presence of calcineurin activation/inactivation. A word of caution, however, is that heterologous reconstitution of L-type Ca^{2+} channels in systems diverse from native cardiomyocytes (e.g. HEK cells), while not only difficult, has often provided contrasting results to similar experiments performed in cardiomyocytes (Ganesan AN et al., 2006; Miriyala J et al., 2008 and discussion therein). In fact, the exclusive use of cardiomyocytes in my studies therefore represents a key strength of the results and conclusions reported in this document.

Finally, my data highlight calcineurin as a potential pro-arrhythmic molecule. In fact, activation of calcineurin *in vivo* is associated with a robust

increase in action potential duration (**Figure 1.3**, Wang Z et al., 2001a), a classic trigger for ventricular arrhythmias and sudden death. As discussed before, however, it is difficult to delineate whether these *in vivo* electrical remodeling events are due directly to calcineurin activation or occur as a by-product of the hypertrophic phenotype. Therefore, a further investigation of the effects of calcineurin on action potential duration should be tested in isolated cardiomyocytes, including the effects of acute calcineurin suppression by cyclosporine A, for example, on action potential duration, contractility and other important factors responsible for arrhythmogenesis (Owen VJ, 2000). Since calcineurin regulates native cardiac L-type Ca^{2+} channels, it would be expected to alter action potential duration and contractility in cardiomyocytes. With the purchase of the IonOptix cardiomyocyte contractility machine, these experiments can now be corroborated in the laboratory.

My findings reported in this document uncover a previously uncharacterized regulatory mechanism governing cardiac L-type Ca^{2+} channel activity by calcineurin and thereby raise the prospect of novel means of modulating this critical element in cardiac biology for therapeutic gain. In conclusion, calcineurin regulates the cardiac L-type Ca^{2+} channel. Thus, it would be expected to contribute to pathological electrical remodeling – and predisposition to malignant arrhythmia – in patients with cardiac hypertrophy and failure.

Table 3. Summary of reports in the literature on the effects of calcineurin on L-type Ca^{2+} current.

Author	Journal	Compound/Method	Effect	Species	Cell/Tissue
Studies that report inhibitory effects of calcineurin on $I_{Ca,L}$					
Chad JE and Eckert R	*J. Physiol.* (1986) **378**:31-51.	40 µg/mL PP2B dialysis	"small" ↓ $I_{Ca,L}$	*Helix*	Neuron
Schuhmann K et al.	*J. Gen. Physiol.* (1997) **110**:503-513.	1 µg/mL PP2B diffusion (inside-out patch)	~50% ↓ $I_{Ca,L}$	Human	smooth muscle
Lulkyanetz EA et al.	*J. Physiol.* (1998) **510**:371-385.	PP2B over-expression	~60% ↓ $I_{Ca,N}$ No effect on $I_{Ca,L}$	Rat/mouse hybrid	NG108-15 cells
Burley JR et al.	*Eur. J. Neurosci.* (2000) **12**:2881-2891.	PP2B over-expression	~30% ↓ I_{Ca} (L, T, N-type)	Rat/mouse hybrid	NG108-15 cells
Santana LF et al.	*J. Physiol.* (2002) **544**:57-69.	100 nM CsA	~33% ↑ $I_{Ca,L}$	Mouse	Ventriculocyte
Studies that report no effect of calcineurin on $I_{Ca,L}$					
Frace AM et al.	*J. Physiol.* (1993) **472**:305-326.	µM inhibitory peptide	No effect on $I_{Ca,L}$	Frog	Cardiomyocyte
McCall E et al.	*Circ. Res.* (1996) **79**:1110-1121	5 µM FK-506	No effect on $I_{Ca,L}$	Rat	Ventriculocyte
duBell WH et al.	*J. Physiol.* (1997) **501**:509-516	25 µM FK-506	No effect on $I_{Ca,L}$	Rat	Ventriculocyte
Zeilhofer HU et al.	*Neurosci.* (2000) **95**:235-241.	10 µM FK-506	No effect on $I_{Ca,L}$	Rat	GH3 cells
Yatani A et al.	*J. Mol. Cell Cardiol.* (2001) **33**:249-259.	1 µM CsA	No effect on $I_{Ca,L}$	Mouse	Ventriculocyte
Su Z et al.	*J. Pharmacol. Exp. Ther.* (2003) **304**:334-341.	10 µM FK-506	No effect on $I_{Ca,L}$	Rabbit/Mouse	Ventriculocyte
Studies that report activating effects of calcineurin on $I_{Ca,L}$					
Mijares A et al.	*J. Mol. Cell Cardiol.* (1997) **29**:2067-2076.	15 mg/kg/day CsA subcutaneous injection 100 pM – 80 µM CsA	~25% ↓ $I_{Ca,L}$ ~11 – 98% ↓ $I_{Ca,L}$	Guinea pig	Cardiomyocyte
Yasutsune T et al.	*Br. J. Pharmacol.* (1999) **126**:717-729.	10 µM FK-506 30 µM FK-506	No effect on $I_{Ca,L}$ ~22% ↓ $I_{Ca,L}$	Pig	Coronary artery strips
Norris CM et al.	*Neurosci.* (2002) **110**:213-225.	0.5 – 50 µM FK-506 20 µM CsA	~10 – 50% ↓ $I_{Ca,L}$ ~25% ↓ $I_{Ca,L}$	Rat	hippocampal cells
Fauconnier J et al.	*Am. J. Physiol. Heart Circ. Physiol.* (2005) **288**:778-786	25 µM FK-506	~25% ↓ $I_{Ca,L}$	Rat	Ventriculocyte

APPENDICES

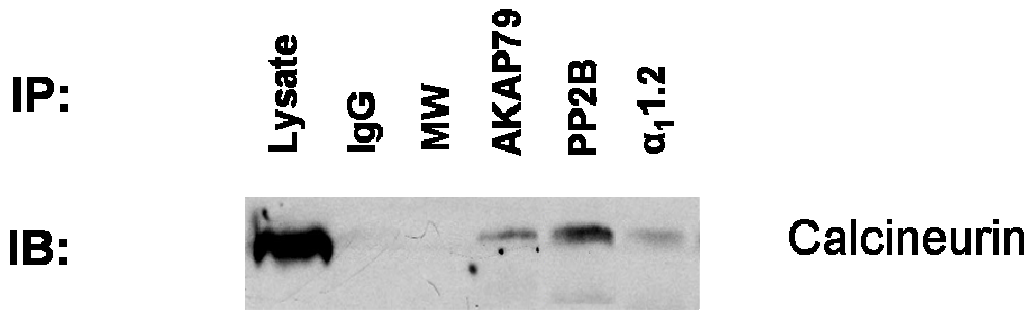


Figure A1: Calcineurin associates with neuronal L-type Ca^{2+} channel. Co-immunoprecipitation from rat brain lysate demonstrates interaction between calcineurin and the $a_1$1.2 subunit of the L-type Ca^{2+} channel. Immunoprecipitations (IP) contained antibodies against $a_1$1.2 and calcineurin (PP2B), as well as AKAP79. Immunoblot (IB) against calcineurin revealed a robust signal at the expected size for CnA (61 kD). No non-specific co-immunoprecipitations were observed with pre-immune serum (IgG) used as a negative control. MW = molecular weight marker.

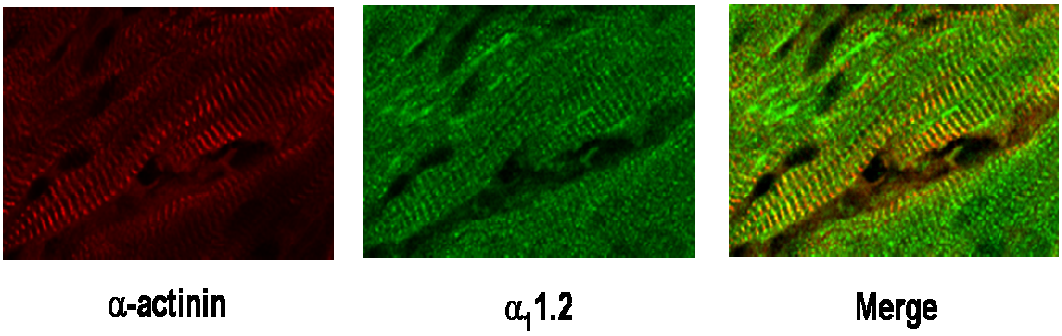


Figure A2: L-type Ca^{2+} channel $\alpha_1$1.2 subunit co-localizes at Z-bands. Immunohistochemistry of mouse cardiac tissue co-stained for $\alpha_1$1.2 (green) and α-actinin (red), a marker for cardiomyocyte Z-band staining, shows co-localization both proteins (yellow; merge) by confocal microscopy.

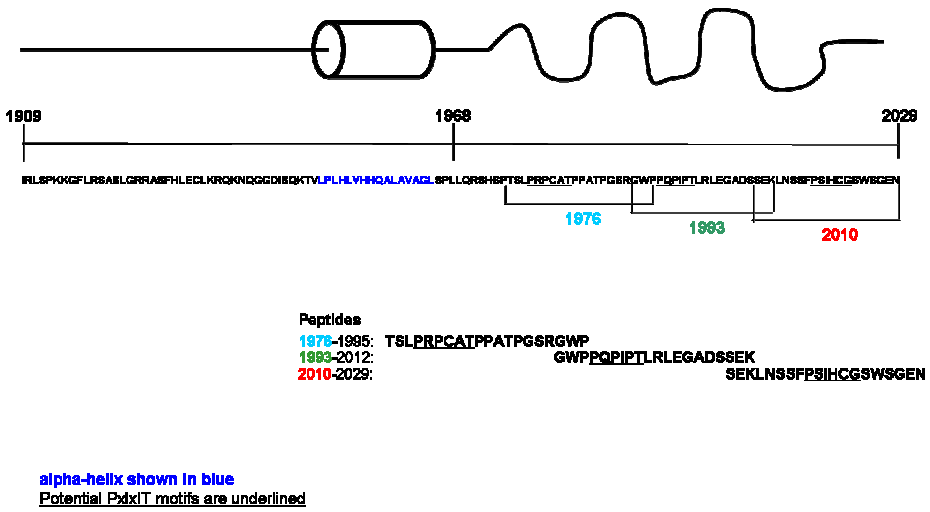


Figure A3: Prediction of CT-8 secondary structure based on Robson-Garnier algorithm analysis of primary amino acid sequence. CT-8 comprises of aa. 1909-2029, with two distinct halves – aa. 1909-1968 comprised of one alpha-helix (cylinder), and aa. 1969-2029 containing no definitive secondary structure but containing potential PxIxIT motifs (underlined). Overlapping peptides (1976, 1993 and 2010) of 20 aa. length comprised of the $\alpha_1$1.2 primary amino acid sequence are shown, with their respective PxIxIT motifs underlined.

Table A1: Putative sites of $\alpha_1$1.2 phosphorylation determined using two bioinformatics algorithms.

PROSITE (ExPASy):

N	I	II	III	1	B	C	D	E	4	7	8	23
				PKA		PKA			PKA		PKA	PKA
PKC		PKC		PKC	PKC	PKC	PKC		PKC		PKC	PKC
CK2	CK2	CK2	CK2	CK2	CK2	CK2	CK2	CK2	CK2	CK2		CK2
						YPhos						

Scansite (MIT) – high stringency:

N	I	II	III	1	B	C	D	E	4	7	8	23
						PKA			PKA		PKA	
							GSK3		GSK3		GSK3	
				PKCmu								

See Figure 2 (Chapter Two) for locations of fusion proteins; CK2: casein kinase 2; GSK: glycogen synthase kinase; PKA: protein kinase A; PKC: protein kinase C; YPhos: tyrosine phosphorylation.

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