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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mechanisms for Non-canonical PKA Signaling Regulation in the Heart

A dissertation submitted in partial satisfaction
of the requirements for the degree Doctor of Philosophy

in

Chemistry with a Specialization in Multi-Scale Biology

by

Kristofer Jean Haushalter

Committee in charge:

Professor Susan S. Taylor, Chair
Professor Hemal H. Patel, Co-Chair
Professor Daniel Donoghue
Professor Elizabeth Komives
Professor Andrew McCulloch
Professor William Trogler

2016

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Co-Chair

Chair

University of California, San Diego

2016

DEDICATION

To my family and friends.

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LIST OF ABBREVIATIONS

AKAP	A-Kinase anchoring protein
AKAP1	Dual specific A-kinase anchoring protein 1
AKAR	A-Kinase activity reporter
AKB	A-Kinase binding domain
AKIP1	A-Kinase Interacting protein 1
AMVM	Adult mouse ventricular myocyte
ARVM	Adult rat ventricular myocytes
ATP	Adenosine triphosphate
Bis-Tris	Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane
BSA	Bovine serum albumin
CBD	cAMP binding domain
C-subunit	Catalytic subunit of PKA
C-terminus	Carboxy-terminus
cAMP	Adenosine 3',5'-cyclic monophosphate.
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cGMP	Guanosine 3',5'-cyclic monophosphate
CNC	Carney complex
COXIV	Cytochrome c oxidase (complex IV)
CytC	Cytochrome c
D/D	Dimerization/docking domain
Diamide	Diazene dicarboxylic acid bis-(N,N-dimethylamide)
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EPK	Eukaryotic protein kinase
ETC	Electron transport chain
FPLC	Fast protein liquid chromatography
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
H₂O₂	Hydrogen peroxide
HEK293	Human embryonic kidney 293 cells
IBMX	Isobutylmethylxanthine
IPTG	Isopropyl-β-D-thiogalactopyranoside
I/R	Ischemia/Reperfusion
IS	Inhibitor site
kDa	Kilodaltons
LB	Luria-Bertani broth
LDH	Lactate dehydrogenase
LVDP	Left ventricular developed pressure

MEF	Mouse embryonic fibroblast
MES	2-(N-morpholino)ethanesulfonic acid
MgCl₂	Magnesium chloride
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
N-terminus	Amino-terminus
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40
OXPHOS	Oxidative phosphorylation
PARP	Poly (ADP-ribose) polymerase
PBC	Phosphate binding cassette
PBS	Phosphate buffered solution
PBST	Phosphate buffered solution with Tween-20
PDB	Protein Data Bank
PDE	Phosphodiesterase
PKA	Protein kinase A (cyclic AMP-dependent protein kinase)
PKI	Protein kinase inhibitor
PP	Protein phosphatase
PTM	Post-translational modification
RSK	p90 ribosomal S6 kinase
R-subunit	Regulatory subunit of PKA.
R1α	PKA regulatory subunit RI alpha
R1β	PKA regulatory subunit RI beta
RIIα	PKA regulatory subunit RII alpha
RIIβ	PKA regulatory subunit RII beta
RLuc	Renilla luciferase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAT	HIV transactivator of transcription
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween-20
Tris	Tris(hydroxymethyl)aminoethane
YT	Yeast-tryptone broth

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FIELDS OF STUDY

Major Field: Chemistry with a Specialization in Multi-Scale Biology

Studies in Chemistry and Biochemistry (Professor Susan S. Taylor)

Studies in Cell Biology and Physiology (Professor Hemal H. Patel)

ABSTRACT OF THE DISSERTATION

Mechanisms for Non-canonical PKA Signaling Regulation in the Heart

by

Kristofer Jean Haushalter

Doctor of Philosophy in Chemistry with a Specialization in Multi-Scale Biology

University of California, San Diego, 2016

Professor Susan S. Taylor, Chair

Professor Hemal H. Patel, Co-Chair

cAMP-dependent protein kinase (PKAc) is a pivotal cell signaling protein in eukaryotes. The catalytic activity of this enzyme is in part controlled by four functionally non-redundant regulatory subunit proteins of PKA (RI α , RI β , RII α and RII β), which bind PKAc in tetrameric “holoenzyme” complexes to

maintain kinase inactivity until stimulation with cyclic-AMP (cAMP). The allosteric activation mechanism triggered by cAMP binding to R-subunits is well understood. However, unique biochemical features and subcellular localizations of R1 α and other PKA proteins in the heart may serve to activate PKAc via non-canonical, cAMP-independent mechanisms. The aim of this work was to examine the role of 1) disulfide bond oxidation at the dimerization/docking (D/D) domain of R1 α , 2) localization of PKA proteins at mitochondria in the heart, and 3) phosphorylation of R1 α by cGMP-dependent protein kinase (PKG) at the linker region. It was hypothesized that these mechanisms result in enhancement of PKAc activity through decreased regulation by R1 α .

First, the effect of cardiac ischemia/reperfusion (I/R) injury on R1 α protein expression, oxidation, and regulation of PKAc activity was assessed. Results show that R1 α is decreased in expression upon extended time periods of reperfusion injury. Analysis in cardiomyocyte cultures showed that oxidant stress alone is sufficient to modify and down-regulate R1 α protein, while simultaneously triggering PKAc activity. Furthermore, overexpression of R1 α increases apoptosis in oxidant stressed cells. In correlation with these results, PKA proteins are specifically enriched within subsarcolemmal mitochondria (SSM) of the heart, wherein the expression of R1 α is specifically decreased with I/R stress. In our study of R1 α phosphorylation, we validated that PKG targets Ser101 in R1 α under both *in vitro* and in cell conditions. Phospho-

mimetic mutation of Ser101 (S101E) displayed enhanced PKAc activity without cAMP while maintaining holoenzyme complex formation.

Taken together, these findings support the notion that Type I holoenzymes can be alternatively activated by means other than classical cAMP-mediated processes. Future experiments will be aimed at delineating whether these mechanisms can work synergistically within the context of oxidative stress. The implications of this work translate to a better understanding of the role PKA signaling in heart disease.

Chapter 1:

Introduction

1.1: cAMP-dependent protein kinase (PKA) signaling

Eukaryotic organisms have developed several methods of cellular signal transduction as a means of sensing and responding to stimuli from the extracellular environment. Biochemical research over the past 60-70 has identified numerous networks of signaling pathways in eukaryotes, most of which are defined based on 1) the major stimulus that acts upon the pathway, 2) the main effector molecule that regulates the pathway, and/or 3) the manner of change or adaptation that the pathway induces within the cell. Among these various modes of signal transduction, eukaryotic protein kinase (EPK) signaling is one of the most widely appreciated in biology, where protein phosphorylation via EPKs has been shown to be involved in nearly every critical biological process required for life ¹, as well as in the pathology of numerous diseases like cancer ².

The cAMP-dependent protein kinase (PKA) signaling pathway is one of the first discovered instances of EPK signal transduction, where as the name suggests it was determined that activation of this particular signaling entity required intercellular stimulation to generate intracellular adenosine 3',5'-cyclic monophosphate (cAMP) ³. Studies concerning this pathway have shown that regulation of PKA signaling is manifested in the interdependent behavior of two protein families: PKA catalytic subunits (C-subunit; PKAc, with α , β and γ homologs) ^{4 5 6}, PKA regulatory subunits (R-subunit; RI and RII, each with α and β isoforms; see **section 1.2**) ^{7 8 9 10}, and protein kinase inhibitors (PKI,

with α , β , and γ homologs)^{11 12}. In this sense, PKA signaling is unique within the family of EPKs due to the nature of R-subunits and PKI as a separate gene products required for inhibition of PKAc under non-stimulated conditions, as opposed to other EPKs which usually contain auto-inhibitory domains within the kinase protein itself to maintain regulation¹³. Together, PKAc and R-subunits proteins are found ubiquitously in nearly every cell type of eukaryotic organisms¹⁴, whereupon they function to control numerous cellular functions including metabolism^{15 16}, growth/proliferation^{17 18}, and gene expression¹⁹.

PKAc is classified as an AGC kinase protein²⁰, functioning to phosphorylate target substrates containing a consensus recognition sequence R-R-X-S/T (where R is arginine, X is any amino acid, and S/T corresponds to serine or threonine residues)³. As with all EPKs, the phospho-transfer reaction in PKAc is dependent on the high affinity binding of two magnesium (Mg^{2+}) ions and adenosine triphosphate (ATP) in addition to substrate, whereupon PKAc catalyzes the hydrolysis of the gamma-phosphate of ATP to form the products, phosphorylated substrate and ADP²¹. In non-stimulated cells, PKAc is kept in an inactive state via inhibitory binding of R-subunit, where association of an R-subunit homodimer to two C-subunits allows for formation of the tetrameric “holoenzyme” complex²². As illustrated in **Figure 1.1**, full activation of PKAc requires binding of two cAMP molecules to allosteric cyclic nucleotide binding (CNB) domains in each inhibitory R-subunit, which decreases binding affinity between the R and C-subunits and thus

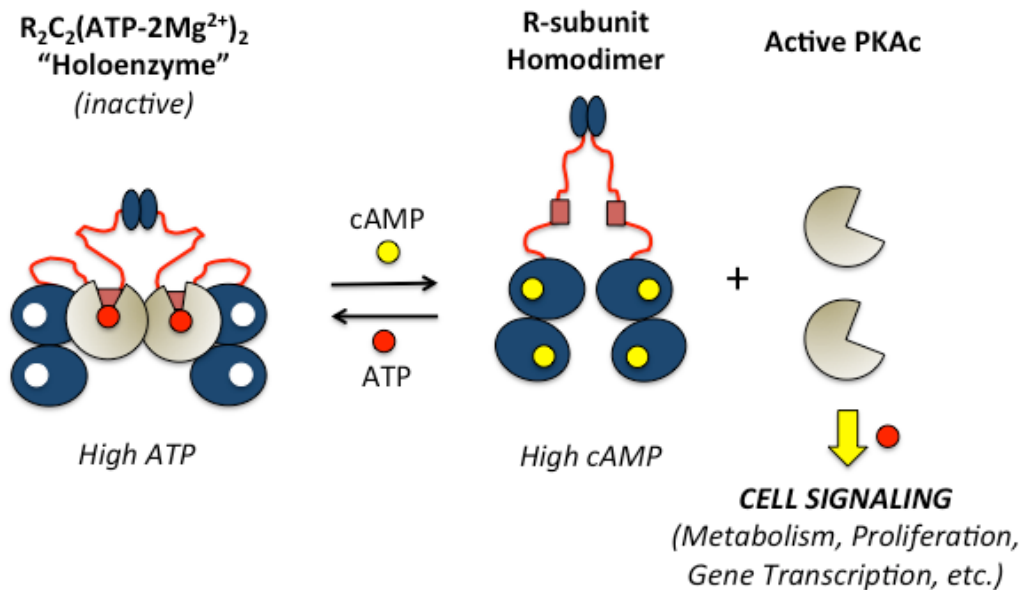


Figure 1.1: Activation of the PKA “holoenzyme” complex via cAMP.

Cartoon diagram representing the process of PKA holoenzyme activation with cAMP. Homodimer regulatory subunits (R-subunits) maintain inactivity of the catalytic subunit of PKA (PKAc) by formation of a “holoenzyme” complex, wherein two molecules of R-subunit directly bind two molecules of PKAc. In the case of $R1\alpha$, the highest affinity for PKAc is achieved when one molecule of ATP and two magnesium (Mg^{2+}) ions (i.e. $R_2:C_2(ATP-Mg^{2+})_2$) are bound. High affinity binding of R-subunit is manifested by both 1) direct binding of the inhibitory sequence (IS) within the linker region of R-subunits to the active site cleft of PKAc, and 2) distal binding of two tandem cyclic-nucleotide binding (CNB) domains to the C-lobe of PKAc. Sequential binding of cAMP to the R-subunit CNB domains lowers its affinity for PKAc, and thus triggers holoenzyme dissociation to promote catalytic activity of PKAc. High affinity binding of $ATPMg_2$ is a unique feature of the $R1\alpha$ holoenzyme, and like PKI is due exclusively to the pseudo-substrate nature of its IS.

allows for dissociation of the holoenzyme complex²³. Recent studies indicate that under physiological conditions full dissociation may not be required to unleash catalytic activity²⁴. Maintaining precise control of PKAc activity is critical for proper cellular signaling, requiring a high degree of regulation to control its behavior under both homeostatic and stress conditions. Thus continuing studies in the field are aimed at understanding the functional role of R-subunits and other proteins related to PKAc, and how these proteins are able to allow for such variable and diverse outcomes for PKA signaling in the cell.

1.2: PKA Regulatory Subunits (R-subunits)

Mammalian isoforms of the regulatory subunits of PKA (RI and RII homologs, each with α and β isoforms) have been well characterized with regard to their allosteric mechanism for inhibition of PKAc²³. For clarity, holoenzymes containing RI subunits are termed as “Type I” PKA, and respectively RII subunit holoenzymes are referred to as “Type II” PKA. The protein domain architecture of the regulatory subunits is highly conserved throughout the evolution of eukaryotes²³. As shown in **Figure 1.2**, all R-subunit isoforms contain 1) an N-terminal dimerization/docking (D/D) domain that allows for both R-subunit homo-dimerization as well as docking to A-kinase anchoring proteins (AKAPs; see **section 1.3**)²⁵⁻²⁶, 2) a flexible loop domain known as the “linker-hinge” (or simply linker) region, which contains

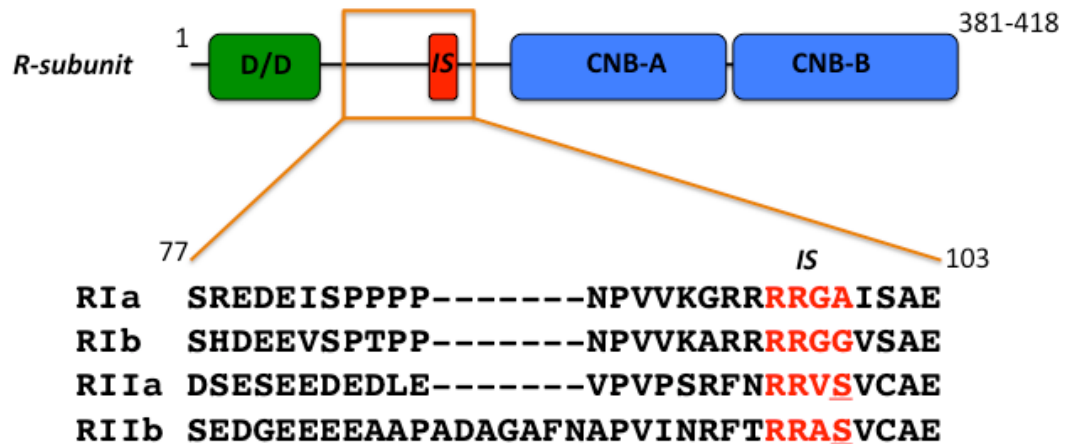


Figure 1.2: Comparison of inhibitor sequence (IS) motif in RI and RII subunits

Cartoon diagram representing the domain layout of regulatory subunit (R-subunit) protein, with specific comparison of aligned sequence features within the linker region of the four non-redundant homologs of R-subunit (namely, RI α , RI β , RII α , RII β) expressed in humans. The R-subunit architecture involves three major domains: 1) The N-terminal dimerization/docking (D/D) domain (*green*) which affords both R-subunit homodimerization as well as specific binding with A-kinase anchoring proteins (AKAPs), 2) the linker region that contains the inhibitory sequence (IS, sequence shown in *red*) that binds and inhibits the PKAc active site, and 3) two tandem cyclic nucleotide binding (CNB-A and CNB-B) domains (*blue*) that allow for allosteric activation of PKA holoenzyme complex dissociation via cAMP binding.

the inhibitory sequence (IS) that binds and effectively inhibits PKAc at its active site cleft²⁷⁻²⁸, and 3) two tandem cyclic nucleotide binding (CNB-A and CNB-B) domains which are responsible for binding cAMP for allosteric activation of PKA holoenzyme complexes^{23, 29}. In terms of common features shared between RI and RII subunits, the CNB domains have been shown to have the most similarity. Previous studies have illustrated that both RI and RII adopt a similar ordered, cooperative mechanism of cAMP binding between CNB domains, where binding of cAMP to one CNB domain increases probability of cAMP binding at the other CNB domain²³.

Despite the overall similarity in sequence and tertiary structure for all R-subunit proteins, they are functionally non-redundant, where RI and RII subunits each have a range of capabilities that differ significantly in many ways²³. Initial biochemical characterization of these proteins has shown significant differences in their relative kinetic parameters for cAMP-dependent activation. Initial work in the field was focused on assessing the differential response of RI and RII subunits to cAMP, where RI subunits were found to be more responsive to cAMP as compared to RII³⁰. More recent structural studies have shown that while RI and RII proteins have very similar interactions with PKAc on the level of a 1:1 heterodimer interaction^{31 32 33 34}, tetrameric (R₂:C₂) complexes show strikingly different quaternary structure of holoenzyme assembly (**Figure 1.3**)^{35 36}.

From a cellular perspective, RI and RII subunits can be easily

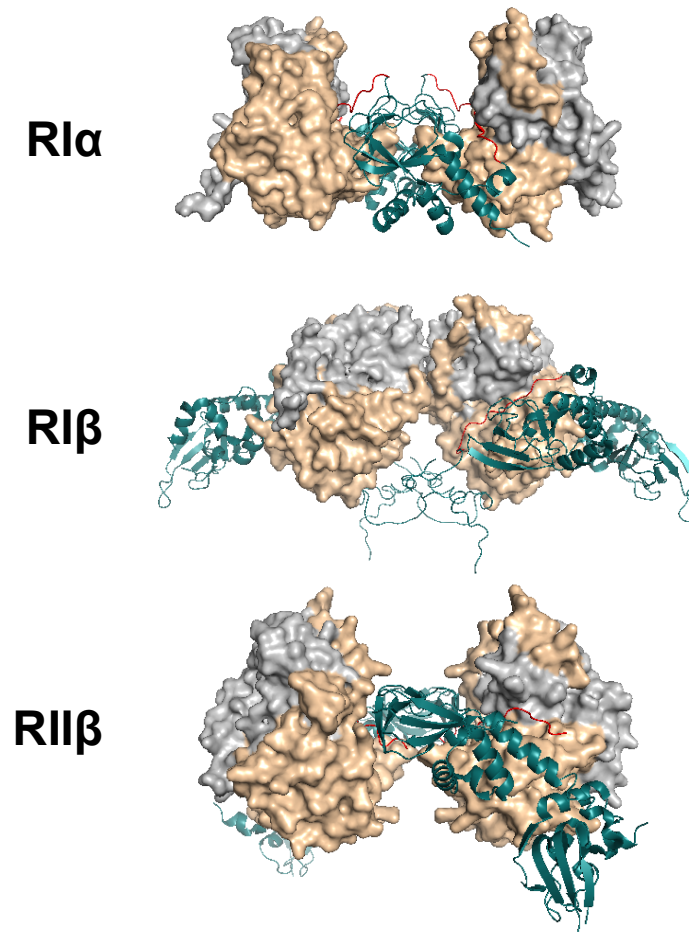


Figure 1.3: Structural comparison of RI α , RI β , and RII β holoenzymes.

Structural data from previous studies in the Taylor laboratory, depicting tetrameric holoenzyme structures for RI α , RI β , and RII β (*teal cartoon*, regulatory subunit CNB domain(s); *red cartoon*, regulatory subunit inhibitor sequence; *light gray surface*, PKAc N-lobe; *tan surface*, PKAc C-lobe). Images represent a “face-on” view.

differentiated based on their sub-cellular localization. As a general trend, RI subunits are primarily localized diffusely within the cytosol, whereas RII subunits are more specifically recruited to sub-cellular compartments such as the plasma membrane¹⁴. As described in *section 1.3*, A-kinase anchoring proteins (AKAPs) are thought to play a critical role in R-subunit localization³⁷. These data suggest that R-subunit proteins are non-redundant in their function, and thus further understanding of how RI and RII could be differentially regulated *in vivo* may shed insight into the pathology of disease related to dysregulation of PKA.

In order to differentiate RI and RII subunit at the level of protein sequence, a major classifying distinction can be observed within the IS. While the IS of RII subunits contains the consensus recognition sequence (R-R-X-S) that allows for phosphorylation by PKAc (making RII subunits *bona fide* substrates of PKAc), the IS motif in RI subunits does not contain a serine or threonine residue capable of phosphorylation (R-R-X-A/G), thus classifying RI subunits as “pseudo-substrate” inhibitor proteins of PKAc²³. As described in previous studies in the Taylor laboratory, the nature of the pseudo-substrate inhibitor in RI subunits significantly affects the nature of kinetic activation of Type I PKA holoenzymes, where an out-competing substrate is required in addition to cAMP agonism in order to fully induce protein complex dissociation^{28, 38}. However, the additional step of substrate competition for RI α can be circumvented by mutation of the pseudo-substrate inhibitor site to either a

serine (capable of phosphorylation) or a phospho-mimetic aspartate residue. This information implies that the linker region in RI α is sensitive to modification, and therefore activation of RI α holoenzymes will likely be significantly affected upon alteration of the biochemistry near the inhibitor site.

Another major distinction between RI and RII subunits occurs within the D/D domain. While both RI and RII adopt a similar “helix bundle” tertiary structure that allows for homo-dimerization between anti-parallel chains, only the RI subunits contain highly conserved cysteine residues which allow for oxidant-sensitive covalent disulfide bonds at the RI homo-dimer interface (see **Figure 1.4**)²⁶. The significance of this post-translational modification (PTM) in RI α , in terms of either its biochemical function in RI α holoenzyme activation or its role in the pathology of diseases *in vivo*, has yet to be fully characterized.

1.3: PKA signaling: regulation by AKAPs

As mentioned previously, there are several additional proteins other than PKAc and the R-subunits that are involved with PKA signaling; most notably, we must consider interactions between PKA holoenzyme complexes with A-kinase anchoring proteins (AKAPs). AKAPs were originally identified based on their strong interaction to R-subunit proteins³⁹. Since their discovery, they've been shown to encompass a large family of diverse proteins that serve as PKA-specific scaffolding proteins that bind PKA holoenzymes via

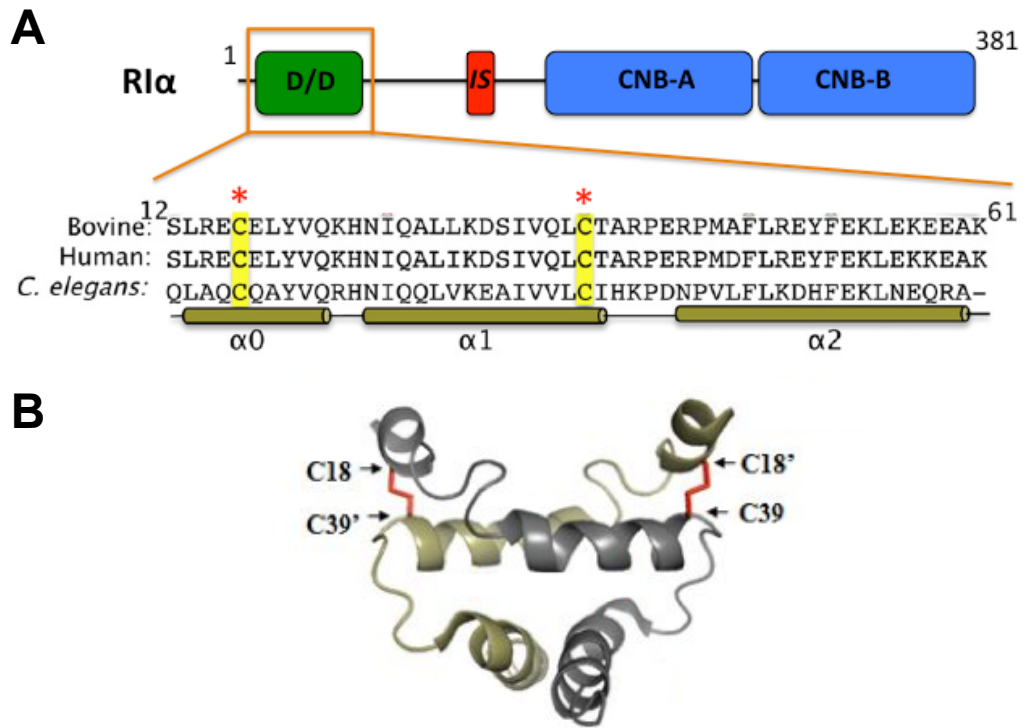


Figure 1.4: R1 α dimerization/docking (D/D) domain is oxidant sensitive.

A) Cartoon diagram depicting the domain layout of R1 α protein, emphasizing the sequence of the dimerization/docking (D/D) domain (numbers indicate bovine sequence). Two cysteine residues (Cys18 and Cys39 in human R1 α , *highlighted in yellow and labeled above with red asterisks*) have shown to be highly conserved throughout evolution of RI proteins in eukaryotes. **B)** Structural representation of R1 α D/D domain, depicting the anti-parallel homo-dimerization between R1 α protein chains. Disulfide bonds between Cys18 and Cys39 are shown in red.

the D/D domain of R-subunit homodimers^{40 41}. As seen in **Figure 1.5**, all AKAP proteins contain an A-kinase binding (AKB) domain, denoted by a general consensus sequence encoding an amphipathic helical motif that directly binds to D/D domains of both RI and RII subunits⁴². Initially it was thought that AKAP proteins were largely RII-specific, with only a few examples of dual specific AKAPs that could bind either RI or RII^{42 43}. This assertion fit well with cell imaging studies that suggested that RI protein localization was more diffuse and limited to the cytosol and nucleus, whereas RII subunits have more compartmentalized localization in cells. However, recent evidence has shown the existence RI-specific AKAPs, suggesting that this family of AKAP proteins have very specific roles for each regulatory subunit isoform^{44 45}.

In addition to binding PKA, an AKAP's primary function is to localize PKA proteins to specific sub-cellular compartments such as mitochondria, the nucleus, or the plasma membrane⁴⁶. Studies have shown that some AKAPs can be anchored into membranes via acyl modifications with fatty acids (either palmitoylation or myristoylation) near the N-terminal residues in the protein⁴⁵. As mentioned above, both RI and RII subunits can bind AKAPs, and therefore all isoforms of R-subunit can be differentially localized in cells via interactions with AKAPs. Furthermore, most AKAPs have accessory protein domains that recruit other effector proteins, such as phosphodiesterase (PDE) and protein phosphatases (PP), in order to modulate PKA signaling in a localized fashion⁴⁶. All of these factors contribute to a concept referred to as the PKA

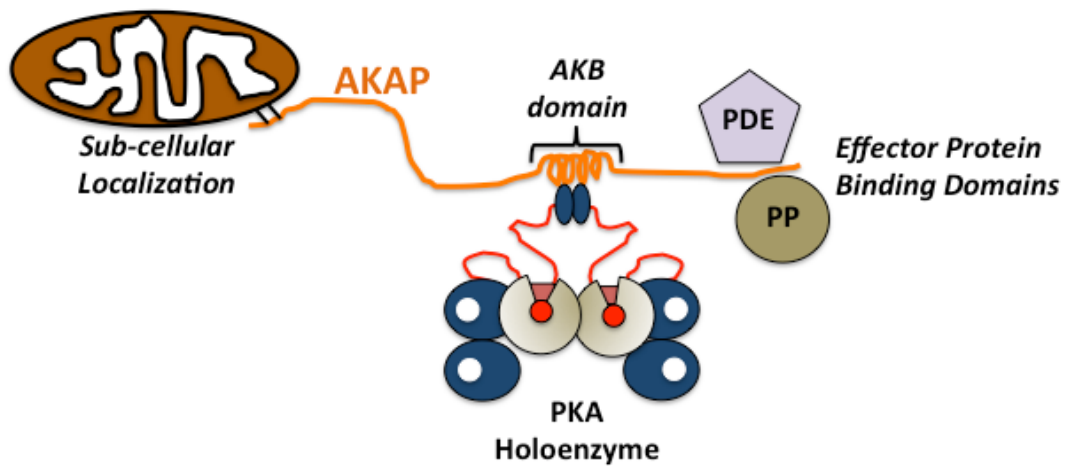


Figure 1.5: AKAPs and effector proteins.

Cartoon depicting the nature of interaction between PKA holoenzyme complexes with A-kinase anchoring proteins (AKAPs). AKAPs serve as PKA-specific scaffold proteins that fulfill three major functions: 1) binding of PKA holoenzymes via the A-kinase binding (AKB) domain, an amphipathic helical motif that directly binds to assembled D/D domains of R-subunit homodimers, 2) localizing PKA proteins to specific cellular sub-compartments such as mitochondria, the nucleus, or the plasma membrane, and 3) recruiting effector proteins, such as phosphodiesterase (PDE) and phosphatase (PP) enzymes, that work to modulate PKA signaling.

“signalosome”, where the concerted effort of these AKAP-associated proteins within confined space and time can be thought of as a cohesive signaling machine with many working molecular parts. In this manner, it is thought that AKAP-mediated complexes function as independent signaling hubs that can be acted upon by the specific stimulus of the particular subcellular environment. Taking into consideration the diversity of R-subunit isoforms, coupled with the potential variety in sub-cellular localization and effector protein binding, AKAPs can create a wide array of specified PKA signaling regimes within the cell. In *chapter 2 and 3* of this dissertation, we explore the role of AKAP1 (a.k.a. D-AKAP1 or mitochondrial AKAP121), a dual-specific AKAP that is localized to the outer mitochondrial membrane (OMM)⁴⁷, with regard to its potential regulation of mitochondrial PKA signaling in the setting of oxidative injury in the heart.

1.4: Activation of PKA via GPCRs

Many other cellular processes have direct roles in the activation and modulation of PKA signaling. Most importantly, we must consider the process of PKA activation via G-protein coupled receptor (GPCR) pathways, wherein sensitization by extracellular stimuli results in a cascade of signaling events in order to generate cAMP and thus trigger PKA holoenzyme dissociation⁴⁸. This represents the first example of second messenger signaling where binding of a ligand such as a hormone or a neurotransmitter to the outside of

the cell leads to the synthesis of an intracellular second messenger. As their name implies, GPCRs utilize various combinations of heterotrimeric G-proteins ($G\alpha$, $G\beta$ and $G\gamma$) to transduce the signal input of receptor-ligand binding interaction to the output of downstream pathway activation⁴⁹. Due to the diversity of $G\alpha$ isoforms, and given the large family of genes that encode for GPCRs along with many characterized ligands, GPCRs facilitate many critical cellular processes including growth/proliferation and neurotransmission⁵⁰. The GPCRs are, in fact the largest gene family and most are still orphan GPCRs where the activating ligand is unknown⁵¹.

In terms of activation of PKA, $G_{\alpha s}$ (or stimulatory $G\alpha$ protein) leads to activation of cAMP dependent pathways, whereas $G_{\alpha i}$ (or inhibitory $G\alpha$ protein) inhibits cAMP production. These two $G\alpha$ isoforms assert their regulatory effects via interaction with adenylyl cyclases (ACs), the family of enzymes that catalyze the formation of cAMP from ATP, where $G_{\alpha s}$ and $G_{\alpha i}$ act to either activate or inhibit the function of these cyclases, respectively⁴⁹. As discussed above, binding of cAMP to regulatory CNB domains triggers the PKA holoenzyme to unleash the PKAc phosphorylation activity; however, in addition to PKA, cAMP acts upon numerous other effector proteins, including cyclic-nucleotide gated ion channels⁵² and exchange proteins activated by cAMP (EPACs)⁵³. This pathway of activation has been described for many GPCRs within the context of numerous tissue and cell types, and thus is a target of study in regard to dysregulation of these pathways in disease. In the

case of the heart, which is a focus of work presented in *chapter 2 - 4* of this dissertation, beta-adrenergic receptors (β ARs; particularly isoforms β 1 and β 2) are a class of GPCRs that have been characterized to mediate cAMP production via G α s stimulation of AC in heart tissues⁵⁴. Their role in cardiac disease, particularly mediated via PKA-dependent processes has been extensively studied but is not fully understood, in particular regard to isoform specificity.

1.5: Canonical roles of R1 α in the cell

In this dissertation, research is focused on the characterization of Type I PKA regulatory subunit alpha (R1 α ; encoded by the *PRKAR1a* gene), with specific emphasis on how modification of this protein could lead to new methods of PKA signaling activation or regulation. Before we explore novel modes of regulation, we first consider all of the previously described roles for R1 α in the cell. With regard to PKA, R1 α has classically been defined as the “tissue specific extinguisher” of cAMP-dependent signaling, serving as the most predominantly expressed regulatory subunit protein in most mammalian tissues and cell types. Evidence accumulated over the last 25 years has contributed to the theory that R1 α acts as the essential regulator of PKAc activity in cells⁵⁵. One particular indication of the importance of R1 α is that increases in its protein expression can compensate for either increases in PKAc expression or activity, or for loss of other R-subunit isoforms⁵⁵.

Furthermore, RI α is unique amongst the R-subunit protein isoforms in that genetic knockout of the *PRKAR1a* gene results in embryonic lethality in mice, caused by morphogenic defects in cardiac organ development⁵⁶. While other R-subunit genetic knockouts lead to severe phenotypes where RI β knockout leads to cognitive impairment⁵⁷, and knockout of RI β causes a lean phenotype due to changes in liver metabolism⁵⁸, only RI α is required for mammalian survival. These findings indicate that RI α has a special role in the cell that is necessary for regulation of PKAc mediated cell stress.

In addition to its direct role in inhibiting PKAc activity, RI α also has also been characterized to have accessory roles in the cell towards the regulation of critical cellular processes like the cell cycle, DNA replication and autophagy. In a series of studies performed by Titora et al., it was shown that alteration of RI α subunit expression had tremendous effects on the progression of the cell cycle. They demonstrated that decrease of RI α expression prevented the transition to S phase and thus resulted in growth arrest and accumulation in the G0/G1 phase of the cell cycle⁵⁹. In a separate publication, overexpression of RI α resulted in a transformed phenotype marked by accumulation of cells in S phase with the capacity for serum-independent growth⁶⁰. These findings suggest that RI α expression must be very strictly controlled in order to maintain proper progression of the cell cycle. In relation to this work, a study from Gupte et al. showed a direct interaction between RI α and the second subunit of Replication Factor C (RFC40), wherein it was

shown that binding of RFC40 to the N-terminus of R1 α allowed for nuclear localization of both proteins ⁶¹. These authors also showed the direct binding of RFC40 to R1 α in a C-subunit-independent manner, and furthermore that the interaction between R1 α and RFC40 is dependent on R1 α phosphorylation by CDK2/cyclin E (see *section 1.7* for more information) ⁶². The involvement of R1 α in other parts of cell metabolism regulation has also been suggested. Particularly, R1 α has more recently been shown to have a role in the process of autophagy, a mechanism in which cells undergoing metabolic stress (e.g. starvation) trigger the lysosomal degradation of cellular components for the purpose of recycling amino acids and other nutrient molecules. Mavrakis et al. reported that loss of R1 α expression leads to down-regulation in autophagy, manifested by increased phosphorylation and activity of mTOR (mammalian target of rapamycin) and the decrease in autophagosome formation ⁶³. All of these findings compel us to believe that R1 α is a critical regulator of homeostatic cellular signaling, and thus novel mechanisms involving protein modification will likely contribute to unique cellular signaling outcomes.

1.6: Deregulation of R1 α in disease

Considering that R1 α is such a crucial regulator of so many processes in the cell, it is easy to imagine that dysregulation of its function could cause aberrant cell signaling and thus lead to disease. PKAc and R1 α have been implicated together in the pathogenesis of numerous human illnesses,

including many differing cancer phenotypes, as well as many diseases that are cardiac-specific in nature. Most diseases associated with alterations in R1 α protein usually assert their harmful effects through deregulation of PKAc phosphorylation activity, which itself is commonly a result of malfunctions in regulatory subunit expression, function, or subcellular localization.

The most notable example of a disease state directly mediated by defects in R1 α protein is Carney complex (CNC), where mutations in *PRKAR1a* produce RNA transcripts either that undergo nonsense mediated decay (NMD) or generate abnormal forms of mutant R1 α protein which in effect cause haploinsufficiency or a deficit of wild type protein^{64 65}. This deficiency creates a state of hyperactive PKAc, leading to detrimental effects throughout the body, especially in the heart and in endocrine glandular systems^{66 67}. The heart phenotype is characterized with generation of hyperplastic cardiac myxomas that eventually lead to heart failure or embolic strokes⁵⁶.

Previous studies have attempted to characterize select CNC mutations that result in stable protein products in order to understand how these mutations result in PKAc hyperactivity. One unpublished study in the Taylor lab was focused on Arg74Cys (R74C), a mutation that introduces a cysteine to the linker region of R1 α . This mutation did not lead to changes in PKAc activity measured *in vitro*. However, upon coexpression of differentially tagged versions of wild-type and R74C variants of R1 α in cells, it was demonstrated

that these proteins undergo heterodimerization between WT and R74C protomers, effectively decreasing the content of wild-type homodimer complexes⁶⁸. In another recent study, crystallization of the wild-type R1 α homodimer complex revealed that CNC mutations (involving residues Arg144 and Ser145) occur at a critical interface of the dimer complex⁶⁹. Another set of disease mutations in *PRKAR1a* result in a deformative disease called acrodysostosis (ACRO), which interestingly has the opposite functional effect as compared to CNC towards reducing PKAc activity. This effect has shown to be largely due to mutations in R1 α that lead to a reduced responsivity to cAMP, and thus decreased holoenzyme dissociation⁷⁰. Mapping of *PRKAR1a* mutations onto the structure of R1 α shows a trend of localizing CNC mutations to the N-terminal portion of the protein up to and including the CNB-A domain, whereas ACRO mutations tend to be concentrated to the CNB-B domain. All of these data demonstrate that modification of R1 α protein by mutation can lead to drastic effects in protein function, possibly manifested by differences in holoenzyme complex formation.

R1 α has also been implicated in the progression of cancer. For the most part, the general consensus among many various cancer cell lines and tumor models is that transformative cell phenotypes are usually associated with increases in R1 α expression. In accordance with this assertion, a handful of studies have demonstrated that treatment of cancer cells with antisense R1 α oligonucleotides can trigger growth arrest⁷¹. While data from these

investigations suggest a more proto-oncogenic role for R1 α , there is also evidence to implicate R1 α as a tumor suppressor^{72 73}. In either case, these findings further highlight the importance of upholding proper ratio of expression between R1 α subunit and PKAc, where chronic states of dysregulation can induce large-scale transformations in cell functionality.

1.7: Post-Translational Modifications (PTMs) in R1 α

In addition to the already complex nature of Type I PKA signaling, we must also consider the role of post-translational modifications (PTMs) in R1 α as a means of dynamically altering the function of this protein pathway. Previous work from our laboratory and others has shown that R1 α has unique biochemical and structural features targeted for oxidation and phosphorylation. Therefore, we presume that PTMs of specific sites unique to R1 α protein would lead to major functional differences in its regulation of PKAc, and thus induce non-canonical modes of PKA signaling.

The first PTM explored in this dissertation is the oxidation of the conserved cysteine residues in the D/D domain of R1 α . As mentioned above, this disulfide bridging modification has been shown to enhance dimerization of R1 α , thus allowing for oxidant-sensitive covalent linkage of protein monomers (**Figure 1.4**)⁷³. A more thorough review of the potential significance of this PTM in the heart, especially within the context of oxidative stress, is presented in the introductory section of *chapter 2*. To briefly summarize, evidence of the

oxidant-sensitive nature of R1 α makes it an interesting protein to target or understand in regard to cellular responses to ischemia/reperfusion (I/R) injury, a well described oxidative pathology resulting during myocardial infarction (MI).

Another modification type of interest with regard to Type I PKA signaling is phosphorylation of the R1 α subunit. While there have been many particular sites in R1 α that have been suggested to be phosphorylated, we have chosen to focus on three particular serine residues that are clustered within the linker region of R1 α (namely Ser77, Ser83, and Ser101). As depicted in **Figure 1.6**, each site has a suggested upstream kinase that targets it for phosphorylation. In *chapter 4* of this dissertation, we present a further characterization of Ser101 phosphorylation in R1 α by cGMP-dependent protein kinase (PKG), an effect that we show results in increased PKAc activity in the absence of cAMP. Please see the introductory section of this chapter for a more in-depth description of this particular regime of phosphorylation in R1 α protein.

In addition to Ser101, Ser77 and Ser83 are particular sites of interest due to their juxtaposition to other recognition sites in the R1 α linker region. One previous mass-spectrometry (MS) based phospho-proteomics study assessed the presence of phosphorylation at Ser83⁷⁴, but limited research has been conducted to determine either the upstream kinases that target these sites, or the relevance of these modifications in the context of R1 α

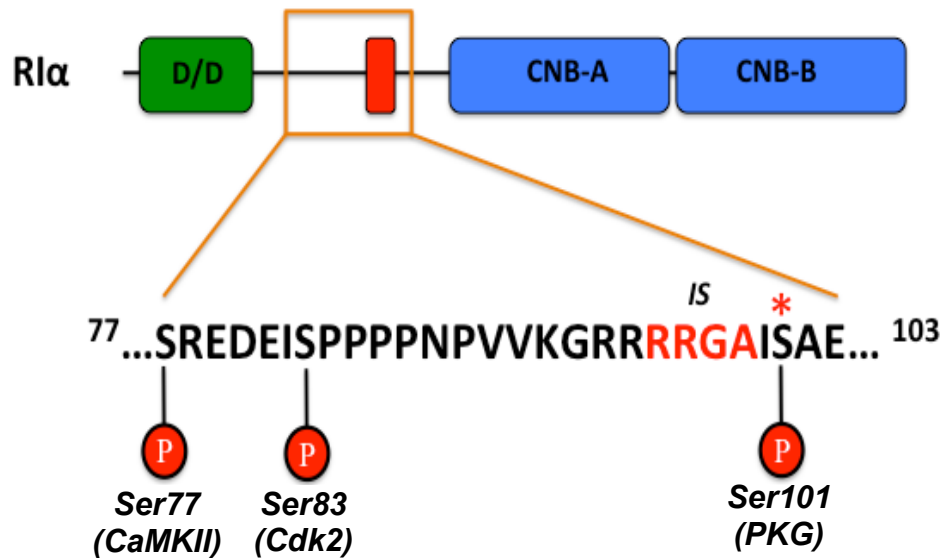


Figure 1.6: Phosphorylation sites in R1 α linker region.

Cartoon diagram of R1 α domain layout, with emphasis on the linker region sequence and phosphorylation sites Ser77, Ser83, and Ser101. The pseudo-substrate inhibitory sequence (IS) is shown in red text. Presumed kinases for each site are listed below. PKG phosphorylation of Ser101 (*red asterisk*) is the focus of the work in *chapter 4*; CaMKII phosphorylation of Ser77 will be discussed in *chapter 5*.

function in the cell. For Ser83, it has been proposed that Cdk2/Cyclin E are involved in R1 α phosphorylation toward the regulation of R1 α interaction with RFC40⁷⁴. In this setting, Ser83 phosphorylation would therefore serve as a switching mechanism to regulate cell cycle processes. In a more recent study from Dr. Ogut et al., increases in Ser77 and Ser83 phosphorylation were shown to occur in the heart tissues of human patients afflicted with heart failure⁷⁵. A comprehensive review of this investigation, along with recent unpublished data and intended future directions for this project, are discussed in *chapter 5*.

Mechanisms of R1 α protein modification for means of protein turn-over have also been previously explored, particularly with regard to the sensitivity of R1 α to proteolytic degradation by either proteases or components of the proteasome-dependent pathway. The linker region of R1 α includes a “PEST sequence” motif (noted for enrichment of proline, glutamate, serine and threonine residues) that has been correlated with higher protein degradation rates⁷⁶. Sites within this cryptic region of the sequence (i.e. those that interact with PKAc upon holoenzyme formation) have been shown to be recognition targets for both ubiquitination and protease-mediated proteolysis⁷⁷. There is also specific evidence of ubiquitin e3-ligases (responsible for catalyzing ubiquitination of proteins selected for proteasomal degradation) that specifically function to target PKA subunits or PKA associated proteins for degradation. AKAP1, for example, has been shown to be a target of seven in-

absentia homolog 2 (Siah2), an E3-ligase that is activated upon hypoxia in cells⁷⁸. With regard to R-subunit targeting for the proteasome, Feliciello and his colleagues have identified the RING ligase Praja2 as a specific regulator of R-subunit proteolysis⁷⁹. PKAc mediated phosphorylation of Praja2 enhances the activity the activity of the E3-ligase, therefore acting as a positive feedback regulation point to allow for prolonged activity of PKAc. While we have not directly assessed the role of E3-ligases in our studies, it is possible that their activity could be either directly or indirectly involved in the signaling process investigated in this work.

1.8: Significance of this study

In these doctoral thesis studies, research has been focused on determining alternative mechanisms for PKA activation outside the well-characterized cAMP-mediated activation pathway. As stated previously, the rationale guiding this work is that these mechanisms, including post-translational modifications specific to Type I regulatory subunits and unique localization to sub-cellular organelles, will have significant effects on regulatory subunit function and compartmentalization, which therefore lead to novel modes of PKA signaling. Given our findings from these studies, it has been demonstrated that these specific processes can contribute to activation of PKAc in differing settings of cellular stimulus, particularly under conditions of cellular oxidative stress.

In *chapter 2*, we show that R1 α is specifically targeted for protein down-regulation within the framework of cardiac ischemia/reperfusion. We also show that this effect is ROS-dependent in nature within cardiac myocytes, and furthermore that it appears to be correlated with direct oxidation of R1 α as well as enhancement of PKAc activity. Lastly, we show that overexpression of R1 α in cardiomyocytes via introduction of adenoviral transgene leads to exacerbated cell death upon oxidative stress.

To further characterize the nature of PKA signaling in the heart, *chapter 3* is centered on an analysis of PKA signaling components (namely R-subunits, PKA, and AKAP1) localized to mitochondria sub-compartments of heart tissues. Here, we review published work on this topic of study, wherein A-kinase interacting protein 1 (AKIP1) was determined to be a mediator of cardioprotective signaling in stressed heart tissue. Upon fractionation of wild-type mouse heart tissue, PKA signaling proteins were found to have distinct localization within specific mitochondrial sub-populations. Using similar methodologies as in *chapter 2* for simulating ischemic stress in the heart, it was also determined that ischemia/reperfusion (I/R) injury induces significant changes in expression at these organelles. Preliminary investigations of these mitochondria within genetic knockout mice for AKAP1 (AKAP1^{-/-}) were also conducted.

Experiments presented in *chapter 4* demonstrate that Ser101 in the linker region of R1 α , juxtaposed to the pseudo-substrate IS, is indeed a

phosphorylation target of PKG as observed both *in vitro* and in cells. Creation and examination of a phospho-mimetic mutation of this site (S101E) illustrated that modification can promote enhanced PKAc activity via altering the affinity of the linker region to PKAc. Nonetheless, other data from this project also suggested that Ser101 mutants R1 α maintain the capacity to form holoenzyme complexes with PKAc *in vitro* and in cells.

Lastly, in *chapter 5*, we overview preliminary data from these and associated projects ongoing in our laboratories in order to outline the future directions of this field of study.

Chapter 2:

ROS-dependent loss of PKA regulatory subunit R1 α in cardiac ischemia/reperfusion injury

2.1: Introduction

Oxidation of cellular signaling proteins such as eukaryotic protein kinases (EPKs) and protein phosphatases (PPs) is of particular importance due to the oxidative nature of many disease pathologies, including cancer, neurodegenerative disorders, and most notably heart disease. Examples of kinase pathways that can be non-canonically activated via oxidative stress in the heart, such as Ca^{2+} /calmodulin-dependent kinase II (CaMKII)⁸⁰, led us to explore the potential for similar oxidant-mediated activation pathways for the PKA pathway.

Earlier investigations from the Taylor laboratory showed that PKAc itself is a target of oxidation. In a series of publications by Humphries et al., it was shown that Cys199, a reactive cysteine found near the active site of PKAc and adjacent to the activation-loop phosphorylation site Thr197, plays a critical role in regulating PKAc activity. First, it was shown that oxidative modification of Cys199 by the chemical diamide or by oxidized glutathione directly inhibited PKAc activity, and furthermore that alanine mutation of this site prevented oxidant-mediated inhibition⁸¹. This work was followed by an analysis of the effect that Cys199 oxidation has on Thr197 phosphorylation, wherein it was demonstrated that modification of Cys199 leads to the enhanced dephosphorylation of Thr197 by phosphatase proteins⁸². The last study in this series was aimed at characterizing the interplay between kinase and phosphatase oxidation in the regulation of PKAc activity in cells. After conducting diamide oxidant treatment experiments in a variety of time-course

and dose response regimes in HeLa cells, it was proposed that PKAc activity undergoes a “biphasic” response to oxidation. In this case, lower doses or shorter exposure time of oxidation treatment led to heightened PKAc activity, whereas higher dose or prolonged exposure time to oxidant lead to PKAc inhibition⁸³. The interpretation proffered by the authors is that this biphasic response is mediated by initial oxidation and thus inhibition of PPs at lower levels of oxidant stress (as PPs have been shown to be very oxidant sensitive)⁸⁴, followed by PKAc oxidation and therefore inactivation upon high levels of oxidant.

While this previous work clearly outlines a role for oxidation in the regulation of PKA signaling, the potential role of R-subunit involvement during oxidative stress was not assessed. As mentioned above, the D/D domain of R1 α has two highly conserved cysteine residues (Cys18/Cys39) that have been shown to generate inter-protein disulfide bonds between anti-parallel monomers of R1 α , thus allowing for stabilization of the R1 α homodimer via covalent linkages. Studies from our lab and others have shown that these disulfide bonds are redox sensitive both *in vitro* and in cells and tissues, where treatment with oxidants promote increased disulfide bond formation^{26 85}. However, the overall significance of this particular modification in R1 α in terms of its effect on PKA signaling in ischemic heart disease has not been fully explored. Therefore the aim of this work is to characterize the effect of cardiac ischemia/reperfusion (I/R) injury, a disease pathology involving acute oxidative

stress in the heart, upon R1 α protein oxidation and subsequent activation of PKAc signaling.

I/R injury is a pathology that occurs in many tissue types, but most research in the field has been focused on its direct association to myocardial infarction (MI; a.k.a. heart attack) and non-hemorrhagic stroke⁸⁶. **Figure 2.1** illustrates the physiological effects of the independent phases of ischemia and reperfusion upon cell function. First, ischemia is defined as the state of oxygen and nutrient deprivation due to lack of blood perfusion. In this setting, decreases in cellular oxygen levels lead to termination of aerobic respiration. Glycolytic mechanisms are activated in order to meet ATP demand, leading to the accumulation of lactic acid (i.e. acidosis). Prolonged ischemia will eventually lead to cell death; however, restoration of blood flow (i.e. reperfusion or re-oxygenation) can also lead to exacerbated cell stress. The dramatically increased flux of oxygen that occurs upon reperfusion leads to the accumulation of reactive oxygen species (ROS) both from mitochondrial and extra-mitochondrial sources. Additionally, rapid calcium influx ensues upon reperfusion, an effect that both disrupts normal ion gradients required for regulation of contraction and causes hypertonic swelling of subcellular organelles. Mitochondria are a target of both of these detrimental signaling responses, whereupon deregulation of critical cellular processes upon reperfusion triggers cytochrome c (CytC) release and the induction of mitochondria-intrinsic apoptosis.

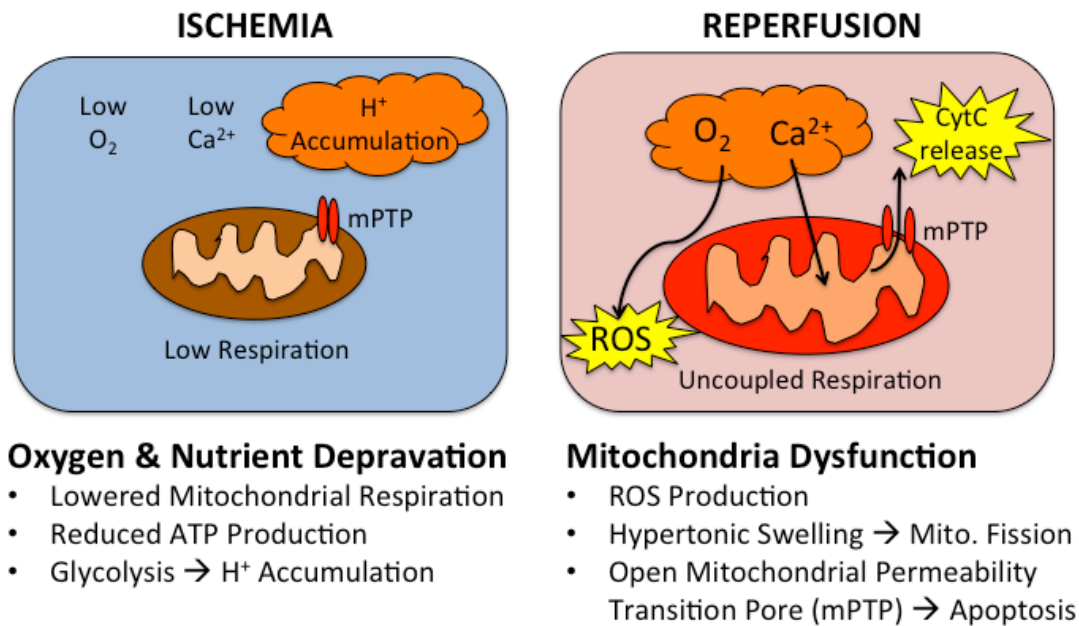


Figure 2.1: Hallmarks of Ischemia/Reperfusion (I/R) Injury

Cartoon diagram of the pathological symptoms associated with the two separate phases of ischemia/reperfusion (I/R) injury. Ischemia is characterized as the state of oxygen and nutrient deprivation due to lack of blood perfusion. Loss of oxygen leads to lowered mitochondrial respiration, leading to a dormant metabolic state in the cell. As a result, glycolytic mechanisms are increased to meet ATP demand, and thus acidosis ensues as a result of proton (H^+) accumulation. Prolonged ischemia will of course lead to cell death; however, re-introduction of blood flow (i.e. reperfusion) can also lead to cell stress in the form 1) heightened oxygen flux leading to accumulation of reactive oxygen species (ROS), 2) rapid calcium influx that both disrupts normal ion gradients required for regulation of contraction and causes hypertonic swelling of subcellular organelles. These two effects resulting from reperfusion injury manifest themselves at mitochondria, where both ROS and calcium stress lead to mitochondrial dysfunction and eventually induction of mitochondria-intrinsic apoptosis via opening of the mitochondrial permeability transition pore (mPTP) and release of cytochrome c (CytC).

As mentioned above, *in vivo* cardiac I/R injury is usually manifested in the form of acute MI, which itself is frequently caused by occlusion of the coronary arteries (possibly by embolic or atherosclerotic plaques). When studying I/R injury in the laboratory, many *in vivo* models have been developed towards simulating coronary artery occlusion via surgical methodologies in mice⁸⁷. These models therefore more closely resemble the physiology of how infarction in the heart would occur as a result of MI. However, to assess the effect of I/R injury on a global scale, *ex vivo* perfusion models have also been developed wherein the entirety of the heart tissue sampled is exposed to I/R stress. One such model employed in this work makes use of the Langendorff perfusion apparatus, which allows for perfusion of isolated mouse hearts for extended periods of time. A schematic of how *ex vivo* I/R injury is conducted in mouse hearts via this method is depicted in **Figure 2.2** (see *experimental procedures for more details*). In brief, mouse hearts are excised and cannulated on the perfusion apparatus, whereupon nutrient and oxygen-rich buffer is pumped retrograde into the left ventricle, thus allowing for perfusion of the coronary arteries. After a period of equilibration and electronic pacing, ischemia is simulated by temporarily stopping perfusion flow to the heart tissue, and reperfusion is therefore initiated upon restoration of perfusion buffer. This global ischemia model has been established within the Patel laboratory as an effective means to assess the role of I/R injury upon cell physiology and heart function⁸⁸.

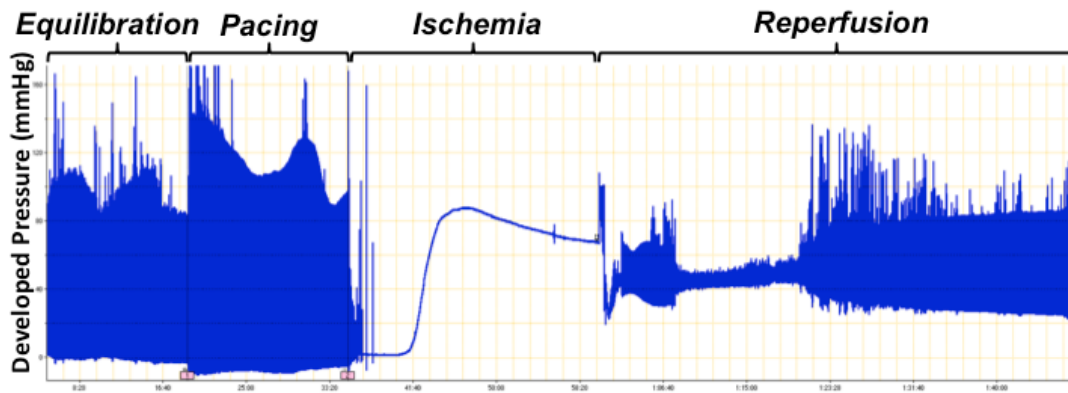


Figure 2.2: *Ex vivo* global I/R injury (via Langendorff perfusion apparatus)

Graph depicting mouse heart function during the onset of our model of *ex vivo* global I/R injury on the Langendorff perfusion apparatus. Signal amplitude (y-axis) represents the left ventricular developed pressure in perfused hearts as measured over time (x-axis) of I/R injury. Heart function is significantly reduced from control perfusion during “equilibration” and “pacing” time-points to that observed upon restoration of contractile rhythm after “ischemia” and “reperfusion” time-points of injury. Please see *Experimental procedures* for more details.

While the aforementioned studies have shed light into the complexity of PKA signaling upon oxidative injury, investigation of PKA and its associated proteins, particularly regulatory subunits, has been neglected within the context of I/R injury. As acknowledged above, previous work from the Eaton laboratory has suggested that exposure to oxidants like hydrogen peroxide (H₂O₂) and nitric oxide (NO) in cardiac systems contributes to cAMP-independent mechanisms for activating PKAc towards regulation of ischemic stress signaling⁸⁵⁻⁸⁹. More recently, using mass-spectrometry (MS) based proteomic comparisons of cAMP-enriched heart proteins, Heck *et al* have shown that human patients suffering from dilated cardiomyopathy (DCM) have significantly down-regulated expression of regulatory subunit proteins⁹⁰. Lastly, in regard to recent studies published during the development of this work, other groups have indicated potential roles for RI α during oxidative signaling in the heart towards regulation of p90 ribosomal S6 kinase 1 (Rsk1)⁹¹. All of these findings point to RI α as a critical regulator of stress signaling in the heart, yet there is still little mechanistic explanation of how RI α specifically regulates PKA signaling upon onset of I/R injury.

The theory explored in the work presented here is that the acute oxidative stress that occurs during the reperfusion phase of I/R injury will have direct effects upon PKA signaling, manifested in the form of RI α and/or PKAc protein oxidation. Along these lines, and in accordance with previous work, we proposed that a “biphasic” PKAc activation response could similarly occur

during I/R injury, possibly toward the regulation of cell fate in the context of this cellular stress. In light of recent *in vitro* studies⁹² and other evidence cited in this dissertation, it can be inferred that changes in R1 α oxidation, protein expression and sub-cellular localization may be critical to adaptation of the heart from ischemic stress.

To summarize, the aim of this study is to integrate theories from previous work in the field into a single cohesive mechanistic understanding of how R1 α influences PKA signaling in cardiac I/R injury. In this work, it is suggested that the oxidation of R1 α is an explanation to the “early phase” of PKAc activation upon oxidative stress, and that further loss of R1 α upon extended oxidant stress likely causes deactivation of PKAc in the “later phase” of the injury. Characterizing this phenomenon within the setting of the disease pathology of ischemic heart disease may eventually lead to methods of therapy for acute MI in the future.

2.2: Results

This study began by using an established *ex vivo* model of cardiac I/R injury (facilitated by Langendorff perfusion apparatus) to examine the protein expression of PKA related proteins within sequential time-points in the onset I/R injury. In this experiment, immunoblot analysis of wild-type C57bl/6 mouse heart ventricle tissues from samples treated either with 25min ischemic alone, or with 25min ischemia and additionally 15min intervals of reperfusion (15min,

30min, 45min) was performed. Control (non-treated) samples were simply perfused in buffer in parallel with treated samples. As shown in **Figure 2.3**, R1 α protein is specifically down-regulated in its global expression in heart ventricular tissues upon exposure to I/R injury. At the ischemia time point (without reperfusion), R11 α appears to have a potential compensatory effect in raised expression; however, upon further reperfusion injury R11 protein does not undergo a significant decrease in protein levels. We also analyzed expression of PKAc and AKAP1; While AKAP1 underwent a decrease in expression, we also observed that PKAc underwent a small down-regulation in expression as well. The effect of R1 α protein decrease manifested most strikingly after 30min of reperfusion injury, whereas 45min time-points showed more highly variable results often with mild recovery of R1 α expression. Therefore we focused on the 30min reperfusion time-point for relative quantification of protein expression in our model, as depicted in **Figure 2.4**. Again, comparing these time-points illustrated that R1 α protein, but not R11 protein, seems to be specifically down-regulated upon reperfusion injury.

Before moving on to experiments aimed at more mechanistic understanding of this phenomenon, we simply wished to establish that the effect of R1 α down-regulation upon ischemic stress was reproducible in isolated cell culture. Our preliminary efforts for this line of experimentation involved the use of HL-1 cells, an immortalized cardiomyocyte cell lineage developed by Dr. William Claycomb and his laboratory. While numerous

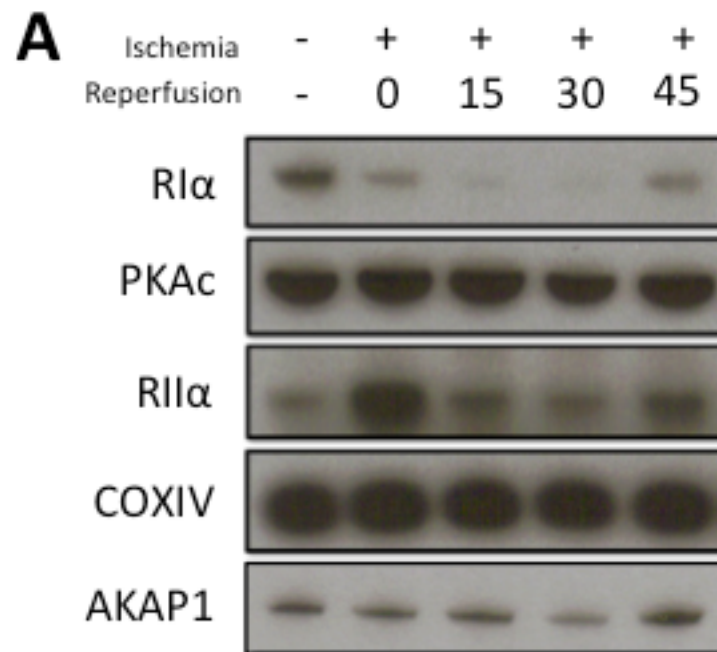


Figure 2.3: R1 α protein is decreased upon time-course of *ex vivo* global I/R injury in mouse hearts.

Immunoblot analysis of tissue homogenate taken from adult C57bl6 mouse hearts exposed to *ex vivo* global I/R injury via Langendorff perfusion apparatus. Control perfusion samples were compared to hearts treated either with 25min ischemia alone, or with 25min ischemia followed by the indicated time (min) of reperfusion injury. R1 α undergoes a marked down-regulation in total protein expression during the course of I/R injury, with a maximum decrease occurring at 30min of reperfusion. Expression of PKAc protein is only moderately decreased at the 30min time-point. R11 α appears to undergo an increase with ischemia, which then decreases back to baseline levels upon reperfusion. AKAP1 was also most significantly down-regulated at 30min reperfusion injury.

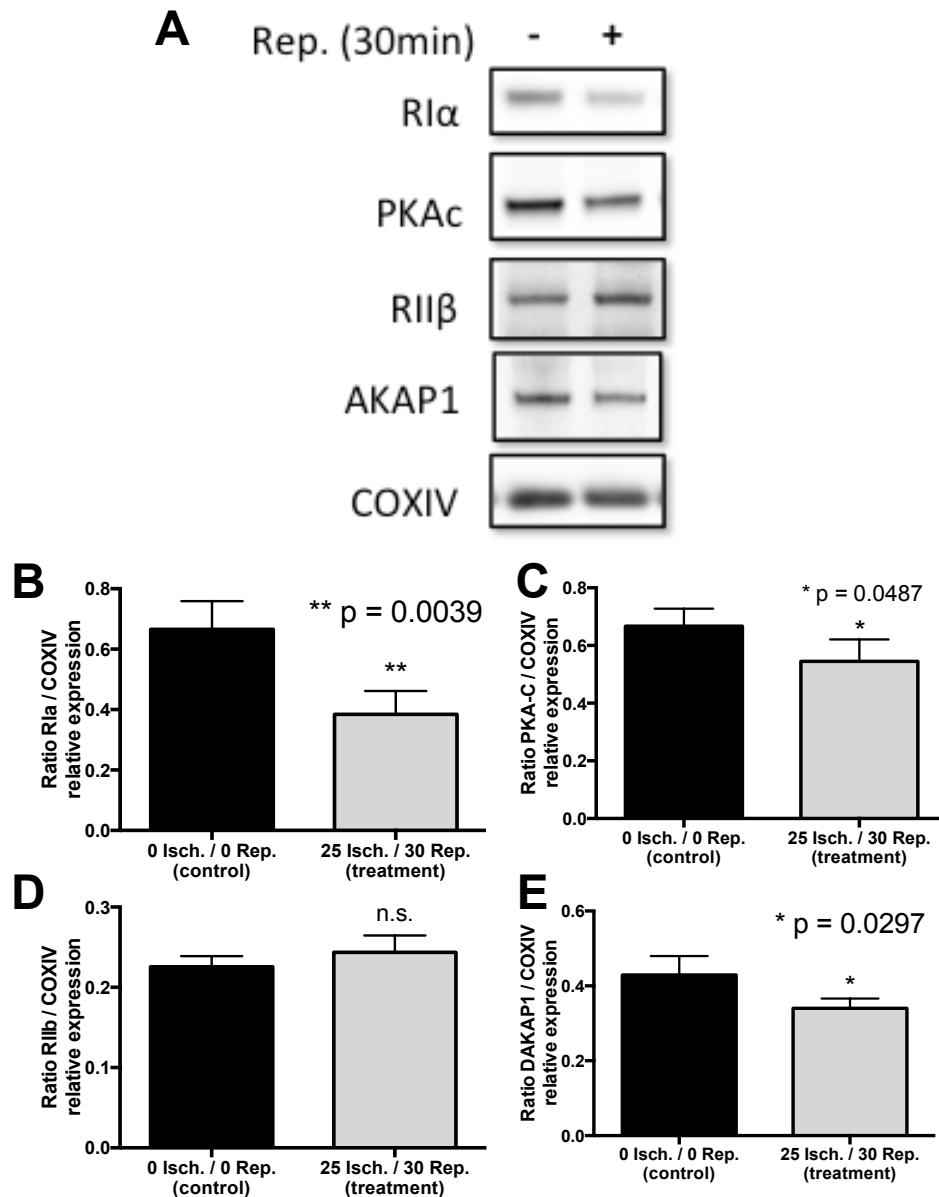


Figure 2.4: R1α protein is most significantly reduced at 30min reperfusion time-point of *ex vivo* I/R injury.

A) Immunoblots comparing control perfused mouse heart tissue to hearts treated with 30min reperfusion injury (using *ex vivo* global I/R injury via Langendorff perfusion apparatus). Corresponding bar graphs below show protein expression quantification for **B)** R1α, **C)** PKAc **D)** RIIβ, and **E)** AKAP1 (N=4; * p < 0.05; ** p < 0.01; n.s., not significant).

models have been developed to study the role of I/R injury in cell culture, we employed hypoxia/re-oxygenation (H/R) stress, a procedure that involves placing cells into a low oxygen (<1% O₂) environment to induce oxidative stress, followed by returning to normal culture conditions to mimic the effects of reperfusion. When we performed hypoxia/re-oxygenation stress on cultured HL-1 cells in an acute manner (i.e. with 30min of hypoxia and up to 60min of re-oxygenation) as shown in **Figure 2.5**, we observed that we could replicate the effect of R1 α protein down-expression within time-frame of 30min re-oxygenation stress. This effect is similar as compared to the 30min reperfusion stress we observed in our *ex vivo* Langendorff model of I/R injury. Also alike to our *ex vivo* model, we observed increases in R11 α expression during the hypoxia phase of injury, but protein levels returned to nominal levels upon re-oxygenation stress. Upon the use of cell culture models, we have used hypoxia inducible factor 1 α (HIF-1 α), a transcription factor that is overexpressed in cells upon induction of hypoxia, as an indicator of the induction of oxidative stress. In this particular experiment, we observed the highest degree of HIF-1 α expression upon the longest time-point of H/R stress examined (i.e. upon 60min of re-oxygenation).

Findings from these initial experiments inspired us to further examine the cause of R1 α down-regulation in cardiac I/R injury. Given the oxidative nature of I/R injury, particularly within the context of the heart as an oxidant-sensitive tissue type, we next wished to investigate the specific role of reactive

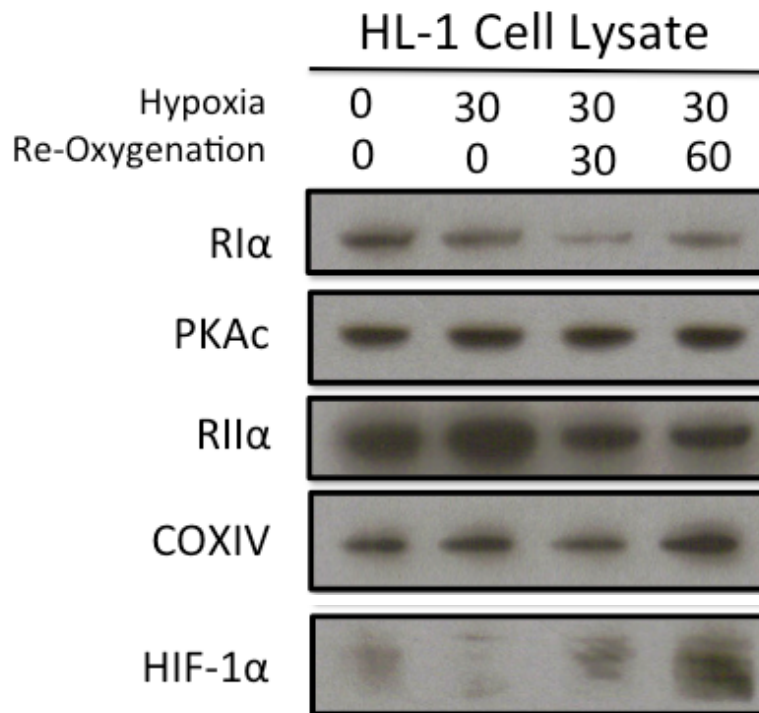


Figure 2.5: R1 α protein is decreased upon *in vitro* hypoxia/re-oxygenation stress in HL-1 cardiomyocyte cell cultures.

Immunoblot analysis of HL-1 cardiomyocyte cell cultures treated with *in vitro* hypoxia/re-oxygenation for the indicated time periods. Hypoxia was performed by first exchanging growth media for serum-free media, and then placing cell cultures into <1% oxygen environment (5%CO₂, balanced with nitrogen; 37°C) for 30min. Re-oxygenation was administered by replacing growth media and returning cell cultures to normal incubation conditions (5% CO₂, balanced with air; 37°C). R1 α protein expression is decreased upon treatment compared to non-treated controls, where we observed most significant reduction at 30 minutes of re-oxygenation stress. Similarly as in the *ex vivo* I/R injury model, we observed slight increase in R11 α levels at hypoxia followed by normalization of protein levels upon re-oxygenation. HIF-1 α is used in these experiments as an indicator of hypoxic stress.

oxygen species (ROS) upon extended oxidant exposure in cardiomyocytes and its effect on RI α . We therefore moved to isolated cell culture models in order to assess the independent effect of oxidation upon RI α protein down-regulation. Primarily, we have used hydrogen peroxide (H₂O₂) treatment in AMVMs as a model of ROS-mediated injury. Given the observation that RI α protein expression was decreased upon extended time-points of ischemic injury in our *ex vivo* and in-cell models, we first moved to assess the effect of increasing dose of oxidant within a 30min timeframe. As seen in **Figure 2.6**, RI α protein expression was decreased as a function of increasing concentration of oxidant treatment, with maximal loss of RI α occurring at the highest dose used (100 μ M H₂O₂). Upon investigation of other PKA related proteins, we noticed that the high oxidant concentration samples mimicked many of the findings we previously saw in our examples of ischemic injury: AKAP1 protein follows a similar degree of protein down-regulation as RI α , PKAc protein expression is partially decreased, and RII subunit expression appears to be unchanged within all the doses tested. This data suggests that ROS stress specifically targets RI, but not RII regulatory subunits.

We next chose to perform time-course treatments (5, 10, 15 and 30 minutes) in AMVM cells, using the 100 μ M H₂O₂ dose in which we previously observed significant changes in RI α protein expression. In accordance to findings from Humphries *et al.*, we hypothesize that PKAc activity will follow a similar trend of “early phase” activation followed by “late phase” deactivation

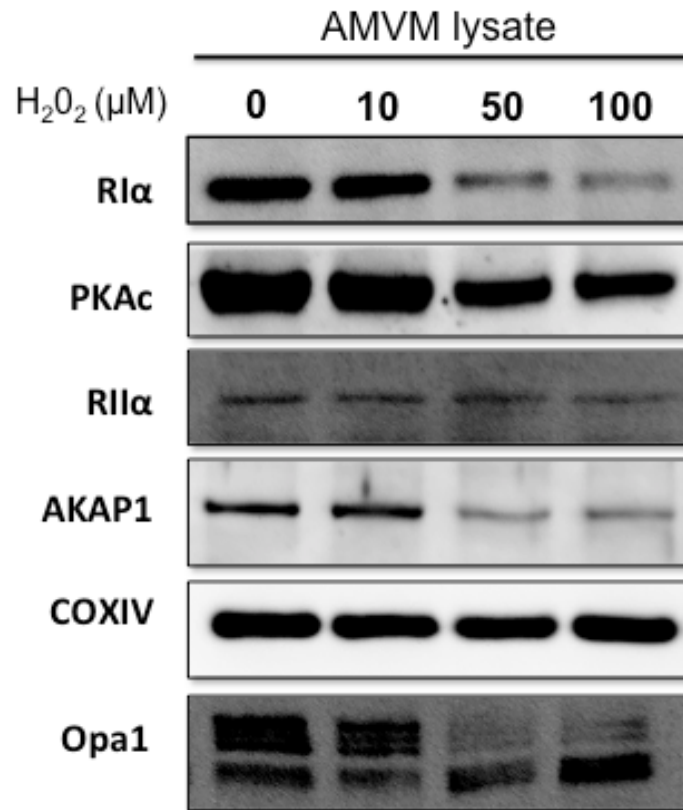


Figure 2.6: Oxidant stress alone in adult mouse ventricular myocytes (AMVMs) reduces RIα protein expression in a dose-dependent manner.

Immunoblot analysis of AMVM cell cultures treated for 30 minutes with the oxidant hydrogen peroxide (H₂O₂) at the indicated concentrations (with samples conducted in triplicate for each condition). RIα protein expression was decreased as a function of increasing concentration of oxidant treatment, with maximal loss of RIα occurring at 100μM H₂O₂. PKAc protein expression is slightly down-regulated and RII subunit expression is unaffected in the same context. AKAP1 expression was decreased in a similar profile to RIα.

as reported previously⁸³. In this regime (see **Figure 2.7**), R1 α protein is steadily decreased as a function of increased exposure time to oxidant, where 30 minutes of treatment lead to the most significant down-regulation in expression. PKAc appears to have stable protein expression throughout majority of the time-course, up until the 30-minute time-point where we observe significant reduction in PKAc. Levels of RII subunit protein expression were not significantly changed in this model. Induction of oxidative stress was validated by both the loss of AKAP1 as well as the appearance of HIF1 α expression with increasing time of oxidant exposure.

These experiments were also aimed to determine the extent of PKAc activity within the context of oxidant stress. Using an antibody raised against phosphorylated substrates of PKAc (phospho-RRX(S/T) PKAc substrate), we observed significant phosphorylation occurring at 10 and 15 minute time-points of H₂O₂ stress. This signal correlated with a band of molecular weight approximately 28kDa. Future work will be aimed at determining the identity of this phosphorylated substrate, which we assume to be cardiac troponin I (cTnI) a well characterized protein in the contractile apparatus of heart sarcomeres that directly regulates cell contraction.

To better understand the role of R1 α subunit in the process of PKAc activation in these oxidant stressed cells, we next compared the extent of R1 α protein oxidation upon time-course treatment with 100 μ M H₂O₂ in AMVM cell cultures. Coming back to the theme of D/D domain oxidation, we hypothesized

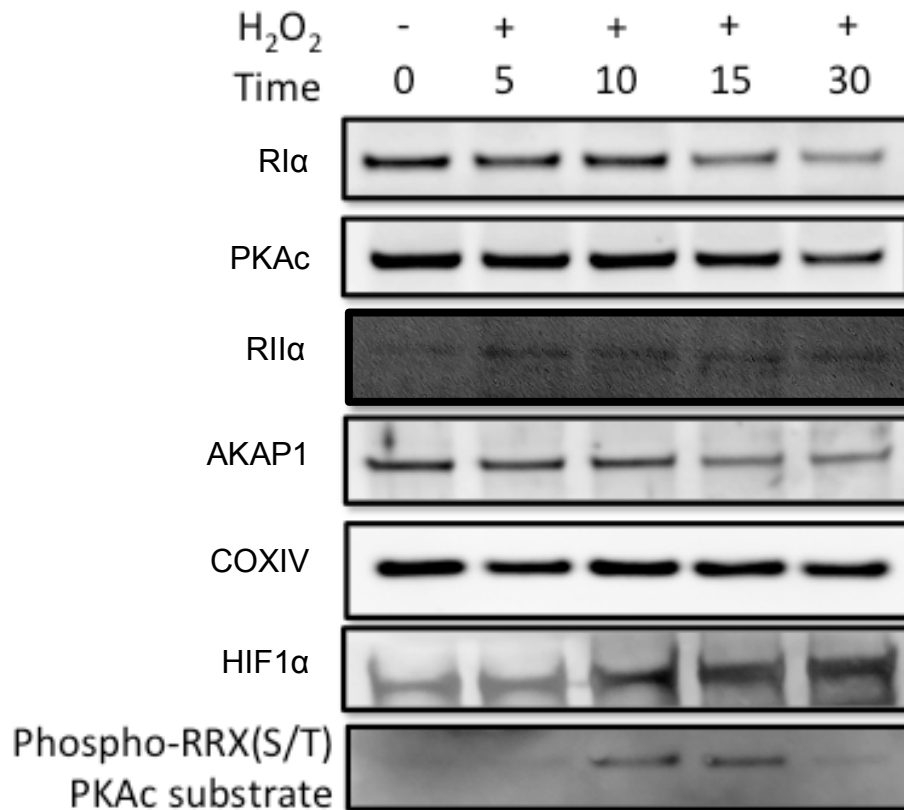


Figure 2.7: Time-course of oxidant stress in AMVMs reveals correlation of PKAc activity with oxidation of R1α protein.

A) Immunoblot analysis of AMVM cell cultures treated with 100μM H₂O₂ for the indicated time periods. R1α protein is steadily decreased as a function of increased exposure time to oxidant, where 30 minutes of treatment lead to the most significant down-regulation in expression. PKAc appears to have stable protein expression throughout majority of the time-course, up until the 30-minute time-point where we observe significant reduction in PKAc. Levels of R11 subunit protein expression were not significantly changed in this model. Induction of oxidative stress was validated by both the loss of AKAP1 as well as the appearance of HIF1α expression with increasing time of oxidant exposure. Using an antibody raised against phosphorylated substrates of PKAc (phospho-RRX(S/T) PKAc substrate), we observed significant phosphorylation occurring at 10 and 15 minute time-points of H₂O₂ stress. This signal correlated with a band of molecular weight approximately 28kDa.

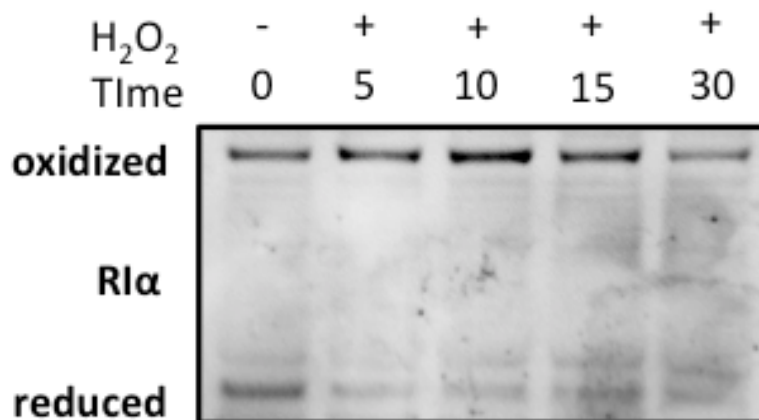


Figure 2.7: Time-course of oxidant stress in AMVMs reveals correlation of PKAc activity with oxidation of R1α protein (continued)

B) Immunoblot analysis comparing the extent of R1α protein oxidation upon time-course treatment with 100μM H₂O₂ in AMVM cell cultures. A previously developed in-house antibody that can recognize both monomer and dimer forms of R1α was used under non-reducing SDS-PAGE conditions in order to differentiate “reduced” monomer bands from “oxidized” dimer bands. Dimer bands are considered oxidized due to the requirement of covalent disulfide bonds to maintain dimerization upon denaturing conditions of non-reducing SDS-PAGE. R1α exists as a mixed population of reduced and oxidized protein in untreated control samples, but then nearly all of the protein becomes oxidized upon stimulus with oxidant. The highest degree of oxidized protein is observed at the 10 minute time-point of H₂O₂ treatment. Total levels of R1α protein expression is then decreased upon extended periods of stress (>15min), with minimum expression observed at 30min.

that modification of R1 α by ROS in this context may be triggering the process of PKAc activation and the subsequent reduction in R1 α protein expression. To assess this, we used a previously developed in-house antibody that can recognize both monomer and dimer forms of R1 α . Under non-reducing SDS-PAGE conditions, this antibody can be used to differentiate “reduced” monomer protein from “oxidized” dimer protein; Dimer bands in this experimental set-up are considered oxidized due to the requirement of covalent disulfide bonds to maintain dimerization upon denaturing conditions of non-reducing SDS-PAGE. As shown in panel B of **Figure 2.7**, we observed that R1 α protein was in a mixed population of reduced and oxidized protein within control (non-treated) AMVM cells; whereas upon introduction of oxidant, the entirety of R1 α protein was oxidized to dimeric form even at the earliest timepoint (5 minutes). We detected the highest extent of oxidized protein at the 10 minute time-point of H₂O₂ stimulus. However, as observed under reducing conditions, total levels of R1 α protein expression were then down-regulated with oxidant stress longer than 15 minutes, where R1 α protein was similarly reduced at 30min of oxidative injury.

To summarize, the findings from these hydrogen peroxide stress studies compellingly illustrate that the nature of R1 α protein down-regulation in these AMVM cells is ROS-dependent. This effect seems to be directly correlated with the oxidation of R1 α protein itself, and furthermore it is also inversely correlated with a transient increase of PKAc activity, similar to results

from previous studies. However, upon characterization of this signaling paradigm, we have yet to understand the general repercussions of R1 α down-regulation upon the fate of the cardiomyocyte cell in the context of oxidative stress in the heart (i.e. what occurs downstream of oxidant-mediated R1 α protein loss and subsequent PKAc activation). To this end, we have been working with differing methods of intervention to fully elucidate the mechanistic role of R1 α in the context of cardiac I/R injury.

Our initial strategy for direct modulation of this pathway within our AMVM model system was to employ adenoviral vectors to overexpress an exogenous version of R1 α protein in these cells in order to counterbalance the effect of ROS-mediated R1 α down-regulation. Viral methods of overexpression are required for AMVMs, as conventional methods (e.g. use of such as cationic liposomes) are unable to engender the transfer of genetic material into these cells. The overall hypothesis guiding this work is that AMVMs overexpressing R1 α will maintain a higher degree of R1 α protein expression, which will in turn reduce the extent of ROS-mediated PKAc activation observed in previous studies. Given this assertion, we also wished to determine whether or not the inhibition of oxidant-mediated PKAc activity (via R1 α overexpression) leads to either pro-survival or pro-apoptotic signaling. As PKAc is often associated with cellular stress signaling, we speculate that R1 α overexpression could actually be harmful to AMVM cell fate in the framework of oxidative injury.

To address these points, adenoviral constructs for the overexpression of human R1 α (Ad-R1 α), as well as green fluorescent protein (Ad-GFP) for controls, were developed (in collaboration with Dr. Atsushi Miyanohara, UCSD medicine). Upon generation of these viruses, optimization of a virus dosage protocol that would lead to robust overexpression of R1 α protein without vastly compromising AMVM cell integrity (i.e. maintain healthy cell function) was required. As shown in **Figure 2.8**, we established a working procedure where cells were exposed to virus for 1hr and then media was exchanged and cells were allowed to culture overnight. Upon testing two particular doses (administered in viral particles per milliliter, or vp/mL), we observed that both GFP and R1 α were significantly increased in protein expression upon the lower dose of adenovirus (1×10^6 vp/mL). We were surprised to note that overexpression lead to a significant appearance of R1 α dimer signal in these cells, even upon reducing (100mM DTT) SDS-PAGE conditions. Within these cell treatments, Ad-R1 α overexpression does not appear to alter expression of PKAc protein. High doses of Ad-GFP (1×10^8 vp/mL) lead to the appearance of a lower molecular weight band on immunoblots for GFP (labeled with *), indicating a possible degradation product. Therefore, we went ahead with the 1×10^6 vp/mL dosage in further experiments.

Once we were able to successfully introduce exogenous R1 α into AMVMs, we next moved to couple Ad-R1 α overexpression with oxidant stress wherein we could test the consequence of R1 α overexpression upon AMVM

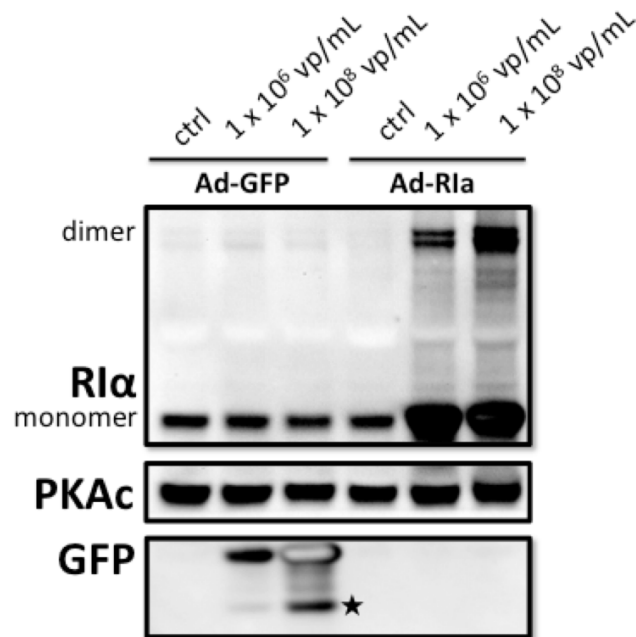


Figure 2.8: Over-expression of RI α in AMVMs via adenoviral infection.

Immunoblot analysis of cultured AMVMs infected with adenovirus to overexpress human RI α (Ad-RI α) or green fluorescent protein (Ad-GFP) for controls. Virus dosage was administered in viral particles per milliliter (vp/mL), where cells were exposed to virus for 1hr and then media was exchanged and cells were allowed to culture overnight. Both GFP and RI α were significantly increased in protein expression upon the lower dose of adenovirus (1×10^6 vp/mL). Note that overexpression lead to a significant appearance of RI α dimer signal in these cells, even upon reducing (100mM DTT) SDS-PAGE conditions. Ad-RI α overexpression does not appear to alter expression of PKAc protein. High doses of Ad-GFP (1×10^8 vp/mL) lead to the appearance of a lower molecular weight band on immunoblots for GFP (labeled with *), indicating a possible degradation product.

cell signaling. In preliminary experiments, we found that 30 minute treatment with the 100 μ M H₂O₂ oxidant dose used in previous experiments lead to detrimental cell death in cells infected with either Ad-R1 α or Ad-GFP controls at the 1x10⁶ vp/mL dosage (data not shown). We attributed this effect to an increased sensitivity of adenoviral-infected cells to stress, especially in the form of oxidative injury. Therefore, we lowered the hydrogen peroxide dose to 10 μ M to increase the likelihood of cell viability after stress treatment. Despite this adjustment, treatment with 10 μ M H₂O₂ was sufficient for significant reduction in R1 α expression in Ad-GFP control cells (see **Figure 2.9**). In Ad-R1 α overexpressing cells, we saw that R1 α protein expression is not significantly reduced upon oxidant stress; moreover, expression levels of PKAc and RII subunit proteins were not altered upon expression of Ad-R1 α . Furthermore, analysis of apoptosis marker Cleaved Poly-ADP Ribose Polymerase (Cl. PARP) showed that cells expressing Ad-R1 α had significant increase in cleaved PARP under basal conditions, and additionally that induction of oxidant stress in these cells lead to a highly exacerbated increase in apoptosis. Evidence presented in these adenoviral overexpression experiments implies that specific down-regulation of R1 α protein upon oxidative injury may have a direct role in initiating protective (i.e anti-apoptotic) responses in cardiomyocytes.

While these experiments did show a correlation between R1 α protein decrease and the up-regulation of PKAc activity, it remains unknown whether

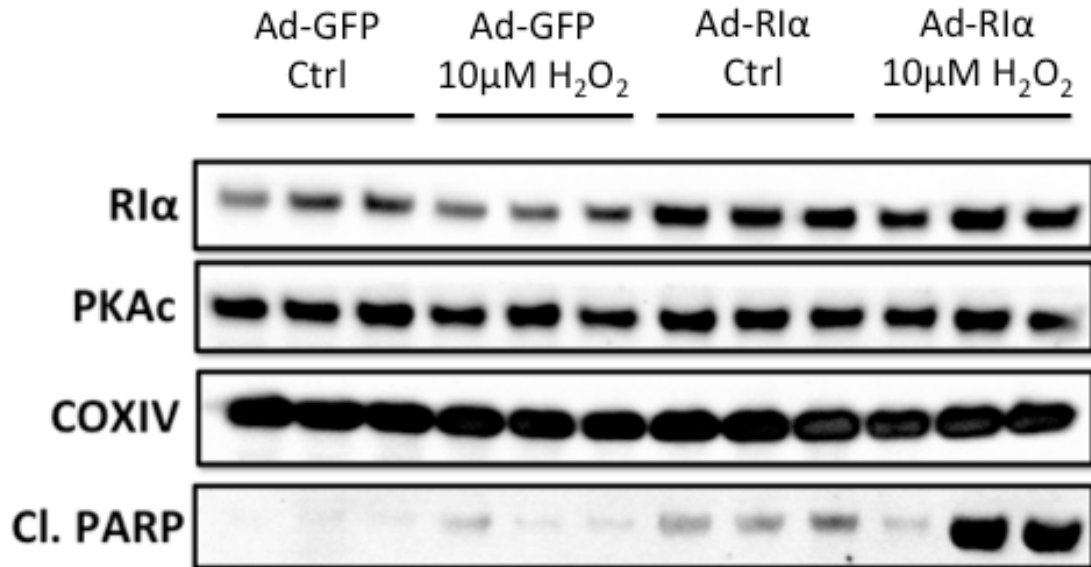


Figure 2.9: Oxidant stress in Ad-R1α overexpressing AMVMs increases induction of apoptosis

Immunoblot analysis of AMVM cell cultures first infected with adenovirus to overexpress R1α or GFP controls (Ad-R1α and Ad-GFP respectively), and then exposed to oxidant stress for 30 minutes (with samples conducted in triplicate for each condition). 10μM H₂O₂ was used as the stress treatment in these experiments because it was determined that higher doses of oxidant lead to significantly exacerbated cell death in adenovirus-infected AMVMs (data not shown); however, treatment with 10μM H₂O₂ was sufficient for significant reduction in R1α expression in Ad-GFP cells. In Ad-R1α overexpressing cells, R1α protein expression was not significantly reduced upon oxidant stress; moreover, expression levels of PKAc and RII subunit proteins were not altered upon expression of Ad-R1α. Analysis of apoptosis marker Cleaved Poly-ADP Ribose Polymerase (Cl. PARP) showed that cells expressing Ad-R1α had significant increase in cleaved PARP under basal conditions, and furthermore that induction of oxidant stress in these cells lead to a highly exacerbated increase in apoptosis.

oxidant-mediated PKAc activation is involved (either directly or indirectly) in anti-apoptotic signaling. Oppositely phrased, it is unknown if the over-expression of Ad-R1 α leads to induction of apoptosis because of its inhibition of PKAc signaling, or because of some other uncharacterized protective role for R1 α . To address this concern, more recent efforts have been aimed towards using specific PKAc inhibitory peptides in order assess the mechanistic significance of oxidant-mediated PKAc activation in the context of I/R injury.

To this end, we have developed a specific PKAc inhibitory peptide by aligning the protein sequences of the basic region of the HIV transactivator of transcription (TAT) protein that confers cell-permeability, and the inhibitory sequence of Protein Kinase Inhibitor (PKI). Our first goal was to compare the inhibitory capacity of either specific PKAc inhibitor peptide (TAT-PKI) or scrambled peptide control (TAT-Scr) within an *in vitro* setting. Using components from the PepTag[®] assay for PKAc activity analysis (see *Experimental procedures*), 200nM PKAc was challenged with either TAT-PKI or TAT-Scr peptide control in a dose-dependent manner (100nM-100 μ M). With this assay, agarose gel electrophoresis of peptide assay samples allows for separation of phosphorylated (active) versus non-phosphorylated (inactive) Kemptide substrate peptides. As shown in **Figure 2.10**, TAT-PKI peptide completely inhibited PKAc phosphorylation of substrate at concentrations of 10 μ M or higher, and partial inhibition was observed at doses as low as 1 μ M.

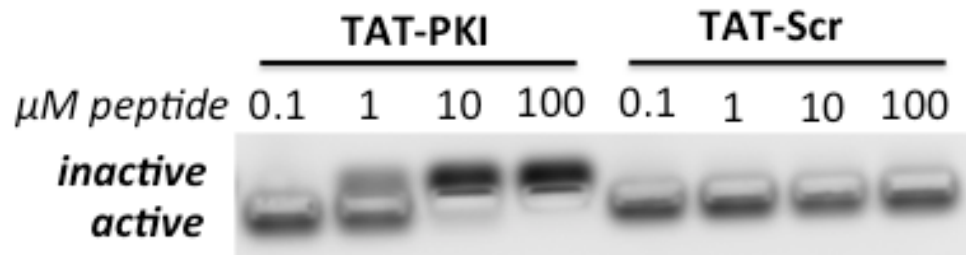


Figure 2.10: TAT-PKI peptide inhibits PKAc *in vitro*.

Qualitative analysis of *in vitro* PKAc kinase activity using the PepTag® activity assay kit, comparing the inhibitory capacity of either specific PKAc inhibitor peptide (TAT-PKI) or scrambled peptide control (TAT-Scr). Electrophoresis of assay samples allows for separation of phosphorylated (active) versus non-phosphorylated (inactive) substrate peptides. In brief, 200nM PKAc was incubated peptide in a dose-dependent manner (100nM-100 μM) with either TAT-PKI or TAT-Scr. While TAT-Scr control peptide had no inhibitory effect on PKAc activity, TAT-PKI peptide completely inhibited phosphorylation of substrate at concentrations of 10 μM or higher, and partial inhibition was observed at doses as low as 1 μM .

In contrast, TAT-Scr control peptide had no inhibitory effect on PKAc activity.

The next step of this work is to utilize this peptide in the AMVM cell cultures to directly assess the role of PKAc activity in regulating cell death within our oxidant stress model. A preliminary experiment comparing cells acutely pre-treated with 10 μ M of either TAT-PKI inhibitor peptide or TAT-Scr scrambled control peptide is shown in **Figure 2.11**. In this experiment, we compared control samples to cells treated for 15 and 30min with 100 μ M H₂O₂ such that we could observe the effect of peptide upon PKAc phosphorylation at earlier time-points of oxidant-mediated activation. It appears that use of 10 μ M TAT-PKI did slightly reduce the degree of substrate phosphorylation, but clearly higher concentrations of peptide will be required to completely inhibit PKAc activity in these cell cultures. If we do observe higher apoptosis induction upon TAT-PKI treatment, we will have bolstered the theory that R1 α loss and activation of PKAc activity upon oxidative stress is a protective signaling regime to prevent cell death in cardiac I/R injury.

2.3: Discussion

Throughout the course of this study, we have worked to combine multiple convergent theories within the field to define a role for the oxidation of R1 α protein in the setting of ischemic heart disease. We aimed to show that oxidative stress resulting from I/R injury is capable of inducing modification of R1 α subunit and therefore dynamic changes in PKAc activity. In review of our

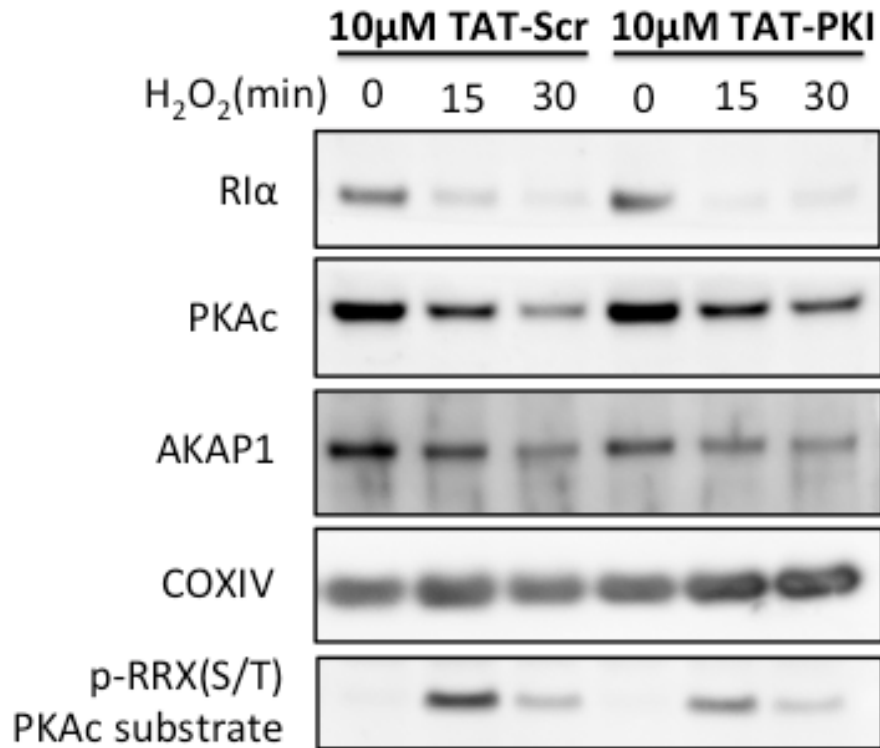


Figure 2.11: PKAc inhibition with TAT-PKI increases apoptosis in oxidant-stressed AMVMs.

Immunoblot analysis of AMVMs treated in a time-course of H₂O₂ stimulus, comparing cells acutely pre-treated with 10 μ M of either TAT-PKI inhibitor peptide or TAT-Scr scrambled control peptide. As observed previously, PKAc activity is highest at 15min of oxidant stress. Treatment with 10 μ M TAT-PKI blunted, but did not abolish, phosphorylation of PKAc substrates in this model.

findings, we began our work with the discovery that R1 α subunit undergoes specific down-regulation in global protein expression as a function of reperfusion injury (**Figures 2.3 & 2.4**). Because this effect occurs on such a rapid timeframe, we have made the assumption that the loss of R1 α protein expression is actualized at the level of protein synthesis and degradation, rather than being due to transcriptionally-regulated effect that would require longer time periods to manifest. Future analysis of mRNA expression levels could definitively support this hypothesis, if indeed mRNA levels are not significantly changed upon oxidative stress.

Previous evidence has suggested that R1 α may have several regulatory mechanisms for higher protein turnover. As mentioned above, the linker region of R1 α includes a “PEST sequence” motif (noted for enrichment of proline, glutamate, serine and threonine residues) that has been correlated with higher protein degradation rates. The linker region of R1 α , especially within cryptic regions of the sequence that interact with PKAc upon holoenzyme formation, has also been shown to be a recognition target for both ubiquitination and protease-mediated proteolysis⁷⁷. With these findings in mind, future efforts in this area should be oriented toward uncovering the mechanism by which R1 α is selectively down-regulated in cardiomyocyte cells.

Upon shifting to primary cell culture models, we have shown that oxidant stress alone is sufficient to 1) induce oxidation of R1 α disulfide bonds as well as promote activation of PKAc kinase activity within short time-frames

(10-15min), and 2) trigger specific down-regulation of R1 α protein expression at extended time-points (30min), which interestingly appears to be correlated with decrease in PKAc activity (see **Figures 2.6 & 2.7**). We believe that these findings corroborate well with the previously described model for regulation of PKAc activity upon oxidation, where the “early phase” (<15 min) of cell oxidation promotes specific modification of R1 α and therefore activation of PKAc, and then the “later phase” (>15min) of oxidation eventually leads to PKAc oxidation and thus deactivation of the PKA oxidant-mediated stress response. In order to validate this theory, follow-up experiments associated with this work will be aimed to assess the relative degree of PKAc oxidation upon a previously described reactive cysteine residue (Cys199) within our cell oxidation model.

Finally, experiments focused on strategies for intervention upon R1 α protein down-regulation have shown that introduction of exogenous human R1 α via adenoviral infection leads to exacerbated cell death with our primary AMVMs (**Figures 2.8 & 2.9**). Follow-up experiments using cell-permeable PKAc inhibitor peptides (TAT-PKI) will be conducted to determine whether specific inhibition of PKAc in the background of oxidative stress can lead to a similar cell-death profile as R1 α over-expression, or if the role of PKAc activity is not the cause for apoptosis observed in our adenovirus treated cells (see **Figure 2.10 & 2.11**).

To encapsulate the significance of this study, we believe that concepts

engendered from our investigations have outlined potential mechanisms for therapeutics within the clinical setting of ischemic heart disease. If indeed oxidation and specific down-regulation of R1 α protein, coupled with subsequent activation of PKAc activity, plays a critical role in cardiomyocyte pro-survival response, it is possible that targeting of this pathway during an ischemic event could lead to beneficial outcomes in the onset myocardial infarction. However, a better understanding of the temporal aspect of this stress event will obviously be crucial for directly intervening upon this disease pathology in the clinic. Considering majority of the oxidative injury induces upon I/R stress is due a rapid flux of oxygen upon reperfusion, we hypothesize that a gradual restoration of nominal PKA signaling dynamics (i.e. recovery of normal stoichiometric ratio of R1 α :PKAc in cells) after early phase oxidant-mediated activation of PKAc. However, while this assertion may or may not be true for acute oxidative damage, this work does not shed much insight into whether oxidative damage on a longer time-scale, such as damage from chronic illnesses in the heart like dilated cardiomyopathy and hypertrophic cardiomyopathy resulting from hypertension. Considering similar findings regarding specific down-regulation of regulatory subunit protein have been shown in examples of human heart disease⁹⁰, future efforts in the field will hopefully be focused on differentiating the effects of PKA signaling in both acute and chronic conditions of oxidative stress in the heart.

2.4: Acknowledgements

Concerning Chapter 2, I would like to thank Dr. William Claycomb and his laboratory for the gift of HL-1 cardiomyocytes used in this work.

Concerning Chapter 2, I would like to thank Dr. Atsushi Miyanohara for his help in the generation of adenoviral constructs used in this work.

Chapter 2, in part is currently being prepared for submission for publication of the material. Haushalter, K.J.; Schilling, J.M.; Sastri, M.; Taylor, S.S.; Patel, H.H. *ROS-Dependent loss of PKA Regulatory Subunit R1 α in Cardiac Ischemia/Reperfusion Injury*. The dissertation author is the primary investigator and author of this material.

Chapter 3:
**PKA signaling in cardiac
mitochondria**

3.1 Introduction

Dysregulation of PKA signaling has been implicated in the progression of heart disease, and, as discussed above, regulation of PKAc activity by R1 α may serve as a critical control point for cardiac response to I/R injury. Many studies have focused on the involvement of PKA signaling localized to mitochondria in the development of these disease phenotypes. PKAc itself has been characterized to have roles intrinsic to mitochondria function, whether in terms of regulation of oxidative phosphorylation (OXPHOS), membrane fission/fusion processes, or phosphorylation of mitochondrial proteins. Numerous publications have suggested that PKAc can phosphorylate specific subunits of cytochrome c oxidase (COXIV, or complex IV of the electron transport chain (ETC)) in order to decrease its activity and down-regulate aerobic metabolism⁹³. Studies showed this effect happens upon oxidative stress in the heart^{94 95}, but the role of regulatory subunit was not explored in this instance. PKAc also has been shown to be involved with proteins in the mitochondrial intrinsic apoptosis pathway. It was shown that the pro-apoptotic protein BAD (a member of the Bcl-2 family of proteins) is inactivated upon phosphorylation by PKAc⁹⁶. A more recent study in the Taylor/Insel labs suggested that the interaction of PKAc and R1 α with Bim, an anti-apoptotic Bcl-2 family member, stabilizes the protein and also prevents apoptosis⁹⁷. Another discovery from the Taylor laboratory focused on Chchd3, a coiled-coil helix motif protein that was shown to be a substrate of

PKAc⁹⁸. ChChd3 is localized to the inner membrane space, where it is part of the TOM complex and critical for regulation of crista biogenesis⁹⁹. To explain these signaling phenomena, characterizing the means of compartmentalization of PKAc and other PKA signaling proteins to mitochondria remains an ongoing challenge. The discovery of mitochondrial AKAPs including AKAP1 (discussed in detail below) and sphingosine kinase interacting protein (SKIP, or SPHKAP) gives a rationale for PKA localization to mitochondria⁴⁴, but the intricacies of these differing localized signaling regimes is not fully understood.

To add further complexity to the understanding of PKA signaling localized to mitochondria in the heart, it is important to recognize the nature of two distinct classes of mitochondria within cardiomyocytes. Subsarcolemmal mitochondria (SSM) are distinguished by their localization to near the sarcolemma membrane of myocyte cells, whereas interfibrillar mitochondria (IFM) are defined as the more metabolically oriented group of mitochondria that are found directly between sarcomeres in the contractile apparatus of myocytes to aid in ATP production for contraction. These mitochondria can be separated by differential centrifugation methods¹⁰⁰, thus allowing for isolated study of their biochemical properties. Since SSM have both a lower tolerance to stress (e.g. Ca²⁺ influx) and a smaller energy production capacity as compared to IFM¹⁰¹, it has been proposed that SSM may function as “sensory” mitochondria in the heart. At present, little is known about the sub-

compartmented regulation of PKA signaling components to these differing mitochondria, and how differential localization of PKA proteins may be involved in stress adaptation. In accordance with this idea, it is possible that PKA localization to SSM versus IFM may serve as a sensory mechanism to mediate responses to ischemic stress. To investigate this possibility, work presented in this chapter is centered on three major regulators of PKA signaling regulation in cardiac mitochondria: 1) AKIP1, 2) regulatory subunits (particularly R1 α), and 3) AKAP1.

A-kinase interacting protein (AKIP1; a.k.a. breast cancer associated protein 3 (BCA3)) was initially discovered to be up-regulated in mRNA expression within breast and prostate tumor tissues¹⁰². Sastri et al. showed AKIP1 is involved with trafficking of PKAc to the cell nucleus following interaction with proteins in the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, events that can lead to initiation of gene transcription events¹⁰³. Considering this gene has a precedent for correlation with disease, more recent efforts to study AKIP1 focused on its role in heart disease¹⁰⁴. As part of my thesis work, the role of AKIP1 expression in the heart was examined, resulting in a publication summarized below⁸⁸. A major conceptual point of this work is that increase of AKIP1 expression upon oxidative injury, particularly at IFM sub-compartments, leads to protective effects in the heart, manifested in the improvement of mitochondrial function.

As a follow-up to this study of AKIP1, as well as related findings

presented in *chapter 2*, a characterization of regulatory subunit localization to SSM and IFM was conducted. Given that R1 α is dynamically regulated in expression during I/R injury similarly to AKIP1 although opposite in direction (i.e. AKIP1 is up-regulated while R1 α is down-regulated), it was inferred that loss of R1 α with oxidative injury might contribute to changes in mitochondrial signaling. Therefore the aim of this work was to assess R1 α localization to mitochondria in the heart, both with and without the onset of I/R injury.

As mentioned in *chapter 1*, AKAP1 facilitates localization of PKAc to the outer mitochondrial membrane (OMM) where it contributes to the regulation of membrane fission/fusion. As shown in **Figure 3.1 (panel A)**, splice variants of AKAP1 are differentially targeted to mitochondria or the endoplasmic reticulum (ER) based on the presence of an N-terminal localization signal sequences⁴⁷. Protein domains within AKAP1c are shown in panel B (**Figure 3.1**), including: 1) the N0 motif which allows mitochondrial localization, 2) phosphatase binding domains, 3) the A-kinase binding (AKB) domain, 4) a K homology (KH) domain that binds 3' mRNA, and 5) a Tudor domain that acts to bind proteins with methylated basic residues like lysine and arginine. AKAP1 is also a target of the E3 ligase Siah2 upon hypoxic stress, making it a target for study in the context of ischemia heart disease⁷⁸.

Studies conducted by the Strack laboratory have implicated AKAP1 as a critical regulator of mitochondrial function, particularly during ischemic stress in neurons¹⁰⁵. As depicted in **Figure 3.2**, the direct interplay of PKA and

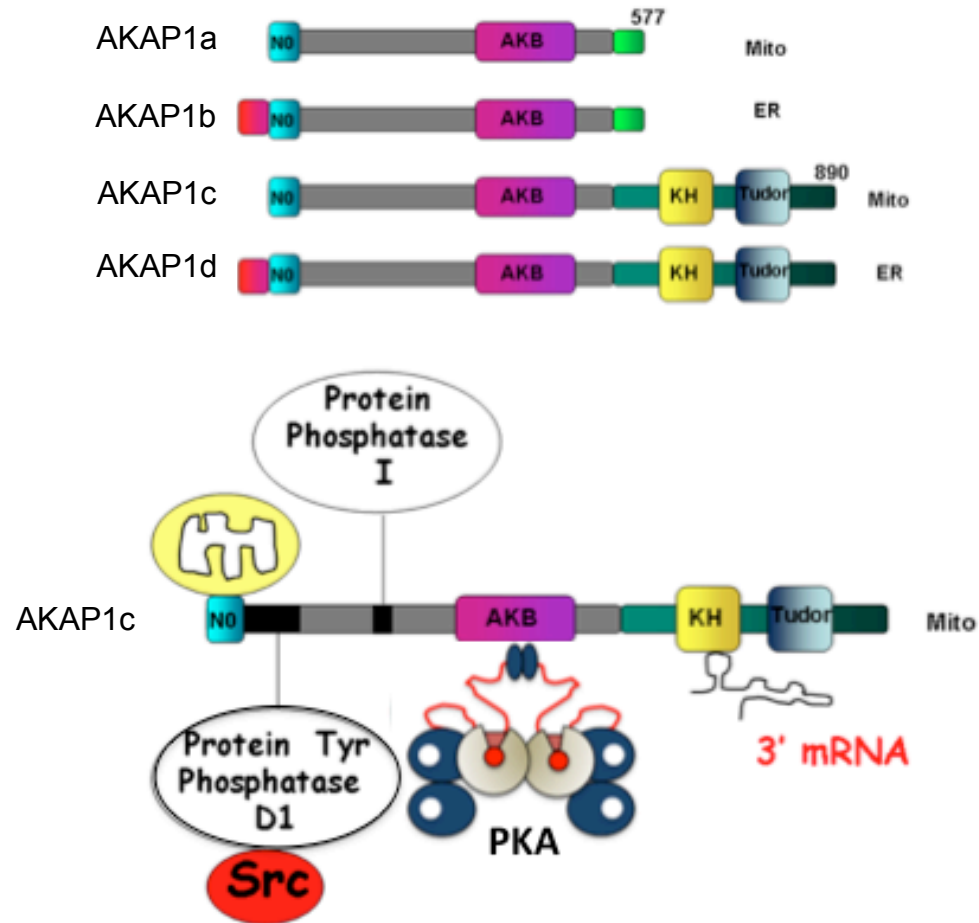


Figure 3.1: AKAP1 localizes PKA to mitochondria

A) Cartoon of AKAP1 splice variants (AKAP1a, AKAP1b, AKAP1c, AKAP1d), highlighting the conservation of protein domains. Subcellular localization of each variant to either mitochondria (mito) or endoplasmic reticulum (ER) is indicated. **B)** Cartoon of AKAP1c, a mitochondria-localized splice variant, showing organization of AKB domain and accessory protein binding domains. (N0, mitochondrial localization motif; AKB, A-kinase binding domain; KH, K homology domain; Tudor, tudor domain; Src, proto-oncogene tyrosine protein kinase Src).

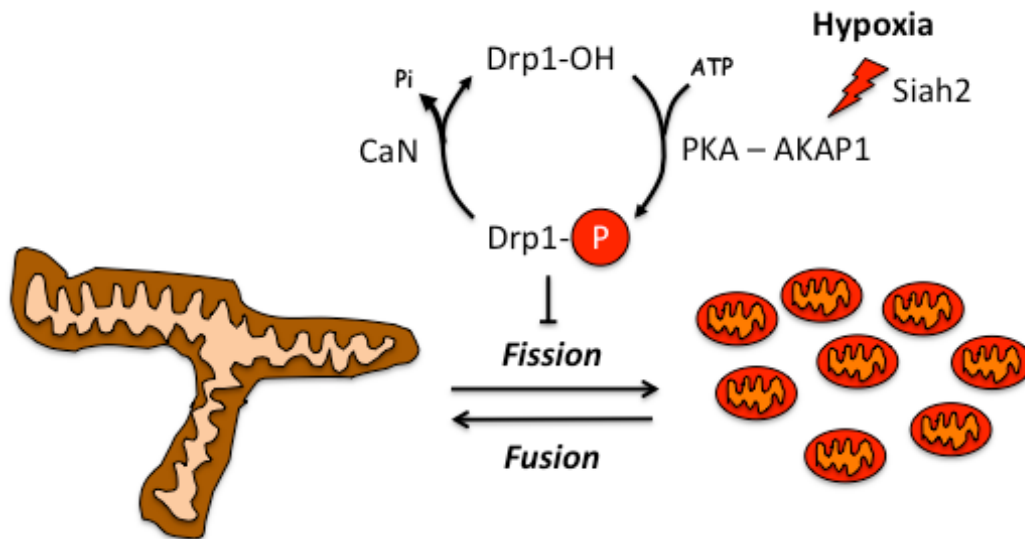


Figure 3.2: AKAP1-PKA regulates mitochondrial fission via Drp1.

Cartoon illustrating the process of mitochondrial membrane fission regulation via inactivation of Drp1 by constitutive phosphorylation by PKA. Upon hypoxic stress, mitochondrial localization of PKA to mitochondria is diminished due to decrease in AKAP1 expression, which in turn promotes activation of Drp1 and induction of membrane fission.

AKAP1 signaling at mitochondria regulates membrane fission/fusion processes, manifested by constitutive phosphorylation of dynamin-related protein 1 (Drp1) to prevent membrane fission¹⁰⁵. It is hypothesized that loss of AKAP1 at mitochondria upon hypoxia results in cell death via increased mitochondria fission activity and in turn induction of apoptosis. From these findings, it can be presumed that AKAP1 could perform similar roles in other oxidative disease phenotypes, particularly ischemic heart disease. Presented below is a preliminary examination of SSM and IFM from mouse heart tissues with or without genetic knockout of AKAP1 (AKAP1^{-/-}). In summary, this transmission electron microscopy (TEM) data shows that AKAP1^{-/-} mice have morphological abnormalities at the SSM, but not at the IFM.

Our global conclusions are that localization of PKA signaling proteins to mitochondria appears to be of critical importance in the regulation of heart physiology, particularly in the setting of oxidative stress. In particular, AKIP1, R1 α , and AKAP1, all associated with mitochondria, have been shown to be PKA signaling entities that are specifically regulated by oxidative stress.

3.2 Results

The first study dealing with the characterization of PKA signaling in heart mitochondria concentrated upon the role of AKIP1 in I/R injury⁸⁸. The illustration in **Figure 3.3** summarizes these findings and the potential significance to cardioprotective mechanisms within mitochondria of the heart.

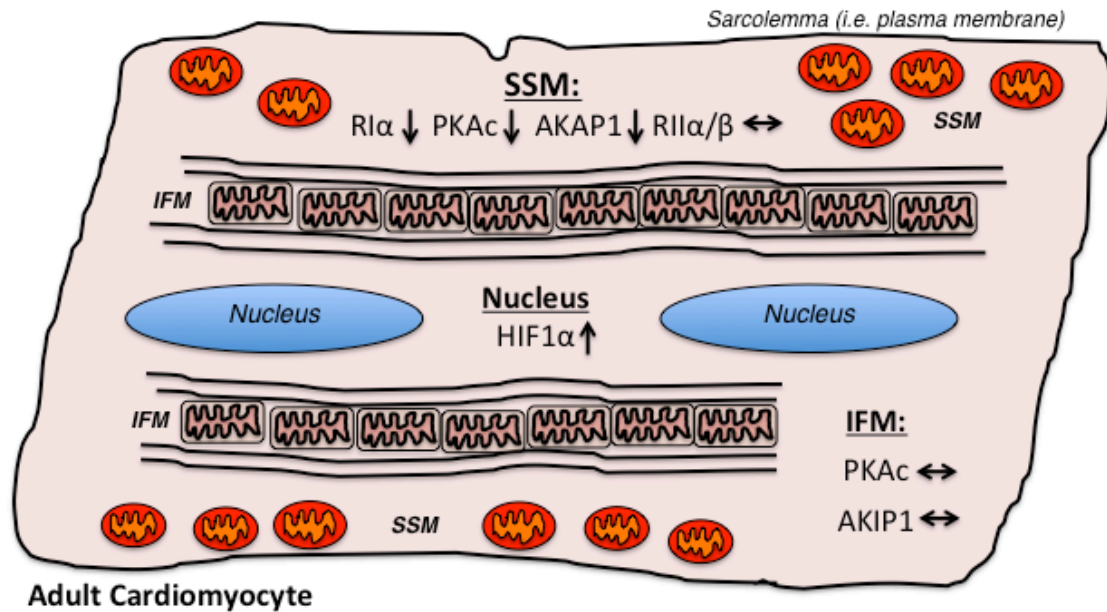


Figure 3.3: Localization changes of PKA proteins in the ischemic cardiomyocyte.

Cartoon depicting the sub-cellular compartmentalization of PKA signaling proteins within adult cardiomyocytes. SSM are peripherally localized near the sarcolemma (i.e. plasma membrane), whereas IFM are found in the myofilaments that allow for muscle cell contraction. Changes in protein expression levels upon I/R injury are indicated by arrows (*up arrow, increased expression; down arrow, decreased expression; sideways double arrow, no significant change*).

Initial characterization of oxidative stress in adult rat ventricular myocytes (ARVMs) showed that oxidant treatment (100 μ M H₂O₂) or hypoxia/re-oxygenation stress induced significant up-regulation of AKIP1 protein levels. This effect was most pronounced at extended time-points of injury (30 minutes or longer), which interestingly coincides with loss of R1 α as observed in other experiments. Overexpression of AKIP1 in mouse hearts (AKIP-OE), as administered by adenoviral transgene delivery via intracoronary injection, was shown to have a significant protective effect upon heart organ physiology in the context of *ex vivo* I/R injury, as measured by improved left ventricular developed pressure (LVDP) and decreased lactose dehydrogenase (LDH) release after injury. To assess the role of AKIP1 in cardioprotection, it was shown that AKIP1 interacts with apoptosis inducible factor (AIF), and that this interaction is increased upon oxidative stress. Furthermore, it was demonstrated that I/R injury increases AKIP1 localization to mitochondria. While present in both SSM and IFM, AKIP1 expression at IFM is more pronounced. Lastly, characterization of mitochondria function showed that AKIP-OE leads to beneficial effects, as exhibited by lower ROS accumulation and decreased susceptibility to calcium-induced hypertonic swelling. Take together, this study uncovered a novel mechanism for PKA regulation within heart mitochondria.

As a further extension of this published work, the localization of other PKA proteins within mitochondria of the heart was further examined.

Differential centrifugation techniques were used in order to isolate SSM and IFM from mouse heart ventricle tissue, either with or without the onset of I/R injury. **Figure 3.4** shows immunoblot analysis of unstressed heart tissues, probing for PKAc, regulatory subunits RI α and RII α , and AKAP1 protein within these organelle populations, using COXIV as a marker for mitochondria loading. While the majority of the catalytic and regulatory subunit protein is found in cytosolic fractions, we observed that all of the PKA proteins examined were strikingly enriched within SSM fractions of the untreated heart tissue. In corroboration with previous work⁸⁸, we did detect expression of PKAc within the IFM fraction as well. Concerning AKAP1, we observed that this protein was also expressed only in the SSM fraction and not found in IFM. We then performed the same mitochondrial fractionation technique upon I/R treated mouse heart ventricle tissues. For this particular data-set, a reperfusion time-point of 10min was used to ensure that mitochondrial preparations were not compromised from the osmotic swelling and overall dysfunction that occurs upon extended time-points of reperfusion injury. Immunoblot analysis depicted in **Figure 3.5** shows that RI α protein down-regulation upon onset of I/R injury was most striking at the SSM fraction. We also observed no change in PKAc levels in the IFM fraction upon I/R injury as previously reported⁸⁸.

These findings demonstrated that PKA signaling proteins seem to be heavily enriched within SSM, and furthermore that expression of these

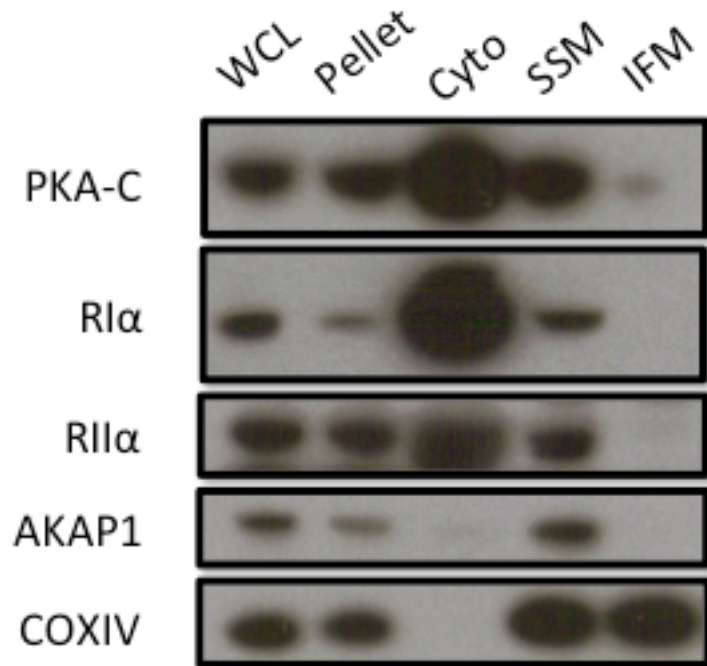


Figure 3.4: PKA-associated proteins are enriched in subsarcolemmal mitochondria (SSM) from mouse hearts.

Immunoblot analysis of PKA-associated proteins from untreated adult mouse heart ventricular tissue that have been fractionated by differential centrifugation methods modified from Palmer *et al.*¹⁰⁰ (WCL, Whole Cell Lysate; Pellet, nuclear/cytoskeleton pellet; Cyto, cytosol; SSM, subsarcolemmal mitochondria; IFM, interfibrillar mitochondria).

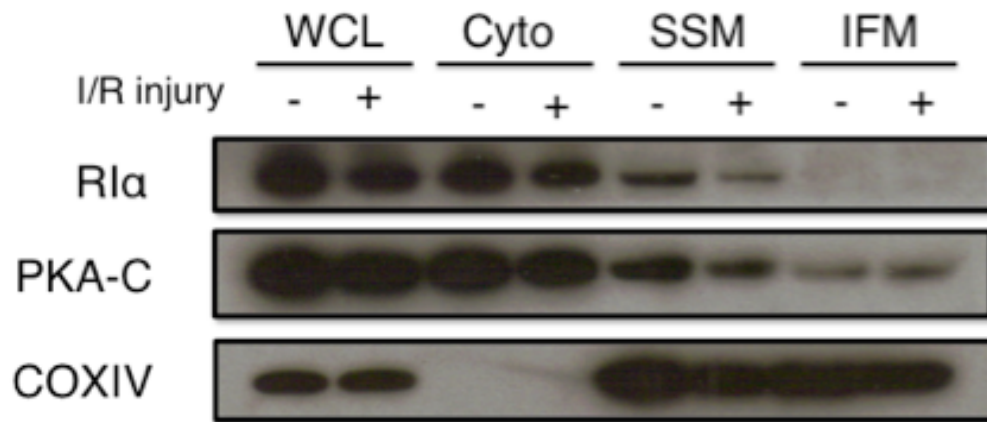


Figure 3.5: R1 α protein undergoes compartmented down-regulation upon *ex vivo* I/R injury.

Immunoblot analysis of PKAc, R1 α and COXIV from fractionated adult mouse heart ventricular tissue, comparing hearts that received either control perfusion or I/R injury (WCL, Whole Cell Lysate; Cyto, cytosol; SSM, subsarcolemmal mitochondria; IFM, interfibrillar mitochondria). Reduction of R1 α and PKAc proteins occurs most strikingly at the SSM compartment upon I/R injury, whereas we observed PKAc levels slightly increase in the IFM with stress as previously reported⁸⁸.

proteins within these unique localizations is dynamically regulated with ischemic stress. Based on these observations, we hypothesized that subsarcolemmal mitochondria would be distinctly sensitive to AKAP1 deficiency, and thus would lead to malfunction of this particular subset of mitochondria. To address this question, preliminary TEM analysis of SSM and IFM morphology was performed comparing untreated tissues of AKAP1^{-/-} mice versus wild-type (WT) controls. As seen in **Figure 3.6**, abnormal mitochondrial morphology was observed in AKAP1^{-/-} mice, but only within the SSM fraction. The subsarcolemmal mitochondria in these mutant animals appear small and fragmented, with evidence of multi-membrane structures. In contrast, the interfibrillar mitochondria appear regularly organized between the sarcomeres of the contractile apparatus similar to WT specimens. This dataset leads to the conclusion that the unique localization of PKA signaling proteins (particularly AKAP1) to SSM is critical for maintenance of mitochondrial ultrastructure in myocytes.

3.3 Discussion

In review of the data presented in *chapter 3*, there are numerous mechanisms by which PKA signaling is localized to mitochondria in the heart. Moreover these mechanisms are specifically modified upon ischemic injury. Our earlier investigation of AKIP1 in I/R injury revealed that the expression of this protein is uniquely up-regulated with oxidative stress, and concluded that

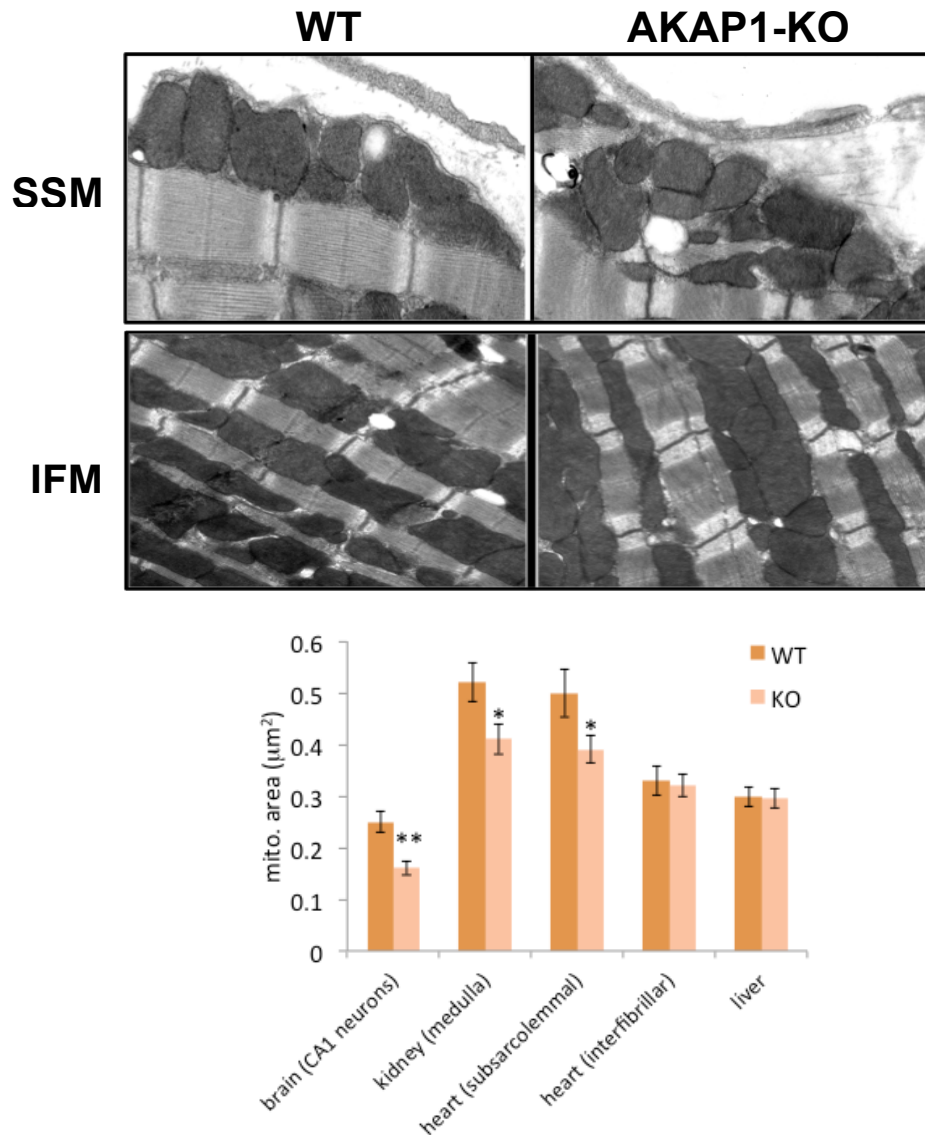


Figure 3.6: AKAP1 knockout perturbs SSM ultrastructure in mouse hearts.

A) Image analysis from transmission electron microscopy (TEM) performed on untreated mouse heart tissues from either wild-type (WT) or AKAP1 knockout (AKAP1-KO) mice. Examination of both subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) showed that SSM were uniquely perturbed in their morphology in AKAP1-KO mice. **B)** Bar graph showing quantification of mitochondrial area (μm^2) in tissues of WT and AKAP1-KO mice. Carried out by G. Perkins at the National Center for Microscopy and Imaging Research (NCMIR).

this effect is likely a cardioprotective signal in the response to oxidation⁸⁸. In this chapter we explored the localization of other PKA proteins including AKAP1 and R1 α . Characterization of fractionated mouse hearts showed that these proteins are markedly enriched within subsarcolemmal mitochondria (**Figure 3.2**). We then showed that levels of R1 α protein in the SSM decrease upon I/R treatment (**Figure 3.3**). Lastly, TEM analysis of mitochondrial ultrastructure from AKAP1^{-/-} mice showed that loss of AKAP1 expression induces aberrant morphology in SSM but not IFM.

Obviously the particular role of R1 α and PKAc proteins localized to SSM in cardiac cells, including the dynamic turnover of for these proteins at this organelle sub-compartment upon I/R stress, is still not fully understood. As mentioned earlier, previous studies have shown numerous roles for PKAc activity at mitochondria, but we cannot conclude from our data whether the effect of ROS-mediated loss of R1 α has a direct outcome in regard to SSM function. To define these biological consequences will require a more comprehensive characterization of the differing functional changes that occur at these unique mitochondrial sub-compartments. Utilization of established protocols for metabolic analysis in isolated SSM and IFM will be a key resource for identifying potential bioenergetic consequences of PKA signaling modification upon I/R injury. Future experiments will assess aerobic metabolism of AMVM cell cultures as well as isolated SSM and IFM from heart tissue using the *Seahorse Bioscience* extracellular flux analyzer. One can

also measure ROS accumulation via electron paramagnetic resonance (EPR) spectroscopy in the context of Ad-R1 α overexpression. As a general prediction for these experiments, we would expect that Ad-R1 α would show indications of cell stress and death, likely being manifested in the form of disrupted SSM morphology, decreased SSM aerobic capacity, and increased ROS production.

A particular important question that has remained unanswered is if there is a close signaling relationship between R1 α and AKIP1; the correlative effects observed in cardiac I/R injury, where R1 α protein decrease occurs within the exact same timeframe and dosing regimen of oxidant stress that AKIP1 expression is increased, is unlikely to be simply coincidence. To our knowledge, no study to date has provided direct evidence of an interaction of AKIP1 with a PKA regulatory subunit, so identifying an interdependence of these two proteins would be a novel discovery. Future efforts should therefore investigate the effect of transgenic manipulation of both R1 α and AKIP1 in the regulation of cardio-protective signaling. Results of these studies could also potentially reveal a purpose for R1 α compartmentalization to SSM, as we still do not know whether loss of R1 α during cardiac I/R injury alters SSM functionality. Given the finding that AKIP1 affords cardioprotection upon overexpression in the heart, our working hypothesis at present is that loss of R1 α protein at SSM upon I/R injury facilitates the interaction between AKIP1 and PKAc. It will be important to determine if increases in AKIP1 expression

upon oxidative stress is in any way directly correlated with the loss of R1 α protein, or whether these events are mutually exclusive in terms of their control of PKAc function.

To assess these questions, the Patel lab will continue to utilize their AMVM cell culture system to define general differences in mitochondria function. However, better understanding of the compartmented nature of SSM and IFM would be achieved through *in vivo* models, where we would not be as limited by our ability to probe these differing pools of mitochondria in the setting of cardiac tissue. As done previously with AKIP1, overexpression of R1 α in mouse hearts can be achieved via intracoronary injection of Ad-R1 α , whereupon we could perform a wider variety of analyses of the effect of this treatment in the context of ischemic stress. It would be interesting to see if Ad-R1 α administration induced significant changes in heart organ physiological parameters upon various models of stress (I/R injury, β AR stimulation, inhibitor treatment, etc.). Assessment of mitochondrial function in this model could be pursued from multiple angles as described above.

The relationship between R1 α and AKAP1 should also be further explored, as it is likely that the interaction between these proteins that fosters mitochondrial localization of PKA proteins to the outer mitochondrial membrane at SSM compartments in cardiomyocytes will be important. With regard to AKAP1^{-/-}, a very recent report from Schiattarella *et al.*, produced concurrently with the work presented here, has shown that deficiency in

AKAP1 expression leads to mitochondrial dysfunction in the heart, especially upon induction of *in vivo* myocardial infarction¹⁰⁶. This effect was attributed to an increase in mitophagosome production, resulting in the induction of apoptosis. However, in contradiction to the results described here, this study asserts that AKAP1 knockout effects SSM and IFM equivalently in the *in vivo* model of myocardial infarction. Future efforts will be aimed at ascertaining the significance of the differences between these two opposing datasets. As mentioned previously, AKAP1 has been shown to undergo hypoxia-mediated protein down-regulation via targeting by Siah2, a hypoxia-sensitive E3-ligase protein⁷⁸. Furthermore, the genetic knockout of AKAP1 in a mouse model has been shown to increase sensitivity to oxidative damage in neurons and the heart¹⁰⁵, so it seems clear that pursuing this topic of study further will be important. Is R1 α loss similarly triggered by hypoxia-initiated proteasomal degradation and possibly directly correlated with AKAP1 loss? Studies defining the proteolytic regulation of R1 α will clearly be required to ascertain the precise mechanism by which this signaling phenomenon is triggered in ischemic heart disease.

3.4 Acknowledgements

Concerning Chapter 3, I would like to thank Dr. Guy Perkins for granting permission to use unpublished material from his laboratory research

for the purposes of describing work intended for future directions of this thesis project.

Chapter 3, in part is currently being prepared for submission for publication of the material. Haushalter, K.J.; Schilling, J.M.; Sastri, M.; Taylor, S.S.; Patel, H.H. *ROS-Dependent loss of PKA Regulatory Subunit R1 α in Cardiac Ischemia/Reperfusion Injury*. The dissertation author is the primary investigator and author of this material.

Chapter 4:

PKG phosphorylation of PKA regulatory subunit R1 α : Crosstalk between PKG/PKA pathways

4.1: Introduction

The linker-hinge region of PKA regulatory subunit proteins contains the auto-inhibitory motif that allows for selective inhibition of PKAc at the kinase active site; however, this inhibitor sequence (IS) differs significantly between RI and RII. While RII subunits have a PKA consensus phosphorylation sequence (RRXS) that is phosphorylated by PKAc, RI subunits have a “pseudo-substrate” IS (RRXX) that is unable to be phosphorylated by PKAc, therefore requiring substrate competition to trigger full PKA holoenzyme dissociation. Additionally, RI subunits have a serine residue (Ser101, P+2 to the pseudo-substrate IS) that has been shown previously to be an *in vitro* substrate of cGMP-dependent protein kinase (PKG)¹⁰⁷. Given the nature of the *in vitro* methodology used, as well as the lack of identity of a well-defined PKG consensus phosphorylation sequence, the physiological relevance of this putative phosphorylation site has not been characterized. To expand upon this work, experiments were aimed to validate *in vitro* studies with purified recombinant proteins, and also to investigate this phenomenon using cell culture models to show whether this modification in RI α can lead to unique PKA signaling in cells.

Given more recent structural information acquired in the last 10-15 years concerning the nature of binding between PKAc and RI α , we can make a more educated assertion about how modification of Ser101 could lead to changes in Type I PKA activity. The protein structure of the RI α :PKAc heterodimer was solved by X-ray crystallography techniques by Kim *et al.*, and

this structure showed how the linker region of RI α binds the active site cleft of PKAc³¹. As shown in **Figure 4.1**, the linker region containing the inhibitory sequence (IS, *red cartoon*) makes direct contacts with residues from both the N-lobe and C-lobe of PKAc, while the CNB-A domain (*teal cartoon*) binds distally to the C-lobe. Due to pseudo-substrate nature of the IS in RI α , the high affinity binding of this motif to PKAc (in complex with ATP and two magnesium ions, Mg₂ATP) presents a kinetic barrier for activation, whereas this effect would not be important for RII subunits capable of phosphorylation by PKAc. The binding affinity of RI α and PKAc in presence of Mg₂ATP is 0.1nM, versus 200nM in the absence of nucleotide^{ref}. ATP also binds with an affinity of 60nM in the RI α holoenzyme, whereas the K_m/K_d is 25 μ M for the free protein. High affinity binding is exclusively to the second metal ion^{ref}. This gives a rationale for why modification of Ser101 could possibly perturb the binding interaction of the linker region of RI α to PKAc.

To address this hypothesis, we can look more closely at the Ser101 site at this interfacial region to better understand how introduction of a phosphate moiety on the hydroxyl group of this serine could significantly alter RI α linker region binding. The close-up view depicted in **Figure 4.2** helps to illustrate the importance of this residue in maintaining proper binding interactions with PKAc. Upon analysis of polar interactions in this region, we can see that Ser101 makes hydrogen bond interactions with residues from the α C-helix of PKAc (particularly residues Gln84 and His87). We can infer that

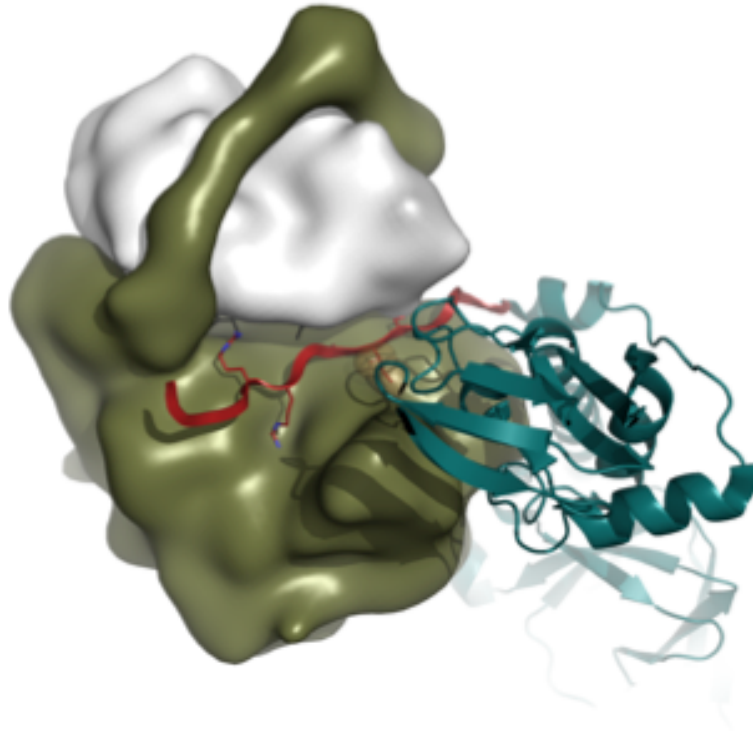


Figure 4.1: The RI α :PKAc heterodimer complex (PDB 2qcs).

PyMOL structural representation of the RI α :PKAc heterodimer complex (PDB 2qcs) (*light-gray surface*, PKAc N-lobe; *olive-green surface*, PKAc C-lobe; *teal cartoon*, RI α cyclic nucleotide binding (CNB) domains; *red cartoon with sticks*, RI α linker region containing the inhibitory sequence (IS) to PKAc). This structure illustrates the “bimodal” nature of RI α binding to the catalytic subunit of PKA; the active site of PKAc is directly bound by the IS of RI α (*red*), whereas distal sites in PKAc are bound by the CNB domains of RI α (*teal*).

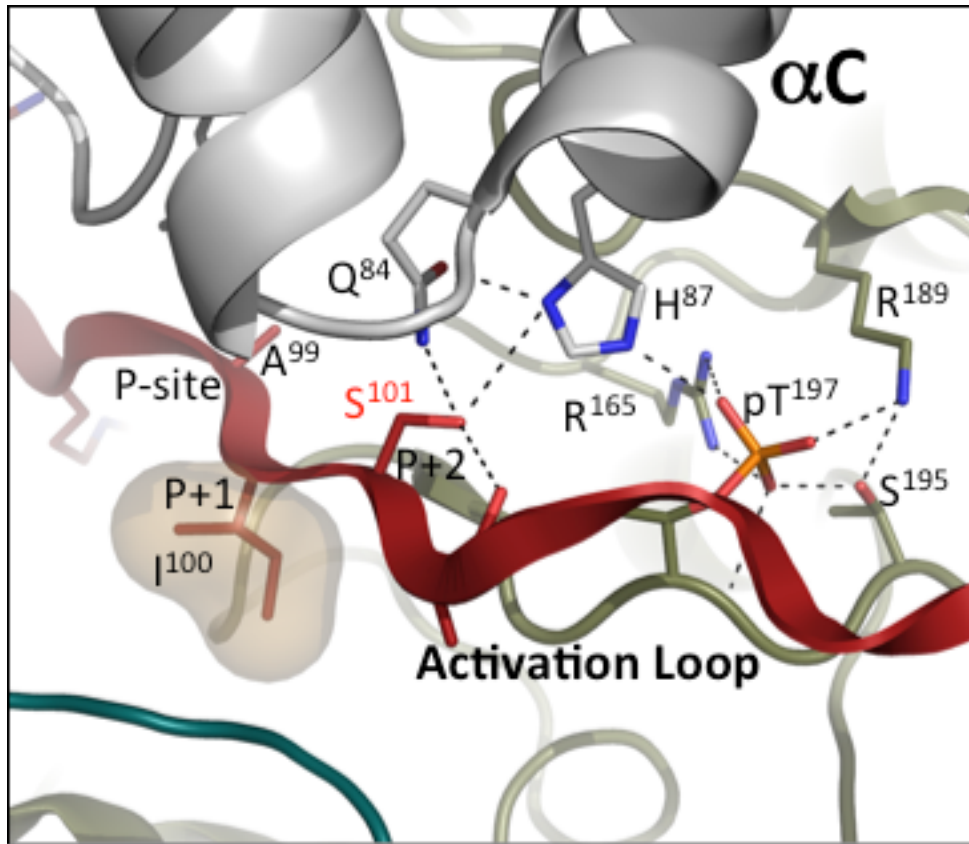


Figure 4.2: Structural insights to the role of serine 101 in R1 α .

PyMOL structural representation of the R1 α :PKAc heterodimer complex (PDB 2qcs), focusing on the binding interface between the active site of PKAc and the linker region IS of R1 α (light-gray cartoon, PKAc N-lobe; olive-green surface, PKAc C-lobe; teal cartoon, R1 α cyclic nucleotide binding (CNB) domains; red cartoon with sticks, R1 α linker region containing the inhibitory sequence (IS) to PKAc). The serine residue of interest in R1 α (S¹⁰¹, red) is depicted along with other critical residues at this binding interface, including: 1) the pseudo-substrate alanine (A⁹⁹) and P+1 residue (I¹⁰⁰) from the R1 α linker region, 2) two residues from the α C-helix of PKAc (Q⁸⁴ and H⁸⁷) that form direct hydrogen bonds with the hydroxyl group of serine 101, and 3) activation-loop phosphorylation site (pT¹⁹⁷) and its neighboring residues in PKAc (R¹⁶⁵, R¹⁸⁹, and S¹⁹⁵). Hydrogen bonds are depicted as dashed lines.

introduction of a phosphate group at this position will introduce steric hindrance that will likely alter the interaction of these residues. Furthermore, we see that the interaction of Ser101 with Gln84 and His87 facilitates a hydrogen bonding network near the activation-loop phosphorylation site Thr197 in PKAc. Thus we can further presume that phosphorylation of Ser101 will also bring about a negative charge-charge repulsion effect caused by the juxtaposition of two phosphate groups within this binding interface. This would lend to opening of the active site cleft.

Given these observations, we hypothesized that phosphorylation of this site by PKG would promote enhanced PKAc activity by removing the additional requirement of substrate competition in R1 α holoenzyme activation. We thus conducted this project to ascertain if R1 α is indeed a substrate for PKG in cells, and to see if this modification could potentially lead to novel modes of PKA signaling in cells expressing these two related proteins. In summary, we find that PKG does phosphorylate R1 α at Ser101, and furthermore we have used a phospho-mimetic mutation of this site (S101E) to show that this modification leads to decreased binding of PKAc and enhanced PKAc activity. However, our data showed that mutants of Ser101 can still form cAMP-sensitive holoenzyme complexes with PKAc in cells, suggesting PKG phosphorylation of this site could in effect “prime” Type I PKA complexes for cAMP-dependent dissociation.

4.2: Results

Our first goal was focused on establishing that RI α is indeed a substrate of PKG. To corroborate previous reports, we initially performed *in vitro* PKGI α phosphorylation reactions using purified recombinant bovine RI α (bRI α). The intent of these experiments was to show that PKGI α can use radio-labeled ATP to incorporate phosphate onto RI α at the Serine 101 site.

As shown in panel A of **Figure 4.3**, we began our *in vitro* investigations by comparing the extent of PKGI α phosphorylation of either “apo” (i.e. cAMP-free, unbound) bRI α regulatory subunit (RI α , lanes 1-2) or RI α in a holoenzyme complex with PKAc, two Mg²⁺ ions, and ATP (Holoenzyme, lanes 3-6). In this experiment, phosphorylation reactions were conducted with or without cGMP stimulation, and protein samples were then resolved by SDS-PAGE and analyzed by autoradiography. Our data shows that 3 μ M cGMP leads to robust phosphorylation of apo bRI α protein, as evidenced by the degree of autoradiography signal at the correct molecular weight corresponding to reduced RI α . We also observed that bRI α protein is phosphorylated in reactions containing PKAc; however, at high concentrations of cyclic nucleotide, cGMP will bind RI α and thus influence the affinity between of RI α to PKAc. Therefore the phosphorylation observed may be dissociated RI α .

After characterizing RI α phosphorylation by PKGI α *in vitro*, we wanted to determine if PKGI α could phosphorylate RI α within a cellular environment.

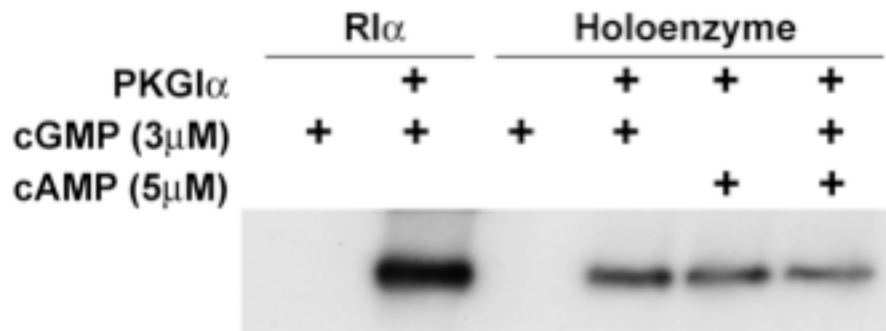


Figure 4.3: PKGI α phosphorylates R1 α *in vitro*

Autoradiography of *in vitro* PKGI α phosphorylation reactions conducted with purified recombinant bovine R1 α protein, either with free regulatory subunit (R1 α , lanes 1-2) or in complex with PKAc (Holoenzyme, lanes 3-6). For all reactions, 70ng of PKGI α was incubated with 5 μ g of R1 α or Holoenzyme. Stimulation with 3 μ M cGMP leads to phosphorylation of both free R1 α and holoenzyme under the conditions tested.

To answer this, we utilized HEK293T cells as a model cell culture system for over-expressing PKG α as well as flagged-tagged versions of either wild-type (WT) or S101A mutant variants of human R α . As displayed in **Figure 4.4**, we tested the differences between WT and S101A R α phosphorylation in the presence or absence of PKG α , with and without 4hr stimulus with 8-CPT-cGMP, a membrane permeable analog of cGMP. For samples over-expressing WT R α , we only observed a significant amount of phosphorylation signal in cells that were both overexpressing PKG α and treated with 8-CPT-cGMP. Under similar conditions using the S101A mutation of R α , we did not observe phosphorylation, suggesting that this particular residue is a specific phosphorylation target of PKG α in cells. The lower panel is an immunoblot using anti-Flag antibodies, showing that we had an even loading of transfected flag-tagged R α in all samples tested.

In these HEK293T cell-based phosphorylation experiments, we also noticed that a very faint phosphorylation signal can be observed for R α in cells not overexpressing PKG α , indicating the potential presence of other phosphorylation sites in R α that are targeted in cells. Given that we also saw *in vitro* phosphorylation of R α extraneous to the serine 101 site, we wished to investigate other putative phosphorylation sites within R α protein, namely serines 77 and 83. These two sites have been shown to be phosphorylated in human heart tissues in the context of ischemic heart disease ⁷⁵ (please see *Chapter 5* for more discussion), and thus may be contributing to the

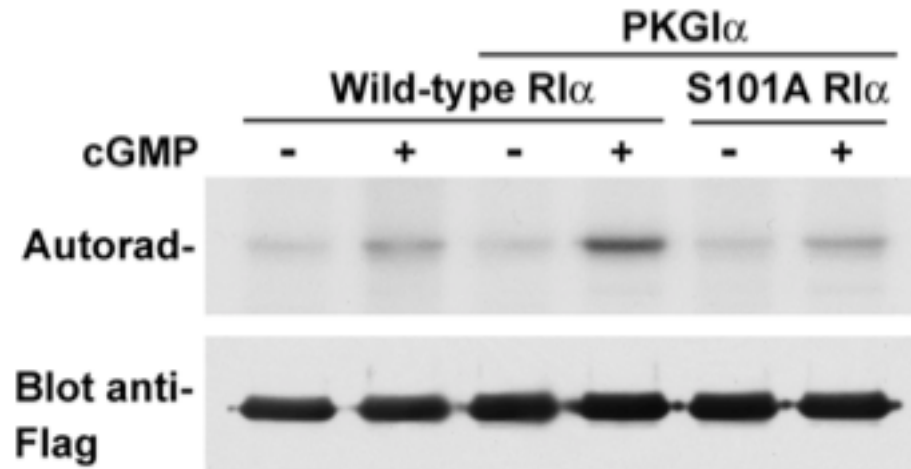


Figure 4.4: PKGI α overexpression in HEK293T cells yields phosphorylation of wild-type RI α , but not RI α (S101A) mutant.

Autoradiography and Immunoblot analysis of phosphorylation reactions performed in HEK293T cells over-expressing flagged-tagged versions of either wild-type or S101A mutant RI α protein, with and without the presence of PKGI α and/or 4hr cyclic-GMP (cGMP) stimulus. In cells over-expressing wild-type protein, robust phosphorylation of RI α was only observed upon combination of over-expression of PKGI α and cGMP stimulus. Similar conditions using the S101A mutation of RI α did not yield robust phosphorylation signal, suggesting this residue is a specific phosphorylation target of PKGI α in cells. Note: Low amounts of phosphorylation signal can be observed for RI α in cells not over-expressing PKGI α , indicating the potential presence of other phosphorylation sites in RI α that are targeted in cells. The lower panel is an immunoblot using anti-Flag antibodies as a loading control.

background signal in our phosphorylation assays. As shown in **Figure 4.5**, phosphorylation reactions were performed in HEK293T cells over-expressing flagged-tagged constructs of either wild-type (WT; lanes 1-2) or a mutant version of RI α protein with both serine 77 and serine 83 mutated to alanine (lanes 3-4). All reactions were conducted with over-expressed PKGI α either with or without 4hr cGMP stimulus. In cells over-expressing WT protein, we again observed low levels of RI α phosphorylation in cells without cGMP stimulus as seen in previous experiments. However, in cells expression the S77/83A mutation of RI α did not yield phosphorylation signal without cGMP stimulus, indicating that all background signal observed in non-stimulated samples is due to extraneous in-cell phosphorylation of these two particular sites in RI α . As before, immunoblots using anti-Flag antibodies were used as a loading control for RI α protein transfection.

These initial phosphorylation experiments performed under both *in vitro* and in-cell conditions have shown that serine 101 in RI α is indeed a *bona fide* phosphorylation target of PKGI α . However, to extend our understanding of the role of serine 101 phosphorylation, our next pursuit in this project was to determine the biochemical effects that this modification in RI α presents, particularly in terms of its binding of PKAc and the relative state of PKAc catalytic activity. As mentioned in the introductory section of this chapter, previous evidence in the field combined with more recent structural insights suggest that phosphorylation of serine 101 would reduce the affinity between

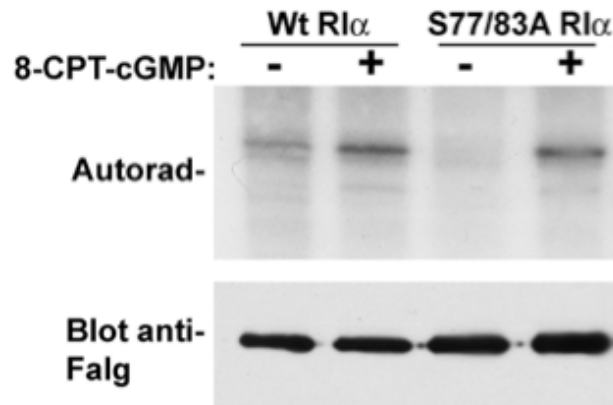


Figure 4.5: PKGI α independent phosphorylation of R1 α at serine 77 and serine 83 causes background phosphorylation signal in HEK293T cells.

Autoradiography and immunoblot analysis of phosphorylation reactions performed in HEK293T cells over-expressing flagged-tagged constructs of either wild-type (Wt R1 α ; *lanes 1-2*), or mutant version of R1 α protein with both Serine 77 and serine 83 mutated to alanine (S77/83A R1 α ; *lanes 3-4*). All reactions were performed with over-expressed PKGI α with or without 4hr 8-CPT-cGMP stimulus. In cells over-expressing wild-type protein, low levels of phosphorylation of Flag-R1 α was observed in cells without cGMP stimulus, suggesting the possibility of other putative phosphorylation sites in R1 α . Similar conditions using the S77/83A mutation of Flag-R1 α did not yield phosphorylation signal without cGMP stimulus, indicating that all background signal observed in non-stimulated samples is due to extraneous in-cell phosphorylation of these other putative sites in R1 α . The lower panel is an immunoblot using anti-Flag antibodies as a loading control.

the RI α inhibitory sequence (IS) and the PKAc active site due to the introduction of a larger, charged moiety at this interfacial region. Therefore we hypothesized that creating a phospho-mimetic mutation at this site in RI α by changing the serine to a glutamate residue (S101E) would similarly weaken IS binding to the active site of PKAc, and thus allow the competition of substrates to bind and be phosphorylated. After generating S101E mutant constructs for both recombinant protein purification (bovine RI α (1-379) and (91-379), in pRSET vector) and for mammalian cell transfection (human RI α (1-379), in pcDNA3.1 vector), we next used components of the PepTag[®] phosphorylation assay to assess differences in PKAc activation for wild-type (WT) versus S101E mutant under both *in vitro* and in-cell conditions.

For our *in vitro* activity studies of S101E, we utilized both full-length (amino acids 1-379) and truncated variants (amino acids 91-379) of bRI α . The 91-379 construct is missing the D/D domain and the majority of the N-terminal linker region, beginning three residues before the auto-inhibitory sequence (IS), which eliminates the capability to form tetrameric holoenzyme complexes. Instead it forms an R:C heterodimer. In **Figure 4.6**, we show PepTag activity assay data for RI α (1-379) in panel A and data for RI α (91-379) in panel B. In these experiments, purified R-subunit is added to PKAc in a 1.2:1 stoichiometric ratio, and then incubated for 30min in the presence of fluorescently-conjugated “Kemptide” peptide substrate, either with or without cAMP. Phosphorylation introduces a negative charge, which allows for

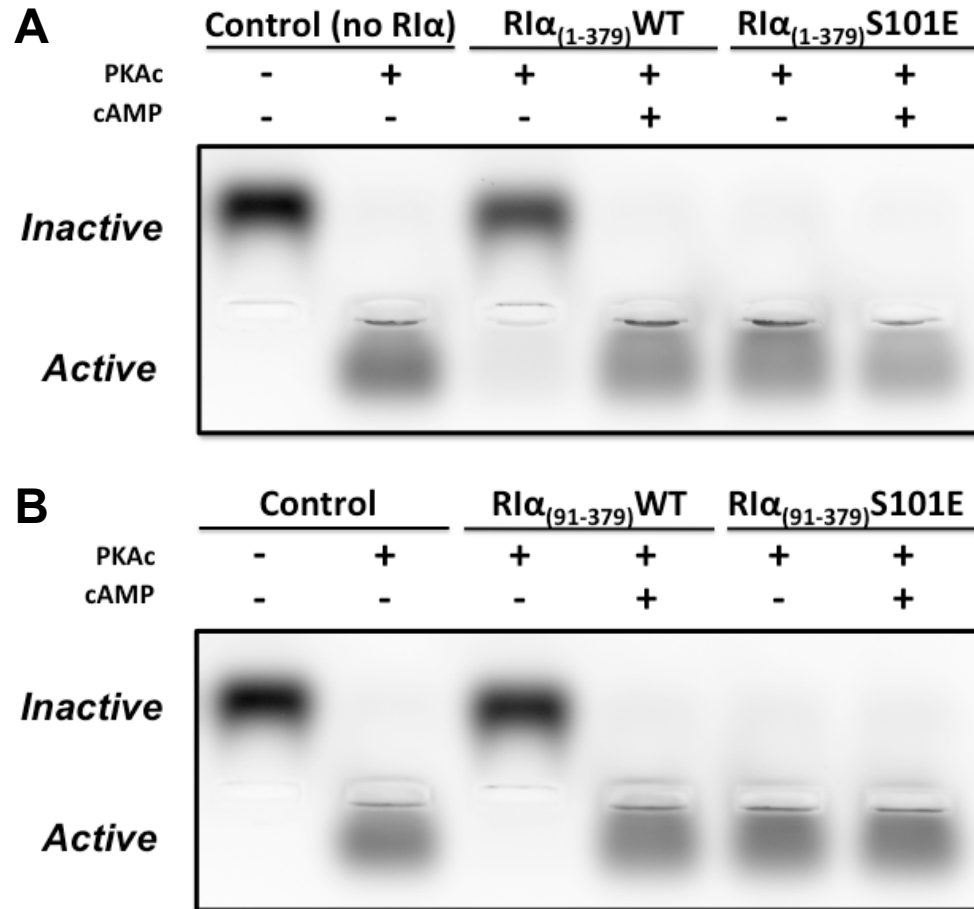


Figure 4.6: Phospho-mimetic mutation of bovine R1 α at serine 101 (S101E) induces PKAc activity without cAMP stimulus *in vitro*.

Qualitative analysis of *in vitro* PKAc kinase activity using the PepTag® activity assay kit, comparing the inhibitory capacity of either wild-type (WT) or phospho-mimetic mutant (S101E) variants of purified bovine R1 α protein. Electrophoresis of assay samples allows for separation of phosphorylated (active) versus non-phosphorylated (inactive) substrate peptides. In panel **A**), a full-length construct of R1 α (1-379) protein was used, while in panel **B**), an N-terminal truncation construct of R1 α beginning at the inhibitory sequence (91-379) was used. While wild-type R1 α protein maintains PKAc in an inhibited state without cAMP stimulus, we observed that S101E mutant R1 α displays high PKAc activity under basal conditions. Full-length and truncated versions of R1 α protein behaved identically, suggesting that the S101E mutant behaves similarly whether in a heterodimer (R:C) state or in tetrameric (R₂:C₂) complex.

separation via gel electrophoresis; migration of phosphorylated (active) and non-phosphorylated (inactive) peptides thus serves as a qualitative measure of PKAc activity. As expected, wild-type R1 α protein maintains PKAc in an inhibited state without cAMP stimulus. However, we discovered that S101E mutant R1 α displays high PKAc activity under basal (cAMP-free) conditions. Full-length and truncated versions of R1 α protein behaved identically, indicating that the S101E mutant behaves similarly whether in a heterodimer (R:C) state or in a tetrameric (R₂:C₂) complex. We then moved to increase the S101E protein from 2x, 5x, and 10x the relative dosage of WT protein (120nM -1.2 μ M S101E dimer protein, incubated with 200nM PKAc catalytic subunit). As seen in **Figure 4.7**, when using only the full-length (1-379) constructs of WT and S101E, the mutant protein still displayed high PKAc activity with every concentration of protein tested.

For our in cell analysis of PKAc activity, we decided to test our WT and S101E variants of human R1 α by over-expressing them in MEF R1 α ^{-/-} cells, mouse embryonic fibroblasts with a genetic knock-out of *PRKAR1 α* . Our rationale for the use of these cells is that the R1 α ^{-/-} cell line allows for a null background to compare the intervention of exogenous R1 α over-expression. cAMP production was stimulated in these cells by the addition of forskolin and IBMX (Fsk/IBMX; 10 μ M and 100 μ M respectively) 10min prior to cell lysis and the subsequent activity assay. Similarly as above, components of the PepTag assay were used, but in these experiments we used cell lysate as our

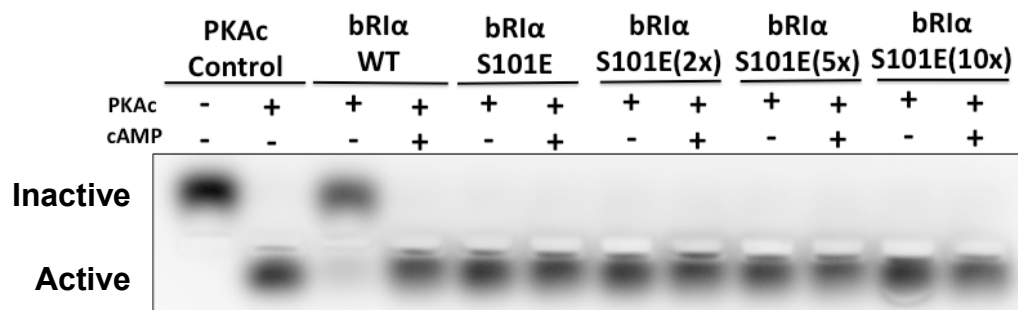


Figure 4.7: S101E mutant induces PKAc activity without cAMP stimulus *in vitro*.

Qualitative analysis of *in vitro* PKAc kinase activity using the PepTag® activity assay kit, comparing the inhibitory capacity of either wild-type (WT) or phospho-mimetic mutant (S101E) variants of purified bovine RI α protein. Electrophoresis of assay samples allows for separation of phosphorylated (active) versus non-phosphorylated (inactive) substrate peptides. In this experiment, full-length RI α S101E dimer protein was added in doses of progressively higher molar stoichiometry as compared to PKAc protein (lane 1, no protein control; lanes 2-12 contain 200nM PKAc, lanes 3-4, 120nM WT dimer; lanes 5-6, 120nM S101E dimer; lanes 7-8, 240nM S101E dimer; lanes 9-10, 480nM S101E dimer; lanes 11-12, 1.2 μ M S101E dimer). RI α S101E was unable to keep PKAc inhibited under every concentration of R-subunit protein investigated.

kinase sample instead of purified protein. In **Figure 4.8**, we compare the relative degree of PKAc phosphorylation activity between non-transfected cells (Ctrl R1 α ^{-/-}), and cells overexpressing either WT or S101E human R1 α . Control cells displayed high substrate phosphorylation under non-stimulated conditions, thus serving as a positive control for PKAc activity, and also confirming earlier observations that in the absence of R1 α , PKA activity is not well regulated even though other R-subunit isoforms are present. In close corroboration with *in vitro* data, over-expression of WT R1 α in cells was able to inhibit the majority of PKAc activity in the absence of Fsk/IBMX. In contrast, cells expressing the S101E mutant showed high activity without cAMP stimulus. We also conducted immunoblot analysis of R1 α and PKAc expression in MEF R1 α ^{-/-} lysates for the corresponding activity assay samples examined in panel A. Two major trends were observed: 1) in both cases the levels of R1 α were slightly decreased upon Fsk/IBMX stimulation, and 2) R1 α expression appeared to be slightly higher in S101E cells as compared to cells expressing WT R1 α .

The competitive nature of the phosphorylation reactions employed suggests that the IS of R1 α (S101E) must have a lowered affinity for the PKAc active site cleft, otherwise the PKA substrate would not be able to bind effectively to allow for phospho-transfer activity of PKAc. Two explanations are possible for our observed results. On the one hand it may be that the R1 α (S101E) mutant is simply unable to form a complex with PKAc, or

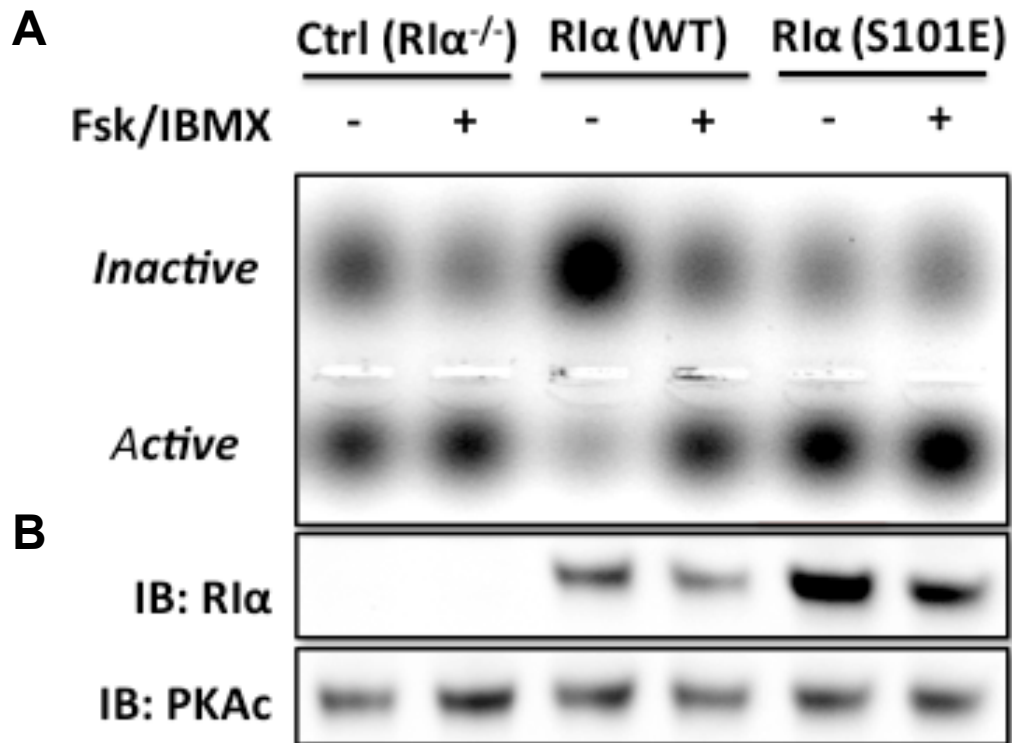


Figure 4.8: Over-expression of S101E mutant in MEF $R\alpha^{-/-}$ cells exhibits high basal PKAc activity without cAMP stimulus.

A) Qualitative analysis of PKAc kinase activity using the PepTag® activity assay kit, comparing wild-type (WT) or phospho-mimetic mutant (S101E) variants of human $R\alpha$ over-expressed in MEF $R\alpha^{-/-}$ cells (mouse embryonic fibroblasts with genetic knock-out of *PRKAR1α*). To stimulate cAMP production in these cells, forskolin and IBMX (Fsk/IBMX; 10 μ M and 100 μ M) were added for 10min prior to cell lysis and subsequent activity assay. Electrophoresis of assay samples allows for separation of phosphorylated (active) versus non-phosphorylated (inactive) substrate peptides. Control cells not expressing human $R\alpha$ (Ctrl $R\alpha^{-/-}$) displayed high substrate phosphorylation under not stimulated conditions, thus serving as a positive control for PKAc activity. Over-expression of WT $R\alpha$ inhibits PKAc in the absence of Fsk/IBMX, whereas cells expressing the S101E mutant showed high activity without cAMP stimulus. **B)** Immunoblot analysis of $R\alpha$ and PKAc protein expression in MEF $R\alpha^{-/-}$ lysates, corresponding to activity assay samples examined in panel **A**. Note: Two major trends were observed: 1) $R\alpha$ protein was slightly decreased upon Fsk/IBMX stimulation, and 2) $R\alpha$ protein expression was slightly higher in S101E cells as compared to WT.

alternatively the mutant protein may still form a holoenzyme complex via its two CNBs domains, even though the modified sequence in the inhibitor sequence is loosely anchored to the PKAc active site cleft. We therefore explored several other techniques to evaluate the relative capacity of S101E and S101A mutants of R1 α to form holoenzyme.

To demonstrate that the mutant R1 α subunits formed holoenzyme complexes in cells, we overexpressed flag-tagged versions of the human R1 α in HEK29T cells, and looked to see whether the S101A and S101E mutant variants of R1 α were able to “pull-down” overexpressed HA-tagged PKAc protein from cell lysates via co-immunoprecipitation. By immunoblotting for HA-tagged protein in our IP samples, we can qualitatively determine the state of holoenzyme complex formation in cells. As shown in **Figure 4.9**, we observed HA-tagged PKAc in the immunoprecipitate of all R1 α proteins tested, signifying that these mutations do not influence the binding of PKAc to R1 α . We further showed that co-immunoprecipitation of PKAc was also possible in the presence of excess substrate (Kemptide), thus indicating that excess substrate does not promote holoenzyme dissociation.

To further confirm that mutant R1 α forms stable holoenzyme complex in cells, we also made use of a bioluminescent protein complementation assay ((PCA) developed by our collaborator Stefan *et al.*; see *Experimental procedures*)¹⁰⁸, where differential tagging of regulatory and catalytic subunit proteins with fragments of Renilla luciferase (RLuc) creates a “split-reporter”

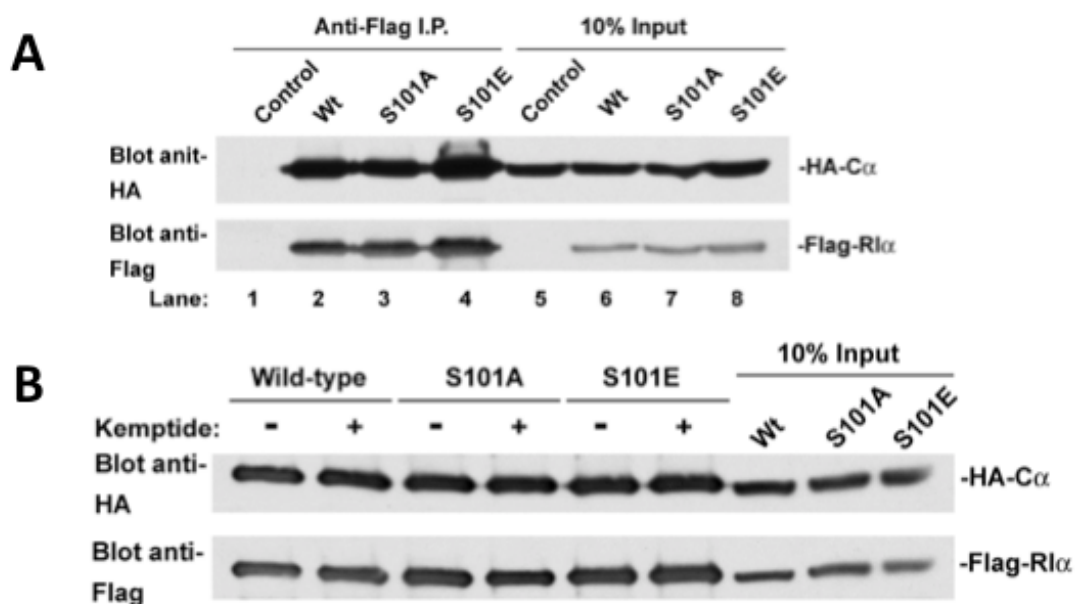


Figure 4.9: S101 mutant variants of Rl α pull-down PKAc from HEK293T cell lysates via co-immunoprecipitation (co-IP).

Immunoblot analysis of co-immunoprecipitation (co-IP) reactions from HEK293T cell lysates, comparing cells over-expressing Flag-tagged versions of either wild-type (WT) or serine 101 mutant variants (S101A and S101E) of Rl α . The presence of HA-tagged PKAc protein (C α) was examined in co-IP samples to qualitatively determine the state of Rl α :PKAc complex formation in cells. In panel **A**, co-IP experiments were performed on untreated lysates, whereas in panel **B**, co-IP experiments were performed in the presence of Kemptide substrate peptide. Both S101A and S101E mutants maintained binding to PKAc under all conditions tested, indicating that these mutations do not influence complex formation in cells.

system which can be used to monitor R:C complex formation as a function of luciferase-fragment complementation. Upon cellular overexpression of RLuc-tagged versions of PKAc and either wild-type, S101A, or S101E variants of R1 α , we can measure the relative degree of R1 α :PKAc protein-protein interaction in cells mediated by holoenzyme association. Moreover, PKA holoenzyme dissociation can be measured by luminescence signal decrease upon cellular stimulation with isoproterenol (a beta-adrenergic receptor (β AR) agonist known to stimulate cAMP production in cells). Data displayed in **Figure 4.10** established that Ser101 mutants of R1 α still displayed a similar degree of complex formation (as compared to wild-type) in non-stimulated cells. Furthermore, bioluminescence signal was diminished upon addition of isoproterenol in all protein constructs tested, thus showing that the Ser101 mutant variants of R1 α :PKAc complexes are still sensitive cAMP and thus allow for holoenzyme dissociation under stimulatory conditions.

For a more quantitative understanding of PKAc binding affinities for wild-type and mutant R1 α subunits *in vitro*, we utilized the ligand regulated competition (LiReC) fluorescence polarization assay (see *Experimental procedures*)¹⁰⁹. This competitive inhibitor binding assay is used to detect the relative fluorescence polarization (FP) of FAM-IP20, a fluorescein-conjugated PKA inhibitor peptide that is derived from the heat stable protein kinase inhibitor, PKI¹¹⁰. PKI, like R1 α , it is a high affinity pseudo-substrate inhibitor of PKAc, and in the presence of ATP and two magnesium ions both bind with

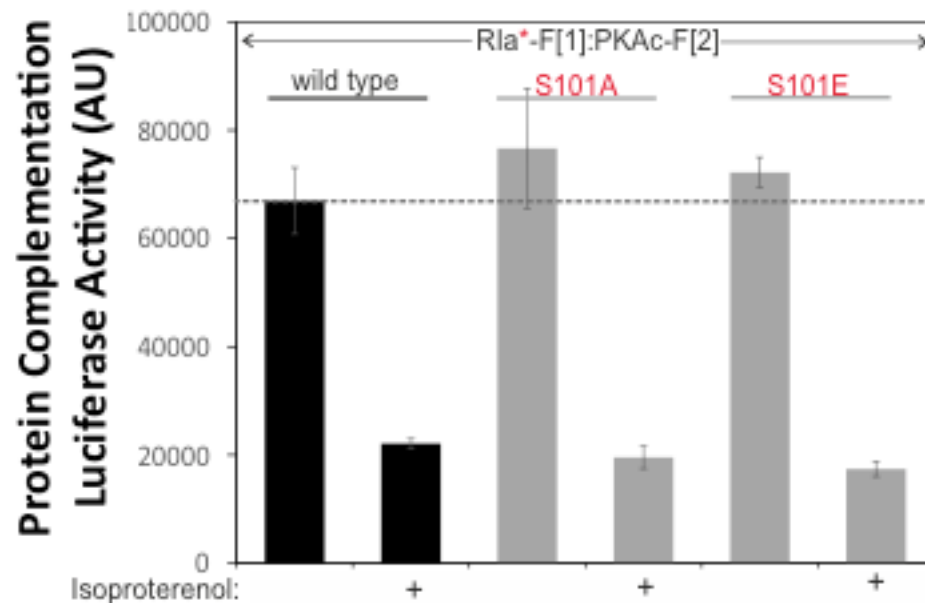


Figure 4.10: Protein complementation assay (PCA) shows Ser101 mutants form cAMP-sensitive complexes in cells.

Protein complementation assay (PCA; see *Experimental procedures*), comparing R1a:PKAc protein association in isoproterenol-stimulated osteoblast cells. In this assay, increased bioluminescence signal indicates complementation of the luciferase “split reporter” due to protein-protein interaction mediated by holoenzyme association. Moreover, PKA holoenzyme dissociation is measured by luminescence signal decrease upon cellular stimulation with isoproterenol (a beta-adrenergic receptor (β AR) agonist known to stimulate cAMP production in cells). Mutants of serine 101 (S101A and S101E) showed no significant differences compared to wild-type (WT) protein in either context; S101A and S101E mutants displayed high bioluminescence signal under basal conditions as well as lowered signal upon isoproterenol stimulation, suggesting that these proteins can form functional, cAMP-sensitive complexes in cells. Carried out by E. Stefan at the University of Innsbruck, Austria.

sub-nanomolar affinity¹¹¹. In the case of WT R1 α holoenzyme, FAM-IP20 cannot bind in the absence of cAMP¹¹². In the LiReC assay, the relative increase of the fluorescence polarization signal measured as the IP20 probe binds PKAc is indicative of the decrease in anisotropic tumbling of the immobilized inhibitor. For the initial experiments we used a constant R1 α :PKAc stoichiometry (14.4nM R1 α to 12nM PKAc) and performed a cAMP dose-response curve to determine whether FAM-IP20 binding to the R1 α (S101E) holoenzyme was cAMP-dependent. We first compared WT and S101E proteins using the bR1 α (91-379) construct as seen in **Figure 4.11**. We found that wild-type R1 α only allows for IP20 binding to PKAc following cAMP stimulation as previously described. However, samples with R1 α (S101E) protein revealed high levels of IP20 probe binding to PKAc even in the absence of cAMP. This most likely reflects an equilibrium between holoenzyme and dissociated R and C subunits. Nevertheless, complete binding was achieved in a dose dependent manner in the presence of cAMP. The S101E mutant also showed slightly increased sensitivity to cAMP, where EC50 values were decreased from 49.1nM in WT to 23.5nM in S101E. Hill Slope values were slightly different between samples. We also repeated these experiments using full-length bR1 α constructs of WT, S101E and S101A mutant proteins as exhibited in **Figure 4.12**. While the S101A mutant behaved more or less like WT protein, the S101E mutant displayed even higher levels of inhibitor binding in the absence of cAMP as compared to the

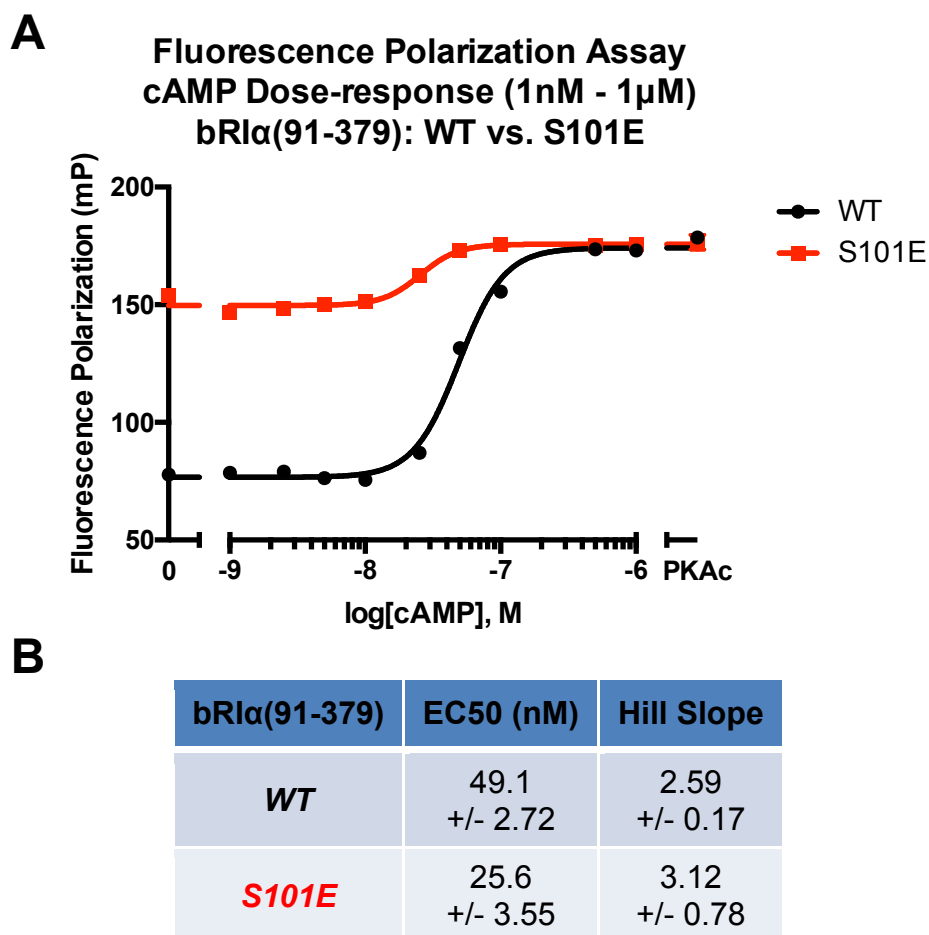


Figure 4.11: cAMP dose-response reveals high basal inhibitor binding for bR1α(91-379) S101E.

Ligand regulated competition (LiReC) fluorescence polarization assay (see *Experimental procedures*), comparing cAMP dose-response between wild-type (WT, *black*) and S101E mutant (*red*) forms of truncated bovine R1α (91-379).

A) Graph of fluorescence polarization data reported in millipolarization units (mP). **B)** Table of EC50 values and Hill Slope. (Each condition was performed in quadruplicate, [N=4]; error bars and table data are representative of +/- standard error of mean (SEM)).

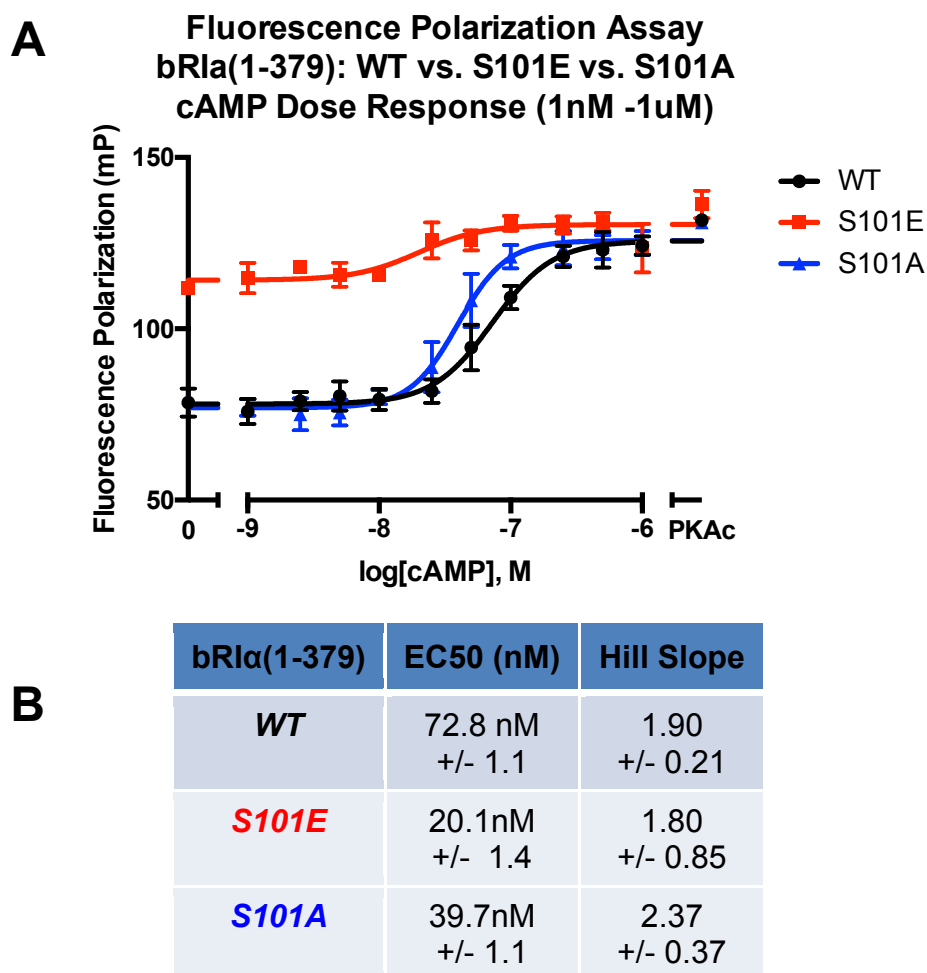


Figure 4.12: cAMP dose response of full length bR1 α (1-379) proteins shows high inhibitor binding for S101E, but not S101A mutant.

Ligand regulated competition (LiReC) fluorescence polarization assay (see *Experimental procedures*), comparing cAMP dose-response between wild-type (WT, *black*), S101E mutant (*red*), and S101A mutants (*blue*) forms of full-length bovine R1 α (1-379). **A**) Graph of fluorescence polarization data reported in millipolarization units (mP). **B**) Table of EC50 values and Hill Slope. (Each condition was performed in quadruplicate, [N=4]; error bars and table data are representative of +/- standard error of mean (SEM)).

91-379 truncation variants. In this instance, the S101E and S101A mutants reported lower EC₅₀ values as compared to WT, yet hill slope values for both mutants were not significantly changed. These data reveal more information concerning the nature of R1 α S101E binding to PKAc.

To more conclusively determine whether R1 α (S101E) with its modified linker region can functionally bind PKAc, we next modified our assay to assess a rough dose-response curve for the R1 α subunit in the presence of a constant concentration of PKAc (10nM) and in the absence of cAMP. Again, we chose to compare full-length and truncated versions of both WT and S101E proteins (as well as S101A in full-length form) in order to discern any differences in formation of holoenzyme complexes. Data shown in **Figures 4.13 and 4.14** compare the R-subunit dose response regimes for truncated R1 α (91-379) and full-length R1 α (1-379) proteins, respectively. We found that R1 α (91-379) S101E mutant protein was unable to out-compete FAM-IP20 probe within the dosing range tested (1nM -1 μ M R1 α protein) whereas the truncated WT protein achieved functional inhibition at low nanomolar concentrations. For the full-length R1 α , significantly higher levels of S101E mutant were required to out-compete FAM-IP20 for binding to PKAc (IC_{50}^{S101E} = 212.5nM +/- 1.55). In contrast, S101A behaved very similar to WT R1 α (IC_{50}^{WT} = 4.5nM +/- 1.05; IC_{50}^{S101A} = 5.05nM +/- 1.08), indicating that both proteins have an affinity for PKAc of less than 10 nM, which is the concentration of PKAc in the assay. These preliminary data suggest that presence of the glutamic acid residue

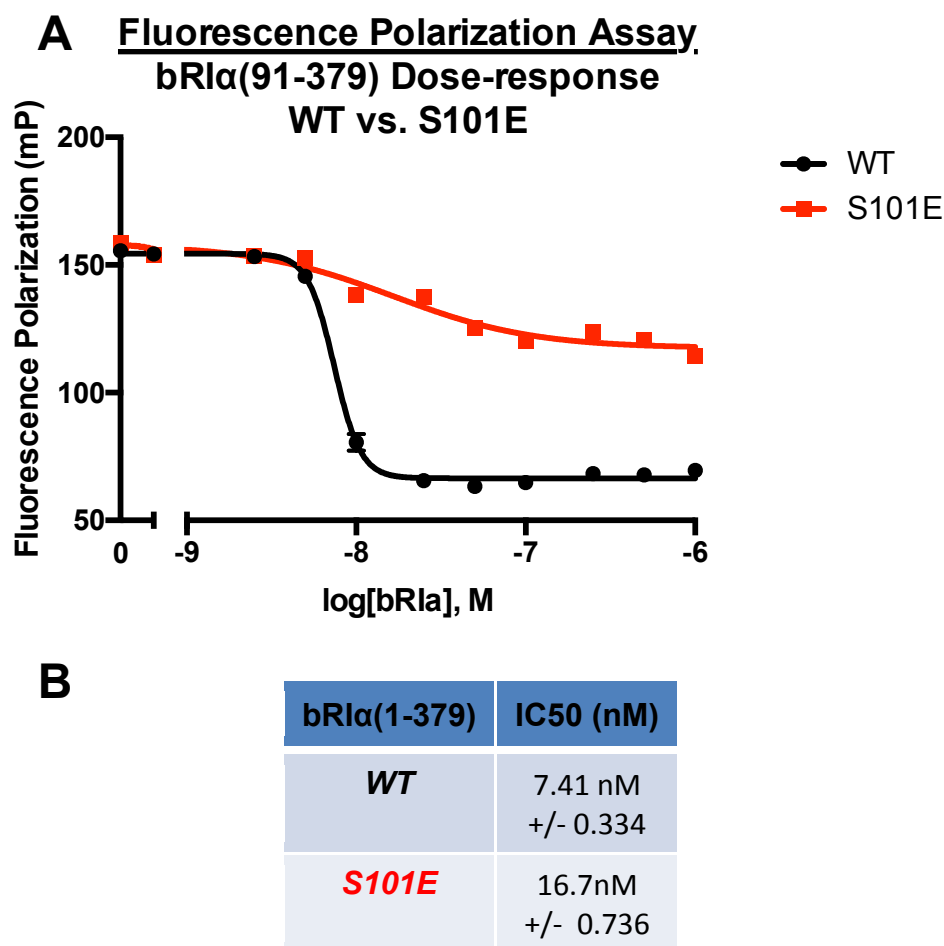


Figure 4.13: R1 α (91-379) dose response shows S101E binds PKAc poorly

Ligand regulated competition (LiReC) fluorescence polarization assay (see *Experimental procedures*), comparing dose-response of regulatory subunit protein concentration between wild-type (WT, *black*) and S101E mutant (*red*) forms of truncated bovine R1 α (91-379). **A**) Graph of fluorescence polarization data reported in millipolarization units (mP). **B**) Table of IC₅₀ values and Hill Slope. (Each condition was performed in quadruplicate, [N=4]; error bars and table data are representative of +/- standard error of mean (SEM)).

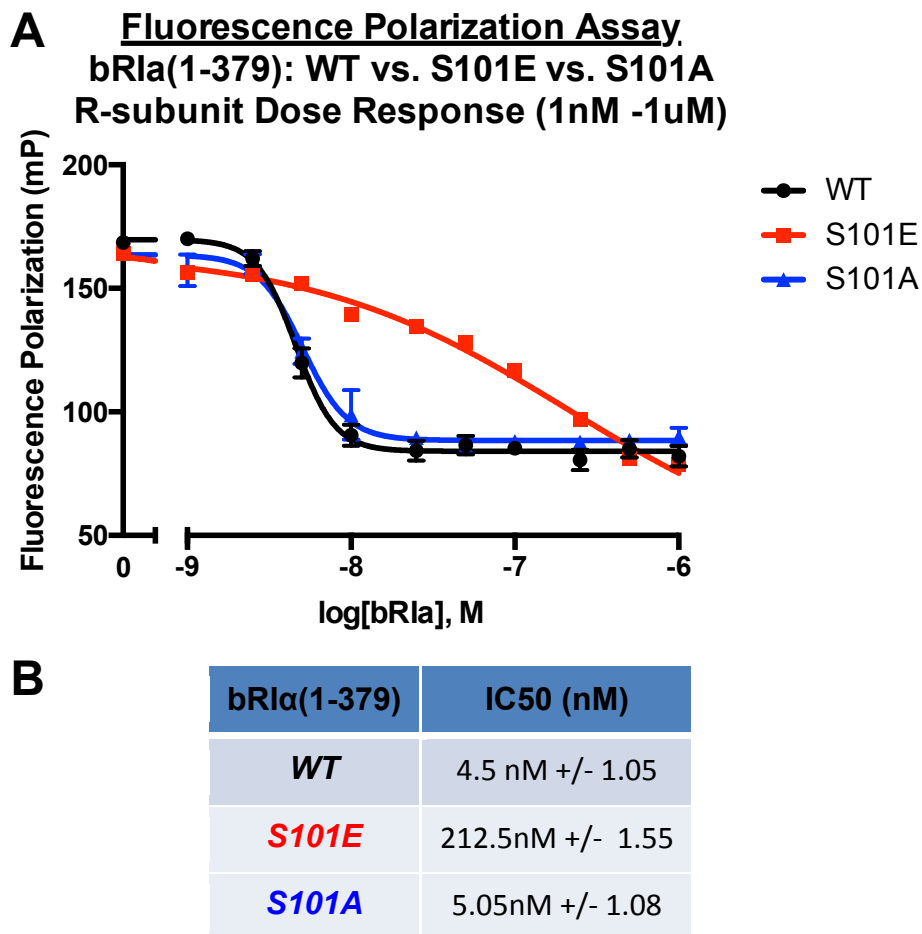


Figure 4.14: R-subunit dose response shows S101E can bind PKAc.

Ligand regulated competition (LiReC) fluorescence polarization assay (see *Experimental procedures*), comparing dose-response of regulatory subunit protein concentration between wild-type (WT, *black*), S101E mutant (*red*), S101A mutant (*blue*) forms of truncated bovine R1 α (1-379). **A**) Graph of fluorescence polarization data reported in millipolarization units (mP). **B**) Table of IC50 values and Hill Slope. (Each condition was performed in quadruplicate, [N=4]; error bars and table data are representative of +/- standard error of mean (SEM)).

specifically and significantly affects the affinity of the R1 α subunit to the PKAc active site, whereas the more conservative alanine substitution can function similar to unmodified wild-type protein at 10nM concentrations. Thus these experiments were able to illustrate that R1 α (S101E) can functionally bind PKAc to out-compete FAM-IP20, but the relative inhibitory equilibrium constant was approximately two orders of magnitude higher compared to the wild-type R1 α control. We are now collaborating with the Herberg lab to confirm the affinities of the S101E and S101A mutants for PKAc both in the presence and absence of ATP using Surface Plasmon Resonance (SPR).

While this work provides a more extensive biochemical characterization of PKG phosphorylation of Ser101 in R1 α , a final goal is to assess the significance of this mechanism for cell signaling crosstalk within *in vivo* systems. As both PKG and PKA signaling play critical roles within cardiac, skeletal muscle, and smooth muscular tissue physiology, our ongoing research is aimed at detecting the endogenous phosphorylation of R1 α in adult cardiomyocytes, either treated with or without 8-CPT-cGMP. Using AMVM cells described in *chapter 2*, we have since developed methods for isolating endogenous mouse R1 α protein from AMVM lysates, which will allow us to measure the protein phosphorylation state by mass spectrometry. As evidenced by **Figure 4.15**, we have shown that we can use cAMP-affinity resin to selectively enrich R1 α from AMVM lysates, which will allow us to detect modified R1 α within cGMP-treated cells using mass spectrometry. Upcoming

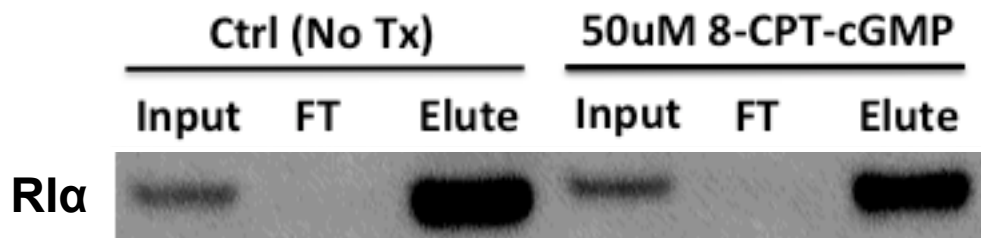


Figure 4.15: cAMP-affinity pull-down of R1 α from AMVM lysates.

Immunoblot of R1 α protein enriched from AMVM lysates after performing cAMP-affinity pull-down (*Input*, cell lysate input; *FT*, flow thru; *Elute*, cAMP-resin elution). Cells treated with 50 μ M 8-CPT-cGMP (a membrane permeable analog of cGMP) were compared to non-treated control cells. R1 α protein was significantly enriched from AMVM cells by this method. We will therefore employ this process in order to detect endogenous phosphorylation of R1 α upon stimulation of PKG activity in AMVM cells.

data from these experiments will conclusively determine whether or not this phenomenon occurs within heart tissues, therefore bolstering the *in vivo* significance of the crosstalk between PKG and PKA pathways.

4.3: Discussion

To summarize the work presented in *chapter 4*, introduction of a phosphorylation modification (or phospho-mimetic mutation) at the serine 101 site in R1 α leads to a state of heightened PKAc activity while still maintaining a R1 α :PKAc complex, as observed both *in vitro* and in cells. This assertion could very well explain discrepancies observed in the field, where it has been shown that elevation of cAMP under physiological conditions may not be sufficient to explain how PKA signaling is activated upon stimulatory signaling in the cell.

To summarize the data presented in this chapter, we have revisited and additionally built upon a former concept within the field of protein kinase signaling, which highlights a potential crosstalk mechanism between the key contributors of cyclic nucleotide signaling in the cells (PKA and PKG). In the development of this project, we first demonstrated that PKG phosphorylation of R1 α does indeed occur on serine 101 both *in vitro* and in cells (**Figures 4.3 - 4.5**). The advent of mutagenesis and the creation of alanine mutations at the serine 101 site in R1 α (S101A) allowed us to conclusively show that removing the capability of phosphorylation at this position lead to specific reduction of

phosphate incorporation in cells. Interestingly, we observed the presence of alternative phosphorylation sites in RI α . For our in-cell experiments, use of S77/83A mutant variants of RI α was able to reduce the background phosphorylation signal, indicating that these other serine residues in the linker region of RI α are indeed being targeted for phosphorylation in cells. Further studies of these two phosphorylation sites is outlined in *Chapter 5*.

The remainder of the experiments in this work was centered on characterizing the glutamic acid phospho-mimetic mutants of RI α (S101E), where we wished to ascertain the effect this mutation has on the binding of RI α to PKAc. As predicted, the introduction of this modification within the linker region of RI α lead to heightened PKAc activity under cAMP-free conditions, as observed both upon *in vitro* activity analysis and within cell overexpression models (**Figures 4.6 - 4.8**). The direct correspondence between these two datasets implies that the S101E mutation has a significant effect on the capacity of RI α to inhibit the activity of PKAc, even if the holoenzyme does not completely dissociate. Therefore, the consequences of PKG phosphorylation of serine 101 *in vivo* would likely lead to a state of activated PKA signaling in the cell, as similarly observed with the phospho-mimetic modification at this site.

As mentioned previously, this data alone was insufficient to determine whether the S101E mutation interfered with holoenzyme formation and promoted dissociation of the complex, or whether this modification simply

lowers the affinity of the linker region to PKAc while still maintaining allosteric binding via the CNB domains. Thus further studies were conducted to determine that S101E mutation is indeed able to functionally bind PKAc, both *in vitro* and in cells (**Figures 4.9 – 4.14**). Using co-immunoprecipitation as our first means of assessing R1 α protein-protein interactions with PKAc in cells, we discerned that flag-tagged versions of our mutant variants of R1 α S101A and S101E could enrich PKAc from lysates of over-expressing HEK293T cells (**Figure 4.9**). While this data does not allow us to ascertain the specific interactions taking place between R1 α and PKAc (with regard to which portions of the protein remain bound or what their relative affinity for binding may be), we do get a qualitative impression that the binding of mutant R1 α to PKAc is strong enough to withstand the stringent washing procedures of the co-immunoprecipitation protocol.

This project also applied a bioluminescence reporter technology specifically designed for assessing the extent of R1 α :PKAc complex formation in cells (developed by our collaborators Dr. Edi Stefan *et al.*, from the University of Innsbruck, Austria). Comparison of WT versus S101A and S101E mutants illustrated that these variants of R1 α were indeed capable of forming holoenzyme complexes in cells, which are furthermore functionally dissociated upon isoproterenol stimulus (**Figure 4.10**). We only tested full-length proteins in this context, but our speculation would be that we would observe similar results with the 91-379 truncation variants of these proteins,

given the assumption that this version of the protein would likely be able to bind via the CNB domains, while having poor active site binding (as observed in our FP assays). We also did not quantify PKAc protein activity within the context of these PCA experiments; if we had, we would also speculate that we would observe a higher amount of PKAc activity in the S101E expressing cells, but we still would be able to detect high amounts of complex formation.

We next made use of the ligand regulated competition (LiReC) fluorescence polarization assay (developed within the Taylor lab) to get a more a comprehensive understanding of the nature of R1 α mutant proteins binding to PKAc. cAMP dose-response experiments (**Figure 4.11 & 4.12**) first revealed that S101E proteins, whether in full-length or truncated form, had a significant amount of basal IP20 probe binding in the absence of cAMP. This finding indicated that S101E must have a lower affinity for PKAc as compared to WT protein or that FAM-IP20 can promote dissociation of the complex. Most likely this is due to a difference in IS accessibility at the active site of PKAc that allows FAM-IP20 to bind and promote dissociation of the holoenzyme even in the absence of cAMP and/or to an altered K_d for interaction between the mutant R1 α subunits and PKAc. R-subunit dose response experiments (**Figure 4.13 & 4.14**) showed that full-length S101E could out-compete IP20 probe at approximately 100x fold increases in protein concentration, whereas the R1 α (S101E) 91-379 truncation variant was never able to fully inhibit PKAc at any concentration tested. As mentioned above,

SPR methods will be used in future experiments to quantitatively determine the binding affinity of Ser101 mutants for PKAc. Utilization of alternative biophysical methodologies, such as hydrogen/deuterium exchange (HDX) mass spectrometry, could also help to better explore the relative degree of dynamics of between WT and S101E protein by quantifying the degree of solvent exposure upon differing timescales of protein-protein interactions.

This data significantly shifts the paradigm in regard to our understanding of Type I PKA signaling, in that modification of the R1 α linker region can serve to circumvent previously described kinetic restrictions of holoenzyme dissociation and thus trigger what we denote as “desensitized” PKAc activity. We have thus created a revised model of R1 α holoenzyme activation (see **Figure 4.16**) in order to better illustrate how the effect of PKG phosphorylation could lead to drastic changes in the equilibrium of R1 α :PKAc association and dissociation. In this model, modification of R1 α leads a “sensitized intermediate” state of the R1 α holoenzyme that is capable of phospho-transfer activity due to the lack of linker region accessibility at the PKAc active site. Addition of physiological levels of cAMP thus can trigger rapid dissociation of the holoenzyme complex, therefore creating a “desensitized” state of activity because of the inability of the modified R1 α to re-associate with PKAc. In terms of chemical equilibrium, we propose that the modified R1 α subunit (i.e. the sensitized intermediate) has a decreased binding affinity to PKAc as compared to the 0.1nM K_d of the inactive holoenzyme

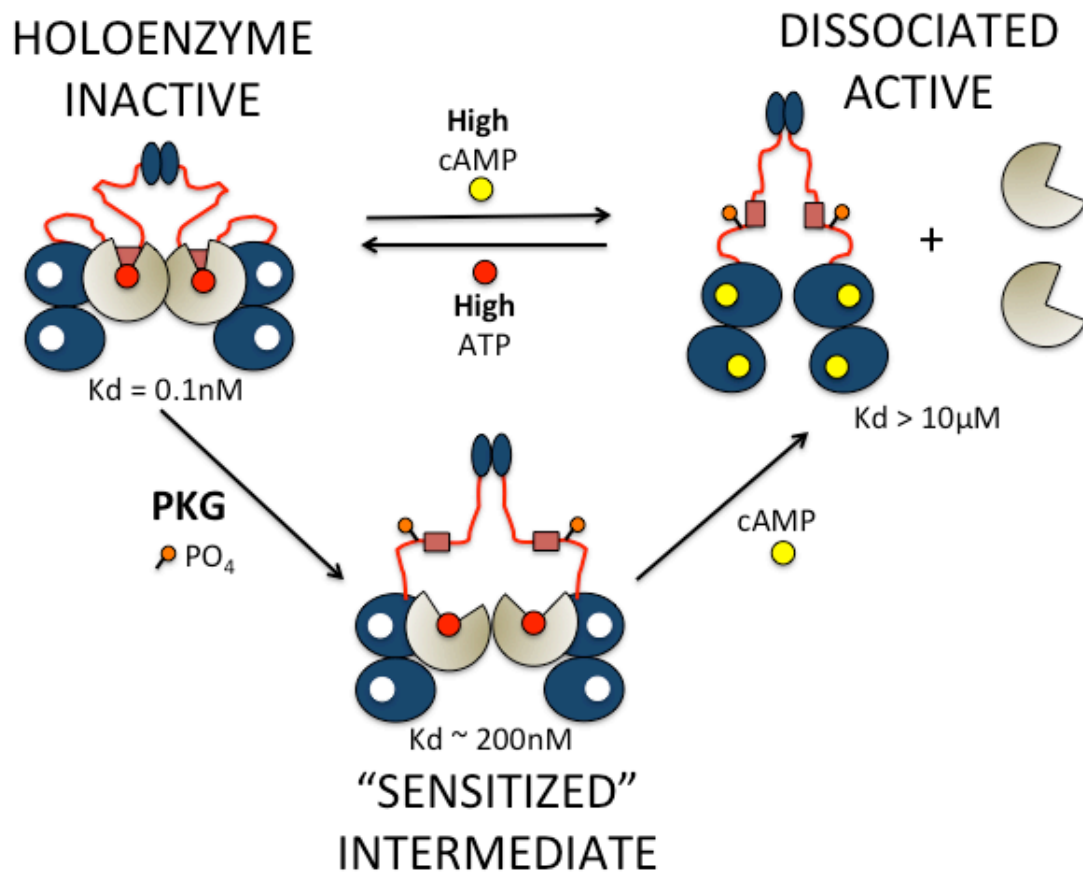


Figure 4.16: PKG phosphorylation model of Type I PKA activation

Cartoon diagram of a modified, 3-state model of Type I PKA holoenzyme activation based on $\text{R}\alpha$ phosphorylation by PKG. Our model illustrates that Type I holoenzyme may not transition directly from an inactive holoenzyme-complex state to a completely dissociated and active state. Instead, our data suggests that phosphorylation of serine 101 in $\text{R}\alpha$ lowers the affinity between the IS of $\text{R}\alpha$ and the active site of PKAc, thus leading to a “sensitized intermediate” state that displays PKAc catalytic activity while maintaining a holoenzyme complex configuration. This intermediate would then respond to physiological levels of cAMP towards dissociation of the holoenzyme complex.

complex. Much like RII subunits, this reduced affinity of the modified linker region can likely be attributed to the loss of high affinity binding to ATP at the active site cleft.

4.4: Acknowledgements

Concerning Chapter 4, I would like to thank Dr. Alexandr Kornev for help with PyMOL figures presented in the introductory section of this chapter.

Chapter 4, in part is currently being prepared for submission for publication of the material. Haushalter, K.J.; Schilling, J.M.; Casteel, D.E.; Stefan, E.; Lapek, J.; Gonzalez, D.; Patel, H.H.; Taylor, S.S. PKG *Phosphorylation of PKA Regulatory Subunit RI α : Crosstalk between PKG/PKA Pathways*. The dissertation author is the primary investigator and author of this material.

Chapter 5:

Concluding remarks and future directions

5. Concluding remarks and future directions.

The information gained from the work described in this dissertation has expanded our understanding of how multiple methods of regulation, including post-translational modification in R1 α subunit as well as interaction with a diverse cohort of regulatory proteins in differing sub-cellular localizations, can lead to activation of Type I PKA signaling independent of canonical cAMP-driven activation mechanisms. Despite the divergent nature of the differing projects described here, the overarching theme of non-canonical modes of PKA signaling manifested in the heart unites this work into one coherent thesis.

As discussed earlier, we believe that the implications of the data presented in this discourse lead to a better understanding of ischemic heart disease. However, there is much to be done to improve upon our theory of dynamic regulation of Type I PKA signaling following oxidative stress. We hypothesize that multiple forms of ROS and RNS (reactive nitrogen species) signaling could theoretically lead to synergistic effects manifested at R1 α in the form of 1) direct oxidant-mediated disulfide modification of the D/D domain, as well as 2) classic activation of PKG by nitric oxide (NO) stimulation of cGMP production which in turn leads to phosphorylation of R1 α . NO signaling increases upon I/R injury, as do other oxidative species like H₂O₂ and superoxide¹¹³. In other words, future endeavors should be aimed at assessing whether R1 α oxidation at the D/D domain and R1 α phosphorylation

by PKG at Ser101 are two mechanisms that work cooperatively towards enhancing the sensitivity of Type I PKA signaling to ischemic injury and potentially promoting the targeted degradation of R1 α to create a “desensitized” PKAc.

In addition to continuing the projects initiated in these doctoral studies, we also hope to elucidate other studies involving alternative modes of modification in R1 α . As mentioned earlier, we are especially interested in the role of Ser77 and Ser83 phosphorylation within the linker region of R1 α , as these two sites have been previously implicated in the progression of heart failure ⁷⁵. To briefly review the research from Ogut *et al.*, cardiac tissues from heart failure patients were examined with regard to the expression of PKA proteins as well as the various phosphorylation state of PKAc substrates. The most interesting observation from this work was that R1 α protein from heart failure tissue could be separated into multiple states of phosphorylation by isoelectric focusing. Phospho-specific antibodies were generated and characterized in order to validate that Ser77 and Ser83 were phosphorylated in these samples. Furthermore, the level of phosphorylation for both of these residues was significantly increased in heart failure in comparison to control tissues. Therefore it was concluded that that modification of R1 α at these positions would likely be an indicative of aberrant downstream PKA signaling in the setting of chronic heart disease, possibly leading to changes in PKAc activity or in R1 α cellular localization as discussed earlier.

While this study unveiled a novel means of regulation in Type I PKA signaling, the cellular mechanisms that cause these modifications (i.e. what kinases target these phospho-sites) in the failing heart were not delineated at the time. While Ser83 was shown to be a target of Cdk2/Cyclin E phosphorylation in a previous publication⁶², the identity of the specific kinase that targets Ser77 remained a mystery. In recent correspondence, Dr. Ogut shared new information with us regarding the phosphorylation of Ser77. His preliminary experiments, which we are now in the process of confirming, indicate a connection to Ca²⁺/calmodulin-dependent kinase II (CaMKII). As shown in panel A (**Figure 5.1**), several kinases were surveyed in an initial *in vitro* trial to determine if increased phosphorylation of Ser77 could be detected. Incubation with both CaMKII α and CaMKII δ isoforms led to robust phosphorylation (as measured from phospho-specific antibody immunoblotting), whereas alternative kinases tested gave no signal increase. To further support this data, mutants of RI α protein were analyzed for their ability to be phosphorylated by CaMKII α . This experiment demonstrated that either removal of the P-3 arginine residue that is part of the CaMKII recognition motif (R74C) or substitution at the Ser77 site with alanine (S77A) prevents CaMKII α phosphorylation of RI α subunit (**Figure 5.1**, panel B).

This evidence provides a clue that could be used to guide further research in the framework of ischemic injury, as CaMKII has already been noted as a critical target in heart disease¹¹⁴. As mentioned previously,

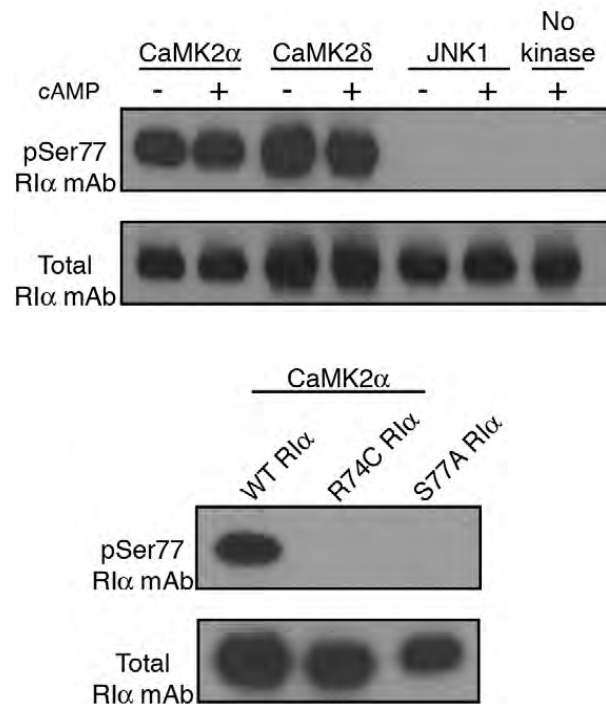


Figure 5.1: Ser77 in R1α is phosphorylated by CaMKII isoforms *in vitro*.

A) Immunoblot of *in vitro* kinase phosphorylation reactions, where kinases targeting Ser77 in R1α were examined. Use of a phospho-specific antibody (pSer77 R1α mAb) allows for detection of R1α protein phosphorylation at this site. CaMKIIα and CaMKIIδ isoforms were shown to modify this site, whereas no phosphorylation was observed in reaction containing mitogen activated protein kinase 8 (MAPK8; a.k.a. JNK1) or no kinase negative control samples.

B) Immunoblot of CaMKIIα phosphorylation of R1α, comparing wild-type protein (WT R1α) versus mutant versions modifying either the P-3 recognition site (R74C R1α) or the Ser77 phospho-site itself (S77A R1α). Both of these mutant forms of R1α protein were unable to be targeted for phosphorylation by CaMKIIα.

CaMKII can be activated by oxidant-mediated mechanisms similar to what we described for Type I PKA in *chapter 2*, further highlighting the importance of understanding potential connections between these various stress signaling pathways. Along these lines, Ser77 phosphorylation has been analyzed in the context of perfused rat hearts treated with ischemic injury. As seen in **Figure 5.2**, levels of phosphorylated Ser77 are significantly increased upon the ischemia stress treatment. While not directly confirmed in this pilot experiment, we can infer that CaMKII is likely activated in this model with R1 α being one of its downstream targets. Future research should therefore be dedicated towards elucidating the link between CaMKII and PKA signaling within in the heart, as establishing a linkage between these two crucial signaling networks would be a very novel and important contribution to the field of research. We hope to confirm these experiments and also to follow up on the finding that the mutation of Arg74 blocks CamKII phosphorylation. This has potential added relevance because Arg74Cys is now known to be a point mutation that is actually found in patients with CNC. Blocking phosphorylation by CamKII would be a functional consequence of this potential activating mutation. As discussed in the Introduction we have studied this mutant extensively and searched for a functional consequence.

In conclusion, the interdisciplinary nature of the research presented in this thesis has allowed for a better understanding of Type I PKA signaling via novel modes of activation and regulation. In essence, this work builds upon

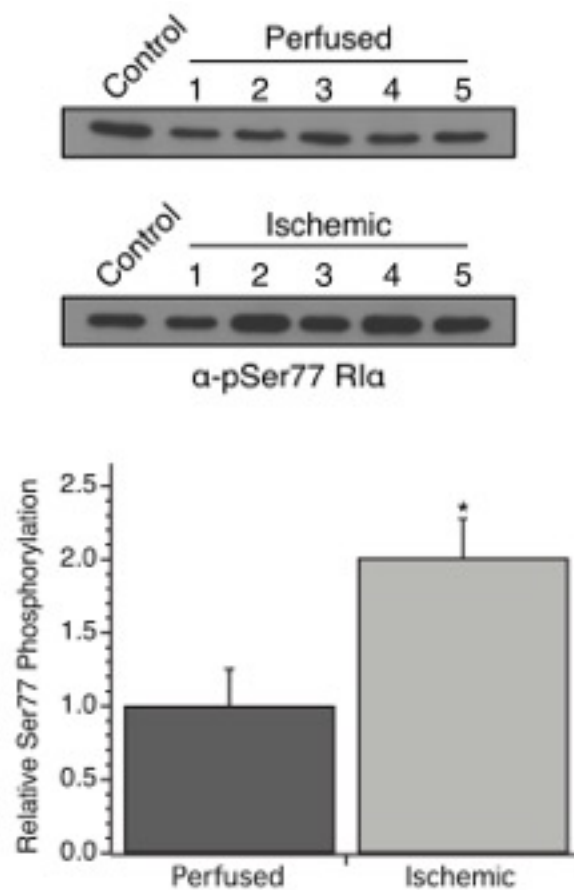


Figure 5.2: Phosphorylation of Ser77 in R1 α is increased with ischemia.

Immunoblot analysis of R1 α phosphorylation in rat hearts, comparing control perfused hearts with those treated with ischemic injury. Ser77 phosphorylation is significantly increased upon treatment, a finding that implies the targeting of this site by CaMKII during oxidative stress.

knowledge of the molecular-scale events using protein kinase biochemistry to make connections to higher orders of biological complexity in terms of cellular biology and physiology, particularly in the context of ischemic heart disease. While the convergence of basic science and translational/pre-clinical research can be challenging both methodologically and ideologically, it is clear that these types of collaborations are essential and can lead to novel discoveries critical to the understanding of biochemical processes in cellular signaling.

Acknowledgments

Concerning Chapter 5, I would like to thank Dr. Ozgur Ogut for granting permission to use unpublished material from his laboratory research for the purposes of describing work intended for future directions of this thesis project.

Experimental Procedures

Materials

Antibodies for PKA-C and RI α were from BD Biosciences (*San Jose, CA, USA*); antibodies for COXIV were from Abcam (*Cambridge, MA, USA*); antibodies for Cleaved PARP and pS/T-PKA substrate were from Cell Signaling Technologies (*Danvers, MA, USA*). Anti-species IgG Secondary antibodies were obtained from Santa Cruz Biotechnology (*Dallas, TX, USA*). Other chemicals and reagents were obtained from Sigma (*St. Louis, MO, USA*) unless otherwise stated.

Ethics statement

Animals were treated in compliance with the U.S. National Academy of Science Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Veterans Affairs San Diego Healthcare System Animal Care and Use Committee.

Animals

Animals were kept on a 12-h light-dark cycle in a temperature- controlled room with ad libitum access to food and water. Wild-type male C57BL/6 mice of age 8-12 weeks were used throughout this study, including within Langendorff procedures as well as adult cardiomyocyte isolations (as described below).

Ex vivo ischemia-reperfusion (I/R) injury using Langendorff perfusion

Intact heart tissue from pentobarbital (80 mg/kg)-anesthetized mice was excised, and retrograde-perfusion with an oxygen-saturated perfusion buffer was initiated using a Langendorff perfusion apparatus. Global ischemia was induced via the prevention of perfusion flow to the heart for a period of 25 minutes (30). After 25 min ischemia, reperfusion was induced by reintroduction of perfusion flow to the heart for time course period between 0-45min. After completion of I/R injury protocols, the heart was immediately removed from perfusion flow and subsequent procedures were initiated.

Mitochondrial isolation

Mice were sacrificed, and hearts were removed. Ventricles were placed in ice-cold mitochondrial isolation medium (MIM: 0.3 M sucrose, 10 mM HEPES, 250 μ M EDTA), minced, and homogenized with a TissueMiser (Fisher Scientific, Waltham, MA, USA). Homogenates were rinsed in MIM. Samples were centrifuged at 600 g to clear nuclear/membrane debris. The resulting supernatant was spun at 8000g for 15 min. The resulting pellet was resuspended in MIM in the presence of 1 mM BSA, followed by another 8000g spin for 15 min. The resulting pellet was resuspended in isolation buffer with BSA and spun again at 8000 g. Metabolically active mitochondria were then suspended in 150 μ L MIM for functional studies.

Isolation of subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM)

Cardiac SSM and IFM populations were isolated using a method modified from Palmer *et al.*¹⁰⁰. Chappell–Perry buffer was used (CP1: 100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄, and 1 mM ATP, pH 7.4; CP2: same as CP1, supplemented with 1mg/mL BSA) for mitochondrial isolation. Upon heart tissue homogenization, samples were centrifuged at 600 g to clear nuclear/cytoskeletal pellet that contains the IFM fraction. The resulting supernatant was spun at 8000g for 15 min, then resuspended in 2mL CP1 and centrifuged at the same condition to isolate the SSM fraction. For isolation of IFM, the first pellet was resuspended in 3mL CP1 and then treated with 0.5mL of 5mg/mL trypsin enzyme for 15min (on ice, with constant stirring) in order to digest the sarcomeric proteins and thus release the IFM fraction from the cytoskeletal components. Trypsin digest was quenched by adding 3mL CP2, and the sample was centrifuged at 600 g to remove contaminants. The resulting supernatant was spun at 8000g for 15 min, then resuspended in 2mL CP1 and centrifuged at the same condition to isolate the IFM fraction. Metabolically active mitochondria fractions were then suspended in 150µL CP1 for functional studies, or directly resuspended in lysis buffer (20mM Tris pH 7.4, 1mM EGTA, 0.1% Triton X-100) for biochemical studies.

Hypoxia/re-oxygenation stress treatments in HL-1 cardiomyocytes

HL-1 cardiomyocyte cells, a gift from the laboratory of Dr. William Claycomb, were cultured according to prescribed conditions. For hypoxia/re-oxygenation stress treatments, HL-1 cells were changed into serum-free media 2hr prior to treatment, and then placing cell cultures into <1% oxygen environment (5%CO₂, balanced with nitrogen; 37°C) for 30min. Re-oxygenation was administered by replacing growth media and returning cell cultures to normal incubation conditions (5% CO₂, balanced with air; 37°C). After removal of treatment media, cells were lysed in 20mM Tris pH 7.4, 1mM EGTA, 0.1% Triton X-100.

Preparation of Adult Mouse Ventricular Myocytes (AMVMs)

In brief, young (8-12 weeks aged) adult male C57BL/6 mice were treated with heparin (XXX mg/kg) to prevent blood clotting, and then anesthetized with pentobarbital (100 mg/kg) before hearts were excised and retrograde-perfused with Joklik medium (*Sigma Aldrich, cat.# 56449C*) containing 1.2mg/mL collagenase II enzyme to digest extracellular matrix material for isolation of ventricular AMVMs. Isolated cells were gradually acclimatized to 100µM CaCl₂ concentration, and then exchanged in to “plating media” (M199 media ® (*Thermo Fisher, Waltham, MA*) supplemented with 4% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic solution) before being plated onto 35mm dishes or 6-well plates coated with 0.01mg/mL mouse laminin

protein (in 1x PBS). After incubation for 2hr to allow cell adherence, plating media was exchanged for “maintenance media” (M199 media ® (*Thermo Fisher, Waltham, MA*) supplemented with 1% culture-grade BSA and 1% penicillin/streptomycin antibiotic solution) in order to allow overnight culture.

AMVM cell treatments

For AMVM cell treatments with hydrogen peroxide (H_2O_2), adherent cells were simply exposed to “maintenance media” supplemented with the desired concentration of H_2O_2 for the indicated period of time. After removal of treatment media, cells were lysed in 20mM Tris pH 7.4, 1mM EGTA, 0.1% Triton X-100.

Immunoblotting

SDS-PAGE samples were prepared using 5x laemelli buffer (100mM Tris-HCl pH 7.0, 10% SDS, 50% Glycerol, 0.01% Bromophenol Blue). Protein concentrations were normalized between samples, and then SDS-PAGE was performed using NuPAGE 4-12% gradient gels (either 10, 12 or 15 well) in MES running buffer (55mM MES, 10mM Tris-base, 1mM EDTA, 0.1% SDS). Gels were resolved at 150V for 70min, and then subsequently transferred to 0.45uM nitrocellulose membranes (200mA/blot for 60min). Membranes were blocked using 5% milk protein (or with 5% BSA for phospho-blot) in 1x PBS-0.05%Tween for 60min at room temperature, then primary antibodies were

incubated overnight at 4°C in 1x PBS-0.05% Tween20 or 1x TBS-0.1% Tween20 at the concentrations specified by the manufacturer.

Generation of adenoviral vector for overexpressing hRI α (Ad-hRI α)

Adenoviral RI α (Ad-RI α) and adenoviral green fluorescent protein (Ad-GFP) were generated in-house by amplifying human RI α cDNA by PCR (contained within the pEGFP-N1 vector), and then subsequently sub-cloning the genetic material (via the BamHI and XbaI sites) into the multi-cloning site reading frame of the Ad-shuttle adenoviral expression vector.

Transmission electron microscopy

Mouse heart muscle was perfusion fixed in the Strack lab and shipped overnight with cold packs in solution containing 2.5% glutaraldehyde + 2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4. The muscle tissue was then diced and washed with ice-cold 0.1 M sodium cacodylate 3x10 min on ice followed by post-fixation in 1% osmium tetroxide, 0.8% potassium ferrocyanide in 0.1 M sodium cacodylate for 3 hours on ice. After 3 washes in ice-cold ddH₂O for 10 min each, the tissue was stained in 2% uranyl acetate for 2 hours. Samples were dehydrated in an ethanol series of ice-cold 20, 50, 70, 90% then 3 washes in 100% ethanol at RT for 10 min each. Samples were infiltrated in 50% ethanol/50% Durcupan ACM (*Fluka/Sigma*) for 3 hours at RT with agitation, followed by 3 changes of 100% Durcupan for 8 hours each at

RT with agitation. The Durcupan resin was polymerized at 60°C for 2 days under vacuum. Sectioning was performed using a Leica ultramicrotome. Ultrathin (80 nm) sections were post-stained with uranyl acetate (5 min) and lead salts (2 min) prior to imaging using an FEI spirit transmission electron microscope operated at 120 kV. The magnification was 4400x corresponding to a pixel resolution of 2.9 nm.

Recombinant DNA and Site-directed Mutagenesis

Constructs of full-length human R1a gene (hR1a 1-379) incorporated into the pCDNA3.3 mammalian expression vector were used for cellular transfection experiments in MEFs. Point mutations (hR1a[S101A], hR1a[S101E]) were introduced by site-directed mutagenesis via Quickchange® PCR as per manufacturers instructions (*Stratagene, La Jolla, CA*). The same genetic material was also incorporated into pRSET-Xa3 vector for bacterial recombinant protein expression. Both full-length (1-379) and truncation (91-379) constructs of bovine R1a gene (bR1a) incorporated into the pRSET bacterial expression vector were used for recombinant protein expression and purification. Point mutations (bR1a[S101A], bR1a[S101E]) were introduced into both constructs by site-directed mutagenesis via Quickchange® PCR as per manufacturers instructions (*Stratagene, La Jolla, CA*). Flag-tagged R1 α was constructed by PCR using an untagged R1 α vector as a template. The PCR product was digested and inserted into the pFLAG-D expression vector. The

S101A and S101E mutant RI α constructs were generated using overlapping extension PCR. All constructs that went through a PCR step were sequenced to ensure that the expected coding sequence was present.

Expression and Purification of Recombinant Proteins from *E. coli*

The C-subunit was expressed in *E. coli* and purified as described¹¹⁵. Both full-length (1-379) and truncation variants (91-379) of either wild-type, S101E, and S101A mutants of bRI α (in pRSET vector) were expressed in BL21(DE3) cells and purified by cAMP affinity chromatography in 20 mM MES (pH 6.5), 100 mM NaCl, 2 mM EGTA, 2mM EDTA, and 5 mM DTT. RI α proteins were further purified on a gel filtration column using Superdex 200 and concentrated for purposes of biochemical assays using amicon® centrifugal filters (*EMD Millipore, Billerica, MA*).

In Vitro PKA RI α Phosphorylation by PKGI α

To examine RI α phosphorylation by PKGI α , purified PKGI α (70 ng in 5 μ L) was added to a 45 μ L reaction mix containing: 5 μ g wild-type RI α , 30 mM Hepes (pH 7.4), 10 mM MgCl₂, 10 mM β -glycerol phosphate, 100 mM ATP, and 0.05 mCi ³²P- γ -ATP. Reactions were incubated at 30°C and at the indicated times 10 μ L aliquots were removed and added to 10 μ L 2x SDS loading buffer containing 50 mM EDTA. To examine how the RI α /PKGI α stoichiometry effected RI α phosphorylation 200ng PKGI α was incubated with

increasing amounts of RI α (as indicated) for 2 h at 30°C in a reaction mix containing 30 mM Hepes (pH 7.4), 10 mM MgCl₂, 10 mM β -glycerol phosphate, 100 mM ATP, and 0.05 mCi ³²P- γ -ATP. After 2 h, beads were washed in PBS and boiled in 1x SDS sample buffer. Phosphate incorporation was determined by SDS-PAGE/autoradiography.

Cell Culture: Growth, Transfection, and Treatments

HEK293T cell cultures as well as mouse embryonic fibroblast (MEF) cells were used throughout this study. Propagating cultures were grown in 10cm dishes using DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, unless otherwise stated.

Phosphorylation of RI α in HEK293T cells

HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) in a 37°C incubator with a 5% CO₂ atmosphere. The day before transfection, the cells were split into a 6-well cluster dish such that the cells were 90-95% confluent at the time of transfection. The cells were transfected with the indicated expression constructs using Lipofectamine 2000 using conditions recommended by the manufacturer (*Thermo Fisher, Waltham, MA*). The next day, the media was removed and 1 mL phosphate free media and 100 μ Ci ³²PO₄ was added to each well. The cells were incubated at 37°C for 2 h and then 250 μ M 8-CPT-

cGMP was added to the appropriate wells. The cells were incubated for an additional 2 h 37°C, at which point the media was removed, the cells were washed once with ice cold Phosphate Buffered Saline (PBS) and the cells were lysed on the plate in: PBS, 0.1% NP40, 1 X protease inhibitor cocktail (*Calbiochem, San Diego, CA*), and 1x HALT phosphatase inhibitor (*Cell Signaling Technology, Danvers, MA*). Cleared lysates were added to 20 μ L anti-Flag affinity gel and incubated for 1h, 4C, with constant mixing. The beads were washed, boiled in 30 μ L 1x SDS sample buffer, and phosphorylation was analyzed by SDS-PAGE/autoradiography. The relative amount of immunoprecipitated Flag-tagged RI α was determined by immunoblotting.

PepTag® Non-radioactive PKA phosphorylation assay

Assays using both cell lysates and recombinant proteins were performed in a modified procedure from manufacturer directions.

For cell based experiments:

MEF cell samples were plated on 6-well dishes at passage 3. Transfections were conducted using lipofectamine 2000® (*Thermo Fisher, Waltham, MA*) as per manufacturer's directions. For activity experiments, MEF cells were serum-starved for 2 hours before conducting 10min Fsk/IBMX stress treatment (i.e. 20 μ M forskolin and 100 μ M IBMX). 10 μ L of cell lysate (of normalized protein concentration) was added to 5 μ L each of "5x peptide", "5x buffer", and

distilled water for all reactions. Samples were mixed and incubated at room temperature for 30min, then heat-inactivated at 95°C for 10min.

For recombinant protein experiments: R1 α and PKAc proteins were incubated *in situ* at a 1.2:1 molar stoichiometry within a 10 μ L volume (for R1 α (1-379) dimer protein, 5 μ L of 1.2 μ M R-subunit was added to 5 μ L of 2 μ M PKAc; for R1 α (91-379) monomer protein, 5 μ L of 600nM R-subunit was added to 5 μ L of 500nM PKAc). Then, 5 μ L each of “5x peptide”, “5x buffer”, and either 5mM cAMP or distilled water was added to start the reactions. Samples were mixed and incubated at room temperature for 30min, then heat-inactivated at 95°C for 10min. For all experiments, phospho-peptides were separated by 0.8% agarose gel as per manufacturer’s directions.

Protein Complementation Assay (PCA) using fragmented RLuc

Plasmid constructs of PKAc tagged with F1 fragment (PKAc-F1) and R1 α tagged with F2 fragment (R1 α -F2) were generated previously¹⁰⁸. S101E and S101A mutations of R1 α were introduced into R1 α -F2 by site-directed mutagenesis via Quickchange® PCR as per manufacturers instructions (*Stratagene, La Jolla, CA*). PKAc-F1 and either wild-type (WT), S101E, or S101A versions of R1 α -F2 plasmids were co-transfected into HEK293T cells, and bioluminescence signal was compared under basal conditions as well as with 10nM isoproterenol stimulus.

Ligand regulated competition (LiReC) fluorescence polarization assay

Use of the LiReC assay has been described previously. All reactions were conducted in assay buffer (50mM MOPS, pH 7.0, 35mM NaCl, 10mM MgCl₂, 1mM ATP, 2mM DTT, and 0.005% TritonX-100). End-point fluorescence polarization measurements were performed using a GENios Pro ® plate-reader spectrometer (*Tecan, Mannendorf, Switzerland*), configured with 485nm excitation and 535nm emission filters and using optimal gain settings. Concentrations of proteins, [5/6-FAM]-IP20, and cAMP for each experimental method is listed below:

cAMP dose-response experiments

12.0nM PKA Catalytic Subunit, 14.4nM PKA Regulatory Subunit bR1 α (91-379) or bR1 α (1-379) (based on monomer concentration), 2.0nM [5/6-FAM]-IP20, 1nM-1uM cAMP (dissolved in 1x buffer w/o ATP or DTT)

R-subunit dose-response experiments

10.0nM PKA Catalytic Subunit, 1nM-1 μ M PKA Regulatory Subunit bR1 α (91-379) or bR1 α (1-379) (based on monomer concentration), 1.67nM [5/6-FAM]-IP20

Rat Ischemia Model:

The in vivo rat ischemia model has been described in greater detail previously¹¹⁶ and follows an experimental protocol approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. In brief, adult male Sprague-

Dawley rats (body wt. 250–350 g) were anesthetized using a mixture of ketamine and xylazine (10:2; 0.5–0.7 mL/kg) administered intramuscularly. An endotracheal tube was inserted for ventilation with O₂-supplemented room air using a positive pressure respirator. Body temperature was maintained at 37°C during the procedure. The animal was allowed to stabilize for 15 min and then the heart was exposed by a midline thoracotomy and a ligature was placed near the bifurcation of the left coronary artery, restricting blood flow through the left anterior descending (LAD) and circumflex arteries for 30 min. Coronary occlusion was monitored by immediate pallor of the left ventricular free wall. For the time-matched perfused condition, the animal underwent surgery but without ligation. Following the procedure, the anterolateral papillary muscle, which derives blood supply from the LAD (Voci et al., 1995), was excised from the left ventricle and flash frozen in liquid nitrogen.

***In vitro* kinase reactions**

All reactions were conducted at 30°C for 60 minutes. Kinase reactions were quenched by freezing at -80°C. Buffer conditions for each kinase reaction conducted are listed below:

CAMK2 Delta (without cAMP)

10 uL PKA Regulatory I (~5.5 ug of 12.5 uM Stock), 1 uL of 0.5 M TCEP (0.5 mM), 50 uL of 2x Reaction Buffer (2x is 50 mM PIPES, pH 7 and 10 mM

MgCl₂), 10 uL of 10 mM ATP (1 mM Final), 10 uL (1 ug) of CaMKII, 10 uL (1000 units) of Calmodulin, 1 uL (1 mM) CaCl₂, 8 uL Water

CAMK2 Delta (with cAMP)

10 uL PKA Regulatory I (~5.5 ug of 12.5 uM Stock), 1 uL of 0.5 M TCEP (0.5 mM), 50 uL of 2x Reaction Buffer (2x is 50 mM PIPES, pH 7 and 10 mM MgCl₂), 10 uL of 10 mM ATP (1 mM Final), 10 uL (1 ug) of CaMK2, 10 uL (1000 units) of Calmodulin, 1 uL (1 mM) CaCl₂, 1 uL (1 mM) of 0.1 M 8-Br-cAMP, 7 uL Water

JNK1 (without cAMP)

10 uL PKA Regulatory I (~5.5 ug of 12.5 uM Stock), 1 uL of 0.5 M TCEP (0.5 mM), 50 uL of 2x Reaction Buffer (2x is 50 mM PIPES, pH 7 and 10 mM MgCl₂), 10 uL of 10 mM ATP (1 mM Final), 10 uL (1 ug) of JNK1, 19 uL Water

JNK1 (with cAMP)

10 uL PKA Regulatory I (~5.5 ug of 12.5 uM Stock), 1 uL of 0.5 M TCEP (0.5 mM), 50 uL of 2x Reaction Buffer (2x is 50 mM PIPES, pH 7 and 10 mM MgCl₂), 10 uL of 10 mM ATP (1 mM Final), 10 uL (1 ug) of JNK1, 1 uL (1 mM) of 0.1 M 8-Br-cAMP, 18 uL Water

No Kinase

Identical to the CaMK2 + cAMP reaction above, but with omission of CaMK2 and CaM. Water was used to make up the balance of the volume

Statistics

All data are presented as means \pm sem. GraphPad Prism 4 software (*GraphPad Software, Inc., San Diego, CA, USA*) was used for all statistical analysis. Statistical analyses were performed by unpaired Student's t test. cAMP and R-subunit dose-response experiments (*chapter 4*) were analyzed using Variable slope (four parameters) algorithms.

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