Cellular Retinoic Acid Binding Protein-I as a Drugable Target to Dampen Calcium-Calmodulin Dependent Protein Kinase II Activity

A Thesis SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Stanislas Iheanacho Ogokeh

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Li-Na Wei

May 2016

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Acknowledgements

I would like to thank my Adviser, Dr. Li-Na Wei and the members of my Committee: Dr. Timothy Walseth and Dr. Sundaram Ramakrishnan.
I would like to also extend my gratitude to my Mentor Dr. Sung-Wook Park and the members of the Wei Lab:

Current Members:
- Shawna Persaud
- Bomi Lee
- Dr. Yu-Lung Lin
- Yi-Wei Lin
- Mariana Teixeira

Former Member
- Dr. Cheng-Ying Wu

Thanks to the Hiroyuki Kagechika laboratory for providing the retinoid analogs and to Dr. Joe Metzger's Lab for providing the primary rat cardiac myocytes.

Thanks to our undergraduate volunteers:

- Jennifer Nhieu
- Vibhav Singh
- Adnan Khan
- Brian Cheng
- Wasif Khan

Finally, thanks to the University of Minnesota Pharmacology Department for this learning experience.

Dedication

This thesis is dedicated to my parents, Theodore O. Ogokeh and Cecilia C. Ogokeh and my siblings, Immaculata, Justina, Constance and Emmanuela Ogokeh.

Abstract

Heart failure is the leading cause of death in developed countries. Current therapeutic approaches target the autonomic nervous system's β and α adrenergic receptors to rescue the failing heart. Despite these approaches, heart failure is responsible for 600,000 deaths in the U.S as of 2004. For this reason new drugs need to be developed. Over the past 10 years, mounting evidence has identified Ca^{2+}-calmodulin dependent protein kinase-II delta (CaMKII-δ) over activation as a main culprit in heart failure, but no groups have been able to develop a strategy to decrease this over activation. In this current study cellular retinoic acid binding protein I (Crabp-I) is shown to be a viable drugable target to dampen the activation of CaMKII-δ. Furthermore, using *in vitro* phosphorylation, western blot and qPCR assays, a novel retinoid analog (compound 11) has been shown to selectively mitigate CaMKII-δ induced hypertrophic damage. This current study sets the tone for the development of a new family of cardiovascular drugs that could help in decreasing the morbidity of heart failure.

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Chapter 1| Introduction

Heart failure is a chronic malady that affects 11.9% of male and 10% of female ages 20-39 as well as 84.7% of male and 85.9% of female ages 80 and above (Figure 1). Moreover, the toll of deaths due to cardiovascular diseases (CVDs) has gradually increased from 38,000 deaths in the 1900s to 600,000 deaths in 2004. When diet and exercise alone are not sufficient to remediate the failing heart, pharmacological intervention in the form of β and α adrenergic antagonists are implemented. While these drugs, have been approved by the FDA and have been used for extended period of times, they also produce unwanted side effects that could aggravate cardiac functions. For instance, the β blocker *pindolol* while being indicated for cardiac arrhythmia can also cause bradycardia and heart failure as side effects. Due to the staggering increase in morbidity over the past decades, and due to the counterintuitive side effects caused by current treatments, it is evident that novel selective xenobiotics need to be developed.

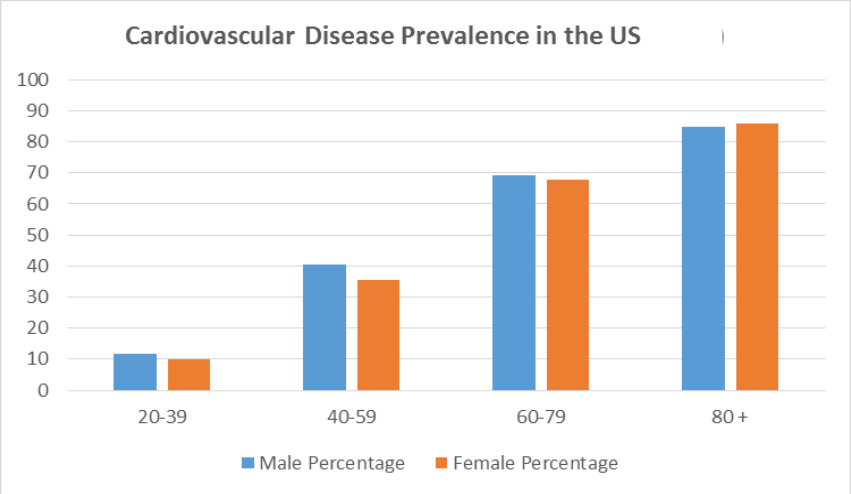

Figure 1: Cardiovascular disease prevalence in the U.S as of 2004.

Over the years CaMKII-δ has caught the attention of numerous independent research groups and accumulating evidence has shown that this enzyme is a universal culprit in heart failure. Indeed, because of its maladaptive role in excitation-contraction coupling and in excitation-transcription coupling, CaMKII-δ plays major roles in the onset of heart failure by inducing arrhythmias, and in the end stage of the disease by increasing the expression of hypertrophic genes. While the negative functions of CaMKII-δ in heart failure has now been widely accepted, no clinically relevant small molecule has been found to selectively curb its over-activation.

Our group has previously published evidence showing that the vitamin A derivative, all trans retinoic acid has cytoplasmic non-canonical activity through Crabp-I. One of these roles involves associating and activating ERK1/2. In an attempt to discover other un-precedent proteins that could associate with Crabp-I, senior members of our team performed co-immunoprecipitation assays, screening for potential complexes. Through this screening process, Crabp-I and CaMKII-δ were found to co-immunoprecipitate. The outcome of this association has not been studied previously. In order to shed some light on this interaction, the following hypothesis was tested: **Crabp-I regulates CaMKII phosphorylation in the presence of retinoic acid or its analogs.**

This hypothesis was tested by the following three specific aims:

Aim 1: Test the effects of Crabp-I and ATRA on CaMKII-δ activation using *in vitro* phosphorylation.

Aim 2: Identify ATRA analogs capable of lowering CaMKII-δ activation using *in vitro* phosphorylation.

Aim 3: Test candidate compounds in biologically relevant models (In a cell line and in primary rat cardiac myocyte cultures).

These studies will lead to the characterization of a potential drug target for cardiovascular diseases.

Chapter 2| All-Trans Retinoic Acid

1. ATRA Synthesis

All-trans retinoic acid (ATRA) is one of multiple biologically active derivatives of the fat-soluble vitamin A (retinol) (Giguère, 1994; Theodosiou, Laudet, & Schubert, 2010; Zhang, Zhao, & Huang, 2012). Retinol is not synthesized *de novo* and must be acquired via dietary intake (Giguère, 1994; Theodosiou et al., 2010). The ingested retinol then undergoes a myriad of biochemical reactions, in the intestine, the liver and target tissues resulting in the formation of ATRA among other by-products (Theodosiou et al., 2010). The metabolized ATRA subsequently drives multiple biological processes including vision, cell growth, and differentiation (Giguère, 1994; Theodosiou et al., 2010; Zhang et al., 2012).

a. Intestine

The first step in ATRA biosynthesis occurs after its precursors are ingested from plants or animals (Giguère, 1994; Theodosiou et al., 2010). Dietary intake of plants provide β-carotene and/or readily available retinol while, dietary intake of animal tissues provide retinyl esters (Giguère, 1994; Theodosiou et al., 2010). On the one hand, once in the intestine, β-carotene is cleaved at its 15,15' carbon double bond by the enzyme β,β-carotene-15,15'-monooygenase-I (BCO-I) to form two all-trans-retinaldehyde (also known as retinal) (Theodosiou et al., 2010). Retinal is then converted to retinol by the enzyme retinal dehydrogenase (Theodosiou et al., 2010). On the other hand, retinyl esters from animal tissues

are directly converted to retinol by the enzyme retinyl ester hydrolase (REH) (Theodosiou et al., 2010). The intestinal retinol subsequently binds to cellular retinol binding protein-II (CRBP-II) which solubilizes it and protects it from being degraded (Theodosiou et al., 2010). Moreover, CRBP-II also delivers its ligand (retinol) to the enzyme lecithin:retinol acetyltransferase (LRAT) which esterifies retinol converting it to retinyl esters (Theodosiou et al., 2010). Those retinyl esters are packed into chylomicrons (cluster of triacyglycerols, phospholipids, carotenoids, retinyl esters retinol, cholesteryl esters and apolipoproteins) and are secreted in the intestinal lymph (Theodosiou et al., 2010).

b. Liver

The circulating retinyl esters ultimately reach the liver (Theodosiou et al., 2010). In the liver's hepatocytes, retinyl esters are hydrolyzed back to retinol by the enzyme REH, and again is attached to CRBP for solubility (Theodosiou et al., 2010). CRBPs attached to retinol can either sequester retinol in the stellate cells of the liver or transfer them to retinol binding protein (RBP) (Theodosiou et al., 2010). Retinol is stored in the stellate cells by being reversely converted to retinyl esters by the LRAT and REH (Theodosiou et al., 2010). In the hepatocytes, RBP protects retinol from being degraded. RBPs bound to retinol are secreted in the plasma en-route to retinol targets tissues (Theodosiou et al., 2010). It is also important to note that the plasma level of retinol bound to RBP is rigorously maintained at 2 μM (Theodosiou et al., 2010). In the plasma, retinol-RBP complex binds to transthyretin (TTR) which prevents retinol from being excreted via glomerular filtration (Theodosiou et al., 2010).

c. Target Tissues

Upon close vicinity of its target tissues, the TTR-RBP-retinol complex binds to the surface receptor Stimulated by Retinoic Acid Gene 6 Receptor (STRA6). Inside the target tissues, retinol has two possible fates. The retinol can bind to CRBP and can be reversibly converted to retinyl esters by the enzymes LRAT and REH (Theodosiou et al., 2010). Additionally, retinol can be converted to retinaldehyde by the enzyme retinol dehydrogenase (RDH) (Theodosiou et al., 2010). Retinal then serves as a substrate for the enzyme retinaldehyde dehydrogenase (RALDH) which irreversibly converts it to all trans retinoic acid (ATRA) (Theodosiou et al., 2010). The newly formed ATRA binds to cellular RA binding proteins type I and II (Crabp -I and Crabp -II) whose purposes are to solubilize ATRA, protect it from cytosolic degradation and transport it to its appropriate cellular compartments (Dong, Ruuska, Levinthal, & Noy, 1999; Theodosiou et al., 2010). Furthermore, Crabp -I and Crabp -II modulate ATRA physiological activities that range from differentiation, apoptosis and growth arrest (Das et al., 2014; Persaud et al., 2016). The physiological functions of ATRA can be grouped into two main categories: a canonical genomic function mediated by Crabp -II and a non-canonical cytoplasmic function mediated by Crabp -I.

2. ATRA Canonical Pathway

During canonical signaling, Holo- Crabp -II (Crabp -II-ATRA) binds to nuclear Retinoic Acid Receptors (RARs) which then forms a hetero dimer with

retinoid X receptor (RXR). The RAR-RXR heterodimer then translocates into the nucleus, binds to specific DNA sequences referred to as Retinoic Acid Response Elements (RARE) and initiates the transcription of those genes through the activation of the co-activator histone acetyl transferase (HAT) (Das et al., 2014).

3. ATRA Non-Canonical Pathway

The non-canonical signaling of ATRA occurs independently of RARs-RXRs by binding to Crabp -I instead of Crabp -II. Moreover, unlike the genomic response seen in the previous pathway, the target proteins of this pathway are located in the cytoplasm. For instance, Crabp -I coordinates ATRA degradation by transporting it to cytochrome P450 enzymes including CYP26A1, and CYP26B1 (Bilbija et al., 2012; Theodosiou et al., 2010). Furthermore recent studies from our group have shown that holo- Crabp -I (Crabp -I-ATRA) can activate ERK1/2 in embryonic stem cells leading to the elongation of the cell cycle by extending the G1 phase (Persaud, Lin, Wu, Kagechika, & Wei, 2013).

While extensive work has been done on the genomic activity of ATRA, little is known about its cytoplasmic activities mediated by Crabp -I. For example, in addition to the interactions with ERK1/2 and the CYP enzymes, a novel protein has been shown to associate with holo- Crabp-I through pathways that have yet to be elucidated. This protein, called calcium-calmodulin depending protein kinase II (CaMKII), is implicated in cardiovascular pathology as will be discussed in the following chapter.

Chapter 3| Calcium Calmodulin Dependent Protein Kinase II

1. Structural Description of CaMKII

Calcium calmodulin dependent protein kinase II or CaM Kinase II (CaMKII) is a serine threonine protein kinase (Mollova, Katus, & Backs, 2015). The gene that encodes for this protein is found in position 22 of the long arm of chromosome 10 (10q22) (NCBI, 2016). The CaMKII gene produces four different isoforms; CaMKII-α, CaMKII-β (found in the brain), CaMKII-ɣ and, CaMKII-δ (found in the heart and in peripheral tissues) (Table 1) (Mollova et al., 2015; Stratton, Chao, Schulman, & Kuriyan, 2013). Crystallographic analysis of the holoenzyme structure of CaMKII hinted at an assembly made of two stacked hexametric rings forming a dodecameric structure (Stratton et al., 2013). The isoform of the monomers that make up this structure are not exclusive. Indeed, one holoenzyme can be made of monomers from a single isoform (homo-dodecarmeric structure) or monomers from different isoforms (hetero-dodecameric) (Mollova et al., 2015; Stratton et al., 2013).

Table 1: The Distribution of the four CaMKII gene products (adapted from Erickson and Anderson, 2008).(Erickson & Anderson, 2008)

CaMKII gene product	Gene product Tissue distribution
α	Brain
β	Brain
ɣ	Heart, Peripheral
δ	Heart, Peripheral

Despite the diversity of the overall architecture of CaMKII, each monomer shares three common subunits: a catalytic, a regulatory, and an association domain (Mollova et al., 2015). The catalytic or kinase domain is situated toward the N terminus and contains an ATP binding site and interacts with CaMKII target proteins (Mollova et al., 2015; Stratton et al., 2013). The regulatory domain is located downstream of the catalytic domain and upstream of the association domain. This domain is composed of an auto-inhibition site and a calmodulin binding site (also known as a calmodulin footprint) (Mollova et al., 2015; Stratton et al., 2013). The regulatory domain also has multiple post-transcriptional sites such as an auto-phosphorylation site at the threonine 287 residue. Downstream of the regulatory domain and toward the C terminus lies the association domain. As its name implies, the association domain is used as a platform to assemble each monomers into the holoenzyme (Mollova et al., 2015; Stratton et al., 2013). The difference between each isoform lies on the length of the linker sequence connecting the catalytic domain and the association domain (Mollova et al., 2015; Stratton et al., 2013). Those linker sequences range from no sequence to around 30 amino acid residues (Mollova et al., 2015; Stratton et al., 2013).

2. CaMKII Conformational States

CaMKII monomers can assume two conformational states depending on the intracellular calcium concentration ($[Ca^{2+}]_i$): an inactive conformation and an active conformation. When $[Ca^{2+}]_i$ is under basal level, for instance during diastole (0.1 μM of Ca^{2+}), CaMKII is in its inactive conformation (Lars S. Maier, 2012; Mollova et al., 2015). In this conformational state, the regulatory domain

folds over the catalytic domain. The pseudo-substrate found in the regulatory domain of CaMKII constrains the catalytic domain thus, auto-inhibiting it (Anderson, Brown, & Bers, 2011; Mattiazzi et al., 2015; Mollova et al., 2015). Contrastingly, when $[Ca^{2+}]_i$ is elevated during systole to 0.5 μM-1 μM, calcium binds to calmodulin (Lars S. Maier, 2012). The calcium-bound calmodulin (Ca^{2+}/CaM) then binds to the CaM footprint region on the regulatory domain of CaMKII. This interaction activates CaMKII by causing a conformation change that frees the catalytic domains from auto-inhibition (Anderson et al., 2011). Subsequently, Ca^{2+}/CaM binding induces the phosphorylation of the Thr 287 domain. This post transcriptional modification locks CamKII in its active conformation. When the $[Ca^{2+}]_i$ returns to basal level during diastole (0.1 μM), and if the frequency of action potential is low, Ca^{2+}/CaM's affinity for the CaMKII decreases (Anderson et al., 2011). Ca^{2+}/CaM then dissociates from the regulatory domain causing CaMKII to be inactivated. In addition, protein phosphatases such as PP2A can also dephosphorylate CaMKII to inactive it (Kreusser & Backs, 2014; Mattiazzi et al., 2015; Stratton et al., 2013).

3. CaMKII-δ Function in the Heart

In the heart, CaMKII-delta (CaMKII-δ) and CaMKII-gamma (CaMKII ɣ) are the predominant isoforms (Anderson et al., 2011; Mattiazzi et al., 2015; Mollova et al., 2015). As mentioned previously, the holoenzymes in cardiac tissues can be composed of both isoforms, creating a hetero-dodecameric (Anderson et al., 2011; Mattiazzi et al., 2015; Mollova et al., 2015). Furthermore, CaMKII-δ can produce two distinct splice variants: CaMKII-δ_B and CaMKII-δ_c . CaMKII-δ_B

contains an extra 11 amino acids sequence used for nuclear localization while, CaMKII-δ_C lacks this nuclear localization sequence (Anderson et al., 2011; Mollova et al., 2015). In the myocardium CaMKII-δ modulates two main functions: excitation-contraction coupling (ECC) and excitation transcription coupling (ETC) (Bers, 2011; L. S. Maier & Bers, 2007).

a. Excitation-Contraction Coupling

Two of the most fundamental attributes of a functioning heart are its ability to contract and relax in tandem in order to orchestrate the rhythmic distribution of blood to and from peripheral tissues. The dynamic process, where electrical input in the form of an action potential is converted to mechanical output in the form of contraction is called excitation-contraction and is mediated by the secondary messenger calcium (Lars S. Maier, 2012).

Indeed, during systole, the initial cardiac action potential depolarizes the sarcolemma. This depolarization predominantly activates the L-type voltage-dependent calcium channel (LTCC), (T-type Ca^{2+} channels plays a marginal role in Ca^{2+} influx) and increases its probability of assuming an open state conformation (Lars S. Maier, 2012; L. S. Maier & Bers, 2007). With the LTCC open, Ca^{2+} enters the cardiac myocytes producing an inward Ca^{2+} current (I_{Ca2+}) (Bers, 2002; Lars S. Maier, 2012). Moreover, CaMKII-δ_C can phosphorylate the α_{1c} subunit of the LTCC at its amino and carboxyl tails and can also phosphorylate the Thr-498 residue of the β_{2a} subunit of the LTCC(L. S. Maier & Bers, 2007). This series of phosphorylation cause the LTCC to remain open longer thus allowing more Ca^{2+} to enter the cell (L. S. Maier & Bers, 2007). The

incoming Ca^{2+} is able to directly interact with the SR's Ca^{2+} release channels; the Ryanodine Receptors (RyRs) (L. S. Maier & Bers, 2007). The activation of the RyRs subsequently leads to the release of stored Ca^{2+} from within the SR, through the Ca^{2+}-induced Ca^{2+}- release (CICR) pathway (Lars S. Maier, 2012; L. S. Maier & Bers, 2007). The release of sarcoplasmic reticulum Ca^{2+} causes a net increase in overall free intracellular calcium concentration $[Ca^{2+}]_i$ (Bers, 2002; Lars S. Maier, 2012). It has also been reported by multiple sources (Witcher et al, Maier et al) that CaMKII can directly phosphorylate RyRs at the Ser-2815 residue and that overexpression of CaMKII-δc in cardiac myocytes induced an increase of SR Ca^{2+} release (L. S. Maier & Bers, 2007). The free calcium produced by the events aforementioned binds the myofilament protein, troponin C leading to heart contraction (Bers, 2002; Lars S. Maier, 2012).

Following systolic contraction, the heart enters the diastolic phase. The diastolic phase is characterized by the relaxation of the heart muscle. For this event to occur, Ca^{2+} bound to the myofilament troponin C must be dislodged and the free cytoplasmic Ca^{2+} must be expelled from the cell in order to decrease the overall $[Ca^{2+}]_i$ (Bers, 2002). The decrease in $[Ca^{2+}]_i$ is initiated in the early stage systole through the negative feedback inhibition of the LTCC by the release of sarcoplasmic Ca^{2+}. As described by Puglisi et al, calcium induced calcium release of the SR causes a 50% decrease in Ca^{2+} influx via the LTCC. In addition to LTCC inhibition, four additional pathways contribute to cytosolic Ca^{2+} removal which include the SR Ca^{2+} ATPase (SERCA), the sarcolemmal Na+/ Ca^{2+} exchange (NCX), the sarcolemmal Ca^{2+}-ATPase or the mitochondrial Ca^{2+}

uniporter(Bers, 2002). In humans cytosolic Ca^{2+} is mainly removed via SERCA2a (70%), and the Na+/ Ca^{2+} exchange (28%). The remaining 1% of cytosolic Ca^{2+} are then removed by the “slow systems”, referring to Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter (Table 2) (Bers, 2002). Calcium reuptake into the SR is dependent of the SR Ca^{2+}-ATPase, which is in turn is controlled by phospholamban (PLB) (Bers, 2002). PLB inhibits SR Ca^{2+} ATPase endogenously. However, when PLB is phosphorylated in its threonine 17 residue by CaMKII-δ_C, it undergoes a conformation change which releases SERCA from its inhibition (L. S. Maier & Bers, 2007). This favors the conformation equilibrium of SERCA to lean towards its active state, allowing cytosolic Ca^{2+} to enter the SR where it is stored until the next action potential. Besides PLB, CaMKII-δ_C can also phosphorylate other Ca^{2+} channels in order to allow more calcium inside the cell (Bers, 2011).

Table 2: Intracellular calcium removal systems.

Species	SR Ca^{2+}-ATPase	Na+/ Ca^{2+} Exchanger	Sarcolemmal Ca^{2+}-ATPase	Mitochondrial Ca^{2+} Uniporter
Human	70%	28%	1%	
Rat	92%	7%	1%	
Mouse	92%	7%	1%	

b. Excitation-Transcription Coupling (ETC)

In addition to modulating the conversion of cardiac electrical signal to mechanical output in the cytoplasm as shown in ECC, CaMKII-δ regulates the transcription of certain genes within the nucleus. This process is termed excitation-transcription coupling and involves the nuclear variant; CaMKII-δ_B.

ETC generally occurs in the compensatory stage of cardiac hypertrophy and during the end stage of heart failure (Lars S. Maier, 2012). ETC is a maladaptive series of events that is marked by an increase in CaMKII-δ_B expression and activation.

At the chromatin level, multiple groups have demonstrated that CaMKII-δ_B is able to moderate the transcription of several hypertrophic genes at multiple levels. For instance, cardiac Myocytes Enhancer Factor 2 (MEF2) and class II histone deacetylases (i.e., HDAC4) have been shown to be controlled by CaMKII-δ_B (Lars S. Maier, 2012). Indeed, during basal activity HDAC4 binds to and inhibits the transcription of MEF2 (Bers, 2011; Lars S. Maier, 2012). However, when CaMKII-δ_B is activated, it phosphorylates two serine residues in HDAC4 (Bers, 2011). The phosphorylated HDAC4 detaches from MEF2 and is translocated out of the nucleus into the cytoplasm. Consequently, the uninhibited MEF2 is then activated leading to the expression of gene products involved in hypertrophy and ultimately heart failure (Bers, 2011; Lars S. Maier, 2012).

Going beyond the chromatin and looking at the histone level, Dr. Awad and her team deduced a new interaction between activated CaMKII-δ_B and histone H3 in hypertrophic induced cardiac myocytes (Awad et al., 2013). Indeed, CaMKII-δ_B from primary rat neonatal cardiomyocytes immuno-precipitated with endogenous histone H3. Moreover, the *in vitro* phosphorylation assay between CaMKII-δ_B and histone H3 showed that CaMKII phosphorylated Histone at the serine 10 residue (Awad et al., 2013). When primary rat neonatal cardiomyocytes were treated with the hypertrophy inducer phenylephrine, the level of the

hypertrophic marker α-actinin was elevated while cell proliferation markers such as Cdk4, cyclin E and Cdc2 were relatively unchanged (Awad et al., 2013). A ChIP Q-PCR assay showed that the CaMKII-histone H3 interaction occurred adjacent of genes involved in cardiac remodeling, i.e., ANF and ϐ-MHC (Awad et al., 2013). Finally, histone H3 phosphorylation through CaMKII-δ_B lead to the increase expression of MEF2 hinting at a crosstalk signal with HDAC4 phosphorylation (Awad et al., 2013).

In this chapter the unique architecture of CaMKII-δ and its role in cardiac ECC and ETC were described. In the following chapter, CaMKII-δ implication in heart failure will be discussed.

Chapter 4| CaMKII-δ in Heart Failure

1. Heart Failure Pathophysiology

Heart failure (HF) is the leading cause of death in both men and women in developed countries with cancer being a close second (American Heart Association, 2016). HF is characterized by cardiac myocyte contractile dysfunction that provokes arrhythmias via an asynchronous systolic and diastolic cycle (Luo & Anderson, 2013). This pathological phenotype is caused by a deviation in intracellular Ca^{2+} homeostasis and an increase in ROS production, and leads to the activation of maladaptive mechanisms that are meant to offset this imbalance. Those mechanisms include the expression of myocardial hypertrophic genes, increase filling pressure as well as enhanced neuro-humoral signals which ultimately leads to myocardial remodeling (Luo & Anderson, 2013). During the past 10 years, the over activation of CaMKII-δ has been identified as a culprit in the development of heart failure

2. CaMKII as a Potential Pharmacological Target in Heart Failure

In the previous chapter, key proteins involved in myocardial Ca^{2+} handling during contraction and relaxation were presented. Those proteins included the LTCC, the sarcoplasmic reticulum's RYR2, PLB and SERCA2, the NCX, the Na^{+} uniporter Ca^{2+}-ATPase and the mitochondrial Ca^{2+} uniporter. Furthermore, proteins involved in the process of cardiac remodeling due to an increase in $[Ca^{2+}]_i$ (HADC4, H3, MEF2) were also described. Since these calcium handling

proteins are modulated by the active form of CaMKII-δ_C or CaMKII-δ_B, It is not surprising that CaMKII-δ plays a pivotal role in maintaining $[Ca^{2+}]_i$ homeostasis in basal condition or during the fight of flight response (Luo & Anderson, 2013). Over-activation of CaMKII-δ negatively affects the proteins aforementioned causing them to mishandle the influx and efflux of calcium. This phenomena causes the $[Ca^{2+}]_i$ to deviate from equilibrium, and foster an environment ideal for arrhythmia, and ultimately heart failure to develop. It is because of this mechanism that multiple independent groups have provided mounting evidence pointing at CaMKII-δ as bona fide malefactor in arrhythmogenic diseases, making it an attractive target for drug development.

This is true in catecholaminergic polymorphic ventricular tachycardia (CPVT) among other diseases. CPVT is an autosomal dominant arrhythmogenic disease caused by a mutation in the RyR2 gene (Di Pasquale et al., 2013). Due to this mutation, Ca^{2+} leaks out of the sarcoplasmic reticulum during diastole causing a potentially fatal arrhythmia called delayed after depolarization (DAD) (Di Pasquale et al., 2013). Using induced pluripotent stem cells (iPSC) from a patient suffering from CPVT, Dr. Di Pasquale and his team where able to differentiate patient specific cardiomyocytes with the CPVT phenotype (Di Pasquale et al., 2013). Inhibiting CaMKII-δ in the iPSC-derived CM with the drug KN-93 (a CaMKII antagonist) abolished DAD in the presence of the β-adrenergic agonist isoproterenol (Di Pasquale et al., 2013).

Moreover, CaMKII-δ auto phosphorylation via pathways other than the classical Ca^{2+} activation have proven to be detrimental in the failing heart. For

instance, in isolated rabbit cardiac myocytes, addition of the nitric oxide (NO) donor SNAP caused arrythmogenic spontaneous SR Ca^{2+} leak and activated CaMKII-δ (Curran et al., 2014). Co-administration of SNAP and the CaMKII inhibitor KN-93 remedied the arrhythmias induced by the calcium leak (Curran et al., 2014).

Those previous findings provided 3 pieces of key information: 1) CaMKII-δ over-activation causes heart failure, 2) inhibiting CaMKI-δ alleviates heart failure syndromes and that 3) there is no clinically approved selective drug capable to lower CaMKI-δ over-activation (KN-93 produces detrimental side effects and is not approved for clinical use). Bearing this in mind, experiments were design to discover compound that could selectively lower CaMKI-δ activity by targeting Crabp-I as will be described in the next chapter.

Chapter 5| Materials

1. All Trans Retinoic Acid

ATRA in the form of Tretinoin were purchased from *Sigma Aldrich.*

2. Retinoid candidates

The retinoid compounds were synthesize by our Japanese collaborators in the Hiroyuki Kagechika laboratory.

3. Plasmid Constructs

The following plasmids were used in this project:

- His-Crabp-I
- Flag-CaMKII
- GST-Calmodulin

The plasmid were desgined following the protocol used in previous publications (Persaud et al., 2013; Persaud, Lin, Wu, Kagechika, & Wei, 2013).

4. Protein Expression

HIS-Crabp-I and GST-Calmodulin plasmids were transfected in T7 competent *E.coli*. Protein expression was induced with Isopropyl β-D-1-thiogalactopyranoside. HIS-Crabp-I and GST-Calmodulin were then affinity purified using NiNTA-resin and Glutathione-resin, respectively according to protocol uses in previous publications (Persaud et al., 2013; Persaud, Lin, Wu, Kagechika, & Wei, 2013).

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FLAG-CaMKII was produced via an *in vitro* coupled transcription/translation reaction using the TnT Coupled Reticulocyte Lysate Systems from *Promega*. 50 µL reactions were prepared according to the protocol in Table 3 and incubated at 30^{o}C for 90 minutes. FLAG-CaMKII was then affinity purified with M2-FLAG affinity beads from *Sigma-Aldrich* as described in the method section.

Table 3: TNT-reaction protocol.

TnT Reagent	Volume
TnT Rabbit Reticulocyte Lysate	25 µL
TnT Reaction Buffer	2 µL
TnT RNA Polymerase	1 µL
Amino Acid (minus methionine)	1.5 µL
Amino Acid (minus Leucine)	1.5 µL
RNAsin Ribonuclease Inhibitor (40u/ µL)	1 µL
DNA template (≤ 2 µg)	2
DEPC H2O	16 µL
Total	50 µL

5. *In Vitro* Phosphorylation

- The Co-immunoprecipitation (Co-IP) buffer was made of 50 mM Tris pH 8, 150 mM NaCl, 0.2% NP40, 10% glycerol and 1 mM EDTA.
- The *in vitro* phosphorylation reaction buffer was made of 20 mM HEPES pH 7.4, 10 mM MgCl2, 1 mM DTT 150 µM ATP.
- M2-Flag affinity beads were used to immuno-precipitate FLAG-CaMKII as will be described in the method section.

6. Western Blot

The following antibodies were used to perform western blot analyses:

- Anti-Phospho-CaMKII-δ_B (from *Cell Signaling*)
- Anti- CaMKII-δ (from *Santa Cruz*)
- Anti-Crabp-I (from *Abcam*)
- Anti-actin (from *Santa Cruz*)
- Anti-FLAG (from *Santa Cruz*)
- Anti-HIS (from *Santa Cruz*)
- Anti-mouse (from *Santa Cruz*)
- Anti-rabbit (from *Santa Cruz*)

The primary antibodies were used at a concentration of 1:1000 while the secondary antibodies were used at concentration of 0.5:1000.

7. QPCR Assay

RNA from rat cardiomyocytes were extracted following the *Trizol* protocol from *Applied Biosystems.*

Reverse transcription was performed using the High capacity cDNA Reverse transcription kits from *ThermoFisher*.

QPCR was performed using Applied *Biosystems'* SYBR green PCR master mix.

8. Cell Culture

The following cell lines were used for this project:

- 293T HEK Cells from *ATCC*
- COS-1 cells from *ATCC*
- C2C12 Cells from *ATCC*
- Primary rat cardiomyocytes from the Metzger lab.

The cells were grown in DMEM media in a 30^{o}C incubator.

Chapter 6| Methods

1. Holo-Crabp1 Dampen CaMKII-δ *In Vitro* CaMKII Phosphorylation

The relationship between Crabp-I, ATRA and CaMKII-δ has not been studied. To better understand the interaction between these 3 molecules, an *in vitro* phosphorylation assay was performed. 5 µL of TNT-FLAG-CaMKII-δ and 10 µL of M2-Flag affinity beads were added to 1.5 ml Eppendorf tubes containing 500 µL Co-IP buffer supplemented with 1% protease inhibitor and 1% of PMSF. The tubes were then incubated on a rocker for a minimum of 2 hours at 4ºC. Following the incubation time, the tubes were centrifuged at 5000G for 5 min. The supernatant was removed and 500 µL of Co-IP buffer was added to each tube and let to incubate on a rocker for 15 min at 4ºC followed by another centrifugation cycle. The Co-IP wash was performed 2 more times for a total of 3 washes. After the last spin, the supernatant of the last wash was carefully removed from the tubes. 40 µL of the *in vitro* phosphorylation buffer with the proper concentration of GST-CaM, and HIS-Crabp-I proteins were added according to the experimental design found in Table 4. The negative control only contained TNT-FLAG-CaMKII-δ and GST-calmodulin without $CaCl_2$ and the positive control contained TNT-FLAG-CaMKII-δ, GST-calmodulin and $CaCl_2$. The experimental tubes received increasing concentration of HIS-Crabp-I in addition of the TNT-CaMKII-δ, and GST-Calmodulin. Those tubes were also pretreated with 2 µM of ATRA for 10 minutes, at 30ºC together with tubes that did not received any ATRA treatment in order to keep the condition constant for all treatments. After the 10 minutes pretreatment, 1 mM of $CaCl_2$ was added to the appropriate tubes and incubated for an additional 20 minutes for a

total of 30 minutes. The reaction was stopped by adding 15 µL of 4X SDS sample loading buffer. The tubes were then boiled on a water bath for 5 minutes. The tubes were briefly spun and loaded on a 10% SDS polyacrylamide gel. Downstream western blot procedures were followed and the membranes were immunoblotted with the following antibodies: Phospho-CaMKII from cell signaling, and anti-Flag.

Table 4: Holo-Crabp-I and CaMKII in vitro phosphorylation assay.

Reagents	1	2	3	4	5	6	7	8
FLAG-CaMKII (5 µL)	+	+	+	+	+	+	+	+
GST-Calmodulin (1 µM)	+	+	+	+	+	+	+	+
Ca^{2+} (0.5 µM)	-	+	+	+	+	+	+	+
HIS-Crabp-I (µM)	-	-	0.5	1	3	0.5	1	3
ATRA (2 µM)	-	-	-	-	-	+	+	+

2. Biologically Active Retinoic Acid Analogs and Their Effects on CaMKII-δ Phosphorylation

a. Compound Library

ATRA while being essential in a cornucopia of physiological events ranging from heart development to vision, can be toxic at high concentration or during chronic use. Due to its adverse side effects collectively known as retinoid toxicity, ATRA is not an attractive option in the development of a novel cardiovascular xenobiotic targeting CaMKII-δ. For this reason a library of 49 synthetic retinoid compounds were acquired from our collaborator, the Hiroyuki Kagechika laboratory. Those compounds were selected because they shared a similar structure with ATRA.

b. Luciferase Reporter Assay

ATRA can mediate two main activities as mentioned in the previous chapters. The canonical activity can activate multiple RARE genes which can lead to unexpected side

effects. Targeting only the non-canonical pathway of ATRA can eliminate those side effects. Furthermore, bearing in mind that most proteins involved in maladaptive CaMKII-δ signaling leading to HF, are located in the cytoplasm, it was important to use compounds selective only for the carrier protein capable of sequestering ATRA or its analogs in the cytoplasm, i.e., Crabp-I. For these reasons and despite finding that ATRA could dampen the phosphorylation of CaMKII-δ through Crabp-I, other safer retinoids similar to ATRA needed to be used for further investigation. To achieve this purpose, the 49 retinoid compounds from the Hiroyuki Kagechika laboratory were tested using a luciferase reporter assay in COS-1 cells. The COS-1 cells were transfected with the RAR-ligand binding domain plasmid and were treated with 250 nM of each candidate compounds for 24 hours.

c. Selection of Candidate Compounds

The compounds that did not activate the canonical pathway of ATRA through RAR were further investigated. *In vitro* phosphorylation assays were performed on those compounds to select the ones that could decrease CaMKII-δ phosphorylation through Crabp-I. The protocol was similar to the first *in vitro* phosphorylation assay but, the concentration of HIS-Crabp1 and candidate compounds were kept constant. The first half of this assay investigated the activity of each compound on CaMKII-δ in the presence of Crabp-I and the second half investigated the activity of those compounds on CaMKII-δ without Crabp-I as seen in Table 5. The negative control did not receive any $CaCl_2$. Two positive controls were used one with 1 mM of $CaCl_2$ and another was pretreated with 2 μM ATRA for 10 minutes and then challenged with 1 mM of $CaCl_2$. The other experimental tubes were pre-treated with each candidates and challenged

with 1 mM $CaCl_2$ in a manner similar to the ATRA treatment. All compounds were tested in the presence and absence of 3 μM HIS-Crabp-I. The compounds that had no effect or further activated CaMKII-δ, and the compounds that mitigated CaMKII activation in the absence of Crabp-I were excluded from the project.

Table 5: Drug screening experimental design. Only compound 1 through 4 are shown here. The same design was used to screen the other compounds.

Reagents	1	2	3	4	5	6	7	8	9	10	11
FLAG-CaMKII-δ	+	+	+	+	+	+	+	+	+	+	+
GST-CaM	+	+	+	+	+	+	+	+	+	+	+
$CaCl_2$	-	+	+	+	+	+	+	+	+	+	+
HIS-Crabp1	+	+	+	+	+	+	+	-	-	-	-
Candidate	-	-	Ra	#1	#2	#3	#4	#1	#2	#3	#4

3. Validation of Positive Hits in a Cellular Context

In order to validate those compounds in a more biologically relevant model, a cell line that expressed both CaMKII-δ and Crabp-I needed to be identified. C2C12 cells are murine myoblasts that can be differentiated into skeletal muscle. Those cells are grown into DMEM culture media containing 1% penicillin/streptomycin (PS) and 1 % fetal bovine serum. Differentiation is induced in the presence of a media containing 1% PS and 2% horse serum.

To ascertain if the proteins of interest were expressed in that cell line, we performed a western blot analysis on cell lysate from C2C12 cells that were

differentiated for various period of time. The western blot membranes were probed for CaMKII-δ and Crabp-I.

a. Holo-Crabp-I Effects on CaMKII-δ Activation in Differentiated C2C12 Cells

Once the ideal differentiation time necessary for the expression of CaMKII-δ and Crabp-I was identified, these cells were pre-treated with 2 μM of ATRA for different amount of time. After pre-treatment, these cells were challenged with the drug 10 μM of ionomycin for 10 minutes. Ionomycin is a drug that increases the intra-cellular calcium concentration, a known activator of CaMKII-δ. The control did not receive any treatments. After the incubation time, the cells were harvested and washed with PBS by spinning them in a centrifuge for 5 minutes at 5000xG. After removal of the PBS supernatant, the pellets were incubated with 200 μL RIPA lysis buffer supplemented with 1% protease inhibitor, 1% NaF, 1% Na_3VO_4 and 1% PMSF for 30 minutes. Afterwards, the cells were centrifuged once more at maximum speed for 15 minutes. The supernatant was then collected into new tubes and protein estimation was performed using the Bradford assay. Downstream western procedures on a 10% polyacrylamide SDS gel were then performed followed by immunoblotting with phospho-CaMKII-δ, CaMKII-δ and actin antibodies.

Additionally, to find the optimum conditions for ionomycin and ATRA treatments, a time course experiment was performed with ionomycin treated cells and a concentration dependent assay was performed with ATRA pre-treated cells that was were challenged with 2 μM of ionomycin for 30 minutes.

b. Compounds 1, 2 and 11 Effects on CaMKII-δ Phosphorylation in C2C12 Cells

Once the optimum conditions showing CaMKII-δ activation and mitigation through Crabp-I were deduced in C2C12 cells, the candidate compounds from the *in vitro* phosphorylation assays were tested in a concentration dependent manner, with and without ionomycin challenge. The negative control did not receive any treatments. The cells that were treated with ionomycin were pre-treated with increasing concentrations of the compounds for 30 minutes and challenged with 2 μM of ionomycin for 30 minutes in tandem with 2 mM of filtered $CaCl_2$. The cells that were not challenged with ionomycin received only increasing amount of the compounds. The compounds that were able to protect CaMKII-δ from the ionomycin challenge were used for further experiments.

4. Animal Study

a. Holo-Crabp-I Action on CaMKII-δ Activation *Ex vivo*

While the C2C12 assays were indicative of which compounds would work in a cellular context, those cells were not ideal to study cardiovascular diseases since they were differentiated into skeletal muscles. For this reason, the compounds that were able to decrease the activation of CaMKII-δ in C2C12 were also tested in primary rat cardiac myocytes. Cardiac myocytes are the functional unit of the heart and are a better suited model for testing the candidate compounds *ex vivo*. Moreover, those cells also express CaMKII-δ and Crabp-I. The primary cardiac myocytes were isolated using the Langerdorf retrograde perfusion method by our collaborator, Dr. Joe Metzger.

Additionally Salma Awad et al, successfully demonstrated that cardiac myocytes treatment of phenylephrine (PE) activated CaMKII-δ which lead to the transcription of hypertrophic genes such as β-MHC. A time course assay showed that PE induced CaMKII-δ phosphorylation and expression of hypertrophic markers as early as 1 hour with a maximum activation time of 24hrs. Following this experiment, the rat primary cardiac myocytes were pre-treated with compound 11 for 30 minutes by itself. Another plate were pre-treated with compound 11 for 30 minutes then received 100 μM PE. The positive control did not receive any treatment while the negative control received 100 μM of PE. The cells were then incubated overnight. Following the incubation period the cells were lysed with Trizol reagent and downstream RNA isolation procedure was performed according to the protocol provided by *Life technology.* The RNA extract were converted to cDNA via reverse transcription using *Applied Biosystems* High Capacity cDNA Reverse Transcription Kits protocol. Next, the cDNAs were used in a qPCR reaction and the hypertrophic marker β-MHC was amplified. The amplicons where normalized with GAPDH.

Chapter 7| Results

1. Crabp-I Mediates ATRA Mitigation of CaMKII-δ Activation

The first *in vitro* phosphorylation assay was performed to determine if holo-Crabp-I had any effects on the phosphorylation of CaMKII-δ. As discussed in the previous sections, CaMKII-δ is unable to auto-phosphorylate in the absence of Ca^{2+}/CaM, so it was not a surprise that in the presence of CaMKII-δ and calmodulin alone, CaMKII-δ was not phosphorylated as seen in lane 1 of Figure 2. As expected, in the presence of $CaCl_2$ the phosphorylation of CaMKII-δ was detected (lane 2). In lanes 3 through lanes 5 CaMKII-δ, calmodulin, $CaCl_2$ and increasing amounts of Crabp-I was added. In those lanes, Crabp1 alone was not able to activate or decrease the phosphorylation of CaMKII-δ as compared to lane 2. The same components were present in lanes 6 through 8 however equimolar ATRA was added in those tubes. Interestingly, in the presence of ATRA holo-Crabp-I decreased the phosphorylation of CaMKII-δ induced by Ca^{2+}/CaM (comparing lanes 6-8 to lane 2). Through this experiment we were able to

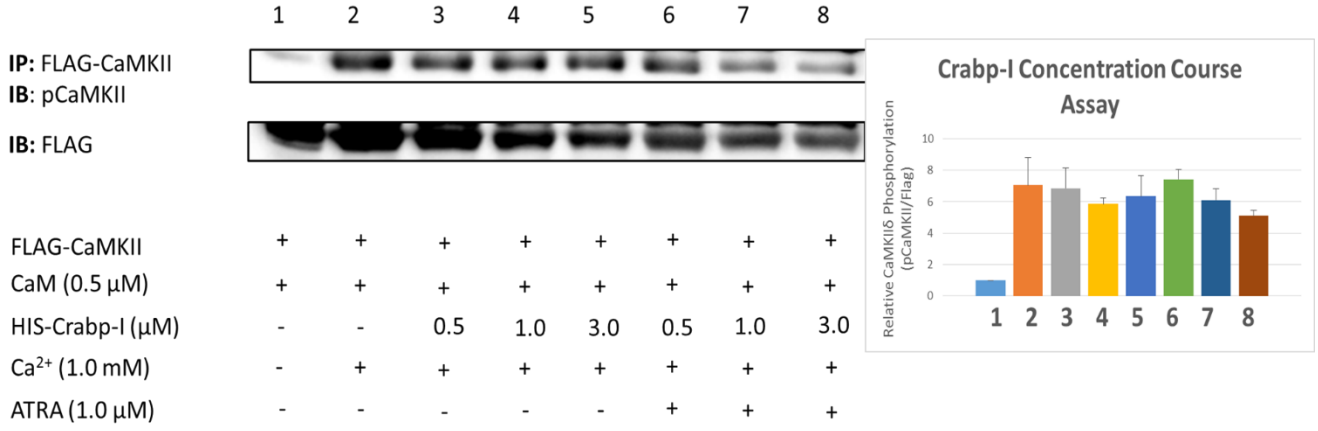

Figure 2: In vitro phosphorylation assay showing that holo-Crabp-I decreases CaMKII-δ Phosphorylation.

deduce that Crabp-I alone does not activate or decrease the phosphorylation of CaMKII-δ. However, in the presence of ATRA, Crabp-I is able to decrease the phosphorylation of CaMKII-δ as seen in Figure 2.

2. Sixteen Candidate Retinoid Compounds were Selected Through the Screening Process

The main goal of the luciferase reporter assay was to identify compounds that did not activate the canonical pathway of ATRA. The candidate compounds able to bind to the transfected RAR-LBD triggered the transcription of the luciferase gene and emitted a luminescent signal. This signal was detected using a luminometer and the signal was normalized with the protein Renilla. Out of the initial 49 compounds, 16 did not trigger any significant genomic activity (Figure 3):

#1	#8	#20	#31
#2	#9	#23	#47
#3	#11	#25	#48
#4	#13	#29	#4

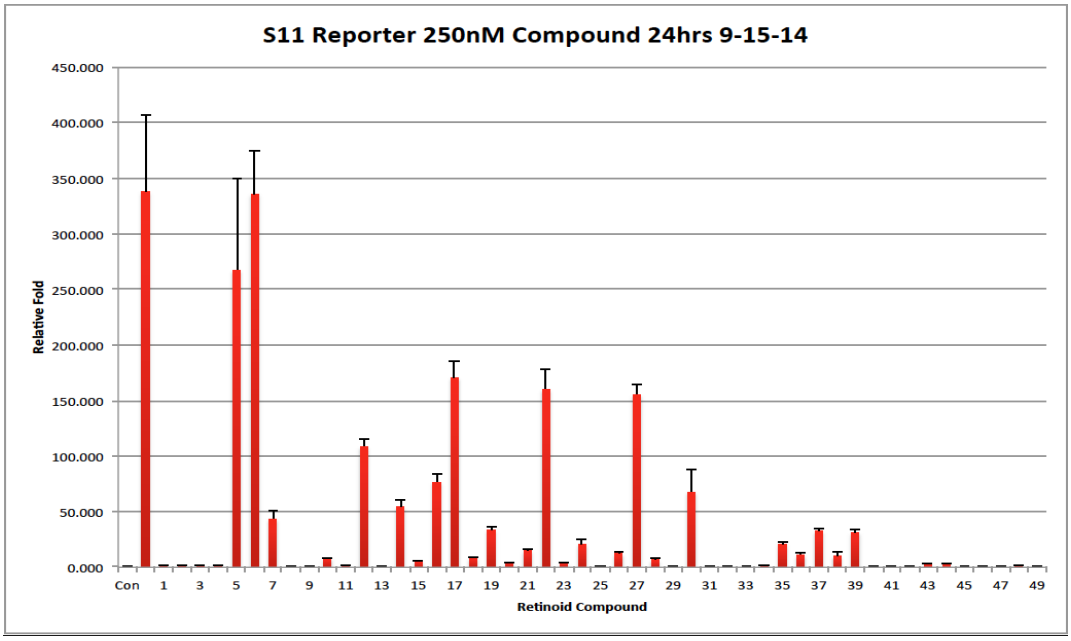

Figure 3: Luciferase assay result showing compounds that did not bind to RAR-LBD.

3. Compounds #1, #2 and #11 Decreased CaMKII-δ Phosphorylation Through Crabp-I

The compounds that did not trigger the genomic activity of ATRA were tested in using an *In vitro* phosphorylation assay similar to that of the first experiment. After screening the 16 candidates, only 3 compounds were found to decrease CaMKII-δ in the presence of Crabp-I: #1, #2 and #11 as seen in Figure 4.

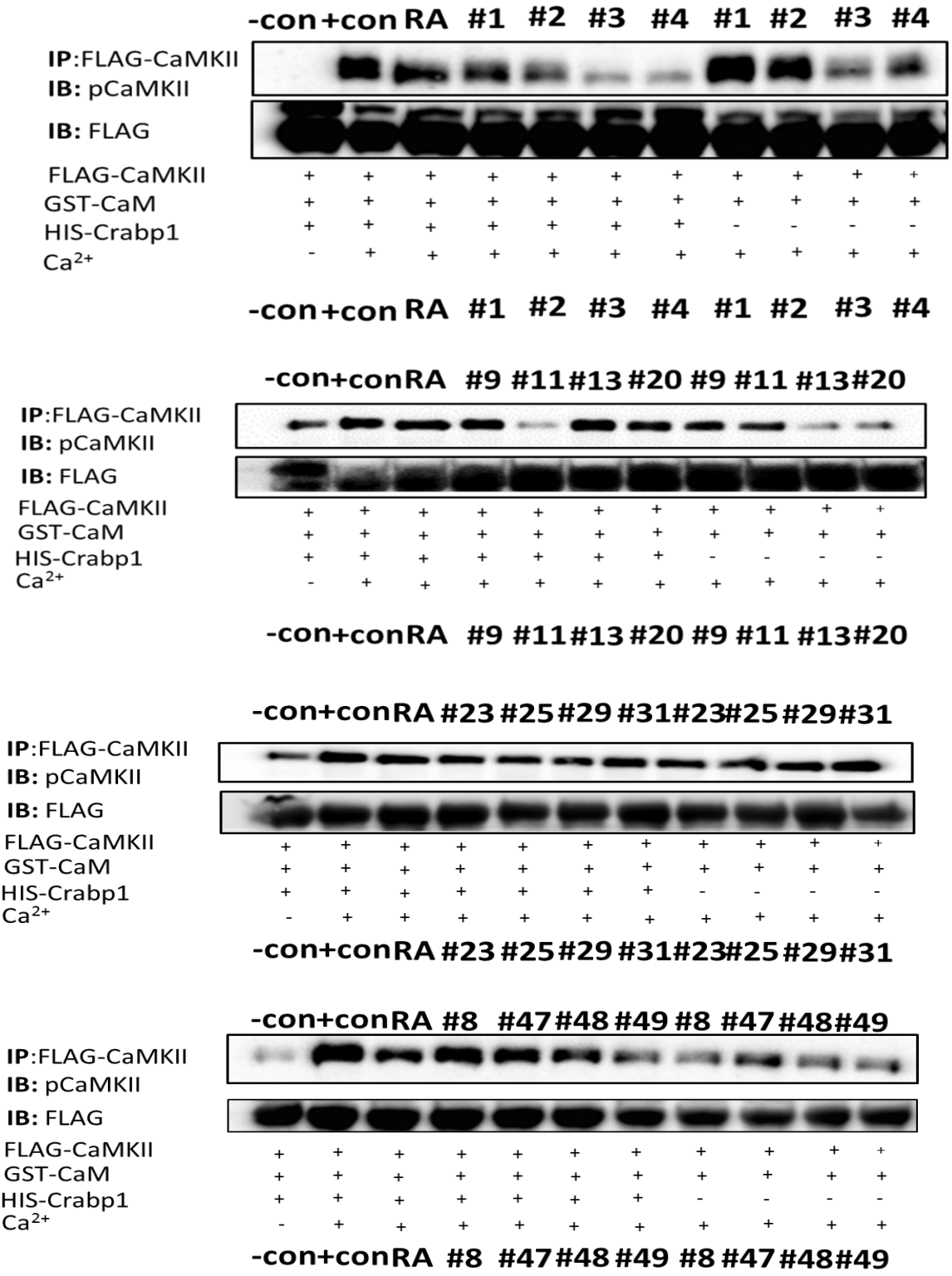

Figure 4: Western blots showing in vitro phosphorylation assays screening for compound capable of decreasing CaMKII-δ through Crabp-I. Compounds 1, 2, and 11 were the only positive hits in this series of assays.

4. Validation of Positive Hits in a Cellular Context

a. CaMKII-δ and Crabp-I are Expressed in Differentiated C2C12 Cells

The previous assays were performed in controlled conditions and they did not accurately represent real biologically relevant milieu. For this reason, the compounds that dampened the phosphorylation of CaMKII-δ were tested in a cell line. C2C12 were investigated as a potential cell for this aim. Figure 5 shows that Crabp-I and CaMKII-δ are both expressed in the differentiated C2C12 cells.

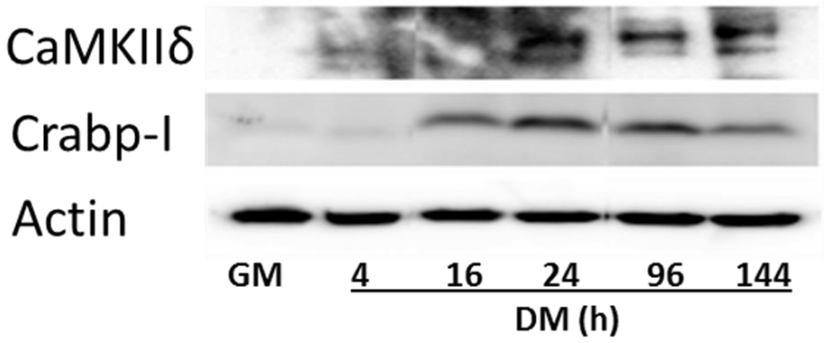

Figure 5: CaMKII-δ and Crabp-I are induced in differentiated C2C12 cells.

b. Holo-Crabp-I Decreased CaMKII-δ Phosphorylation in C2C12 Cells

To confirm that the dampening effect on CaMKII-δ was also true in a biologically relevant environment, a time course assay was performed using cells pre-treated with ATRA and challenged with the CaMKII-δ activator ionomycin (Figure 6). Lane 1 did not receive any ionomycin or any ATRA and showed only basal level activity of CaMKII-δ. Lane 2 was treated with ionomycin only, and as expected showed an appreciable increase in CaMKII-δ phosphorylation (compare lane 1 and 2). However, in the presence of 2 μM ATRA for different time increments, CaMKII-δ was activation was mitigated.

In order to determine if lower concentration of ionomycin could be used to activate CaMKII-δ, a time course using only 2 µM was performed and in conjunction with an ATRA concentration dependent assay. These series of assays provide us with the following information:

- 2 µM ionomycin treatment for 10 minutes is sufficient to activate CaMKII-δ in differentiated C2C12 cells
- Pre-treating differentiated C2C12 with 0.1 µM of ATRA for 30 minutes is sufficient to decrease CaMKII-δ activation induced by ionomycin.

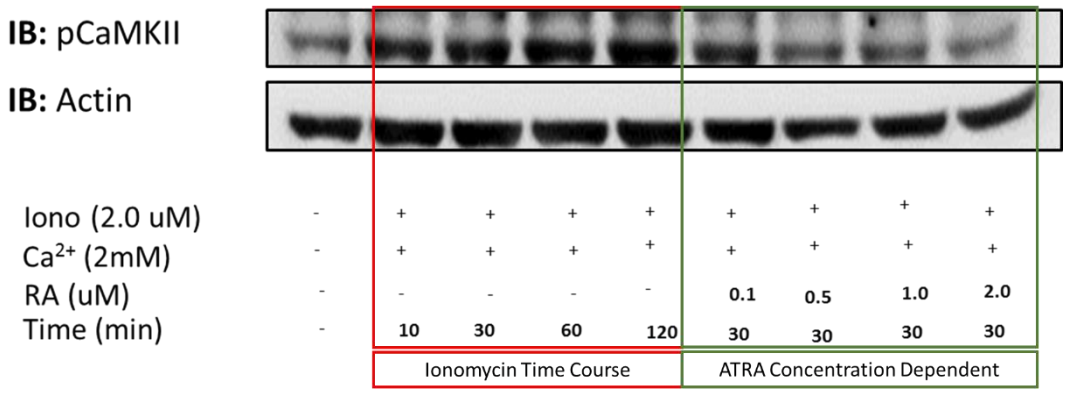

Figure 6: Ionomycin time course and concentration dependent change in C2C12 cells.

c. Compound 11 Decreases CaMKII-δ Phosphorylation in C2C12 Cells

The previous screening assays were done in well controlled conditions. In order to understand how those compounds could behave in biological environment, C2C12 were treated with the positive hits. Compounds #1, 2 and 11 were only tested on C2C12 since they were able to dampen CaMKII-δ in through Crabp-I.

i. Compound 1

Compound 1 did not activate CaMKII-δ in the absence of ionomycin as shown in Figure 7a (compare lane 1 to lanes 2-4). 2 μM of ionomycin with 2 mM of $CaCl_2$ activated CaMKII-δ as seen in lane 5. Increasing amount of compound 1 co-treated with ionomycin and $CaCl_2$ did not decrease the phosphorylation of CaMKII-δ as seen in the *in vitro* phosphorylation assay (compare lanes 6-8 to lanes 1 and 5).

ii. Compound 2

Similarly, compound 2 had no effects on CaMKII-δ activation in the absence of ionomycin as shown in Figure 7c (compare lane 1 to lanes 2-4). Likewise, increasing amount of compound 2 co-treated with ionomycin and $CaCl_2$ did not decrease the phosphorylation of CaMKII-δ as seen in the *in vitro* phosphorylation assay (compare lanes 6-8 to lanes 1 and 5).

iii. Compound 11

Finally, compound 11 was consistent with the two previous compounds in that it showed no significant activities on CaMKII-δ in the absence of ionomycin and $CaCl_2$ as compared to the control as shown in Figure 7e. Unlike previous compounds, compound 11 decreased CaMKII-δ phosphorylation in the presence of ionomycin and $CaCl_2$ in a concentration dependent manner (compare lanes 6-8 to lanes 1 and 5).

Considering those results, it is clear that compound 11 activity in the *in vitro* phosphorylation assays are also true in a biological context, making it a promising compound for further studies.

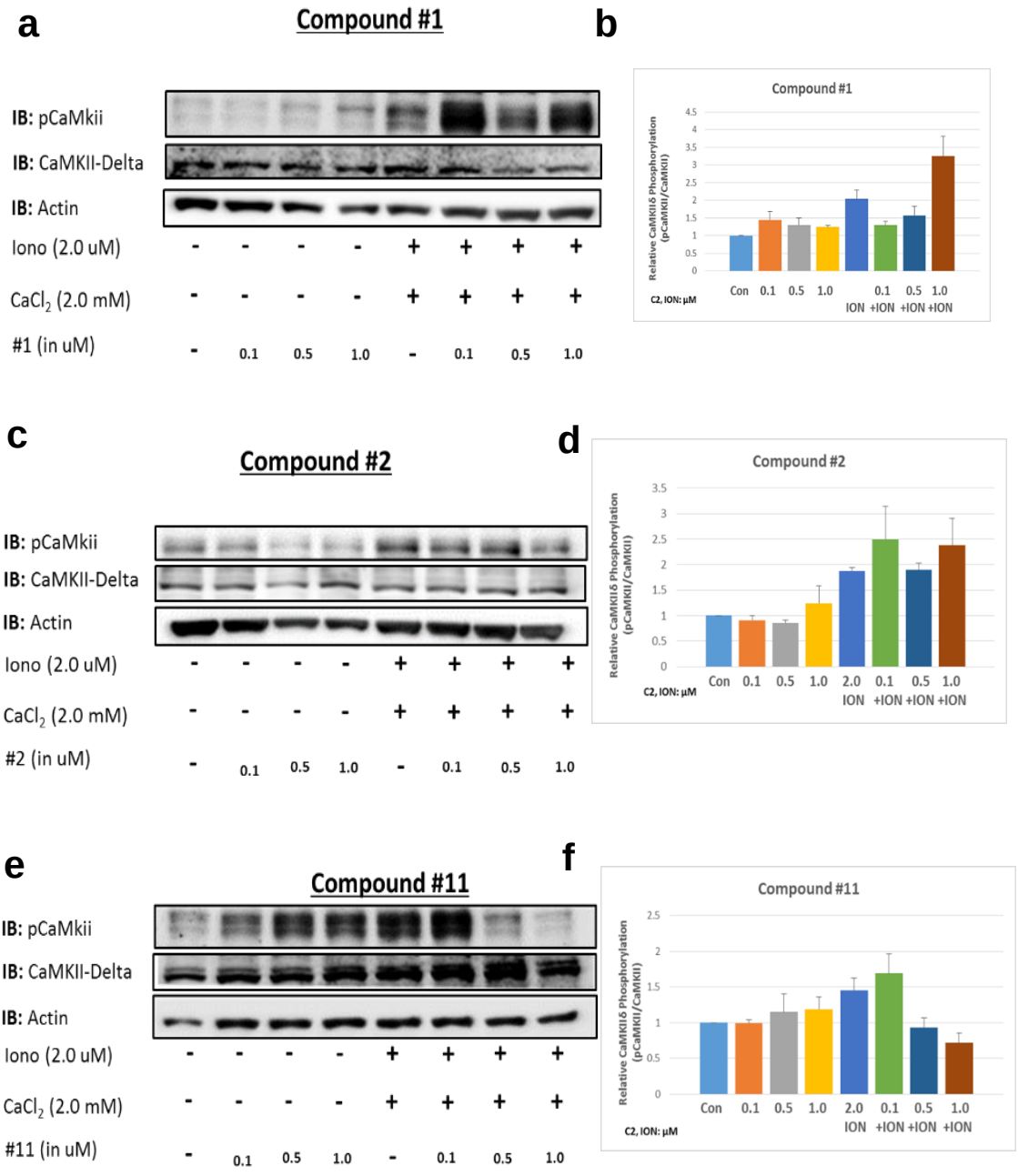

Figure 7: Compound 11 decreased CaMKII-δ phosphorylation in differentiated C2C12 cells.

5. Crabp-I and ATRA Protects Murine Hearts from Hypertrophy Induced by CaMKII-δ Mediated ETC

Following the validation of Crabp-I and ATRA role in modulating CaMKII-δ activation within differentiated murine skeletal muscles, the role of these molecules were also investigated in primary rat cardiac myocytes. Cardiomyocytes are better suited cells to study cardiovascular diseases since they are the functioning unit of the heart. Additionally, successful isolation of cardiomyocytes produce excitable cells that are able to contract independently and respond to Ca^{2+} influx making them relevant to this study.

The development of hypertrophy is marked by expression of many maladaptive genes that leads to the development of fibrosis in cardiac tissue and ultimately to a loss of contractility. CaMKII-δ is a player in this maladaptive signaling pathway and has been to shown to induce hypertrophic markers such as β-MHC in the presence of PE treatment. To ascertain the effect of compound 11 on the PE induction of β-MHC, rat cardiac myocytes were tested according to the method mentioned in the previous chapter. In this assay compound 11 treated cells did not induce the expression of β-MHC as compared to the control treatment. In the presence of PE β-MHC was dramatically induced as compared to the control. Interestingly, when compound 11 was co-treated with PE, the level of β-MHC was reverted back to basal level. This assay indicated that compound 11 protected rat cardiac myocytes against PE induced hypertrophy through Crabp1 and CaMKII-δ as seen in Figure 8.

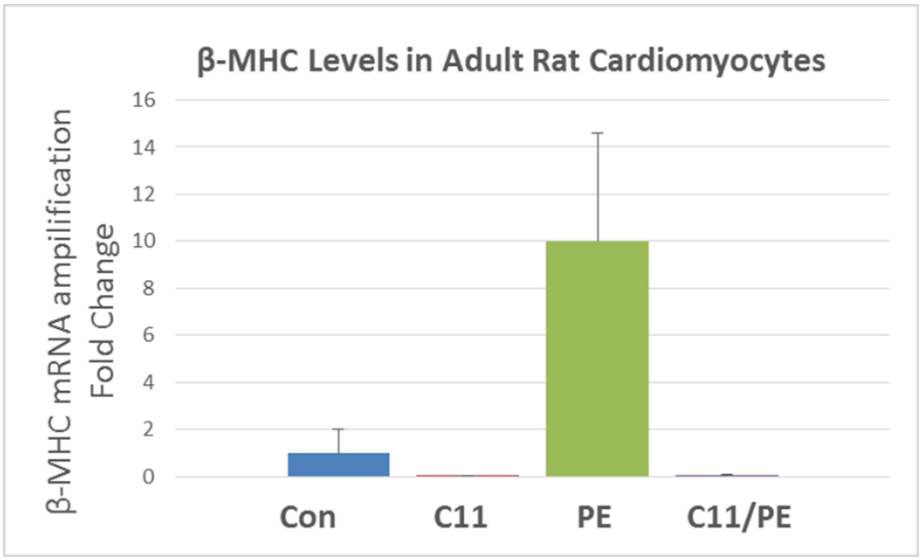

Figure 8: Compound 11 protects rat cardiomyocytes from PE and CaMKII-δ_B induced hypertrophy.

Chapter 8| Discussion

The finding that holo-Crabp-I associated with CaMKII-δ was unprecedented and the outcome of this interaction has not been previously explored. To understand the result of this interaction the hypothesis that Crabp-I regulates the phosphorylation of CaMKII-δ in the presence of ATRA was proposed. This hypothesis was tested by three aims. In the first aim, CaMKII-δ phosphorylation was examined in an *in vitro* phosphorylation assay in the presence and absence of holo Crabp-I. In this aim it was found that Crabp-I decreased CaMKII-δ phosphorylation in the presence of ATRA. The next aim tested if other compound similar to ATRA could decrease CaMKII-δ through Crabp-I in order to avoid the side effects seen with the genomic pathway of ATRA. Compounds 1, 2, and 11 were shown to decrease CaMKII-δ phosphorylation through Crabp-I in the *in vitro* phosphorylation assay. The third aim had two main purposes: 1-to test the candidates selected in the previous aim in a biologically relevant context, i.e., in C2C12 cells, and 2-to test the positive hit in a physiological relevant cell line pertaining to heart failure, i.e., primary rat cardiac myocytes. These aims established compound 11 as a promising selective Crabp-I candidate capable to dampen CaMKII-δ phosphorylation seen especially in hypertrophic heart tissues and in HF as a whole.

1. Possible Mechanism of Action

This study is a preliminary work on the development of novel cardiovascular drugs targeting CaMKII-δ through Crabp-I. While the data shown

here are promising more experiments need to be performed to address many questions left unanswered by this project. Those include:

- Do holo-Crabp-I and CaMKII-δ directly bind together, if so where are their binding sites?
- Where does compound 11 binds to Crabp-I? And what is the binding affinity of that interaction?
- How does the decrease in CaMKII-δ phosphorylation through holo-Crabp-I affect CaMKII-δ target proteins during ECC and ETC (e.g., RYR2, PLB and HDAC4)?
- What are the pharmacodynamics and pharmacokinetic profile of compound 11?
- Can compound 11 work *in vivo*?

While more work needs to be done, this current study gives us a glimpse at the possible mechanism present in the interaction between CaMKII-δ and holo-Crabp-I. As mentioned in the chapter discussing CaMKII-δ, four events need to occur prior to its activation:

1. Cytoplasmic calcium needs to be elevated
2. Calcium needs to bind to calmodulin
3. Calmodulin needs to bind to the CaMKII-δ calmodulin binding site found in the regulatory domain
4. Finally CaMKII-δ needs to undergo a conformation change that leads to auto phosphorylation at the Thr 287 residue.

a. Compound 11 and CaMKII-δ Activation in ECC

Since ATRA or compound 11 were unable to have any effects on CaMKII-δ phosphorylation in the absence of calcium we can infer that direct interaction of holo-Crabp-I with CaMKII-δ does not lead to a decrease in CaMKII-δ phosphorylation. Conversely, when calcium is present holo-Crabp-I decreases CaMKII-δ activation but does not totally inhibit it. One possible explanation could be that holo-Crabp-I could sequester the calcium-calmodulin complex away from CaMKII-δ. Furthermore, holo-Crabp-I could also interact with Ca^{2+}/CaM bound to CaMKII-δ and can prevent it from assuming its full active conformation state and decrease to extend to which it auto-phosphorylates at the Thr-287 residue as disucessed in chapters 3 and 4 (Figure 9). Holo-Crabp-I bound to Ca^{2+}/CaM that is already attached to CaMKII-δ could also explain why both proteins (CaMKII-δ and Crabp-I) co-precipitated in previous Co-IP assays.

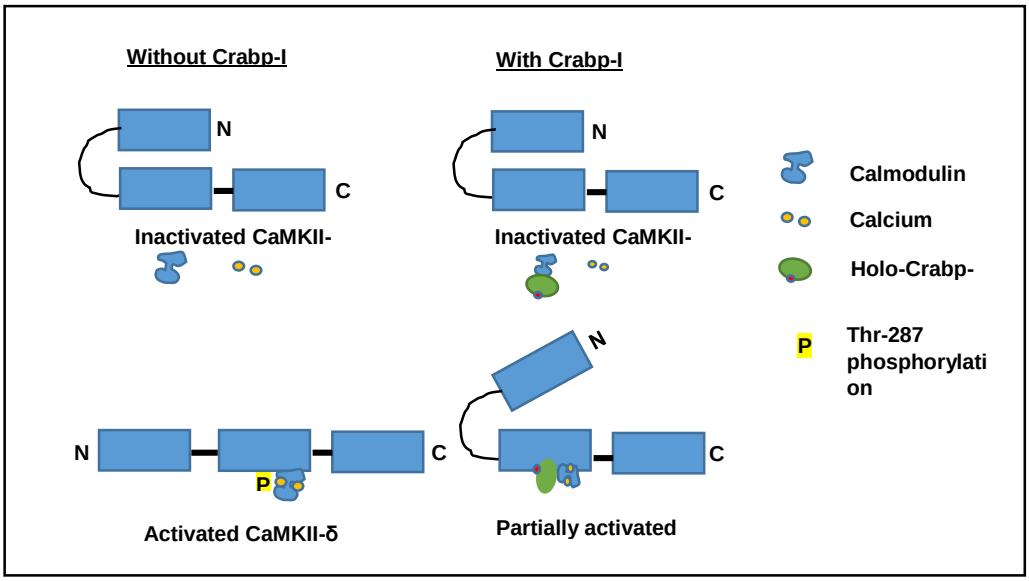

Figure 9: Proposed mechanism of holo-Crabp-I dependent CaMKII-δ activation mitigation.

b. Compound 11 and CaMKII-δ Activation in ETC

Phenylephrine is an α-adrenergic receptor agonist that can induce the production of inositol 1, 4, 4-triphosphate (IP3) through the G-coupled receptor pathway. IP3 binds to the intra-nuclear IP3 receptor (IP3R) of the nuclear envelop, causing calcium stored in the nuclear envelop to enter the nucleus. The influx of nuclear calcium binds to nuclear calmodulin which subsequently activates the nuclear isoform CaMKII-δ_B. This leads to the activation of hypertrophic genes such as MEF2 or β-MHC. Since Crabp-I does not translocate into the nucleus Persaud et al., 2013; Persaud, Lin, Wu, Kagechika, & Wei, 2013) and considering all the steps needed for CaMKII activation listed in the previous section, the effect of compound 11 on CaMKII-δ_B has to be through the modulation of $[Ca^{2+}]_i$. Indeed, the over activated cytoplasmic CaMKII-δ_C can further increase $[Ca^{2+}]_i$ by increasing SR calcium $Ca2^+$ release via RyR2 receptors or by increasing Ca^{2+} influx via Ca^{2+} channels. Normalizing the overall $[Ca^{2+}]_i$ by dampening CaMKII-δ_C can bring the nuclear envelop Ca^{2+} storage to basal levels. This will prevent CaMKII-δ_B from being over activated even in the presence of IP3 and ultimately inhibits the transcription of hypertrophic genes.

In conclusion, our studies have demonstrated that CaMKII-δ over-activation could be mitigated by targeting Crabp-I. While, more experiments needs to be done to better understand this interaction, this current work, has laid the foundation for the development of new cardiovascular drugs.

Chapter 9|References

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